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Thomas C. Südhof
Klaus Starke
Editors

Pharmacology of Neurotransmitter Release

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Preface

This book is intended to provide an overview of the pharmacology of neurotransmitter release. Neurotransmitter release initiates synaptic transmission, the major mechanism by which neurons communicate with each other and with effector cells. Although a larger number of drugs act on the postsynaptic receptors that are the targets of the released neurotransmitters than on the release process itself, some of the oldest drug agents in medicine influence the release of subsets of neurotransmitters, for example, reserpine, which empties synaptic vesicles containing catecholamines and thereby blocks catecholamine release. Furthermore, some long-recognized compounds that act on neurotransmitter release are being increasingly used for new applications. For example, botulinum toxins are now among the most frequently administered cosmetic drugs employed to counteract the development of wrinkles; they act by inhibiting neurotransmitter release.

Dramatic progress has been made over the last decades in our understanding of neurotransmitter release. The principal mechanism that mediates release was elucidated by Bernhard Katz more some 50 years ago, but the molecular events remained obscure until the components and functions of nerve terminals were studied in recent years (reviewed in Südhof 2004). The basic mechanisms of release are discussed in the book's first part.

For a long time it was tacitly assumed that the amount of transmitter released per action potential was constant – at least at a given action potential frequency. However, this is not so – an almost baroque diversity of presynaptic plasticity mechanisms has emerged over the last two decades. Axon terminals are not only passively transmissive structures, but also represent actively computational elements. Synaptic neurotransmitter release changes as a function of use, often dramatically, in a manner that depends both on the release machinery and on extrinsic inputs. Indeed, nerve terminals are endowed with a large number of receptors for endogenous chemical signals – presynaptic receptors which, when activated, modulate the amount of transmitter being released.

Interestingly, the first experiment that retrospectively must be explained by presynaptic receptors was published in this handbook – in its second volume, in 1924, by the British pharmacologist Walter E. Dixon. Figure 1 shows that he injected

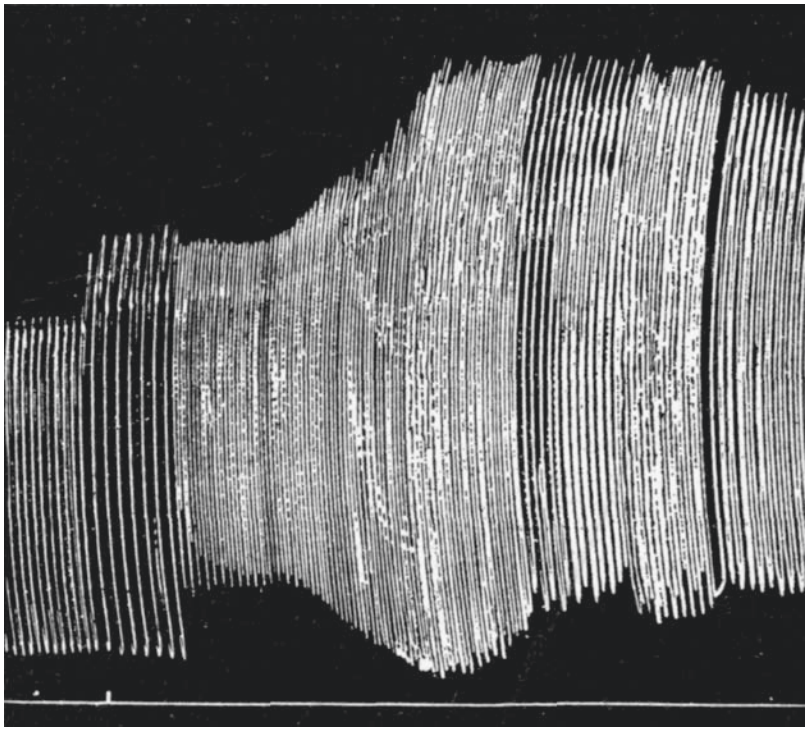

Fig. 1 Effect of nicotine on a rabbit isolated heart. From Dixon (1924).

nicotine into the isolated perfused heart of a rabbit. Immediately on injection, nicotine slowed the heart rate by stimulating intracardiac vagal ganglion cells. After a few seconds, however, bradycardia was replaced by marked tachycardia and an increase in contraction amplitude. Because the isolated heart does not contain sympathetic ganglion cells (and because an effect on the myocardium can be excluded), nicotine must have acted on the cardiac sympathetic axon terminals, on what we now call presynaptic nicotinic receptors.

Presynaptic nicotinic receptors are ligand-gated ion channels. Many other presynaptic receptors couple to G-proteins. Presynaptic receptors may be targets of bloodborne substances or substances secreted from neighboring cells, including neighboring axon terminals. In 1971 it was noticed with some surprise that many axon terminals even possess receptors for their own transmitter–presynaptic autoreceptors, the α_2-autoreceptors for noradrenaline being a prominent example (reviewed in Starke 2001). The various presynaptic ligand-gated ion channels and G-protein-coupled receptors are discussed in the second part of this volume. Questions regarding where the receptors' signal transduction pathways hit the exocytosis cascade and whether the receptors have therapeutic potential will be addressed in all chapters.

We attempt a synthesis of a large amount of information and cannot be expected to be totally successful. Nevertheless, we hope that the various contributions will be useful, and that the book will be of help to scientists in a wide number of fields.

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Neurotransmitter Release

Thomas C. Südhof

Abstract Neurons send out a multitude of chemical signals, called neurotransmitters, to communicate between neurons in brain, and between neurons and target cells in the periphery. The most important of these communication processes is synaptic transmission, which accounts for the ability of the brain to rapidly process information, and which is characterized by the fast and localized transfer of a signal from a presynaptic neuron to a postsynaptic cell. Other communication processes, such as the modulation of the neuronal state in entire brain regions by neuromodulators, provide an essential component of this information processing capacity. A large number of diverse neurotransmitters are used by neurons, ranging from classical fast transmitters such as glycine and glutamate over neuropeptides to lipophilic compounds

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and gases such as endocannabinoids and nitric oxide. Most of these transmitters are released by exocytosis, the i.e. the fusion of secretory vesicles with the plasma membrane, which exhibits distinct properties for different types of neurotransmitters. The present chapter will provide an overview of the process of neurotransmitter release and its historical context, and give a reference point for the other chapters in this book.

1 Principles of Neurotransmitter Release

Neurons communicate with each other and their target cells via two principal mechanisms: the secretion and reception of chemical messengers called neurotransmitters, and the direct transfer of intercellular signals via gap junctions. Communication via neurotransmitters occurs in several forms that range from classical synaptic transmission at synapses to diffuse secretion of neuromodulators which mediate volume transmission. Communication via gap junctions occurs at so-called electrical synapses. Almost all of the neuronal communication is mediated by neurotransmitters, and electrical synapses are exceedingly rare in vertebrate brain. Both types of communication are not unique to neurons. Secretion of neuromodulators and neuropeptides is also mediated by endocrine cells and even some highly differentiated cells such as adipocytes, and diffusible neurotransmitters such as nitric oxide are released by many non-neuronal cells. Only the presynaptic secretion of classical neurotransmitters in the context of a synapse is specific to neurons, although the postsynaptic cell can be either a neuron (most of the time) or an effector cell (e.g., a muscle cell). The present book will only deal with communication by neurotransmitters, and only with the release of such transmitters and the pharmacology of this release.

What is a neurotransmitter, and how many different "types" of neurotransmitter release exist? At least five types of neurotransmitter release can be defined.

1. Synaptic neurotransmitter release occurs in a classical, electron microscopically observable synapse, and is mediated by synaptic vesicle exocytosis from nerve terminals (Figure 1; Katz, 1969; Südhof, 2004; note that a "nerve terminal" is not necessarily the end of an axon, but generally is formed by axons en passant as they arborize throughout the brain). Synaptic neurotransmitter release, the first step in synaptic transmission, transfers information extremely rapidly (in milliseconds) in a highly localized manner (restricted to an area of less than a square micrometer; reviewed in Südhof, 2004). Synaptic release secretes "classical" neurotransmitters: GABA, glycine, glutamate, acetylcholine, and ATP. It has been suggested that in addition to neurons, astrocytes also secrete classical neurotransmitters by a similar mechanism (**?**), but this type of secretion has not been directly demonstrated.
2. Monoaminergic neurotransmitters (dopamine, noradrenaline, adrenaline, histamine, and serotonin) are released by exocytosis of small dense-core vesicles from

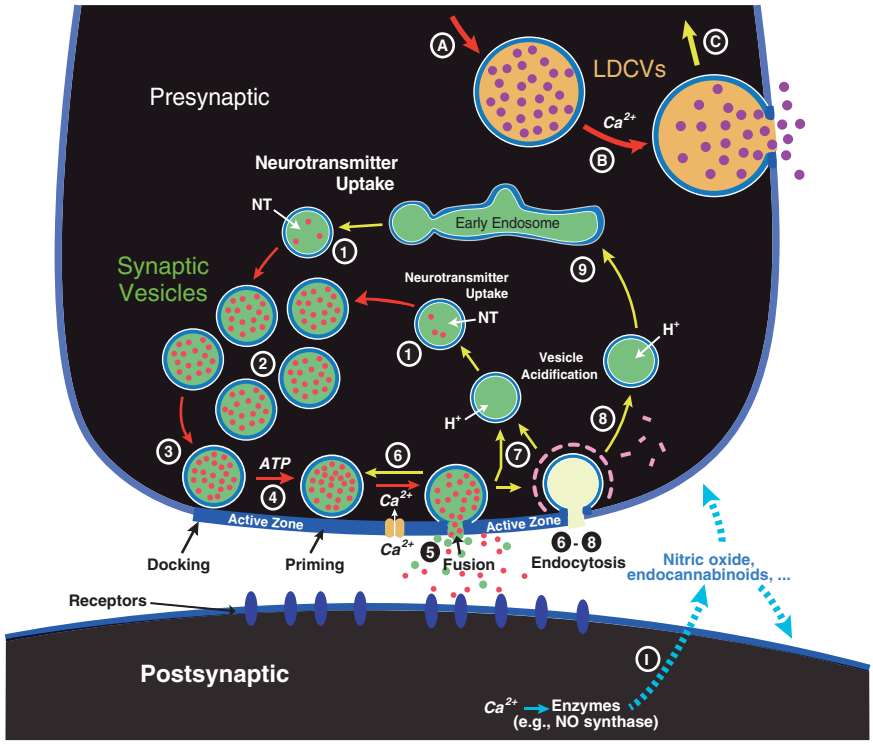

Fig. 1 Secretory pathways in neurons. The drawing schematically illustrates the three major neurotransmitter release pathways. (**a**) Release of classical neurotransmitters by synaptic vesicle exocytosis (center; steps 1–9). Classical neurotransmitter release depends on an underlying synaptic vesicle cycle that starts when synaptic vesicles are filled with neurotransmitters by active transport (step 1), and form the vesicle cluster (step 2). Filled vesicles dock at the active zone (step 3), where they undergo a priming reaction (step 4) that makes them competent for Ca^{2+}-triggered fusion-pore opening (step 5). After fusion-pore opening, synaptic vesicles undergo endocytosis and recycle via three alternative pathways: local reuse (step 6; also called kiss-and-stay), fast recycling without an endosomal intermediate (step 7; also called kiss-and-run), or clathrin-mediated endocytosis (step 8) with recycling via endosomes (step 9). Steps in exocytosis are indicated by red arrows, and steps in endocytosis and recycling by yellow arrows. (**b**) Release of neuropeptides and biogenic amines by LDCV exocytosis. LDCVs are generated in the cell body by budding from the Golgi complex filled with neuropeptides (not shown). LDCVs are then transported from the cell body to the axons or dendrites (step A, as shown for nerve terminals). A Ca^{2+}-signal triggers the translocation and fusion of LDCVs with the plasma membrane outside of the active zone (step B). After exocytosis, empty LDCVs recycle and refill by transport to the cell body and recycling via the Golgi complex (step C). (**c**) Release of gaseous or lipidic neurotransmitters, which are synthesized in either the pre- or the postsynaptic neuron (only the postsynaptic synthesis is shown), and secreted by diffusion across the plasma membrane (step I) to act on local extracellular receptors (e.g., CB1 receptors for endocannabinoids) or intracellular targets (e.g., guanylate cyclase for nitric oxide). (Modified from Südhof, 2004).

axonal varicosities that are largely not associated with a specialized postsynaptic structure (i.e., are outside of synapses; Brock and Cunnane, 1987; Stjärne, 2000). However, at least in the case of dopamine, postsynaptic specializations can occur with presynaptic small dense-core vesicles.

3. Neuropeptides are secreted by exocytosis of large dense-core vesicles (LDCVs) outside of synapses (Figure 1; Salio et al., 2006). LDCVs undergo exocytosis in all parts of a neuron, most often in axon terminals and dendrites. Monoamines are often co-stored with neuropeptides in LDCVs and co-secreted with them upon exocytosis. For all intents and purposes, LDCV-mediated secretion resembles hormone secretion in endocrine cells.
4. Classical neurotransmitters and monoamines may rarely be secreted by neurons, not by exocytosis, but by transporter reversal. This mechanism involves the transport of neurotransmitters from the cytosol to the extracellular fluid via transporters that normally remove neurotransmitters from the extracellular fluid. This mechanism appears to account for the burst of dopamine released by amphetamines (Fleckenstein et al., 2007), but its physiological occurrence remains unclear.
5. A fifth pathway, finally, is the well-established secretion of small membrane-permeable mediators by diffusion. This mechanism is used for the secretion of nitric oxide, endocannabinoids, and other important lipidic or gaseous neurotransmitters. The major point of regulation of release here is the synthesis of the respective compounds, not their actual secretion.

Only the first type of neurotransmitter release mediates the fast point-to-point synaptic transmission process at classical synapses (sometimes referred to as wiring transmission). All of the other types of neurotransmitter release effect one or another form of "volume transmission" whereby the neurotransmitter signal acts diffusely over more prolonged time periods (Agnati et al., 1995). Of these volume transmitter pathways, the time constants and volumes involved differ considerably. For example, diffusible neurotransmitters such as nitric oxide act relatively briefly in a localized manner, whereas at least some neuropeptides act on the whole brain, and can additionally act outside of it (i.e., function as hormones). There is an overlap between wiring and volume neurotransmission in that all classical neurotransmitters act as wiring transmitters via ionotropic receptors, and also act as "volume transmitters" via G-protein-coupled receptors. Moreover, neuromodulators in turn feed back onto classical synaptic transmission.

Quantitatively, synaptic transmission is the dominant form of communication between neurons. A single look at an electron micrograph reveals that synapses with their appendant organelles, especially synaptic vesicles, are abundant in brain, whereas LDCVs are only observed occasionally (Figure 2). However, this does not mean that synaptic transmission is more important than the volume transmission pathways. The two principally different signaling pathways play distinct roles in information processing by the brain, and both are essential for brain function.

With the multitude of different types of transmitters, the question arises whether a single neuron can release more than one transmitter. Dale's principle stated that

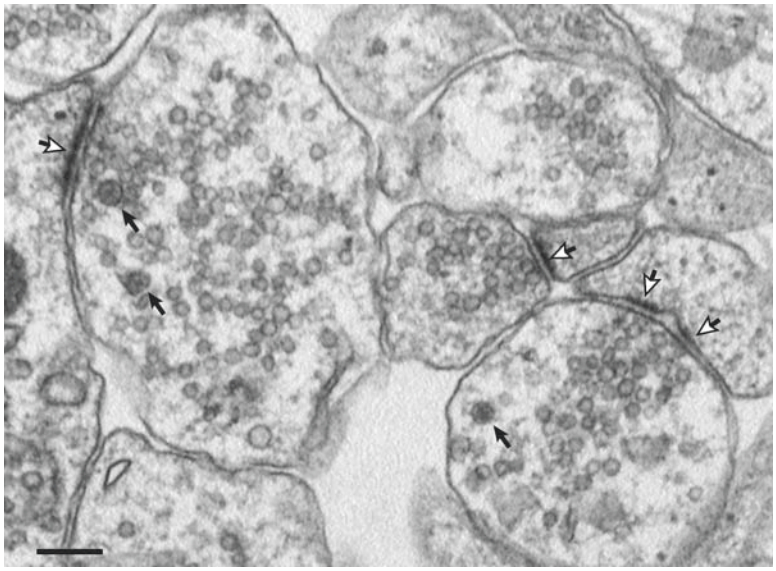

Fig. 2 Electron micrograph of synapses. The image shows synapses formed by cultured cortical neurons from mouse. Note abundant synaptic vesicles in nerve terminals adjacent to synaptic junctions that are composed of presynaptic active zones and postsynaptic densities (open arrows point to postsynaptic densities of synaptic junctions; synapse on the right contains two junctions). In addition to synaptic vesicles, two of the nerve terminals contain LDCVs (closed arrows). Calibration bar = 500 nm. (Image courtesy of Dr. Xinran Liu, UT Southwestern).

this is not the case, but seems to be incorrect given the fact that virtually all neurons secrete neuropeptides and either classical neurotransmitters or monoamines (Salio et al., 2006). Moreover, many neurons additionally secrete diffusible neurotransmitters. Thus, a neuron usually operates by multiple neurotransmitter pathways simultaneously. To add to the complexity of these parallel signaling pathways, the relatively small number of neurons that secrete monoamines from axonal varicosities may also secrete classical neurotransmitters in separate classical synapses (Trudeau, 2004). Despite this complexity, however, Dale has to be given credit for his principle because the multiple transmitters secreted by a given neuron generally operate in distinct secretory and effector pathways. A given neuron usually releases only one type of classical neurotransmitter (with a few exceptions), suggesting that a modified Dale principle is still correct cotransmission.

2 Very Short History of the Analysis of Neurotransmitter Release

Our current concept of synaptic transmission, as mediated by intercellular junctions formed by one neuron with another neuron or target cell, is fairly recent. This concept was proposed in the second half of the 19th century, and proven

only in the 20th century. It was embedded in a larger debate of whether neurons form a "reticular" network of connected cells, or a network of cells whose connections are discontinuous (the so-called neuron theory). Like with everything else in neuroscience, Ramón y Cajal is usually credited with the major discoveries in this field, but the actual concept predates him, and the development of the current view of synaptic transmission is due to a team effort. When Ramón y Cajal followed in the footsteps of scientists like Kühne, Koelliker, and His, who had formulated the first concept of synapses, even though the actual term was coined much later, Cajal's elegant prose and the fortunate opposition of Emilio Golgi to the neuron theory enhanced the influence of his writings and somewhat obscured the fact that the actual concepts that Cajal was presenting were already well established in the literature.

The term synapse was coined in 1897 by the physiologist Charles Sherrington in M. Foster's *Textbook of Physiology*, but the idea of the chemical synapse was developed almost half a century earlier in studies on the neuromuscular junction. As always in science, technical advance spawned conceptual breakthroughs. The three technical advances that fueled the progress in neuroscience in the second half of the 19th century were the improvements in light microscopy, chiefly due to Lister's invention of apochromatic lenses, the continuous development of staining methods culminating in Golgi's epynomous stain, and the application of more precise electrical recordings, allowing the emergence of electrophysiology to complement anatomy. Each historical stage in the discovery process is coupled to a particular preparation and technical approach, and major progress was usually achieved when a new technique was applied to a new preparation. This pattern also applies to the discovery of the synapse which was first described, without naming it, at the neuromuscular junction.

In the middle of the 19th century, it was known from the work of Volta, Galvani, and others that the nerve stimulates muscle contractions at the neuromuscular junction, and that electrical signals were somehow involved. Using the tools of cellular neuroanatomists, Kühne (1862) and Krause (1863) first demonstrated that the neuromuscular junction is not composed of a direct cellular connection between nerve and muscle as had been believed, but is discontinuous. Fifteen years later, the electrophysiologist Emil du Bois-Reymond (1877) proposed that the transmission of a synaptic signal is chemical. Subsequent work by Koelliker, Cajal, and Sherrington generalized this concept of a discontinuous synaptic connection that mediates intercellular signaling to the interneuronal synapses. Although the concept of the synapse continued to be disputed until well into the 20th century (e.g., see Golgi's Nobel lecture), the very existence of these disputes should not prevent us from recognizing that the actual description of synaptic transmission, and at least its proof for one particular synapse, the neuromuscular junction, had been established 50 years earlier.

The next major step forward in deciphering the mechanisms of synaptic transmission occurred in the neuropharmacological studies of Henry Dale, Otto Loewi, Wilhelm Feldberg, and their colleagues. Although, as in the discovery of the synapse as an intercellular noncontinuous junction, many individuals contributed, Loewi is generally credited with the single decisive experiment. This is probably fair, since

Loewi demonstrated directly that a chemical mediator (acetylcholine) is responsible for the transmission of the signal from the vagus nerve to the heart (Loewi, 1921). Despite Loewi's, Dale's, and Feldberg's advances, however, doubts lingered as to whether a chemical signal could be fast enough to account for the speed of synaptic transmission. Many scientists, with John Eccles (one of Sherrington's last pupils) as the most vocal protagonist, continued to espouse the view that fast synaptic transmission is essentially electrical, whereas chemical signaling serves only as a slow modulatory event. In other words, these views proposed a clean division of transmission into fast synaptic wiring transmission that is electrical, and slow volume transmission that is chemical. The doubts about the speed of chemical neurotransmission, and its general validity, were only definitively laid to rest by Bernhard Katz's seminal experiments on the frog neuromuscular junction, demonstrating that synaptic transmission operates as a quantal chemical event (Katz, 1969). It is remarkable that from Kühne's to Katz's studies, the major contributions to establishing synaptic transmission as the major mechanism by which neurons communicate came from the neuromuscular junction. The concept of the synapse was first postulated at the neuromuscular junction, the first genuine neurotransmitter was identified with acetylcholine as the neuromuscular junction neurotransmitter, and the chemical quantal nature of synaptic transmission was revealed at the neuromuscular junction.

The findings of Katz and colleagues raised two major questions: what are the mechanisms that allow the fast secretion of neurotransmitters from presynaptic terminals in response to an action potential? What molecules mediate the fast recognition of these neurotransmitters by the postsynaptic cell? The elucidation of the basic mechanisms of release again started with the cholinergic system in the description and isolation of synaptic vesicles as the central organelle, chiefly by Victor Whittaker (Whittaker and Sheridan, 1965). The progress in the field, however, then shifted to central synapses, with the identification of the major molecules involved in release of neurotransmitters, and the description of the mechanism by which Ca^{2+}-influx into nerve terminals achieves the fast triggering of release via binding to synaptotagmins (reviewed in Südhof, 2004). The discovery of neurotransmitter receptors and their properties was initiated by classical pharmacological approaches dating back to the British school founded by Langley (1921), but the definitive description of these receptors was enabled by the simultaneous development of patch clamping by Neher and Sakmann (1976) and of molecular cloning of these receptors by S. Numa (Noda et al., 1982).

3 Basic Mechanisms of Release by Exocytosis

Most neurotransmitter release occurs by exocytosis of secretory vesicles, which involves the fusion of the secretory vesicles (synaptic vesicles and LDCVs) with the plasma membrane. All intracellular membrane fusion (except for mitochondrial fusion) is thought to operate by the same fundamental mechanism that involves a core machinery composed of four classes of proteins: SNARE-proteins, SM-proteins (for

Sec1/Munc18-like proteins), Rab-proteins, and Rab-effectors (Jahn et al., 2003). The specific isoforms of these proteins that are being used vary tremendously between fusion reactions, but the general principle by which these proteins act seems to be always similar: Rab and Rab-effector proteins appear to proofread the docking and fusion reaction between the two target membranes and may even mediate the docking at least in part, whereas SNARE- and SM-proteins catalyze the actual fusion reaction.

3.1 Rab-Proteins and Rab-Effectors

Rab-proteins are GTP-binding proteins that interact with effectors in a GTP-dependent manner. Rab3A, 3B, 3C, and 3D represent a family of Rab-proteins that are highly enriched on synaptic vesicles and other secretory organelles throughout the body. In addition, Rab27A and 27B are also generally found on secretory vesicles, although it is unclear whether they are present on synaptic vesicles (Südhof, 2004). Rab3/27 proteins together function in exocytosis, and mediate vesicle docking at least in part. Two classes of Rab3/27 effectors were described: rabphilins and RIMs. Both effector classes include multiple members encoded by distinct genes. Rabphilins are cytosolic proteins that are recruited to secretory vesicles by Rab3/27, but their function has remained largely obscure. RIMs are components of the detergent-insoluble protein complex that makes up the active zone, the part of the presynaptic plasma membrane where synaptic vesicles dock and fuse (Figure 3). The active zone is composed of the RIM-containing protein complex that includes several other large proteins, in particular Munc13s, piccolo/bassoon, ELKS, and α-liprins, all of which are crucial for normal synaptic vesicle exocytosis. It is noticeable that in most intracellular fusion reactions, Rab-effectors are composed of large complexes that do more than just bind the Rab-protein, but perform several functions in the fusion process, with the Rab-protein often being involved in the docking of the membranes for fusion and in the regulation of the other activities of the complex during the fusion reaction. The same appears to be true for Rab3/27 binding to the RIM-containing active zone protein complex. The whole active zone complex could be considered as a single large Rab-effector complex (Figure 3), and is likely involved not only in the docking of synaptic vesicles, but also in organizing the actual fusion reaction and in synaptic plasticity (see below).

3.2 SNARE Proteins

Membrane fusion consists of merging two negatively charged phospholipid bilayers, and thus requires overcoming a major energy barrier (Jahn et al., 2003). SNARE proteins represent a family of membrane proteins that are present on opposing membranes destined to fuse. As first proposed by Jahn, Heuser, Rothman and colleagues

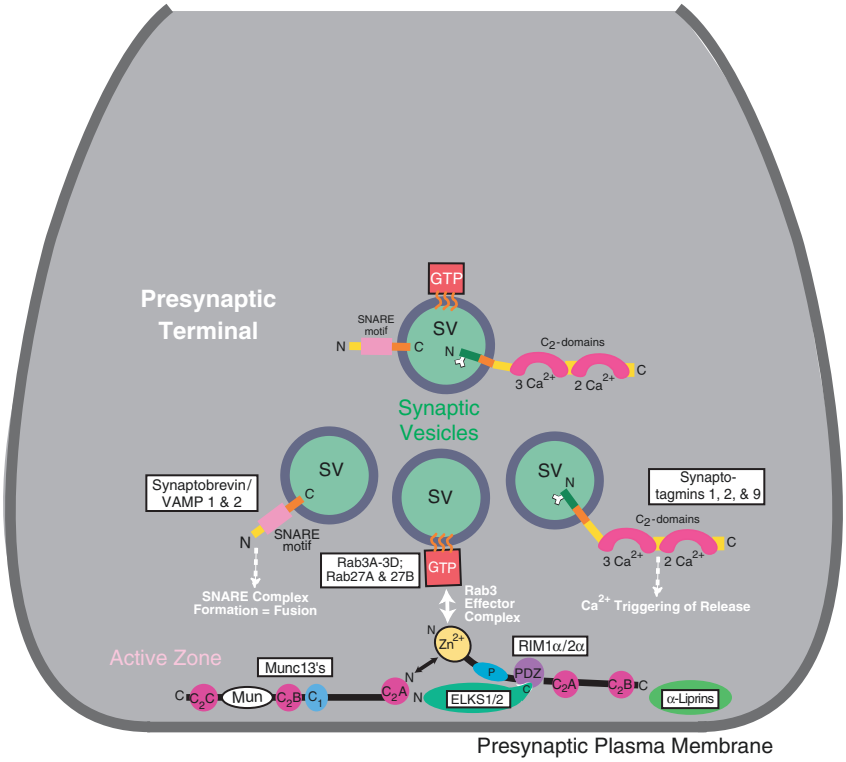

Fig. 3 Interaction of Rab3 and Rab27 on synaptic vesicles with the active zone protein complex containing Munc13s, RIMs, ELKS, and liprins. The schematic drawing depicts a nerve terminal with a few synaptic vesicles containing the three vesicle proteins that mediate exocytosis: the SNARE protein synaptobrevin/VAMP that participates in fusion (see Figure 4), the Rab-proteins Rab3 and Rab27 that attach synaptic vesicles to the active zone protein complex as shown, and the Ca^{2+}-sensor protein synaptotagmin that translates the Ca^{2+}-signal into release (Figure 5). The active zone protein complex is composed of Munc13, RIM, ELKS, and liprins, so that RIM binds to all of the three other active zone proteins, and additionally interacts with Rab3/27 via its N-terminal domain. The active zone protein complex likely contains other protein components that are not shown, in particular piccolo/bassoon. (Modified from Südhof, 2004).

(Hanson et al., 1997; Weber et al., 1998), formation of a "trans-complex" by SNARE proteins on opposing membranes forces these membranes together, thereby overcoming the energy barrier (Figure 4). SNARE proteins contain a characteristic 60-residue sequence, the so-called SNARE motif. SNARE complexes are assembled from four types of SNARE motifs (called R, Qa, Qb, and Qc, classified based on sequence homologies and the central residue) that fold into a tight four-helical bundle which always contains one copy for each type of SNARE motif. The close approximation of two membranes by SNARE-complex assembly destabilizes their negatively charged surfaces, thereby initiating the intermixing of their hydrophobic lipid interiors. This is thought to provide the energy for membrane fusion.

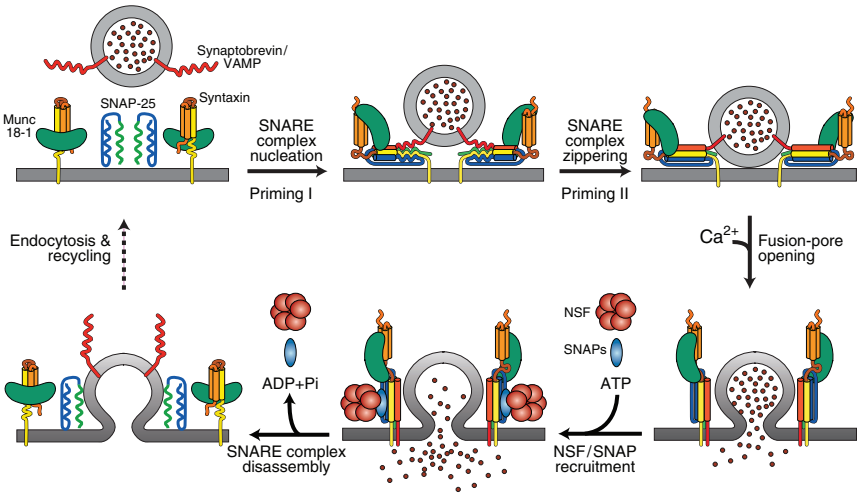

Fig. 4 Schematic diagram of the SNARE protein/Munc18 cycle. Docked synaptic vesicles (top left) may be attached to the active zone via the Rab/RIM interaction (see Figure 3) but contain SNARE proteins that have not yet formed a complex with each other (synaptobrevin/VAMP on synaptic vesicles and SNAP-25 and syntaxin-1 on the plasma membrane; note that syntaxin-1 is thought to be complexed to the SM-protein Munc18-1). Priming is envisioned to occur in two steps that involve the successive assembly of SNARE-complexes (priming I and II). During priming, Munc18-1 is thought to be continuously associated with syntaxin-1, shifting from a heterodimeric binding mode in which it was attached to syntaxin-1 alone to a heteromultimeric binding mode in which it is attached to the entire SNARE complex (top right). After priming, Ca^{2+} triggers fusion-pore opening to release the neurotransmitters by binding to synaptotagmin (see Figure 5). After fusion-pore opening, SNAPs (no relation to SNAP-25) and NSF (an ATPase) bind to the assembled SNARE complexes, disassemble them with ATP-hydrolysis, thereby allowing synaptic vesicles to undergo re-endocytosis and to recycle with synaptobrevin on the vesicle, while leaving SNAP-25 and syntaxin-1/Munc18-1 on the plasma membrane. Note that the overall effect is that SNARE/Munc18-proteins undergo a cycle of association/dissociation that fuels the membrane fusion reaction which underlies release. (Modified from Rizo and Südhof, 2002).

Synaptic exocytosis involves three SNARE proteins: the R-SNARE synaptobrevin/VAMP (isoforms 1 and 2) on the vesicle, and the Q-SNAREs syntaxin (isoforms 1 and 2) and SNAP-25 on the plasma membrane (Figure 4). Since SNAP-25 has two SNARE-motifs, synaptobrevin, syntaxin, and SNAP-25 together have four SNARE-motifs. Synaptobrevins and SNAP-25 are relatively simple SNARE proteins that are composed of little else besides SNARE motifs and membrane-attachment sequences (a transmembrane region for synaptobrevin, and a cysteine-rich palmitoylated sequence for SNAP-25). Syntaxins, in contrast, are complex proteins. The N-terminal two-thirds of syntaxins include a separate, autonomously folded domain (the so-called H_{abc}-domain), while the C-terminal third is composed of a SNARE motif and transmembrane region just like synaptobrevin.

3.3 SM Proteins

Genes for SM-proteins were discovered in genetic screens in *C. elegans* (unc18) and yeast (sec1), and their connection to membrane fusion was identified when the SM-protein Munc18-1 was found to directly bind to syntaxin-1 (Brenner, 1974; Novick et al., 1980; Hata et al., 1993). SM-proteins are composed of a conserved ∼600 amino acid sequence that folds into an arch-shaped structure. With seven members in mammals and four in yeast, SM-proteins constitute a small family of highly homologous proteins. SM proteins have essential roles in all fusion reactions tested. Three SM proteins (Munc18-1, −2, and −3) are involved in exocytosis, where they are at least as essential as SNARE proteins. For example, deletion of Munc18-1 in mice has more severe consequences for synaptic vesicle exocytosis than deletion of synaptobrevin or SNAP-25 (Verhage et al., 2000).

Initially, Munc18-1 was found to bind only to monomeric syntaxin-1 in a manner that is incompatible with SNARE-complex formation. Puzzlingly, however, other SM proteins were subsequently found to bind to assembled SNARE complexes. This puzzle was resolved with the discovery that Munc18-1 (and presumably −2) participates in two distinct modes of SNARE interactions: the originally defined binding to monomeric syntaxins, and a novel mode of direct binding to assembled SNARE complexes (Dulubova et al., 2007; Shen et al., 2007). These results suggested that all SM-proteins directly or indirectly interact with assembled SNARE complexes in fusion. The additional binding of Munc18-1 to the closed conformation of syntaxin prior to SNARE complex formation renders Ca^{2+}-triggered exocytosis unique among fusion reactions, possibly in order to achieve a tighter control of the fusion reaction.

3.4 Mechanism of SNARE and SM Protein Catalyzed Fusion

Both SNARE and SM proteins are required as components of the minimal fusion machinery. At the synapse, for example, deletion of Munc18-1 leads to a loss of all synaptic vesicle fusion, revealing Munc18-1 as an essential component of the fusion machine (Verhage et al., 2000). It is likely that SNARE proteins first force membranes together by forming trans-complexes, thereby creating a fusion intermediate that at least for synaptic vesicles appears to consist of a hemifusion stalk (Figure 4). Since the unifying property of SM proteins is to bind to assembled SNARE complexes, they likely act after such a fusion intermediate has formed, but their exact role remains unknown.

Each intracellular fusion reaction exhibits characteristic properties, and involves a different combination of SM and SNARE proteins. The specificity of fusion reactions appears to be independent of SNARE proteins because SNARE complex formation is nonspecific as long as the Q/R-rule is not violated (i.e., the fact that SNARE complexes need to be formed by SNARE proteins containing R-, Qa-, Qb-, and Qc-SNARE motifs), and of SM proteins because SM proteins often function in

multiple fusion reactions. Fusion specificity must be determined by other mechanisms, possibly GTP-binding proteins of the rab family.

4 Mechanism of Ca^{2+}-Triggering: Ca^{2+}-Channels, Ca^{2+}-Buffering, and Synaptotagmin

Neurotransmitter release is triggered by Ca^{2+} when an action potential invades the nerve terminal and gates the opening of voltage-sensitive Ca^{2+}-channels. Thus, there are two determinants of neurotransmitter release: (1) The Ca^{2+}-dynamics in the nerve terminal that are dictated by the properties and location of the Ca^{2+}-channels; the concentration, affinities, and kinetics of local Ca^{2+}-buffers; and the Ca^{2+}-extrusion mechanisms and (2) the action of the Ca^{2+}-receptors that translate the Ca^{2+}-signal into release, with most release being mediated by Ca^{2+}-binding to synaptotagmins (see below).

4.1 Ca^{2+}*-Dynamics*

The Ca^{2+}-concentration in a nerve terminal depends on the number and temporal pattern of action potentials, the effectiveness of these action potentials to open Ca^{2+}-channels, and the properties and concentrations of Ca^{2+}-buffers. Not only the time course of changes in Ca^{2+}-concentrations, but also the spatial distribution of Ca^{2+}, is important because Ca^{2+} is not uniformly distributed in a nerve terminal. Moreover, the Ca^{2+}-dynamics of a nerve terminal differ between nerve terminals, and play a central role in synaptic plasticity (e.g., see Rozov et al., 2001; Zucker and Regehr, 2002).

Ca^{2+}-channels are well investigated, have proven to be great drug targets, and will be discussed at length in the chapter by Kisilevsky and Zamponi. Two types of Ca^{2+}-channels, the so-called P/Q- and N-type channels (referred to as Cav2.1 and 2.2) account for the vast majority of releases. These Ca^{2+}-channels are located in the active zone of the presynaptic terminal (Llinas et al., 1992), although their precise location is unknown. Ca^{2+}-channels are – not surprisingly – tightly regulated by several signaling systems. As a result of their non-uniform localization and their stringent regulation, the Ca^{2+}-signal produced by the opening of Ca^{2+}-channels by a given action potential cannot be predicted, but varies greatly between synapses in amplitude, space and time (Rozov et al., 2001). This variation is increased by differences in Ca^{2+}-buffering between synapses. Ca^{2+}-buffers are much less understood than Ca^{2+}-channels because of the large number of different types of buffers, the difficulty in manipulating them pharmacologically or genetically, and the problems in measuring them. The most important nerve terminal Ca^{2+}-buffer likely is ATP, which has a relatively low Ca^{2+}-affinity but a high concentration and is highly mobile, rendering it an effective buffer at peak Ca^{2+}-concentrations (Meinrenken et al.,

2003). In addition, a number of Ca^{2+}-binding proteins of the EF-hand class probably function as nerve terminal Ca^{2+}-buffers, including parvalbumin and calbindin. Interestingly, these proteins exhibit a highly selective distribution in the brain, with inhibitory interneurons generally expressing much higher concentrations of selected Ca^{2+}-buffer proteins than excitatory neurons, a difference that may account for the distinct short-term plasticity properties of GABA release (Silberberg et al., 2005). Ca^{2+}-extrusion, finally, is mediated by multiple mechanisms. Although uptake of nerve terminal Ca^{2+} into mitochondria and endoplasmic reticulum has been widely discussed, the most important step is the transport of Ca^{2+}-ions from the cytosol into the extracellular space against a vast concentration gradient. Several mechanisms for this transport exist, of which the Na^{+}/Ca^{2+}-exchanger and the plasma membrane Ca^{2+}-ATPase appear to be the most important (Meinrenken et al., 2003).

4.2 Synaptotagmins as Ca^{2+}-Sensors for Fast Neurotransmitter Release

At resting Ca^{2+}-concentrations, release occurs spontaneously at a low rate; Ca^{2+} increases this rate $>10,000$-fold in less than a millisecond, faster than most diffusion-controlled chemical reactions. Ca^{2+} thus increases the probability of a rare event that normally occurs all the time. Studies in the Calyx of Held synapse – a large vertebrate synapse that allows simultaneous recordings from pre- and postsynaptic neurons – showed that Ca^{2+} triggers release at micromolar concentrations with an apparent cooperativity of 5 (Meinrenken et al., 2003). For each active zone, the Ca^{2+}-signal produced by an action potential has a finite probability of triggering exocytosis (usually between 0.05 and 0.30) that changes as a function of the previous use of a synapse (i.e., is subject to plasticity), and additionally depends on signaling mediated by neuromodulators. In addition to this fast synchronous type of neurotransmitter release, synapses exhibit a second slower, more asynchronous type of release that is also triggered by Ca^{2+}. Both types are likely important, but under most physiological conditions the fast synchronous type predominates.

The speed of Ca^{2+}-triggered exocytosis suggests that Ca^{2+} acts at a stage at which part of the fusion reaction has already been completed, most likely on vesicles in a hemifusion state that is presumably created by the combined action of SNARE proteins and Munc18-1 (i.e., after priming II in Figure 4). Much of synaptic vesicle exocytosis likely operates in the "kiss-and-run" mode (Harata et al., 2006). Kiss-and-run exocytosis predominates during low-frequency stimulation, while full exocytosis is more important during high-frequency stimulus trains. The kiss-and-run mode allows fast recycling of synaptic vesicles for reuse after exocytosis, but probably has only a minimal effect on the kinetics or amount of release because the total size of synaptic vesicles ($\sim$20 nm radius) is only 5- to 10-fold larger than that of the fusion pore.

The definition of the primary structure of synaptotagmin-1, composed of an N-terminal transmembrane region and two C-terminal C_2-domains (Figure 3), led

to the hypothesis that synaptotagmin-1 functions as the Ca^{2+}-sensor for fast neurotransmitter release (Perin et al., 1990). Subsequent studies revealed that synaptotagmin-1 and two other synaptotagmin isoforms, synaptotagmin-2 and -9, act as Ca^{2+}-sensors in exocytosis by a strikingly simple mechanism: Ca^{2+} flowing into the nerve terminal when Ca^{2+}-channels open during an action potential binds to the two C_2-domains of synaptotagmins (Xu et al., 2007). Ca^{2+}-binding to the C_2-domains induces the interaction of synaptotagmins with phospholipids and with SNARE proteins with a micromolar apparent Ca^{2+}-affinity, consistent with the affinity of release, which in turn opens the fusion pore mechanically by pulling the SNARE complexes apart (Südhof, 2004). This model was supported by a host of biochemical and genetic experiments, most importantly the finding that point mutations in the endogenous synaptotagmin gene in mice that alter the apparent Ca^{2+}-affinity of synaptotagmin also alter the apparent Ca^{2+}-affinity of release in an identical manner (Fernandez-Chacon et al., 2001). However, these findings also raised a crucial question that is only now beginning to be answered: Why doesn't the fusion pore just pop open after priming II (Figure 4)? Recent results suggest that another nerve-terminal protein, a soluble protein called complexin, plays a central role here (Giraudo et al., 2006; Tang et al., 2006). Complexins bind in a Ca^{2+}-independent manner only to the C-terminal part of assembled SNARE complexes where the SNARE complexes are anchored in the membrane (Figure 5). Deletion of complexins in mice revealed a selective impairment of fast synchronous Ca^{2+}-triggered release, whereas other forms of fusion were unchanged (Reim et al., 2001). This phenotype resembles the synaptotagmin-1 knockout phenotype in its selectivity, but was milder because the complexin knockout phenotype can be rescued by boosting Ca^{2+}-influx into the nerve terminal, whereas the synaptotagmin-1 knockout phenotype cannot. Thus complexins function as activators of SNARE complexes for subsequent synaptotagmin-1 action. The mechanism by which the functions of synaptotagmin-1 and complexins interact was revealed in biochemical experiments demonstrating that Ca^{2+}-induced binding of synaptotagmin-1 to SNARE complexes displaces complexin from the SNARE complexes (Tang et al., 2006), indicating that synaptotagmin-1 serves as the trigger of a complexin-cocked gun.

These observations suggest a model for how synaptotagmin-1 triggers exocytosis (Figure 5): By binding to SNARE complexes during assembly, complexins force completion of SNARE-complex assembly that creates an activated, frozen fusion intermediate, likely a hemifusion state. When Ca^{2+} binds to synaptotagmin, this induces binding of synaptotagmin to both fusing membranes and to the SNARE complexes. We envision that this binding pulls open the fusion pore by two independent mechanisms: a mechanical force on the membrane by coupling phospholipids to SNARE complexes via the simultaneous binding, and a disinhibition of fusion-pore opening by displacing from the SNARE complexes the complexins that at the same activate and stabilize the complexes. Note that the complexin/synaptotagmin-dependent fusion reaction mediates fast synchronous release, and is distinct from the second type of release, slower asynchronous release which also depends on SNARE-proteins and Munc18-1 but is independent of complexins and synaptotagmin.

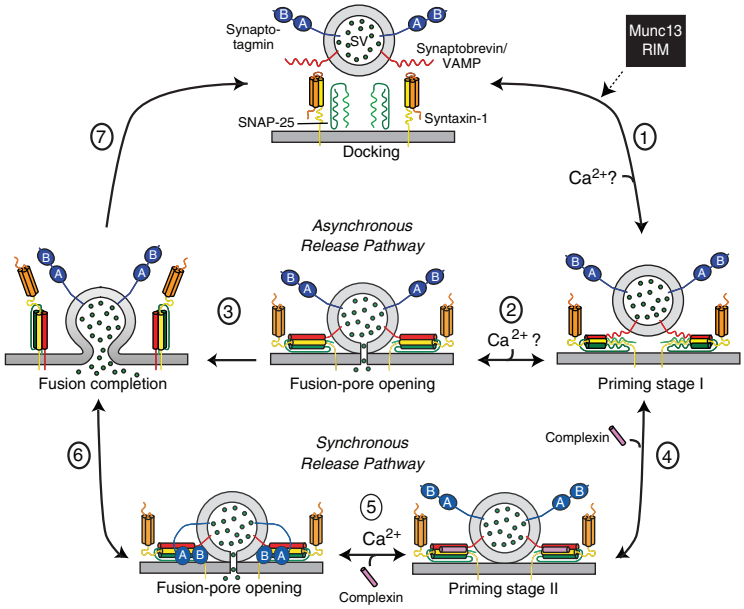

Fig. 5 Model for the interplay between SNARE, complexin, and synaptotagmin function in Ca^{2+}-triggered neurotransmitter release. Docked vesicles containing unassembled SNARE complexes (top) are attached to the active zone via the Rab3/27 interaction with RIMs (Figure 3), and are primed in a two-stage reaction by SNARE-complex assembly (step 1; see also Figure 4). The resulting primed vesicles form the substrate for two release pathways: asynchronous release that is synaptotagmin- and complexin-independent (steps 2 and 3), and synchronous release that depends on synaptotagmin and complexins (steps 4–6). Note that quantitatively, the asynchronous pathway is less important for release than the synchronous pathway which mediates >90% of all release under most conditions. Asynchronous release is triggered by Ca^{2+} by a poorly understood mechanism that, like synchronous release, requires assembly of SNARE complexes, but differs from synchronous release in that it does not appear to stop fusion-pore opening by a "clamp" after SNARE complexes are fully assembled. Asynchronous release becomes important when the Ca^{2+}-concentration rises for prolonged time periods to intermediate levels (0.1–1.0 µM) that are too low to trigger synchronous release, but sufficient to trigger asynchronous release. This occurs, for example, during the accumulation of residual Ca^{2+} during high-frequency stimulus trains (Zucker and Regehr, 2002). Synchronous release involves "superpriming" of synaptic vesicles by binding of complexins to assembled SNARE complexes (step 4). Complexin binding activates and freezes SNARE complexes in a metastable state (priming stage II). Superprimed vesicles in which the SNARE complexes have been clamped by complexin are then substrate for fast Ca^{2+}-triggering of release when Ca^{2+}-binding to synaptotagmin triggers the simultaneous interaction of synaptotagmin with phospholipids and SNARE complexes, with the latter reaction displacing complexin and resulting in fusion-pore opening (step 5). Opened fusion pores can then dilate to complete fusion (step 6), although both steps 2 and 5 are potentially reversible, i.e., lack of dilation of the fusion pore could lead to "kiss-and-stay" or "kiss-and-run" exocytosis in these pathways. Note that steps 1 and 4 are also probably reversible, with a much faster forward than backward speed. It is likely that step 1 is Ca^{2+}-dependent, but it is unclear whether or not step 2 is Ca^{2+}-dependent, since it is possible that asynchronous release is Ca^{2+}-dependent solely because Ca^{2+} accelerates step 1, and step 2 has a finite probability. Thus the nature of Ca^{2+}-triggering of asynchronous release could operate either at the priming or at the actual fusion step. Note that the function of Munc18-1 is not included in this diagram, but is thought to be central to the actual fusion reaction (see Figure 4). (Modified from Tang et al., 2006).

In addition to functioning as Ca^{2+}-sensors for vesicle exocytosis, synaptotagmins may be involved in vesicle endocytosis, particularly the decision between kiss-and-run versus full exocytosis. Such a role would be economical in linking fusion-pore opening (which is triggered by Ca^{2+}-binding to synaptotagmin) to fusion-pore expansion or contraction, but the precise mechanisms involved have not yet been explored.

As mentioned above, three different synaptotagmin isoforms mediate fast synchronous release: synaptotagmin-1, -2, and -9. Why does the vertebrate brain express three different proteins to do the same job? As it turns out, these three different synaptotagmins mediate the Ca^{2+}-triggering of release with distinct properties, and are differentially distributed (Xu et al., 2007). Of the three synaptotagmins, synaptotagmin-2 is the fastest, and synaptotagmin-9 the slowest, corresponding with the selective presence of synaptotagmin-2 in the calyx synapse, one of the fastest synapses in brain involved in sound localization, and synaptotagmin-9 in the limbic system involved in emotional reactions. Thus, the properties of the synapses formed by a neuron depend among others on the isoform of synaptotagmin which that neuron expresses, adding additional complexity to the neural circuits formed by synaptic networks.

5 Regulation of Release Beyond Ca^{2+}-Triggering

We already alluded to the fact that release is not constant, but is highly plastic, i.e., regulated by extrinsic signals and by the intrinsic previous activity of a nerve terminal. Both forms of regulation constitute a type of synaptic memory: the history of the activity of other surrounding neurons and of the neuron to which a terminal belongs strongly influence to what extent this terminal translates an action potential into a neurotransmitter release signal (Zucker and Regehr, 2002). Many different forms of synaptic plasticity exist. Although traditionally postsynaptic forms of plasticity have been appreciated more than presynaptic forms, recent studies have revealed the existence of purely presynaptic forms of synaptic plasticity, and have moreover shown that many forms of plasticity are mediated by the combined action of pre- and postsynaptic mechanisms.

A complete discussion of presynaptic modulation and plasticity of release is beyond the scope of this chapter. Instead, three exemplary forms of presynaptic plasticity will be discussed because of their importance for the pharmacology of neurotransmitter release.

5.1 Acetylcholine-Receptor-Mediated Ca^{2+}*-Influx into Presynaptic Nerve Terminals*

Nicotine is an addictive drug that activates a diverse subset of ionotropic acetylcholine receptors. Most cholinergic actions in brain, both by ionotropic and metabotropic receptors, are modulatory, and very few fast synapses exist that utilize

acetylcholine as a transmitter. Interestingly, although some ionotropic acetylcholine receptors are postsynaptic, a relatively large proportion, much larger than observed for other neurotransmitters, appears to be presynaptic. A possible pathway accounting for the addictive actions of nicotine was discovered in the observation that presynaptic nicotinic acetylcholine receptors, when activated, mediate the influx of Ca^{2+} into presynaptic terminals, and thereby stimulate the release of neurotransmitters (Wannacott, 1997). Interestingly, this effect appears to operate via a class of nicotinic acetylcholine receptors containing α6 subunits that are relatively enriched in the nigrostriatal pathway, suggesting that nicotine may be addictive by increasing dopamine release (Quik and McIntosh, 2006). Thus the presynaptic facilitation of neurotransmitter release by cholinergic nicotinic receptors is one of the best and physiologically most important systems illustrating how presynaptically acting receptors can regulate release.

5.2 Ca^{2+}-Channel Modulation by Presynaptic Receptors

Presynaptic G-protein coupled receptors for a large number of neurotransmitters, both autoreceptors and receptors for extrinsic signals, suppress Ca^{2+}-channel gating in response to an action potential. This mechanism of action appears to be the dominant mechanism involved in short-term plasticity mediated by presynaptic receptors. A typical example is depolarization-induced suppression of inhibition (DSI), which is the short-term suppression of presynaptic GABA-release induced by the depolarization of the postsynaptic cell (Diana and Marty, 2004). DSI is caused when the postsynaptic depolarization causes the release of endocannabinoids from the postsynaptic cell, and the endocannabinoids then bind to presynaptic CB1 receptors whose activation suppresses presynaptic Ca^{2+}-channels. Like many other forms of presynaptic suppression mediated by activation of presynaptic receptors, this effect is short-lasting (in the millisecond range). The precise mechanisms by which Ca^{2+}-channels are suppressed appear to vary between receptors, but the outcome is always a very effective short-term decrease in synaptic signaling.

5.3 Presynaptic Long-Term Plasticity Mediated by cAMP-Dependent Protein Kinase A (PKA)

PKA-dependent long-term potentiation and depression was initially discovered in the mossy-fiber synapses of the hippocampus, and later demonstrated in parallel fiber synapses of the cerebellum and corticothalamic synapses of the forebrain (Malenka and Siegelbaum, 2001). This widespread form of plasticity does not involve changes in Ca^{2+}-influx, but operates via a direct increase or decrease, respectively, of the amount of vesicle exocytosis that can be triggered by a given Ca^{2+}-signal. Interestingly, this form of plasticity appears to depend on the interaction

of the synaptic vesicle GTP-binding protein Rab3 with its effectors, RIM-proteins (Figure 3), since deletion of either Rab3A or of RIM1α abolishes LTP in the hippocampal mossy fibers (Castillo et al., 2002). Moreover, recently it was revealed that a special form of endocannabinoid-dependent long-term depression of GABAergic synapses in the hippocampus also operates as a presynaptic process that requires PKA activation and RIM1α. This form of LTD, referred to as i-LTD for inhibitory LTD, is distinct from the DSI discussed above; indeed, DSI is normal in mice lacking RIM1α. Thus, endocannabinoids can trigger two different forms of plasticity depending on the precise conditions of the postsynaptic depolarization. Overall, these results highlight the role of the core release machinery in presynaptic long-term plasticity.

6 Ca^{2+}-Induced Exocytosis of Small Dense-Core Vesicles and LDCVs

Biogenic amines are generally secreted from small dense-core vesicles (SDCVs) in varicosities of axons that are not associated with classical synapses, and additionally from LDCVs, whereas neuropeptides are secreted – usually together with biogenic amines – only from LDCVs. Different from synaptic vesicle exocytosis where Ca^{2+} is thought to trigger fusion of predocked and primed vesicles, a significant part of SDCV and LDCV exocytosis involves the Ca^{2+}-dependent mobilization of vesicles before Ca^{2+}-triggered fusion. As a result, dense-core vesicle exocytosis requires more sustained Ca^{2+}-transients, but is also slower and longer lasting than synaptic vesicle exocytosis (Stjarne, 2000).

Ca^{2+}-triggered exocytosis of SDCVs, LDCVs, and their endocrine equivalents has been examined in several systems (e.g., chromaffin cells, cultured melanotrophic neurons, pancreatic β-cells, and PC12 cells), but the best-studied system is that of chromaffin cells because they allow the highest measurement resolution, and are the only system that has been investigated extensively by a loss-of-function approach. Ca^{2+}-triggered chromaffin granule exocytosis is ∼10-fold slower than synaptic vesicle exocytosis, but otherwise the two systems are similar. Chromaffin granule exocytosis, like synaptic vesicle exocytosis, involves SNARE and Munc18-proteins. Knockout experiments revealed, however, that Ca^{2+}-triggering of chromaffin granule and synaptic vesicle exocytosis are mechanistically distinct. Specifically, chromaffin cell exocytosis is mediated by two different synaptotagmins: synaptotagmin-1, which is shared with synaptic vesicle exocytosis, and synaptotagmin-7, which does not function in synaptic vesicle exocytosis. The synaptotagmin-7 selectively functions as a major Ca^{2+}-sensor for chromaffin granule but not for synaptic vesicle exocytosis, indicating a fundamental difference between the two types of exocytosis. However, beyond this difference, little is known about the other molecular components that direct SDCV and LDCV exocytosis and make it different from synaptic vesicle exocytosis, a question that is of obvious importance in understanding the release of biogenic amines and neuropeptides in brain.

7 Presynaptic Drug Targets

This introductory chapter discussed the fundamental mechanisms of release because understanding these fundamental mechanisms is crucial for insight into the actions of drugs that act on release. Overall, the neurotransmitter release machinery is a poor drug target, as judged by the number of successful drugs that act on it, and in contrast to the neurotransmitter reception machinery (i.e., neurotransmitter receptors) that is the target of many different drugs. In fact, the quantitatively largest number of drugs that influence neurotransmitter release act on presynaptic neurotransmitter receptors. Nevertheless, several well-established drugs exist that act on the release machinery: reserpine on the vesicular catecholamine transporter, leviracetam on the vesicle protein SV2, and cocaine on the presynaptic re-uptake of dopamine.

It is clear that the presynaptic machinery is an underutilized target for drugs. The actual membrane-trafficking components of that machinery, such as the SNARE, SM, or Rab-proteins or synaptotagmins, are probably not good drug targets because they operate via protein-protein interactions that are difficult to influence with small molecules. However, several synaptic proteins with receptor, transport, or enzyme functions are likely drug targets, but have not yet been explored. These proteins include synapsins, which are ATP-binding proteins (Hosaka and Südhof, 1998), CSPα as a synaptic vesicle co-chaperone (Tobaben et al., 2001), and vesicular GABA- and glutamate transporter (Gasnier, 2000; Takamori, 2006). We hope that the material presented in this book will not only help understand the actions of current drugs, but also stimulate the development of new ones.

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Pharmacology of Neurotransmitter Release: Measuring Exocytosis

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Abstract Neurotransmission in the nervous system is initiated at presynaptic terminals by fusion of synaptic vesicles with the plasma membrane and subsequent exocytic release of chemical transmitters. Currently, there are multiple methods to detect neurotransmitter release from nerve terminals, each with their own particular advantages and disadvantages. For instance, most commonly employed methods monitor actions of released chemical substances on postsynaptic receptors or artificial substrates such as carbon fibers. These methods are closest to the physiological setting because they have a rapid time resolution and they measure the action of the endogenous neurotransmitters rather than the signals emitted by exogenous probes. However, postsynaptic receptors only indirectly report neurotransmitter release in a form modified by the properties of receptors themselves, which are often nonlinear detectors of released substances. Alternatively, released chemical substances

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can be detected biochemically, albeit on a time scale slower than electrophysiological methods. In addition, in certain preparations, where presynaptic terminals are accessible to whole cell recording electrodes, fusion of vesicles with the plasma membrane can be monitored using capacitance measurements. In the last decade, in addition to electrophysiological and biochemical methods, several fluorescence imaging modalities have been introduced which report synaptic vesicle fusion, endocytosis, and recycling. These methods either take advantage of styryl dyes that can be loaded into recycling vesicles or exogenous expression of synaptic vesicle proteins tagged with a pH-sensitive GFP variant at regions facing the vesicle lumen. In this chapter, we will provide an overview of these methods with particular emphasis on their relative strengths and weaknesses and discuss the types of information one can obtain from them.

1 Electrophysiological Detection of Secretion

1.1 Electrical Detection of Neurotransmitter Release Using Postsynaptic Ionotropic Receptors

During fast chemical neurotransmission, released neurotransmitter substances cross the synaptic cleft and activate a set of neurotransmitter receptors on the postsynaptic side (Figure 1). These neurotransmitter receptors are ligand-gated ion channels that are activated rapidly within milliseconds upon neurotransmitter binding. Ion fluxes through neurotransmitter-gated channels in turn can be measured electrically via whole cell recording from the somata or dendrites of postsynaptic neurons (Hamill et al., 1981; Stuart et al., 1993). This electrophysiological method is commonly used to detect synaptic transmission and estimate the properties of neurotransmitter exocytosis. The principal advantage of this method is its direct physiological relevance. The main impact of fast neurotransmitter release is to cause electrical signaling in the postsynaptic neuron and in the case of Ca^{2+} permeable channels such as NMDA receptors to activate second messenger signaling cascades. Therefore, measuring neurotransmitter release by postsynaptic recordings provides a direct readout of its impact on postsynaptic signaling.

The second advantage of this method is its rapidity; electrical signals can be detected reliably with a millisecond time resolution. Furthermore, these whole cell recordings can be performed on any cell type or neuronal process in culture, in brain slices as well as in vivo (Margrie et al., 2002).

Dissociated neuronal cultures provide a versatile system for analysis of mechanisms underlying neurotransmitter release. These cultures can be prepared from fetal or postnatal brain tissue. This preparation has been particularly instrumental in analysis of synapses deficient in key components of the release machinery. For instance, genetic deletion of synaptic SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins such as synaptobrevin-2 and SNAP-25

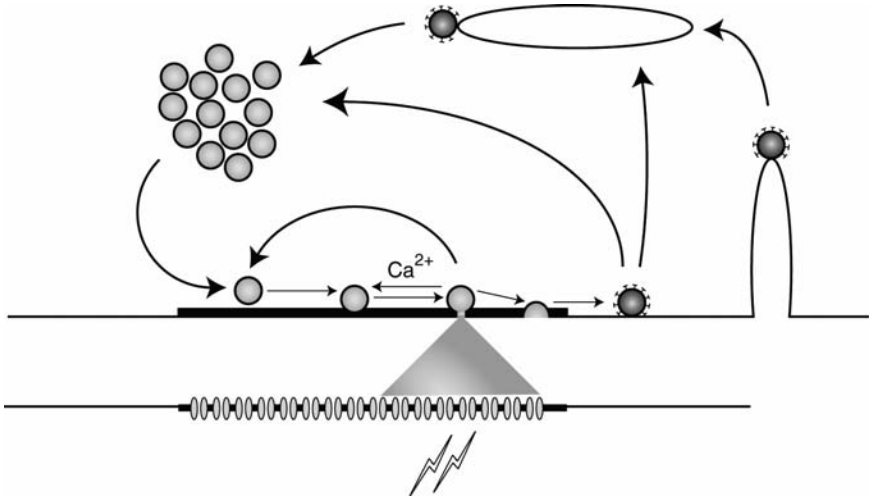

Fig. 1 The synaptic vesicle cycle. During neurotransmission a rise in Ca^{2+} level leads to vesicle fusion and neurotransmitter release at the active zone, after which vesicles completely collapse onto the plasma membrane. During this process vesicle membrane components are thought to intermix with their plasma membrane counterparts. Subsequently clathrin, through its adaptor proteins, is recruited to the membrane (typically at the periphery of the active zone) and forms coated vesicles by reclustering vesicle membrane components, which eventually bud off the plasma membrane with the help of the GTPase dynamin. Besides the plasma membrane, endocytosis of clathrin-coated vesicles may also occur from membrane infoldings or endosomal cisternae. This classical pathway is thought to operate within 40 and 90 seconds time scale. During an alternative fast (within seconds) pathway of vesicle recycling, synaptic vesicles retain their identity and do not intermix with the plasma membrane or endosomal compartments. This pathway is also referred to as "kiss and run" pathway in which the fusion pore opens transiently without complete collapse of the vesicle and without intermixing of synaptic vesicle membrane components with the plasma membrane. Most commonly used methods to study neurotransmitter release either measure the action of released neurotransmitters or they use exogenous fluorescent probes to monitor membrane and protein turnover in the presynaptic terminal.

lead to lethality at birth, therefore analysis was performed in neuronal cultures obtained from fetal brains (Schoch et al., 2001; Tafoya et al., 2006). In the absence of systemic effects arising from the loss of these proteins essential to neurotransmitter secretion, mutant synapses formed in these cultures readily attain morphological maturity. A critical advantage of neurotransmitter release measurements in dissociated neuronal cultures is their high sensitivity for detecting very low probability fusion events. Other major advantages of synaptic preparations include triggering fusion using non-Ca^{2+}-dependent means of release, such as hypertonicity or alpha-latrotoxin. Such approaches are instrumental for identifying selective effects of mutants on Ca^{2+} sensitivity of fusion. A form of dissociated neuronal cultures can be prepared by low-density plating of neurons on isolated glial islands that gives rise single neurons forming synapses onto themselves that are called "autapses." Use of autaptic cultures substantially simplifies the whole cell recording configuration by

enabling a single electrode to perform stimulation and recording at the same time. However, local stimulation methods applied to nonautaptic neuronal cultures also bring in several advantages (Maximov et al., 2007).

A key advantage of dissociated neuronal cultures is their amenability to molecular manipulations either through high-efficiency transfections using the calcium phosphate technique (Virmani et al., 2003) or via viral gene delivery, especially by lentiviral infections (Deak et al., 2006). Low-efficiency transfection methods can be useful to assess alterations in postsynaptic parameters after gene delivery to a target neuron. However, when presynaptic properties need to be assessed, then a high-efficiency transfection setting provides a significant advantage, as in this setting nearly all neurons in a culture are transformed and thus all nerve terminals synapsing onto a given target neuron can carry the exogenous protein of interest. Comparison of high-efficiency versus low-efficiency transfections, therefore, can help distinguish cell-autonomous versus non-cell-autonomous effects of a particular gene product (Burrone et al., 2002). Furthermore, this setting may help to sort out pre-versus postsynaptic effects of a particular protein, which are typically hard to deduce from autaptic cultures, as the protein of interest can be present in both pre- and postsynaptic compartments of a given cell (Nelson et al., 2006).

1.2 Detection of Probability of Neurotransmitter Release

During a whole-cell voltage clamp recording experiment an alteration in synaptic efficacy can be detected as an increase or a decrease in the postsynaptic current triggered in response to a presynaptic action potential. However, this change in postsynaptic response may arise from multiple sources. There can be an increase in the number of active synapses or conversion of silent synapses to active ones. There may also be an alteration in the number or neurotransmitter sensitivities of postsynaptic receptors. All-or-none changes in synaptic strength as well as postsynaptic mechanisms typically scale synaptic responses without substantial alterations in their frequency-dependent characteristics. In contrast, an alteration in the probability of neurotransmitter release may result in the same outcome (i.e., an increase or a decrease in synaptic strength) but in turn can fundamentally alter synaptic responses to physiological trains of action potentials. A change in the probability of neurotransmitter release is the most common way neurotransmitter release kinetics are altered during synaptic plasticity. Such a change may arise from an alteration in the number of vesicles available for release or a modification in their fusion propensity in response to an action potential. These alterations can alter the rate of short-term synaptic plasticity. For instance, an increase in the probability of neurotransmitter release, in the absence of rapid replenishment of fused vesicles, may result in faster depletion of readily releasable vesicles and cause enhanced depression. A decrease in the release probability may make synapses less prone to vesicle depletion and tend to decrease the extent of depression. Therefore, systematic measurement of the

extent of synaptic depression induced by a train of action potentials at moderate to high frequency stimulation may yield critical information in regard to neurotransmitter release kinetics (Zucker and Regehr, 2002).

In addition to measuring the kinetics of short-term synaptic depression or facilitation from a population of synapses, one can also obtain a more direct measure of neurotransmitter release probability if vesicle fusion from a single release site can be detected. Such a setting can be achieved during paired recordings between sparsely connected neurons (albeit in a labor-intensive way), or using the minimal stimulation method via application of low-intensity stimulation to activate a single release site. Under these conditions one can typically observe a substantial amount of neurotransmission failures arising from the probabilistic nature of vesicle fusion in single synapses. Over a large number of independent trials, a decrease in the number of failures would suggest an increase in probability of release or vice versa.

Use-dependent block of NMDA receptors by MK-801 provides an additional method to estimate the probability neurotransmitter release. MK-801 is a well-characterized blocker of NMDA receptors that penetrates open NMDA channel pores and impairs the ion flux through NMDA receptors (Huettner and Bean, 1988). This highly selective open channel block renders MK-801-dependent inhibition of NMDA currents extremely use-dependent. Therefore, the time course of MK-801 block of synaptically evoked NMDA currents is proportional to the activation of NMDA receptors by vesicle fusion events, providing a measure of the probability of neurotransmitter release (Hessler et al., 1993; Rosenmund et al., 1993). However, recent studies suggest that NMDA receptors in given synapse may not be saturated by the glutamate released in response to a single action potential (Mainen et al., 1999; Oertner et al., 2002). This observation may complicate direct estimation of the absolute release probability. Nevertheless, this method may still allow a relative measure by comparing different experimental conditions as long as there are no alterations in the number of NMDA receptors and in their sensitivities to glutamate between the conditions in question.

As indicated above, detection of evoked quantal responses (either through minimal stimulation or paired recordings) provides a suitable setting to determine neurotransmitter release probability and alterations in rate of vesicle fusion. However, in synapses with multiple release sites, such as the calyx of Held, isolation of evoked quantal responses is nearly impossible and truly quantal release is hard to detect except in the case of spontaneous neurotransmission. Therefore, under these conditions, the rate of synaptic vesicle fusion can be determined by deconvolution of synaptic currents with the quantal unitary current. This approach is valid only when the synaptic current can be assumed to result from the convolution between a quantal current and quantal release rates. This assumption is not valid in cases where postsynaptic mechanisms, such as receptor saturation and desensitization, alter quantal events and thus shape synaptic responses during repetitive stimulation (Neher and Sakaba, 2001).

Despite its wide use and versatility, electrophysiological approaches also possess several caveats. First, this form of detection is limited to fast neurotransmitters that are released rapidly and activate closely juxtaposed ligand-gated channels.

Using this approach it is not possible to examine release kinetics of neuromodulatory transmitters such as dopamine or cathecholamines, as they typically activate G-protein-coupled receptors and only generate slow electrical responses that are not necessarily linearly proportional to release kinetics. Second, in the case of ionotropic receptors the reliance of this physiological setting on receptor properties such as receptor desensitization and saturation brings in a major caveat that hinders direct estimation of presynaptic release properties. Third, synaptic events, which occur at distal sites as well as on thin dendritic branches, are hard to detect due to dendritic filtering. Fourth, in most cases information is difficult to obtain from single release sites; therefore, experimental readout typically provides an analysis of a population of synapses. Lastly, electrical detection of neurotransmitter release is not inherently sensitive to use history of vesicles (i.e., synaptic vesicle recycling). However in cases when endocytosis is impaired (Delgado et al., 2000) or vesicle re-acidification is inhibited using blockers of vacuolar ATPase, electrophysiological readout of neurotransmitter release can provide information in regard to synaptic vesicle recycling (Ertunc et al., 2007).

1.3 Studying Synaptic Vesicle Recycling Using Electrophysiological Techniques

In order to determine the reliance of neurotransmission on synaptic vesicle endocytosis and recycling, one needs to estimate the time point when exocytosed vesicles become re-available for release. This parameter can be estimated from the kinetic difference between the rate of FM dye destaining (see section 2.2) and the time course of neurotransmitter release from a set of synapses. The rationale behind these experiments stems from previous observations that during stimulation, FM dyes, especially the least hydrophobic FM dye FM2-10, can be cleared out of a fused vesicle within a second by departitioning into solution (Ryan, 1996; Klingauf et al., 1998; Kavalali et al., 1999; Pyle et al., 2000), or within milliseconds by lateral diffusion in the neuronal membrane (Zenisek et al., 2002). These time frames are typically faster than the rate of fusion pore closure and endocytic retrieval. Therefore, under most circumstances recycled vesicles would not contain significant amounts of FM dye that could be detected as further destaining, whereas the same vesicles would be refilled with neurotransmitter following endocytosis that could give rise to further synaptic responses. This difference between the two reporters of vesicle mobilization should result in a deviation between the kinetics of FM dye destaining and neurotransmitter release at the time when recycled vesicles start to be reused. The method has been successfully applied to estimate the kinetics of synaptic vesicle recycling in several preparations, including the frog neuromuscular junction and dissociated hippocampal cultures (Betz and Bewick, 1992, 1993; Sara et al., 2002). However, applicability of this technique relies on two basic assumptions. First, loss of FM dye from vesicles upon fusion should be unrestricted leading to negligible dye retention. There are several lines of evidence that this may not be the case especially

for the more lipophillic dye FM1-43. Second, to estimate the time course of synaptic vesicle recycling from the kinetic comparison of dye release and postsynaptic electrical signals, we have to assume a linear relationship between the electrical and optical measures. This assumption may also be a problem under circumstances where relative occupancy of postsynaptic receptors changes during trains of action potentials (Ertunc et al., 2007). In the case of glutamatergic neurotransmission, this assumption can be tested by examining the extent of block in the presence of rapidly dissociating AMPA receptor antagonists kynurenate or γ-DGG. These compounds compete with the endogenous levels of glutamate (Diamond and Jahr, 1997), and therefore their effectiveness decreases as the amount of glutamate concentration in the synaptic cleft increases. Furthermore, FM dye release kinetics typically has a low time resolution $(\sim 1\,\mathrm{s})$, which may lead to underestimation of rapid alterations in neurotransmitter release kinetics.

An alternative method to estimate the time course of synaptic vesicle recycling takes advantage of the naturally occurring macrolide antibiotics such as bafilomycin A1 and folimycin (also called concanamycin A). These compounds can reduce synaptic output by selectively decreasing the neurotransmitter refilling (Roseth et al., 1995; Fonnum et al., 1998). This effect is attributed to their blockade of v-ATPase, which acidifies the lumen of recently endocytosed vesicles and generates an electrical and pH gradient (Moriyama and Futai, 1990). The neurotransmitter refilling process is maintained by transporters coupled to this proton pump to create the necessary driving force (Drose and Altendorf, 1997). After inhibition of this vesicular proton pump, endocytosed vesicles cannot be reloaded with neurotransmitter and therefore, when reused, these vesicles cannot evoke synaptic responses for a second time. In this way, postsynaptic recordings from synapses treated with proton pump inhibitors only exhibit responses originating from previously unused vesicles within the recycling pool. Although bafilomycin has recently been used in studies that investigate the synaptic vesicle endocytosis with optical pH sensitive probes (Sankaranarayanan and Ryan, 2001), folimycin's affinity $(\mathrm{Ki} = 20\,\mathrm{pM})$ for v-ATPase is 25-fold higher than that of bafilomycin $(\mathrm{Ki} = 500\,\mathrm{pM})$. This property of folimycin requires lower concentrations of vehicle (DMSO), which may have effects of its own at higher concentrations. This approach recently enabled examination of the kinetics of vesicle recycling in synapses onto CA1 pyramidal neurons using electrophysiological measures, which allows measurements at a milliseconds time scale (Ertunc et al., 2007).

1.4 Detection of Neurotransmitter Secretion Through Amperometric Recordings

As indicated in the previous sections, whole-cell recordings are widely used to detect neurotransmission in multiple settings; however, they possess some shortcomings, which hinder their applicability to all forms of neurotransmission. Whole-cell recordings can only be effective to detect fast neurotransmitters that are released

rapidly and activate closely juxtaposed ligand-gated channels. Using this approach it is not possible to examine release kinetics of neuromodulatory transmitters such as dopamine, serotonin, or cathecholamines as they typically activate G-protein coupled receptors and only generate slow electrical responses that are not linearly proportional to release kinetics. Furthermore, in the case of ionotropic receptors the reliance of this physiological setting on receptor properties such as receptor desensitization and saturation hinders direct estimation of presynaptic release properties.

The chemical structures of catecholamine or monoamine transmitters make them susceptible to rapid oxidation. These oxidation current signals can be detected using carbon fiber electrodes held at DC voltage above the redox potential of these neurotransmitters. This method proves an accurate depiction of vesicular neurotransmitter content free of nonlinear properties of postsynaptic ionotropic receptors (Bruns et al., 2000). Furthermore, detailed analysis of individual fusion event profiles can provide information in regard to diffusion kinetics of neurotransmitters out of fused vesicles (Chow et al., 1992). For instance, initial application of this method to catecholamine secretion of adrenal chromaffin cells revealed prominent slowly rising "foot" signals preceding the actual rapid rise of neurotransmitter concentration after fusion (Chow et al., 1992). The characteristics of this foot signal have been routinely analyzed in multiple settings to examine mechanisms underlying fusion pore expansion (Jackson and Chapman, 2006). In addition to amperometry, fast cyclic voltametric methods can be used to identify the released substances. In fast cyclic voltametry, a periodic voltage pattern is applied to the carbon electrode to extract the characteristic redox signatures of oxidizable substances in a solution. This method can be useful in distinguishing individual components within a mixture of neurotransmitters in cases where there is co-release of multiple oxidizable neurotransmitters (Zhou et al., 2005).

1.5 Detection of Membrane Fusion via Presynaptic Capacitance Measurements

Synaptic vesicles are membranous organelles, which release their neurotransmitter content after fusion with the plasma membrane. This process can be directly monitored via capacitance measurements. Alteration in total membrane capacitance can be used as a reporter of membrane fusion or endocytosis as long as the magnitude of the capacitance change is above the background noise. A critical requirement for employment of this method is direct electrical access to the secretory cellular component. This requirement significantly impairs the applicability of this method to small synapses of the central nervous system, which typically cover a $1\,\mu m^2$ surface area. Therefore, most capacitance measurements have been limited to secretion from cell bodies of mast cells, adrenal chromaffin cells, or large pituitary nerve terminals. Recent studies on a large presynaptic terminal, called the calyx of Held, located within the medial nucleus of the trapezoid body region of the brain stem, have employed capacitance measurements in examining the coupling between synaptic

vesicle exocytosis and endocytosis (Sun and Wu, 2001). In the hippocampus, large mossy fiber terminals originating from dentate granule cells have also been studied using this method, revealing a large pool of recycling vesicles (Hallermann et al., 2003).

Despite its limited applicability to synaptic vesicle fusion, the capacitance measurement method, when available, provides several advantages due to its ability to report the kinetics of exocytosis and endocytosis as well as in some cases properties of fusion pore openings. Nevertheless, capacitance measurements also possess several shortcomings. First, measurement of fusion during repetitive stimulation is problematic, as rapid changes in membrane potential induce massive charge movements across the membrane and render capacitance measurements difficult to interpret. Therefore, the best setting to examine evoked release using this method takes advantage of light-induced Ca^{2+} uncaging within the nerve terminal bypassing voltage-gated Ca^{2+} influx rather than direct depolarization of the nerve terminal. Second, like every method, capacitance measurements are prone to artifacts. These artifacts may originate from multiple sources, including inaccurate estimation of the electrotonic properties of the nerve terminal, which is needed to extract capacitance values from phase changes in the output signal. In some cases fusion events not relevant to neurotransmitter release may complicate direct interpretation of the signals in terms of synaptic vesicle fusion. Some of these artifacts can be alleviated by appropriate control experiments such as testing capacitance changes in the absence of secretion using application of clostridial toxins that block membrane fusion. Recently, capacitance measurements using the cell-attached configuration of the patch clamp technique have been achieved on or near the active zones of the calyx of Held nerve terminals. This configuration has several advantages over traditional whole terminal recoding configuration due to electrical isolation of a small membrane area, thus limiting background noise (He et al., 2006). This setting allowed resolution of single-vesicle fusion events and their properties of endocytosis. However, establishing a tight giga-ohm seal on a release site may also modify the properties of vesicle fusion events by altering membrane tension or the presynaptic scaffolding matrix present in active release sites.

2 Fluorescent Visualization of Synaptic Vesicle Fusion and Recycling

2.1 Optical Detection of Neurotransmitter Release

Despite their versatility, electrophysiological methods typically report neurotransmitter release from a population of synapses or release sites. In most cases, unitary quantal transmission can only be detected using recordings of spontaneous neurotransmission or under settings that activate a very small number of synapses. Moreover, these methods usually provide little or no spatial information about the origin of release. Optical experiments using fluorescent probes, on the other hand, report

release at the level of individual synapses. These methods either monitor Ca^{2+} influx through postsynaptic NMDA receptors or can directly monitor presynaptic properties using styryl dye imaging and GFP-tagged synaptic vesicle proteins.

NMDA receptor mediated Ca^{2+} influx provides a valuable reporter of postsynaptic receptor activation in response to presynaptic glutamate release. NMDA receptors have a very high affinity for glutamate ($\sim$1 μM range), thus they can detect even subtle fusion events that may not release sufficient glutamate to activate AMPA receptors. In addition, in response to a single quantal fusion event postsynaptic NMDA receptors are not saturated, which enables measurements of release with a large dynamic range despite their high affinity. Therefore, this approach has recently been quite informative for providing evidence for multivesicular release in central synapses (Oertner et al., 2002). Ca^{2+} indicator dyes with adequate linear response range and affinity can be used to detect Ca^{2+} signal in a single dendritic spine evoked by quantal release events. This method, by its very nature, is limited to glutamatergic synapses or synapses with reliable postsynaptic Ca^{2+} selective channels (e.g., some subtypes of nicotinic acetylcholine receptors or purinergic receptors such as P2X receptors). Moreover, the properties of neurotransmitter release can only be inferred postsynaptically; therefore, the same caveats that apply to electrophysiological recordings in principle apply to this method as well. However, the ability to monitor neurotransmission at the level of individual synapses and availability of a wide variety of Ca^{2+} indicators makes this setting a method of choice for investigation of synaptic activation in intact tissues such as brain slices using two-photon imaging.

Recently, in addition to postsynaptic Ca^{2+} imaging, several fluorescence imaging modalities have been introduced which primarily report synaptic vesicle fusion and endocytosis. These methods either take advantage of styryl dyes that can be loaded into recycling vesicles or they utilize exogenous expression of synaptic vesicle proteins tagged with a pH-sensitive variant of GFP at regions facing the vesicle lumen. Styryl dyes, such as FM1-43 and FM2-10, are amphipathic compounds that label recycling synaptic vesicles in an activity-dependent manner. This optical approach is also useful for monitoring the kinetics of replenishment for the release-ready pool of vesicles in a synapse. Furthermore, uptake and release of these dyes provide a faithful readout for synaptic vesicle reuse during activity. GFP-tagged synaptic vesicle proteins such as synaptobrevin and synaptophysin, on the other hand, provide a molecularly specific way to examine surface exposure and re-internalization of these proteins during synaptic vesicle exo- and endocytosis.

2.2 Functional Analysis of Exocytosis and Vesicle Recycling Using Styryl Dyes

In the last decade significant advances in our understanding of the dynamics of the synaptic vesicle cycle were made possible by the availability of new optical probes. The first set of probes was the styryl dye FM1-43 and its analogs (Betz et al., 1996).

FM1-43 is a partially lipophilic dye, which increases fluorescence 100-fold when it partitions into membranes; thus it is virtually invisible in aqueous solution. The chemical structure of FM1-43 makes it difficult for it to cross lipid membranes, thus limiting the fluorescence labeling to outer membranes and recycling vesicles. Using this methodology, one can detect activity-dependent vesicular dye uptake and release in synaptic connections in multiple preparations, including central neuronal cultures. Studies using FM dyes revealed that activity-induced dye uptake and release operates with high fidelity. Specifically, the extent of fluorescent dye endocytosed during a particular stimulation matches the magnitude of fluorescence loss due to exocytosis in response to the same stimulation. This observation suggests that synaptic vesicles recycle without intermixing with endosomal compartments and thus the fluorescence probes do not get diluted (Murthy and Stevens, 1998).

The properties of styryl dyes make them useful tools for revealing quantitative information on vesicular recycling within a presynaptic terminal (Figure 2). These dyes can be rapidly inserted into membranes within milliseconds; however, with the increasing lengths of their hydrocarbon chains, they have decreasing rates of dissociation from membranes (Ryan, 1996; Klingauf et al., 1998; Kavalali et al., 1999). Previous measurements have shown that FM1-43 departitions from lipid to aqueous environment with a time constant of 2.5s, whereas its analogs, FM2-10 and FM1-84, departition with time constants of 0.6s and 4.8s, respectively. These differential departitioning properties of FM dyes can be exploited to estimate the kinetics of endocytosis in hippocampal synapses (Ryan, 1996; Klingauf et al., 1998; Kavalali et al., 1999). Because of FM2-10's membrane dissociation rate, destaining experiments with the dye provide the closest estimates of vesicle mobilization and exocytosis. In contrast, the same experiments performed with FM1-43 may be vulnerable to dye trapping due to fast endocytosis. A significant proportion of endocytosis takes place following the cessation of stimulation, albeit on a slower time scale. This slow endocytosis may require formation of clathrin coats, followed by a recycling pathway where vesicles go through an endosomal intermediate structure and recover all of the necessary molecular components. Alternatively, recycling vesicles can be formed from membrane invaginations called "cisternae" (Richards et al., 2000) that develop adjacent to the active zone. Reformation of synaptic vesicles from these endosomal structures requires clathrin and clathrin-adaptor proteins that bind to specific synaptic vesicle membrane proteins. Recent studies in the frog neuromuscular junction have shown that a slow endocytosis pathway, characterized by large membrane invaginations formed in response to prior activity, operates at rest for up to at least 10 minutes or more after cessation of exocytosis and selectively traps FM1-43 but not FM2-10. The slowly endocytosed membrane can form synaptic vesicles, which can in turn mix with the reserve pool and become re-available for release with a delay. FM2-10 uptake, in contrast, occurs in rapidly endocytosing vesicles, which preferentially populate the readily releasable pool and give rise to the fast component of destaining.

In a typical experiment to estimate slow endocytosis, one can stimulate synaptic terminals with 10- to 30-Hz field stimulation for 30 to 60 seconds and rapidly perfuse FM1-43 onto the preparation (for 60 seconds and up to 10 minutes) at gradually

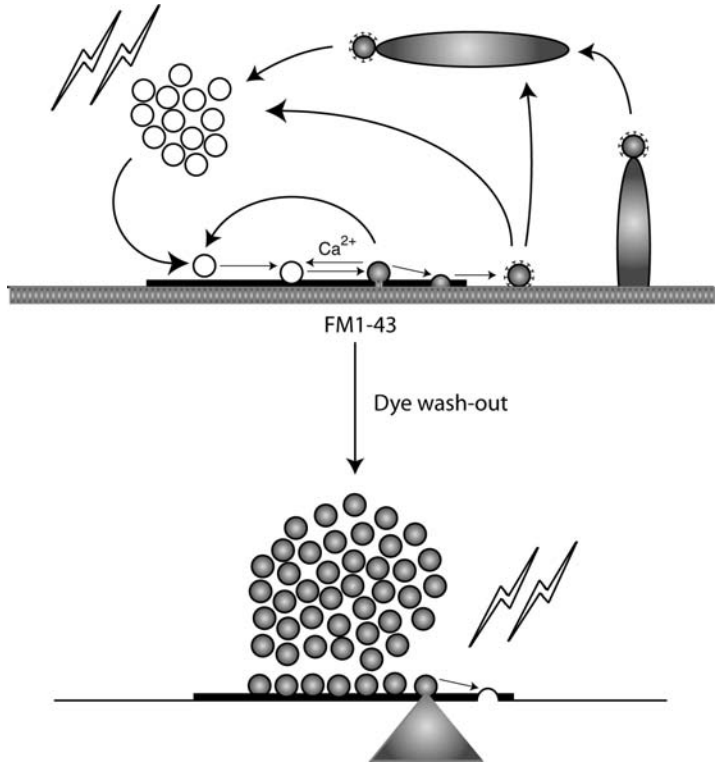

Fig. 2 Optical monitoring of vesicle recycling using FM dyes. FM1-43 and its analogs are partially lipophilic dyes which fluorescence increases almost 100-fold when they partition into membranes. Therefore, they are virtually invisible in aqueous solution. The chemical structure of FM dyes makes it difficult for them to cross lipid membranes, thus limiting the fluorescence labeling to outer membranes and recycling vesicles. During a typical experiment, stimulation in the presence of extracellular dye leads to uptake of dye molecules into vesicles that have undergone exocytosis followed by endocytosis. Subsequent washout of extracellular dye uncovers fluorescent staining specific to actively recycling vesicles during the stimulation paradigm. Consequent stimulation results in fusion of dye-loaded vesicles and loss of fluorescence trapped in synapses. This fluorescence loss is due to departitioning of the dye into aqueous solution or lateral diffusion of dye in neuronal membrane.

increasing delays following the cessation of stimulation. The time course of decrease in the amount of fluorescence trapped within synapses provides an estimate of the time constant of slow endocytosis. This time constant has previously been shown to slow with increasing extent of depletion of vesicles in the neuromuscular junction (Wu and Betz, 1996). However, in these experiments, parallel control experiments are essential to account for the potential effect of changes in the level of exocytosis. Some evidence indicates that the number of fused vesicles can regulate the rate of endocytosis. Under the same setting more rapid endocytosis can be quantified when dye is applied concomitantly during stimulation and washed out rapidly afterward with no delay. Fast endocytosis is typically tightly coupled to exocytosis,

and it is thought to be critical for maintenance of neurotransmission during sustained neurotransmission (Sara et al., 2002).

The measurement of the size of functionally distinct vesicle pools is a critical step in linking the structure and function of presynaptic terminals. Styryl dyes are also quite useful in determination of vesicle pool sizes at the level of individual synapses (Mozhayeva et al., 2002). In these measurements, it is important to distinguish changes in the Ca^{2+} dependence of the release machinery from changes in numbers and maturational (release competent) states of synaptic vesicles. This distinction can be achieved by using saturating levels of Ca^{2+}-dependent stimulation or Ca^{2+}-independent stimulations, such as hypertonic sucrose application (typically $+500$ mOsm), to mobilize vesicles. The recycling kinetics of readily releasable pool (RRP) vesicles can be determined using uptake and release of the styryl dye FM2-10 in response to hypertonic stimulation. To determine the total recycling pool, uptake and release of styryl dyes can be induced by sustained depolarization achieved via application of elevated potassium solution. This strong stimulation can induce mobilization of virtually all vesicles capable of recycling within individual synapses. The comparison of the two fluorescence measurements (RRP versus the total recycling pool) can reveal critical information in regard to functional allocation of vesicles to a particular pool in given condition.

Detecting release of FM dyes rapidly after their uptake into the synapse provide a commonly used setting to quantify synaptic vesicle reuse in synapses (Ryan and Smith, 1995). In these experiments, FM dye is applied during and/or immediately after stimulation to label endocytosing vesicles. The dye-containing solution is then swiftly washed out to prevent further dye uptake. This dye application stains a number of vesicles, depending on the stimulation strength as well as the duration of dye presence. Application of a second stimulation at gradually delayed time points (typically 0 to 120 s) causes dye loss due to the re-availability of labeled vesicles. However, the proximity of stimulations inducing dye uptake and release makes it difficult to image actual dye loss during the second stimulation because of the high fluorescence background originating from residual dye. This shortcoming is usually circumvented by monitoring the remaining detectable staining at a later time point (typically $\sim$5 minutes) after complete dye washout. The releasable fluorescence trapped in the synapse gradually decreases following stimulations applied at longer intervals or for longer durations after dye washout. The time course of this decrease in fluorescence provides an estimate of the time constant of vesicular reuse, which is the time it takes for an endocytosed vesicle to become release-ready again. This is a very sensitive method to detect vesicle reuse. As stated above, the major caveat is the background fluorescence, while usually not sufficient to restain vesicles during stimulation, it makes fast imaging nearly impossible. Because of this problem, the vesicle reuse kinetics is typically reconstructed from a population of synapses rather than from repeated measurements at individual presynaptic terminals.

2.3 Detection of Synaptic Vesicle Exocytosis and Endocytosis Using pHluorin-Tagged Synaptic Vesicle Proteins

SynaptopHluorin (spH) is another fluorescent probe that is commonly used to monitor exocytosis and endocytosis of synaptic vesicles (Miesenbock et al., 1998; Sankaranarayanan and Ryan, 2000). Synaptophluorin is a fusion construct of the synaptic vesicle protein synaptobrevin (also called vesicle associated membrane protein, or "VAMP") with a pH-sensitive EGFP at its C-terminal (located in the synaptic vesicle lumen). Synaptic vesicle lumen normally has an acidic pH of approximately 5.5, at which spH fluorescence is quenched (Figure 3). When vesicles fuse, lumenal EGFP is exposed to the extracellular pH, which results in a marked increase in its fluorescence. Studies using spH have provided valuable information on the tight coupling between exocytosis and endocytosis. One advantage of the spH probe is the molecular specificity of the fluorescence signal, since the signal originates from a synaptic vesicle protein as opposed to nonspecific lipid-based fluorescence of FM dyes. Proton diffusion from a synaptic vesicle is not expected to be limited, and the time course of vesicle re-acidification is thought to be fast (see below), without imposing a significant delay in the fluorescence signal during spH endocytosis. Although most studies so far used synaptobrevin as a carrier for the pHluorin tag, recent work took advantage of other synaptic vesicle proteins as pHluorin attachments (Granseth et al., 2006; Voglmaier et al., 2006). Surprisingly, in these experiments, different synaptic vesicle proteins tagged with pHluorin gave different results with respect to their background fluorescence (i.e., surface exposure) and retrieval. For instance, both synaptophysin (a four-transmembrane domain synaptic vesicle protein) and the vesicular glutamate transporter reveal

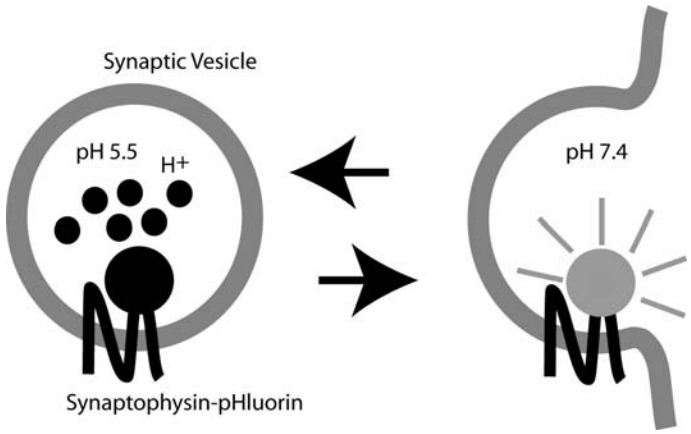

Fig. 3 Synaptophysin-pHluorin is a fusion construct of the synaptic vesicle protein synaptophysin (a four-transmembrane domain synaptic vesicle protein) with a pH-sensitive EGFP at its C-terminal (located in the synaptic vesicle lumen). Synaptic vesicle lumen normally has an acidic pH of approximately 5.5 at which spH fluorescence is quenched. When vesicles fuse, lumenal EGFP is exposed to the extracellular pH, which results in a marked increase in its fluorescence. During endocytosis, pHluorin fluorescence is re-quenched as vesicle lumen becomes acidic.

very little surface exposure (thus fluorescence) in the absence of stimulation. Once stimulated they show a swift increase in fluorescence without significant lateral diffusion upon fusion, which was a salient feature of synaptopHluorin-based measurements. The dependence of the properties of pHluorin-based measurements on the identity of the tagged protein complicates a straightforward interpretation of these experiments in terms of synaptic vesicle recycling. Despite this caveat, these measurements reveal critical information in regard to copy numbers and specific cell biological attributes of synaptic vesicle proteins.

The vacuolar ATPase blockers discussed above are also useful in dissecting the kinetics of exocytosis and endocytosis in pHluorin-based measurements. In response to brief field stimulation, synaptophysin-pHluorin (or synaptopHluorin) fluorescence shows a swift increase and a gradual slow return to baseline (Granseth et al., 2006), presumably due to slow compensatory endocytosis. However, when the same experiment is repeated in the presence of a vacuolar ATPase blocker (such as folimycin) synaptophysin-pHluorin fluorescence shows a larger increase detectable within the first 500 ms (Ertunc et al., 2007). The difference between these two signals can be taken as a readout of endocytosis with the assumption that re-acidification of vesicles upon endocytosis proceeds rapidly and requenches internalized pHluorin. However, current estimates of synaptic vesicle re-acidification widely differ. According to some estimates re-acidification may take place rapidly within 500 milliseconds or less upon endocytosis (Gandhi and Stevens, 2003; Ertunc et al., 2007). In contrast, recent findings in hippocampal cultures suggest a relatively slow time course ($\sim$5 seconds) (Atluri and Ryan, 2006). These slower estimates have originated from measurements of synaptic vesicle re-acidification during delayed endocytosis following stimulation and, therefore, they may have overlooked a fast component of vesicle re-acidification. The vesicles that are endocytosed with a delay after stimulation may end up being slowly re-acidified due to slower reassembly of their protein complements. Whereas vesicles that are endocytosed rapidly during stimulation may also be re-acidified rapidly, as their protein components (including the vacuolar ATPase) are more likely to be intact.

2.4 Comparison of Styryl Dye Imaging and pHluorin-Based Visualization of Exo-Endocytosis

While FM dyes and spH are commonly used to measure endocytosis and exocytosis, there are advantages and disadvantages to both. FM dye measurements have been instrumental in tracing vesicles during their cycle in the synapse. Using uptake and release of FM dyes one could reach conclusions on vesicle mixing with endosomal compartments (Murthy and Stevens, 1998) or on repeated use of the same vesicles during activity (Aravanis et al., 2003), which would be difficult to attain using probes like spH. FM dyes report lipid cycling; spH reports the fate of synaptic vesicle protein synaptobrevin (or others) upon fusion and endocytosis. SpH is a genetically expressed probe, which facilitates more reproducible

repeated measurements on the same set of synapses (as it does not require dye uptake). However, measurement of endocytosis with spH is confounded by two factors. First, lateral diffusion of vesicular spH following fusion is commonly observed (Sankaranarayanan and Ryan, 2000; Li and Murthy, 2001). It is unclear whether reuptake of spH truly reflects endocytosis of vesicles or reclustering and re-internalization of synaptobrevin. It is also unknown whether endocytosis of lipid and protein components of synaptic vesicles are distinct processes or whether they are coupled. Second, the dependence of endocytosis measurements on vesicle reacidification may delay the fluorescence signal after spH internalization. There are also concerns with using FM dyes. Typically, FM dye measurements suffer from nonspecificity. FM dyes stain the surface membrane indiscriminately, and thus they are less specific to synaptic vesicles. Moreover, changes in FM dye fluorescence typically occur slowly, in seconds, making it difficult to detect events in the millisecond time scale, which more closely match the time course of neurotransmission. In addition, after FM dye uptake, preparations need to be washed with dye-free solution for a significant period to reduce background fluorescence, which limits the rapid detection of synaptic vesicle re-availability after endocytosis. In summary, spH provides a molecularly specific probe to monitor the coupling between exocytosis and endocytosis, whereas FM dyes are better at tagging vesicles during their cycling within the synapses. Thus these two optical probes complement each other in monitoring distinct aspects of the synaptic vesicle cycle.

The experiments using these optical tools consistently supported the premise that synaptic vesicle exocytosis and endocytosis are tightly coupled processes. This largely kinetic coupling is also backed up by recent molecular evidence that proteins critical for exocytosis such as synaptotagmin and synaptobrevin, are also essential for triggering endocytosis (Poskanzer et al., 2003; Deak et al., 2004; Nicholson-Tomishima and Ryan, 2004).

3 Biochemical Approaches to Measuring Neurotransmitter Release

Synaptic neurotransmission in brain occurs mostly by exocytic release of vesicles filled with chemical substances (neurotransmitters) at presynaptic terminals. Thus, neurotransmitter release can be detected and studied by measuring efflux of neurotransmitters from synapses by biochemical methods. Various methods have been successfully employed to achieve that, including direct measurements of glutamate release by high-performance liquid chromatography of fluorescent derivatives or by enzyme-based continuous fluorescence assay, measurements of radioactive efflux from nerve terminals preloaded with radioactive neurotransmitters, or detection of neuropeptides by RIA or ELISA. Biochemical detection, however, lacks the sensitivity and temporal resolution afforded by electrophysiological and electrochemical approaches. As a result, it is not possible to measure individual synaptic events and apply quantal analysis to verify the vesicular nature of neurotransmitter release.

Carefully controlled experiments are required to ensure that most of the measured neurotransmitter efflux is vesicular and is not significantly contaminated by release from nonvesicular sources (e.g., leakage from the cytoplasmic pool, reversal of plasma membrane neurotransmitter transporters). Use of biochemical methods to measure neurotransmitter release also has a number of significant advantages: it is instrumentally simpler and less expensive; release is averaged over large number of terminals, thus eliminating synapse to synapse variability; detection does not depend on postsynaptic terminal function.

In principle, high-density primary neuronal cultures and acute or chronic brain slice preparations can be used to measure neurotransmitter release biochemically. However, by far the most commonly used preparation is synaptosomes, resealed broken-off nerve terminals obtained by homogenization of brain tissue in isotonic solutions of low ionic strength and differential centrifugation. Synaptosomes retain most of the cytoplasm, synaptic vesicles, mitochondria, and other organelles found in presynaptic terminals. When incubated in oxygenated balanced salt solutions containing glucose, synaptosomes remain metabolically active for several hours ex vivo, generating ATP and maintaining membrane potential and normal channel function (Nicholls, 2003). Depending on the method used, synaptosomes can be prepared quickly (under 30 min) in crude form from a large portion of the brain (e.g., neocortex) or in highly pure form from specific brain region (e.g., CA3 region of hippocampus) after several step gradient centrifugations. Unlike dissociated neuronal cultures and brain slices, synaptosomes are electrically inaccessible. To stimulate neurotransmitter release, synaptosomes are treated with substances modifying cation-selective channels, e.g., by applying high potassium solutions or potassium channel blockers (4-aminopyridine) to open voltage clamped calcium channels or by substances stimulating neurotransmitter release independent of channel function, e.g., hypertonic solutions (0.5 M sucrose), calcium ionophores (ionomycin), or presynaptic neurotoxins (α-latrotoxin).

Glutamate release from synaptosomes can be measured by enzyme-based continuous fluorescence assay (Nicholls and Sihra, 1986; Nicholls et al., 1987). This experimental setup allows direct measurement of glutamate efflux from synaptosomes but cannot be easily adapted to measure release of other neurotransmitters. In addition, synaptosomes are subjected to stimulation for prolonged period of time, which results in nonvesicular glutamate release, thus complicating interpretation of data obtained by this method. A different approach is used in superfusion release assays developed to measure neurotransmitter release from synaptosomes preloaded with radioactive neurotransmitters (Lonart et al., 1993; Lonart and Sudhof, 1998). Synaptosomes are captured on glass fiber filters, thus avoiding mechanical damage caused by stirring and sedimentation (Figure 4). This method allows measurements of all major neurotransmitters, and in some cases can be used to monitor release of several neurotransmitters simultaneously (Khvotchev et al., 2000). Superfusion release setup enables rapid and transient stimulation of synaptosomes, which can be applied multiple times. Preloading of synaptosomes with radioactive neurotransmitter and assaying neurotransmitter release should be carried out relatively quickly to avoid metabolization of radioactive compounds.

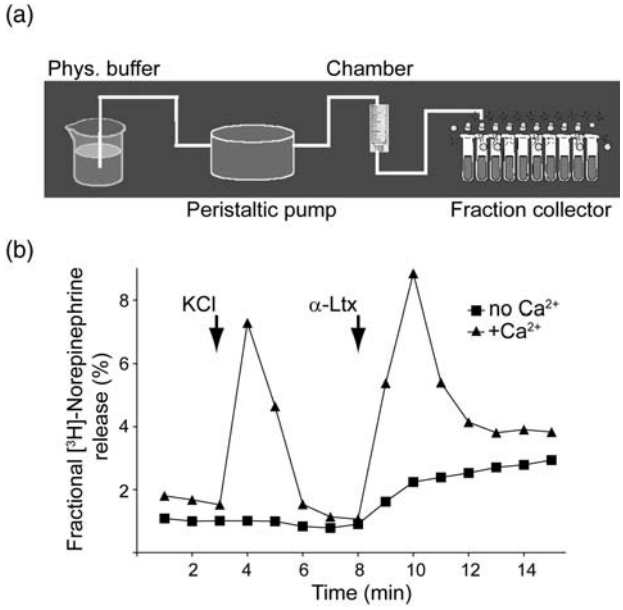

Fig. 4 Superfusion neurotransmitter release assay in synaptosomes. (**a**) Schematic drawing of a superfusion setup. Synaptosomes are preloaded with radioactive neurotransmitter and captured on fiberglass filters in superfusion chambers under continuous superfusion with gassed physiological salt solution (e.g., Krebs bicarbonate buffer) using a peristaltic pump. After a 10- to 15-minute wash, neurotransmitter release is triggered by rapid switching of superfusion lines to a stimulating buffer (e.g., high-potassium solution). Superfusate is collected on a fraction collector, and radioactivity is measured by liquid scintillation. (**b**) Typical trace recording of tritium-labeled norepinephrine fractional release in rat cortical synaptosomes stimulated by high potassium and α-latrotoxin in the presence or absence of external calcium.

Significant advances in studying secretion were obtained with help of permeabilized cell preparations, e.g., cracked PC12 cells that allows direct biochemical access to intracellular release machinery (Klenchin et al., 1998; Martin and Grishanin, 2003). Similar approaches were relatively unsuccessful in neuronal preparations, possibly due to the complexity and small size of synaptic terminals. However, better-controlled permeabilization (using bacterial toxins) may be a promising approach to develop reliable and reproducible assays to measure neurotransmitter release in these preparations.

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Presynaptic Calcium Channels: Structure, Regulators, and Blockers

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Abstract The central and peripheral nervous systems express multiple types of ligand and voltage-gated calcium channels (VGCCs), each with specific physiological roles and pharmacological and electrophysiological properties. The members of the Ca_v2 calcium channel family are located predominantly at presynaptic nerve terminals, where they are responsible for controlling evoked neurotransmitter release. The activity of these channels is subject to modulation by a number of different means, including alternate splicing, ancillary subunit associations, peptide and small organic blockers, G-protein-coupled receptors (GPCRs), protein kinases, synaptic proteins, and calcium-binding proteins. These multiple and complex modes of calcium channel regulation allow neurons to maintain the specific, physiological window of cytoplasmic calcium concentrations which is required for optimal neurotransmission and proper synaptic function. Moreover, these varying means of channel regulation provide insight into potential therapeutic targets for the treatment of

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pathological conditions that arise from disturbances in calcium channel signaling. Indeed, considerable efforts are presently underway to identify and develop specific presynaptic calcium channel blockers that can be used as analgesics.

1 Subtypes and Physiological Functions of Calcium Channels

The entry of calcium ions into the cytosol mediates a wide range of cellular responses, including the activation of calcium dependent enzymes, gene transcription, and the initiation of calcium triggered membrane fusion – a key aspect of vesicular neurotransmitter release (Dolmetsch et al. 2001; Fields et al. 2005; Martin-Moutot et al. 1996; Reid et al. 2003; Sutton et al. 1999; Wheeler et al. 1994). Calcium may enter cells by a number of different mechanisms, including ligand and voltage-gated calcium channels (VGCCs). The nervous system expresses a number of different types of VGCCs, each with specific physiological functions and electrophysiological and pharmacological properties (Table 1). These include the L-type ($Ca_v1.1$-1.4), P/Q-type ($Ca_v2.1$), N-type ($Ca_v2.2$), R-type ($Ca_v2.3$), and T-type ($Ca_v3.1$-3.3) channels, which are broadly classified into low- and high-voltage activated channels (LVA and HVA channels, respectively) (Catterall 2000). LVA (a.k.a. T-type) calcium channels activate in response to small membrane depolarizations, display rapid gating kinetics, exhibit a small unitary conductance, and play a major role in neuronal pacemaker activity (Mangoni et al. 2006; Nelson et al. 2006). In contrast, HVA channels require larger membrane depolarizations in order to open and can be further categorized, based on their functional characteristics, into N-, P-, Q-, R-, and L-types. These HVA calcium channel subtypes are distinguished by their pharmacological profiles: L-type channels are sensitive to dihydropyridines (Fox et al. 1987), N-type channels are selectively inhibited by ω-conotoxins GVIA, MVIIA, and CVID (Adams et al. 1993; Feng et al. 2003; Olivera et al. 1984; Reynolds et al. 1986), and P- and Q-type channels are differentially sensitive to ω-agatoxin IVA (Adams et al. 1993). R-type channels were originally identified based on their resistance to these pharmacological tools (Randall and Tsien 1995) but have since been shown to be potently inhibited by the spider toxin SNX-482 (Bourinet et al. 2001; Newcomb et al. 1998). These calcium channel subtypes also support distinct physiological functions and exhibit specific subcellular distributions (Table 1). For example, L-type calcium channels are expressed on cell bodies and support calcium-dependent gene transcription (Bading et al. 1993; Dolmetsch et al. 2001; Weick et al. 2003), while both N-type and P/Q-type channels are expressed at presynaptic nerve terminals, where they control evoked neurotransmitter release (Ishikawa et al. 2005; Westenbroek et al. 1992; Westenbroek et al. 1995). Recent evidence also implicates R-type calcium channels in the release of neurotransmitters at certain synapses (Kamp et al. 2005). For the purposes of this chapter, we will focus predominantly on these presynaptic VGCC subtypes.

Table 1 Comparison of the physiological, pharmacological, and electrophysiological characteristics of voltage-gated calcium channel subunit subtypes

	Channel Family	Calcium Current	Subunit	(Sub)-Cellular Distribution	Physiological Function	Pharmacological Sensitivity	Knockout Phenotype
	$Ca_v1.1$	L-type	α_{1S}	skeletal muscle	excitation-contraction coupling, gene transcription	dihydropyridines (DHPs)	lethal
	$Ca_v1.2$	L-type	α_{1C}	cardiac muscle, endocrine cells	excitation-contraction coupling, hormone secretion	DHPs	lethal
	$Ca_v1.3$	L-type	α_{1D}	cochlear neurons, endocrine cells	tonic neurotransmitter release, hormone secretion	DHPs	cardiac arrythmias, deaf
HVA	$Ca_v1.4$	L-type	α_{1F}	retinal neurons, immune cells	tonic neurotransmitter release, T lymphocyte activation	DHPs	blind
	$Ca_v2.1$	P/Q-type	α_{1A}	presynaptic nerve terminals	evoked neurotransmitter release	ω-agatoxin IVA, IIIA ω-grammotoxin SIVA	ataxic, absence seizures
	$Ca_v2.2$	N-type	α_{1B}	presynaptic nerve terminals	evoked neurotransmitter release	ω-conotoxin GVIA, MVIIA and CVID, ω-grammotoxin SIVA, farnesol, peptidylamines	hyponociceptive, reduced anxiety, reduced withdrawal symptoms
	$Ca_v2.3$	R-type	α_{1E}	dendrites, presynaptic nerve terminals	evoked neurotransmitter release, neuronal excitability	SNX-482	seizure resistant, hyponociceptive

(*Continued*)

Table 1 Continued

	Channel Family	Calcium Current	Subunit	(Sub)-Cellular Distribution	Physiological Function	Pharmacological Sensitivity	Knockout Phenotype
LVA	$Ca_v3.1$	T-type	α_{1G}	neurons, cardiac tissue	pacemaker activity, burst firing, oscillatory behaviour, hormone secretion, smooth muscle contraction	Kurtoxin, ethosuximide, nickel (low sensitivity)	seizure resistant, increased visceral pain perception
	$Ca_v3.2$	T-type	α_{1H}	neurons, cardiac tissue, endocrine cells, smooth muscle		Kurtoxin, ethosuximide, nickel (high sensitivity)	compromised vascular function, reduced pain sensitivity
	$Ca_v3.3$	T-type	α_{1I}	neurons		Kurtoxin, nickel (low sensitivity)	–
ancillary subunits	common to all α_1 subunits		β	common to α_1 subunit distribution	membrane targeting, alteration of channel kinetics and current densities	–	lethal ($\beta_{1,2a}$), reduced neuronal function (β_3), ataxic and absence seizures (β_4)
			α_2-δ			gabapentin, pregabalin	ataxic, absence seizures

2 Molecular Structure of Voltage-Gated Calcium Channels

HVA calcium channels are formed by association of a multimeric protein complex that is comprised of α_1, β, and $\alpha_2 - \delta$ subunits, with, as far as we know, a 1:1:1 stoichiometry (Figure 1) (Catterall 2000). In addition, although its presence remains controversial, some types of HVA calcium channels appear to contain an additional γ subunit (Kang and Campbell 2003; Moss et al. 2002). The α_1 subunit is the major pore-forming subunit and is by itself sufficient to form a functional channel. However, the ancillary subunits are important for proper membrane targeting, and alteration of the functional characteristics of α_1 (Bichet et al. 2000; Stea et al. 1993; Walker and De Waard 1998; Zamponi et al. 1996). All α_1 subunits share a common architecture of four major transmembrane domains (I through IV), connected by cytosolic linker regions and cytoplasmic N- and C-termini. Each major domain contains six membrane spanning helices (S1 through S6) and a re-entrant p-loop that is thought to contain the amino acids that line the pore and form the ion

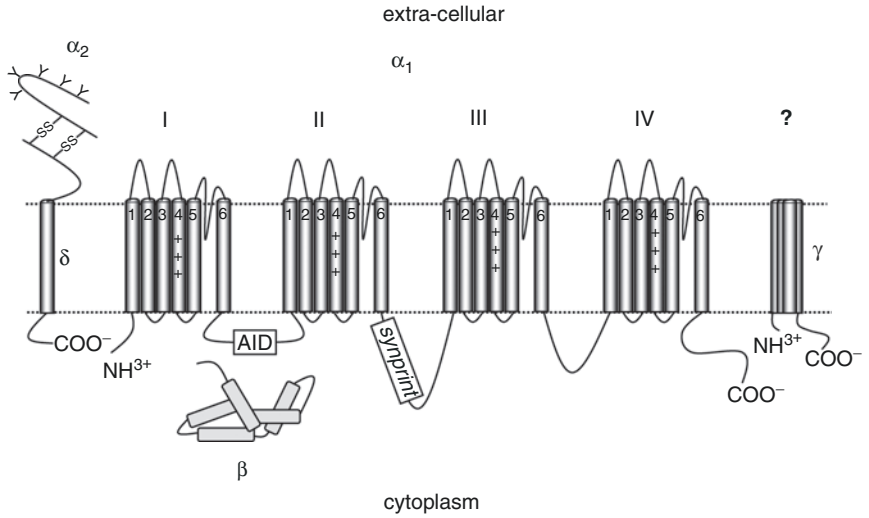

Fig. 1 Topology of the voltage-gated calcium channel protein complex. The main pore-forming subunit, α_1, is composed of four homologous domains (I through IV), connected by cytosolic linkers and flanked by cytosolic N- and C-termini. Each domain consists of six membrane-spanning helices (S1 through S6) and a p-loop between the S5 and S6 segments. The S4 segment contains several positively charged amino acids which act as the voltage sensor. The II-III linker contains a *synprint* region which is important for interactions with synaptic proteins essential for neurotransmitter release. The calcium channel β subunit is a cytoplasmic protein that associates with the α_1 subunit on the I–II linker alpha interaction domain (AID). The calcium channel α_2-δ subunit is composed of the membrane-spanning δ-subunit, connected via di-sulfide linkages to the heavily glycosylated α_2 subunit. Finally, the calcium channel γ subunit is comprised of four transmembrane helices with cytoplasmic N- and C-termini. The presence of this subunit in synaptic calcium channels is controversial, hence it is depicted with a question mark (?) overhead. The auxiliary calcium channel subunits are important for proper membrane targeting of the channel as well as for alteration of channel function.

selectivity filter (Figure 1) (Catterall 2000). Moreover, it is the calcium channel α_1 subunit that defines the calcium channel subtype. To date, 10 different α_1 subunits have been identified and shown to correspond to the native calcium channel isoforms when expressed functionally. They fall into three gene families, Ca_v1, Ca_v2, and Ca_v3 (Table 1). The Ca_v1 family encodes four different L-type channel isoforms (Koschak et al. 2003; Mikami et al. 1989; Tomlinson et al. 1993; Williams et al. 1992), the Ca_v3 family encodes three different T-type channel isoforms (Cribbs et al. 1998; Klockner et al. 1999; Lee et al. 1999b; McRory et al. 2001; Monteil et al. 2000; Perez-Reyes et al. 1998), and most relevant to this chapter, the Ca_v2 family includes the synaptic calcium channel subtypes. $Ca_v2.2$ and $Ca_v2.3$, respectively, correspond to N-type and R-type channels (Dubel et al. 1992; Soong et al. 1993; Williams et al. 1994), and different splice isoforms of $Ca_v2.1$ give rise to P- and Q-type channels (Bourinet et al. 1999). Each of the other types of calcium channel α_1 subunits undergoes alternate splicing, often producing calcium channel isoforms with very distinct electrophysiological properties (Lipscombe et al. 2002), and in the case of N-type calcium channels, differential cellular expression patterns (Bell et al. 2004).

The mammalian brain expresses four different types of β subunits (β_1 through β_4), which can undergo alternate splicing (Dolphin 2003a; Richards et al. 2004). They share a similar structural arrangement with two highly conserved regions (C1 and C2) of high overall sequence homology (75% and 85%, respectively), separated and flanked by a total of three variable regions (V1 through V3) of much lower homology (35%-55%) (Walker and De Waard 1998). These subunits are cytoplasmic proteins, with the exception of β_{2a}, whose N-terminus contains a pair of cysteine residues that can be palmitoylated, thus leading to membrane insertion of some splice isoforms of this subunit (Qin et al. 1998). Each of the four β subunits physically binds to a region within the α_1 subunit domain I-II linker, which is highly conserved among all HVA calcium channels and is termed the alpha interaction domain (AID) (Figure 1) (Pragnell et al. 1994). In addition, there have been reports of a second calcium channel β subunit interaction site within the C-terminus of α_1 (Qin et al. 1997), however, this role of this site remains unclear. The functional effects of β subunit co-expression include changes in channel kinetics and, depending on the channel subtype, an increase in current densities (Bichet et al. 2000; Chien et al. 1995; Yasuda et al. 2004). The latter observation has been linked to the masking of an endoplasmic reticulum (ER) retention signal on the calcium channel α_1 subunit (Bichet et al. 2000); however, our recently published work indicates that, at least in mammalian expression systems, the calcium channel α_1 subunit can give rise to robust current activity even when expressed alone (Yasuda et al. 2004). Perhaps the most obvious effect of the different β subunits is their regulation of channel inactivation rates, with β_{2a} dramatically slowing inactivation, and β_3 and β_{1b} accelerating inactivation (Arikkath and Campbell 2003; Yasuda et al. 2004).

The calcium channel β subunit is the only calcium channel subunit for which there is crystal structure information. The core of this subunit is homologous to membrane-associated guanylate kinases (MAGUKs), with conserved, interacting SH3 and guanylate kinase (GK) domains (Takahashi et al. 2004). Residues in the

GK domain form a hydrophobic groove for high-affinity binding of HVA calcium channels. Binding of the β subunit to the AID region is critically dependent on functional association of the SH3 and GK regions (Opatowsky et al. 2003; Van Petegem et al. 2004). The functional significance of the intramolecular SH3-GK interaction is supported by electrophysiological data showing that coexpression of separate cDNA constructs that encode the SH3 and GK domains, respectively, results in normal β subunit function, whereas either one of the regions alone is incapable of regulating channel activity (Takahashi et al. 2005).

There are also four different types of calcium channel α_2-δ subunits, each of which is encoded by a single gene that is postranslationally cleaved into α_2 and δ peptides which are then reconnected via a disulfide bond (De Jongh et al. 1990; Klugbauer et al. 2003). The α_2 subunit is extracellular and can be heavily glycosylated, whereas the δ subunit spans the plasma membrane with a single helix (Figure 1).

It has been known for a long time that the skeletal muscle L-type calcium channel complex also contains a γ subunit – a four transmembrane helix with cytoplasmic N- and C-termini (Arikkath and Campbell 2003). Seven additional potential candidates for neuronal calcium channel γ subunits have been identified; however, it is not clear if these are bona fide calcium channel subunits. Indeed, the first identified neuronal calcium channel γ subunit "stargazin" has also been linked to AMPA receptor trafficking and pharmacology (Chen et al. 2000; Tomita et al. 2005). Hence, it remains unclear to what extent these subunits associate with the calcium channel complex in neurons.

Calcium channel α_1 and β subunits are subject to further heterogeneity due to alternate splicing, thus giving rise to a potentially vast number of different types of calcium channel molecules whose expression and distribution may be dynamically regulated to suit a particular physiological function. It is important to bear this in mind when comparing native calcium channel currents to data obtained in transient expression systems.

3 Consequences of Calcium Channel Gene Knockout

The differential physiological roles of individual calcium channel subunits are perhaps best highlighted by knockout (KO) mouse experiments (Table 1). KO animals lacking each of the major calcium channel α_1 and β subunits have been characterized in detail. KO of the $Ca_v1.1$ channel is lethal due to inability of mice to contract their diaphragm (Strube et al. 1996). KO of the $Ca_v1.2$ channel is embryonic lethal due to compromised cardiac function (Seisenberger et al. 2000). Mice lacking $Ca_v1.3$ exhibit cardiac arrhythmias and are deaf (Platzer et al. 2000), whereas mice lacking $Ca_v1.4$ are blind (Mansergh et al. 2005). These two calcium channel subtypes, respectively, control tonic glutamate release at ribbon synapses in cochlear hair cells and photoreceptors – this type of neurotransmitter release thus differs from the N- and P/Q-type channel mediated action potential evoked release, which

is transient. Mice lacking $Ca_v3.1$ channels are resistant to certain types of pharmacologically induced seizures and may have an altered perception of visceral pain (Kim et al. 2001b), whereas mice lacking $Ca_v3.2$ channels are subject to compromised vascular function (Chen et al. 2003). $Ca_v2.3$ KO mice behave normally but show reduced pain responses, as well as resistance to certain types of epileptic seizures (Saegusa et al. 2000; Weiergraber et al. 2006). Although both N-type and P/Q-type channels control neurotransmitter release, they are clearly not created equal. Mice lacking the N-type $(Ca_v2.2)$ channel are hyposensitive to pain and show reduced ethanol reward behavior (Hatakeyama et al. 2001; Kim et al. 2001a; Newton et al. 2004). Other than a slightly compromised control of blood pressure, these mice are behaviorally normal and viable. In contrast, mice lacking $Ca_v2.1$ channels are severely ataxic and show absence seizures (Jun et al. 1999). These mice usually die before reaching maturity, which is in stark contrast to the findings with $Ca_v2.2$ KO mice. The ataxic and epileptic phenotype of the $Ca_v2.1$ KO mice is consistent with data from several mouse lines with missense and frame shift mutations (Doyle et al. 1997; Wakamori et al. 1998), or polyglutamine expansions (Zhuchenko et al. 1997), in $Ca_v2.1$, as well as with several $Ca_v2.1$ human mutations found in patients with episodic ataxia type 2 (Denier et al. 2001; Friend et al. 1999; Guida et al. 2001; Ophoff et al. 1996) (see Section 4).

KO of the calcium channel β_1 subunit is lethal due to skeletal muscle paralysis that results from reduced membrane targeting of the $Ca_v1.1$ channel (Gregg et al. 1996; Strube et al. 1996). KO of the β_{2a} subunit is also lethal due to compromised development of the cardiac vasculature (Ball et al. 2002). Mice lacking the β_3 subunit are viable but show reduced neuronal L-type and N-type channel activity (Namkung et al. 1998). In the context of synaptic physiology, KO of the calcium channel β_4 subunit is of interest. *Lethargic* is a mouse line with a mutation in the calcium channel β_4 subunit that leads to a premature stop codon and *de facto* elimination of this subunit. The consequences of this KO are strikingly similar to those observed with KO or mutated $Ca_v2.1$ animals (Burgess et al. 1997), hinting at a potential common effect of these subunits on synaptic function. Similar considerations apply to α_2-δ subunits where a frameshift mutation leads to the elimination of this subunit in the *ducky*^{2j} mouse strain – again, as with $Ca_v2.1$ KO animals, these mice are ataxic and have absence seizures (Brodbeck et al. 2002). These observations suggest that KO of either β_4 or α_2-δ may lead to altered P/Q-type channel targeting and/or function.

The notion that N-type and P/Q-type channel knockout mice are phenotypically distinct suggests that these two calcium channel subtypes contribute to different aspects of synaptic function. Indeed, is has been reported that P/Q-type channels are more frequently linked to excitatory transmission, whereas N-type channels may contribute more often to inhibitory transmission (Potier et al. 1993). This is an important point to consider in the context of channel modulation as outlined below.

4 Calcium Channelopathies Involving P/Q-Type Calcium Channel α_1 Subunits

Mutations in the α_1 subunits of voltage gated calcium channels have been linked to a number of disorders. These include hypokalemic periodic paralysis ($Ca_v1.1$) (Lapie et al. 1997), Timothy syndrome ($Ca_v1.2$ and $Ca_v3.2$) (Splawski et al. 2005; Splawski et al. 2004; Splawski et al. 2006), congenital stationary night blindness ($Ca_v1.4$) (Bech-Hansen et al. 1998; Hoda et al. 2005), and various forms of absence epilepsy ($Ca_v3.2$) (Khosravani and Zamponi 2006), none of which we will elaborate on further. Mutations in P/Q-type calcium channels of both mice and humans are also associated with a number of pathological conditions. As mentioned earlier, point mutations in $Ca_v2.1$ have been associated with episodic ataxia type 2, as well as with familial hemiplegic migraine (FHM) in humans (Ophoff et al. 1996). When introduced into recombinant channels and expressed in either transient expression systems or neurons, FHM mutations tend to result in decreased channel function (Pietrobon and Striessnig 2003). In contrast, data from knock-in mice carrying certain FHM mutations are consistent with a gain of function mediated by these mutations. To date, this issue has not been resolved. Similarly, patients with spinocerebellar ataxia type 6 show an ataxic phenotype due to a polyglutamine expansion in the $Ca_v2.1$ C-terminus (Zhuchenko et al. 1997) but, as in the case of FHM mutations, it is unclear whether this is due to increased or decreased P/Q-type channel activity. However, in general terms, any alteration in P/Q-type channel function is expected to produce pronounced effects on neurotransmitter release and synaptic function. Thus, increases and decreases in P/Q-type channel activity may well produce similar alterations in neuronal network function depending on whether inhibitory or excitatory inputs are affected.

There are also a number of murine models of P/Q-type channelopathies. *Leaner*, *Tottering, rolling Nagoya*, and *Rocker* mice all carry mutations in the $Ca_v2.1$ calcium channel α_1 subunit that result in either the substitution of individual amino acids or premature truncations of the channel protein. These mice tend to show an ataxic phenotype and, in some cases, absence seizures (Lorenzon et al. 1998; Mori et al. 2000; Noebels and Sidman 1979; Zwingman et al. 2001). In addition, several of these mutations cause cerebellar atrophy (Herrup and Wilczynski 1982). When introduced into recombinant channels, these mutations tend to reduce channel activity which is consistent with reduced P/Q-type currents in neurons isolated from these mouse lines.

To our knowledge, there are no identified N-type or R-type calcium channelopathies, which is consistent with the mild phenotype observed upon KO of the $Ca_v2.2$ or $Ca_v2.3$ genes.

5 Calcium Channel Pharmacology

5.1 Peptide Blockers

As mentioned earlier, the pharmacological profile of a given calcium channel is a key means of identifying a particular calcium channel subtype. While small organic molecules such as dihydropyridines have been used to definitively characterize L-type calcium channels (Bean 1984), peptide toxins isolated from fish-hunting marine snails, spiders, scorpions, and snakes have been an invaluable source of selective inhibitors of synaptic calcium channels (Doering and Zamponi 2005). The archetypal N-type calcium channel inhibitor is ω-conotoxin GVIA, a peptide isolated from the fish-hunting cone snail *Conus geographus*. This toxin docks at the outer vestibule of the channel and physically occludes the channel pore, thus preventing calcium entry (Ellinor et al. 1994). Block occurs with high affinity and selectivity over other types of calcium channels and is poorly reversible upon washout (Feng et al. 2001b). ω-Conotoxins MVIIA and CVID are isolated, respectively, from the venoms of *Conus magus* and *Conus catus*, and they too selectively inhibit N-type calcium channels via a pore blocking mechanism (Feng et al. 2001b; Fox 1995; Monje et al. 1993; Olivera et al. 1984). Compared with MVIIA, CVID has even higher selectivity for N-type over P/Q-type calcium channels. Both MVIIA and CVID have been used clinically to treat pain, via intrathecal injection, underscoring the importance of N-type calcium channels in synaptic transmission in the dorsal horn of the spinal cord. Although multiple structural domains likely contribute to conotoxin block, efforts to map the contoxin interaction site on the N-type calcium channel indicate that the domain III S5-S6 region is critical (Ellinor et al. 1994; Feng et al. 2001b). In addition, the ancillary α_2-δ subunits have been shown to reduce the affinity of N-type calcium channels for MVIIA and CVID (Mould et al. 2004).

Perhaps the best known peptide blocker of P/Q-type calcium channels is ω-agatoxin IVA, a peptide isolated from the venom of the North American funnel web spider *Agelenopsis aperta*. Unlike the conotoxins which are pore blockers, aga IVA functions as a gating inhibitor (McDonough et al. 1997). Application of aga IVA at concentrations as low as several nanomolars causes rapid inhibition of P-type channel activity and typically does not reverse upon washout (Adams et al. 1993; Turner et al. 1992). However, application of strong membrane depolarizations removes aga IVA block so that the channel effectively undergoes a large depolarizing shift in half activation potential – in essence, the toxin prevents activation gating but can be dislodged from its binding site by membrane depolarization (Mintz et al. 1992). The site of action of aga IVA appears to involve extracellular regions within domain IV of the $Ca_v2.1$ subunit (Winterfield and Swartz 2000). Indeed, alternate splicing of an asparagine-proline motif within the domain IV S3-S4 linker accounts for a 50-fold difference in aga IVA affinity between P-type and Q-type calcium channels (Bourinet et al. 1999). Other examples of peptide gating inhibitors include ω-grammotoxin SIVA (an N- and P/Q-type channel blocker) and SNX-482 (an R-type channel blocker), both isolated from different species of

tarantula. Interestingly, the effects of aga IVA and grammotoxin on P/Q-type channel activity are additive, indicating that they bind to separate sites on the channel (McDonough et al. 2002).

The 78 amino acid ω-agatoxin IIIA blocks P/Q-type channels with nanomolar affinity, but also potently inhibits L-type and N-type channels (Mintz 1994; Mintz et al. 1991). This toxin is much larger than the ω-conotoxins (which are typically 25–30 amino acids in length), but nonetheless acts by a pore blocking mechanism and is thus distinct in action from aga IVA (Mintz 1994). Yet, pore block by this peptide is incomplete suggesting that toxin-bound channels can still conduct a small amount of calcium. Due to its nonselective nature, and its failure to elicit complete channel block, this toxin is not very useful experimentally.

Aga IVA and ω-conotoxin GVIA are standard tools in elucidating the roles of P/Q-type and N-type calcium channels in synaptic transmission. In many types of synapses, application of either toxin may mediate moderate inhibition of neurotransmitter release, whereas co-application of both blockers may almost abolish synaptic transmission due to the nonlinear dependence of synaptic release on intracellular calcium concentration. On a final note, we should add that there are many other species of cone snails and spiders that produce active toxins which selectivity inhibit specific calcium channel subtypes (for example, ω-conotoxins GVIB, GVIC, GVIIA, SVIA, SVIB), and it is likely that many more remain to be discovered (Olivera et al. 1994).

5.2 Small Organic Compounds Blocking N-type Channels

Besides peptide toxins, presynaptic calcium channels can also be inhibited by divalent metal ions such as cadmium, and by a number of different classes of small organic molecules (Doering and Zamponi 2005). While there are, to our knowledge, no selective small organic inhibitors of P/Q-type calcium channels, substantial efforts are being made to develop small organic N-type channel blockers for use as analgesics which can be administered orally. Such compounds include 4-benzyloxyaniline piperidine derivatives, fatty acid like compounds such as farnesol, and peptidylamines (Hu et al. 1999a; Hu et al. 1999b; Hu et al. 1999c; Roullet et al. 1999) . High throughput screening continues to identify novel compounds which block N-type channel activity and which show efficacy as analgesics. However, in many cases the degree of selectivity of these compounds for N-type channels over other calcium channel subtypes is much less than that observed for peptide toxins.

The anticonvulsant gabapentin (1-(aminomethyl) cyclohexaneacetic acid) and its second-generation cousin pregabalin are highly effective as analgesics in rats and humans (Cheng and Chiou 2006; Zareba 2005). These compounds are unique in that they bind to the calcium channel α_2-δ subunit (Gee et al. 1996). Transgenic animals in which the putative gabapentin binding site on this subunit has been eliminated are resistant to the effects of gabapentin. It remains controversial as to whether the

gabapentin-α_2-δ interaction does indeed reduce voltage gated calcium currents, or whether the analgesic effects of gabapentin are mediated by a different mechanism.

6 Regulation by G Protein–Coupled Receptors

It is well established that activation of G protein–coupled receptors (GPCRs), in particular those that couple to the $G\alpha_{i/o}$ subunit, can result in potent inhibition of both N-type and P/Q-type calcium channels (Figure 2) (Beech et al. 1992; Bernheim et al. 1991; Caulfield et al. 1994; Dunlap and Fischbach 1981; Golard and Siegelbaum 1993; Ikeda 1992; Ikeda and Schofield 1989; Lipscombe et al. 1989; Mintz and Bean 1993; Shapiro and Hille 1993; Zhu and Ikeda 1993). The physio-

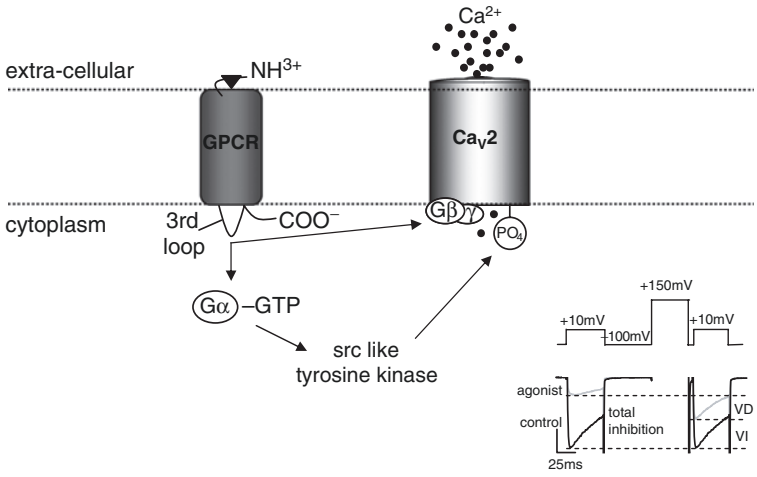

Fig. 2 Voltage-dependent (VD) and voltage-independent (VI) G-protein mediated inhibition of synaptic calcium channels. In the absence of agonist, the heterotrimeric G-proteins, $G\alpha\beta\gamma$, bind GDP and associate with the third intracellular loop of seven trans-membrane helix spanning G-protein coupled receptors (GPCRs). Agonist (▼) activation of the receptor results in an exchange of GDP for GTP on the G protein α-subunit. This exchange leads to dissociation of $G\alpha$-GTP and $G\beta\gamma$ subunits, which are subsequently free to act on downstream effectors such as enzymes or ion channels. $G\beta\gamma$, which remains associated with the plasma membrane, binds to N- and P/Q-type calcium channels on the α_1 subunit, stabilizing the closed conformation of the channel and resulting in loss of current activity which is voltage-dependent (VD). Liberated $G\alpha$-GTP is cytoplasmic and activates enzymes such as adenylyl cyclase. Subsequent phosphorylation of synaptic calcium channels on the α_1 subunit leads to voltage-independent (VI) inhibition of channel activity. **Inset**: In the presence of GPCR agonist (grey trace), a step to $+10\,\mathrm{mV}$ results in inhibition of control current (black trace). VD and VI inhibition can be separated by applying a strong, depolarizing pre-pulse (i.e. $+150\,\mathrm{mV}$) immediately prior to the test pulse. This pre-pulse relieves $G\beta\gamma$-mediated VD inhibition, by physical removal of $G\beta\gamma$ from the channel, and remaining current inhibition can thus be attributed to VI inhibition.

logical significance of this modulation is underscored by the action of opiates in the pain pathway, where activation of μ-opioid receptors by morphine mediates analgesia in part by inhibition of N-type calcium channel activity (Altier and Zamponi 2004). GPCR-mediated inhibition may have both voltage-dependent (VD) and voltage-independent (VI) components (Figure 2). VD inhibition involves the binding of G protein βγ subunits to a cytoplasmic site on the calcium channel α_1 subunit, thereby stabilizing the closed conformation of the channel (Figure 2) (Herlitze et al. 1996; Ikeda 1996). This inhibition is referred to as VD because it is favored at hyperpolarized potentials and can be relieved by strong membrane depolarizations or rapid trains of action potentials, thus allowing for activity-dependent dis-inhibition (Bean 1989; Hille 1994; Zamponi and Snutch 1998a, 1998b). The degree of VD inhibition depends on the calcium channel subtype, with N-type channels typically undergoing a larger degree of inhibition than P/Q-types (Currie and Fox 1997), and on the G protein β subunit isoform (Arnot et al. 2000; Garcia et al. 1998; Ruiz-Velasco and Ikeda 2000; Zhou et al. 2000). There may also be a subtle G protein γ subunit dependent component to N-type channel regulation (Ruiz-Velasco and Ikeda 2000). Furthermore, distinct Gβ subunit structural determinants appear to underlie modulation of these two channel types (Doering et al. 2004; Ford et al. 1998; Mirshahi et al. 2002a; Mirshahi et al. 2002b; Tedford et al. 2006). With different types of GPCRs coupling to specific subsets of G protein βγ subunits, differing degrees of VD inhibition of both calcium channel subtypes can thus be elicited. In addition, the extent of VD inhibition of both N- and P/Q-type channels appears to be dependent on the type of β subunit that is present in the calcium channel complex (Barrett and Rittenhouse 2000; Bourinet et al. 1996; Canti et al. 2000; Feng et al. 2001a). While many of the molecular details governing this regulation have been described over the past decade and have been reviewed in recent literature (Dolphin 2003b) new and important details concerning the structural underpinnings of VD inhibition are still emerging. For example, although both the calcium channel domain I-II linker and N-terminal regions have long been implicated in G protein modulation, it is now known that an interaction between these two regions is necessary for VD modulation (Agler et al. 2005). It is not completely resolved as to whether G protein βγ subunits facilitate the interaction between the N-terminus and the I-II linker region, or whether this interaction is needed to allow Gβγ binding to the channel. It has also been recently demonstrated that the C-terminal region of N-type calcium channels serves to enhance the affinity of the channel for Gβγ (Li et al. 2004), suggesting that a complex interplay among multiple cytoplasmic regions of the channel underlies VD inhibition. The aforementioned calcium channel β subunit dependence of VD G protein modulation may be due to the close proximity of the calcium channel β subunit interaction site and putative G protein binding sites within the domain I-II linker region (De Waard et al. 1997; Zamponi et al. 1997). However, the underlying mechanism remains subject to investigation. Indeed, there are differing views as to whether G protein βγ subunits compete with the calcium channel β subunit for binding, and whether or not G protein interactions with the channel cause dissociation of the calcium channel β subunit from the channel complex. However, irrespective of these molecular details, it is clear that G protein βγ subunits interact with cyto-

plasmic regions of the calcium channel α_1 subunit resulting in a reduction of N- and P/Q-type channel activity that culminates in reduced calcium entry into the cell and thus reduced neurotransmitter release.

VD G protein modulation can be fine tuned by activation of other intracellular signaling cascades. For example, the activation of protein kinase C (PKC), which regulates channel activity directly, also interferes with the ability of N-type channels to undergo VD inhibition (Barrett and Rittenhouse 2000; Swartz 1993; Swartz et al. 1993). Intriguingly, this effect is dependent on the G protein $\beta\gamma$ subunit subtype, so that only $G\beta_1$-mediated responses are antagonized (Cooper et al. 2000). This is due to a unique pair of asparagine residues localized to the N-terminal helix of the $G\beta_1$ subunit (Doering et al. 2004). The structural basis for this effect has been resolved, as it has been shown that a single threonine residue located in the putative G protein $\beta\gamma$ subunit interaction site is the key locus for PKC-mediated effects (Hamid et al. 1999). In essence, this cross talk allows the channel to integrate two separate second messenger pathways in order to modulate current activity in a kinase- and $G\beta$ subunit-specific manner. The complexity of VD G protein modulation may allow synapses to precisely regulate the amount of calcium entering cells, and thus to adjust synaptic output.

In addition to VD $G\beta\gamma$ mediated inhibition of N-type channel activity, GPCR-induced VI modulation has been described for N-type calcium channels (Figure 2) (Beech et al. 1992; Beedle et al. 2004; Bernheim et al. 1991; Delmas et al. 1998a; Delmas et al. 1998b; Kammermeier et al. 2000; Schiff et al. 2000; Shapiro et al. 1999; Surmeier et al. 1995). VI inhibition is insensitive to membrane depolarizations and occurs over a slower time course than VD inhibition, implicating intracellular signaling cascades such as tyrosine kinase activation in this process. Furthermore, for P/Q-type channels, a form of VI modulation has been shown to involve an association of the G protein α_o subunit with the $Ca_v2.1$ C-terminus (Kinoshita et al. 2001). More recently, two studies have revealed a novel form of VI inhibition of N-type calcium channels that involves physical removal of calcium channels from the plasma membrane. N-type calcium channels and nociceptin (or ORL1) receptors have been shown to form stable, physical signaling complexes (Beedle et al. 2004). Prolonged agonist application results in internalization of the entire receptor-channel complex and to its subsequent degradation in lysosomes (Altier et al. 2006). The resultant loss of channel numbers in the plasma membrane and reduced current densities are, in effect, a form of VI inhibition. Channel internalization has also been shown to occur in response to $GABA_B$ receptor activation, although by an entirely different molecular mechanism that involves tyrosine kinase activity and cytoskeletal elements (Tombler et al. 2006). Moreover, this effect spontaneously reverses with receptor desensitization. That said, as in the case of nociceptin receptor activation, numbers of channels in the plasma membrane are reduced. It is likely that other types of GPCRs show similar receptor-mediated channel trafficking, and that this mechanism may emerge as a novel means of regulating N-type channels and thus synaptic activity.

7 Regulation of Calcium Channels by Synaptic Proteins

The activity of N-type and P/Q-type calcium channels is also regulated by proteins that form part of the synaptic vesicle release machinery (Figure 3). These channel subtypes contain a specific synaptic protein interaction site (termed *synprint*) in the

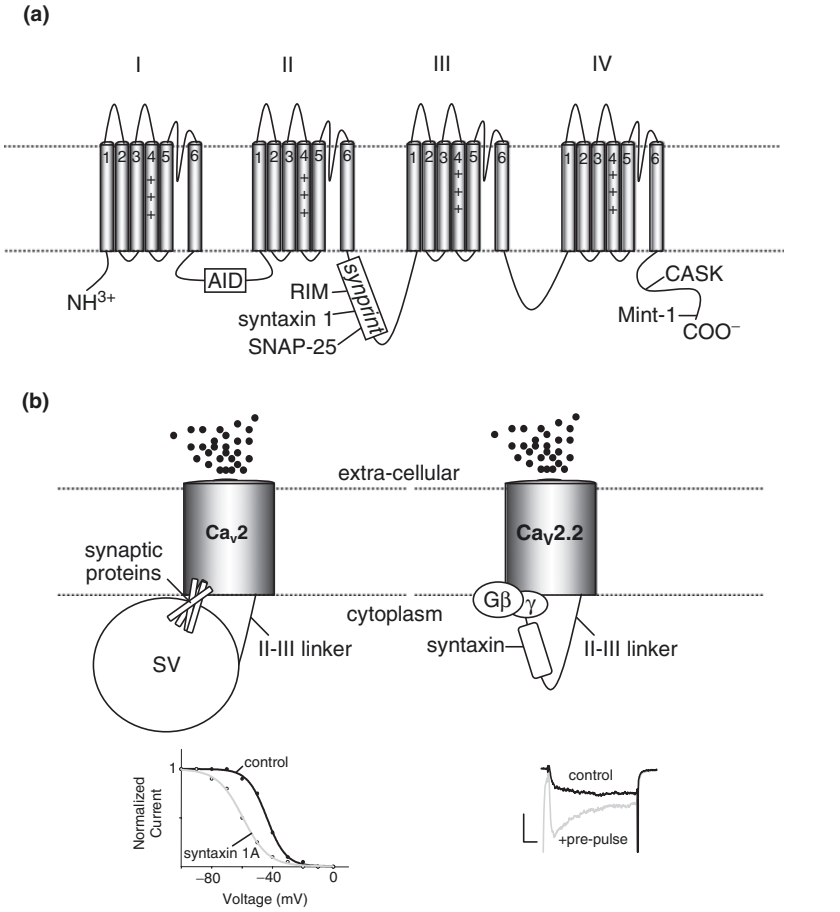

Fig. 3 Regulation of calcium channels by synaptic proteins. (**a**) Putative interactions sites of synaptic and adaptor proteins with Ca_v2 channels are indicated on the calcium channel α_1 subunit. RIM, syntaxin 1, and SNAP-25 are believed to interact with synaptic calcium channels on the II-III linker *synprint* region, while CASK and Mint-1 are thought to interact with the α_1 C-terminus. (**b**) The physical associations between calcium channels and synaptic proteins are believed to colocalize synaptic vesicles (SVs) to sources of cellular calcium entry (left). In addition, several of these Ca_v2-synaptic protein interactions are believed to reduce channel activity directly by shifting half-inactivation channel kinetics to more hyperpolarized potentials (bottom, left). In the absence of GPCR activation, N-type calcium channel interactions with syntaxin 1A are speculated to result in tonic, Gβγ-mediated channel inhibition. This tonic, Gβγ-mediated inhibition is evident following its removal by a strong depolarizing pre-pulse (bottom, right).

intracellular loop linking domains II and III (Sheng et al. 1994). However, it is important to note that there are channel splice variants that lack this region and that, although invertebrates are capable of neurotransmission, no equivalent *synprint* region is found in invertebrate synaptic calcium channel homologs (Spafford and Zamponi 2003; Zamponi 2003). In the case of the N-type calcium channel, the *synprint* region is known to bind syntaxin 1, SNAP-25, and Rim (Coppola et al. 2001; Jarvis and Zamponi 2001b; Sheng et al. 1994; Sheng et al. 1997). The P/Q-type channel *synprint* region also interacts with syntaxin 1 and SNAP-25, however, unlike the N-type channel, it also binds synaptotagmin 1 (Charvin et al. 1997). The *synprint* motif in these channels appears to serve multiple functions. It has been suggested that the interactions between the *synprint* region and the synaptic proteins help to co-localize synaptic vesicles to sources of calcium entry (Figure 3ii) (Zamponi 2003). There is also evidence that the *synprint* region is involved in targeting calcium channels to presynaptic sites, as substitutions or deletions to this region lead to a loss of presynaptic localization of both N- and P/Q-type channels (Harkins et al. 2004; Mochida et al. 2003). However, it should be noted that other regions of the channels, such as the C-terminus by virtue of its interactions with the adaptor proteins Mint-1 and CASK, appear equally critical for synaptic targeting (Maximov and Bezprozvanny 2002; Spafford et al. 2003). Finally, the *synprint* region appears to serve as an important modulatory site that allows synaptic proteins to regulate channel activity *per se*. In both transient expression systems and neurons, binding of syntaxin 1 and SNAP-25 results in a hyperpolarizing shift in the half-inactivation potential of both P/Q-type and N-type calcium channels, thus reducing channel availability (Figure 3ii) (Bezprozvanny et al. 1995; Jarvis and Zamponi 2001a; Stanley 2003). This negative shift is ablated in the concomitant presence of both proteins (and in the case of P/Q-type channels, in the presence of synaptotagmin) (Zhong et al. 1999), is abolished in the presence of munc-18, and does not occur when syntaxin is in its "open" conformation. Because the association between syntaxin 1 and munc-18, as well as the conformational state of syntaxin, vary during the vesicle release cycle, channel availability can be dynamically regulated at various stages in the neurotransmitter release process. This in turn may help to ensure the appropriate amount of calcium entering the nerve terminal during neurotransmission.

Our group also identified a second action of syntaxin 1A (but not 1B) on N-type calcium channels. Co-expression of this protein with N-type calcium channels results in a tonic G protein–mediated inhibition of channel activity that does not involve receptor activation (Jarvis et al. 2000). This appears to be due to a syntaxin-mediated co-localization of the channel and Gβγ that ultimately culminates in tonic channel inhibition (Figure 3ii). Indeed, syntaxin 1A binds to Gβγ *in vitro* on a region that is distinct from the Gβγ site involved in interactions with the *synprint* motif. These data are consistent with the idea of a sandwich formed by the channel, syntaxin and Gβγ (Jarvis et al. 2002). Intriguingly, although syntaxin 1B can also bind to Gβγ *in vitro*, no tonic G protein inhibition of channel activity ensues (Lu et al. 2001). This suggests that, compared with syntaxin 1A, syntaxin 1B adopts a slightly different channel-bound conformation. The syntaxin-mediated effect on G protein inhibition appears to be much more robust than the effect on channel

availability as it occurs when syntaxin is in the open conformation and is maintained upon coexpression of SNAP-25. Furthermore, although binding of syntaxin 1A to the *synprint* region is destabilized upon activation of PKC (Yokoyama et al. 2005; Yokoyama et al. 1997), the functional effects of PKC activation are limited to elimination of syntaxin-mediated channel availability and not to G protein inhibition (Jarvis and Zamponi 2001a, 2005). For completeness, we note that a similar SNARE protein dis-inhibition of P/Q-type calcium channels is observed for cyclin-dependent kinase 5, which has been shown to increase P/Q-type channel activity by blocking interactions of the channel with SNAP-25 (Tomizawa et al. 2002).

It is worth noting that the binding interaction between synaptic proteins and $G\beta\gamma$ may allow G proteins to regulate synaptic activity independently of N-type channel activity. Taken together, the interactions between calcium channels, G proteins, kinases, and synaptic proteins such as syntaxin provide for complex mechanisms that regulate N-type calcium channel activity both in expression systems and in neurons.

8 Regulation of Presynaptic Calcium Channel Activity by Protein Kinases

Many types of kinases have been shown to regulate calcium channel activity, and this topic in itself would be sufficient for a free-standing chapter. Here, we will give only a few select examples of the major pathways of this type of presynaptic calcium channel regulation.

PKC is an important regulator of presynaptic calcium channel activity. PKC activity has been shown to upregulate R-type calcium channels (Kamatchi et al. 2000; Shekter et al. 1997; Stea et al. 1995), as well as to increase N-type calcium channel activity (Hamid et al. 1999; Stea et al. 1995). This, in addition to the aforementioned antagonistic effects of PKC on syntaxin and VD G protein inhibition of N-type channels, produces an increase in N-type current activity in response to PKC activation. It has recently been shown that the PKC binding protein, enigma homolog (ENH) serves to co-localize PKC-ε and N-type channels to facilitate PKC-dependent phosphorylation of the $Ca_v2.2$ subunit (Maeno-Hikichi et al. 2003), indicating the formation of a macromolecular signaling complex. In contrast, there is little direct PKC regulation of P/Q-type calcium channels (Wu et al. 2002).

Protein kinase A (PKA) has been reported to increase P/Q-type channel activity in both neurons (Huang et al. 1998) and in transient expression systems (Fournier et al. 1993; Fukuda et al. 1996; Kaneko et al. 1998). In contrast, N-type calcium channels appear to be relatively insensitive to PKA. PKA has been shown to regulate P/Q-type calcium channel activity indirectly by interfering with phosphatidylinositol 4,5 biphosphate (PIP_2) mediated regulation (Wu et al. 2002). Under normal circumstances, PIP_2 slows rundown of P/Q-type channel activity and produces VD current inhibition. Activation of PKA selectively antagonizes the VD inhibition mediated by PIP_2, perhaps by preventing PIP_2 binding to the inhibitory site on the channel while sparing PIP_2 action on the site linked to current rundown. Once again,

this cross talk underscores the ability of voltage-gated calcium channels to integrate multiple signaling pathways.

Calcium channel β subunits are also targets for phosphorylation events. It was recently reported that a phosphatidylinositol 3-kinase dependent elevation of phosphatidyl-inositol-4,5 triphosphate (PIP_3) is critical for the ability of the calcium channel β_{2a} subunit to promote N-type calcium channel trafficking to the plasma membrane (Viard et al. 2004). Increased levels of PIP_3 result in activation of protein kinase B, which phosphorylates β_{2a} and allows for trafficking of N-type channels to the plasma membrane. Along similar lines, it has been shown that Ras/extracellular signal–regulated kinase (ERK) phosphorylation of both the α_1 and the β_{1b} subunit mediates an upregulation of $Ca_v2.2$ current activity (Martin et al. 2006). Collectively, these findings clearly underscore the importance of ancillary calcium channel subunits as targets for second messenger regulation.

9 Feedback Regulation by Calcium Binding Proteins

Another important regulatory mechanism of calcium channel activity is via calcium binding proteins such as calmodulin (Figure 4) (Halling et al. 2005). It is well established that L-type calcium channels undergo robust calcium-dependent inactivation (CDI) – a reduction of current activity in response to elevated intracellular calcium levels which serves to protect cells form calcium overload (Imredy and Yue 1994). In the absence of calcium ions, calmodulin is pre-associated with the C-terminus of the channel at a highly conserved region containing pre-IQ and IQ motifs. Upon calcium entry into the cell, calmodulin binds Ca^{2+}, accelerating the inactivation kinetics of the channel. CDI in L-type channels requires the high-affinity calcium binding sites of calmodulin, thus being relatively resistant to standard calcium buffers (Soldatov 2003). Although CDI was once believed to be a unique property of L-type channels, it was subsequently shown that all types of HVA channels are subject to this process (Lee et al. 1999a; Liang et al. 2003). However, unlike L-type channels, R-, N-, and P/Q-type channels undergo CDI only when calcium is weakly buffered (Figure 4) (Liang et al. 2003). Indeed, the CDI process in these channels involves the low-affinity calcium binding sites of calmodulin. This key difference means that calcium entry through an individual L-type channel is sufficient to induce CDI, whereas CDI in non-L-type HVA channels requires global rises in intracellular calcium, increases generated by calcium entry though numerous channels.

P/Q-type channels undergo a second calcium-dependent process whereby current activity increases following a strong membrane depolarization or a train of action potentials. This increase is only observed in the presence of external calcium, is insensitive to calcium buffers, and requires the high-affinity calcium binding sites on calmodulin (Chaudhuri et al. 2004; DeMaria et al. 2001). This process, termed calcium-dependent facilitation (CDF), is not observed with N-type or R-type channels and is $Ca_v2.1$ channel splice isoform dependent (Chaudhuri et al. 2004). The

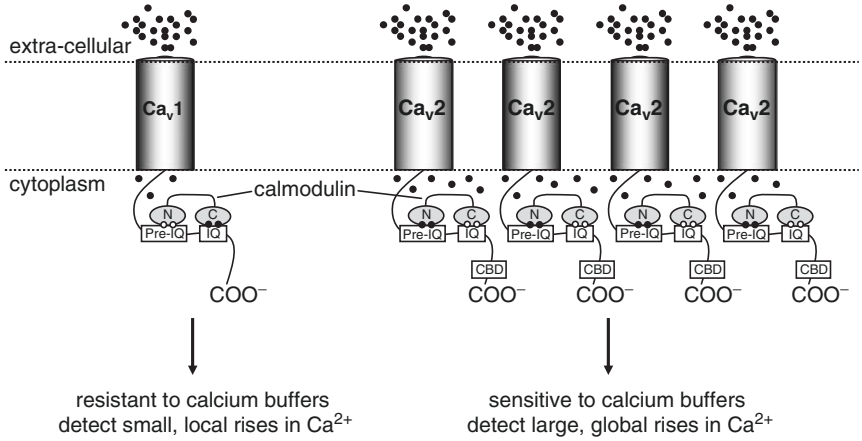

Fig. 4 Modulation of calcium channels by calcium binding proteins. Calcium-dependent inactivation (CDI) in L-type channels (Ca_v1) involves binding of calcium ions (•) to the high-affinity binding sites located on the C-lobe of calmodulin. In contrast, CDI in synaptic calcium channels involves binding of calcium ions to the low-affinity binding sites located on the N-lobe of calmodulin. Both types of CDI result in a loss of current activity, in response to calcium, as the inactivation kinetics of the channel are accelerated. However, because of the difference in occupation of low- versus high-affinity calcium binding sites, between channel types, different forms of CDI exhibit different properties. In the case of L-type channels, CDI is resistant to calcium buffers and detects small, local rises in calcium generated by a single ion channel (left), whereas, in the case of synaptic channels, CDI is sensitive to standard calcium buffers and detects large, global rises in calcium which require calcium entry through numerous channels (right). P/Q-type channels are also subject to calcium-dependent facilitation (CDF), and to modulation by the calcium binding proteins, calcium binding protein 1 (CaBP1) and visin-like protein 2 (VILIP-2). Both of these proteins bind to the calcium binding domain (CBD) on the channel's C-terminus. In contrast to calmodulin, CaBP1 antagonizes calcium channel opening in a calcium-independent manner, and VILIP-2 slows the inactivation kinetics of the channel.

distinct roles of the high- and low-affinity sites of calmodulin in CDF and CDI may thus manifest themselves in an initial upregulation of P/Q-type channel activity, followed by negative feedback inhibition as intracellular calcium concentration is increased.

We now know of other types of calcium binding proteins that mediate similar feedback regulation of P/Q-type calcium channels, including calcium binding protein 1 (CaBP1) and visin-like protein 2 (VILIP-2). Both of these proteins bind to the calcium binding domain (CBD) within the channel C-terminus and, compared with calmodulin, differentially regulate channel activity. CaBP1 antagonizes channel opening in a calcium-independent manner (Lee et al. 2002), whereas VILIP-2 slows the inactivation kinetics of the channel (Lautermilch et al. 2005). Both proteins must undergo myristoylation in order to mediate their effects (Few et al. 2005). To our knowledge, this type of regulation has only been described for P/Q-type calcium channels.

Overall, the regulation of presynaptic calcium channels by different types of calcium binding proteins may provide for mechanisms by which neurons can fine tune the amount of calcium entering presynaptic nerve terminals, shift the relative contributions of N-type and P/Q-type channels to calcium entry, and thus regulate the amount of neurotransmitter that can be released from the synapse.

10 Summary

The entry of calcium into neurons via presynaptic calcium channels is a key step in evoked neurotransmitter release. Compromised calcium channel function can lead to severe neurological consequences, and yet the pharmacological inhibition of specific calcium channel subtypes can be beneficial in the treatment of conditions such as neuropathic pain. Because of the importance of these channels, neurons have evolved complex means for regulating calcium channel activity, including activation of second messenger pathways by G protein coupled receptors and feedback inhibition by calcium binding proteins. By these means, neurons are able to maintain the fine balance of cytoplasmic calcium levels that is required for optimal neurotransmitter release.

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Pharmacology of Neurotransmitter Transport into Secretory Vesicles

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Abstract Many neuropsychiatric disorders appear to involve a disturbance of chemical neurotransmission, and the mechanism of available therapeutic agents supports this impression. Postsynaptic receptors have received considerable attention as drug targets, but some of the most successful agents influence presynaptic processes, in particular neurotransmitter reuptake. The pharmacological potential of many other presynaptic elements, and in particular the machinery responsible for loading transmitter into vesicles, has received only limited attention. The similarity of vesicular transporters to bacterial drug resistance proteins and the increasing evidence for regulation of vesicle filling and recycling suggest that the pharmacological potential of vesicular transporters has been underestimated. In this review, we discuss the pharmacological effects of psychostimulants and therapeutic agents on transmitter release.

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1 Introduction: Neurotransmitter Recycling

Synaptic transmission involves the exocytotic release of neurotransmitter from a presynaptic site and the activation of specific receptors on the membrane of postsynaptic or surrounding cells. Given the high rates of firing observed for many neuronal populations, sustained signaling also requires replenishment of the released transmitter. Indeed, the nerve terminal expresses a series of transport activities designed to recycle transmitter. Among these activities, one class confers the reuptake of dopamine and other classical transmitters across the presynaptic plasma membrane, regulating their levels in the synaptic cleft, and recycling them for subsequent release (Figure 1) (Raiteri et al., 2002; Torres et al., 2003). Although the amino acid transmitters glutamate and particularly GABA recycle in part by reuptake into the nerve terminal, most of the released glutamate and a substantial proportion of the released GABA undergo transport into surrounding glial cells, where they are converted into glutamine (Danbolt, 2001; Bak et al., 2006). Transported back to the nerve terminal, glutamine serves to regenerate both glutamate and GABA (through conversion from glutamate by glutamic acid decarboxylase) (Figures 1C, 1D) (Chaudhry et al., 2002). Essentially all of the plasma membrane neurotransmitter transporters rely on a Na^+ gradient produced by the Na^+ pump, cotransporting Na^+ with transmitter, but vary in the stoichiometry of ionic coupling and in coupling to other ions such as chloride.

The exocytotic release of classical transmitters requires their transport into synaptic vesicles. In contrast to the plasma membrane transporters, the vesicular transport activities depend on a H^+ gradient created by the vacuolar-type H^+-ATPase rather than a Na^+ gradient, and function as H^+ exchangers. Biochemical studies have also revealed multiple, distinct vesicular transport activities. The monoamines dopamine, norepinephrine, and serotonin enter secretory vesicles through a common carrier. Distinct activities have been identified for the vesicular transport of acetylcholine (ACh), GABA, and glutamate (Schuldiner et al., 1995; Liu and Edwards, 1997). Well-characterized drugs acting on plasma membrane transporters do not affect the vesicular activities, but a variety of other drugs inhibiting vesicular transport have dramatic effects on brain function, and psychostimulants also interact with vesicular transport mechanisms.

Molecular identification of the proteins mediating vesicular neurotransmitter transport has revealed similarity to bacterial proteins, rather than to plasma membrane transporters (Figure 2). In contrast to concentrative uptake by plasma membrane transporters, the direction of vesicular transport resembles the efflux of many drugs from bacteria. Indeed, the first vesicular transmitter transporter was identified by expression cloning using selection in the neurotoxin N-methyl-4-phenylpyridinium (MPP^+) (Liu et al., 1992). The vesicular monoamine transporters protect against MPP^+ by sequestering the toxin inside vesicles, away from its primary site of action in mitochondria. The vesicular transporters thus represent important drug targets with a diversity of potential applications.

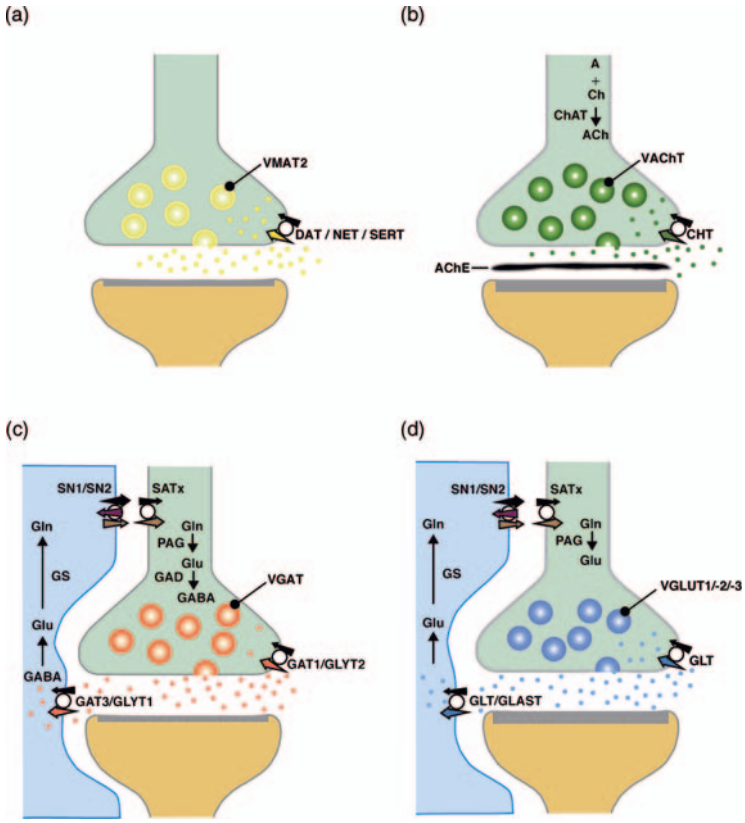

Fig. 1 Neurotransmitter recycling. Classical neurotransmitters are released by exocytosis from the nerve terminal, and act on specific postsynaptic receptors. The signal is terminated and transmitter recycled by specific transporter proteins located at the nerve terminal and/or in surrounding glial cells. Transport by these proteins is generally energized by the electrochemical gradient of Na^+. (**a**) Three distinct transporters mediate transport of monoamines across the plasma membrane. The dopamine transporter DAT, the norepinephrine and epinephrine transporter NET and the serotonin transporter SERT, are responsible for the reuptake of released monoamine at dopaminergic, noradrenergic and serotonergic nerve terminals (yellow arrow), respectively. The vesicular monoamine transporter VMAT2 then recognizes all of these monoamines and transports them inside synaptic vesicles for subsequent exocytotic release. (**b**) Cholinergic signaling is terminated by metabolism of acetylcholine (ACh) to the inactive choline and acetate by acetylcholinesterase (AChE) located in the synaptic cleft. Choline (Ch) is transported back into the nerve terminal (green arrow) by the choline transporter (CHT), where choline acetyltransferase (ChAT) subsequently catalyzes acetylation (**a**) of choline to reform ACh. (**c**) At GABAergic and glycinergic nerve terminals, the GABA transporter (GAT1) and the glycine transporter (GLYT2) recycle GABA and glycine (red arrow), respectively. In addition, GABA may be taken up by surrounding glial cells (e.g. by GAT3), and converted to glutamine which is transported back to the nerve terminal by the concerted action of the system N (SNs) and system A transporters (SATs) (brown arrows). The glial transporter GLYT1 also contributes to the clearance of glycine. (**d**) Reuptake of glutamate at glutamatergic terminals has recently been demonstrated for a GLT isoform (blue arrow). However, the majority of glutamate is accumulated by surrounding glial cells (e.g., by GLT and GLAST) for conversion to glutamine which is subsequently transported back to the nerve terminals. System N transporters and a system A transporter (SATx) enriched in glutamatergic neurons mediate glutamine transfer from glia to excitatory neurons (brown arrows).

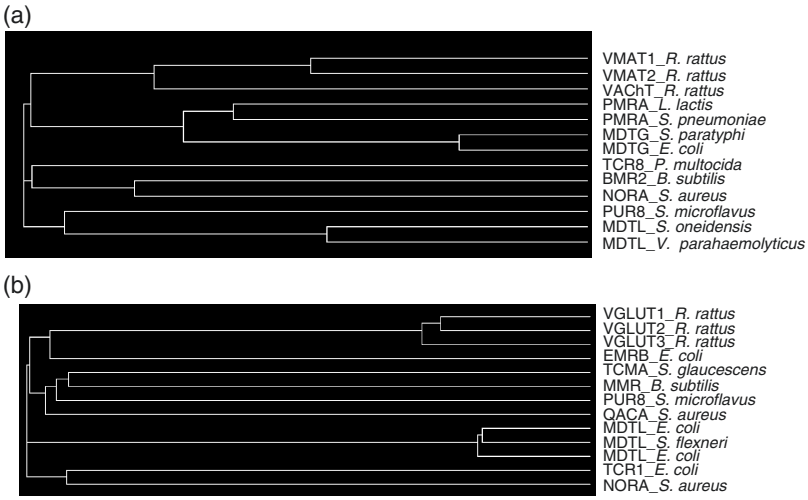

Fig. 2 Cladogram showing sequence relationship of the vesicular transporters to a selected number of bacterial drug resistance proteins. (**a**) VMAT1, VMAT2 and VAChT are highly homologous. They are more distantly related to a variety of bacterial drug resistance proteins. PMRA, multidrug resistance efflux pump pmrA; MDTG, multidrug resistance protein mdtG; TCR8, a tetracycline resistance protein, class H (TETA(H)); BMR2, multidrug resistance protein 2; NORA, quinolone resistance protein norA; MDTL, multidrug resistance protein mdtL. (**b**) The rat homologs VGLUT 1, 2 and 3 show sequence similarity to a set of bacterial drug resistence proteins: EMRB, multidrug resistance protein B; TCMA, tetracenomycin C resistance and export protein; MMR, methylenomycin A resistance protein (MMR peptide); PUR8, puromycin resistance protein pur8; QACA, antiseptic resistance protein; MDTL, multidrug resistance protein mdtL; TCR1, tetracycline resistance protein, class A (TETA(A)); NORA, Quinolone resistance protein norA. *L. lactis*: *Lactococcus lactis*; *S. pneumoniae*: *Streptococcus pneumoniae*; *S. paratyphi*: *Salmonella paratyphi*; *E. coli*: *Escherichia coli*; *P. multocida*: *Pasteurella multocida*; *B. subtilis*: *Bacillus subtilis*; *S. aureus*: *Staphylococcus aureus*; *S. microflavus*: *Streptomyces microflavus*; *S. oneidensis*: *Shewanella oneidensis*; *V. parahaemolyticus*: *Vibrio parahaemolyticus*; *S. glaucescens*: *Streptomyces glaucescens*; *S. flexneri*: *Shigella flexneri*..

2 H^+ Electrochemical Gradient

2.1 Vacuolar H^+*-ATPase*

An H^+ electrochemical gradient ($\Delta\mu_{H+}$) provides the energy required for active transport of all classical neurotransmitters into synaptic vesicles. The Mg^{2+}-dependent vacuolar-type H^+-ATPase (V-ATPase) that produces this gradient resides on internal membranes of the secretory pathway, in particular endosomes and lysosomes (vacuole in yeast) as well as secretory vesicles (Figure 3). In terms of both structure and function, this pump resembles the F-type ATPases of bacteria, mitochondria and chloroplasts, and differs from the P-type ATPases expressed at the plasma membrane of mammalian cells (e.g., the Na^+/K^+-, gastric H^+/K^+- and muscle Ca^{2+}-ATPases) (Forgac, 1989; Nelson, 1992). The vacuolar and F0F1

ATPases are multisubunit complexes comprising a cytoplasmic complex (V_1 or F_1) that hydrolyzes or synthesizes ATP and a membrane-bound complex (V_0 or F_0) which translocates H^+ (Wilkens, 2005; Beyenbach and Wieczorek, 2006). Subunit c of V_0 consists of six proteolipid subunits that form an H^+-binding ring, and the ring rotates during ATP hydrolysis and H^+ pumping. The macrolide antibiotics bafilomycin and concanamycin inhibit the V-ATPase by intercalating between helices of the c subunit and preventing their rotation (Figure 3) (Bowman et al., 2004).

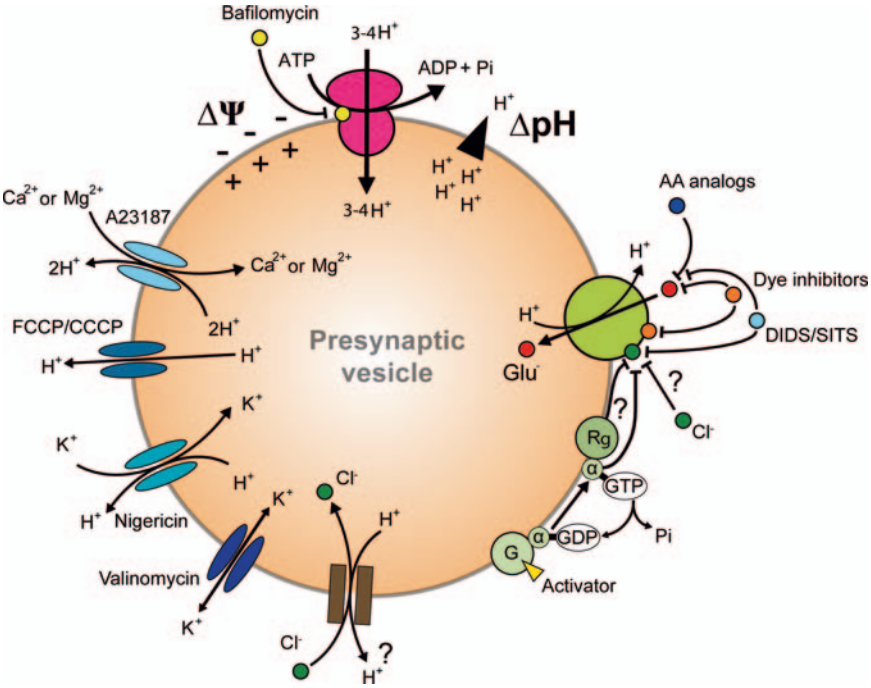

Fig. 3 Pharmacological targeting of VGLUT containing vesicles. Schematic representation of a VGLUT-expressing vesicle and the main activators/inhibitors of vesicular glutamate uptake. The Mg^{2+}-dependent v-ATPase creates an electrochemical gradient of protons ($\Delta\mu_{H+} = \Delta\Psi + \Delta pH$) by coupling the energy produced by hydrolysis of an ATP to proton translocation into synaptic vesicles. Bafilomycin inhibit the V-ATPase by intercalating between helices of the c subunit of V_0 and preventing their rotation. The vesicular glutamate transporter couples the transport of H^+ down its electrochemical gradient to glutamate flux into synaptic vesicles. Several glutamate analogues, dyes and other compounds compete at the glutamate binding sites, or noncompetitively at distinct sites on the transport proteins. VGLUTs have an allosteric chloride binding site and may mediate chloride flux. Several inhibitors, such as DIDS/SITS, block the chloride binding site. Heterotrimeric G proteins can interact directly or through an as yet unidentified regulator (Rg) protein of the chloride conductance. Chloride channels, such as ClC-3, have been demonstrated on the vesicle membrane. Recent studies suggest that these proteins may be H^+ exchangers rather than channels, and yet still mediate net vesicle acidification. Finally, different ionophores such as valinomycin, nigericin, FCCP/CCCP and A23187 can act on the vesicle membrane to perturb the electrochemical gradient of protons and thus the vesicular uptake of glutamate.

These drugs have thus been used extensively to generate models for the function of V-ATPases. Hydrolysis of ATP by the vesicular H^+-ATPase is coupled to movement of H^+ into the vesicle lumen, creating a H^+ electrochemical gradient. Measurements from cholinergic and aminergic vesicles show a ΔpH $\sim$1.4 units and an electrical potential $\sim +39\,\mathrm{mV}(\Delta\Psi)$ (Whittaker, 1987; Schuldiner et al., 1998).

2.2 Chloride Channels

The vesicular H^+-ATPase is potently regulated by Cl^-. Influx of Cl^- neutralizes the positive charge on luminal H^+ and hence dissipates the electrical gradient, allowing the H^+ pump to transport more H^+ and thereby increase ΔpH. Other mechanisms may serve to increase $\Delta\Psi$ at the expense of ΔpH. The two components of the H^+ electrochemical gradient may thus be regulated independently. Interestingly, the activity of different vesicular neurotransmitter transporters depends to differing extents on the two components of the H^+ electrochemical gradient. The transport of monoamines and acetylcholine (ACh) depends primarily on the chemical component ΔpH, glutamate transport mostly on the membrane potential $\Delta\Psi$, and GABA transport more equally on both components of the H^+ electrochemical gradient (Johnson, Jr., 1988b; Parsons et al., 1993; Carlson et al., 1989; Maycox et al., 1988; Kish et al., 1989; Hell et al., 1990; Chaudhry et al., 2008). The expression of vesicular chloride channels or activities that influence $\Delta\mu_{H+}$ might thus be expected to vary in vesicles that store different neurotransmitters, but this remains unknown. The intracellular chloride channels (ClC-3, -4, -5, -6 and -7) are widely expressed, but ClC-3 has a particularly high level of expression in the brain, and disruption of ClC-3 leads to impaired vesicular acidification and to neural degeneration (Stobrawa et al., 2001) through mechanisms that remain unclear and may involve expression on the postsynaptic plasma membrane (Wang et al., 2006). Indeed, it has recently become clear that many of the intracellular ClCs may function as Cl^-/H^+ exchangers rather than Cl^- channels (Figure 3) (Accardi et al., 2004; Scheel et al., 2005; Picollo and Pusch, 2005). Although it seems counterintuitive that influx into the vesicles of Cl^- through a Cl^-/H^+ exchanger would mediate vesicle acidification, the shown stoichiometry of 2 Cl^-: 1 H^+ would move relatively large amounts of charge and still enable net acidification by secondary activation of the H^+ pump. Why then couple the entry of chloride to proton efflux? Given the activity of the H^+-ATPase, perhaps it serves to concentrate chloride inside vesicles, for a function that is not yet understood (Jentsch, 2006).

2.3 Ionophores

In addition to drugs acting on the V-ATPase, several agents are capable of dissipating the electrochemical gradient for H^+ (Figure 3). The ionophore nigericin dissipates the chemical gradient for H^+ (ΔpH) by exchanging H^+ for K^+. Similarly,

A23187 exchanges H^+ for Ca^{2+} or Mg^{2+}. FCCP/CCCP allows H^+ to run down their electrochemical gradient, but will be limited by the development of membrane potential. In contrast, valinomycin is a K^+ ionophore, influencing specifically $\Delta\Psi$. These compounds dissipate the electrochemical gradient of H^+ and would therefore be expected to reduce all vesicular neurotransmitter transport activities. However, they will have different effects, depending on the proportion of ΔpH and $\Delta\Psi$ expressed in a given vesicle population, and on the stoichiometry of ionic coupling by the neurotransmitter transporters.

3 Vesicular Transport Proteins

3.1 Vesicular Monoamine Transporters (VMAT1 and VMAT2)

Studies on monoamine uptake by chromaffin granules from the adrenal medulla show that the activity relies more on ΔpH than $\Delta\Psi$. In particular, the uptake of one cytoplasmic monoamine is coupled to the movement of two H^+ in the opposite direction (Knoth et al., 1981). However, the activity recognizes the protonated and hence charged form of the substrates, resulting in a net efflux of only one charge despite the two protons. Due to the H^+ electrochemical gradient and this coupling stoichiometry, chromaffin granules accumulate monoamines at molar concentrations. Indeed, monoamines interact with other vesicular components to form insoluble aggregates within chromaffin granules (Johnson, 1988a).

Vesicular monoamine transport can also protect against exogenous and endogenous toxins by sequestering them into synaptic vesicles. Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetragydropyridine (MPTP) in rats has been used as an animal model for idiopathic Parkinson's disease (Langston et al., 1983). The lipophilic MPTP penetrates the blood-brain barrier relatively easily and is metabolized by glial monoamine oxidase B (MAO-B) to the active metabolite N-methyl-4-phenylpyridinium (MPP^+) (Heikkila et al., 1984; Langston et al., 1984). MPP^+ resembles protonated monoamines, and is recognized by plasma membrane monoamine transporters that drive its accumulation in monoamine cells (Javitch et al., 1985), where it enters mitochondria, inhibits oxidative phosphorylation, and triggers apoptosis (Ramsay et al., 1986; Nicklas et al., 1985; Yang et al., 1998). However, the vesicular monoamine transporters have a high affinity for MPP^+, sequester the toxin inside vesicles, and thus protect against its mitochondrial toxicity. Indeed, selection in MPP^+ was used to isolate the first cDNA encoding a vesicular neurotransmitter transporter (Liu et al., 1992). Since dopamine is itself toxic due to the generation of hydrogen peroxide and production of highly reactive quinones (Michel and Hefti, 1990), the vesicular monoamine transporters (VMATs) may also protect against intrinsic toxicity of the endogenous transmitter, but the role of cytosolic dopamine in the pathogenesis of Parkinson's disease remains unknown.

Molecular cloning has identified two closely related but distinct vesicular monoamine transporters, VMAT1 and VMAT2 (Liu et al., 1992; Erickson et al., 1992; Liu and Edwards, 1997). Sequence analysis predicts 12 transmembrane domains with N- and C-termini in the cytoplasm, and the proteins show no sequence similarity to plasma membrane monoamine transporters (Nguyen et al., 1983; Neal and Chater, 1987; Neyfakh et al., 1991). Rather, they show similarity to bacterial proteins involved in detoxification (Figure 2). These bacterial proteins all function as H^+ exchangers, and several are even inhibited by the same drugs that inhibit VMATs (e.g., reserpine).

VMAT1 is expressed by endocrine tissues (e.g., chromaffin cells in the adrenal medulla) and paracrine cells (e.g., enterochromaffin cells in the stomach and intestines, and small intensely fluorescent (SIF) cells in sympathetic ganglia) (Erickson et al., 1996). In contrast, VMAT2 is expressed by monoamine neurons in the central nervous system (e.g., substantia nigra, locus coeruleus, and the raphe nucleus), and in selected peripheral endocrine populations (Table 1) (Weihe et al., 1994; Peter et al., 1995; Nirenberg et al., 1996). In general, VMAT2 has a higher affinity for most monoamines (by ∼three-fold) than VMAT1 (Peter et al., 1994; Erickson et al., 1996), but both VMAT1 and 2 can protect against MPP^+ toxicity (Table 1). However, only VMAT2 appears to recognize histamine, which may bind to a site different from that of other monoamines (Merickel et al., 1995).

The VMATs are also among the very few vesicular neurotransmitter transporters whose turnover number is known. At 29° C, they transport ∼5 molecules of serotonin per second and up to 20 molecules of dopamine (Peter et al., 1994). Since synaptic vesicles contain 5 to 20,000 molecules of transmitter and can recycle within at least 20 seconds (Ryan and Smith, 1995; Rizzoli et al., 2003), this rate has important implications for quantal size. At 5 molecules/second, the vesicle would contain only 100 molecules of transmitter after 20 seconds—if there were only one transporter per vesicle. Recent estimates suggest several transporters per vesicle (Takamori et al., 2006), but these might still not suffice to fill a rapidly cycling vesicle with monoamine unless the turnover was substantially higher at 37° C, where it is more difficult to measure transport accurately due to increased membrane leakiness. Indeed, the ability to determine the turnover of VMATs has been enabled by the availability of ligands to quantify the transporter and hence provide a denominator for measurements of transport.

3.1.1 Reserpine

The alkaloid reserpine is very effective in treating hypertension, but frequently produces the side effect of depression. Both actions are due to the inhibition of VMAT, the former in the peripheral sympathetic nervous system, and the latter in the brain. Indeed, the inhibition of VMAT by reserpine gave rise to the monoamine hypothesis of affective disorders (Carlsson et al., 1965; Beers and Passman, 1990). In terms of mechanism, reserpine competes with monoamines for binding to the VMATs and has three-fold higher affinity for VMAT2 than for VMAT1 (Ki 12 nM

Table 1 A summary of the vesicular transporters, their differential cellular localization, properties and inhibitors. For details and references, see text

	Main cellular localization	Substrates	Specific inhibitors
VMAT1	Endocrine tissue Sympathetic ganglia Enterochromaffin cells	Monoamines (Km ∼ low μM range) Monoamine analogues Toxins (e.g. MPP+)	Reserpine (Ki ∼ 34 nM) Psychostimulantia (e.g. Amphetamine, cocaine)
VMAT2	Locus ceruleus Substantia nigra Arcuate nucleus Groups of cells in the midbrain Raphe nuclei Postganglionic sympathetic neurons	Monoamines (Km ∼ low μM range) Monoamine analogues Histamine Toxins (e.g. MPP+)	Reserpine (Ki ∼ 12 nM) Tetrabenazine Psychostimulantia (e.g. Amphetamine, cocaine)
VAChT	Medial septum - diagonal band of Broca Magnocellular forebrain nuclei Striatum Preganglionic neurons Postganglionic parasympathic neurons Motoneurons	Acethylcholine (Km ∼ high mM range) Variety of cationic compounds	vesamicol (Kd ∼ 5 nM) variety of ACh analogues
VGAT	Majority of interneurons of the CNS A- and B-cells of islets of Langerhans	GABA (Km ∼ 5 mM) Glycine (Km > 10 mM)	Nipecotic acid γ-vinyl-GABA β-alanine

(*Continued*)

Table 1 Continued

	Main cellular localization	Substrates	Specific inhibitors
VGLUT1	Cerebral cortex Hippocampus Cerebellar cortex Glial cells A − cells of islets of Langerhans	Glutamate (Km ∼1 − 3 mM)	***Amino acid analogs (Ki for glutamate)*** Erythro-4 Methyl-L-glutamate (Ki ∼ 0.7 mM) Trans aminocyclopentane-1,3-dicarboxylic acid (t-ACDP) (Ki ∼ 0.7 mM) 4-methylene-L-glutamate (Ki ∼ 3 mM) Kynurenic acid (Ki ∼ 1.3 mM) Xanthurenic acid (Ki ∼ 0.19 mM) 7-chloro-kynurenic acid (Ki ∼ 0.59 mM) 6-biphenyl-4-yl-quinoline-2,4-dicarboxylic acid (Ki ∼ 41 uM) 6-(4′-phenylstyryl)-quinoline-2,4-dicarboxylic acid (Ki ∼ 64 uM)
VGLUT2	Thalamus Brainstem Cerebellar nuclei Glial cells A − cells of islets of Langerhans	Glutamate (Km ∼1 − 3 mM)	***Biphenyl derivative of naphthalene disulfonic acid*** Evans blue (Ki ∼ 40 nM) Chicago sky blue (Ki ∼ 190 nM) Trypan blue (IC50 ∼ 20 nM) Naphtol blue black, Reactive blue 2, Benzopurpurin 4B, Ponceau SS, Direct blue 71, Acid red 114 — IC50 ∼ 330 to 1670 nM
VGLUT3	Subset of GABAergic, cholinergic, aminergic and glutamatergic neurons Motoneurons Glial cells B − cells of islets of Langerhans Muscle	Glutamate (Km ∼1 − 3 mM)	***Stilbene derivatives*** 4-acetamido-4′-isothiocyanostilbene-2,2′- disulfonic acid (SITS) 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) 5-nitro-2-(3-phenylpropylamino)benzoic acid (N144) ***Polyhalogenated fluorescein derivative*** Rose bengal (IC50 ∼19 nM)

and 34 nM, respectively), consistent with differences in the recognition of substrates by the two transporters (Peter et al., 1994; Erickson et al., 1996). Interestingly, the imposition of $\Delta\mu_{H+}$ accelerates reserpine binding to the VMATs and monoamines inhibit reserpine binding with potencies similar to their apparent affinity as substrates (Weaver and Deupree, 1982; Rudnick et al., 1990; Schuldiner et al., 1993a). Moreover, the membrane-impermeable analog, reserpic acid, cannot inhibit transport from the vesicular lumen (Chaplin et al., 1985). Thus, the binding of reserpine and monoamines occurs when the substrate recognition site is oriented to the cytoplasmic face of the vesicle membrane. With monoamine bound, the transporter reorients to the inside of the vesicle, releasing monoamine (possibly due to a reduction in affinity for the substrate) and rebinding H^+, which reorients the substrate recognition site back to the cytoplasm (Liu and Edwards, 1997). At high concentrations, reserpine can act like a detergent, and recent studies have revealed other targets for reserpine in neurons, including sites on other proteins. Indeed, reserpine inhibits the plasma membrane dopamine transporter function in a noncompetitive manner at high concentrations (Metzger et al., 2002). In vivo, reserpine administration does not alter the level of VMATs, but single injections can block the binding site for another major VMAT inhibitor, tetrabenazine (Naudon et al., 1996).

3.1.2 Tetrabenazine

In contrast to reserpine, the binding of tetrabenazine to VMAT is affected only by very high concentrations of monoamines, indicating a distinct site of interaction (Darchen et al., 1989). However, there is an interaction between the binding of reserpine and tetrabenazine, suggesting either that the sites are overlapping, or that the drugs interact with conformationally distinct stages of the transport cycle (Darchen et al., 1989; Liu and Edwards, 1997). In addition, tetrabenazine shows much higher affinity for VMAT2 than VMAT1, both in human and particularly in rat, where VMAT1 is essentially resistant to inhibition by tetrabenazine (Schuldiner et al., 1993a; Erickson et al., 1996; Adamson et al., 1997). Ketanserin, a serotonin receptor antagonist, binds to the same site as tetrabenazine, and a photoactivatable analog (7-Azido-8-iodoketanserin) has been used to demonstrate that the binding site is located at the N-terminus of bovine VMAT2 (Sievert and Ruoho, 1997; Sagne et al., 1997). Lobeline, an alkaloid resembling nicotinic receptor agonists (Dwoskin and Crooks, 2002), also inhibits VMAT by interacting with the tetrabenazine binding site (Teng et al., 1998). Thus, VMAT inhibitors appear to fall into two classes, the ones that bind to the reserpine site and those that bind to the tetrabenazine site.

3.1.3 Psychostimulants

Psychostimulants generally act by increasing the level of extracellular monoamine. The mechanisms responsible include inhibition of plasma membrane monoamine transport and, in particular, stimulation of flux reversal, which may occur by

exchange or by efflux promoted in the case of the dopamine transporter by phosphorylation of the N-terminus (Fischer and Cho, 1979; Rudnick and Wall, 1992; Schuldiner et al., 1993b; Sulzer and Galli, 2003; Wall et al., 1995; Khoshbouei et al., 2004; Fog et al., 2006). However, psychostimulants also influence the flux of monoamine across the vesicle membrane. Amphetamine is a lipophilic weak base that can deplete monoamine stores by dissipating the $\Delta\mu_{H+}$ which drives monoamine uptake. Second, amphetamines may inhibit VMAT2 directly (Peter et al., 1994; Gonzalez et al., 1994; Sulzer and Rayport, 1990; Sulzer et al., 1993; Sulzer et al., 1995). The analysis of VMAT2 knockout mice illustrates the importance of interaction with VMAT for the effect of amphetamine. In the absence of VMAT2, amphetamine still induces dopamine release in culture, and administration in vivo can increase survival of the knockout mice, which usually die within a few days after birth (Fon et al., 1997). Presumably, dopamine efflux promoted by amphetamine bypasses the defect in exocytotic release in the VMAT2 knockout. However, dopamine release in response to amphetamine is greatly reduced in the knockout, indicating that amphetamine acts at least in part by releasing monoamine stores. In addition, mice heterozygous for VMAT2, which appear grossly normal, exhibit an increased locomotor response to amphetamine that occludes the usual increase seen with the sensitization to repeated administration (Wang et al., 1997).

Fleckenstein and co-workers have also demonstrated that amphetamine and its analogs (methamphetamine [METH], 3,4-methylenedioxymethamphetamine [MDMA]) alter the subcellular location of VMAT2-containing vesicles (Riddle et al., 2005). The "reuptake blockers" cocaine and methylphenidate (MPD), which inhibit the plasma membrane dopamine transporter (DAT), mobilize VMAT2-expressing vesicles. In vivo, cocaine exposure increases the Vmax of VMAT2 for dopamine and the number of binding sites for dihydrotetrabenazine (Brown et al., 2001). In addition, it mobilizes a synapsin-dependent reserve pool of dopamine-containing synaptic vesicles, thereby increasing the release of dopamine (Venton et al., 2006). MPD increases VMAT2 immunoreactivity and dihydrotetrabenazine (DHTBZ) binding, apparently through the redistribution of VMAT2-containing vesicles. The vesicle-mobilizing action of both the "releasers" (such as amphetamines) and the "reuptake blockers" (such as cocaine) has been shown to involve dopamine receptors since antagonists abolish the effects (Sandoval et al., 2002; Riddle et al., 2005).

3.1.4 Toxins

The VMATs were identified through their ability to protect against toxicity of MPP^+, presumably by sequestering the compound inside secretory vesicles, and away from its primary site of action in mitochondria. Indeed, the sequence of the VMATs shows homology to many bacterial drug resistance genes, including the toxin-extruding antiporters (TEXANs) (Figure 2). These transporters recognize many of the same drugs as the mammalian multidrug resistance (MDR) transporters,

including P-glycoprotein, but use an H^+ exchange mechanism rather than ATP hydrolysis to provide the energy for efflux. Several of the transporters, including the tetracycline resistance protein from Tn10, even show inhibition by reserpine (Ruiz et al., 2005). Considerable functional as well as structural analysis of the multimeric mini-TEXAN EmrE has begun to elucidate the mechanism of active transport by this family of proteins (Schuldiner et al., 2001; Gottschalk et al., 2004). In addition, cytotoxic compounds such as doxorubicin, rhodamine, ethidium, isometamidium, and tacrine are recognized by the VMATs as well as the TEXANs (Yelin and Schuldiner, 1995). The VMATs also recognize several environmental toxins, such as brominated flame retardants (BFRs), which inhibit vesicular dopamine transport (Mariussen and Fonnum, 2003). Finally, meta-iodobenzylguanidine (MIBG), a noradrenaline analog, is used for scintigraphy and radiation therapy of neuroendocrine (NE) tumors, and accumulation of this compound is inhibited by reserpine, indicating its accumulation in secretory vesicles in vivo (Kolby et al., 2003).

3.1.5 Nonspecific Effects

Pharmacological agents acting primarily on other proteins may also influence the VMATs. Phencyclidine, a noncompetitive NMDA receptor antagonist and DAT inhibitor, at high doses increases vesicular dopamine transport and binding of tetrabenazine (Crosby et al., 2002). However, the effect of phencyclidine is not mimicked by dizocilpine (another NMDA antagonist), but is antagonized by eticlopride, a D2 receptor blocker. Clozapine, an atypical antipsychotic, is very effective at reducing negative symptoms of schizophrenia as well as depression (Buchanan et al., 1998; Buckley, 1999), and leads to increased tetrabenazine binding, whereas haloperidol has no effect on VMAT binding capacity (Rehavi et al., 2002).

The antiarrythmic drug amiodarone inhibits reserpine binding in a concentration-dependent manner and has a sympatholytic, anti-adrenergic effect in the heart, inhibiting the uptake of norepinephrine (Haikerwal et al., 1999). Blockers of VMAT and L-type calcium channels have also been reported to exert reciprocal inhibitory actions (Mahata et al., 1996).

3.2 Vesicular Acetylcholine Transporter (VAChT)

A distinct synaptic vesicle transport activity for acetylcholine (ACh) has biochemical properties that resemble the VMATs. Vesicular ACh transport depends more on ΔpH than $\Delta\Psi$, but is nonetheless electrogenic (Anderson et al., 1981). Since ACh is permanently positively charged, the transport activity is thus believed to move more than one proton, similar to vesicular monoamine transport. However, vesicular ACh transport appears much less efficient than transport by VMAT in generating high concentration gradients of transmitter. Since the coupling would predict extremely high gradients, it has been speculated that the protein itself leaks protons and hence dissipates the driving force for transport (Parsons et al., 1993).

Organophosphorus toxins inhibit the enzyme acetylcholinesterase (AChE) which degrades ACh in the synaptic cleft (Figure 1). In *Caenorhabditis elegans*, resistance to such a toxin reflects a defect in ACh release and was thus used to screen for genes required for the release of ACh. Mutations in a sequence similar to VMAT (*unc-17*) led to identification of the vesicular acetylcholine transporter (VAChT) (Alfonso et al., 1993). The rat ortholog (rVAChT) shows 40% identity and 65% similarity to the rat VMATs (Roghani et al., 1994; Erickson et al., 1994), and the predicted topology also suggests 12 transmembrane domains. However, the Km for VAChT is several orders of magnitude higher (mM) than that for the VMATs (low μM) (Varoqui and Erickson, 1996). As anticipated for a protein with these properties, VAChT associates with synaptic vesicles in cholinergic neurons of the central nervous system, and the mRNA co-localizes with mRNA for the biosynthetic enzyme choline acetyltransferase (ChAT) (Schafer et al., 1994; Roghani et al., 1994; Roghani et al., 1996). The turnover of VAChT has also been determined, and it appears to be even slower than the VMATs, with a rate $\sim$1/second (Varoqui and Erickson, 1996).

3.2.1 Pharmacology of VAChT

Although closely related to the VMATs in sequence (Figure 2), the pharmacological profile of VAChT differs markedly (Table 1). The substrate selectivity is poor, and many cationic compounds are recognized by the transporter (Clarkson et al., 1992; Rogers and Parsons, 1989; Rogers et al., 1993). On the other hand, it does not recognize the precursor choline, and is not inhibited by reserpine (Bravo et al., 2005; Scheunemann et al., 2004). Rather, $(-)$-trans-2-(4-phenylpiperidino)-cyclohexanol (vesamicol) inhibits ACh uptake in synaptic vesicles from the electric organ of *Torpedo californica* (Anderson et al., 1983), and is the best characterized inhibitor of VAChT in higher organisms. Vesamicol does not inhibit the VMATs. Vesamicol is stereospecific with the $(-)$-enantiomer 25 times more potent than the $(+)$ isomer (Rogers and Parsons, 1989), and it has a dissociation constant of $\sim$5 nM (Bahr et al., 1992). The inhibition of transport is noncompetitive (Bahr and Parsons, 1986), although substrates compete with vesamicol for binding at equilibrium (Bahr et al., 1992), perhaps due to a distinct ACh binding site that regulates the access of vesamicol (Van der Kloot, 2003). During transport, the binding site for ACh translocates inward, releasing ACh into the vesicle lumen. This allows vesamicol to bind in two distinct states, to either the inwardly facing substrate recognition site or, after H^+ exchange, to the outwardly facing recognition site (Bravo et al., 2004). This model predicts more binding of vesamicol during steady-state transport than at rest (Bravo et al., 2004). In addition, the inhibition of VAChT with vesamicol appears to generate an efflux of the most recently accumulated ACh (Anderson et al., 1986)—presumably due to blocking of uptake of ACh that has leaked out of the vesicle nonspecifically. Indeed, cationic transmitters such as monoamines and ACh appear to leak nonspecifically out of synaptic vesicles, presumably across the lipid bilayer, and this may account for the regulation of quantal size by the number of vesicular

transporters. Overexpression of the VMATs and VAChT increases quantal size (Pothos et al., 2000; Song et al., 1997), presumably by off-setting the leak across the vesicle membrane.

A variety of other ACh analogs have been identified (Kaufman et al., 1989; Rogers and Parsons, 1989). Some of these, such as cetiedil (Israel et al., 1987; Rogers and Parsons, 1989), inhibit VAChT competitively, presumably by interfering with the binding of ACh to VAChT (Van der Kloot, 2003). Drugs acting on proteins involved in the ACh metabolism may also influence VAChT. Rivastigmine, an AChE inhibitor used clinically for memory disorders, increases VAChT immunoreactivity (Tayebati et al., 2004).

3.3 Vesicular GABA and Glycine Transporter (VGAT)

A distinct vesicular transport activity for GABA relies on both ΔpH and $\Delta\Psi$ (Kish et al., 1989; Takamori et al., 2000; McIntire et al., 1997), presumably because as a zwitterion with no net charge, the number of protons exchanged for GABA equals the amount of charge moved. In terms of substrate recognition, vesicular GABA uptake was not inhibited by glycine in several studies, suggesting distinct transport proteins for the two inhibitory neurotransmitters (Kish et al., 1989). However, other studies have suggested that the same activity recognizes both GABA and glycine (Christensen et al., 1990; Christensen and Fonnum, 1991; Burger et al., 1991; Roseth and Fonnum, 1995; McIntire et al., 1997; Sagné et al., 1997). Further, physiological studies have documented the corelease of glycine and GABA from the same synaptic vesicles (Jonas et al., 1998).

Consistent with the differences in ionic coupling, vesicular GABA transport is encoded by a protein with no discernible sequence similarity to the VMATs or VAChT. Genetic analysis in *C. elegans* identified a protein with multiple transmembrane domains (UNC-47) required for GABAergic transmission. Expressed in a heterologous expression system, the mammalian ortholog VGAT conferred GABA uptake dependent on both ΔpH and $\Delta\Psi$ (McIntire et al., 1997). The expressed transporter exhibits low-affinity uptake of GABA (Km $\sim$5 mM), consistent with results from native synaptic vesicles, and glycine inhibits GABA transport (IC50 $\sim$25 mM) (Sagne et al., 1997; McIntire et al., 1997). Furthermore, immunogold electron microscopy demonstrated VGAT expression in at least a subpopulation of GABAergic, glycinergic as well as mixed terminals (Chaudhry et al., 1998). Indeed, disruption of VGAT reduces release of GABA as well as glycine (Wojcik et al., 2006), consistent with the evidence for co-release in vivo (Jonas et al., 1998). However, it is important to note that the release of glycine depends heavily on the high-affinity neuronal glycine transporter GLYT2. GLYT2 presumably generates the high cytoplasmic concentrations of glycine necessary to drive the low-affinity vesicle transporter (Figure 1) (Gomeza et al., 2003).

3.3.1 Pharmacology of VGAT

No drugs acting specifically on VGAT have thus far been identified. However, several structural analogues of GABA have been tested for inhibition of VGAT (Table 1). Nipecotic acid and N-butyric acid weakly inhibit VGAT activity (McIntire et al., 1997). The potent anticonvulsant γ-vinyl-GABA (vigabatrin), known to inhibit GABA transaminase, was also found to inhibit VGAT with low affinity (McIntire et al., 1997).

Interestingly, the fate of GABA differs from that of monoamines after genetic disruption of the cognate vesicular transporter. In mice, the loss of VMAT2 results in the depletion of all monoamines (to 1%-5% wild-type levels), apparently due to metabolism of the cytosolic transmitter by monoamine oxidase, as well as other factors (Fon et al., 1997). In contrast, GABA accumulates to levels above wild type in the *C. elegans* mutant *unc-47* (McIntire et al., 1993). In addition, a vesicle membrane-bound form of the biosynthetic enzyme glutamic acid decarboxylase (GAD65) can apparently be inhibited by disrupting vesicular $\Delta\mu_{H+}$ with the proton pump inhibitor bafilomycin A1, the protonophore carbonyl cyanide m-chorophenylhydrazone or the ionophore gramicidin. Direct coupling between GAD and VGAT has thus been suggested, although the mechanism remains unclear (Jin et al., 2003).

3.4 Vesicular Glutamate Transporters (VGLUT1-3)

Characterization of the glutamate transport activity in native synaptic vesicles has demonstrated that it recognizes specifically glutamate (not aspartate) and depends predominantly on $\Delta\Psi$ rather than ΔpH (Naito and Ueda, 1985; Maycox et al., 1988; Carlson et al., 1989; Carlson and Ueda, 1990; Roseth, 1995). In addition, a low concentration of Cl^- (2–10 mM) is required for optimal vesicular glutamate transport, with no or high Cl^- reducing but not entirely eliminating transport activity. Although chloride entry might be expected to dissipate the membrane potential that drives glutamate uptake, it is thus also required to activate transport and apparently interacts with an allosteric regulatory site (Figure 3) (Naito and Ueda, 1983; Hartinger and Jahn, 1993; Wolosker et al., 1996).

Molecular cloning has identified three vesicular proteins that exhibit the biochemical properties described for glutamate uptake by native synaptic vesicles from brain (Reimer and Edwards, 2004; Erickson et al., 2006; Takamori, 2006). They all show stereoselectivity for L-glutamate and a Km ∼1–3 mM. None of them recognizes aspartate or the inhibitory neurotransmitters GABA or glycine. Dissipation of $\Delta\Psi$ with the K^+ ionophore valinomycin substantially reduces transport, but addition of the H^+ ionophore nigericin further reduces glutamate uptake, suggesting a reliance on ΔpH as well. Interestingly, dissipation of ΔpH alone can sometimes increase vesicular glutamate transport, presumably by increasing $\Delta\Psi$, but the loss of ΔpH in addition to $\Delta\Psi$ clearly reduces transport further, supporting the H^+

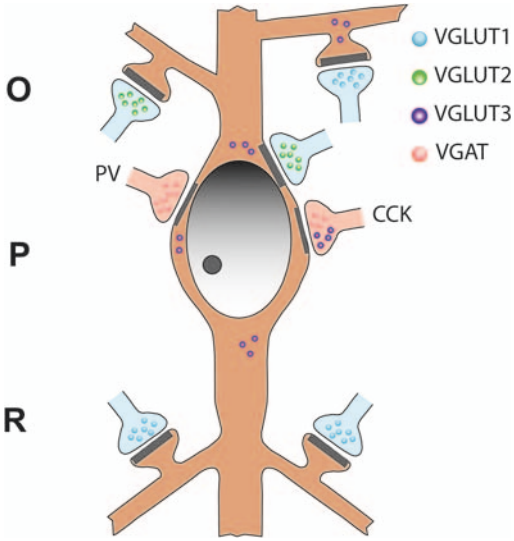

Fig. 4 Differential subcellular localization of the three VGLUTs. Nerve terminals containing VGLUT1, −2 or −3, all target the pyramidal cells of hippocampal region CA2. However, the inputs differ in origin and they target distinct regions of the pyramidal cell membrane. VGLUT1$^+$ nerve terminals derive from Schaffer collaterals and form synapses on the spines in stratum radiatum and oriens. VGLUT2$^+$ terminals originate in subcortical areas and form synapses onto spines, proximal denrites or cell bodies. VGLUT3 localizes to the subset of interneurons that contain cholecystokinin (CCK), and form synapses onto the cell body and proximal dendrites. VGLUT3 also mediates glutamate release from the cell bodies and dendrites of selected neurons in the cortex and striatum.

exchange mechanism (Bellocchio et al., 2000). Moreover, all three isoforms show a biphasic dependence on $[Cl^-]$, with the optimal activity ∼2–4 mM. However, the isoforms differ in expression at cellular and subcellular levels (Figure 4; Table 1), as well as through development (Boulland et al., 2004). VGLUT1 and VGLUT2 show a complementary expression pattern, occurring in different subpopulations of glutamatergic nerve terminals (Fremeau, Jr. et al., 2001; Schafer et al., 2002). In particular, VGLUT1 is expressed by glutamatergic cells in cerebral cortex, hippocampus, and cerebellar cortex, whereas VGLUT2 predominates in glutamatergic cells of the thalamus, brainstem, and deep cerebellar nuclei. The two homologs are also involved in glial glutamate release (Bezzi et al., 2004; Haydon and Carmignoto, 2006). In contrast, VGLUT3 is expressed throughout the brain but restricted to a subpopulation of glial cells and neurons, including serotonergic, cholinergic, GABAergic and glutamatergic cells (Gras et al., 2002; Fremeau, Jr. et al., 2002). In contrast to VGLUT1 and 2, which localize only to axon terminals, VGLUT3 localizes as well to cell bodies and dendrites, where it participates in retrograde signaling (Harkany et al., 2004). VGLUT2 and VGLUT3 are also transiently expressed at high levels in specific subpopulations of cells early in life, suggesting additional developmental roles for glutamatergic neurotransmission (Boulland et al., 2004).

3.4.1 Pharmacology of Vesicular Glutamate Transport

The drugs that inhibit vesicular glutamate transport (Table 1) differ from those acting on plasma membrane glutamate transporters. However, several of the drugs were initially identified as glutamate receptor blockers, or at least derived from these agents. Evans blue, a biphenyl derivative of naphthalene disulfonic acid, blocks specifically the excitatory AMPA/kainate receptor responses mediated by glutamate receptor subunits GluR1, GluR1,2, GluR1,3, and GluR2,3, and inhibition appears to be noncompetitive (Keller et al., 1993). The group of F. Fonnum has characterized the effects of Evans blue and related dyes on vesicular transport of glutamate (Table 1; Figure 3). At low concentrations, Evans blue inhibits glutamate uptake competitively (Ki $\sim$ 40 nM), while at higher concentrations it affects membrane potential and the pH gradient as well (Fykse et al., 1992; Roseth et al., 1995). The structural analog Chicago sky blue also blocks vesicular glutamate uptake competitively, but is much less effective (Roseth et al., 1995). Neither Evans blue nor Chicago sky blue has any effect on Na^+-dependent glutamate uptake across the cell membrane (Roseth et al., 1995). The authors of this study suggest that the distance of $\sim$6 Å between sulfonic acid groups structurally fixed by aromatic rings and the electron-donating groups in close vicinity of the negative charge are critical for the inhibition of the glutamate uptake (Roseth et al., 1995). Although we still lack the structural information required to interpret the observed structure-activity relationships, the distance between negatively charged groups is smaller in aspartate than glutamate, possibly accounting for the lack of recognition. Further, dyes structurally similar to Evans blue with biphenyl and amino- and sulphonic-acid-substituted naphthyl groups potently inhibit glutamate uptake. The IC_{50} value for Trypan blue is 49 nM, whereas Naphtol blue black, Reactive blue 2, Benzopurpurin 4B, Ponceau SS, Direct blue 71, and Acid Red 114 have IC_{50} values ranging from 330 to 1670 nM (Roseth et al., 1998). However, all of these dyes are cell impermeant and thus of limited use in vivo or even in vitro with intact cells. Some of the dyes (e.g., Evans blue and Chicago sky blue) also inhibit VGAT, albeit less potently.

Another dye derived from polyhalogenated fluorescein, Rose Bengal, acts as a potent noncompetitive inhibitor of vesicular glutamate uptake with Ki $\sim$19 nM (Ogita et al., 2001). The site of Rose Bengal action remains to be determined, but there are indications that it involves an allosteric site (Ogita et al., 2001). Structure-activity analysis has also shown that the phenyl group, multiple iodine groups, and the bridging oxygen of xanthine are required for potent inhibition of vesicular glutamate uptake (Bole and Ueda, 2005). Since Rose Bengal is membrane permeable, it is much more useful in vivo and in brain slice experiments where it has been shown to impair glutamate release (Montana et al., 2004).

Glutamate analogs have also been tested for their ability to interact with the VGLUTs (Table 1). Although several act on plasma membrane transporters, they have either no or only partial effects on vesicular glutamate uptake (Naito and Ueda, 1985; Tabb and Ueda, 1991). Interestingly, several amino acid analogs used to block the glutamate-binding domain of glutamate receptors and/or selectively inhibit plasma membrane glutamate transport also inhibit the VGLUTs (Ortwine

et al., 1992; Bridges et al., 1999). Quinoline-2,4-dicarboxylic acids (QDCs) were tested as inhibitors of vesicular glutamate transport due to their structural similarity to quinoline-2-carboxylic acids (QCAs), which have an embedded glutamate structure (Carrigan et al., 1999). The Ki values of optimized QDCs are as low as 41 μM, and QDCs resembling Evans blue and Chicago sky blue have been reported as particularly promising (Carrigan et al., 2002). Table 1 summarizes the Ki values of these glutamate analogs.

3.4.2 Other Targets in Vesicular Glutamate Transport

Although it is well known that optimal glutamate uptake requires low concentrations of Cl^-, the mechanisms involved are not completely known. The group of F. Fonnum has demonstrated that the stilbene derivatives and plasma membrane anion channel blockers 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (N144) inhibit glutamate uptake through competition directly with glutamate (Figure 3) (Roseth et al., 1995). These organic acids have two centers of negative charge and resemble the dyes mentioned above. The loop diuretics furosemide and bumetanide, acting on the $Na^+/K^+/2Cl^-$ cotransporter, also inhibit glutamate uptake. Like the stilbene derivatives, these drugs block glutamate uptake competitively and the Cl^- conductance noncompetitively (Roseth et al., 1995). However, there is compelling evidence that an allosteric effect not involving the glutamate binding site also contributes. The group of R. Jahn has shown inhibition of vesicular glutamate transport by DIDS which could be overcome by chloride, but not glutamate, suggesting a distinct, DIDS-sensitive chloride binding site on the cytoplasmic face of the vesicular glutamate transporter (Figure 3) (Hartinger and Jahn, 1993). Consistent with this, Wolosker and co-workers have shown that although chloride flux would influence the driving force for glutamate transport, the regulation by chloride does not depend on ΔpH or $\Delta\Psi$, but rather represents an allosteric form of regulation (Wolosker et al., 1996). However, the regulation of glutamate transport may not involve the VGLUTs directly, but an associated intracellular chloride channel (Jentsch et al., 1999; Stobrawa et al., 2001). Genetic inactivation of ClC-3 has been shown to produce severe retinal and hippocampal degeneration (Stobrawa et al., 2001). However, the mechanism for this degeneration and the role of ClC-3 and other intracellular chloride channels in the allosteric regulation of VGLUTs and their observed chloride channel activity remains unknown. Recent reconstitution of purified VGLUT2 from a heterologous expression system has demonstrated the same biphasic dependence on chloride observed for the native protein (Juge et al., 2006), suggesting that the chloride binding site is not encoded by another polypeptide. However, the authors also suggest that VGLUT2 encodes a Na^+-dependent phosphate cotransport activity (Juge et al., 2006). Originally described as phosphate cotransporters (Ni et al., 1994), vesicular glutamate transport catalyzed by the VGLUTs is not affected by phosphate or Na^+ (Bellocchio et al., 2000; Fremeau, Jr. et al., 2001), so it is surprising that they encode a mechanistically

and pharmacologically distinct activity. Indeed, we have observed that heterologous expression of the VGLUTs confers a highly variable phosphate uptake activity that can be Na^+-independent (R.H.E., unpublished observations). Previous work on the related transporter NaPi-1 also demonstrates a poor correlation between expression of the protein and phosphate uptake activity (Busch et al., 1996; Broer et al., 1998).

Heterotrimeric G proteins also appear to regulate vesicular glutamate transport by influencing the allosteric chloride site. Activation of the $G\alpha_{o2}$-subunit shifts the sensitivity of vesicular glutamate transport to lower chloride concentrations (Winter et al., 2005). In the absence of $G\alpha_{o2}$, vesicular glutamate transport exhibits no clear dependence on chloride, a result quite remarkable in light of the reconstitution studies demonstrating preservation of the chloride dependence with the purified protein (Juge et al., 2006). However, it remains unclear whether the effect of G proteins involves a direct interaction with the VGLUTs or with an unidentified "regulator" protein (Figure 3). An Inhibitory Protein Factor (IPF) that blocks vesicular glutamate transport has been purified from bovine brain cytosol, and apparently corresponds to a proteolytic fragment of fodrin (Ozkan et al., 1997).

A variety of other drugs also regulate the activity and/or expression of VGLUTs. Antidepressant agents, including fluoxetine, desipramine, the atypical antipsychotic clozapine, and lithium increase VGLUT1 mRNA and protein in neurons of the cerebral cortex and hippocampus (Moutsimilli et al., 2005; Tordera et al., 2005). Antidepressants and their analogs may thus influence the release of glutamate. Bilirubin, L-homocysteate, L-a-aminoadipate, the alkaloid bromocriptine, several fatty acids, and some other compounds also inhibit vesicular glutamate transport, and their analogs suggest additional strategies for drug development (Roseth et al., 1995; Roseth et al., 1998; Thompson et al., 2005). Nonetheless, despite the wide range of compounds that have been reported to affect VGLUT function, there are few if any specific antagonists, and certainly none that really distinguish between the three isoforms.

3.5 Other Transporters on Vesicles

Since many of the proteins found on synaptic vesicles still lack a clearly identified function, a number of these may contribute to filling the vesicles with transmitter. Indeed, the synaptic vesicle protein SV2 was identified many years ago through the production of monoclonal antibodies to purified synaptic vesicles (Buckley and Kelly, 1985). The sequence derived from molecular cloning (Bajjalieh et al., 1992; Feany et al., 1992) predicted 12 transmembrane domains, and multiple isoforms have since been identified. SV2A appears to be the most ubiquitous isoform, but SV2B and C exhibit an overlapping distribution (Janz et al., 1999; Bajjalieh et al., 1993; Bajjalieh et al., 1994). A related sequence, SVOP, has also been identified in invertebrates as well as mammals (Janz et al., 1998). However, the function of SV2 remains unknown. The knockout of SV2A alone exhibits seizures and early lethality (Crowder et al., 1999), while physiological analysis of the double SV2A

and B knockout shows a calcium-sensitive increase in transmitter release, suggesting a role in Ca^{2+} homeostasis (Janz et al., 1999). On the other hand, the analysis of release from endocrine cells as well as neurons shows a relatively specific role for SV2 in promoting release from the readily releasable pool of vesicles (Xu and Bajjalieh, 2001; Custer et al., 2006). The N-terminal, cytoplasmic domain of SV2 has also been shown to interact with selected isoforms of the synaptotagmin family (Schivell et al., 1996; Pyle et al., 2000), but the polytopic structure of SV2 and its clear relationship to sugar transporters strongly suggests a role as channel, or more likely, transporter. Interestingly, the widely use anticonvulsant levetiracetam (Keppra) has recently been found to bind specifically to SV2A (and not the other isoforms) (Lynch et al., 2004). The affinity of binding by related compounds correlates with their potency as anticonvulsants, further implicating SV2 as the site of action for these drugs. However, it seems paradoxical that the SV2A knockout develops seizures and a drug that binds to the same site can prevent seizures, suggesting that levetiracetam might activate rather than inhibit SV2A.

Remarkably, SV2 also appears to be the cellular receptor for botulinum neurotoxin A (Dong et al., 2006). In particular, a segment of the large lumenal loop binds to the toxin, and all of the SV2 isoforms appear capable of serving as receptors. Thus, levetiracetam, even if it is bound to the same site as the toxin, seems unlikely to protect against poisoning since it interacts only with SV2A.

A number of transporters that function on the plasma membrane also reside on synaptic vesicles, providing a mechanism for their regulated delivery to the cell surface. The Na^+- and Cl^--dependent proline transporter resides primarily on synaptic vesicles (Renick et al., 1999; Crump et al., 1999), but the physiological role of this transporter and hence the biological context for its regulatory exocytosis remains unclear. More recently, it has been found that the Na^+-dependent choline transporter involved in the recycling of choline after cleavage from ACh by extracellular acetylcholinesterase, also localizes predominantly to synaptic vesicles (Ferguson et al., 2003). In this case, it was demonstrated that synaptic vesicle exocytosis produces a concomitant increase in choline uptake. Since loss of the choline transporter dramatically impairs cholinergic transmission in mammals as well as *C. elegans* (Ferguson and Blakely, 2004; Matthies et al., 2006), the biological role of the transporter is clear. Its localization to synaptic vesicles presumably serves to coordinate expression of the transporter with activity, but it remains unclear why this transporter normally resides in vesicles, whereas other proteins involved in reuptake remain primarily on the cell surface. Nonetheless, membrane trafficking of the plasma membrane neurotransmitter transporters remains a major mechanism for their regulation.

4 Conclusions

The vesicular transporters are capable of concentrating neurotransmitters from 10- to 100,000-fold, with important implications for Ca^{2+}-dependent quantal release. These transporters also protect neurons from the toxicity of exogenous compounds

such as the parkinsonian neurotoxin MPTP and against endogenous molecules such as the normal neurotransmitter dopamine. The molecular analysis of vesicular neurotransmitter transport also provides an important new perspective to understand the physiological regulation of transmitter release and its role in disease. The proteins responsible and even orphan transporters of the synaptic vesicle also represent potent targets for the development of therapeutic agents.

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Core Proteins of the Secretory Machinery

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Abstract Members of the Rab, SM- and SNARE-protein families play key roles in all intracellular membrane trafficking steps. While SM- and SNARE-proteins become directly involved in the fusion reaction at a late stage, Rabs and their effectors mediate upstream steps such as vesicle budding, delivery, tethering, and transport. Exocytosis of synaptic vesicles and regulated secretory granules are among the best-studied fusion events and involve the Rab3 isoforms Rab3A-D, the SM protein munc18-1, and the SNAREs syntaxin 1A, SNAP-25, and synaptobrevin 2. According to the current view, syntaxin 1A and SNAP-25 at the presynaptic membrane form a complex with synaptic vesicle-associated synaptobrevin 2. As complex formation proceeds, the opposed membranes are pulled tightly together, enforcing the

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fusion reaction. Munc18-1 is essential for regulated exocytosis and interacts with syntaxin 1A alone or with SNARE complexes, suggesting a role for munc18-1 in controlling the SNARE-assembly reaction. Compared to other intracellular fusion steps, special adaptations evolved in the synapse to allow for the tight regulation and high membrane turnover rates required for synaptic transmission. Synaptic vesicle fusion is triggered by the intracellular second messenger calcium, with members of the synaptotagmin protein family being prime candidates for linking calcium influx to fusion in the fast phase of exocytosis. To compensate for the massive incorporation of synaptic vesicles into the plasma membrane during exocytosis, special adaptations to endocytic mechanisms have evolved at the synapse to allow for efficient vesicle recycling.

1 SNAREs

SNAREs comprise a superfamily of proteins that function in all membrane fusion steps of the secretory pathway within eukaryotic cells. They are small proteins that vary in structure and size (see Section 1.1), but share an evolutionary conserved stretch of 60–70 amino acids containing eight heptad repeats, which is termed SNARE motif (Brunger 2005). The number of different SNAREs varies between different organisms, ranging from 25 in yeast, 36 in mammals, to over 50 in plants. Each fusion step requires a specific set of four different SNARE motifs that is contributed by three or four different SNAREs, and each of the membranes destined to fuse contains at least one SNARE with a membrane anchor.

Membrane traffic usually consists of a sequence of steps involving the generation of a transport vesicle by budding from a precursor compartment, the transport of the vesicle to its destination, and finally the docking and fusion of the vesicle with the target compartment. SNAREs operate in the very last step of this sequence (Jahn and Scheller 2006). SNAREs on opposed membranes form a complex in "trans" that is mediated by the SNARE motifs and that progressively assembles from the N-terminal tips toward the C-terminal membrane anchors, thus clamping the two membranes together. The energy released during assembly is probably used for overcoming the fusion barrier. During fusion, the complex reorients from "trans" to "cis." Cis-SNARE complexes are unusually stable, and disassembly requires the action of an AAA-ATPase and ATP. Hence, SNAREs undergo a conformational cycle that is crucial for fusion. The cycle is controlled by an array of regulatory factors that are only partially understood (see Section 1.2).

While each fusion step appears to be mediated by a specific set of SNAREs, some SNAREs operate in multiple transport steps where they each interact with different SNARE partners. Conversely, SNAREs of the same subfamily (see Section 1.1) can substitute for each other, at least to a certain degree, in a given transport step. In vitro, there is less specificity in SNARE assembly, suggesting that additional con-

trol mechanisms are involved. According to current concepts, specificity in membrane traffic is achieved by successive layers of regulation that operate upstream of SNARE assembly and that involve members of conserved protein families. Rab-proteins are thought to orchestrate the initial contact between membranes destined to fuse (see Section 4) and to assure that only appropriate organelles are tethered. SM proteins are involved in preparing and proofreading SNAREs for trans-complex formation (see Section 2). The combination of these and possibly additional still unknown mechanisms would guarantee the required specificity in intracellular membrane trafficking.

The SNAREs involved in the fusion of synaptic vesicles and of secretory granules in neuroendocrine cells, referred to as neuronal SNAREs, have been intensely studied and serve as a paradigm for all SNAREs. They include syntaxin 1A and SNAP-25 at the presynaptic membrane and synaptobrevin 2 (also referred to as VAMP 2) at the vesicle membrane. Their importance for synaptic neurotransmission is documented by the fact that the block in neurotransmitter release caused by botulinum and tetanus neurotoxins is due to proteolysis of the neuronal SNAREs (Schiavo et al. 2000). Genetic deletion of these SNAREs confirmed their essential role in the last steps of neurotransmitter release. Intriguingly, analysis of chromaffin cells from KO mice lacking synaptobrevin or SNAP-25 showed that these proteins can be at least partially substituted by SNAP-23 and cellubrevin, respectively (Sorensen et al. 2003; Borisovska et al. 2005), i.e., the corresponding SNAREs involved in constitutive exocytosis.

In the following sections, we limit our discussion to the neuronal SNARE complex that, however, is paradigmatic for most SNARE complexes studied so far.

1.1 Structure of the Neuronal SNAREs

1.1.1 Syntaxin 1A

Syntaxin 1A is a protein composed of 288 amino acids (all numbers refer to rat proteins). Its structure is typical for most SNAREs: the SNARE motif is flanked by an independently folded N-terminal domain and a single transmembrane domain at the C-terminus (Figure 1). The N-terminal domain is composed of an antiparallel three-helix bundle with a small N-terminal extension and is linked to the SNARE motif via a long flexible linker region. Syntaxin is able to interact intramolecularly by folding back its N-terminal domain onto the SNARE motif, resulting in the so-called closed conformation of syntaxin in which the linker is structured and part of the SNARE motif assumes a helical conformation. Syntaxin 1 is highly abundant in neurons and neuroendocrine cells (approximately 1% of total brain protein) but lacking in non-neuronal cells.

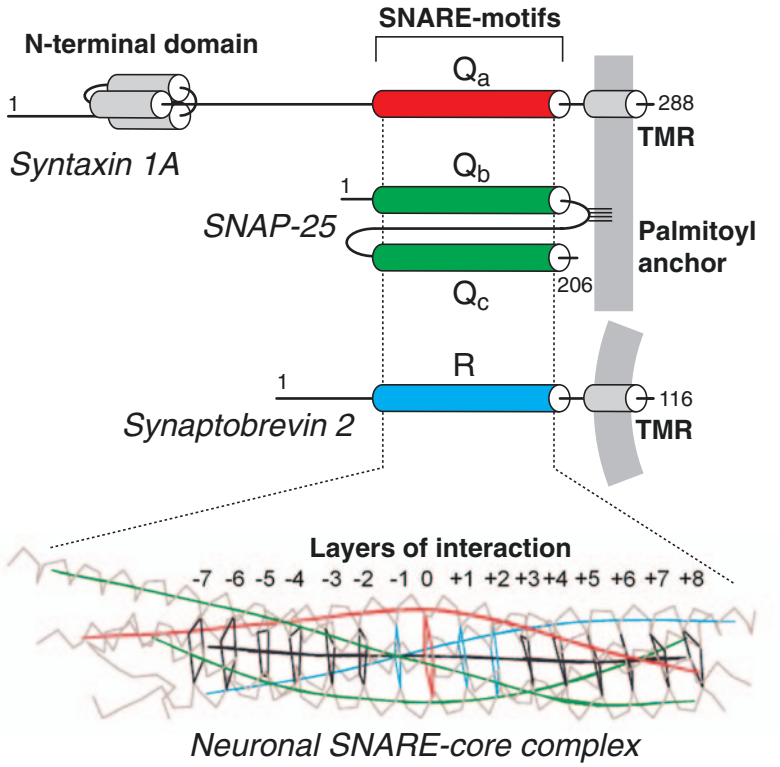

Fig. 1 Structure of the neuronal SNAREs. Upper panel: domain structure of the three neuronal SNARE proteins involved in synaptic vesicle fusion. Syntaxin 1A and SNAP-25 (contains two SNARE motifs) are associated with the presynaptic membrane, whereas synaptobrevin 2 is synaptic vesicle associated. The SNARE motifs form a stable complex (core complex) whose crystal structure has been analyzed (lower panel). In the complex, each of the SNARE motifs adopts an alpha-helical structure, and the four alpha-helices are aligned in parallel forming a twisted bundle (modified from Sutton et al. 1998). Stability of the complex is mediated by layers of interaction (-7 to $+8$) in which amino acids from each of the four alpha-helices participate (see text).

1.1.2 Synaptobrevin 2/VAMP 2

Synaptobrevin 2 is a small protein composed of 118 amino acids. It contains a SNARE motif with a short N-terminal proline-rich extension but lacks an independently folded N-terminal domain. Like syntaxin 1, the protein possesses a C-terminal transmembrane domain that is connected to the SNARE motif by a short linker (Figure 1). Synaptobrevin is palmitoylated at cysteine residues close to its transmembrane domain. Synaptobrevin 2 is highly expressed in neurons and neuroendocrine cells, but unlike syntaxin 1 it is also present in many non-neuronal tissues albeit at low levels.

1.1.3 SNAP-25

SNAP-25, a protein of 208 amino acids, deviates from the typical SNARE structure in that it has two SNARE motifs, joined by a flexible linker region, but lacks a transmembrane domain (Figure 1). The linker contains a cluster of four palmitoylated cysteine residues by which the protein is anchored at the plasma membrane. SNAP-25 can be phosphorylated at positions Thr138 and Ser187 by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), respectively. SNAP-25 represents a small subgroup of SNAREs with a similar structure, including SNAP-23, SNAP-29, and SNAP-47. In contrast to the neuron-specific SNAP-25 these SNAREs are ubiquitously expressed.

1.1.4 The Neuronal SNARE Complex

Syntaxin 1A, SNAP-25, and synaptobrevin 2 undergo structural changes when they assemble during membrane fusion. The crystal structure of the core-region of the neuronal SNARE complex (the assembled SNARE motifs) has been analyzed (Figure 1) and turned out to be paradigmatic for all SNARE-core complexes. In the complex, all SNARE motifs adopt an alpha-helical structure and are aligned in parallel, forming a twisted coiled-coil (Sutton et al. 1998). Along the longitudinal axis in the center of the bundle 16 stacked layers of interacting side chains have been identified (Figure 1). Each layer is formed by four amino acids, each contributed by a different SNARE motif. The layers are largely hydrophobic, with the exception of one ionic central layer that contains three glutamines and one arginine, all highly conserved (Fasshauer et al. 1998). The central layer is used as a center of reference for the remaining layers and termed "0"-layer. The layers from the N-terminal region of the SNARE motif to the 0-layer are termed -7 to -1, those upward from the 0-layer $+1$ to $+8$. An attractive working hypothesis is that SNARE motifs resemble a molecular zipper as they assemble from their N-terminal toward their C-terminal regions. According to this model, layers of interaction form sequentially one after another, pulling the opposed membranes stepwise together. During formation of the last layers, membranes would be forced so closely together that they fuse.

1.1.5 Q/R Classification of SNAREs

Initially, SNAREs were classified functionally into t-SNAREs (target-membrane SNAREs, e.g., syntaxin 1A and SNAP-25) or v-SNAREs (vesicle-membrane SNAREs, e.g., synaptobrevin 2). However, this concept cannot be applied to homotypic fusion events and is misleading because SNAREs are grouped together that belong to different subfamilies. A complete and unambiguous grouping is accomplished by the Q-/R-SNARE classification referring to the conserved amino acids present in the "0"-layer. According to the position of the SNARE motif in the structurally conserved SNARE complex, SNARE motifs are classified into Q_a-,

Q_b-, Q_c- and R-SNAREs (Bock and Scheller 2001). Following this classification, syntaxin 1A, SNAP-25. and synaptobrevin 2 represent the Q_a-, Q_b- and Q_c-, and R-SNAREs, respectively (Fasshauer et al. 1998). It turned out later that actually all functional SNARE complexes assigned to trafficking steps in yeast and mammals have a $Q_aQ_bQ_cR$-composition (Hong 2005; Jahn and Scheller 2006).

1.2 Assembly and Disassembly of SNAREs: Mechanistic Considerations

As discussed above, fusion is driven by the assembly of SNAREs mediated by their SNARE motifs. Assembly is associated with a major release of energy, and consequently the SNAREs need to be refueled with energy by the generation of free SNAREs before they are reusable for another round of fusion. Thus, SNAREs undergo cyclic assembly and disassembly, and together the individual reactions involved are referred to as the conformational cycle of SNAREs (Figure 2).

1.2.1 Assembly and Fusion

Free SNAREs are presumably short-lived, as they can form complexes among themselves, including homophilic oligomerization into clusters or with SNARE interacting proteins. It is becoming apparent that initial trans-contact between SNAREs

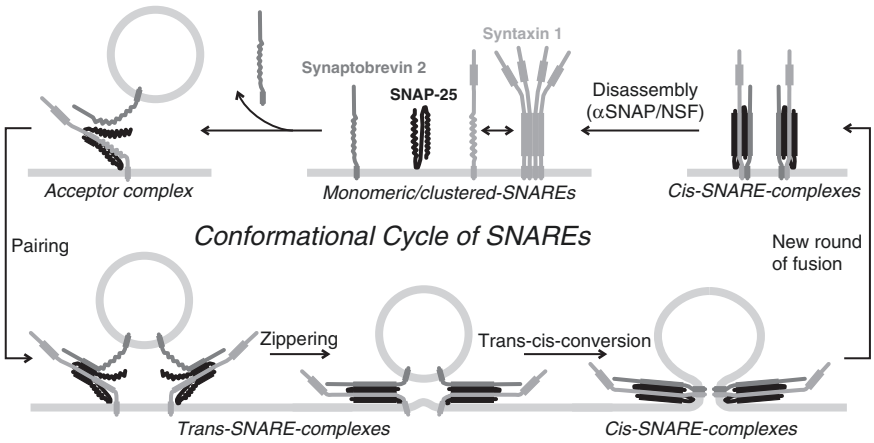

Fig. 2 The conformational cycle of SNAREs. SNAREs cycle between two extreme conformations, the unstructured monomeric SNAREs and the fully assembled cis-SNARE complexes. Initially, SNAREs on the membranes destined to fuse establish trans-SNARE complexes between the opposed membranes. Proceeding SNARE complex assembly forces the membranes tightly together enforcing membrane fusion. The resulting cis-SNARE complexes are disassembled into free SNAREs by the ATPase NSF and its co-factor, a process that consumes ATP and fuels the SNAREs with energy for undergoing a new SNARE cycle (for details see text).

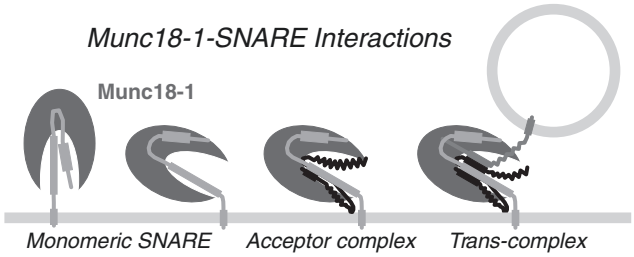

Fig. 3 munc18-1 SNARE-binding modes. The following munc18-1 interactions with monomeric/assembled SNAREs have been proposed. From left, binding of munc18-1 to a closed conformation of syntaxin 1A (Misura et al. 2000), to a half-open conformation of syntaxin or to an acceptor complex formed by syntaxin and SNAP-25 (Zilly et al. 2006), and to an assembled SNARE complex (Dulubova et al. 2007). It is possible that each of the proposed complexes represents an intermediate on a munc18-1 controlled molecular pathway of specific SNARE complex assembly.

(also referred to as "nucleation") is tightly controlled by cellular factors. A key role in preparing SNAREs, and perhaps in controlling nucleation, is assigned to the SM proteins, including its neuronal variant munc18-1, which are discussed more fully below. (see Section 3 and Figure 3).

It is not clear at present whether assembly is regulated after nucleation of trans-complexes or whether nucleation invariantly proceeds toward fusion. In vitro, assembly is completed rapidly after nucleation (Pobbati et al. 2006). However, mutagenesis of side chains in the central layers of the SNARE complex resulted in phenotypes that are best explained by energy minima during SNARE zippering, representing partially zippered and metastable intermediates in the molecular pathway of SNARE assembly. A partially assembled SNARE complex thus may account for the primed state of secretory granules in neuroendocrine cells (Sorensen et al. 2006). A protein thought to act on partially assembled trans-complexes is complexin, a small, soluble protein of 15 kDa (Marz and Hanson 2002). In vitro, complexin binds to SNARE complexes with high affinity, with the central part of complexin forming an α-helix that binds in an antiparallel orientation in the groove between synaptobrevin and syntaxin. While this interaction is thought to stabilize partially assembled SNARE complexes, deletion of complexin in mice lowers the sensitivity of neuronal exocytosis to calcium, resulting in a phenotype resembling that of synaptotagmin knockout mice (see section 3.2). Recently it has been proposed that complexin operates by stabilizing an otherwise metastable trans-SNARE intermediate that requires calcium-dependent displacement of complexin by synaptotagmin to be activated for fusion (Figure 4) (Tang et al. 2006).

The final steps in fusion are only incompletely understood. For instance, it is controversial whether the non-bilayer transition states in fusion are initiated primarily by force, transmitted from the "pull" of the assembling SNARE motifs via the linkers onto the membrane, as suggested by mutagenesis of the linker domain. Alternatively, the function of the SNARE motifs may be confined to close apposition of the membranes, with the final steps being catalyzed by other factors such as a

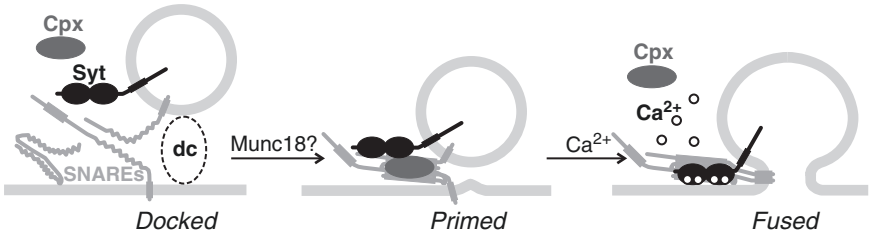

Fig. 4 Stages in synaptic vesicle exocytosis. Putative intermediate steps on the molecular pathway to synaptic vesicle fusion. Vesicle delivery and tethering to the presynaptic membrane most likely involves Rab-proteins and their effectors. So far, the nature of a speculative docking complex (dc) is unclear, but docking appears to be independent from SNARE proteins. In the primed state, SNAREs have assembled into a complex probably stabilized by complexin (Cpx). The fusion reaction is arrested until the intracellular calcium concentration increases. The putative calcium sensor for fast neurotransmitter release, synaptotagmin 1 (Syt), binds to intracellular calcium and in turn triggers fusion by associating with the presynaptic membrane and interacting with the SNARE complex, thereby displacing complexin (Tang et al. 2006).

perturbing "fusogenic" function of the transmembrane domains. For instance, it has been suggested that hetero-dimerization of the transmembrane domains of syntaxin 1A and synaptobrevin 2 facilitate the conversion from a hypothetical hemifusion intermediate state to full fusion (Ungermann and Langosch 2005). Recent evidence supports the involvement of hemifusion intermediates in the SNARE fusion pathway (see, e.g., Yoon et al. 2006). Furthermore, it is still controversial whether the fusion pore, i.e., the first aqueous connection between the vesicles and the extracellular space, is primarily lipidic or whether proteins (e.g., the transmembrane domains of the SNAREs) are part of the transition state.

1.2.2 Other Proteins Involved in the Regulation of SNAREs

In addition to the proteins discussed above, neuronal SNAREs were reported to interact with numerous other proteins in a specific manner, but in most cases both the structural basis and the biological function of these interactions need to be defined. For instance, synaptophysin, a membrane protein of synaptic vesicles, forms a complex with synaptobrevin in which synaptobrevin is not available for interactions with its partner SNAREs syntaxin 1A and SNAP-25, suggesting that this complex represents a reserve pool of recruitable synaptobrevin (Becher et al. 1999) or regulates interactions between the vesicle-associated synaptobrevin and the plasmalemmal SNAREs. Alternatively, it has been suggested that this complex is involved in synaptobrevin sorting to synaptic vesicles.

Munc13 is a 200 kDa protein essential for synaptic vesicle priming. As double knockouts of munc13 and syntaxin in *Caenorhabditis elegans* are rescued by constitutively open syntaxin, it has been suggested that munc13 mediates the transition from closed to open syntaxin (Brunger 2005). Recently, a ternary complex

composed of Rab3, RIM, and munc13 was described, suggesting a function in targeting synaptic vesicles to the priming machinery.

Tomosyn is a soluble protein of 130 kDa with a C-terminal R-SNARE motif that is capable of replacing synaptobrevin in the neuronal SNARE complex. Most available data indicate that tomosyn negatively regulates exocytosis by competing with synaptobrevin in the formation of SNARE complexes (Brunger 2005), thereby leading to the inhibition of synaptic vesicle priming (McEwen et al. 2006).

1.2.3 Disassembly of SNARE Complexes

After membrane fusion, all neuronal SNAREs reside in the plasma membrane. Their assembled SNARE motifs are aligned in parallel and the TMRs of syntaxin 1A and synaptobrevin 2 are close to each other in the same membrane. These cis-SNARE complexes are of remarkable stability and do not disassemble spontaneously into free SNAREs. Reactivation of the SNAREs is mediated by the ATPase NSF (N-ethylmaleimide-sensitive factor). NSF is required for all intracellular trafficking steps, and its function is to disassemble cis-SNARE complexes into free SNAREs. NSF is a member of the AAA-protein family (ATPases associated with other activities) that generally appear to be involved in disentangling protein complexes and protein aggregates (Hanson and Whiteheart 2005). Unlike many other AAA-ATPases, NSF cannot act alone–it needs co-factors termed SNAPs (soluble NSF attachment protein), represented by three isoforms termed α-, β-, and γ-SNAP. SNAPs bind to the cis-SNARE complex first and in turn recruit NSF, followed by stimulation of its ATPase activity. The hydrolysis of ATP induces major conformational changes resulting in the disassembly of the entire complex into its free constituents. In this uncomplexed state, the neuronal SNAREs are probably most susceptible to cleavage by clostridial neurotoxins.

2 Sec1/Munc18 (SM) Proteins

SM proteins were initially discovered during genetic screens in yeast and *C. elegans* for mutants showing defects in membrane traffic and secretion (Toonen and Verhage 2003). They comprise a small family of cytosolic proteins of 650–700 amino acids with seven members in mammals and four members in yeast. Although not yet documented unequivocally in each case, it appears that each trafficking step catalyzed by SNAREs is dependent on one of the SM proteins. Due to the small number of SM proteins, it is evident that some of them operate in more than one fusion reaction. In contrast to SNAREs, no functional redundancy has been observed so far, but SM proteins from distant species are capable of replacing each other provided they participate in the same trafficking step. Wherever investigated, genetic deletion of an SM protein leads to a block of the corresponding fusion reaction, indicating that their role in membrane fusion is essential. SM proteins most likely exert their

function upon regulating SNARE assembly, although also a role in docking has been suggested (Voets et al. 2001). Although significant progress has been made in recent years, it has turned out to be surprisingly tricky to unravel how they work, with major questions still being open.

2.1 SM Protein Interactions with SNAREs

All SM proteins interact with SNAREs, either directly or indirectly in complex with other proteins. Furthermore, strong genetic interactions have been documented between SM proteins and SNAREs. In some cases deletion of an SM protein is associated with a reduced expression level of its respective SNARE binding partner (Gallwitz and Jahn 2003).

The crystal structure of mammalian and squid munc18 and of yeast Sly1p shows a remarkable degree of structural conservation. SM proteins are composed of three domains that form an arch-shaped molecule with a central cleft (Misura et al. 2000; Bracher and Weissenhorn, 2002). Surprisingly, however, a confusing variety of binding modes between SM proteins and SNAREs has been observed (see, e.g., Figure 3). Both munc18 and Sly1p directly bind to the corresponding Qa-SNAREs syntaxin 1 and Sed5p (syntaxin 5), respectively, but in a completely different manner. In the crystal structure of the munc18-1-syntaxin 1A complex, syntaxin is arrested in the closed conformation, being inserted in the central cleft of munc18-1. In stark contrast, Sed5p binds only with a short N-terminal peptide that precedes its helical N-terminal domain, and the binding site on Sly1p is represented by a small groove on the surface of the SM protein, with no involvement of the central cleft. The latter binding mode has also been described for yeast and mammalian SM proteins involved in trafficking steps of the ER, the Golgi, the trans-Golgi network, and early endosomes (Toonen and Verhage 2003; Rizo and Südhof 2002), and it thus appears that the binding mode between munc18 and syntaxin 1 is unique among the family.

2.2 Munc18-1–an Oddity among the SM Proteins?

Munc18-1 (and its ortholog unc-18 in *C. elegans*) are essential for exocytosis. Knockout of munc18-1 in mice results in a nervous system that initially develops normally but in which synapses are totally silent – one of the most dramatic phenotypes of synaptic proteins that highlights the essential role and the lack of redundancy of the protein. Despite this dramatic phenotype, it has been remarkably difficult to reconcile the physiological findings obtained from synapses containing deleted, overexpressed, or otherwise manipulated munc18 with its biochemical properties studied in vitro. As discussed above, munc18-1 binds to syntaxin 1A with high affinity in a manner that clamps the N-terminal domain of syntaxin onto its

SNARE motif, effectively preventing syntaxin from binding to its partner SNAREs SNAP-25 and synaptobrevin 2. However, an inactivation of syntaxins' SNARE function is exactly the opposite of what one would expect from an essential protein, and (except for a debated study in *Drosophila*) even massive overexpression of munc18 does not appear to affect exocytosis (Gallwitz and Jahn 2003). Thus, it has been debated whether the "closed" conformation of syntaxin 1 in the munc18 represents a nonphysiological extreme situation that does not occur in intact cells, particularly since no other SM protein interferes with the formation of SNARE complexes (see below). Hence, the search has been on for munc18 SNARE complexes with different properties that may be closer to the physiological situation. Recently, evidence was provided showing that in native membranes munc18-1 stabilizes a half-closed conformation of syntaxin that still is capable of engaging in SNARE assembly (Zilly et al. 2006). Furthermore, it has been shown that munc18-1 activates SNARE-mediated membrane fusion in a reconstituted liposome system (Shen et al. 2007), and can bind directly to the assembled SNARE complex (Dulubova et al. 2007). Thus it is becoming apparent that munc18, in addition to its ability to form a complex with closed syntaxin 1, is capable of interacting with the neuronal SNAREs in other binding modes that are not inhibitory but rather may promote assembly of SNARE complexes (Figure 3). Such binding modes are more compatible with all other SM proteins that bind to partially or even fully assembled SNARE complexes. Although much more work needs to be done, a picture is emerging according to which SM proteins may assist in the formation of SNARE acceptor complexes needed for trans-SNARE interaction, and in doing so they may also be involved in proofreading of SNAREs, thus differentiating between cognate and noncognate SNAREs (Peng and Gallwitz 2002).

With the focus clearly being on the SNAREs, it needs to be borne in mind that SM proteins interact with a diverse array of additional proteins, in some cases even forming stable complexes (e.g., the SM protein Vps33p is part of the HOPS/VpsC complex needed for vacuole fusion in yeast). For instance, munc18 binds to the cytoplasmic protein Mint and it has been suggested that munc18-1 binding to Mint could regulate exocytosis by syntaxin-independent interactions (Schütz et al. 2005; Ciufo et al. 2005).

3 Synaptotagmins

3.1 Synaptotagmin Family

Synaptotagmins comprise a small family of single-membrane spanning proteins that are expressed in neurons and neuroendocrine cells. So far 16 members have been identified in vertebrates (Craxton 2004). They contain an N-terminal transmembrane domain followed by a variable linker region and two C2 domains which are connected by a short linker (Südhof 2002). Some synaptotagmins have additional short

N-terminal domains that in some cases are glycosylated (Syt 1 and 2) or carry a disulfide bond (Syt 3, 5, 6, and 10). The two C2 domains (C2 stands for second constant sequence, as defined when the first C2 domains were identified in protein kinase C isoforms) are termed C2A and C2B and generally bind three and two calcium ions, respectively, although some synaptotagmins do not bind calcium. Synaptotagmins are found both on synaptic and secretory vesicles (Syt 1, 2, and 9) and on the plasma membrane (e.g., Syt 3 and 7). More recently, additional proteins have been discovered that are similar to synaptotagmins in that they possess C2 domains and membrane anchors. Best characterized are the ferlins which comprise a membrane protein family with four to seven C2 domains and a single TMR at their C-terminus (see, e.g., Washington and Ward 2006). As for the synaptotagmins, recent evidence suggests that ferlins also play a role in Ca^{2+}-dependent exocytosis such as that involved in membrane repair in muscle cells (requiring dysferlin) and in vesicle release in the hair cells of the inner ear (otoferlin).

Synaptotagmin 1 is the founding member of the synaptotagmin family, and it is also the most intensely studied isoform. Together with Syt2, Syt1 functions as synaptic Ca^{2+}-sensor that couples Ca^{2+}-influx with fast transmitter release (Chapman 2002). Intriguingly, some of the plasma membrane associated synaptotagmins have a 10-fold higher binding affinity for Ca^{2+}. Together with the finding that Syt7 can function as Ca^{2+}-sensor for exocytosis in chromaffin cells but not in neurons, it is conceivable that low-calcium-affinity synaptotagmins (Syt1 and 2) trigger fast neurotransmitter release in neurons (see Section 3.2), whereas plasma-membrane-associated Syt7 may function as calcium sensor for exocytosis in neuroendocrine cells. In addition, having synaptotagmins on both the plasma and the vesicle membrane may result in a system of complementary Ca^{2+}-sensors regarding the sensitivity to intracellular calcium signals.

3.2 Synaptotagmin 1 as Ca^{2+}-Sensor for Fast Neurotransmitter Release

Genetic deletion of Syt1 in *Drosophila* and mice leads to the loss of the fast, Ca^{2+}-dependent phase of transmitter release that follows the arrival of an action potential in the presynaptic nerve terminal (Chapman 2002). Disruption of Ca^{2+}-binding to either of the C2 domains severely inhibits the function of synaptotagmin in mediating fast synchronous transmitter release, with the disruption of the C2B-domain being more severe than that of the C2A domain. Furthermore, when mutant synaptotagmins exhibiting either reduced or increased Ca^{2+}-affinity are expressed in mice lacking synaptotagmin I a close correlation was observed between the Ca^{2+}-affinity and the Ca^{2+} -dependence of neurotransmitter release, all confirming that Syt1 is an essential link between Ca^{2+}-influx and the synaptic fusion machinery. Interestingly, lack of synaptotagmin does not abolish exocytosis, as a "normal" SNARE-dependent exocytotic response is attainable when exocytosis is triggered by α-latrotoxin (the active ingredient of black widow spider venom).

3.3 Molecular Mechanism of Synaptotagmin 1

C2 domains are represented by stable, mostly β-stranded folds, with the Ca^{2+}-binding site at one end of the elongated domain. The Ca^{2+}-binding site only incompletely coordinates the Ca^{2+}-ions. Consequently, the affinity of the free C2 domains for Ca^{2+}-is rather low but dramatically increases in the presence of acidic phospholipids. Thus, the C2 domains mediate Ca^{2+}-binding to membranes that in the case of Syt1 is further enhanced in the presence of phosphatidylinositol (4, 5) bisphosphate (PIP_2). Furthermore, synaptotagmin binds to both isolated syntaxin 1 and SNAP-25 as well as to partially (containing SNAP-25 and syntaxin) and fully assembled SNARE complexes in vitro. For SNARE binding, both Ca^{2+}-dependent and Ca^{2+}-independent interactions have been described. Mutagenesis studies involving partial or full disruption of Ca^{2+}-dependent binding to phospholipids and of binding to SNAREs have suggested that each of these interactions is required for the function of Syt1.

As in the case of munc18-1, it has been tricky to delineate the molecular mechanism of Syt1 action, and many details are still unclear (Rizo et al. 2006). The problems in understanding the mechanism of synaptotagmin, despite major efforts, highlight the fact that we do not yet have a good understanding of the status of the fusion machinery including SNAREs and associated proteins in the docked and primed state that synaptotagmin is acting upon. Ca^{2+}-dependent binding of the C2-domains to membranes may clamp the membranes tightly together (as recently suggested). Furthermore, membrane binding may destabilize bilayers at the site of fusion, facilitating the formation of transition states (such as hemifusion) or destabilizing the transition states themselves. Furthermore, as discussed above, Syt1 is capable of displacing complexin from the surface of the SNARE complex in a Ca^{2+}-dependent manner. It was proposed that these proteins act in sequence, with complexin stabilizing a labile SNARE trans-intermediate that then is driven toward fusion by Syt1 upon displacement of complexin.

4 Rab Proteins

Rabs are small (20–29 kDa) ubiquitously expressed proteins. They represent monomeric GTPases which belong to the Ras GTPase superfamily. So far 11 members are known in yeast and more than 60 in mammalian cells (Schultz et al. 2000), whereas the numbers reflect the complexity of membrane trafficking pathways in these different organisms. Rabs cycle between the cytosol and the membrane of the trafficking organelle. This cycle is controlled by conformational changes that are regulated by guanine-nucleotides, thus providing a molecular switch, with the membrane-bound GTP-form being “on” and the GDP-form being “off.” GTP-Rabs bind to proteins termed effectors that only recognize Rabs in their GTP-bound state. A large diversity of Rab effectors is known, many being specific for a single or for small subsets of Rabs.

Rabs function as master regulators of membrane docking and fusion. Fusion can only occur if membrane contact is established, and numerous lines of evidence suggest that contact is orchestrated by Rabs. In fact, selective activation of Rabs at a given site ensures directionality and specificity of membrane docking and fusion (Grosshans et al. 2006), similar to the GTPases Sar1 and Arfs that are involved in coat recruitment during vesicle budding. In some cases, distinct Rab domains are present on the same organelle where they are involved in different transport steps.

As Rabs share a common structure and are highly homologous, the structural diversity of their effectors probably reflects the versatile functions of these GTPases as molecular switches. This diversity is highlighted by the functional diversity of Rab effectors. For instance Rab27a regulates transport of melanosomes to the cell periphery by binding to its effector melanophilin. Melanophilin associates with the actin motor myosin-Va. Rab-mediated tethering of membranes in preparation for fusion involves multimeric complexes. One of the best-studied examples is the exocyst, an octameric protein complex that tethers secretory vesicles to the plasma membrane in yeast. For homotypic membrane fusion of early endosomes (or of yeast vacuoles), Rab effectors interact with SNAREs such as the Rab5 effector EEA1 that binds to syntaxin-13.

4.1 Rab3

Despite major efforts, the precise role of Rab proteins in synaptic exocytosis is still not clear. One of the most abundant synaptic Rab proteins is Rab3, which is selectively localized to synaptic vesicles and that is represented by four homologous isoforms (Rab3A, Rab3B, Rab3C, and Rab3D). Of these, Rab3A is the most abundant and best studied (Südhof 2004). Rab3A undergoes a synaptic vesicle association and dissociation cycle coupled to calcium-stimulated exocytosis and recovery after stimulation.

Like all Rabs, the GTP-bound form of Rab3A is anchored in the synaptic vesicle membrane via a covalently bound geranylgeranyl moiety. When exocytosis is triggered, Rab3A-bound GTP is hydrolyzed to GDP, and the resulting Rab3A-GDP forms a complex with GDI (guanine dissociation inhibitor) in which the geranylgeranyl anchors are enveloped, leading to the dissociation of the GDI-Rab3A-GDP complex from the synaptic vesicle membrane. Rab3A is then recruited again to the synaptic vesicle membrane by a poorly understood mechanism involving binding of a specific GEF (guanine nucleotide exchange factor) to Rab3A and GDP exchange by GTP.

The tight coupling of exocytosis to the Rab3A association and dissociation cycle suggested a key role for Rab3A and its effectors in mediating directionality of synaptic vesicle traffic. However, mice lacking Rab3A are viable and have an only moderate synaptic dysfunction. In neurons derived from the hippocampal CA1 region, an alteration of the short-term plasticity was observed. In contrast, in neurons derived from the hippocampal CA3 region short-term plasticity was unaltered, but

Rab3A was essential for mossy-fiber long-term potentiation. However, a complete genetic analysis in mice showed that Rab3 is essential for survival in mice and that the Rab3 isoforms are functionally redundant (Schlüter et al. 2004).

Presently, it cannot be excluded that despite the apparently highly specific function of certain Rabs in intracellular trafficking pathways there is redundancy with respect to vesicle docking in the synapse. The surprising diversity of Rabs on highly purified synaptic vesicles (more than 30 different Rabs) supports the view that multiple Rabs are required for synaptic vesicle recycling, which may have overlapping functions.

4.2 Rab3 Effectors

Rabphilin and RIM1α/2α represent two different classes of effectors that bind to Rab3-GTP (Südhof 2004). They have structural similarities, as both contain two C_2 domains, an N-terminally located zinc-finger domain that mediates binding to GTP-Rab3, and sites for phosphorylation by PKA that are located in the center of the proteins.

Rabphilin 3A is a soluble protein that is recruited to the membrane of synaptic vesicles by Rab3A and C in a GTP-dependent manner, closely coupling it to the Rab3 cycle. Like synaptotagmin, it has two functional C2 domains in the C-terminal region, but unlike synaptotagmins, it does not contain a transmembrane domain.). Rabphilin is phosphorylated by various kinases in a stimulation-dependent manner. Rabphilin knockout mice are viable and do not show a major synaptic phenotype, thus providing no clue for the function of this protein.

In contrast, genetic deletion of RIM1α in mice revealed that the protein is required for long-term potentiation both in the hippocampus and in the cerebellum. Biochemical experiments revealed that RIM1α is part of a presynaptic protein scaffold containing "active zone proteins" that is required for normal release of neurotransmitters. No change in the number of docked vesicles was observed, however, suggesting that other proteins are needed for the docking of synaptic vesicles at active zones.

5 Endocytic Proteins

Exocytosis of synaptic vesicles or of regulated secretory granules results in incorporation of membrane into the plasma membrane. For maintaining the cell surface area constant, homeostatic mechanisms are required that assure a rapid and efficient re-internalization of the incorporated vesicle membranes. Different types of vesicle recycling pathways are discussed for synapses (Figure 5), including fast retrieval of the vesicle at the site of exocytosis, called "kiss-and-run," a slower clathrin-dependent pathway, and other clathrin-independent retrieval pathways (Royle and

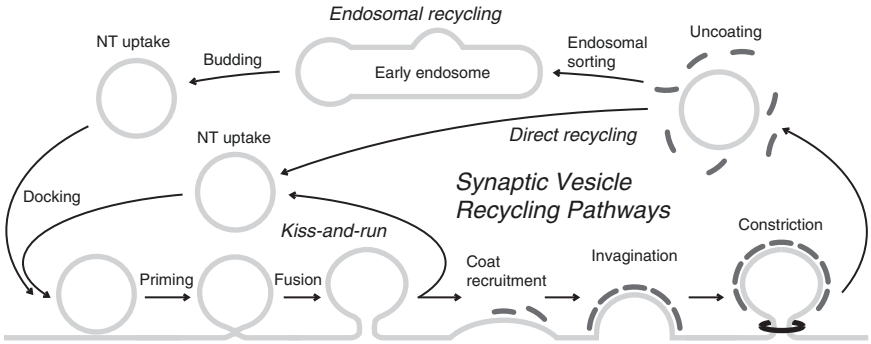

Fig. 5 Synaptic vesicle recycling in the synapse. For synaptic vesicle recycling, several endocytic mechanisms appear to co-exist in synaptic nerve terminals. In the case of fast "kiss-and-run" exocytosis/endocytosis, the fused vesicle does not collapse into the membrane but is retrieved directly by a fast process. The molecular machinery underlying this pathway is unknown. Vesicles that have fully collapsed into the membrane are recycled by clathrin-mediated endocytosis. Clathrin, along with other proteins, is involved in membrane invagination (see figure and text) and leads finally to the formation of a constricted pit. The GTPase dynamin (black ring) mediates membrane scission of the constricted pit. After removal of the clathrin coat, two pathways are possible (direct recycling and recycling via the early endosome). In all cases, before fusion the recycled vesicles have to be loaded with neurotransmitters (NT).

Lagnado 2003). Which of the pathways dominates seems to depend on the type of neuron and the duration and intensity of the exocytotic stimulus. Apparently, the fast "kiss-and-run" pathway is preferred at low stimulation frequency for retrieving vesicles of the readily releasable pool. At higher stimulation frequencies the slow clathrin-dependent, endosomal recycling pathway is activated (Rizzoli and Betz 2005). Whereas no synapse is functional without an intact clathrin-dependent recycling pathway, similarly firm evidence for the need of the "kiss-and-run" pathway is not available. In fact, most of the evidence for "kiss-and-run" is either at the detection limit of the respective techniques, or of indirect nature. Furthermore, no protein machinery that is specific for this pathway has been identified. For these reasons, doubts persist whether such a pathway does exist at all in synapses. Another debated issue relates to the function of endosomal intermediates. Although synapses possess functional early endosomes it is unclear whether endocytosed vesicles must pass through an endosomal intermediate during each recycling or whether endosomes can be bypassed, with synaptic vesicles reforming directly after clathrin uncoating (Südhof 2004).

5.1 Kiss-and-Run Exocytosis/Endocytosis

According to the classical view, synaptic vesicles completely flatten during exocytosis, which is followed by retrieval of the membrane components by clathrin-dependent endocytosis. Evidence for direct retrieval (kiss-and-run) was provided

more than 30 years ago by Bruno Ceccarelli. At the neuromuscular endplate, Ceccarelli and co-workers were unable to observe reduction of vesicle numbers and the appearance of coated vesicles during low-stimulation frequencies, although synaptic vesicles acquired an extracellular fluid-phase marker in a stimulus-dependent fashion (Ceccarelli et al. 1973). When exocytotic events were captured by electron microscopy using rapid shock-freezing of stimulated synapses, exocytotic events were visible, with vesicles opening to the extracellular space by means of a pore. It was proposed that synaptic vesicles do not necessarily flatten into the membrane but instead can undergo rapid and transient fusion with the presynaptic membrane, a mechanism that was termed "kiss-and-run" (Fesce et al. 1994). This mechanism would allow for neurotransmitter release without the loss of synaptic vesicle identity, and therefore without any need of membrane recycling. Indeed, evidence for "reversible" fusion has since been provided from secretory cells with much larger vesicles. For instance, capacitance measurements on a variety of secretory cells showed that exocytosis, observed as a stepwise increase in capacitance, is frequently followed by a decrease of similar size (Fernandez et al. 1984; Breckenridge and Almers 1987). The molecular mechanisms that would mediate the scission of the fused, but incompletely flattened synaptic vesicles are still unclear. In chromaffin cells, in which the regulated organelles are large secretory granules, available data indicate that dynamin-1 may be responsible for the direct rapid retrieval pathway while dynamin 2 is involved in slow clathrin-mediated endocytosis (Artalejo et al. 2002). However, it is unclear how these findings are exactly related to the many types of nonclassical recycling pathways in nerve terminals that may differ with respect to retention of shape, protein, or lipid of the fused vesicle before its reinternalization. However, as a generally accepted terminology for the different fast nonclassical modes is not available, they are usually termed "kiss-and-run" exocytosis/endocytosis.

5.2 Clathrin-Mediated Endocytosis (CME)

As has been shown by genetic approaches and by in vitro reconstitution, the molecular machinery for clathrin-mediated endocytosis (CME) in nerve terminals is generally similar to that involved in CME of non-neuronal cells. However, at the synapse internalization and recycling of vesicle membranes occurs in seconds, much faster than, e.g., ligand-induced, receptor-mediated endocytosis that occurs at a slower time scale. In fact the synapse possesses several adaptations of the CME pathway that may be responsible for these differences. First, endocytic proteins are highly enriched in synapses, including clathrin, AP2, epsin, eps15 (epidermal growth factor pathway subunit 15), amphiphysin, and synaptojanin. In addition, several of the major CME proteins express neuron-specific isoforms, such as dynamin, AP180, syndapin 1, clathrin light chain, and intersectin. Second, within the nerve terminal the endocytic machinery is localized close to sites of vesicle exocytosis (Roos and Kelly 1999). Hence, there is no need for long-distance diffusion from exocytotic

to endocytic sites when the clathrin machinery is activated. Third, recent evidence suggests that the components of synaptic vesicles may remain clustered prior to internalization instead of dispersing into the membrane (see, e.g., Willig et al. 2006), thereby avoiding the need for elaborate sorting to reconcentrate vesicle components in the plasma membrane.

CME involves sequential and morphologically distinguishable steps, including coat recruitment and assembly on the membrane, invagination, formation of a constricted pit, fission, and uncoating. Coat recruitment and invagination are initiated by endocytic adaptors like AP-2 and stonin that function in the selection of cargo molecules and the initiation of the assembly of the clathrin coat and other factors which are required for the shaping of the vesicle. The endocytic adaptors bind to a type of lipid enriched in the cytoplasmic leaflet of the presynaptic membrane (phosphatidylinositol (4,5)-bisphosphate), to clathrin and other accessory proteins, and to cargo-sorting signals of, e.g., the synaptic vesicle transmembrane protein synaptotagmin (Maldonado-Báez and Wendland 2006; Di Paolo and De Camilli 2006). Although it appears that adaptor-lipid binding initiates the process, stable recruitment of adaptors to the plasma membrane requires cooperation between theses three types of adaptor-interactions (coincidence detection). Adaptor binding to clathrin and the neural specific AP180 leads to the formation of lattice-like structures with basic units of a trimer of clathrin (named triskelion). These assemble into larger baskets, and the interaction of epsin and AP-180 with the polymerizing clathrin lattice is supposed to promote membrane curvature, finally leading to the invagination of the membrane. Such invaginations are morphologically visible in electron micrographs as clathrin-coated pits. As has been revealed more recently by using fluorescent proteins for the study of endocytosis, clathrin-coated pits are not static but dynamic structures exchanging components with the soluble pool of endocytic proteins (Edeling et al. 2006).

The next step is the formation of a constricted pit, followed by fission of the membrane. Membrane fission is mediated by the mechanochemical GTPase dynamin that appears to wrap around the neck of the clathrin-coated pit (Takei and Haucke 2001). Recruitment of dynamin to its site of action is facilitated by amphiphysin, a protein that not only binds to dynamin but also to AP2 and clathrin. Furthermore, dynamin binds to phosphatidylinositol (4,5)-bisphosphate by its pleckstrin homology domain. The binding of GTP to dynamin in complex with amphiphysin has been suggested to redistribute dynamin close to the neck region, resulting in the formation of a constricted pit. GTP-hydrolysis leads to a conformational change of dynamin accompanied by a constriction of the dynamin ring around the neck. This mechanism probably generates the membrane fission event, resulting in a clathrin-coated vesicle ready for transport into the cytosol. Dynamin may thus be regarded as the counterpart of the SNAREs, with the SNAREs mediating fusion and dynamin mediating fission.

Uncoating requires an interaction with the uncoating ATPase Hsc70. Apparently, however, hydrolysis of phosphatidylinositol (4,5)-bisphosphate is required, which is carried out by the protein synaptojanin. Synaptojanin has two phosphatase domains, and in its absence clathrin-coated vesicles accumulate. Furthermore, the

coated vesicle-associated protein auxilin is needed for uncoating. This protein recruits Hsc70 and stimulates its ATPase activity.

It is still unclear whether uncoated recycling vesicles must pass through an endosomal intermediate before synaptic vesicles are re-formed. Synaptic vesicles contain high concentrations of the protein machinery required for endosome fusion (Takamori et al. 2006), and synaptic organelles that have just undergone endocytosis are capable of homotypic fusion. However, it is possible that the endosomal intermediate serves as a backup rather than a mandatory intermediate, with vesicles being directly regenerated after uncoating without the involvement of an additional fusion and budding step.

5.3 Coupling Exocytosis to Endocytosis

In the "kiss-and-run" mode exocytosis and endocytosis are directly coupled to each other, while in the case of classical complete vesicle fusion, exocytosis and slow clathrin-mediated endocytosis are timely and spatially separated. However, it appears that also in the latter case exocytosis and endocytosis occur coordinated, as both are stimulated by an increase of the cytoplasmic calcium concentration. It has been shown that after calcium entry the enzyme phospho-inositol-5 kinase Iγ, which is enriched in the synapse, catalyzes the synthesis of phosphatidylinositol (4,5)-bisphosphate and that this mechanism is important for synaptic vesicle trafficking (Di Paolo et al. 2004). As many proteins involved in clathrin-mediated endocytosis are recruited to the plasma membrane by binding to phosphatidylinositol (4,5)-bisphosphate (e.g., amphiphysin, dynamin, epsin, AP-180, and AP-2) it is attractive to speculate that elevated levels of calcium mediate the recruitment of endocytic proteins to the plasma membrane by this mechanism. The increased level of phosphatidylinositol (4,5)-bisphosphate could be in part degraded by synaptojanin that thereby initiates the disassembly of the clathrin coat. Hence, calcium-induced transient increases in the level of phosphatidylinositol (4,5)-bisphosphate appear to play a central role for coupling exocytosis to clathrin-mediated endocytosis. In addition, it has been demonstrated that calcium also leads to the dephosphorylation of endocytic proteins as amphiphysin, dynamin, and synaptojanin, which in vitro is important for efficient coat assembly (Cousin and Robinson 2001).

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Presynaptic Neurotoxins with Enzymatic Activities

Ornella Rossetto and Cesare Montecucco(✉)

Abstract Toxins that alter neurotransmitter release from nerve terminals are of considerable scientific and clinical importance. Many advances were recently made in the understanding of their molecular mechanisms of action and use in human therapy. Here, we focus on presynaptic neurotoxins, which are very potent inhibitors of

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the neurotransmitter release because they are endowed with specific enzymatic activities: (1) clostridial neurotoxins with a metallo-proteolytic activity and (2) snake presynaptic neurotoxins with a phospholipase A2 activity.

Tetanus and botulinum neurotoxins produced by anaerobic bacteria of the genus *Clostridium* enter peripheral cholinergic nerve terminals and cleave proteins of the neuroexocytosis apparatus, causing a persistent, but reversible, inhibition of neurotransmitter release. Botulinum neurotoxins are used in the therapy of many human syndromes caused by hyperactive cholinergic nerve terminals. At variance, some snake presynaptic neurotoxins block nerve terminals by binding to the plasma membrane, where they hydrolyze phospholipids with production of lysophospholipids and fatty acids. These compounds change the membrane conformation causing enhanced neurotransmitter release and, at the same time, inhibition of synaptic vesicle recycling. It is possible to envisage pharmacological applications of the lysophospholipid/fatty acid mixture to inhibit hyperactive superficial nerve terminals.

1 Introduction

Many toxins act selectively on the nervous tissue and the most poisonous toxins are neurotoxins. Given the essential role of the nervous system in animal physiology, even a minor biochemical modification of a few neurons may result in a profound behavioral alteration. In general, neurotoxins block in one way or another the transmission of the nerve impulse. The majority of neurotoxins act by binding specifically to ion channels and the ensuing strong alteration of ion permeability of the plasma membrane results in the blockade of the transmission of nerve signals. In contrast, clostridial neurotoxins with metalloproteolytic activity and snake neurotoxins with phospholipase A_2 activity interfere directly with neurotransmitter release by exploiting their highly specific enzymatic activity, which makes them the most poisonous substances.

Tetanus neurotoxin (TeNT) and botulinum neurotoxins (BoNTs) are the sole cause of tetanus and botulism. This permitted their early detection in culture filtrates of anaerobic and spore-forming bacteria of the genus *Clostridium* (Faber 1890; Tizzoni and Cattani 1890; Van Ermengem 1897). However, it took over a century to achieve a molecular understanding of the pathogenesis of the diseases they cause, and still, some steps of the intoxication process are ill-known. TeNT inhibits neurotransmitter release of synapses of the central nervous system (CNS) causing the spastic paralysis of tetanus; BoNT inhibits the release of acetylcholine (ACh) at peripheral cholinergic nerve terminals causing the flaccid paralysis of botulism. Hence, these neurotoxins cause two diseases (tetanus and botulism) with opposite clinical symptoms, yet they impair the same very essential neuronal function: *neuroexocytosis* (Brooks et al. 1955; Burgen et al. 1949; Rossetto et al. 2006; Schiavo et al. 2000). To date, one TeNT and seven (A–G) serologically distinct BoNTs have been characterized. The latter can be further divided into subserotypes; for example, there are four subserotypes of BoNT/A (termed A1-A4) (Arndt et al. 2006;

Smith et al. 2005), which are approximately 90% identical in their amino acid sequences.

Presynaptic snake neurotoxins endowed with PLA2 activity (SPANs) are major components of the venom of four families of venomous snakes (*Crotalidae, Elapidae, Hydrophiidae, and Viperidae*). These neurotoxins play a crucial role in envenomation of the prey (Harris 1997) by causing a persistent blockade of neurotransmitter release from nerve terminals with a peripheral paralysis very similar to that of botulism (Connolly et al. 1995; Gutiérrez et al. 2006; Kularatne 2002; Prasarnpun et al. 2005; Theakston et al. 1990; Trevett et al. 1995; Warrell et al. 1983).

The botulinum neurotoxins and the snake presynaptic PLA2 neurotoxins share three levels of interest: (1) they are pathogenic to humans and animals, (2) they contribute to the understanding of the molecular steps of neurotransmission, and (3) their present and future clinical applications. In this chapter, these neurotoxins are considered in terms of mode of action and in relation to their potential use in cell biology and neuroscience research as well as therapeutics in some human neurodisorders.

2 Toxicity of BoNTs and of SPANs

Tetanus and botulinum neurotoxins are the most potent toxins known, as few nanograms/Kg are sufficient to kill most mammals. When injected peripherally the mouse LD_{50} s of TeNT and BoNTs are between 0.4 ng and 1 ng of toxin per Kg of body weight (Gill 1982). When BoNTA and BoNT/B are injected into the brain the LD_{50} s are comparable with those determined by intraperitoneal injection in mice (Luvisetto et al. 2003).

The SPANs are comparatively less potent and show a much wider range of toxicity, with mouse LD_{50} values comprised between the 1 µg/kg of textilotoxin (present in the venom of the Australian snake *Pseudonaja textilis*) to the 1300 µg/kg of pseudexin A (present in the venom of *Pseudechis porphyriacus*) (Kini 1997; Montecucco and Rossetto 2000; Pearson et al. 1993), when injected intraperitoneally. In addition, they display largely different toxicities versus different animal classes, probably reflecting different predator-prey relationships in different venomous snakes. Even though SPANs do not cross the blood-brain barrier, they are active on the central nervous system and β-bungarotoxin is three orders of magnitude more potent when administered into rat brain (Hanley and Emson 1979). These high toxicities derive from: (1) the specificity of these toxins for the nervous tissue, whose complete functionality is essential for survival, particularly in the wilderness, and (2) their enzymatic activities localized on nerve terminals.

3 The Diseases

3.1 Tetanus

It is now well established that TeNT is the sole cause of tetanus, though it took many centuries to establish a direct mechanistic link between the neurotoxin and the disease it causes. In fact, tetanus was first described in medical terms by Hippocrates, who defined this paralysis as "tetanus" (τετανοσ in Greek means contraction). Tetanus may develop in different forms, and the most common one is the *generalized tetanus* caused by contamination of even minor wounds or skin scratches with spores of toxigenic *Clostridium tetani*. Between the time of injury and the first symptoms there is a lag phase varying from few days up to four weeks, which corresponds to the time necessary for (1) germination of spores, (2) toxin production and release, and (3) toxin diffusion, binding, transport to its target cells within the spinal cord. Tetanus usually begins with a characteristic lockjaw (*risus sardonicus*), with difficulty in swallowing and neck stiffness (Bleck 1989). With time, the muscle paralysis extends downwards to the muscles of the trunk, abdomen, and legs. The typical tetanic seizure is characterized by a sudden burst of tonic contraction of skeletal muscles with extension to the lower extremities. Seizures are very painful and can be triggered by minor stimuli such as a small light or noise, with the patient remaining completely conscious during such episodes. Later on, autonomic symptoms develop with alterations of blood pressure and of the cardiac rhythm and sweating. Dysphagia and glottal and laryngeal spasm may cause cyanosis and asphyxia, which can be relieved by tracheotomy and mechanically assisted respiration.

A milder form of the disease is *local tetanus*, with rigidity of the muscles close to the site of injury and release of the toxin. This local tetanus may persist for a considerable period of time without further developments or it may proceed to generalized tetanus. It is due to dysfunction of the spinal cord inhibitory interneurons which inhibit the alpha motor neurons of the affected muscles, with little, if any, further spread through the central nervous system. Tetanus is often fatal, and death follows body exhaustion and usually intervenes by respiratory failure or heart failure (Bleck 1989). The mortality has decreased owing to modern intensive care techniques, but it is still high because of the usually advanced age of patients and because their respiration has to be mechanically assisted for long periods of time with the risk of developing pulmonary infections. Following vaccination with tetanus toxoid (formaldehyde-treated tetanus toxin), tetanus has almost disappeared from the more developed countries, but it still takes hundreds of thousand of lives in those regions of the world where vaccination is not performed (Galazka and Gasse 1995). Here, the major form of tetanus is *tetanus neonatorum*, which develops following the nonsterile cut of the umbilical cord of babies born from nonimmunized mothers. This condition is prevalent in communities that employ traditional midwifery practices such as cutting the cord with dirty scissors or rubbing manure on the umbilical stump.

3.2 Botulism

Botulism was described much later than tetanus (Kerner 1817; Midura and Arnon 1976; Pickett et al. 1976), and this delayed recognition is due to its much less evident symptoms, which include a generalized muscular weakness with diplopia, ptosis, dysphagia, facial paralysis, and reduced salivation and lacrimation. The paralysis then progressively descends to affect the muscles of the trunk, including respiratory and visceral muscles. All the symptoms of botulism can be ascribed to the blockade of skeletal and autonomic peripheral cholinergic nerve terminals (Tacket and Rogawski 1989).

The gravity of the illness depends on the amount and type of BoNT, which is usually acquired via the oral route. Death follows the blockade of respiratory muscles, but if ventilated mechanically, the patient will eventually recover completely. In general terms, botulism is much less dangerous than tetanus also because, in most cases, the amount of toxin that reaches the general circulation is not sufficient to block respiration. BoNT/A, /B and /E account for most cases of human botulism, and the disease caused by BoNT/A is more dangerous with symptoms persisting much longer.

The incidence and the types of botulism depend on the occurrence of toxigenic strains of *Clostridium botulinum, C. barati, and C. butyrricum* in the environment and subsequently in foods, and on the cooking practices. The geographical distribution of the different types of botulism corresponds to the distribution of the different toxinotypes of *Clostridium* in the environment. Consequently, type E botulism is mainly found in the colder regions of the northern hemisphere, and aquatic animals are usually involved (Hauschild 1993), while types A and B botulism generally occur in the temperate countries. Most of botulism outbreaks are caused by home-prepared or – stored/fermented food contaminated by spores of BoNT producing strains of *Clostridium* and kept under anaerobic conditions which permit bacterial germination and production of the poison; contamination of foods prepared by food industries is very rare (Hauschild 1993).

A less common form of the disease is that of *infant botulism*, which follows the ingestion of spores of neurotoxigenic *Clostridium* that germinate and multiply within the intestinal tract. Rather than a primary intoxication, this disease is an intoxication following a previous infection of the infant's intestinal tract, which lacks the protective bacterial flora of the adult, allowing colonization by *Clostridium*. It has been estimated that fewer than 100 spores are sufficient to cause such disease (Arnon 1980). The affected infants present constipation, weak sucking, hypotonia, and ptosis. In the more severe cases, the patient becomes lethargic and loses head posture control. The disease progresses to a flaccid paralysis which may extend to respiratory muscles with arrest. Type A toxin is usually associated with more severe diseases and a higher mortality rate than type B or E toxin, and the recovery time is accordingly longer (Arnon 1980). "Wound botulism" following spore contamination of wounds is very rare. On the contrary, animal botulism is rather common, and it can also be caused by BoNT/C and /D.

As a consequence of the fact that a single protein is responsible for all the clinical symptoms of tetanus and botulism, these diseases can be completely prevented by anti-toxin specific antibodies (Galazka and Gasse 1995; Middlebrook and Brown 1995). Toxin neutralizing antibodies can be acquired passively by injection of immunoglobulins isolated from immunized donors or, actively, as a result of vaccination with toxoids, obtained by treating TeNT or BoNT with formaldehyde (Ramon and Descombey 1925; Galazka and Gasse 1995). Tetanus toxoid is very immunogenic, and it is used as a standard immunogen in a variety of immunological studies (Corradin and Watts 1995). Antitetanus and antibotulism vaccines based on the recombinant binding domain (Hc) of TeNT and BoNT have been recently developed based on the observation that the majority of protecting antibodies are directed against this part of the molecule (Byrne and Smith 2000).

3.3 Features of Envenoming from Bites of Snakes Producing Venoms Containing Large Amounts of PLA2 Presynaptic Neurotoxins

At variance from most neurotoxigenic clostridia, which release only BoNT or TeNT, snake venoms contain a multitude of pharmacologically active molecular components. In general, they can be divided into three classes based on their physiological targets: (1) the neuromuscular system, similarly to BoNTs, (2) the blood coagulation system, and (3) the control of blood pressure (Gutiérrez et al. 2006). In the case of many highly poisonous snakes of Asiatic and Australian origin, the major role in envenomation is played by presynaptic neurotoxins endowed with PLA2 activity (SPAN) (Connolly et al. 1995; Prasarnpun et al. 2005). SPANs cause a persistent blockade of neurotransmitter release from peripheral nerve terminals with no evidence of central activities, though they bind to and act on neurons isolated from brain and spinal cord (Kini 1997; Lambeau and Lazdunski 1999; Othman et al. 1982; Rehm and Betz 1982; Rigoni et al. 2004, 2005; Rossetto et al. 2006; Rugolo et al. 1986). Venoms from *Elapidae* and *Hydrophiidae* snakes also contain paralytic α-toxins, which act postsynaptically by binding and inhibiting the acetylcholine receptors. In fact, independently on the anatomical site of biting, patients develops symptoms comparable to those of botulism. They are reported to have ptosis, diplopia, ophthalmoplegia, difficulty in swallowing, respiratory paralysis, abdominal pain, and autonomic symptoms. Mechanical ventilation is often required, but most patients recover within days/weeks (Connolly et al. 1995; Kularatne 2002; Prasarnpun et al. 2005; Theakston et al. 1990; Trevett et al. 1995; Warrell et al. 1983).

There is no accurate estimation of the incidence and number of deaths caused by snake bites in the world. It is possible that tens of thousands of individuals die each year following snake envenomation in Asia, which is the most affected area (Gutiérrez et al. 2006). The death rate has strongly decreased in Australia

following the introduction of first-aid procedures and of anti-venoms animal antisera (Hodgson and Wickramaratna 2002, 2006).

4 Structural Organization of Tetanus and Botulinum Neurotoxins

The similar effect of the eight clostridial neurotoxins (CNTs) on nerve terminals is the result of a closely related protein structure. They are synthesized in the bacterial cytosol as a 150 kDa polypeptide chain without a leader sequence, and are released in the culture medium only after bacterial autolysis. No protein is associated with TeNT, whereas the BoNTs are released as multimeric complexes with a variable set of nontoxic proteins encoded by genes present in the neurotoxin locus (Inoue et al. 1996; Minton 1995; Sakaguchi 1982). Some BoNT-associated proteins have hemagglutinating activity: HA of 17 kDa (HA17), HA of 34 kDa (HA34) and HA of 70 kDa (HA70) (Fujita et al. 1995; Henderson et al. 1997). In addition, a large nontoxic nonhemagglutinating protein (NTNH) of similar size to BoNT is always present, and it has been suggested that it nests the formation of the BoNT-NTNH complex, which then may, or may not, progress to the formation of larger complexes (Figure 1). In fact, three forms of progenitor toxins have been characterized: extra-large size (LL sediments at 19S, approximately 900 kDa); large size (L sediments at 16S, 500 kDa), and medium size (M, sediments at 12S, 300 kDa). It is noteworthy that BoNT interacts with the nontoxic portion in the complex via the H_C domain (Chen et al. 1997), raising the possibility that NTNH prevents its interaction with the presynaptic terminal receptors. Complexed BoNTs are more stable than isolated BoNTs to proteolysis and denaturation induced by temperature, solvent removal, or acid pH (Chen et al. 1998a; Sakaguchi 1982), and therefore they have an increased resistance to the environment in which they are released and to their passage through the alimentary tract (Ohishi et al. 1977). There is evidence that accessory proteins do protect BoNT within the gut, but it is not clear if they also promote their uptake from the intestinal lumen into the general body fluid circulations (Fujinaga et al. 2000; Maksymowych et al. 1999). The BoNTs transcytose from the apical to the basolateral side of intestinal epithelial cells (Ahsan et al. 2005; Maksymowych and Simpson 1998; Simpson 2000), and the toxin form appearing in the lymph is the 150 kDa "naked" protein (Heckly et al. 1960). Once in the tissue fluids, they then reach their specific targets which are the peripheral cholinergic nerve terminals (Montecucco et al. 2004; Rossetto et al. 2006; Simpson 2000). This gastrointestinal passage is bypassed by TeNT, which enters the general circulation directly form the infected wounded site. The target specificity of TeNT and BoNTs is more evident if one considers that they are lethal at pico-femtomolar concentrations and yet they are able to reach their target presynaptic terminals, which represent an infinitesimal part of the body in terms of exposed cell surfaces (Montecucco et al. 2004).

The single chain 150 kDa CNTs are inactive and are activated by specific proteolysis within a surface exposed loop subtended by a highly conserved disulfide bridge.

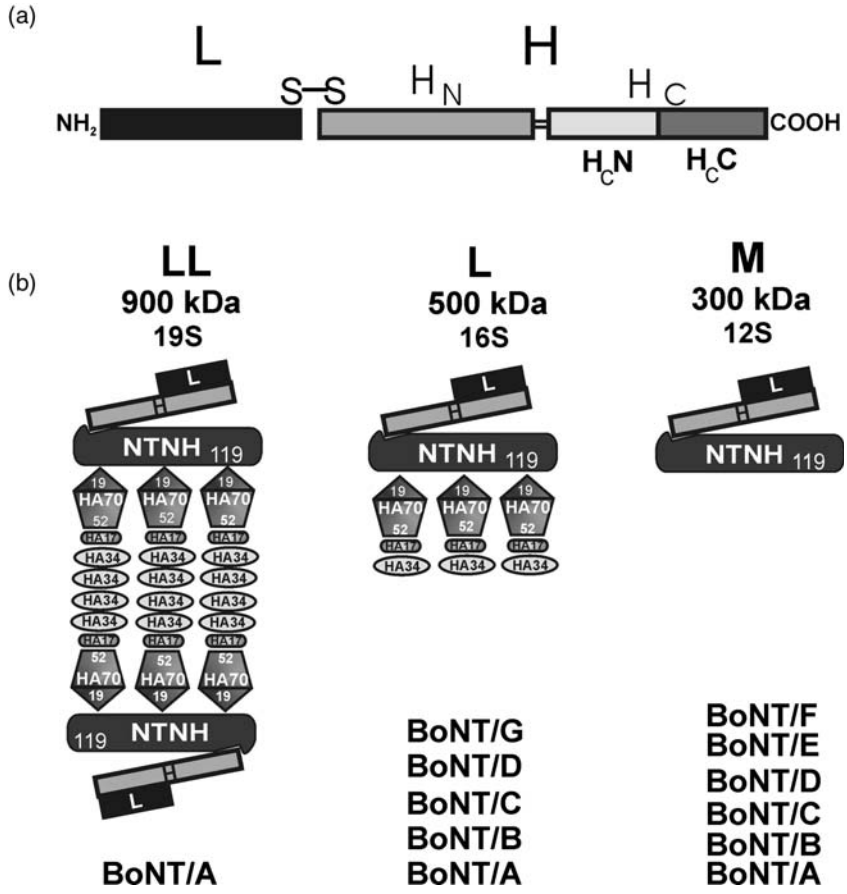

Fig. 1 (**a**) Schematic structure of activated di-chain clostridial neurotoxins. The neurotoxins are composed of a two-polypeptide chain held together by a single disulfide bridge. The C-terminal portion of the heavy chain (H, 100 kDa) is responsible for neurospecific binding (domain H_C), while the N-terminus (H_N) is implicated in the translocation of the light chain in cytosol and pore formation. Structurally H_C can be further subdivided into two portions of 25 kDa, H_CN, and H_CC. The light chain (L, 50 kDa) is a zinc-endopeptidase responsible for the intracellular activity of CNTs. (**b**) Botulinum neurotoxins are produced as multimeric progenitor toxins. The neurotoxin moiety interacts with a large, nontoxic, nonhemagglutinin protein (NTNH) via its C-terminal domain. This dimeric complex (Mw 300 kDa, 12S) serves as a scaffold for the assembly of larger complexes formed by the incorporation of three types of additional proteins with hemagglutinin activity and with Mw of 17 kDa (HA17), 35 kDa (HA35), and 70 kDa (HA70, present in a cleaved state of 19 and 52 kDa). These additional forms are the large L (16S, Mw 500 kDa) and the extra-large LL (19S, Mw 900 kDa) progenitor toxins.

Several bacterial and tissue proteinases are able to generate the active di-chain neurotoxin (Dasgupta 1994; Krieglstein et al. 1991). The heavy chain (H, 100 kDa) and the light chain (L, 50 kDa) remain associated via noncovalent interactions and via the conserved interchain S-S bond, whose integrity is essential for neurotoxicity (Figure 1) (de Paiva et al. 1993; Schiavo et al. 1990; Simpson et al. 2004).

The length of the polypeptide chains of CNTs varies from the 1251 amino acid residues of *C. butyricum* BoNT/E to the 1297 residues of BoNT/G and the 1315 residues of TeNT (Minton 1995; Niemann 1991). The exact length of the L and H chains depends on the site of proteolytic cleavage within the exposed loop. The H chains vary in size from the 829 amino acid residues of BoNT/E to the 857 residues of TeNT. The L chains range in size from the 419 amino acid residues of BoNT/E to the 449 residues of TeNT.

Since the first molecular glimpse of BoNT/A in 1998 (Lacy et al. 1998) there has been a profusion of X-ray crystal structures. There are now 33 BoNT holotoxin or domain structures available, including BoNT/A and BoNT/B holotoxins, TeNT and BoNT/B binding domain, and LC structures of BoNT/A–/G (structures are available at the protein data bank: http://www.rcsb.org/pdb/). CNTs are organized in three distinct 50 kDa domains (Lacy et al. 1998; Swaminathan and Eswaramoorthy 2000): (1) an N-terminal metalloprotease domain (LC), (2) a membrane translocation domain (H_N), and (3) a binding domain (H_C) (see Figure 2) (Lacy et al. 1998; Swaminathan and Eswaramoorthy 2000; Umland et al. 1997).

4.1 The Binding Domain

The H_C binding domain of these CNTs consist of two distinct subdomains: the N-terminal half (H_CN) and the C-terminal half (H_CC), with little protein-protein contacts among them. H_CN is enriched in β-strands arranged in a jelly-roll motif closely similar to some carbohydrate binding proteins (legume lectins). The amino acid sequence of H_CN is highly conserved among CNTs. The H_CC subdomain contains a modified β-trefoil folding motif present in several proteins involved in recognition and binding functions such as IL-1, fibroblast growth factor, and the Kunitz-type trypsin inhibitors. Its sequence is poorly conserved among CNTs. It harbors one binding site for the oligosaccharide portion of polysialogangliosides in BoNT/A and /B (Figure 2), while the H_CC of TeNT has two such sites (Rummel et al. 2003, 2004b). H_CC of BoNT/B harbors a cleft which fits in segment 44–60 of synaptotagmin belonging to the luminal domain of this SV protein (Chai et al. 2006; Jin et al. 2006; Rummel et al. 2007).

4.2 The Translocation Domain

The H_N portions of BoNT/A and /B are very similar, and their sequences are highly homologous among the various CNTs. H_N has an elongated shape determined by a pair of unusually long and twisted α–helices, corresponding to segment 685-827 of BoNT/A (Figure 2) (Lacy et al. 1998; Swaminathan and Eswaramoorthy 2000). At both ends of the pair, there is a shorter α-helix which lies parallel to the main helices and, in addition, several strands pack along the two core helices. The overall

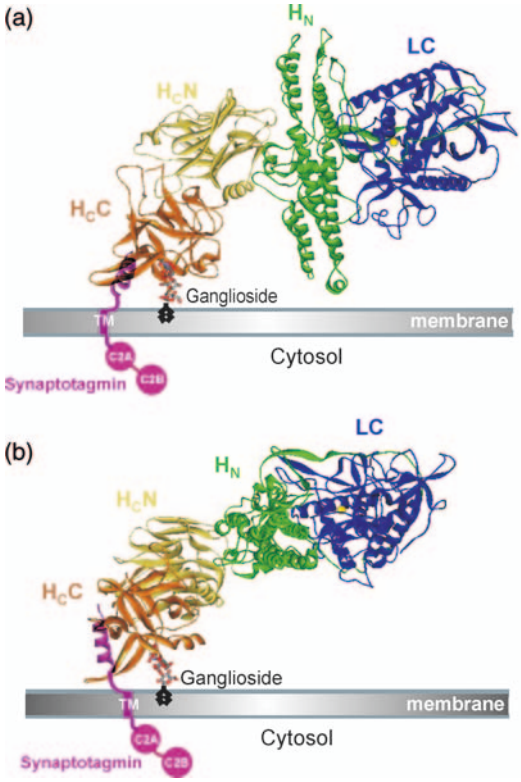

Fig. 2 Binding of botulinum neurotoxin type B to the presynaptic membrane. Two different models have been proposed (Chai et al. 2006; Jin et al. 2006). The C-terminal part of the binding domain (H_CC in orange) interacts specifically both with polysialogangliosides and with the luminal domain of synaptotagmin (magenta), which adopts an helical conformation upon BoNT/B binding. The remaining part of the synaptotamin molecule is drawn as a transmembrane domain (TM) and two C2 cytoplasmic domains (magenta balls). The helices of the toxin translocation domain (H_N) may sit orthogonal to the plane of the membrane surface (panel **a**) (Chai et al. 2006) or may be oriented parallely (panel **a**) (Jin et al. 2006). The light chain (LC), the N-terminal part of the heavy chain (H_N), and the two C-terminal subdomains of the heavy chain (H_C) are shown in blue, green, yellow and orange, respectively. The yellow sphere represents the atom of zinc of the active site of the L chain metalloprotease.

structure of H_N resembles that of some viral proteins undergoing an acid-driven conformational change (Bullough et al. 1994; Kielian and Rey 2006; Weissenhorn et al. 1997). Two models of BoNT/B bound to both Syt-II and ganglioside have been generated with a different orientation of the translocation domain with respect to the cell membrane (Baldwin et al. 2007; Chai et al. 2006; Jin et al. 2006; Rossetto and Montecucco 2007; Schiavo 2006) (Figure 2). Jin et al. (2006) suggest that the helices of the translocation domain sit orthogonal to the plane of the membrane (Figure 2a). On the contrary, Chai et al. (2006) propose a parallel orientation, which would place hydrophobic areas of the helices close to the inner surface of the vesicle, facilitating their insertion into the membrane (Figure 2b).

4.3 The Catalytic Domain

The crystal structures of LC/A (Segelke et al. 2004), LC/B (Hanson and Stevens 2000), LC/E (Agarwal et al. 2004), LC/D (Arndt et al. 2006), LC/F (Agarwal et al. 2005), LC/G (Arndt et al. 2005), and LC/TeNT (Breidenbach and Brunger 2005a) have been determined. They have a unique structure among metalloproteases. The active site zinc atom is coordinated by the imidazole rings of two histidines, a water molecule bound to the glutamic acid of the motif and a glutamic acid which is conserved among CNTs. The Glu residue of the motif is particularly important because it coordinates the water molecule directly implicated in the hydrolytic reaction of proteolysis. Its mutation leads to complete inactivation of these neurotoxins (Li et al. 2000). The active site is similar to that of thermolysin and identifies a primary sphere of residues essential for catalysis, which coincides with the zinc coordinating residues. In addition, there is a secondary layer of residues, less close to the zinc centre, including Arg362 and Tyr365 (numbering of BoNT/A), which appears to be directly involved in the hydrolysis of the substrate (Binz et al. 2002; Rigoni et al. 2001; Rossetto et al. 2001a; Yamasaki et al. 1994). The active site of BoNTs is a long cleft which becomes accessible to the substrate upon reduction of the interchain disulfide bridge.

5 Structure of the Snake Presynaptic PLA2 Neurotoxins

At variance with the high structural similarity of BoNTs, the SPANs vary greatly, both in terms of structure and of PLA2 activity. The crystallographic structure of few of the simpler toxins has been determined (Arni and Ward 1996; Carredano et al. 1998; Kwong et al. 1995; Scott 1997; Singh et al. 2001; Westerlund et al. 1992) (see structures at Protein Data Bank: http://www.rcsb.org/pdb/). They include a phospholipase A2 domain with a flattened ellipsoid shape of approximate dimensions $45\,\text{Å} \times 30\,\text{Å} \times 20\,\text{Å}$. The structure is stabilized by six intrachain disulfide bonds and by a catalytic Ca^{2+} atom. Two long antiparallel and disulfide linked α-helices characterize their structure and include the four key residues of a catalytic site shielded from the water solvent (His48, Asp49, Tyr52 and Asp99) (Figure 3). The histidine residue hydrogen binds the water molecule used for hydrolysis, while Asp49 coordinates and positions the Ca^{2+} ion which binds both the phosphate and the sn-2 carbonyl groups of the phospholipid molecule during hydrolysis. In addition, there is an interfacial binding site, which mediates the absorption of the enzyme onto the lipid-water interface, which is strongly promoted by anionic amphipatic molecules such as fatty acids (Scott 1997). Another characteristic feature of PLA2s is the presence inside the molecule of an hydrophobic channel where a phospholipid molecules diffuses from the membrane into the catalytic site.

Chemical modification and structural comparison studies implicate the extended loop and β region between residues 59 and 100 as the main responsible for neurotoxicity (Dufton and Hider 1983; Kini and Evans 1989; Yang 1997). However,

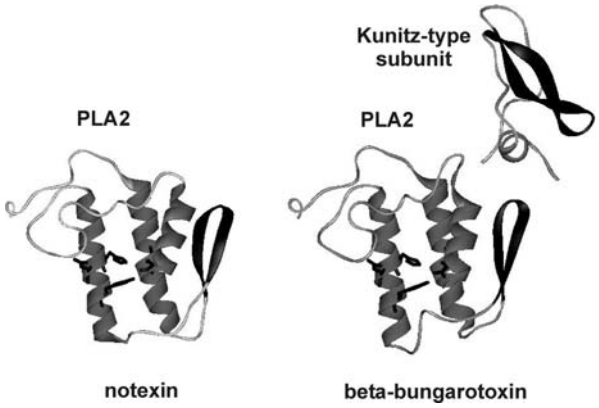

Fig. 3 Ribbon diagram of the single subunit notexin (PDB number 1AE7, Westerlund et al. 1992) and the β-bungarotoxin (PDB number 1BUN, Kwong et al. 1995). The structure of the PLA2 reveals the characteristic three α-helices (dark grey) with two β-strands (black). In β-bungarotoxin the PLA2 subunit is disulfide linked to a 7 kDa protein with a sequence and a structure very similar to that of the Kunitz-type trypsin inhibitor and to the three-foil subdomain H_CN of clostridial neurotoxins. The residues of the catalytic site His48, Asp49, Tyr52, and Asp99 (Asp94 in β-bungarotoxin) are shown.

this leaves open the possibility that additional regions and/or subunits may be involved, particularly in the case of multi-subunit toxins. In fact, SPANs vary greatly in terms of their quaternary structure, from the single subunit notexin (from the Australian tiger snake *Notechis scutatus*), to β-bungarotoxin (from *Bungarus multicinctus*), which consists of two covalently linked subunits, the larger of which is a PLA2 (Figure 3). Similarly, only one of the three subunits of taipoxin (from *Oxyuranus scutellatus*) has PLA2 activity. In contrast, textilotoxin (from the Australian elapid *Pseudonaja textilis*) consists of 5 subunits (A, B, C and two D subunits), all endowed with PLA2 activity (Kini 1997).

6 The Mode of Action of Clostridial Neurotoxins

The structural organization of CNTs is functionally related to the fact that they intoxicate neurons via a four-step mechanism consisting of (1) binding, (2) internalization, (3) membrane translocation, and (4) enzymatic target modification (Figure 4) (Montecucco et al. 1994; Montecucco and Schiavo 1995; Rossetto et al. 2006). The LC is responsible for the intracellular catalytic activity, the amino-terminal 50 kDa domain of the H chain (H_N) is implicated in membrane translocation, and the carboxy-terminal part (H_C) is mainly responsible for the neurospecific binding.

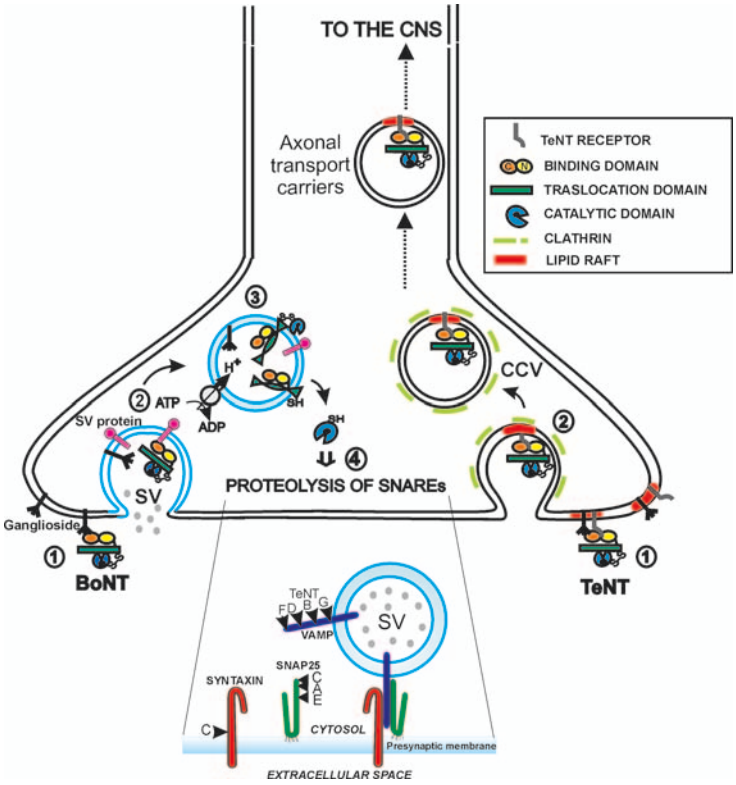

Fig. 4 Binding and entry of BoNTs and TeNT at peripheral nerve terminal. **(1)** The BoNT binding domain associates with the presynaptic membrane of α-motoneurons through interaction with ganglioside and with the exposed luminal domain of a synaptic vesicle protein upon SV membrane fusion. **(2)** BoNTs are endocytosed within synaptic vesicles via their retrieval to be refilled with neurotransmitter. TeNT exploits a pathway requiring lipid rafts and the clathrin machinery by binding to a lipid–protein receptor complex containing the ganglioside GD1b. Once internalized in clathrin-coated vesicles (CCV), TeNT is sorted into vesicle carriers of the axonal retrograde transport pathway. **(3)** At the low pH generated by the v-ATPase in the vesicle lumen, BoNTs change conformation, insert into the lipid bilayer of the vesicle membrane and translocate the L chain into the cytosol. **(4)** Inside the cytosol the L chain catalyses the proteolysis of one of the three SNARE proteins VAMP, SNAP-25 and syntaxin, depicted in the lower panel. The same four-step pathway of entry of BoNTs into peripheral nerve terminals is believed to be followed by TeNT in the inhibitory interneurons of the spinal cord, which are reached after retroaxonal transport and release from the peripheral motoneurons.

6.1 Binding

From the site of adsorption, BoNTs diffuse in the body fluids and eventually bind very specifically to the presynaptic membrane of cholinergic terminals. At variance, the pathway of TeNT is more complex, as it involves an additional retroaxonal transport to the spinal cord and a specific binding to inhibitory interneurons

(Lalli et al. 2003). The H_C domain plays the major role in the neurospecific binding (Chai et al. 2006; Jin et al. 2006). Polysialogangliosides are involved in CNT binding (reviewed in Montecucco et al. 2004; Rossetto et al. 2006; Schiavo et al. 2000; Verderio et al. 2006; Yowler and Schengrund 2004). In fact, the H_CC subdomain of BoNT bind polysialogangliosides, particularly GD1a, GT1b, and GQ1b, via the conserved peptide motif H...SXWY...G (Rummel et al. 2004b). Pre-incubation of BoNTs with polysialogangliosides partly prevents cell intoxication and the pretreatment of cultured cells with polysialogangliosides increases their sensitivity to BoNT/A; conversely, depletion of gangliosides in neuroblastoma or PC12 cells impairs BoNT/A and BoNT/B internalization. Neuraminidase removes sialic-acid residues and decreases BoNT cell binding. Knockout mice defective in the production of polysialogangliosides show reduced sensitivity to BoNT/A and BoNT/B (Bullens et al. 2002; Chai et al. 2006; Kitamura et al. 2005; Schiavo et al. 2000; Yowler et al. 2002). More recently, polysialogangliosides GD1b and GT1b and phosphatidylethanolamine were reported to be the functional receptors of BoNT/C and BoNT/D, respectively (Tsukamoto et al. 2005). At variance, two sugar-binding sites have been identified within the TeNT H_CC fragment and this double binding, possibly to a polysialoganglioside molecule and to a glycoprotein, is essential for the toxicity of TeNT (Rummel et al. 2003; Herreros et al. 2000).

It is well established that both glycolipid and protein receptors are crucial for high-affinity binding to nerve cell membranes (Rummel et al. 2007). As BoNTs and TeNT are toxic at concentrations estimated to be $<$ picomolar (Montecucco et al. 2004), binding has to be interpreted as the simultaneous occupation of the ganglioside binding pocket(s) and of the protein binding site. Indeed, TeNT was shown to interact with a GPI-anchored glycoprotein and to bind to lipid rafts (Herreros and Schiavo 2002; Herreros et al. 2001; Munro et al. 2001). Synaptic vesicle (SV) proteins participate with their luminal domains to the neurospecific binding of BoNT/A, /B, and /G, and this is probably true for all the other BoNTs (for a recent review, see Verderio et al. 2006). BoNT/B and BoNT/G interact with the luminal domain of the SV proteins synaptotagmin I (Syt-I) and Syt-II. BoNT/B has a higher affinity for Syt-II (Dong et al. 2003; Nishiki et al. 1996), whereas BoNT/G interacts preferentially with Syt-I (Rummel et al. 2004a). The crystal structures of BoNT/B in complex with the luminal domain of Syt-II (Chai et al. 2006; Jin et al. 2006) shows that H_CC accommodates the α-helical segment 45–59 of Syt-II in a cleft adjacent to the polysialogangliosides-binding site of the toxin (Figure 2). Mutations in the synaptotagmin-binding cleft and in the polysialoganglioside-binding pocket greatly reduce the toxicity of BoNT/B and /G, and double mutants at the two binding sites abolish neurotoxicity (Rummel et al. 2007).

At variance, BoNT/A binds specifically to the luminal domain of SV2 (Dong et al. 2006; Mahrhold et al. 2006). SV2 is a highly glycosylated transmembrane protein of SV implicated in vesicle recruitment to the plasma membrane in endocrine cells (Iezzi et al. 2005). Vertebrates express three distinct, but homologous, SV2 proteins termed SV2A, SV2B, and SV2C (Bajjalieh et al. 1992; Bajjalieh et al. 1994). Interestingly, motor neuron terminals express the receptor isoforms that bind BoNTs more strongly, such as Syt-II (Juzans et al. 1996; Li et al. 1994; Marquèze et al.

1995) and SV2C (Janz and Südhof 1999). Conversely, high-affinity isoforms are poorly expressed in CNS neurons and terminals, in which Syt-I, SV2A, and SV2B are the prevalent isoforms (reviewed in Verderio et al. 2006). Recently Baldwin and Barbieri (2007) demonstrated that BoNT/A and /B binding domains associate with synaptic vesicle membrane proteins and suggested that BoNT protein receptor may be a component of a larger protein complex.

Both TeNT and BoNTs bind the presynaptic membrane of α-motoneurons, but then TeNT follows a different intracellular trafficking route and this must be determined by yet unidentified specific receptor(s).

6.2 Internalization

Available evidence indicates that CNTs do not enter the cell directly from the plasma membrane, but are endocytosed inside the lumen of vesicular structures in a temperature- and energy-dependent process (Black and Dolly 1986; Critchley et al. 1985; Dolly et al. 1984; reviewed in Schiavo et al. 2000). The finding of SV proteins as receptors of BoNTs support the proposal (Montecucco and Schiavo 1995) that, after binding, BoNTs are endocytosed within synaptic vesicles (SV) via their retrieval to be refilled with neurotransmitter, an hypothesis originally advanced to account for the increased rate of poisoning with NMJ activity (Figure 4) (Hughes and Whaler 1962).

The protein receptor of TeNT would be responsible for its inclusion in an endocytic vesicle that moves in a retrograde direction all along and inside the axon (Figure 4). TeNT-H_C internalization at the motor nerve terminal occurs via a specialized clathrin-dependent pathway, which is distinct from SV endocytosis and is preceded by a lateral sorting from its lipid raft–associated ligand GD1b. (Deinhardt et al. 2006; Roux et al. 2005). The TeNT-carrying vesicles reach the cell body of the motoneurons which reside within the spinal cord and then move to dendritic terminals to release the toxin in the intersynaptic space. TeNT then binds and enters the inhibitory interneurons of the spinal cord via SV endocytosis (Matteoli et al. 1996). Consequently, it blocks the release of inhibitory neurotransmitters and impairs the system which controls the balanced movement of the skeleton (Kandel et al. 2000), giving rise to a spastic paralysis. It is not known if, and if so, to what an extent, BoNTs migrate retroaxonally. Direct measurements of retroaxonal transport indicated that ^{125}I-BoNT/A does not reach the central nervous system (Habermann and Dreyer 1986).

6.3 Membrane Translocation

The L chains of CNTs block neuroexocytosis by acting in the cytosol, and therefore at least this toxin domain must reach the cell cytosol. In order to do so, the L chain

must cross the hydrophobic barrier of the vesicle membrane, and compelling evidence indicates that CNTs have to be exposed to low pH for this step to occur (Matteoli et al. 1996; Simpson et al. 1994; Williamson and Neale 1994). In fact, acidic pH causes a conformational change from a water-soluble "neutral" structure to an "acid" structure with exposure of hydrophobic patches on the surface of both the H and L chains which then enter into the hydrocarbon core of the lipid bilayer (Montecucco et al. 1989; Puhar et al. 2004; Schiavo et al. 2000; Shone et al. 1987). Following this low pH-induced membrane insertion, BoNTs and TeNT form transmembrane ion channels (reviewed in Montecucco and Schiavo 1995) and in PC12 membranes (Sheridan 1998). The cleavages of SNAP-25 by BoNT/A and BoNT/E is differentially affected by inhibitors of the vacuolar ATPase proton pump (Keller et al. 2004), but this is unlikely to be due to a difference in the toxin acid-triggered conformational change, as the low pH-driven toxin conformational change takes place in a very narrow range of pH: 4.4–4.6 for TeNT and BoNT/A, /B, /C, /E, and /F (Puhar et al. 2004). This similarity indicates that the key residues for the conformational change are conserved among CNTs and that they behave very similarly with respect to lowering pH. Currently, membrane translocation is the least understood step of the mechanism of cell intoxication of CNTs, and further investigation is required to uncover the extent and implication of the structural changes that occur at low pH (for a recent discussion see Rossetto and Montecucco 2004). Koriazova and Montal (2003) have proposed that the H chain channel acts as a trans-membrane chaperone for the L chain, preventing its hydrophobicity-driven aggregation and maintaining its unfolded conformation during translocation. The L chain is then released into the neutral cytosol where it refolds into its native enzymatically active conformation. In this vision, additional chaperones are not implicated. However, a complex of cytosolic chaperones and thioredoxin present on the cytosolic face of endosomes was recently found to assist the translocation of the enzymatic chain of other bacterial toxins acting in the cytosol (Haug et al. 2003; Ratts et al. 2003). Four different chaperone systems have been found so far within neuron terminals and are essential for normal function of the synaptic vesicle cycle during neurotransmitter release (Evans et al. 2003; Zinsmaier and Bronk 2001). In light of the proposed role of SVs as vesicular carriers of BoNTs inside peripheral neurons, it is noteworthy that two out of four synaptic terminals chaperone complexes includes the cystein string protein of SVs. It is therefore possible that the membrane translocation and refolding of the L chain is assisted by SV chaperones in addition to the H chain, but this remains to be proven.

6.4 SNARE Proteins' Specific Metalloproteolytic Activity

The L chains of BoNTs and TeNT are highly specific proteases that recognize and cleave only three proteins, the so-called SNARE proteins, which form the core of the neuroexocytosis apparatus (Figure 4) (Schiavo et al. 2000). TeNT, BoNT/B, /D, /F and /G cleave VAMP, a protein of the SV membrane, at different single peptide

bonds; BoNT/C cleaves both syntaxin and SNAP-25, two proteins of the presynaptic membrane; BoNT/A and /E cleave SNAP-25 at different sites within the COOH-terminus (reviewed in Humeau et al. 2000; Schiavo et al. 2000). BoNT-poisoned nerve terminals have a normal size and appearance with normal content and shape of synaptic vesicles and mitochondria (Duchen 1971; Thesleff 1960). Possibly the only morphological sign of BoNT/A poisoning is noticeable at the frog NMJ with the disappearance of the small outward curvatures of the membrane close to the active zones (Harlow et al. 2001; Pumplin and Reese 1977).

VAMP, SNAP-25, and syntaxin form an heterotrimeric coiled-coil complex, termed the SNARE complex, which induces the juxtaposition of vesicle to the target membrane (Jahn et al. 2003; Südhof 2004). Several SNARE complexes assemble into a rosette in order to bring the SV membrane close enough to the cytosolic face of the presynaptic membrane to permit their fusion with subsequent release of the vesicle neurotransmitter content into the synaptic cleft (Montecucco et al. 2005). Proteolysis of one SNARE protein prevents the formation of a functional SNARE complex and, consequently, the release of neurotransmitter. This is not true for BoNT/A and /C, which cleave SNAP-25 within few residues from the C terminus giving rise to a truncated SNAP-25, which can still form a SNARE complex but not a rosette of SNARE complexes. Given the fact that a rosette of SNARE complexes is necessary for the exocytosis of one synaptic vesicle, the presence of a single defective SNARE complex will have a dominant negative effect on neurotransmitter release. This rationale explains the experimental finding that incomplete proteolysis of SNAP-25 at nerve terminals by BoNT/A is sufficient to cause full inhibition of neurotransmitter release (Bruns et al. 1997; Foran et al. 1996; Jurasinski et al. 2001; Keller et al. 2004; Osen-Sand et al. 1996; Raciborska et al. 1998; Williamson et al. 1996). This also explains the fact that the paralysis induced by BoNT/A and /C is long lasting, as the inhibitory activity of C-terminal truncated SNAP-25 on the assembly of the rosette of SNARE complexes overlaps and functionally extends the lifetime of the metalloproteolytic activity of the L chain of these two BoNTs (for a detailed discussion see Rossetto et al. 2006). These explanations, however, may not be sufficient to account for the astonishing fact that the duration of the BoNT/A inhibition of autonomic nerve terminals is more than one year (Naumann and Jost 2004).

The molecular basis of the CNTs specificity for the three SNAREs resides on protein-protein interactions which extend well beyond the proteolysed SNARE regions, and a major role is played by a nine-residue-long motif present within the SNARE proteins. This motif is characterized by three carboxylate residues alternated with hydrophobic and hydrophilic residues (Breidenbach and Brunger 2004; Evans et al. 2005; Pellizzari et al. 1996; Rossetto et al. 1994; Vaidyanathan et al. 1999; Washbourne et al. 1997). This motif is present in two copies in VAMP and syntaxin and four copies in SNAP-25. The various CNTs differ with respect to the specific interaction with the recognition motif (Rossetto et al. 2001b). Only protein segments including at least one copy of the motif are cleaved by the toxins, and mutations within the motif inhibit the proteolysis (Fang et al. 2006; Schiavo et al. 2000). Moreover antibodies against the SNARE motif inhibit the proteolytic

activity of the neurotoxins (Pellizzari et al. 1997). A recent co-crystal structure of LC/A and SNAP25-(146–204) defined additional regions of interaction external to the cleavage site and to the motif (Breidenbach and Brunger 2004; Breidenbach and Brunger 2005b). More recently, the fitting of an extended region of the substrate (residues 189–203) within the long active site cleft was defined following extensive mutagenesis of LC/A and SNAP-25 (Chen and Barbieri 2006; Chen et al. 2007).

There are no quantitative data on the number of L chains required to intoxicate a nerve terminal. In *Aplyisa californica* cholinergic neurons, few molecules of toxin appear to be sufficient to block neuroexocytosis within an hour at room temperature (Poulain, personal communication). It is even more likely that few copies of L chain are sufficient in warm-blooded animals. It is evident that as long as the toxin is present in an active form, the nerve signal cannot be transmitted.

The half-time of action of the different BoNTs has been estimated in mice cerebellar granular neurons in culture by Foran et al. (2003b): BoNT/A (31 days) > BoNT/C (25) > BoNT/B (10) > BoNT/F (2) > BoNT/E (0.8). Comparable data were found in the mice NMJ *in vivo* (Meunier et al. 2003; Morbiato et al., 2007). These data parallel those observed in human patients injected with different BoNTs within skeletal muscles: BoNT/A (2–4 months) > BoNT/C (12–16 weeks) > BoNT/B (5–10 w) > BoNT/F (5–8 w) > BoNT/E (4–6 w). In general, humans recover more slowly than do rodents. The lifetime of LC inside the nerve terminal is clearly involved in determining the duration of paralysis. The finding that LC/A overexpressed in cultured cells binds to the cytosolic face of cell membrane, while LC/E is prevalently cytosolic (Fernández-Salas et al. 2004), has provided a possible explanation, within the limits of the significance of protein overexpression with respect to their intracellular localization. However, it is hard to conceive that this is the sole explanation, particularly if one considers that the duration of the effect of BoNT/A in autonomic disfunction in humans is 12–15 weeks (Naumann and Jost 2004). As discussed above, experimental evidence supports the proposal that an important role is also played by the dominant negative effect on neurotransmission exerted by the BoNT/A- or /C-truncated SNAP-25 (Montecucco et al. 2005; Rossetto et al. 2006). Nevertheless, even the addition of the lifetimes of LC and of truncated SNAP-25 may not be sufficient to provide a satisfactory explanation for the very long time of recovery of intoxicated autonomic terminals of exocrine glands.

7 The Mode of Action of PLA2 Snake Presynaptic Neurotoxins

Contrary to TeNT and BoNTs, which act inside nerve, there is a consensus that, at least in the initial phases of intoxication, SPANs act on the presynaptic plasma membrane. Many investigations performed with different SPANs using different NMJ preparations have documented that the progression of muscle paralysis consists of three phases:

(1) A brief initial phase of weak inhibition of Ach release, lasting few minutes, which is absent with some toxins such as notexin and paradaxin, and which has a different relevance in mice and rats NMJ preparations.
(2) A more prolonged second phase (10–30 min) which consists in a facilitated neurotransmitter release with generation of sufficient intersynaptic Ach to stimulate muscle twitch. The increased ACh release, induced by SPANs, is due to an increased frequency of m.e.p.p.s with no change in m.e.p.p.s amplitude, indicating an unaltered response of endplates; also e.p.p.s are larger in this phase of SPAN action and, in some cases, giant m.e.p.p.s are induced.
(3) A third phase of progressive decline of neurotransmission with strong decrease of e.p.p.s and m.e.p.p.s and no alteration of muscle contractile properties (reviewed in Harris 1997; Howard and Gundersen 1980; Montecucco and Rossetto 2000; Rowan 2001).

Moreover, the time course of the SPAN-induced paralysis is greatly shortened by nerve stimulation, which increases the tightly coupled events of SV exocytosis and endocytosis (Sudhof 2004); on the contrary depressing nerve terminal activity decreases the rate of intoxication (Chang et al. 1973). The action of SPANs is also characterized by the fact that the toxic effects can be neutralized by antitoxin antibodies, or Sr^{2+} or washing with fresh medium only if performed within a short time period after toxin addition (Kamenskaya and Thesleff 1974; Simpson et al. 1993; Wilson et al. 1995).

Phase 1 is promoted by Ca^{2+} and is largely unaffected by temperature and by treatments that depress the PLA2 activity of SPANs. In addition this phase is saturated at low toxin concentrations (Caratsch et al. 1981, 1985; Simpson et al. 1993), and the binding of radioactively labeled SPANs to the central nervous tissue indicates high affinity and limited number of binding sites, mainly located at presynaptic nerve terminals (Lambeau and Lazdunski 1999; Lambeau et al. 1989; Othman et al. 1982; Rehm and Betz 1982; Strong et al. 1977). The different SPANs do not compete one with another for binding to the presynaptic membrane. β-bungarotoxin is rather specific for motor nerve terminals, and sensory discharges are not affected for several hours of incubation with the toxin. At variance in the central nervous system this neurotoxin appear to bind more specifically to cholinergic and GABAergic terminals (Abe et al. 1976; Rehm and Betz 1982). These data suggest that phase 1 represents SPAN binding to the nerve terminal, a biological activity not overlapping the enzymatic one. Why the mere SPAN binding on the extracellular surface of presynaptic terminals should inhibit neuroexocytosis is not clear. This is even more puzzling if one considers that lysophosphatidylserine, lysophosphatidylglycerol, and lysophosphatidic acid also induce a phase 1 in the mouse hemidiaphragm (Caccin et al. 2006). These authors have suggested that SPANs and some lysophospholipids bind to selected portions of the presynaptic membrane corresponding to the active zones of neuroexocytosis (AZ), which face the Ach receptor rich in foldings of the muscle membrane (Couteaux and Pécot-Dechavassine 1970; Harlow et al. 2001). SPAN binding causes by itself, or following a limited and local phospholipid hydrolysis, a structural alteration of these sites of neurotransmitter release resulting in a lowered probability of membrane fusion; it

is conceivable that certain lysophospholipids can as well alter the AZ, making them less prone to membrane fusion with synaptic vesicles. Toxin binding to the AZ also accounts for the lack of binding competition among the different SPANs, and, at the same time, for the presynaptic neurospecificity of SPANs. Additionally, it is possible that SPANs display their hydrolytic activity within AZ boundaries where some lipid mismatching and a reduced amount of cholesterol may exist. This suggestion follows the finding that β-bungarotoxin is most active at the liquid crystalline-gel phase transition of phosphatidylcholines and virtually inactive in the presence of cholesterol (Kelly et al. 1979; Strong and Kelly 1977). Different pharmacokinetics and/or different affinities of binding of SPANs to the AZ also account for the lack of correlation between their PLA2 activity and toxicity (Montecucco and Rossetto 2000) and the very large difference in toxicity between SPANs and the pancreatic PLA2. In fact, in this view, binding with consequent strict localization of the PLA2 activity is a major event in the entire intoxication procession.

The progressive increase of lysophospholipids within the AZ due either to SPAN PLA2 mediated hydrolysis of phospholipids, or to partition of lysophospholipids present in the bathing medium (Rigoni et al. 2005), is suggested to cause a transition from inhibition (phase 1) to promotion of neuroexocytosis (phase 2) with the mechanisms discussed here below.

Most studies indicate that the PLA2 activity is implicated in phase 3, though it is not clear how and to what an extent (Howard and Gundersen 1980; Kini 1997; Montecucco and Rossetto 2000). SPANs were indeed shown to hydrolyse phospholipids with production of lysophosphatidylcholine (lysoPC) and fatty acids (FA) with no preference for a particular FA chain (Rigoni et al. 2005). Moreover, it was found that an equimolar mixture of these two lipids $(\text{lysoPC} + \text{FA})$ inhibit nerve terminals in a manner identical to that of SPANs, conclusively demonstrating that phase 3 is caused by the PLA2 activity of nerve-bound toxins. These findings clearly show that, at least in the initial phase of their action, SPANs acts on the outside of neurons. This does not exclude that later on SPANs may enter the paralyzed nerve terminal and contribute to further damage. Indeed. ammodytoxin (from the venom of *Vipera ammodytes ammodytes*) binds to cytosolic and mitochondrial proteins and has been detected inside hippocampal neurons (Petrovic et al. 2004; Sribar et al. 2001, 2003a, 2003b). In addition, taipoxin was found to bind to a 49-kDa calcium-binding protein located on the endoplasmic reticulum (Dodds et al. 1997; Kirkpatrick et al. 2000) and has been immunodetected inside chromaffin cells (Neco et al. 2003).

The involvement of the PLA2 activity in phase 2 is more controversial, as it was implicated in the case of the frog NMJ (Abe and Miledi 1978; Abe et al. 1977; Caratsch et al. 1981; Kondo et al. 1978; Oberg and Kelly 1976) and in some studies employing rat and mice NMJ preparations (Strong et al. 1976, 1977) but not in others (Chang 1979; Chang and Su 1982; Chang et al. 1977). However, an enhanced ACh release can be also induced by lysophosphatidylserine and lysophosphatidylglycerol with no toxin present, providing compelling evidence for the involvement of PLA2 in phase 2 (Caccin et al. 2006).

Electron microscopic pictures taken during phase 2 show an increased number of clathrin-coated Ω-shaped plasma membrane invaginations, which were initially attributed to synaptic vesicle caught in the process of fusion with the presynaptic membrane (Abe et al. 1976; Chen and Lee 1970), but later studies interpreted them as incomplete endocytosis of SV (Dixon and Harris 1999; Harris et al. 2000). We have suggested that SPAN halts endocytosis at its very last step, the fission of the vesicle from the plasma membrane which requires the neck closure, i.e., exactly the reverse process of membrane fusion (Montecucco and Rossetto 2000; Rossetto et al. 2006).

Frog, mice, and rat preparations fixed in phase 3 show enlarged axon terminals with large depletion of synaptic vesicles, including those close to the membrane (ready to release pool) and those occupying a more internal position (reserve pool). There are several clathrin-coated Ω-shaped plasma membrane invaginations, also in areas not facing the muscle membrane foldings. Mitochondria are swollen with altered cristae, so much as to appear in some cases as large vacuoles. Smaller vacuoles are also present (Abe et al. 1976; Chen and Lee 1970; Cull-Candy et al. 1976; Dixon and Harris 1999; Gopalakrishnakone and Hawgood 1984; Hamilton et al. 1980; Harris et al. 2000; Lee et al. 1984; Prasarnpun et al. 2004). It should be emphasized that even at this late stage of SPAN intoxication, no damage to muscle, fibroblasts, and Schwann cells is noticeable. Observations made at later stages (several hours after treatment) both in frog and rat muscles show that the nerve terminal of the motor neuron axon virtually disappears from the endplate. This is accompanied by protrusions of Schwann cells establishing extensive contacts with the muscle fiber, similar to what happens after several days of denervation achieved by transecting the motor axon or by crushing the nerve terminal (Abe et al. 1976; Dixon and Harris 1999; Harris et al. 2000; Kang et al. 2003; Son and Thompson 1995). Prasarnpun et al. 2005 also documented a substantial axonal degeneration with loss of mitochondria, neurofilaments, and synaptophysin staining. The robust extracellular matrix structure of the NMJ, which binds ACh esterase, remains in place.

Loss of SVs and alterations of mitochondria are also caused by SPANs on neurons in culture or by incubation with the LysoPC + OA lipid mixture (Bonanomi et al. 2005; Rigoni et al. 2004, 2005, 2007). A stricking and typical morphological alteration of cultured neurons and of uncovered *Drosophila* NMJ is the bulging of their nerve terminals (Megighian et al. 2007; Rigoni et al. 2004). The kinetics and the morphological changes induced by SPANs at NMJs and these *in vitro* findings led us to propose that SPANs promote fusion of SV with the presynaptic membrane, and at the same time, inhibit their retrieval (Figure 5). These alterations are the consequence of the same membrane lesion which consists in the hydrolysis of the cylindrically shaped phospholipids into the cone-shaped fatty acids and the inverted cone-shaped lysophospholipids. At least in the initial stages of intoxication, this hydrolysis takes place on the external leaflet of the presynaptic membrane, close to the SPAN binding sites, as indicated by the finding that LysoPC is the major product of SPAN hydrolysis with very little hydrolysis of phospholipids present on the cytosolic membrane leaflet (Rigoni et al. 2005). FAs rapidly equilibrate by

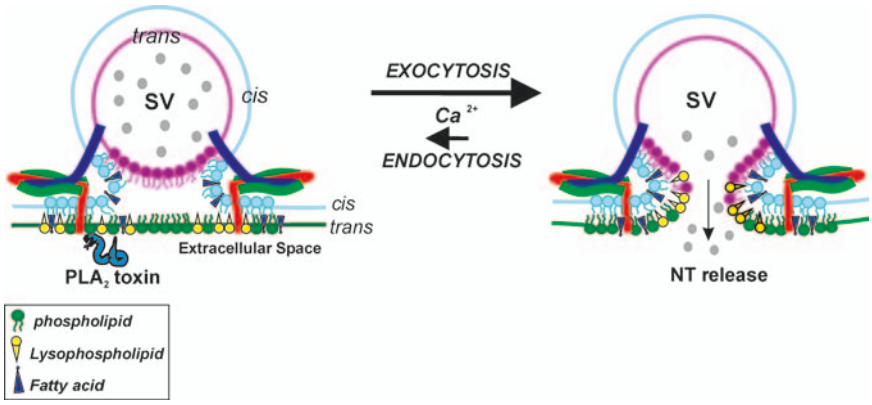

Fig. 5 The snake PLA2 neurotoxin is depicted here as a snake, which binds to an active zone, i.e., a synaptic vesicle (SV) release site, and hydrolyses the phospholipids of the external layer of the presynaptic membrane (green) with formation of the inverted-cone shaped lysophospholipid (yellow) and the cone-shaped fatty acid (dark blue). Fatty acids rapidly equilibrate by trans-bilayer movement among the two layers of the presynaptic membrane. In such a way lysophospholipids, which induce a positive curvature of the membrane, are present in trans and fatty acid, which induce a negative curvature, are present also in cis, with respect to the fusion site. This membrane conformation facilitates the transition from a hemifusion intermediate to a pore. Thus, the action of the toxin promotes exocytosis of neurotransmitter (NT) (from the left to the right panel) and, for the same membrane topological reason, it inhibits the opposite process, i.e., the fission of the synaptic vesicle.

trans-bilayer movement among the two layers of the presynaptic membrane (Kamp et al. 1995), while lysoPC remains largely confined to the outer membrane leaflet. LysoPC, which induces a positive curvature of the membrane, is present in trans, and FAs, which induce a negative curvature, are also present in cis with respect to the synaptic vesicle-membrane fusion site. This membrane conformation is fusogenic because it facilitates the transition from an hemifusion lipid intermediate to a pore, which develops by intermixing the cis monolayers of the two membranes that have come together following the formation of the rosette of SNARE complexes (Chernomordik and Kozlov 2003; Chernomordik et al. 2006) (Figure 5).The existence of a lipid hemifusion intermediate has been theoretically predicted long ago (Kozlov and Markin 1983) and experimentally documented in the case of viral membrane fusion (Chernomordik et al. 1997), and, more recently, in the case of SNARE-mediated fusion (Giraudo et al. 2005; Reese and Mayer 2005; Xu et al. 2005, Yoon et al. 2006). It is possible that the ready-to-release SVs are indeed already hemifused with the presynaptic membrane. and this would account for the ultra-fast event of neuroexocytosis that takes place within few hundreds of microseconds from the entry of calcium (Zimmerberg and Chernomordik 2005). For the same reason, the membrane configuration induced by the SPAN hydrolytic action inhibits membrane fission, which is the reverse of the membrane fusion, and is essential for the recycling of the empty SV (Figure 5). This scenario accounts for the bulging of nerve terminals and the Ω-shaped membrane indentation induced by SPANs. It also

explains the acceleration of SPAN intoxication induced by electrical stimulation of the NMJ, mentioned above, as there is a tight coupling between SV exocytosis and retrieval and increasing nerve stimulation increases the rate of SV exo-endocytosis (Cremona and De Camilli 1997; Sudhof 2004). In addition, it explains the finding that SPAN injected into the cytosol inhibit exocytosis (Wei et al. 2003) because this generates an anti-fusogenic membrane configuration with LysoPC on the cytosolic leaflet and FA on both sides.

The SPANs PLA2 activity is not fatty acid specific. Eicosopentanoic acid and arachidonic acid were recently found to promote exocytosis as deduced from increased membrane capacitance in PC-12 cells (Ong et al. 2006). Moreover, arachidonic acid, and some detergents, were found to stimulate the release of munc18 from syntaxin, thus allowing formation of SNARE complexes, a passage which is expected to increase exocytosis (Rickman and Davletov 2005). Moreover, omega-3 and omega-6 polyunsaturated fatty acids were found to stimulate membrane expansion at the nerve growth cone by a mechanism requiring syntaxin 3 (Darios and Davletov 2006). Accordingly, it is possible that these fatty acids contribute to the increased neuroexocytosis triggered in phase 2, but their role may be limited *in vivo* as the lysophospholipid was found to be much more effective than the fatty acid in the induction of paralysis (Caccin et al. 2006; Rigoni et al. 2005).

This rationale explains the induced neuroexocytosis of the ready-to-release SV pool, but does not well account for the massive vesicle depletion observed in the later phases of intoxication. However, one should consider that, with time, more and more lysophospholipids and FAs are generated and their accumulation may cause additional phenomena. Among them, increased plasma membrane permeability to ions caused by lysophospholipids (Leung et al. 1998; Wilson-Ashworth et al. 2004; Woodley et al. 1991) and partition of FAs inside mitochondria where they act as uncouplers of respiration with inhibition of ATP generation (Wojtczak and Schönfeld 1993). A specific, site-directed permeabilization of the membrane was proposed to be implicated in the intoxication by β-bungarotoxin (Rugolo et al. 1986), but how this would generate the observed alterations of morphology was not discussed. Clearly the FA uncoupling of mitochondria proposed by Wernicke et al. 1975 plays little role, if any, in SPAN intoxication because FA alone does not inhibit the NMJ (Rigoni et al. 2005). The products of SPAN phospholipid hydrolysis are likely to act at the plasma membrane level, as the SPAN-induced NMJ paralysis is rapidly reversed by albumin washing (Caccin et al. 2006). It was recently found that the bulged nerve terminals accumulate calcium independently on the agent responsible for bulging, whether it is SPAN or the LysoPC + FA mixture (Rigoni et al. 2007). Ca^{2+} ions entered from the medium and an increased cytosolic Ca^{2+} concentration within the nerve terminal of a synapse is by itself sufficient to trigger the release of all vesicles, including the SV reserve pool (Ceccarelli et al. 1972; Rizzoli and Betz 2005). Such high Ca^{2+} has an additional pathological consequence owing to the accumulation of this ion into the mitochondrial matrix with precipitation of calcium phosphate (Carafoli 1986).

The last event of this SPAN nerve terminal intoxication scenario may be the entry of the SPAN inside the nerve terminal, as suggested by two studies performed with

cells in culture (Neco et al. 2003; Petrovic et al. 2004). A SPAN inside a nerve terminals in the presence of Ca^{2+} could damage profoundly mitochondria and other organelles via its PLA2 activity as shown with isolated mitochondria (Wagner et al. 1974; Wernicke et al. 1975). In fact, β-bungarotoxin is much more active on low cholesterol membranes than on the plasma membrane (Lee et al. 1972; Strong and Kelly 1977; Kelly et al. 1979).

8 Regeneration of the Skeletal Neuromuscular Junction and the Innervated Muscle Fibers after Poisoning by Botulinum or Snake Neurotoxins

None of the clostridial or snake neurotoxins, when used at concentrations corresponding to the mouse lethal doses, kill intoxicated neurons *in vivo*, although they are extremely toxic to the animal because of the unique role of synaptic transmission in animal physiology and behavior. A small amount of BoNT, dissolved in a minimal volume of carrier solution and injected into a muscle, does not spread around significantly. NMJs present at and around the injection site become paralyzed and lose their functionality, but the motoneuron and the innervated muscle fiber remain alive. Anatomical contacts between nerve and muscle are maintained, and there is no apparent loss of motor axons (Eleopra et al. 2002). However, the muscle undergoes a transient atrophy with loss of acetylcholine esterase staining and dispersion of acetylcholine receptors from the end plate (Angaut-Petit et al. 1990; Bambrick and Gordon 1994; Comella et al. 1993). Thin, amyelinated neuritic processes are emitted from their original parent nerve ending (terminal sprouting) or from the most proximal node of Ranvier (nodal sprouting), ultimately growing into the muscle (de Paiva et al. 1999; Son and Thompson 1995). Sprouting from the poisoned nerve terminal is preceded and guided by the nerve terminal Schwann cells, which are, at least in part, activated to migrate by the lack of supply of ACh consequent to the persistent inhibition of ACh release caused by the toxin (Kang et al. 2003; Son and Thompson 1995). Nerve sprouting is a remarkable example of synaptic plasticity. The number of motor end plates on single muscle fibers increases as well as the number of fibers innervated by a single motor axon. Moreover, single muscle fibers may become innervated by more than one motor axon. Following recovery of the normal function by the original NMJ, the sprouts degenerate and ACh esterase and ACh receptors reconcentrate exclusively at the junctions; the muscle eventually regains its normal size (Borodic et al. 1994; de Paiva et al. 1999; Meunier et al. 2003; reviewed in Foran et al. 2003a; Meunier et al. 2002).

The time necessary to recover the function after BoNT paralysis mainly depends on the type of nerve terminal and the type of BoNT. The entire process of recovery from BoNT/A intoxication typically requires two to four months at the mammalian NMJ, but much longer ($>$1 year) at glands (Naumann and Jost 2004). BoNT/A has been the first type of BoNT used for therapy in humans (Schantz and Johnson 1997; Scott et al. 1989), and luckily it is the one that gives the longest lasting effects.

In fact, the other BoNT serotypes are very effective paralyzing agents, but their action is much shorter, except in the case of BoNT/C, which is comparable to BoNT/A (Eleopra et al. 1997, 1998a, 2002, 2004; Ludlow et al. 1992; Morbiato et al. 2007). This parallels the well-known fact that botulism caused by BoNT/A in humans is more dangerous than that caused by BoNT/B and /E (Smith and Sugiyama 1988) and observations performed at the rat NMJ (Kauffman et al. 1985; Molgó et al. 1989; Sellin et al. 1983).

The biochemical/cellular events at the basis of the different duration of action of the different toxins are unknown, but several factors may contribute: (1) the lifetime of the L chain in the cytosol, (2) the turnover of the truncated SNARE proteins, (3) secondary biochemical events triggered by the production of truncated SNARE proteins and/or the released peptides, and (4) other effects. The lifetime of intracellular acting toxins is usually quite long, and it is estimated to be on the order of weeks (Bartels et al. 1994; Habig et al. 1986; Keller et al. 1999; Marxen and Bigalke 1991; O'Sullivan et al. 1999). Moreover, it was documented that in chromaffin cells and at the frog NMJ, the truncated SNAP-25 generated by BoNT/A or /C persists for a long time and does interfere with exocytosis (Criado et al. 1999; Huang et al. 1998; O'Sullivan et al. 1999; Raciborska and Charlton 1999).

The cotreatment of human endplates with BoNT/A and E results in a rapid recovery of neuromuscular function, equivalent to that of BoNT/E alone (Eleopra et al. 1998b). This suggests that the lifetime of the protease activities does not account for the duration of paralysis at the motor nerve endings in situ. Meunier et al. (2003) have recently shown that the prolonged paralysis induced by BoNT/A arises from the slow replacement of BoNT/A truncated SNAP-25 fragment 1–197 (SNAP-25A), which is known to be inhibitory when overexpressed (O'Sullivan et al. 1999). Moreover, type E was shown to hasten the removal of inhibitory SNAP-25A from BoNT/A-treated mouse neuromuscular synapses by converting it to SNAP-25E (fragment 1–180). SNAP-25E can be replaced rapidly by newly synthesized SNAP-25, thereby accelerating resumption of synaptic transmission. Co-injection of BoNT/A and BoNT/E in rats and mice does not produce the same effect as in humans (Keller et al. 1999), raising the question of how comparable are data obtained in different animal species. Even larger are the differences found with cultured neurons (Foran et al. 2003b; O'Sullivan et al. 1999).

Fewer data are available on the regeneration of the NMJ intoxicated by SPANs (Dixon and Harris 1999; Gopalakrishnakone and Hawgood 1984; Grubb et al. 1991; Harris et al. 2000). As mentioned above, SPANs impair the nerve terminal function more rapidly than BoNTs, and morphological alterations are much more extensive. Within few hours nerve terminals degenerate with loss of neurofilaments to such an extent that some terminals are barely discernible. Schwann cells are unaffected and extend their processes into the synaptic cleft. Also, the muscle fibers may degenerate and be invaded by phagocytic cells. Re-innervation of the muscle fibers is achieved by pre- and postterminal sprouts within three to five days from intoxication. This is accompanied by muscle regeneration with the appearance of smaller fibers with a typical central nucleus, which persists in this location for months. By 7 to 10 days more than 90% of the muscle fibers are capable of generating an indirect action

potential, and by three to four weeks the process of regeneration is complete and few examples of multiple innervation originated by the sprouting process persist. The short duration of the effects of SPAN with the relatively rapid recovery with respect to BoNT/A or /C can be rationalized by considering the different nature of the SPAN hydrolysis products, which are highly diffusible. Moreover, phagocytic cells are recruited, possibly following the release of inflammatory mediators, either directly by the SPAN via release of arachidonic acid or indirectly by other chemotactic substances, or both.

9 Clostridial Neurotoxins in Cell Biology

It is presently very difficult to isolate and culture peripheral motoneurons, and this has hampered research on the morphological and functional consequences of CNT intoxication. However, dissociated spinal cord neurons in culture, including primary motorneurons, are very sensitive to CNTs. In addition, brain neurons derived from the cortex or the hippocampus or the cerebellum are sensitive to these neurotoxins. In these cells, BoNT/A was found to cause no detectable morphological changes. In contrast BoNT/C, which cleaves both SNAP-25 and syntaxin, brings about rapid swelling of synaptic terminals followed by degeneration of axons and dendrites followed by cell death (Williamson and Neale 1998) and the collapse of growth cones in chick dorsal root ganglia (Igarashi et al. 1996). BoNT/C also kills cortical and hippocampal neurons (Osen-Sand et al. 1996) and cerebellar granular cells (Berliocchi et al. 2005; Foran et al. 2003b). In contrast, when injected in different human muscles, BoNT/C does not cause loss of motoneurons (Eleopra et al. 2002). Taken together, these results demonstrate the central role played by syntaxin in the control of the integrity of synaptic contacts, in addition to its essential function in exocytosis.

Given the general role of SNAREs in vesicular trafficking, which is paralleled by a strong structural similarity, the use of CNTs is not limited to neuronal cells possessing receptors (for comprehensive reviews see Rossetto et al. 2001b; Schiavo et al. 2000). If the simple incubation with CNTs is sufficient to cause inhibition of neurotransmitter release in nerve cells or synaptosomes following SNARE cleavage, exocytosis can be inhibited in many non-neuronal cells as well, provided that they are permeabilized or microinjected. Incubation with very high doses of CNTs may be also sufficient to elicit effects with cells characterized by a large fluid phase endocytosis. Alternatively, cells can be transfected with the gene encoding for the light chain. The fact that the SNARE protein isoforms are involved in a variety of intracellular vesicle fusion events (Jahn and Scheller 2006; Takamori et al. 2006), in addition to exocytosis, has extended their potential range of use, but a word of caution is called for because more than one SNARE isoform can be inactivated by a given toxin within the same cell. It is not straightforward to predict the cleavability of a SNARE by a CNT from its sequence. Moreover, low sensitivity of GABA release to BoNT/A and BoNT/E intoxication, relative to the release of ACh, noradrenaline or glutamate has been reported by different studies (Ashton and

Dolly 1988; Bigalke et al. 1981). This could result from the different expression of the proteolytic substrate in different neuronal populations. Indeed, the virtual absence of SNAP-25, as detected by immunofluorescence at inhibitory terminals of CNS neurons (Frassoni et al. 2005; Verderio et al. 2004, 2007) parallels the low sensitivity of GABAergic exocytosis to BoNT/A and BoNT/E. On the same line, the higher sensitivity to these neurotoxins of synaptic vesicle recycling in immature GABAergic cells parallels the higher degree of SNAP-25 expression in developing inhibitory neurons as compared to the mature neurons (Frassoni et al. 2005; Verderio et al. 2007). Different amounts of the expressed SNAREs or their different intracellular distributions relative to the toxin location, as well as the expression of toxin-insensitive SNARE isoforms, could therefore explain the distinct properties or the different efficacy of intoxication in specific neuronal subpopulation. The finding that BoNT/A and BoNT/E, used at suitable concentrations, only partially affect GABAergic transmission, while impairing glutamatergic signaling, opened the possibility of using these toxins to treat CNS diseases characterized by imbalances between excitatory and inhibitory transmission such as epilepsy (Costantin et al. 2005; Verderio et al. 2007).

The absolute neurospecificity of clostridial neurotoxins, and the ability of TeNT to undergo axonal retrograde transport, make them ideal tools to study endocytosis and sorting at the synapse, and of retro-axonal transport both *in vitro* and *in vivo* (reviewed in Deinhardt and Schiavo 2005). These processes, which are still poorly understood at the molecular level, represent an exciting area of application for clostridial neurotoxins and their binding fragments.

10 Therapeutic Uses

The demonstration that the inhibition of the nerve-muscle impulse is followed by a functional recovery of the NMJ provides the scientific basis of the rapidly growing use of BoNTs in the therapy of a variety of human diseases caused by hyperfunction of cholinergic terminals and other neurodiseases (Jankovic 2006; Montecucco et al. 1996; Scott et al. 1989; Truong and Jost 2006). Injections of minute amounts of BoNT into the muscle(s) to be paralyzed lead to a depression of the symptoms lasting months. Owing to the long lasting duration of its effect, BoNT/A has almost invariably been used. Since the NMJ paralysis is reversible, the injection has to be repeated, and the possibility of an immune response with production of BoNT/A-neutralizing antibodies can occur (Brin 1997; Hanna et al. 1999; Jankovic and Schwartz 1995; Sankhla et al. 1998; Zuber et al. 1993). The injection of a different BoNT serotype could overcome this drawback. BoNT/E and BoNT/F were tested in humans and were found to have beneficial effects of short duration (Aoki 2001; Billante et al. 2002; Chen et al. 1998b; Eleopra et al. 1998b, 2006; Mezaki et al. 1995). Also BoNT/B has a rather short duration of action, and longer paralysis can only be achieved with very high doses, thus increasing the possibility of an immune response (Brin et al. 1999; Dressler and Bigalke 2005; Dressler and Eleopra

2006; Lew et al. 1997; Sloop et al. 1997). Studies performed in humans (reviewed in Eleopra et al. 2006) and in mice (Morbiato et al. 2007), show that BoNT/C has a general profile of action similar to that of BoNT/A.

Injection of BoNT is currently recognized as the best available treatment for dystonias and for certain types of strabismus, and new uses are continuously found (Bhidayasiri and Truong 2005; Montecucco and Molgó 2005). In addition, BoNT/A inhibit ACh release at autonomic nerve terminals which innervate the glands and smooth muscle, and it is currently used to treat diseases such as hypersalivation and hypersudoration (Brisinda et al. 2004; Naumann and Jost 2004).

The use of BoNT/A has been increasingly reported in many conditions of pathological pain, including migraine and other headache disorders (Aoki 2003; Binder and Blitzer 2003), musculoskeletal pain, such as myofascial pain, low back pain, and other chronic pain syndromes (Luvisetto et al. 2007; Reilich et al. 2004; Sycha et al. 2004).

BoNTs are not known to be retrotransported to the CNS, but they are highly toxic when injected directly in the brain (Luvisetto et al. 2003). Both the intraventricular and the hind paw injection of minute amounts of BoNT/A in mice strongly depress the perception of inflammatory pain induced by formalin (Luvisetto et al. 2006). On the basis of the differential effect of BoNT on glutamatergic and GABAergic nerve terminals in hippocampal neurons (Verderio et al. 2004), BoNT/E was tested as a potential therapeutic of epilepsy, and it was found to effectively lower the symptoms induced in mice by the epileptogenic kaininic acid (Costantin et al. 2005).

TeNT has not yet been used as a therapeutic but it has a series of interesting biological properties. When injected in the hippocampus of rats it induces an epileptic-like syndrome (Bagetta and Nisticò 1994). The carboxy-terminal third of TeNT, which retains most, but not all, of the neuronal binding and uptake properties of the entire toxin, has been used as a carrier of lysosomal hydrolase (Dobrenis et al. 1992; Jiang et al. 2005), superoxide dismutase inside cells in culture (Figueiredo et al. 1997; Francis et al. 1995), or β-galactosidase in mouse embryos (Coen et al. 1997). These studies open the possibility of using TeNT as carrier of various biologicals from peripheral sites of injections to selected areas of the CNS.

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α-Latrotoxin and Its Receptors

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Abstract α-Latrotoxin (α-LTX) from black widow spider venom induces exhaustive release of neurotransmitters from vertebrate nerve terminals and endocrine cells. This 130-kDa protein has been employed for many years as a molecular tool to study exocytosis. However, its action is complex: in neurons, α-LTX induces massive secretion both in the presence of extracellular Ca^{2+} ($Ca^{2+}{}_e$) and in its absence; in endocrine cells, it usually requires $Ca^{2+}{}_e$. To use this toxin for further dissection of secretory mechanisms, one needs an in-depth understanding of its

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functions. One such function that explains some α-LTX effects is its ability to form cation-permeable channels in artificial lipid bilayers. The mechanism of α-LTX pore formation, revealed by cryo-electron microscopy, involves toxin assembly into homotetrameric complexes which harbor a central channel and can insert into lipid membranes. However, in biological membranes, α-LTX cannot exert its actions without binding to specific receptors of the plasma membrane. Three proteins with distinct structures have been found to bind α-LTX: neurexin Iα, latrophilin 1, and receptor-like protein tyrosine phosphatase σ. Upon binding a receptor, α-LTX forms channels permeable to cations and small molecules; the toxin may also activate the receptor. To distinguish between the pore- and receptor-mediated effects, and to study structure-function relationships in the toxin, α-LTX mutants have been used. At least one non-pore-forming α-LTX mutant can activate latrophilin, a G protein-coupled receptor, causing release of Ca^{2+} from intracellular stores. Latrophilin action still requires $Ca^{2+}{}_e$ and may trigger transmitter secretion either by itself or by activating Ca^{2+}-channels and/or inducing Ca^{2+} waves. These results reveal two $Ca^{2+}{}_e$-dependent mechanisms of α-LTX action (membrane pore formation and signalling via latrophilin), but how α-LTX triggers $Ca^{2+}{}_e$-independent neurotransmitter release still remains unexplained. Hypotheses for this action include direct interaction with intracellular components involved in exocytosis or the effects of α-LTX pores.

1 α-LTX and Release of Neurotransmitters

The notorious black widow spider (*Latrodectus* genus) and its venom, first studied by ancient Greeks (Aristotle 350 B.C.), entered the era of modern science in the 1930's with the discovery of its proteinacious active principles (D'Amour et al. 1936) and, following the demonstration that it affects synaptic neurotransmitter release (Longenecker et al. 1970) and the isolation of toxic components (Frontali et al. 1976), gripped the attention of neurobiologists. It played a role in the debate leading to the Ca^{2+} (Augustine et al. 1987) and vesicular quantum (Ceccarelli and Hurlbut 1980) paradigms of neurotransmission. However, some details of α-LTX actions still remain mysterious, despite its widespread use as a stimulant of neurosecretion.

The venom contains at least 86 unique proteins (Duan et al. 2006), including several homologous LTXs which play a role in its toxicity to insects and crustaceans (Grishin 1998), with only one, α-LTX, targeting vertebrates specifically; reviewed by Rosenthal and Meldolesi (1989). α-LTX is usually isolated from spider venom by conventional chromatography (Frontali et al. 1976; Tzeng et al. 1978), but to achieve homogeneity and remove contaminants (Volkova et al. 1995; Pescatori et al. 1995) that may endow the preparation with uncharacteristic properties (Umbach et al. 1998), preparative native electrophoresis should ideally be used (Ashton et al. 2000).

1.1 Target Cells

The toxin induces strong and sustained release of neurotransmitters and hormones from secretory cells capable of regulated exocytosis (Rosenthal et al. 1990). The effects of α-LTX on neurosecretion were first described on a cellular level in the 1970s at frog neuromuscular junctions (NMJ) (Longenecker et al. 1970; Frontali et al. 1976); and later in mouse brain slices (Tzeng et al. 1978), in synaptosomes (isolated brain nerve terminals) from rat (Grasso et al. 1978), dog (Tzeng and Siekevitz 1979), and guinea pig (Nicholls et al. 1982); primary cerebellar granule cell cultures (Grasso and Mercanti-Ciotti 1993); and in hippocampal organotypic cultures (Capogna et al. 1996). In addition, catecholamine-secreting chromaffin cells (Picotti et al. 1982) and PC12 cell line (Robello et al. 1987), insulin-secreting pancreatic β-cells in primary or derived cell cultures (Lang et al. 1998), oxytocin- and vasopressin-secreting neurohypophysis cells (Hlubek et al. 2003) and luteinizing hormone-secreting rat gonadotropes (Tse and Tse 1999) were also used to characterize the toxin's effects on secretion from non-neuronal excitable cells. A secretory cell not sensitive to α-LTX still remains to be found.

1.2 Site and Mode of Action

From the earliest description of the toxin's actions on neuronal systems, it emerged that α-LTX affects specifically the presynaptic element, from which it causes massive neurotransmitter release (e.g., Longenecker et al. 1970). The toxin has no major enzymatic activities (Frontali et al. 1976). Crucially, α-LTX has been discovered to create Ca^{2+}-permeable channels in lipid bilayers (Finkelstein et al. 1976), and a large body of evidence shows that Ca^{2+} influx through membrane channels induced by α-LTX in the presynaptic membrane accounts for a major part of its effect. Pore formation occurs in all the biological systems mentioned above, but the features of α-LTX-triggered release cannot be fully explained by the toxin pore.

The effect of α-LTX at NMJs is usually delayed and develops fully after ∼10 min, although synaptosomes react much faster. It is detected electrophysiologically as an increase in the frequency of spontaneous miniature postsynaptic potentials at NMJs (Longenecker et al. 1970; Misler and Hurlbut 1979; Ceccarelli and Hurlbut 1980; Tsang et al. 2000) and excitatory or inhibitory postsynaptic currents at central synapses (Capogna et al. 1996). In addition, α-LTX affects action potential-evoked, synchronous release, and does this in a time-dependent manner: initially, it enhances evoked potentials, but eventually inhibits (Capogna et al. 1996) or blocks them (Longenecker et al. 1970; Hurlbut and Ceccarelli 1979; Liu and Misler 1998). Finally, when used in higher concentrations, α-LTX can cause morphological deformation and cell death, as reviewed by Südhof (2001).

However, the most surprising feature of the α-LTX-evoked secretion is that it can occur both in the presence and absence of $Ca^{2+}{}_e$.

1.3 Ca^{2+}*-Independent Release*

The ability of α-LTX to trigger neurotransmitter exocytosis in the absence of extracellular Ca^{2+} remains particularly interesting and inexplicable to the field (Longenecker et al. 1970; Ceccarelli et al. 1979; see also Südhof (2001) and Ushkaryov et al. (2004) for review). This is clearly different from depolarization-induced exocytosis, which is Ca^{2+}-dependent, but not unlike the effect of hypertonic sucrose. The possibility that α-LTX-induced release involves an unknown, Ca^{2+}-independent mechanism which may also occur during normal synaptic activity has provided the *casus belli* for many a quest for α-LTX structure and receptors that could trigger neurotransmission *via* intracellular mechanisms.

In neurons, the Ca^{2+}-independent secretion is restricted to small synaptic vesicles, as demonstrated by synaptosomal and NMJ experiments, where glutamate, GABA, and acetylcholine are released in the absence of Ca^{2+}, while catecholamines or peptides are not (Matteoli et al. 1988; Davletov et al. 1998; Khvotchev et al. 2000). Ca^{2+}-independent release does not normally occur in endocrine cells (Grasso et al. 1980; Michelena et al. 1997; Silva et al. 2005), although in some cultured cells it does (Meldolesi et al. 1983; Lang et al. 1998; Tse and Tse 1999).

The characteristics of Ca^{2+}-independent release are peculiar: it requires the presence of divalent cations, such as Mg^{2+}, which can be added or removed in succession, causing respective bouts of secretion or its cessation (Misler and Hurlbut 1979). In the absence of Mg^{2+}, this release can be supported by slightly hypertonic sucrose, by itself insufficient to cause secretion (Misler and Hurlbut 1979). The Ca^{2+}-independent release can be blocked by millimolar La^{3+} (Rosenthal et al. 1990) or concanavalin A (Grasso et al. 1978; Boehm and Huck 1998). It may involve release of Ca^{2+} from mitochondria, as observed in peripheral (Tsang et al. 2000) and not central synapses (Adam-Vizi et al. 1993), but it is unclear if stored $Ca^{2+}{}_i$ itself can trigger release.

As Ca^{2+} is important for endocytosis, the sustained secretory activity in its absence eventually depletes NMJ terminals (Longenecker et al. 1970; Ceccarelli and Hurlbut 1980), but not synaptosomes (Watanabe and Meldolesi 1983), of all vesicles. Electrophysiological recordings at NMJs show that in the absence of Ca^{2+} α-LTX causes a large but slow rise in the frequency of spontaneous exocytotic events, which then slow down and cease altogether; bursts of miniatures are never observed.

1.4 Ca^{2+}*-Dependent Release*

In the presence of $Ca^{2+}{}_e$, α-LTX also causes a slow and large increase in the frequency of miniature events, overlaid by bursts of release (Ceccarelli et al. 1979). As in the absence of Ca^{2+}, depletion of vesicles and block of exocytosis also occurs, but because vesicles are able to recycle for some time, the total number of quanta released is twice higher in Ca^{2+} than in its absence (Fesce et al. 1986; Auger and

Marty 1997). It appears that the gradual rise and fall of the frequency of vesicle fusion events is a common feature of α-LTX-induced release both with and without $Ca^{2+}{}_e$, and that Ca^{2+} adds an extra component, increasing the total release.

Influx of Ca^{2+} through α-LTX pores might explain Ca^{2+}-dependent secretion, but not the bursts of miniatures. These may be due to Ca^{2+} waves caused by activation of phospholipase C (PLC) (Vicentini and Meldolesi 1984; Davletov et al. 1998) and release of intracellular Ca^{2+} ($Ca^{2+}{}_i$). In fact, U73122 (blocks PLC activation by G proteins), thapsigargin (depletes Ca^{2+} stores), and 2-APB (inhibits activation of Ca^{2+} stores) block α-LTX action (Davletov et al. 1998; Ashton et al. 2001; Capogna et al. 2003), implicating a G protein cascade in Ca^{2+}-dependent toxin-induced release.

2 The Structure of α-LTX

In order to understand and explain the complex activities of α-LTX, its primary and three-dimensional (3D) structure has been thoroughly studied.

2.1 Sequence Analysis

α-LTX is synthesized as a 157 kDa polypeptide (Kiyatkin et al. 1990) (Figure 1a) by free ribosomes in the cytosol of secretory epithelial cells of the venom glands (Cavalieri et al. 1990). These cells disintegrate (Smith and Russell 1966) and expel toxin into the gland lumen together with various proteases (Duan et al. 2006). Here, it is cleaved at both termini by a furin-like protease (Volynski et al. 1999), producing an active α-LTX of ∼131 kDa (Kiyatkin et al. 1990; Ichtchenko et al. 1998).

The most striking feature of the α-LTX primary structure is a series of 22 ankyrin repeats that account for about two thirds of the sequence (Figure 1b) (Kiyatkin et al. 1995). Ankyrin repeats are found in a wide variety of proteins and generally mediate protein-protein contacts but, unlike other protein-binding motifs, they take part in a wide range of interactions and do not have a specific target (Sedgwick and Smerdon 1999). The N-terminal third of α-LTX shows no significant homology to other proteins. This region contains three conserved cysteines important for structural stability and activity of all LTXs (Kiyatkin et al. 1995; Ichtchenko et al. 1998).

α-LTX is a mostly hydrophilic protein that does not have a classical hydrophobic signal peptide or clear transmembrane regions (TMRs). The hydrophilic nature of α-LTX seemed for a long time at odds with its membrane insertion capabilities, but a study of its 3D structure (Orlova et al. 2000) shed light on this issue (Section 2.2).

2.2 Higher-Order Structures

So far, crystallization of α-LTX has not been reported. However, the toxin has been successfully studied by cryo-electron microscopy (cryo-EM) (Orlova et al. 2000),

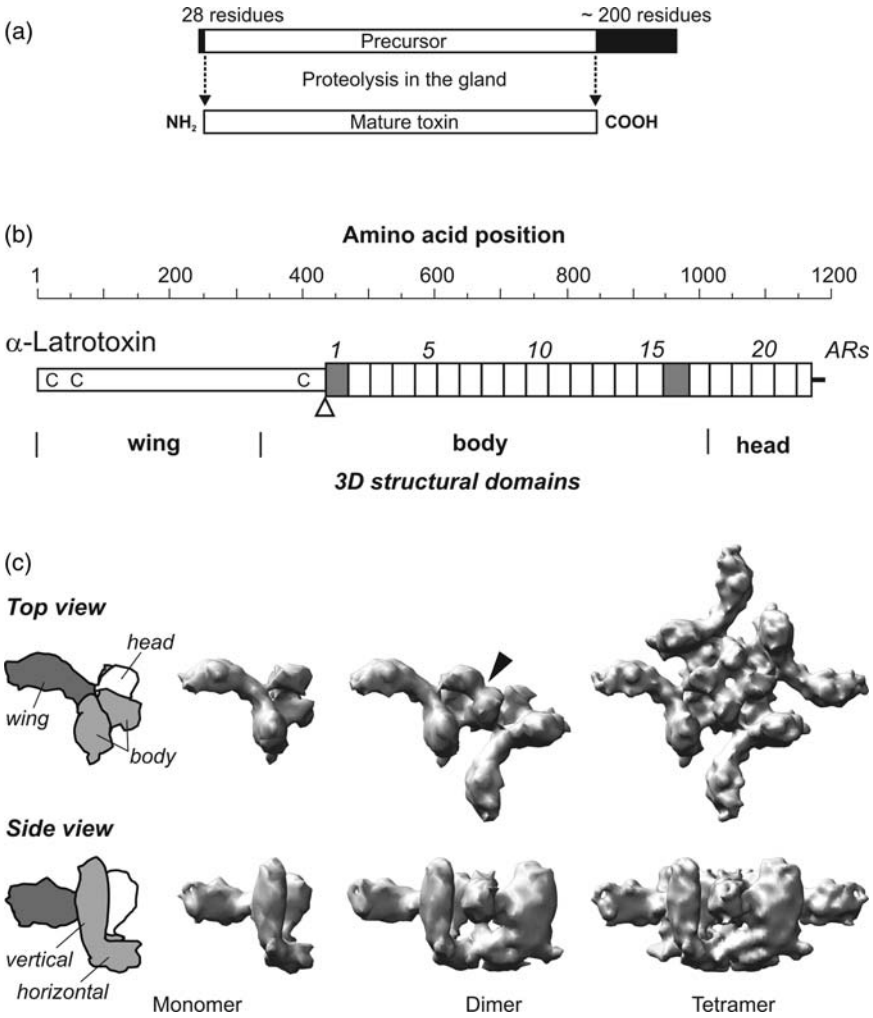

Fig. 1 The structure of α-LTX. (**a**) Schematic of α-LTX processing in the venom gland. (**b**) Primary and domain structure. The numbered boxes, ankyrin repeats (ARs). Grey, imperfect repeats; C, conserved cysteines residues in the N-terminal domain; open arrowhead, insert in the mutant α-LTX^{N4C}. Protein domains identified from the 3D structure (Orlova et al. 2000) are delimited below. (**c**) 3D reconstructions of the α-LTX monomer, dimer and tetramer, viewed from the top and side. The monomer has been computationally extracted from the experimentally determined tetramer structure. Left-most image, a scheme of the monomer, with the domains designated by different shades of grey. Filled arrowhead, strong association of the head domains in the dimer.

a method that allows visualization of proteins instantly frozen in their native conformations. The size of the toxin tetramer (∼520 kDa) proved ideal for this approach to reveal medium-resolution (14 Å) information, and even the dimer (∼260 kDa) was reconstructed to below 20 Å resolution, revealing detailed information on the domain organisation of the toxin in its two oligomeric states (Orlova et al. 2000).

2.2.1 Domain Structure of the Monomer

The 3D cryo-EM reconstructions of the α-LTX tetramer and dimer (Orlova et al. 2000) show that the monomer consists of three domains (Figure 1c): (1) The wing, at the N-terminus, has an estimated molecular mass of 36 kDa. Thought to comprise mainly α-helices, it has no close homologues among proteins of known 3D structure. The wing links to the rest of the toxin molecule through a narrow rigid connection. (2) The body comprises the first 17 ankyrin repeats of the α-LTX sequence. Its estimated molecular mass is 76 kDa. The ankyrin repeats forming the "vertical" part of the body are tightly packed into a characteristic structure, reminiscent of ankyrin. However, the lower part of the body is bent and has a more complex arrangement. (3) At the C-terminus is the head, the smallest domain, with an estimated 18.5-kDa mass. It consists of 4.5 ankyrin repeats and demonstrates high spatial homology to ankyrin-repeat-containing proteins with known 3D structures (Orlova et al. 2000).

Despite having a large number of ankyrin repeats (superseded only by the 24 repeats in ankyrin itself), α-LTX does not assume the monotonous arch-like shape so characteristic of proteins with multiple ankyrin repeats, such as the 12-repeat ankyrin fragment (Michaely et al. 2002), the human protein phosphatase HEAT (15 repeats), or the porcine ribonuclease inhibitor LRR (16 repeats) (Andrade et al. 2001). Instead, the ankyrin repeat-containing part of α-LTX is broken in several places and forms clearly delimited domains and subdomains, probably reflecting sequence divergence of some repeats and the functional specialization of the subdomains.

2.2.2 Quaternary Structures

Although some monomers have been observed by cryo-EM in EDTA-treated α-LTX, the toxin almost always exists as a stable dimer (Orlova et al. 2000; Ashton et al. 2001). In the asymmetric dimer, the two monomers are associated "head to tail" by tight multipoint contacts mediated by the "horizontal" parts of the bodies and by the sides of the head domains (arrowhead in Figure 1c).

Association of dimers, strongly catalyzed by Mg^{2+}, produces a cyclical structure (Figure 1c) that can contain four monomers only. The tetramer has C4 rotational symmetry and resembles a bowl, in which the bottom is formed by the "horizontal" parts of the bodies. This part is important for penetration into lipid bilayers, and it is likely that structural rearrangements required for tetramerization expose the surface regions favorable to interaction with lipid bilayers. In addition, this part represents the intracellular mouth of the channel, with a large (30 Å) central hole in its center.

Above this, in the centre of the "bowl," the four heads form a cylindrical assembly surrounding the channel (Figure 1c), which is restricted at one point to 10 Å (Figure 2; see also Section 3.4.7). This constriction most probably corresponds to the cation binding site (selectivity filter) of the α-LTX channel (Section 3.4.1).

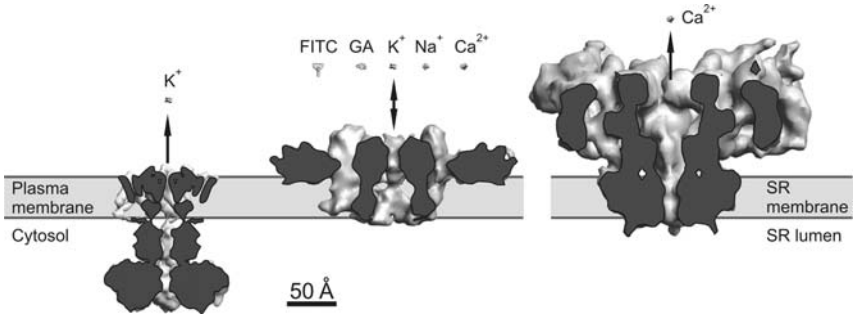

Fig. 2 Membrane topography of the α-LTX pore. Cross-section of the α-LTX tetramer embedded in a membrane (as observed in liposomes) (Orlova et al. 2000) is shown alongside the cut-open voltage-dependent K^+ channel (Kv1.2) (Long et al. 2005) and Ca^{2+} release channel (ryanodine receptor) (Serysheva et al. 2005). Fully hydrated cations and molecules known to permeate through the respective channels are shown next to each reconstruction (FITC, fluoresceine isothiocyanate; NE, norepinephrine). The narrowest part of the α-LTX channel is 10 Å. Molecular images were produced using the UCSF Chimera package (Pettersen et al. 2004).

The wings extend sideways from the body domains perpendicular to the central symmetry axis of the tetramer and could participate in the binding to some receptors (see Sections 2.3 and 4). They also seem to mediate homotypic interactions, causing tetramers to assemble into flat 2D crystals, often containing large numbers of tetramers (Lunev et al. 1991). These lattices could underlie the frequently described phenomenon of α-LTX channel clusterization (Robello et al. 1987; Krasilnikov and Sabirov 1992; Filippov et al. 1994; Van Renterghem et al. 2000).

2.3 Recombinant α-LTX

Detailed structure-function analysis of α-LTX is impossible without generating mutants. The difficulty inherent to this approach is to ensure proper folding of this large protein. Two groups relied on baculovirus expression and successfully purified active recombinant toxins. Ichtchenko et al. (1998) used two 8-histidine tags for purification, whereas Volynski et al. (1999, 2003) utilized a monoclonal antibody.

Recently, active recombinant α-LTX has been generated using bacteria in which both thioredoxin reductase and glutathione reductase are inactivated to improve the formation of disulphide bonds in expressed proteins (Li et al. 2005). The toxin is expressed as a fusion with glutathione-S-transferase (GST), which is used for affinity purification of the recombinant toxin and can be subsequently removed by selective proteolysis. Considering the relative ease of generating recombinant proteins in bacteria, this approach will facilitate structure-function studies of α-LTX.

Using these methods, several useful observations regarding the functions of the α-LTX domains have been made by analyzing various mutants of recombinant toxin. These results are discussed in relevant sections below.

3 Membrane Pore

How the hydrophilic α-LTX inserts into lipid membranes and makes cation-permeable pores is not fully known, but an in-depth insight into the mechanisms of channel formation has been gained by combining cryo-EM, biochemical and biophysical studies with toxin mutagenesis. α-LTX pore formation consists of at least three steps: toxin tetramerisation, interaction with a specific cell-surface receptor and, finally, membrane insertion. Many experimental procedures can affect some of these steps and thereby prevent or assist channel formation.

3.1 Tetramerization

The tetrameric state of α-LTX is divalent cation-dependent. Accordingly, treatment with EDTA renders purified α-LTX mostly dimeric, while subsequent addition of millimolar Ca^{2+} or Mg^{2+} promptly restores tetramerization (Ashton et al. 2000). This transition, requiring conformation changes (Orlova et al. 2000) and catalyzed by divalent cations, may explain the dependence of α-LTX pore-mediated actions on Mg^{2+} (e.g. Misler and Hurlbut 1979). Tetramerization can also be triggered by amphipathic molecules (Ashton et al. 2000), possibly membrane lipids.

Interestingly, La^{3+} at a concentration higher than 100 μM also greatly reduces the number of tetramers (Ashton et al. 2001). In addition, this trivalent cation is able to block the channels of membrane-inserted tetramers (see Section 3.4.6), making La^{3+} useful in studying pore-independent toxin effects.

The role of tetramerization in toxin pore formation has been vividly illustrated by mutagenesis of α-LTX. In particular, Ichtchenko et al. (1998) have generated an interesting mutant (α-LTX^{N4C}) that contains a four-amino-acid insert between the N-terminal domain and the ankyrin repeats (Figure 1b). This insert apparently causes a conformational change enabling α-LTX^{N4C} to form dimers but not cyclical tetramers (Volynski et al. 2003). Accordingly, although α-LTX^{N4C} still binds its receptors (Ichtchenko et al. 1998), it fails to incorporate into the membrane and form pores (Ashton et al. 2001; Volynski et al. 2003), providing a powerful argument that the tetramer is the molecular species that inserts into membranes.

3.2 Receptor Interaction

Although α-LTX is able to insert into pure lipid membranes (Finkelstein et al. 1976), reconstituted receptors greatly enhance the rate of insertion (Scheer et al. 1986). Biological membranes seem even more refractive to the toxin: when cells do not possess α-LTX receptors, no pore formation can be detected (Hlubek et al. 2000; Van Renterghem et al. 2000; Volynski et al. 2000), whereas expression of exogenous receptors allows abundant α-LTX insertion and concomitant channel

formation. Receptors, thus, confer specificity to the pore-mediated effects of α-LTX. It is not clear whether receptors are directly involved in membrane insertion, simply concentrate toxin near the membrane, or organize membrane lipid domains to make them accessible to α-LTX. Importantly for some aspects of toxin-evoked secretion, interaction with one of the receptors, neurexin (NRX), is Ca^{2+}-dependent, while interaction with the other two, latrophilin 1 (LPH1) and protein tyrosine phosphatase σ (PTPσ), is completely Ca^{2+}-independent (see Section 4 for more details).

The role of the structural domains of α-LTX in receptor interaction has been studied by mutagenesis. Ichtchenko et al. (1998) have generated three point-mutants by replacing conserved cysteines in the N-terminal domain (Figure 1b) with serines. These mutants (C14S, C71S, C393S) are inactive in release, probably because they do not bind α-LTX receptors. Accordingly, chemical reduction of disulphide bonds in α-LTX abolishes receptor binding (Ichtchenko et al. 1998). Thus, proper folding of the N-terminal domain, aided by disulphide bonds, is essential for toxin-receptor interactions.

In another work, Li et al. (2005) have deleted the C-terminal ankyrin repeats 15 to 22, comprising the head and part of the body (Figure 1b), and found that this mutant fails to bind LPH1 but still interacts with NRX Iα. This suggests that α-LTX complex with LPH1 requires an intact C-terminal quarter of the toxin molecule. Whether this sequence binds LPH1 directly or affects the conformation of other α-LTX regions that actually interact with LPH remains to be addressed. Clearly, the C-terminal region is not necessary for binding of NRX Iα.

3.3 Membrane Insertion

The insertion of the α-LTX channel into black lipid membranes is unidirectional (Robello et al. 1984), suggesting that the toxin has evolved specialized domains to help it enter lipid bilayers. The direct observation of tetramers inserted into artificial lipid membrane (Orlova et al. 2000) indicates that the "bowl" formed by the body domains is immersed deeply in the lipid, while the wing domains remain splayed on, and slightly buried into, the extracellular surface of the bilayer, while the heads are exposed to the extracellular space (Figure 2). In this model, some regions of the body domains are exposed to the cytosol, consistent with the observation that protease treatment from that side of the lipid bilayer modifies the inserted channel (Robello et al. 1984; Chanturia and Lishko 1992) and supportive of the hypotheses that α-LTX could interact with intracellular release machinery (Khvotchev et al. 2000) or act as a fusogen catalyzing vesicle fusion (Sokolov et al. 1987; Lishko et al. 1990).

In addition to the presence of receptors, the ionic composition of buffer (Finkelstein et al. 1976) and the makeup of lipid bilayer (Robello et al. 1984) can influence the rate of α-LTX insertion into lipid membranes. It is possible that the membrane microenvironment affects the insertion of a receptor-bound tetramer also in a physiological context. For example, at 0° C, α-LTX binds to receptor-containing membranes, but it is not inserted (Khvotchev et al. 2000; Volynski et al. 2000).

Another feature of α-LTX is that it co-purifies with a small protein, called LMWP (Volkova et al. 1995) or latrodectin (Pescatori et al. 1995). It does not affect the toxicity of α-LTX nor has secretory activity of its own (Volkova et al. 1995). However, when LMWP is removed (Ashton et al. 2000), the toxin becomes unable to form channels in artificial lipid bilayers, although its receptor-mediated permeation of biological membranes is unhindered (Volynski et al. 2000). It is tempting to speculate that LMWP helps wild-type α-LTX to penetrate lipid bilayers (see also Grishin et al. 1993). To test this, recombinant toxins lacking LMWP need to be studied in pure lipid membranes.

The receptor-aided insertion of α-LTX into biological membranes has been recently investigated using recombinant toxin N-terminally fused to GST and expressed in bacteria (Li et al. 2005). When, after purification, the GST moiety is removed, the resulting wild-type recombinant α-LTX inserts into the plasma membrane and induces strong cation currents in tsA cells (HEK-293-derived) transfected with NRX Iα, whereas the GST-fused toxin is much less effective. As both the GST-free and GST-fused toxins bind well to the receptors (Li et al. 2005), it is clear that GST attached to the α-LTX N-terminus decreases the efficiency of toxin incorporation into membranes. However, despite its large size and hydrophilicity, GST does not block channel formation completely, indicating that when α-LTX inserts into the membrane, the wing does not need to penetrate the bilayer.

This idea is further supported by the analysis of a series of α-LTX truncation mutants, lacking 1, 2, 3, or 8 C-terminal ankyrin repeats (Li et al. 2005). When tested in NRX Iα-transfected tsA cells, some of these constructs induce cation conductance; others completely lack this ability. As all these mutants possess unaltered N-terminal wing domains, the wing apparently does not participate in pore formation, while the structure of the C-terminal head is crucial for membrane insertion. This is consistent with the model of the membrane-inserted α-LTX tetramer in Figure 2.

3.4 Features of the Pore

3.4.1 Ion Selectivity

Once formed, the α-LTX channel only mediates cationic currents, probably because negatively charged acidic side chains line the channel (Finkelstein et al. 1976). Most significantly, the α-LTX channel is permeable to Ca^{2+} (Finkelstein et al. 1976; Krasil'nikov et al. 1982; Mironov et al. 1986), and it is this aspect of the α-LTX channel that is most often considered. However, this channel is not very selective, and Ba^{2+}, Sr^{2+}, Mg^{2+} as well as Li^{+}, Cs^{+} and, importantly, Na^{+} and K^{+} currents are also carried by the channel (Krasil'nikov et al. 1982; Mironov et al. 1986).

A detailed analysis of the permeability of α-LTX channels to cations and non-electrolytes (Mironov et al. 1986; Robello 1989; Krasilnikov and Sabirov 1992) postulates that the negatively charged selectivity filter of the channel is located close to the extracellular side of the membrane, fitting in with the hypothesis that the C-terminal head domains form the selectivity filter (see Section 2.2.2).

This hypothesis finds additional confirmation in the features of the α-LTX truncation mutants described above (Section 3.3) (Li et al. 2005). These mutants have 1 to 8 ankyrin repeats removed from their C-termini and form cation channels of dramatically different conductivities. For example, α-LTxΔ1 mutant mediates an enormous conductance, by far exceeding that of wild-type toxin. It is possible that the removal of the last ankyrin repeat lifts an obstruction for cation movements in the extracellular mouth of the channel. In contrast, α-LTxΔ2 and α-LTxΔ3 make very inefficient channels, probably due to perturbations of the channel lining. Finally, α-LTxΔ8 does not induce any cation currents, as it seemingly cannot form tetramers.

3.4.2 Ca^{2+} Currents

Although Ca^{2+} only carries a small proportion of currents through cell membrane-inserted α-LTX channels (Hurlbut et al. 1994; Tse and Tse 1999), the influx of Ca^{2+} through presynaptically-targeted α-LTX channels is most often referred to, because of the well-established link between presynaptic $[Ca^{2+}]$ and neurotransmitter release. There is a wealth of evidence indicating that in conditions favorable to channel formation (e.g., in the presence of divalent cations), influx of extracellular Ca^{2+} through α-LTX channels is an important aspect of α-LTX action.

Increases in $[Ca^{2+}]_i$ upon α-LTX addition in the presence of Ca_e^{2+} have been detected in synaptosomes (Nicholls et al. 1982; Meldolesi et al. 1984), native PC12 (Grasso et al. 1980; Meldolesi et al. 1984), and chromaffin (Barnett et al. 1996) cells, as well as in receptor-transfected COS (Volynski et al. 2000; Ashton et al. 2001), BHK (Krasnoperov et al. 1997) and neuroblastoma cells (Volynski et al. 2004).

While the bulk of this effect is due to calcium influx through the toxin channel, it is possible that the rise in $[Ca^{2+}]_i$ is contributed to by Ca^{2+} release from intracellular stores (Tse and Tse 1999; Tsang et al. 2000; Liu et al. 2005) or influx through endogenous Ca^{2+} channels (Nicholls et al. 1982; Lajus et al. 2006). In LPH-transfected neuroblastoma cells, Ca_i^{2+} waves caused by LPH signalling are observed in response to non-pore-forming α-LTX (Volynski et al. 2004). Nevertheless, influx of Ca^{2+} through the channel formed by wild-type toxin appears more dramatic.

3.4.3 Na^+ Currents

α-LTX channels inserted in artificial membranes are permeable to Na^+ (Krasil'nikov et al. 1982; Robello et al. 1984), which is modulated by Ca^{2+} added to the topologically extracellular side of the bilayer (Mironov et al. 1986). Inward Na^+ currents are observed electrophysiologically in *Xenopus* oocytes, PC12 and neuroblastoma cells (Wanke et al. 1986; Filippov et al. 1994; Hurlbut et al. 1994), and α-LTX elevates $[Na_i^+]$ in synaptosomes detectable with a fluorescent dye (Deri and Adam-Vizi 1993), although not with radioactive Na^+ (Storchak et al. 1994).

Such currents probably account for at least one of α-LTX actions: tetrodotoxin-insensitive depolarization of artificial and biological membranes (Grasso and Senni 1979; Nicholls et al. 1982; Scheer et al. 1986). Although depolarization could cause neurotransmitter release by activating voltage-gated Ca^{2+} channels, Ca^{2+} flow through the α-LTX channel probably overwhelms this effect (Nicholls et al. 1982), so the role of depolarization in α-LTX action is unclear.

In addition, Na^+ currents through α-LTX channels can underlie some of the α-LTX-induced nonvesicular neurotransmitter release by causing, for example, the collapse of the cross-membrane Na^+ gradient and the reversal of some (McMahon et al. 1990) but not all (Deri and Adam-Vizi 1993) transmitter uptake pumps.

α-LTX-mediated Na^+ influx can also induce mitochondria of frog NMJ synapses (Tsang et al. 2000) and neurohypophysis terminals (Hlubek et al. 2003) to release large amounts of Ca^{2+} into the cytosol because of the reversal of their Na^+/Ca^{2+} exchangers, although this seems to be inconsequential for secretion in the absence of Ca_e^{2+}. In contrast, increases in $[Ca^{2+}]_i$, partially dependent on Na_e^+, correlate with acetylcholine release in synaptosomes (Deri and Adam-Vizi 1993). Generally, a rise $[Na^+]_i$ in nerve terminals correlates with enhanced secretion (Meiri et al. 1981). Thus, Na^+ currents represent yet another facet of the α-LTX channel actions at the synapse and could account for some of the toxin-evoked Ca_e^{2+}-independent vesicular release. However, α-LTX may be able to induced release even in Ca^{2+}-free and Na^+-free medium in synaptosomes (Storchak et al. 1994) and frog NMJs (Tsang et al. 2000).

3.4.4 Factors Affecting Conductance

A perplexing feature of the literature that explores α-LTX channels is the extremely wide range of single-channel conductances, ranging from 15 pS (Wanke et al. 1986; Krasilnikov and Sabirov 1992) to 100-300 pS (Finkelstein et al. 1976; Van Renterghem et al. 2000) or even 1100 pS (Krasilnikov and Sabirov 1992). To put these figures in perspective, the voltage-gated K^+ channel Kv1.2 and the ryanodine-sensitive Ca^{2+}-release channels have conductances of 14-18 pS and ∼700 pS, respectively (Lindsay et al. 1994; Gutman et al. 2005) (Figure 2).

Several plausible hypotheses can explain this variability. Firstly, the venom contains a range of pore-forming toxins with varying conductances. Secondly, there is evidence that the make-up of the permeated membrane (Robello et al. 1984; Scheer et al. 1986; Krasilnikov and Sabirov 1992) and variations in lipid packing and order (Chanturia and Lishko 1992), but not the type of receptor present (Hlubek et al. 2000; Van Renterghem et al. 2000), can affect the properties of α-LTX channels.

Interestingly, Ca^{2+} inhibits the conductance of α-LTX channels to monovalent cations, causing a "flickery block," in artificial membranes (Mironov et al. 1986; Krasil'nikov et al. 1988), neuroblastoma cells (Hurlbut et al. 1994), and embryonic kidney cells (Hlubek et al. 2000), although this is not apparent in receptor-expressing oocytes (Filippov et al. 1994). Mg^{2+} positively modulates the conductivity of toxin channel for Ca^{2+} (Davletov et al. 1998; Van Renterghem et al. 2000).

Finally, the frequent observation of bursts of conductance, likely corresponding to synchronized opening and closing events, has led many authors to suggest that α-LTX channels form clusters in the membrane (Robello et al. 1987; Krasilnikov and Sabirov 1992; Filippov et al. 1994; Van Renterghem et al. 2000). Such clustering is plausible from a structural point of view, as crystalline arrays of α-LTX tetramers are observed by EM (Lunev et al. 1991) (see Section 2.2.2), and it may account for some of the variability in conductances reported in the literature.

3.4.5 Gating

The channel is open most of the time, with an open probability of 0.8 (Filippov et al. 1994). Closure events, albeit rare, have been observed in many systems (Finkelstein et al. 1976; Wanke et al. 1986; Hurlbut et al. 1994), and several channels can open and close in concert (Krasilnikov and Sabirov 1992; Filippov et al. 1994; Tse and Tse 1999). Membrane depolarization may increase the likelihood of closure events (Filippov et al. 1994; Van Renterghem et al. 2000), and Ca^{2+} and Mg^{2+} modulate gating (Filippov et al. 1994; Hurlbut et al. 1994). The molecular basis of gating is currently unknown, but is likely to involve head domains.

3.4.6 Divalent and Trivalent Cations

The α-LTX pore is permeable to alkaline earth cations, whose affinities for the channel decrease in the following sequence: $Mg^{2+} > Ca^{2+} > Sr^{2+} \geq Ba^{2+}$ (Mironov et al. 1986). Transition metal cations ($Cd^{2+} > Co^{2+} \geq Ni^{2+} \geq Zn^{2+} > Mn^{2+}$) strongly block Ca^{2+} and K^{+} currents through α-LTX channels in artificial membranes (Mironov et al. 1986). This block is only effective when the cation is applied from the *cis*-side (equivalent to the extracellular side) of the membrane.

Millimolar concentrations of trivalent cations, such as Yb^{3+}, Gd^{3+}, Y^{3+}, La^{3+} and Al^{3+}, block the pore-mediated effects of α-LTX in synaptosomes (Scheer 1989). The nature of this effect is complex. For example, Al^{3+} blocks the binding of α-LTX to synaptosomes (Scheer 1989), while La^{3+} prevents tetramerization (Ashton et al. 2000) and thereby channel insertion and formation.

Most trivalent cations, at 50–100 μM, block previously inserted channels (Scheer 1989; Hurlbut et al. 1994; Van Renterghem et al. 2000) and inhibit α-LTX-mediated Ca^{2+}-uptake, while La^{3+} blocks depolarization as well (Scheer 1989). Channel inhibition by trivalent cations is very important because La^{3+} is essentially the only reagent that blocks the Ca^{2+}-independent α-LTX-evoked neurotransmitter secretion in neurons (Scheer 1989; Capogna et al. 2003).

The use of trivalent cations for the purpose of dissecting the mechanisms of α-LTX action, however, is complicated by their well-described ability to trigger neurotransmitter release in the absence of other stimuli (Scheer 1989). Furthermore, at low concentrations, both La^{3+} (at 15 μM) and Cd^{2+} (at 50 μM) support, rather than block, the secretagogue activity of the toxin (Misler and Falke 1987).

3.4.7 Permeation of Molecules/Leakage

It is possible that the water-filled α-LTX channel, which is relatively wide (∼10 Å at its narrowest (Krasilnikov and Sabirov 1992; Orlova et al. 2000), can pass small molecules. Indeed, α-LTX channels inserted in the membranes of synaptosomes, NMJ nerve terminals, and receptor-transfected COS7 cells appear to pass fluorescein (Stokes-Einstein radius, $R_e = 4.5$ Å) and norepinephrine ($R_e \leq 4$ Å) (Davletov et al. 1998; Rahman et al. 1999; Volynski et al. 2000), shown in Figure 2 for comparison with 8-hydrated calcium ion ($R_e = 4.2$ Å) and the toxin channel. Analysis of "impermeant" cations commonly used in channel studies reveals that α-LTX channels are poorly permeable (Hurlbut et al. 1994) to glucosamine H^+($R_e = 4.6$ Å) and not significantly permeable (Tse and Tse 1999) to N-methyl-D-glucamine ($R_e = 5.2$ Å), thus limiting the pore diameter by ∼10 Å.

These observations are particularly relevant to the experimental use of α-LTX in neurotransmission studies, since α-LTX has been shown in several systems to cause nonvesicular release by allowing leakage of cytoplasmic neurotransmitters (McMahon et al. 1990; Deri et al. 1993; Davletov et al. 1998). This flux could be mediated by the α-LTX channel itself, by local disruptions of cellular membranes, or by reversal of transmitter uptake pumps driven by Na^+ gradient (see Section 3.4.3). Synaptosomes seem to be particularly sensitive to an increase in hydrostatic pressure, which may occur when influx of Na^+ or Ca^{2+} leads to a concomitant influx of water.

3.5 Can the Pore Explain Everything?

It is now universally accepted that α-LTX can insert into, and permeabilize, artificial and biological membranes. Cation currents can explain some, but not all, of the toxin's effects. For example, it is not clear how the α-LTX channel could mediate Ca_e^{2+}-independent exocytosis in neurons. Although it would be tempting to assign some of α-LTX actions in the absence of Ca_e^{2+} to Na^+ currents, lack of Na^+ does not prevent Ca^{2+}-independent secretion (Tsang et al. 2000). Cation flux-associated incursion of terminals by water could be involved, but α-LTX effect on intracellular osmotic pressure has not been characterized yet.

Based on the results of α-LTX mutagenesis, strong correlation exists between pore formation and stimulation of Ca^{2+}-dependent exocytosis from neuroendocrine cells. However, in some experiments with chromaffin cells, α-LTX action does not involve Ca^{2+} entry (Michelena et al. 1997). In addition, α-LTX sensitizes chromaffin cells to Ca^{2+} even when the cells are permeabilized and toxin pores should have no effect; this involves protein kinase C (PKC) activation (Bittner and Holz 2000). Furthermore, the ability of $\alpha\text{-LTX}^{N4C}$ to induce Ca^{2+}-dependent exocytosis without forming pores implicates a stimulating mechanism other than pore formation.

Therefore, α-LTX receptors, which are so crucial for the toxin's action in all biological systems, have been comprehensively studied.

4 Receptors

The three currently known receptors for α-LTX – NRX, LPH (or CIRL) and PTPσ – were all isolated from brain extracts by affinity chromatography on immobilized α-LTX (Petrenko et al. 1990; Davletov et al. 1996; Krasnoperov et al. 1996; Krasnoperov et al. 2002a). Surprisingly, they were purified only as a result of three separate efforts several years apart. In part, this piecemeal discovery of α-LTX receptors reflects differences in purification procedures; in part, it is due to the researchers' inability to explain all the actions of the toxin by the features of each newly found receptor, warranting further attempts at isolating the "ultimate" target of α-LTX. While new proteins interacting weakly/transiently with the toxin and mediating some of its activities may still be found, there is no doubt that NRX Iα, LPH1 and PTPσ exhaust the repertoire of major α-LTX receptors. As these proteins are discussed in detail below, it is important to remember that, with any receptor, wild-type α-LTX can insert into the membrane and form pores (or engage in other interactions), causing strong direct effects that make definitive conclusions regarding receptor signalling, or lack of it, difficult. Only when membrane insertion and pore formation are blocked (as, for example, in α-LTX^{N4C} mutant, see Section 2.3), can the observed effects be attributed to the action of one or the other receptor.

4.1 Neurexin

The first α-LTX receptor to be identified was isolated from solubilized bovine brain by toxin-affinity chromatography in the presence of Ca^{2+} (Petrenko et al. 1990) and termed neurexin (NRX) (Ushkaryov et al. 1992) (Figure 3).

4.1.1 Structure

NRX is a member of a family of polymorphous neuronal cell-surface receptors (Ushkaryov et al. 1992; Ushkaryov and Südhof 1993; Ullrich et al. 1995) (Figure 3a). Three homologous genes encode NRXs I, II, and III. Each gene, in turn, is transcribed from two independent promoters (α and β), leading to a long (α) and a short (β) forms of each NRX (Missler et al. 1998a). The resulting six main NRXs are further diversified by extensive alternative splicing at five splice sites. As a result of independent combinations of variable-size inserts, hundreds of NRX isoforms may be produced (Ullrich et al. 1995; Missler and Südhof 1998). Given the differential expression of the three NRX genes in brain regions (Ullrich et al. 1995), developmental regulation of the two promoters and cell-specific alternative splicing (Ushkaryov and Südhof 1993; Ullrich et al. 1995; Occhi et al. 2002), NRXs could play the role of neuronal recognition tags (Missler and Südhof 1998).

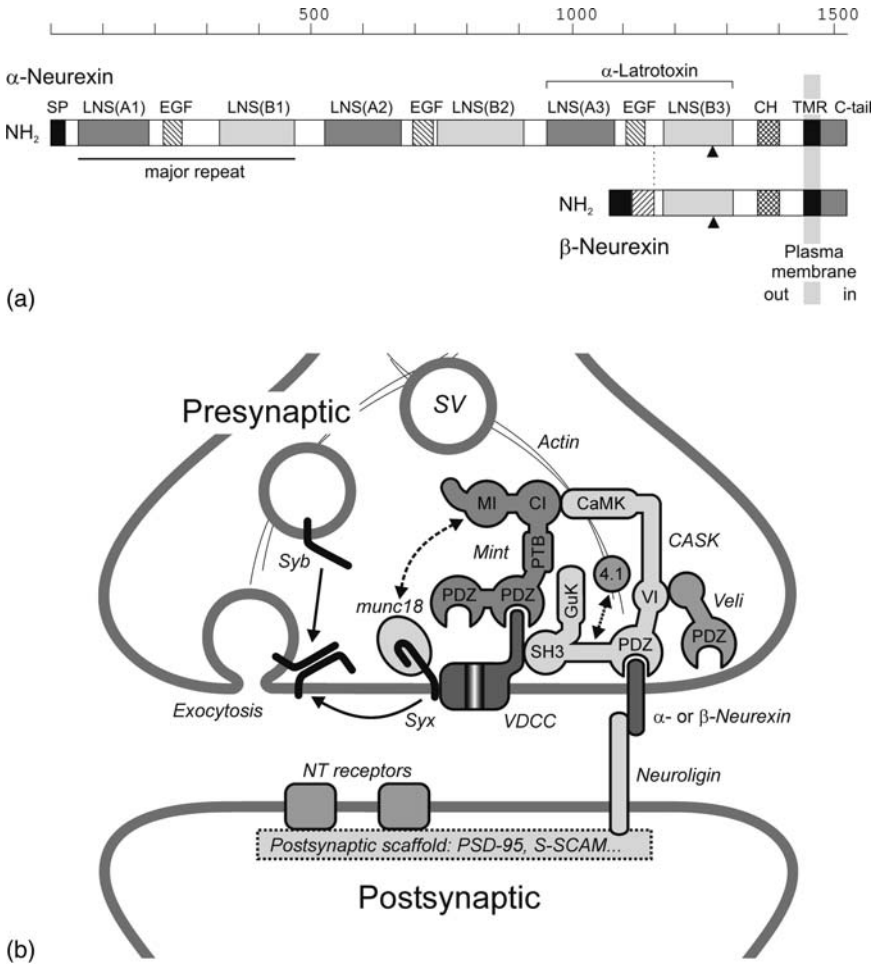

Fig. 3 Generalized structure and functions of neurexins. (**a**) Domain structure of α- and β-neurexins. Scale above shows amino acid positions. Arrowheads, splice site 4; SP, signal peptide; LNS, laminin G/NRX/sex hormone-binding globulin-like domains; EGF, epidermal growth factor-like repeats; CH, O-linked carbohydrate attachment domain; bracket, α-LTX binding site. α- and β-NRXs are identical to the right of the dotted line. (**b**) Scheme of NRX interactions. Domain names: CaMK, Ca^{2+}, calmodulin-dependent protein kinase homology; CI, CaMK-interacting; GuK, membrane-associated pseudo-guanylate kinase; MI, munc18-interacting; VI, Veli (vertebrate Lin7)-interacting. Other abbreviations: 4.1, protein 4.1 bound to actin; Mint, munc18-interacting protein; NT, neurotransmitter; PTB, phosphotyrosine binding; SH3, src homology; S-SCAM, synaptic scaffolding molecule; SV, synaptic vesicle; VDCC, voltage-dependent Ca^{2+}-channel. Dotted arrows, protein interactions; solid arrows, formation of the SNARE complex involved in exocytosis of synaptic vesicles.

All NRXs have a single transmembrane region (TMR) and a short cytoplasmic tail. In α-NRXs, the large ectodomains are composed of six LNS domains (Figure 3a) characteristic of many adhesion molecules. Pairs of LNS(A) and LNS(B) domains, with EGF-like sequences in the middle, form three "major repeats" (Figure 3a). In contrast, β-NRXs have one LNS(B) domain and a unique N-terminal sequence. All NRXs contain an extensively O-glycosylated Thr- and Ser-rich region upstream of the TMR (Ushkaryov et al. 1992; Ushkaryov et al. 1994).

4.1.2 Distribution

NRXs are present mostly in brain (Ushkaryov et al. 1992) but also in pancreas and lung (Occhi et al. 2002). They are differentially expressed in brain regions, showing partially overlapping patterns of distribution of their six main forms and region-specific splice variants (Ullrich et al. 1995). It is difficult to localize NRX proteins at the cellular level, partly because the respective α- and β-forms are almost identical immunologically. As demonstrated by light microscopy, NRXs are expressed at synapses (Ushkaryov et al. 1992; Dean et al. 2003), but in the absence of EM data, their distribution between the pre- and postsynaptic membranes is not clear. Based on the presynaptic action of α-LTX, NRX Iα is implicitly presynaptic; β-NRXs are assigned to presynaptic membranes due to their interaction with neuroligins, indeed localized in the postsynaptic membrane by EM (Song et al. 1999). It remains to be seen whether any NRXs may be expressed postsynaptically.

4.1.3 Binding of α-LTX

Analysis of NRX isolated from brain by α-LTX-affinity chromatography (Petrenko et al. 1990; Petrenko et al. 1993) shows that out of the six main isoforms only NRX Iα binds toxin with sufficient affinity (Davletov et al. 1995), and this interaction strictly requires Ca^{2+} (Petrenko et al. 1990; Davletov et al. 1995).

The α-LTX binding site has not been mapped in a systematic effort, but incidental evidence implicates the third major repeat, LNS(A3/B3). In particular, α-LTX binds neither a NRX Iα mutant lacking the LNS-domains B2, A3, and B3, nor the full-size NRX Iα with an insert in LNS(B3) (Davletov et al. 1995). Although LNS(B3) domains of all three β-NRXs are able to interact with α-LTX in a splicing-dependent manner under mild conditions (Sugita et al. 1999), they are not sufficient for strong toxin binding: NRX Iβ does not pull down α-LTX (Davletov et al. 1995), nor are β-NRXs isolated from brain by α-LTX chromatography (Petrenko et al. 1990). Finally, a NRX Iα mutant, containing only LNS(A3/B3), binds the toxin well (Li et al. 2005). Thus, the strong interaction of α-LTX with NRX Iα is mediated by both LNS(A3) and LNS(B3), while splicing in LNS(B3) regulates this binding.

4.1.4 Proposed Functions

The structure of NRXs is consistent with a function in cell adhesion and/or ligand binding. Indeed, the extracellular domains of α-NRXs interact specifically and strongly with neurexophilins (Petrenko et al. 1993; Petrenko et al. 1996; Missler et al. 1998b), a family of glycosylated, proteolytically processed hormone-like 30-kDa proteins with species-specific differential expression in mammalian tissues (Petrenko et al. 1996; Missler and Südhof 1998). The biological role of neurexophilins is unknown (Missler et al. 1998b), but their tight binding to the LNS(B1) domain of α-NRXs implies long-term/structural, rather than short-term/signaling, interactions.

In addition, both α- and β-NRXs can interact with neuroligins, a family of neuronal proteins that contain a region of homology to esterases in their extracellular domains and a single TMR (Ichtchenko et al. 1995; Ichtchenko et al. 1996) (Nguyen and Südhof 1997; Boucard et al. 2005). Neuroligin-1 has been identified owing to its ability to bind all three β-NRXs – only if they lack an insert at splice site 4 (Ichtchenko et al. 1995) (Figure 3). Neuroligins are also differentially spliced, and the absence of an insert at splice site B enables neuroligins to bind also α-NRXs (Boucard et al. 2005). These proteins are localized on the postsynaptic membrane of excitatory (Song et al. 1999) and inhibitory (Varoqueaux et al. 2004) synapses. Their Ca^{2+}-dependent, splicing-controlled interaction with β-NRXs (Ichtchenko et al. 1996; Nguyen and Südhof 1997; Comoletti et al. 2006) is important for synapse formation (Scheiffele et al. 2000; Dean et al. 2003; Chih et al. 2006), differentiation (Levinson et al. 2005), and maturation (Dresbach et al. 2004; Varoqueaux et al. 2006).

In the cytoplasmic tail of NRXs, there is a short C-terminal site that binds to PDZ domain-containing scaffold proteins, CASK (Hata et al. 1996) and Mint (Biederer and Südhof 2000). These proteins interact with each other and with Ca^{2+} channels; Mint also makes complexes munc18, a protein involved in vesicle exocytosis (Verhage et al. 2000). Therefore, NRXs may participate in arranging transmitter release mechanisms. Indeed, knockout of all three α-NRXs has demonstrated that they play a role in organizing N- and P/Q-type Ca^{2+} channels (Zhang et al. 2005) in nerve terminals (Missler et al. 2003) and endocrine cells (Dudanova et al. 2006). Even though β-NRXs have the same C-terminal sequences as the respective α-NRXs, the two isoforms participate differently in synaptogenesis and transmitter secretion. In particular, α-NRXs are apparently not involved in synapse formation (Missler et al. 2003) but may influence the expression of postsynaptic NMDA (but not AMPA) glutamate receptors (Kattenstroth et al. 2004).

4.2 Latrophilin

Because NRX Iα requires Ca^{2+} to bind α-LTX, it cannot mediate the toxin's effects in the absence of Ca^{2+}. The quest for a Ca^{2+}-independent receptor continued, and a major protein was eventually isolated by α-LTX-chromatography

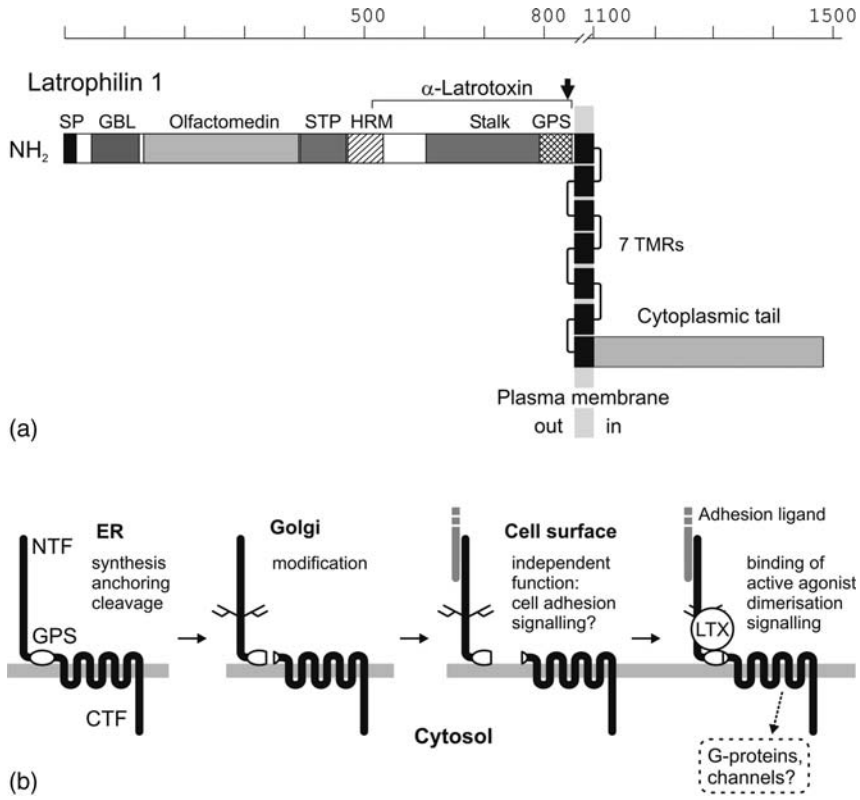

Fig. 4 LPH structure and processing. (**a**) Generalized domain structure of latrophilins. 7TMRs, seven transmembrane regions and corresponding loops; arrow, cleavage point; bracket, the site of α-LTX binding in LPH1; GBL, galactose-binding lectin; GPS, GPCR-proteolysis site domain; HRM, hormone receptor motif; STP, Ser-, Thr- and Pro-rich domain. (**b**) Scheme of LPH processing and functioning (Volynski et al. 2004). See text for details. ER, endoplasmic reticulum.

of solubilized brain proteins and termed latrophilin (LPH) (Figure 4) (Davletov et al. 1996; Lelianova et al. 1997), or Ca^{2+}-independent receptor of α-LTX (CIRL) (Krasnoperov et al. 1996; Krasnoperov et al. 1997). LPH binds toxin in the presence or absence of divalent cations (Davletov et al. 1996). It was not discovered earlier because its 120-kDa toxin-binding fragment was confused on SDS gels with α-LTX, while the second (65 kDa) fragment was lost due to its quantitative precipitation upon boiling with SDS.

4.2.1 Structure

LPH is a large (185 kDa) heptahelical receptor. It comprises three major domains (Figure 4a): (1) a long, glycosylated N-terminal extracellular domain; (2) seven hydrophobic TMRs; and (3) a long cytoplasmic tail. The ectodomain contains

regions of homology to: galactose-binding lectin; the surface-attached extracellular matrix protein olfactomedin (Snyder et al. 1991; Loria et al. 2004); a hormone receptor motif (HRM) found also in other G-protein-coupled receptors (GPCRs) and probably involved in ligand binding; a "Stalk" domain important for proteolytic cleavage (Chang et al., 2003); and the G-protein-coupled receptor proteolysis site (GPS). The seven TMRs are similar to the corresponding regions of the secretin/calcitonin GPCRs that bind peptide hormones and induce release of various substances (Harmar 2001).

Based on this homology and on the length of ectodomains, LPH and similar receptors have been initially classified as "Long N-terminus group B" GPCRs (Hayflick 2000; Stacey et al. 2000). However, it is recognized now (Fredriksson and Schioth 2005) that they form a separate group, termed "adhesion GPCRs." This nomenclature stems from the fact that these receptors contain various cell adhesion modules in their ectodomains. The group includes 33 proteins identified by their function/activity or through genome searches (Fredriksson and Schioth 2005).

LPH has two very similar homologues, LPH2 and LPH3 (Matsushita et al. 1999). The family is also called CIRL1-3 (Ichtchenko et al. 1999) and CL1-3 (an abbreviation combining the two other names of the protein, CIRL and LPH) (Sugita et al. 1998). The LPHs have seven sites of alternative splicing (Sugita et al. 1998; Matsushita et al. 1999). Some splice variants encode receptors with an altered third cytoplasmic loop or a truncated cytoplasmic tail (Matsushita et al. 1999), modifications that are likely to affect coupling to G proteins.

4.2.2 Unusual Architecture

"Adhesion GPCRs" have an unusual architecture (Gray et al. 1996; Krasnoperov et al. 1997; Volynski et al. 2004). They contain two functional parts: an N-terminal cell adhesion receptor-like extracellular domain and a C-terminal domain with seven TMRs and a signaling potential (Hayflick 2000; Stacey et al. 2000). Intriguingly, all these receptors are post-translationally cleaved at the GPS domain (Krasnoperov et al. 1997) (Figure 4a, b) into N- and C-terminal fragments (NTF and CTF), which correspond to the two functional parts. This constitutive proteolysis, occurring in the endoplasmic reticulum, is prerequisite for LPH cell surface delivery (Krasnoperov et al. 2002a; Volynski et al. 2004). The GPS is localized upstream of the first TMR, yet the ectodomain is not released into the medium, apparently because it is attached to the membrane by a hydrophobic anchor (Volynski et al. 2004).

The peculiar biology of LPH has been studied in neuroblastoma cells stably expressing recombinant LPH1 (Volynski et al. 2004). Following delivery to the plasma membrane, the two receptor fragments can dissociate and behave as independent cell-surface proteins. However, under certain conditions, the fragments reassociate. This reassembly, mediated only by seven most N-terminal amino acids of the CTF, is induced by detergents and also by α-LTX binding to the NTF (Volynski et al. 2004). Ternary α-LTX-NTF-CTF complexes form large patches in the plasma membrane, triggering formation of molecular dimers of the CTF. This process temporally

and spatially correlates with signal transduction, which can be visualized using the non-pore-forming mutant α-LTX^{N4C} (see Section 2.3). This signaling requires the full-size CTF and involves activation of PLC and release of Ca^{2+} from intracellular stores (Volynski et al. 2004). It is interesting that both in this model system and in central synapses (Ashton et al. 2001; Capogna et al. 2003), α-LTX^{N4C} induces Ca^{2+} signals only in the presence of Ca_e^{2+}. Calcium ions may serve here as a co-factor for LPH activation or they may enter the cytosol through an LPH-activated Ca^{2+} channel(s).

4.2.3 Distribution

LPHs are differentially distributed in mammalian tissues: while LPH1 and LPH3 are enriched in brain, LPH2 features more prominently in other tissues (Sugita et al. 1998; Ichtchenko et al. 1999; Matsushita et al. 1999). Similar to NRX Iα (Ushkaryov et al. 1992; Occhi et al. 2002), very small levels of LPH1 mRNA can be detected outside brain, especially in kidneys and pancreas, and both receptors reside in endocrine cells, such as pancreatic β-cells (Lang et al. 1998). Based on α-LTX binding and action, which are dramatically inhibited by the LPH gene knockout, this receptor is localized in presynaptic terminals. In a yeast two-hybrid system, the C-terminal cytoplasmic tail of LPH can bind Shank (Tobaben et al. 2000; Kreienkamp et al. 2000), an ankyrin repeat-containing protein of postsynaptic density, although the specificity and significance of this interaction is unclear.

4.2.4 Binding of α-LTX

LPH1 has a very high affinity for α-LTX (Kd ∼0.1–1.5 nM) (Volynski et al. 2000; Ichtchenko et al. 1999), while the interaction of LPH 2 with toxin is more than 10-fold weaker (Ichtchenko et al. 1999). α-LTX binds the Stalk/GPS domains of the NTF (Krasnoperov et al. 1999), while the lectin and olfactomedin regions upstream could participate in adhesion interactions with the extracellular matrix or adjacent cells (Figure 4b). Hence, the NTF is thought to remain on the cell surface at specialized cell-cell or cell-matrix junctions (Volynski et al. 2004) and, thus, define the sites where the LPH fragments can interact with each other. If endogenous CTF-mediated signaling requires NTF-CTF complex formation (as with α-LTX), then LPH signals may be restricted to adhesion foci.

4.2.5 Proposed Functions

The functions of LPH remain to be elucidated. As follows from the results of LPH1 gene knockout (Tobaben et al. 2002), this receptor is not crucial for basic neuronal survival and activity, although LPH2 and 3 may compensate for the lack of LPH1. The structure of all adhesion GPCRs indicates that these receptors can transform

extracellular interactions into intracellular signaling. Being involved in some pathways leading to release of stored Ca^{2+}, LPH may participate in the cell contact-dependent fine tuning of $Ca^{2+}{}_i$ levels and modulation of transmitter secretion.

4.3 Protein Tyrosine Phosphatase σ

In the search for a Ca^{2+} independent α-LTX receptor, affinity chromatography in the absence of Ca^{2+} was used to isolate brain proteins with any affinity for α-LTX. Sequencing of all proteins in the α-LTX column eluate (Krasnoperov et al. 1996; Krasnoperov et al. 2002b) has revealed that, in addition to LPH1, PTPσ can also bind to the column as a set of two minor bands.

4.3.1 Structure

PTPσ is a member of the family of receptor-like PTPs that contain cell adhesion molecule-like extracellular domains, a single TMR, and cytoplasmic phosphatase domains (Tonks 2006). In PTPσ, the extracellular domain comprises three immunoglobulin-like modules and, depending on alternative splicing, from four to eight fibronectin type III-like domains (Figure 5a). The cytosolic portion of the protein contains a catalytically active phosphatase domain and a pseudo-phosphatase.

Reminiscent of LPH, PTPσ is proteolytically cleaved 125 residues upstream of the TMR into two fragments, which remain non-covalently associated (Figure 5a) (Yan et al. 1993; Aicher et al. 1997). The proteolysis occurs inside the cell but, unlike in LPH, is not necessary for cell surface delivery of PTPs (Serra-Pages et al. 1994). On the cell surface, the P-subunit of PTPσ can be further cleaved by the metalloprotease TACE (Ruhe et al. 2006) six residues upstream of the TMR (Aicher et al. 1997). This process, stimulated by treating cells with Ca^{2+} ionophores or phorbol esters (Aicher et al. 1997), or by interaction of PTPσ with the epidermal growth factor receptor (EGFR) (Ruhe et al. 2006), leads to the shedding of the E-subunit and sequestration of the P-subunit (Figure 5b), thus downregulating the PTPσ function.

4.3.2 Distribution

The large splice variant of PTPσ is found in most tissues, while the shorter variant is mostly brain-specific (Yan et al. 1993; Pulido et al. 1995) but, similar to NRX Iα and LPH1, is also found in small amounts in some other tissues (Yan et al. 1993), which may be due to autonomic innervation.

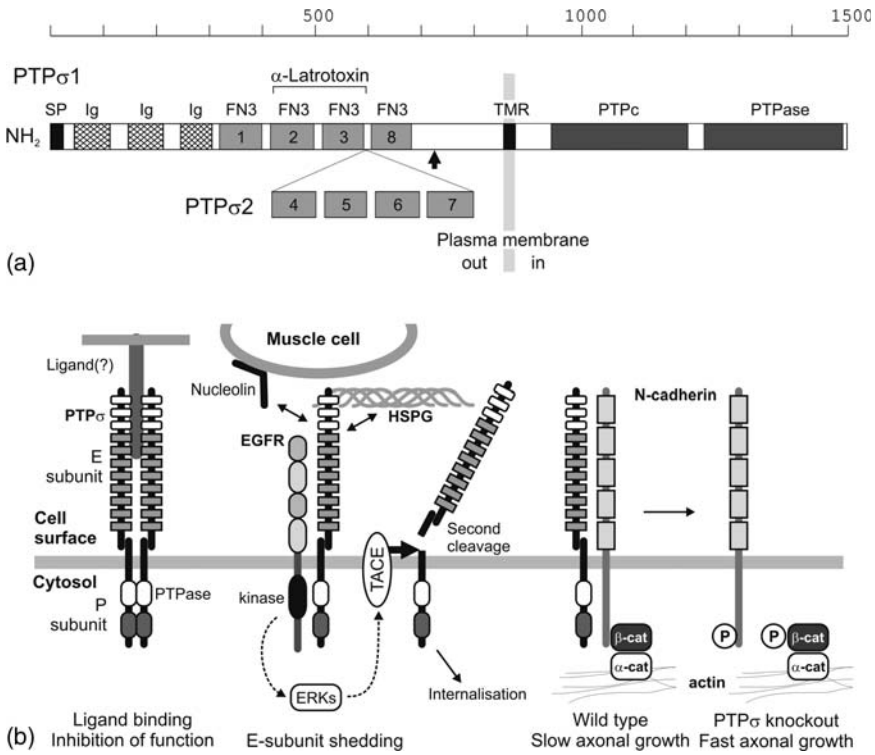

Fig. 5 PTPσ structure and functions. (**a**) Generalized domain structure of receptor-like PTPσ. FN3, fibronectin type III-homology domains; Ig, immunoglobulin-like domains; PTPc, catalytic PTP domain; PTPase, pseudo-phosphatase domain. Arrow, proteolytic cleavage site; bracket, α-LTX binding site. (**b**) Cellular functions and interactions of PTPσ. Ligand binding leads to dimerization and inhibition of the intracellular domains. PTPσ interaction with EGFR activates the ERK-mediated signalling and upregulates the TACE metalloprotease which cleaves the P subunit and releases E subunit. PTPσ may interact with HSPG of the basement membrane and nucleolin on muscle cells. PTPσ binds N-cadherin and dephosphorylates it, causing increased adhesion and inhibition of axonal growth. In mice lacking PTPσ, N-cadherin is hyper-phosphorylated; this reduces adhesion and increases neuronal growth. EGFR, epidermic growth factor receptor; ERKs, MAP kinases ERK1 and ERK2; HSPG, heparin sulphate proteoglycan; cat, catenin.

4.3.3 Binding of α-LTX

Despite the very low binding of this protein to toxin columns, the α-LTX-PTPσ interaction is specific and maps onto the fibronectin domains 2 and 3 (Krasnoperov et al. 2002b). These are present in both PTPσ1 and PTPσ2, but on overexpression of these splice variants in pancreatic β-cells, only PTPσ1 mediates the secretory action of α-LTX (Lajus and Lang 2006). This may be because the toxin binds PTPσ2 too far from the membrane and is then unable to insert into the plasma membrane.

4.3.4 Proposed Functions

As phosphorylation of proteins localized in focal adhesions controls cell-cell and cell-matrix interactions, dephosphorylation is also important for the regulation of cell adhesion. Indeed, PTPσ is involved in negative regulation of axonal growth (McLean et al. 2002; Thompson et al. 2003) and growth cone pathfinding (Meathrel et al. 2002). Apart from EGFR (see Section 4.3.1), PTPσ interacts with N-cadherin and dephosphorylates it, augmenting its catenin-mediated attachment to the cytoskeleton and inhibiting axonal growth (Figure 5b). In PTPσ knockout mice, N-cadherin becomes over-phosphorylated (Siu et al. 2006) and unable to interact with catenins and the cytoskeleton, leading to stimulated axonal growth (McLean et al. 2002).

The structure of PTPσ is consistent with its role in binding ligands on the surface of other cells or in the extracellular matrix. It interacts with heparin sulphate proteoglycan in the basement membrane (Sajnani-Perez et al. 2003). It may also bind to the C-terminal domain of cell surface-exposed nucleolin, a normally nuclear protein that is presented on the surface of developing muscle cells (Alete et al. 2006). However, these complexes seem to mediate structural or long-term regulatory interactions rather than short-term signaling to the neuronal secretory machinery.

4.4 α-LTX Receptors and Signaling

4.4.1 NRX Iα

Can NRXs transduce intracellular an exocytotic signal? All currently known cytoplasmic interactions of α- and β-NRXs appear to be stable, nonenzymatic, structural connections that are essential for neuronal development, synapse organization, and long-term regulation but are unlikely to underlie a massive and relatively fast response induced by α-LTX. However, toxin binding to NRXs might cause conformational changes in Mint, which, by interacting with munc18, might regulate the availability of docking sites for synaptic vesicles. On the other hand, even a truncated NRX Iα that lacks the cytoplasmic tail can mediate the sensitivity of PC12 cells to wild-type α-LTX (Sugita et al. 1999). The most likely explanation of this is that toxin inserts into the membrane on binding any membrane-attached form of NRX Iα (Volynski et al. 2000; Hlubek et al. 2000), and because NRX experiments require Ca^{2+}_e, influx of this cation through the toxin pore can trigger maximal exocytosis irrespective of any NRX Iα-mediated signaling. With this in mind, there is no doubt that NRXs (full-size or C-terminally truncated) constitute functional receptors for α-LTX (Sugita et al. 1999), but only in the sense that they provide binding sites for the toxin, which subsequently exerts its own actions.

4.4.2 LPH1

LPH1 is the only receptor capable of G-protein-mediated signaling induced by α-LTX in central synapses (Davletov et al. 1998; Ashton et al. 2001; Section 1.4). Furthermore, LPH1 is directly involved in α-LTX^{N4C}-induced signaling in LPH-transfected neuroblastoma cells (Volynski et al. 2004). This mutant activates PLC also in neurons, causing release of $Ca^{2+}{}_i$ (Ashton et al. 2001; Volynski et al. 2003; Volynski et al. 2004) and leading to neurotransmitter release, which is blocked when the Gq cascade is inhibited by various drugs (Davletov et al. 1998; Ashton et al. 2001; Capogna et al. 2003). LPH1 also co-purifies with Gq (Rahman et al. 1999).

The endogenous ligand of LPH1 is currently unknown, and it is interesting to consider an invertebrate LPH orthologue from *Caenorhabditis elegans* (Mee et al. 2004; Willson et al. 2004). The nematode protein LAT-1 (B0457.1) lacks the olfactomedin region but contains the domains required for α-LTX binding (HRM, Stalk and GPS). Although its identity to vertebrate LPH1 is not too high (18%–45% in the area of toxin binding), it mediates the lethal effect of an α-LTX homologue, ε-latroinsectotoxin (Mee et al. 2004). *C. elegans* also expresses NRX and PTPσ homologues, but only the knockdown of LPH renders the worms resistant to ε-latroinsectotoxin (Mee et al. 2004). Interestingly, the worm LPH mediates the action of the anthelmintic octadepsipeptide emodepside, which causes paralysis in nematodes (Willson et al. 2004). By the coupling to LAT-1, emodepside activates Gαq and PLC-β (Willson et al. 2004). The product of the PLC activity, IP_3 stimulates release of Ca^{2+} from intracellular stores, known to be important for α-LTX activity in mammals (Davletov et al. 1998; Capogna et al. 2003). In addition, the effect of emodepside involves UNC-13, a protein implicated in control of vesicular exocytosis and activated by diacyl glycerol, another product of PLC. It is possible that such different agonists as emodepside and LTX^{N4C} can stimulate similar signaling from LPH orthologues, suggesting that at least part of the α-LTX effect on exocytosis is due to LPH signal transduction.

Is LPH involved only in one type of signaling, to Ca^{2+} stores? In fact, α-LTX^{N4C}, acting via LPH, can activate Ca^{2+} and inhibit K^+ channels in pancreatic β-cells (Lajus and Lang 2006), raising the possibility that, similar to many other GPCRs, LPH may be linked to ion channels. In addition, in endocrine cells, α-LTX^{N4C} triggers $Ca^{2+}{}_e$-independent release of stored calcium and also activates PKC (Liu et al. 2005; Bittner et al. 2005). Thus, α-LTX can both provide an exocytotic stimulus (by releasing stored Ca^{2+}) and sensitize the secretory apparatus to it (by activating PKC).

4.4.3 PTPσ

Only LTX binding to PTPσ1, but not to PTPσ2, leads to secretory response in β-cells (Lajus and Lang 2006). This indicates that LTX apparently does not evoke any signaling through PTPσ, which probably simply aids toxin pore formation. Indeed, the multimeric toxin must induce the dimerization of PTPσ, leading to

reciprocal inhibition of the catalytic domains (Tonks 2006). Furthermore, while all three receptors are found naturally occurring in pancreatic β-cells, the distribution of PTPσ does not correlate with the endogenous sensitivity of these cells to α-LTX (Lang et al. 1998; Lajus and Lang 2006).

4.4.4 α-LTX Mutants

As α-LTX^{N4C} (see Section 2.3) does not form pores, it probably stimulates exocytosis by receptor mediated signaling. Therefore, the activity of α-LTX^{N4C} has been investigated in Ca^{2+}-dependent and Ca^{2+}-independent exocytosis from neurons and Ca^{2+}-dependent exocytosis from neuroendocrine cells.

In Ca^{2+}-free media, the mutant toxin does not stimulate substantial exocytosis in neurons (Ashton et al. 2001; Volynski et al. 2003; Capogna et al. 2003). This strongly indicates that Ca^{2+}-independent exocytosis, so characteristic of wild-type α-LTX, requires more than receptor activation. However, α-LTX^{N4C}, strongly stimulates transmitter release from neurons in the presence of $Ca^{2+}{}_e$, presumably due to its ability to activate LPH1 (Ashton et al. 2001; Capogna et al. 2003; Volynski et al. 2003). The specific details of how receptor activation triggers exocytosis remain to be established, but it is clear that thapsigargin-sensitive release of $Ca^{2+}{}_i$ plays a pivotal role (Ashton et al. 2001; Capogna et al. 2003).

α-LTX^{N4C} is also able to stimulate Ca^{2+}-dependent exocytosis from endocrine cells: it is active in chromaffin cells in the presence of Ca^{2+} or Sr^{2+} (Volynski et al. 2003) and in pancreatic β-cells (Liu et al. 2005; Lajus and Lang 2006). On the other hand, bacterially expressed α-LTX^{N4C} appears unable to stimulate Ca^{2+}-dependent exocytosis from PC12 cells (Li et al. 2005), but this may be due to mutant's misfolding or by specific characteristics of the PC12 cell line used.

4.4.5 Receptor Knockouts

The genes for all three α-LTX receptors have been knocked out in mice, and these mutations are not lethal. NRX Iα knockout mice show no obvious behavioral phenotype (Geppert et al. 1998). LPH1 knockout causes social problems in mice (Tobaben et al. 2002), consistent with LPH1 involvement in schizophrenia (Chen and Chen 2005). PTPσ knockout leads to developmental abnormalities in the nervous system and outside it (Meathrel et al. 2002; Batt et al. 2002).

In mice lacking the NRX Iα gene, Ca^{2+}-dependent α-LTX binding to brain membranes is inhibited by 60% (Geppert et al. 1998), although the Ca^{2+}-independent binding seems to be affected as well (Geppert et al. 1998). LPH1 knockout leads to the loss of 75% of Ca^{2+}-independent binding of α-LTX but also affects the Ca^{2+}-dependent binding (Tobaben et al. 2002). The NRX-LPH1 double knockout still binds about 25% of α-LTX compared to wild-type mice, both in the presence and absence of Ca^{2+} (Tobaben et al. 2002). Ca^{2+}-dependent release of glutamate induced by wild-type α-LTX in synaptosomes from the NRX Iα, LPH1 and NRX

Iα –LPH1 knockout mice is similarly inhibited, while Ca^{2+}-independent secretion is most grossly affected in the LPH1 single knockout, but not abolished. Surprisingly, the inactivation of both LPH1 and NRX Iα is less detrimental for the toxin-stimulated release than the LPH1 knockout alone (Tobaben et al. 2002). These data suggest that the two α-LTX receptors, NRX Iα and LPH1, may functionally interact (Südhof 2001), e.g., by reciprocally influencing each other's expression or synaptic delivery and, thus, altering the actions of α-LTX. The removal of both NRX Iα and LPH1 from synaptic cleft may allow the pore-forming toxin to access PTPσ more freely, with subsequent membrane insertion and Ca^{2+}-independent effects. It would be interesting to test non-pore-forming α-LTX mutants on neuronal preparations from NRX Iα, LPH1 and PTPσ knockout mice.

5 Overview of Mechanisms

α-LTX causes Ca^{2+}_e-dependent and –independent release of neurotransmitters (Figure 6). Dense core vesicles require Ca^{2+}_e, while synaptic vesicles, containing amino acids or acetylcholine, can be stimulated in its absence. However, in the presence of Ca^{2+}, transmitter is stronger overall due to initially unperturbed vesicle recycling.

All α-LTX actions in biological systems require receptors, which provide binding sites for the toxin on the cell surface. Once the toxin is bound, part of its Ca^{2+}-dependent action is due to pore formation and influx of Ca^{2+}. This mechanism

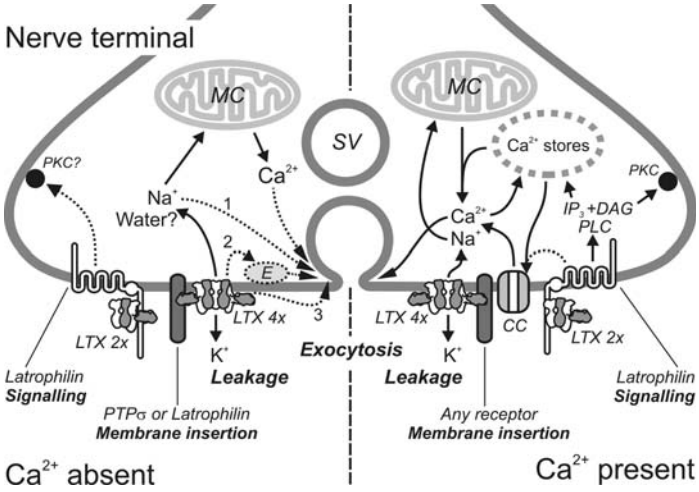

Fig. 6 Diverse mechanisms of α-LTX action. *Right*, Ca^{2+} is present in the medium. The pathways shown are described in the text. CC; Ca^{2+} channels; DAG, diacyl glycerol; LTX 4x, α-LTX tetramers; MC, mitochondria. *Left*, Ca^{2+}-free conditions. For main comments, see text. The possible pathways for Ca^{2+}-independent exocytosis shown include: (1) high concentrations of Na^+ mimicking Ca^{2+}; (2) the internalised domains of α-LTX interacting with components of the exocytotic machinery (E); (3) α-LTX exerting direct fusogenic action.

triggers the release of both readily releasable and reserve pools of vesicles (Ashton et al. 2001). Another action is based on receptor-mediated signaling, which involves stimulation of PLC, production of IP_3 and diacyl glycerol, with respective release of stored Ca^{2+} and activation of PKC. This mechanism, most likely mediated by LPH1, affects readily releasable vesicles only. Both the pore- and receptor-mediated signals can be amplified by the release of $Ca^{2+}{}_i$ and influx of $Ca^{2+}{}_e$, producing Ca^{2+} waves. Mitochondria can contribute to the increase in $[Ca^{2+}]_i$. The pore-mediated component of the Ca^{2+}-dependent action can be blocked by La^{3+}, whereas the receptor-mediated action cannot (Ashton et al. 2001; Capogna et al. 2003).

In the absence of $Ca^{2+}{}_e$, α-LTX only binds to LPH1 and PTPσ. Ca^{2+}-independent exocytosis requires the presence of Mg^{2+} and toxin insertion into the plasma membrane, but these conditions also induce formation of α-LTX channels. Influx of Na^+ and efflux of K^+ through these channels and associated efflux of small molecules and influx/efflux of water may cause secretion. In addition, transmitter release can be caused by membrane perturbation or direct interaction with secretory machinery. Some secretion may be nonvesicular. Receptor-mediated signaling can cause the activation of PKC in some cells. However, Ca^{2+}-independent release is blocked by La^{3+}, indicating that toxin pores play a crucial role in this release.

Finally, although α-LTX receptors (at least LPH1) can transduce a signal, it is obvious that this signaling cannot explain all the effects of α-LTX. So, while the use of the toxin has brought about the discovery of neuronal cell adhesion protein families (NRX and LPH) and new knowledge about PTPσ, the toxin's effect in the absence of $Ca^{2+}{}_e$ remains unclear, and its study may throw light onto the very basic mechanisms of exocytosis and different levels of its regulation.

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Presynaptic Signaling by Heterotrimeric G-Proteins

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Abstract G-proteins (guanine nucleotide-binding proteins) are membrane-attached proteins composed of three subunits, α, β, and γ. They transduce signals from G-protein coupled receptors (GPCRs) to target effector proteins. The agonist-activated receptor induces a conformational change in the G-protein trimer so that the α-subunit binds GTP in exchange for GDP and α-GTP, and βγ-subunits separate to interact with the target effector. Effector-interaction is terminated by the α-subunit GTPase activity, whereby bound GTP is hydrolyzed to GDP. This is accelerated in situ by RGS proteins, acting as GTPase-activating proteins (GAPs). Gα-GDP and Gβγ then reassociate to form the Gαβγ trimer. G-proteins primarily involved in the modulation of neurotransmitter release are G_o, G_q and G_s. G_o mediates the widespread presynaptic auto-inhibitory effect of many neurotransmitters (e.g., via M2/M4 muscarinic receptors, α_2 adrenoreceptors, μ/δ opioid receptors, $GABA_B$ receptors). The G_o βγ-subunit acts in two ways: first, and most ubiquitously, by direct binding to Ca_V2 Ca^{2+} channels, resulting in a reduced

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sensitivity to membrane depolarization and reduced Ca^{2+} influx during the terminal action potential; and second, through a direct inhibitory effect on the transmitter release machinery, by binding to proteins of the SNARE complex. G_s and G_q are mainly responsible for receptor-mediated facilitatory effects, through activation of target enzymes (adenylate cyclase, AC and phospholipase-C, PLC respectively) by the GTP-bound α-subunits. AC generates cyclic AMP which activates protein kinase A (PKA) and PLC hydrolyzes membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) to form diacylglycerol (DAG) to activate protein kinase C (PKC). PKC phosphorylates Ca^{2+} channel proteins to oppose G_oβγ-mediated inhibition, and both PKA and PKC phosphorylate various components of the release machinery to enhance exocytosis. cAMP and DAG can themselves facilitate release by direct, phosphorylation-independent, regulation through second messenger binding proteins, including cAMP-GEFs and Munc-13 (DAG-binding). Finally, membrane levels of PIP_2 play a signaling role throughout the stimulus-secretion cascade.

1 Overview of G Protein Signaling

Heterotrimeric G-proteins are guanine nucleotide-binding membrane-associated proteins that directly intermediate between the G-protein-coupled (heptahelical) receptor and the target effector protein. They are composed of α, β and γ subunits. The trimer is anchored in the membrane via palmitoyl or myristoyl fatty acids at the N-terminus of the α subunit and a prenyl moiety at the C-terminus of the γ-subunit (see Gilman 1987; Oldham and Hamm 2006 for details).

The basic cycle of G-protein activation and inactivation is illustrated in Figure 1. In the basal state (1) the α-subunit of the trimer binds guanosine diphosphate (GDP). On activation by agonist (Ag) the receptor "docks on" to the G-protein (2) and induces a conformational change in the latter that exposes a high-affinity guanosine triphosphate (GTP) binding site on the α-subunit. Since the concentration of GTP in the cytosol exceeds that of GDP, this promotes a GTP:GDP nucleotide exchange (the rate-limiting process for activation). This conformational change also induces a partial (see below) or complete dissociation of the α and βγ subunits, though both usually stay anchored in the membrane (except the α-subunit of rod transducin) (Calvert et al. 2006; Rosenzweig et al. 2007). The α and βγ subunits then separately (or sometimes conjointly) interact with one or more effector proteins, to activate or inhibit them (3). The cycle is completed by the hydrolysis of α-attached GTP to GDP by the GTPase activity of the α-subunit; the GDP-bound α-subunit then reassociates with the βγ-subunit to restore the ground state. Clear dissociation of α and βγ subunits occurs in the visual transduction pathway, and has also been shown for the G_o trimer (Tesmer et al. 2005); however some evidence (Bunemann et al. 2003; Gales et al. 2006; Levitzki and Klein 2002; Rosenzweig et al. 2007) suggests that the α and βγ subunits do not necessarily dissociate in other systems but stay

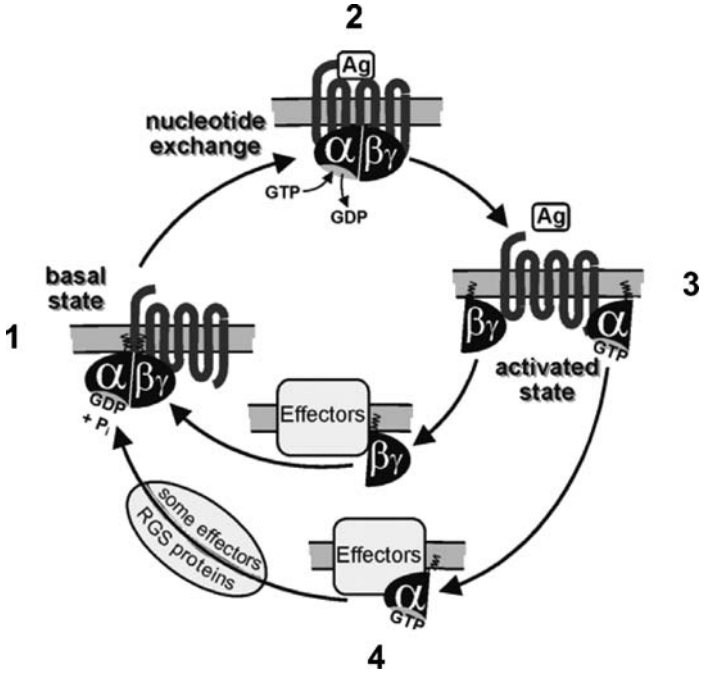

Fig. 1 Diagram of G-protein cycle following receptor activation. See text for description. Ag = agonist. (Adapted from Wettschureck & Offermanns 2005, Physiol Rev 85:1159–1204, with permission of the American Physiological Society).

conjoined and partially separate to open up a pathway for rapid GDP exit from the trimer (Figure 2). Dissociation or otherwise may be receptor-dependent (Digby et al. 2006).

Hydrolysis of GTP is frequently accelerated by GTPase-activating proteins (GAPs). There are two classes of GAPs – effector proteins (e.g., phospholipase Cβ1 (PLCβ1) for G_q; phosphodiesterase-γ (PDEγ) for G_t and adenylate cyclase V (ACV) for G_s); and, more ubiquitously, RGS proteins (Regulators of G-protein Signaling; see further below). Several other ancillary proteins can also modify G-protein activity, either by accelerating GDP:GTP exchange (guanosine nucleotide exchange factors, GEFs) or by inhibiting GDP release (GDI proteins) (Sato et al. 2006).

1.1 Types of G-Protein

Ignoring splice-variants, 16 species of α-subunit have been described, plus 5 species of β-subunit and 12 species of γ-subunit (see Table 2 in Wettschureck and Offermanns 2005). Trimeric G-proteins are characterized by their α-subunit, primarily in

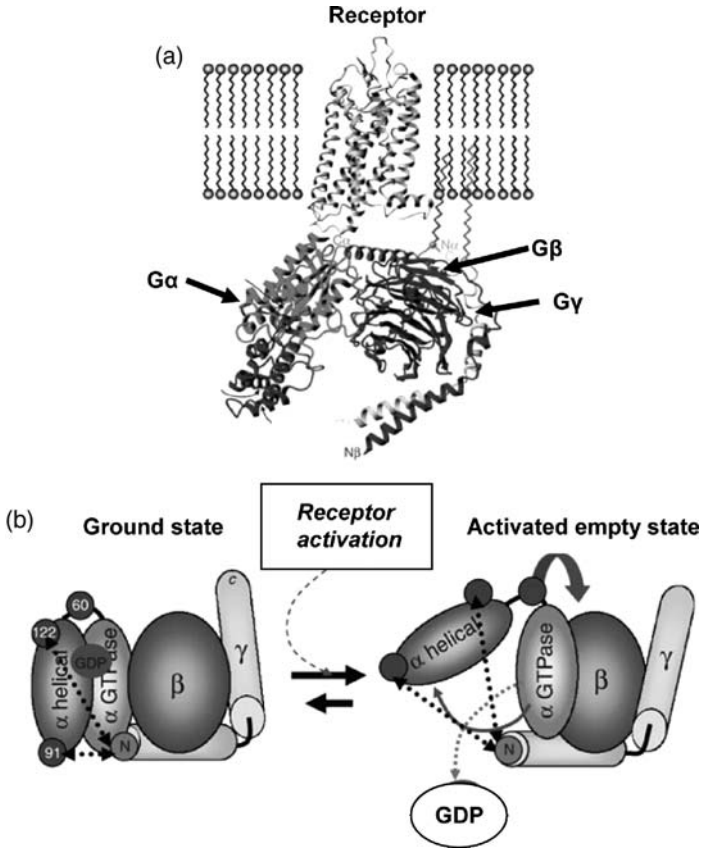

Fig. 2 (**a**) Projection diagram of the complex between a receptor (rhodopsin) and a G-protein trimer (G_i). (**b**) Schematic of the structural rearrangement of the G-protein following receptor activation. Opening of the Gα GTPase and helical domains creates an exit route for GDP. (Adapted from Gales et al. 2006, Nat Struct Mol Biol 13:778–786, with permission from Macmillan Publishers, Ltd.).

terms of their principal effectors and sensitivity to *Pertussis toxin* (Ptx, which ADP-ribosylates a C-terminal cysteine present in certain α-subunits and prevents receptor coupling):

(1) G_s/G_{olf}-activate adenylate cyclase; not inhibited by Ptx;
(2) G_i/G_o – inhibit adenylate cyclase and/or modulate certain Ca^{2+} and K^+ channels (via βγ-subunits); inhibited by Ptx;
(3) G_q/G_{11}, G_{14}, $G_{15/16}$-activate PLCβ (β1,3,4); not inhibited by Ptx;
(4) $G_{12/13}$–multiple downstream targets; not inhibited by Ptx.

Those most relevant to the regulation of transmitter release are G_s, G_i, G_o and G_q/G_11 (and potentially G_{12}/G_{13}); these are widely distributed in the nervous system and are present in nerve terminals. Intraterminal localization of G-protein an-

tibodies has been studied in large calyciform nerve endings in chick and mammal. Isolated calyx terminals stain strongly for G_o/G_i (e.g., Mirotznik et al. 2000); G_o co-localizes therein with $Ca_V2.2$ Ca^{2+} channels (Li et al. 2004). In the mammalian calyx of Held, G_o is most abundant and co-localizes with synapsin, whereas G_i1-3 are predominantly postsynaptic (Kajikawa et al. 2001).

1.2 Receptor Coupling and Selectivity

Upon receptor activation, the GPCR undergoes a conformational change that opens up a pocket on its inner face; the C-terminus of the G-protein α-subunit then binds within this pocket (Bourne 1997; Oldham and Hamm 2006). Thus, the Gα-subunit C-terminus forms the primary determinant of receptor–G-protein selectivity. For example, substitution of the terminal five amino acids from $G\alpha_i$ or $G\alpha_o$ into $G\alpha_q$ enables the α_2-adrenoceptor (which normally couples to $G\alpha_i$ or $G\alpha_o$) to activate G_q and stimulate PLC (Conklin et al. 1993). However, the Gβγ-subunits also contribute to binding, and can modify selectivity. For example, in GH3 pituitary tumor cells, activation of M4 muscarinic receptors preferentially inhibits the Ca^{2+} current via $G\alpha_o\beta(3)\gamma(4)$, whereas somatostatin uses $G\alpha_o\beta(1)\gamma(3)$ for the same effector (Schneider et al. 1997). Although receptor–G-protein coupling is normally interpreted along diffusion-limited collision-coupling lines (e.g., Lamb and Pugh 1992), there is some old (Rimon et al. 1978) and more recent (Gales et al. 2006; Nobles et al. 2005) evidence suggesting some degree of precoupling between inactive receptors and G-proteins (but see also Hein et al. 2005; Lober et al. 2006). Certainly, some form of restricted access is required to explain why, for example, M2 and M4 muscarinic acetylcholine receptors, which readily couple to both G_i and G_o G-proteins in reconstituted systems, selectively activate only G_i or G_o, respectively, in sympathetic neurons (Fernandez-Fernandez et al. 1999; Fernandez-Fernandez et al. 2001), since the density of both G-proteins in these neurons is likely to exceed that of the receptors by an order of magnitude.

1.3 G-Protein–Effector Coupling

As with receptor coupling, specificity of effector protein coupling is primarily determined by the α-subunit. Indeed, where the α-subunit itself acts as the messenger, effector selection is highly specific, and the three-dimensional structures for α-subunit–effector complexes have been solved for $G\alpha_s$–adenylate cyclase, $G\alpha_t$–phosphodiesterase-γ (PDEγ)–RGS9, and $G\alpha_q$–receptor kinase 2 (GRK2)–Gβγ (see Oldham and Hamm 2006). In contrast, there is relatively little selectivity among different β or γ subunits when tested as free β and/or γ combinations on effectors that respond to Gβγ such as $K_{ir}3.1/3.4$ (GIRK) channels (Wickman and Clapham

1995) and $Ca_V2.2$ Ca^{2+} channels (Garcia et al. 1998; Jeong and Ikeda, 2000a; Ruiz-Velasco and Ikeda 2000; Zhou et al. 2000); such selectivity as occurs appears to depend on the β-subunit (Garcia et al. 1998; Ruiz-Velasco and Ikeda 2000). Notwithstanding, Gβγ-responsive effectors do not normally respond to βγ-subunits released indiscriminately from different G-protein trimers but only from receptor-specific αβγ-trimers. Thus, in sympathetic neurons, while $K_{ir}3.1/3.2$ channels can be activated by βγ-subunits associated with either $G\alpha_i$ or $G\alpha_o$ when these two α-subunits are overexpressed, under physiological conditions they can only be activated via $G\alpha_i$, whereas $Ca_V2.2$ channels are only inhibited by βγ-subunits associated with $G\alpha_o$ (Fernandez-Fernandez et al. 2001). Indeed, $K_{ir}3$ channels seem to show a unique response to different receptor–G-protein combinations (Benians et al. 2005). Further, the α-subunit of G_i can directly interact with the channel to modify its response to Gβγ (Peleg et al. 2002). These effects may be best explained in terms of a receptor–G-protein–effector ternary complex (Clancy et al. 2005; Fowler et al. 2007; Huang et al. 1995) with restricted dissociation of the Gαβγ trimer (see above). There may be similar G-protein–effector complexes involving Ca^{2+} channels since these closely co-localize with G_o in chick calyx terminals (Li et al. 2004) and also for G_q – G-protein receptor kinase2 (GRK2) (Tesmer et al. 2005).

1.4 Rates of G-Protein–Mediated Events

G-protein-mediated responses can be quite rapid. The minimal latency for physiological Gβγ activation of $K_{ir}3$ channels by a GPCR (e.g., activation of GIRK channels by M2 muscarinic receptors in cardiac sinoatrial node cells) is around 30–50 msec, with an onset time-constant for channel opening of ∼250 msec and an offset time-constant of ∼800 msec (Trautwein et al. 1980). Similar values have been obtained for the GPCR-activation of $K_{ir}3$ channels in neurons (α_2-adrenoceptors on enteric neurons: latency 33 msec after afferent adrenergic nerve stimulation; rise time 85 msec; decay time-constant 290 msec [Surprenant and North 1988]; $GABA_B$ receptors on hippocampal neurons: latency 29 msec; rise-time ∼110 msec; duration ∼723 msec [Davies et al. 1990]). Corresponding values for Gβγ-mediated α_2-adrenergic inhibition of $Ca_V2.2$ channels in sympathetic neurons are ∼10 msec latency, 390 msec onset time-constant and ∼5.6 sec offset time-constant (Zhou et al. 2000). Agonist-receptor binding appears not to be rate-limiting; instead, the rate-limiting step is the activation of the G-protein since the time-constant for free Gβγ–calcium channel interaction (measured by the time for reinhibition by the Gβγ-subunits following their dissociation from the channel by a large voltage step) was <100 msec (Zhou et al. 2000). Other measures of reinhibition kinetics following adrenergic inhibition in these neurons have yielded values of 103 msec (Ehrlich and Elmslie 1995) and 37 msec (Delmas et al. 1998b). The $GABA_B$-agonist baclofen inhibits calcium currents in cultured hippocampal neurons with comparable kinetics (delay ∼80 msec; onset time-constant 220 msec; offset time-constant 2.2 sec (Pfrieger et al. 1994). This was virtually identical to the kinetics

for baclofen-induced inhibition of excitatory synaptic transmission in this preparation (delay ~80 msec; onset time-constant 170 msec; offset time-constant 3.4 sec (Pfrieger et al. 1994). As pointed out by Zhou et al. (2000), such rapid-onset kinetics require that the receptors, G-proteins, and ion channel effectors be within $<1\,\mu m$ of each other. Indeed, there is a close co-localization of $GABA_B$ receptors and $K_{ir}3.2$ channels in hippocampal neurons (Kulik et al. 2006), and FRET analysis indicates an association between expressed $GABA_B$ receptors, K_{ir} channels and RGS4 in HEK293 cells (Fowler et al. 2007). Also, as noted above, Ca^{2+} channels and G_o show a close co-localization at nerve terminals (Li et al. 2004), which may facilitate their rapid inhibition.

The offset rate is determined, in the first instance, by the GTPase activity of the α-subunit. In situ, this is accelerated by GTPase-activating proteins (GAPs) – sometimes by the effector itself, but more widely by RGS proteins (see further below). Thus, when $K_{ir}3.1 + 3.4$ channels are reconstituted in CHO cells with M2-muscarinic receptors, the offset of the current following removal of agonist (acetylcholine) is >10 times slower that that of the natural cardiac current, but is accelerated to match the native current kinetics on co-expressing RGS4 (Doupnik et al. 1997); similar effects on dopamine D2-receptor activation of $K_{ir}3.1 + 3.2$ channels in oocytes were observed following co-expression of RGS8 (Saitoh et al. 1997). In fact, as noted above, RGS4 appears to participate in a preassembled signaling complex with $K_{ir}3$ channels (see also Fujita et al. 2000; Tinker 2006). RGS proteins also accelerate recovery of Ca^{2+} channels from G-protein-mediated inhibition (Ding et al. 2006; Jeong and Ikeda, 2000b; Mark et al. 2000; Melliti et al. 2001) and – importantly – accelerate recovery from the adenosine-mediated presynaptic inhibition at hippocampal synapses that results from Ca^{2+} current inhibition (Chen and Lambert 2000) (see further below). As with K_{ir} channels, individual RGS proteins may form signaling complexes with Ca^{2+} channels (Richman et al. 2005). However, some RGS proteins are principally cytoplasmic or nuclear and only translocate to the membrane on activating the cognate G-protein, where they bind to the activated α-subunit (Druey et al. 1998; Heximer et al. 2001; Masuho et al. 2004; Saitoh et al. 2001).

1.5 Further Notes on RGS Proteins

RGS proteins (see Abramow-Newerly et al. 2006; De Vries et al. 2000; Druey 2001; Neitzel and Hepler 2006; Tinker 2006; Willars 2006 for reviews) comprise a wide and rather diverse family, with 30+ members, but with a common 120-residue RGS domain that enables binding to the G-protein α-subunit. The effect of this is to lower the energy requirement for GTP hydrolysis and hence to accelerate the GTPase activity of the α-subunit. This is reflected in the faster offset of Gα or Gβγ-mediated effector responses noted above. Because the effective dissociation equilibrium constant for G-protein–effector interaction (K_{diss}) is determined by $k_{off}/k_{on} \times G^*$ (where G^* is the concentration of activated G-protein), RGS-accelerated GTPase activity reduces effector response to agonist, effectively

"antagonizing" agonist-action. For example, expression of an RGS-insensitive $G_o\alpha$ mutant shifts the concentration-response curve for the inhibition of the Ca^{2+} current in sympathetic neurons to noradrenaline by ~tenfold to the left (Jeong and Ikeda, 2000b), implying that the endogenous RGS proteins in these cells reduce noradrenaline sensitivity by an equivalent amount. More importantly, perhaps, RGS proteins also protect the release process from *tonic* G-protein-mediated inhibition that can arise as a result of the spontaneous turnover of the G_o trimer in the absence of GPCR activation (Han et al. 2006). However, superimposed on this uniform GAP activity, RGS proteins show a number of other properties.

1.5.1 Note 1

RGS proteins also accelerate the onset kinetics of G_i-mediated $K_{ir}3$ activation (e.g., Doupnik et al. 1997; Saitoh et al. 1997) and G_o-mediated Ca^{2+} channel inhibition (e.g., Jeong and Ikeda 2000b). This is not predicted from their GAP activity; and indeed, in RGS8, accelerated $K_{ir}3$ activation is driven by a different (amino-terminal) domain from the RGS GAP-domain that determines deactivation (Jeong and Ikeda 2001). This may result from the additional binding of the RGS proteins with the effector ($K_{ir}3$ [Fujita et al. 2000] or $Ca_V2.2$ [Richman et al. 2005]), effectively reducing the diffusion time required for $G\beta\gamma$ to interact with the channel after GTP/GDP exchange on the α-subunit.

1.5.2 Note 2

The binding of RGS proteins to G-protein α-subunits can hinder binding of effectors to the activated α-subunit, and hence antagonize $G\alpha$ (GTP)–effector coupling independently of any GAP activity (e.g., Anger et al. 2004; Hepler et al. 1997; Yan et al. 1997). By this mechanism RGS2 acts as a somewhat specific antagonist to $G\alpha_q$ (GTP)-activated phospholipase C (Hao et al. 2006; Heximer et al. 1997, 1999) – a useful property in view of the absence of Gq-inactivating toxins. Thus, RGS2 expression prevents the inhibition of the M-type (Kv7.2/7.3) K^+ current and the "slow" Pertussis (Ptx) toxin-insensitive inhibition of the N-type $(Ca_V2.2)Ca^{2+}$ current produced by expressed metabotropic mGluR1 glutamate receptors in sympathetic neurons (Kammermeier and Ikeda 1999) and the inhibition of expressed $Ca_V2.2$ calcium channels by M1-muscarinic acetylcholine receptors (Melliti et al. 2001). It also reduces the inhibitory effect of P2Y1 nucleotide receptors on expressed $K_{ir}3.1/3.2$ currents in sympathetic neurons (Filippov et al. 2004).

1.5.3 Note 3

RGS proteins can also modify GPCR–G-protein coupling in an agonist-selective manner. For example, in pancreatic acinar cells, RGS4 inhibits muscarinic signaling

more strongly than that of bombesin or cholecystokinin (Xu et al. 1999b). Of more relevance to presynaptic regulation, RGS9-2 reduces dopamine-D2 inhibition of Ca^{2+} currents without effect on M2-muscarinic inhibition (Cabrera-Vera et al. 2004). Like effector antagonism, GPCR regulation appears to be determined by the N-terminal region (Saitoh et al. 2002), so is dissociated from GAP activity or Gα-binding. This confers an ability to bind directly to the third inner loop of selective GPCRs, e.g., M1-muscarinic receptors (Bernstein et al. 2004), and α_{1B}-adrenoceptors (Wang et al. 2005). However, binding may also be facilitated by other scaffolding proteins (see Neitzel and Hepler 2006; Willars 2006), and it has been suggested that the selectivity of RGS–receptor–G-protein modulation may depend on the formation of a ternary complex with the RGS protein, GPCR, and Gα subunit (Benians et al. 2005).

1.5.4 Note 4

Importantly, RGS protein expression is subject to variable physiological and pathological regulation (Willars 2006). Thus, the modulatory effects of RGS proteins on G-protein kinetics, and on GPCR–G-protein and G-protein–effector coupling discussed above may be subject to substantial variation. One example: upregulation of RGS4 in the striatum following dopamine depletion compromises M4-muscarinic autoregulation of acetylcholine release (Ding et al. 2006), which may be relevant to Parkinson's disease.

2 G-Proteins and Transmitter Release

Here we consider the role of G-proteins in regulating transmitter release. We divide it into two stages – effects mediated through changes in Ca^{2+} entry, and effects on the release process downstream from Ca^{2+} entry (see Figure 3)

2.1 Effects on Ca^{2+} *Entry*

This divides into two parts – direct effects on presynaptic Ca^{2+} currents, and indirect effects mediated through changes in presynaptic nerve ending properties.

2.1.1 Direct Effects on Ca^{2+} Channels

The Ca^{2+} charge required for transmitter release results principally from Ca^{2+} influx through N-, P and/or Q-type Ca^{2+} channels ($Ca_V2.2$, $Ca_V2.1$, and $Ca_V2.3$ channels, respectively). The modulation of these channels by G-proteins has been

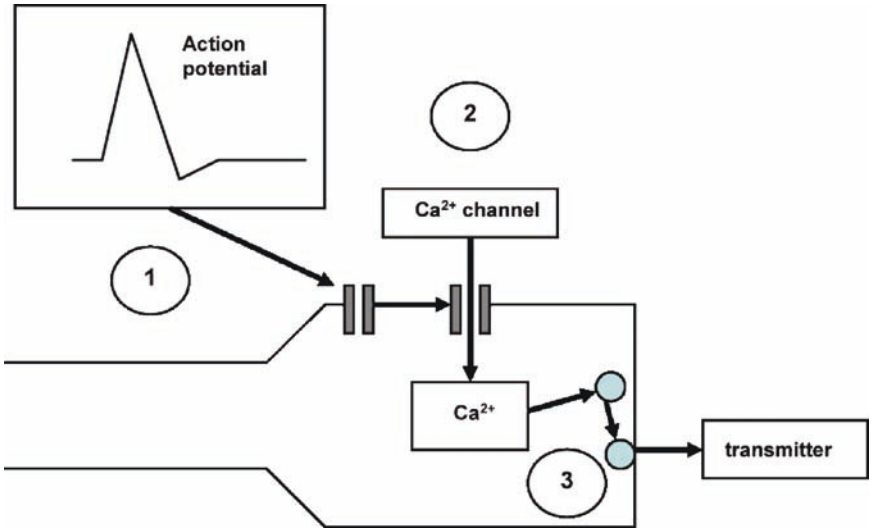

Fig. 3 Some sites for modulation of transmitter release at nerve endings. (1) Invading action potential. (2) Ca^{2+} channel / Ca^{2+} influx. (3) Release mechanism.

studied intensively in both reconstituted systems and in some primary neuron somata, most notably sympathetic and sensory neurons (see DeWaard et al. 2005; Dolphin 2003; Strock and Diverse-Pierluissi 2004; Tedford and Zamponi 2006 and chapter by Zamponi, this volume). Effects on these different channel types are qualitatively similar, though differing quantitatively. Effects of GPCRs or G-protein activation on Ca^{2+} channels in nerve endings have been studied directly in the large calyciform endings in the chick ciliary ganglion (Mirotznik et al. 2000; Yawo and Chuhma 1993) and in the calyx of Held in the mammalian trapezoid body (Kajikawa et al. 2001; Takahashi et al. 1996, 1998), and inferred indirectly from biophysical release parameters or biochemical studies (see below) in other preparations.

G-Protein-Mediated Ca^{2+} Channel Inhibition

Two principal forms of inhibition have been described: (1) "fast" voltage-dependent inhibition, and (2) 'slow" voltage-independent inhibition; the former is sensitive to Ptx, the latter (normally) not (Hille 1994) (see Figure 4). The former is overwhelmingly the most important and widespread, and most pertinent to the feedback auto-inhibition of transmitter release by neurotransmitters.

Fast voltage-dependent inhibition

Voltage-dependent inhibition of neuronal Ca^{2+} currents was first reported by Marchetti et al. (1986) and Tsunoo et al. (1986). It results from the binding of

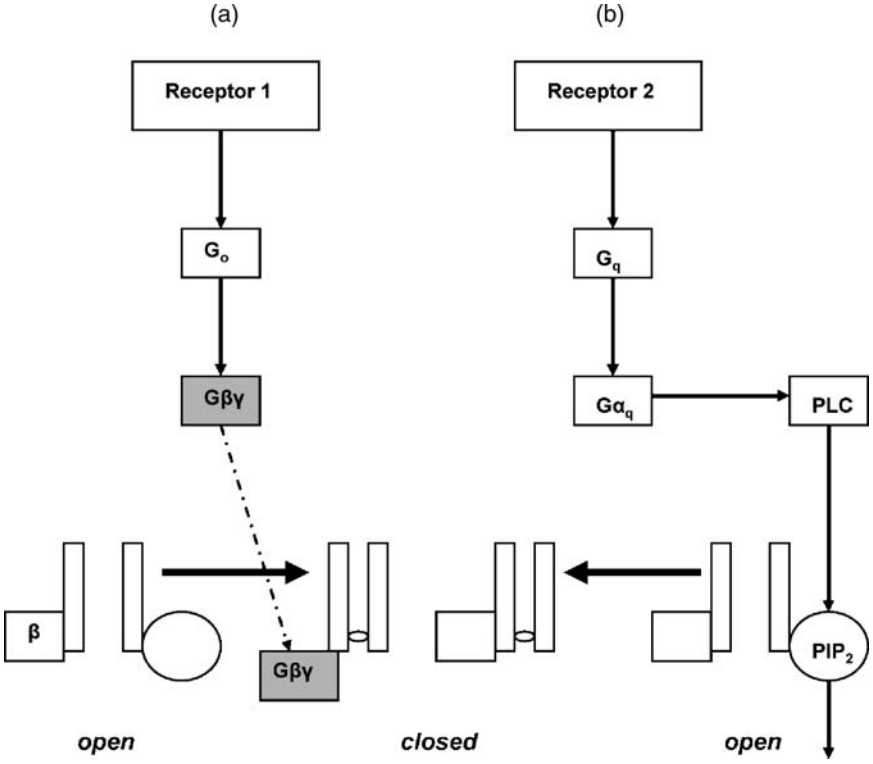

Fig. 4 Two mechanisms for G-protein inhibition of Ca^{2+} channels. (**a**) Gβγ subunits from activated G_oαβγ bind to channel and displace channel β-subunit, thereby reducing channel sensitivity to membrane depolarization. (**b**) Activated $Gα_q$ activates phospholipase C (PLC) so hydrolyzing membrane phosphatidylinositol-4,5-bisphosphate (PIP_2), normally required to stabilize the channel open state.

Gβy-subunits to the $α_1$-subunit of the Ca_V channel (see Tedford and Zamponi 2006). Inhibition is fast (latency $<$100 msec) because of close apposition of G-protein and Ca^{2+}-channel (see above). Voltage-dependence arises because depolarization favors the dissociation of the βγ-subunits from the channel, whereas hyperpolarization favors their association (they are "reluctant" to open at hyperpolarized potentials but become more "willing" at depolarized potentials) (Bean 1989; Kasai and Aosaki 1989; Patil et al. 1996). This voltage-dependence has the important physiological consequence that, during continuous GPCR-induced inhibition, the inhibited Ca^{2+}-current shows a frequency-dependent recovery (Park and Dunlap 1998), with a consequent partial recovery of synaptic transmission (Brody and Yue 2000). Voltage-dependence is obtunded in a splice-variant of Ca_V2.2channels expressing a tyrosine-phosphorylatable form of exon 37 that is expressed in a subpopulation of sensory neurons, so that their response to GPCR-inhibition is more sustained (Raingo et al. 2007).

There is relatively limited selectivity among different βγ-subunits for $Ca_V2.2$ interaction: under different circumstances, βγ-subunits associated with G_o, G_i, G_s, G_q and G_z are capable of inducing voltage-dependent inhibition (see Tedford and Zamponi 2006), but under normal conditions, GPCR-induced inhibition is primarily mediated by βγ-subunits associated with G_o (Delmas et al. 1999; Fernandez-Fernandez et al. 2001). (βγ-subunits associated with $G\alpha_i$ can also contribute to fast noradrenergic inhibition of Ca^{2+} current in both chick embryonic (Diverse-Pierluissi et al. 1995) and rat (Delmas et al. 1999) sympathetic neurons but this component is not voltage-sensitive so is mediated by a different mechanism to that produced by G_o-derived βγ-subunits.)

Slow voltage-independent inhibition

Receptors that couple to G_q, such as M1-muscarinic receptors, initiate a slower, voltage-independent inhibition of Cav2.2 currents (Beech et al. 1992; Hille 1994). This is mediated by the α-subunit of G_q, not the βγ-subunit (Delmas et al. 1998a; Haley et al. 2000). It results primarily from the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by $G\alpha_q$-activated phospholipase-C and the consequent reduction of membrane PIP_2 (Gamper et al. 2004), which is required for Ca_V2 channel opening (see Delmas et al. 2005; Wu et al. 2002). Thus, the "slowness" compared with $G_o\beta\gamma$-mediated inhibition results in the first instance from the presence of an intermediate enzymatic step, while recovery is determined by the rate of PIP_2 resynthesis rather than the GTPase activity of $G\alpha_q$ (Suh et al. 2004). The time-constant for M1-receptor inhibition is ∼14 seconds (Beech et al. 1992), which probably reflects the time required for PIP_2 to fall to a sufficient level to cause channel closure (Suh et al. 2004). However, the latency to onset of inhibition following a pulse-application of agonist may be only around 250 msec or so, as judged from the latency to closure of PIP_2-activated M-type $(K_V7.2/7.3)K^+$ channels following synaptically-released acetylcholine (Brown et al. 1995) – i.e., about 10 times longer than that for Gβγ-mediated inhibition. Other mechanisms further downstream from PIP_2 hydrolysis, such as the production of arachidonic acid from diacylglycerols, formed from PIP_2 hydrolysis (see Delmas et al. 2005) or phosphorylation processes (Strock and Diverse-Pierluissi 2004), may also contribute to G_q-mediated Ca^{2+}-current reduction, though the latter would be on a much slower time-scale than PIP_2-depletion.

The mechanism of Ca-current inhibition at nerve endings

Which (if either) of these processes occurs at nerve endings? Direct recording from mammalian trapezoid calyces of Held, which possess primarily $Ca_V2.1$ (P/Q-type) Ca^{2+} channels (Schneggenburger and Forsythe 2006; Takahashi et al. 1996), revealed that the $GABA_B$ agonist, baclofen, strongly reduced the Ca^{2+} current in a voltage-dependent manner (Takahashi et al. 1998) and that this was imitated, and occluded, by intracellular delivery of free Gβγ-subunits (Kajikawa et al. 2001). Further, baclofen totally occluded the effect of stimulating the endogenous

metabotropic mGluR4 and mGluR7 receptors present at these terminals (Kajikawa et al. 2001). Stimulation of the adenosine A1 receptor produced similar (though weaker) effects, which were also occluded by baclofen (Kimura et al. 2003). Thus, these terminals show the same direct Gβγ-mediated inhibition in response to GPCR stimulation described in (2.1.1) above, with apparently no evidence for the slower G_q-mediated slow inhibition in (2.1.1). The same appears to be true for the calyx endings in the chick ciliary ganglion, even though they appear to contain other G-proteins α-subunits such as α_s, $\alpha_{q/11}$. $\alpha_{12/13}$ and α_z, which might be expected to couple through different pathways (Mirotznik et al. 2000). Recently, direct recordings from mossy fiber terminals in the hippocampus have revealed a very similar action of adenosine, acting on A1 receptors (Gundlfinger et al. 2007): Ca^{2+} current inhibition (of N and P/Q-type currents) showed characteristic Gβγ-type voltage-dependence, and the reduced Ca^{2+} influx was sufficient to account for the reduced transmitter release.

This may well apply to sympathetic nerve terminals, since the effects of stimulating adrenergic, muscarinic, somatostatin, PGE, and secretin receptors on the release of noradrenaline from the processes of cultured sympathetic neurons evoked by a brief pulse of K^+ (recorded amperometrically) were all blocked by Ptx (Koh and Hille 1997). Hence, the slow Ptx-insensitive G_q-mediated form of inhibition recorded in the soma of these cells on stimulating muscarinic (M1) receptors (2.1.1) did not appear to contribute significantly to muscarinic inhibition of release from their processes. Noradrenergic inhibition of transmitter release in these terminals is clearly due to Gβγ-mediated Ca^{2+} current inhibition since, in autaptic connexion made in culture, transmission was blocked by instillation of Gβγ-subunits down the processes and noradrenergic inhibition was prevented by expressing the Gβγ-sequestering protein, transducin (Stephens and Mochida 2005). Further evidence that this form of Ca^{2+}-current inhibition underlies the presynaptic inhibitory action of many G_o/G_i-coupled receptors derives from experiments showing a clear reduction in intra-terminal Ca^{2+} transients at both peripheral and central synapses (Dittman and Regehr 1996; Qian et al. 1997; e.g., Toth et al. 1993; Wu and Saggau 1994, 1995).

Nevertheless, several GPCRs that couple to G_q rather than to Ptx-sensitive G_o G-proteins are clearly able to reduce transmitter release (see, e.g., Boehm and Kubista 2002; Kubista and Boehm 2006; Miller 1998). In one case at least – that of bradykinin – it seems highly probable that this results from an inhibition of the Ca^{2+}-current by the PIP_2-depletion mechanism referred to in (**??**) above (cf. Gamper et al. 2004; Lechner et al. 2005). However, it appears from these experiments that the effects of bradykinin on Ca^{2+} currents – and possibly other G_q-coupled receptors such as P2Y nucleotide receptors – may vary depending on the resting amount of PIP_2 in the membrane and the degree to which they are able to deplete PIP_2 to a level that results in significant Ca^{2+} channel closure (Brown et al. 2007); this might vary between different neurons or under different experimental conditions.

In sum: fast, voltage-dependent Gβγ-mediated Ca^2+ current inhibition triggered by activation of (primarily) G_o makes a major and widespread contribution

to the presynaptic inhibitory and auto-inhibitory effects of neurotransmitters. In contrast, the contribution of the "slow," voltage-independent inhibition produced by Gq-coupled receptors appears more variable. Nevertheless, for both forms of GPCR – G-protein pairing, additional effects downstream of the $Ca^{2}+$ channel may also be significant (see further below).

G-Protein-Mediated Ca^{2+} Channel Enhancement

Effects mediated by protein kinase C (PKC)

Stimulation of G_q-coupled receptors can also enhance Ca_V2 channel activity (e.g., mGluR1 Stea et al. 1995, M1, M3, M5-muscarinic receptors Bannister et al. 2004). This is mediated, not through a direct interaction of $G\alpha_q$ or $\beta\gamma$ subunits with the channel but through the formation of diacylglycerol by PLC hydrolysis and activation of PKC (Bannister et al. 2004). It results from the PKC-mediated phosphorylation of two specific serine and threonine residues in the I-II linker of the $Ca_V2\alpha_1$-subunit; the specific subtype of PKC involved is PKCε, which is anchored to the C-terminus of $Ca_V2.2$ channels through a PKC-binding protein (see Tedford and Zamponi 2006). The principal effect of this is to antagonize Gβγ-induced inhibition, so providing for an interaction between G_q- and G_o-coupled receptors (Cox et al. 2000; Queiroz et al. 2003; Gonçalves and Queiroz, this book). This may itself result in overt enhancement of resting currents because the latter are subject to a varying degree of tonic Gβγ-inhibition as a result of the spontaneous G_o trimer dissociation, but an enhancement of noninhibited currents by PKC has also been noted (Bannister et al. 2004; Stea et al. 1995), probably as a result of dual phosphorylation of the two residues (Hamid et al. 1999). Such effects may underly the enhancement of noradrenaline release from sympathetic neurons by bradykinin, which also involves a Ca^{2+}-independent isoform of PKC (Scholze et al. 2002).

Effects mediated by protein kinase A (PKA)

The principal target for cAMP produced by GPCR activation of AC is the cognate cAMP-dependent protein kinase (PKA), which can alter presynaptic function through protein phosphorylation of multiple substrates at the level of nerve terminal excitability and Ca^{2+}-influx, and at loci downstream of Ca^{2+}-entry. Evidence for regulation at proximal loci comes from synaptosomal studies showing that PKA-mediated phosphorylation underlies the β-adrenoreceptor-stimulated facilitation of glutamate release from isolated nerve terminals (synaptosomes), an effect mimicked by cAMP phosphodiesterase inhibition (Herrero and Sanchez-Prieto 1996; Wang et al. 2002; Wang and Sihra 2003). This facilitation of neurotransmitter release is attributable to an increase in voltage-dependent Ca^{2+}-influx through P/Q- and N-type channels (Millan and Sanchez-Prieto 2002). The presynaptic enhancement of P-type Ca^{2+}-currents by β-adrenoreceptor activation has also been observed in acutely isolated amygdala neurons (Huang et al. 1996, 1998). Additionally, AC/PKA activation

results in an abrogation of the effects of inhibitory GPCRs, e.g., $5HT_{1A}$ (Wang et al. 2002) and adenosine A1 receptors, in synaptosomes (Wang and Sihra 2003). These latter findings reflect a feedback regulation of Gβγ-mediated inhibition by indirect second-messenger-driven signaling in presynaptic terminals. Conversely, inhibitory mGluRs (mGluR7) stimulated by L-AP4 and negatively coupled to AC suppress glutamate release by decreasing voltage-dependent Ca^{2+}-entry (Millan et al. 2002, 2003). In addition to these proximal targets for GPCR/cAMP/PKA signaling, several key loci are posited distal to Ca^{2+}-entry (section 2.2).

2.1.2 Indirect Effects on Ca^{2+} Entry through Changes in Presynaptic Excitability or Action Potential Waveform?

The integral of the presynaptic Ca^{2+} current (and hence amount of Ca^{2+} entry) is strongly dependent on the amplitude and width of the action potential (e.g., Borst and Sakmann 1999; Park and Dunlap 1998)). Because of the well-known power function between the amount of Ca^{2+} entry and the amount of transmitter released ($n = 2$–4: motor nerve terminal [Dodge, Jr., and Rahamimoff, 1967], squid giant axon [Augustine and Charlton 1986; Llinas et al. 1982], calyx of Held [Borst and Sakmann 1999; Lou et al. 2005]), small changes in the amplitude or duration of the invading action potential might have substantial effects on transmitter release. Action potential repolarization (and hence duration) in nerve terminals such as the calyx of Held is determined by K_V1 and K_V3 voltage-gated K^+ channels, and possibly by Ca^{2+}-activated and Na^+-activated K^+-channels (Schneggenburger and Forsythe 2006). These channels are not directly controlled by G-proteins, though K_V3 channels can be regulated by PKC-mediated phosphorylation (Macica et al. 2003), so might be affected by G_q-coupled receptors (see further below). Related $K_V3.2$ channels are modulated by receptor-mediated PKA activation, but these are restricted to interneuron somata and dendrites, not terminals (Atzori et al. 2000) Thus, effects of adenosine and baclofen on evoked glutamate release at cerebellar synapses were not associated with any change in presynaptic action potential waveform (Dittman and Regehr 1996).

The most widely distributed G-protein-regulated K^+ channels are the G-protein-gated inwardly rectifying K^+-channels (GIRK channels, composed in neurons of $K_{ir}3.1 + K_{ir}3.2$ subunits), which are activated by βγ-subunits derived from G_i (see section 1 and Stanfield et al. 2002). Though primarily concentrated in neuron somata and dendrites, they appear to be present in some central terminals at least (Ponce et al. 1996). These channels are also present in some sympathetic (Wang and McKinnon 1995), and parasympathetic (Surprenant and North 1988) neurons, though not necessarily in their terminals. When activated, they generate a hyperpolarization and can suppress spike generation. Since they are activated by the same presynaptic receptors that so widely activate G_o to inhibit Ca^{2+}-currents, they could exert a profound presynaptic inhibitory effect if functionally expressed in nerve terminals. However, this appears to be very rarely (if ever) the case. Thus, there is no change in K^+ current in calyx endings on activating receptors that inhibit Ca^{2+}

currents (Takahashi et al. 1996); Kir3.2 gene-deletion eliminated the postsynaptic effects in the hippocampus produced by activating $GABA_B$, $5HT_{1A}$ or adenosine-A1 receptors without affecting their presynaptic inhibitory actions (Luscher et al. 1997); and $GABA_B$-mediated presynaptic inhibition in the retino-hypothalamic tract is not prevented by tertiapin, a $K_{ir}3$-channel blocker (Moldavan et al. 2006). On the other hand, tertiapin does reduce cannabinoid-induced inhibition of parallel fiber transmission in the cerebellum (Daniel et al. 2004), so the question may not be totally resolved.

In isolated nerve terminals, activation of type 1 mGluRs leads to activation of PKC (Coffey et al. 1994a) and facilitation of glutamate release (Coffey et al. 1993; Coffey et al. 1994a; Sanchez-Prieto et al. 1996). Based on measurements of membrane potential (Barrie et al. 1991), $^{42}K^+$ fluxes (Chaki et al. 1994; Colby and Blaustein 1988) and observations that PKC-mediated modulation is not observed when high-[K^+] is used as the secretagogue (Barrie et al. 1991; Breukel et al. 1998; Cousin et al. 1999), modulation is hypothesized to occur through the inhibition of a K^+ channel. Electrophysiological studies have indeed shown PKC phosphorylation-dependent effects on delayed rectifier (Augustine 1990) and inward rectifier K^+-channels (Takano et al. 1995) – inhibition of which would tend to broaden of the action potential, and thus enhance voltage-dependent Ca^{2+} entry, and thereby facilitate neurotransmitter release.

2.2 *GPCR-Mediated Presynaptic Regulation of the Release Process Distal to* Ca^{2+} *Entry*

Although the principal, and most widespread, effect of stimulating presynaptic G_o-coupled receptors is to inhibit transmitter release by reducing the presynaptic Ca^{2+} current (see Section 2.1), there is abundant evidence that, at some synapses, such inhibitors as adenosine (at adenosine-A1 receptors) and baclofen (at $GABA_B$ receptors) exert additional effects on the release process beyond the point of Ca^{2+} entry (see Thompson et al. 1993; Wu and Saggau 1997). Distal to the entry of Ca^{2+}, alteration of synaptic strength by modulating the exocytotic apparatus would seem a logical molecular locus for presynaptic GPCR function to regulate neurotransmitter release. Although the debate continues as to the relative contributions of voltage-dependent Ca^{2+} influx inhibition (as discussed above) and downstream suppression of the exocytosis itself (Wu and Saggau 1997), both adenosine and GABA reduce the frequency of spontaneous miniature excitatory synaptic currents (mEPSC) (Scanziani et al. 1992; Scholz and Miller 1992), or the probability of such release (Prince and Stevens 1992), in a number of studies pointing to a post-Ca^{2+} entry locus of action for the inhibitory presynaptic GPCRs.

Starting from the classical frog neuromuscular preparation where spontaneous acetycholine release is suppressed by purinergic adenosine receptor activation (Silinsky 1984), studies extending to mammalian central synapses contend a role for GPCR modulation of exocytosis *per se*. Thus, adenosine receptor activation reduces

spontaneous glutamate release, evinced by mEPSC frequency, at synapses on dentate granule cells in hippocampal slices (Prince and Stevens 1992) and pyramidal cells in hippocampal cell cultures (Scholz and Miller 1992). At synapses on hippocampal CA3 cells, both adenosine and $GABA_B$ (baclofen) receptor activation suppresses mEPSCs in slices and this modulation is unaffected by blanket inhibition of any voltage-dependent Ca^{2+} entry by Cd^{2+} (Scanziani et al. 1992) A similar effect of $GABA_B$ (baclofen) receptor (but not adenosine A1 receptor) activation on the mEPSC frequency is observed in cerebellar granule cell-Purkinje cell synapses (Dittman and Regehr 1996; Harvey and Stephens 2004). Baclofen also reduces the tetrodotoxin-resistant release of GABA at hippocampal pyramidal cells (Jarolimek and Misgeld 1997). In most of these studies, the issue of VDCC modulation by the GPCR is obviated, given that during spontaneous transmitter release, there is nominally no change in intraterminal Ca^{2+} concentrations. (However, it is worth noting that spontaneous and evoked transmitter release may derive from different pools of synaptic vesicles (Sara et al. 2005)). Clearly, because evoked release is contingent on voltage-dependent Ca^{2+}-entry, demonstration of a "Ca^{2+}-independent" action of inhibitory GPCR is difficult. Notwithstanding, using protocols obviating voltage-dependent Ca^{2+} channel involving, evoked release is also inhibited by adenosine and $GABA_B$ receptor activation at hippocampal CA3 synapses (Capogna et al. 1996). Notably, baclofen and adenosine inhibited the release of glutamate induced by ionomycin (which raises intracellular Ca^{2+} independently of Ca^{2+} channel activity) (Capogna et al. 1996). Finally, methacholine and *trans*-aminocyclopentane-1,3-decarboxylic acid (*t*-ACPD)-mediated stimulation of muscarinic acetylcholine and metabotropic glutamate receptors, respectively, inhibited spontaneous mEPSCs (Scanziani et al. 1995). At cerebellar synapses, even though baclofen also reduced the Ca^{2+} current, its post-Ca^{2+} entry action contributed a significant component of its presynaptic inhibitory effect because it produced more synaptic inhibition than that resulting from an equivalent inhibition of the Ca^{2+} current by Ca^{2+} channel blocking drugs or by adenosine (Dittman and Regehr 1996; see Wu and Saggau 1997 for further examples). In contrast, the reduced Ca^{2+} influx in mossy fiber terminals is sufficient of itself to account for the presynaptic inhibitory action of adenosine at this site (Gundlfinger et al. 2007). Thus, in general, it appears that post-Ca^{2+} entry effects are primarily limited to the presynaptic action of GABA.

Where post-Ca^{2+} entry modulation does occur, the question arises: what are the molecular targets of this GPCR-mediated regulation of exocytosis? Elucidation of the components of the exocytotic core complex, as well as upstream and downstream players, has opened the way to defining potential targets for regulation by GPCRs (Südhof 1995, 2000, 2004 and see Lang & Jahn, this volume). Thus, there are now good examples of GPCR regulation of neurotransmitter release by action at the level of recruitment of synaptic vesicles from a reserve pool (RP) into a readily-releasable pool (RRP), as well as docking, priming, exocytosis, and recovery processes (Figure 5a). In congruence with the heterotrimeric G-protein-mediated modulation exacted at the level of presynaptic excitability and Ca^{2+}-influx, control of the release process can be considered with respect to the direct effects of $G\beta\gamma$ subunits, and the indirect, $G\alpha$-instigated, regulation-mediated through second messengers activating protein phosphorylation pathways.

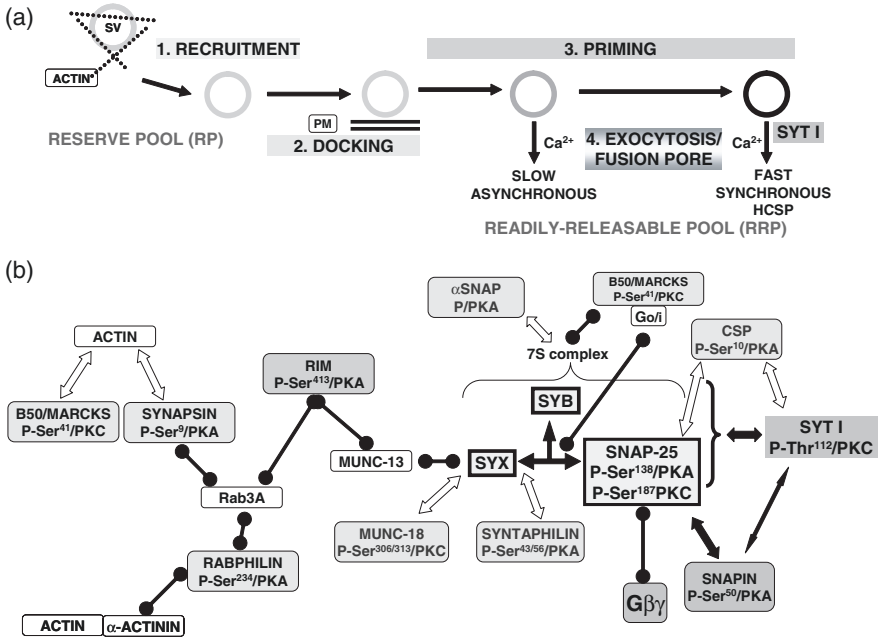

Fig. 5 GPCR regulation of exocytosis downstream of Ca^{2+}-entry. (**a**) Sequence of steps leading from recruitment to maturation of synaptic vesicles from a reserve pool (RP) to a readily-releasable pool (RRP) displaying slow (asynchronous) and fast (synchronous; highly Ca^{2+}-sensitive pool, HCSP; synaptotagmin 1 (SYT 1) supported) components. (**b**) Protein-protein interactions of SNARES (SYX, syntaxin; SYB, synaptobrevin and SNAP-2s-7S complex) and major putative regulatory proteins. Phosphoproteins are shown in shaded boxes (phosphorylation sites for PKA and PKC are indicated where known) with phosphorylation-dependent interactions depicted by arrows (increase indicated by filled arrows; decrease indicated by open arrows). Circle-end connectors indicate a phosphorylation-independent or as yet unspecified interaction. Potential effects of interactions at various points of the sequence in A are discussed in the text.

2.2.1 Actions of Go-Coupled GPCRs: Direct Gβγ–Mediated Regulation of SNARE Complex Assembly and Exocytosis

Based on the effects of a number of inhibitory GPCRs on spontaneous neurotransmitter release, where the involvement of nerve terminal excitability and voltage-dependent Ca^{2+}-influx is obviated, direct effects of GPCRs on the exocytotic apparatus have long been posited (see Wu and Saggau 1997). Supporting the layer of control afforded by Gβγ subunits in the control of presynaptic excitability, there is now accumulating evidence that the Gβγ limb of inhibitory GPCR activation can influence the function of the SNARE complex. An initial study at the turn of the century found that the serotonin (5HT)-mediated inhibition of neurotransmission at the lamprey reticulospinal/motoneuron synapse could be mimicked by injection of Gβγ subunits (Blackmer et al. 2001). Crucially, the effects of the GPCR ligand serotonin itself is abrogated by a Gβγ scavenger based on the C-terminal binding

sequence of G protein-coupled receptor kinase 2. All of the regulation observed occurs in the absence of any changes in presynaptic excitability or Ca^{2+} influx, and indeed, is independent of protein phosphorylation changes.

This exciting first report, unequivocally showing a Gβγ effect on exocytosis, stimulated a hunt for the molecular target for the G-protein. Two more recent studies clarified this issue somewhat in a series of elegant experiments. Firstly, Blackmer et al. (2005) showed that the very rapid Gβγ-mediated inhibition of exocytosis seen in the lamprey synapse could be recapitulated in the permeablized PC12 cell model in which ion-channel activities play no part. In vitro studies had shown that Gβγ binds the target SNAREs, SNAP-25 and syntaxin individually, and the SNARE complex as a whole (Figure 5b). Intriguingly, specific cleavage of SNAP-25 by the prior action of the Zn^{2+}-protease botulinum toxin (BoNT A), substantially reduces the Gβγ binding and, in toxin-treated cells, the inhibitory effect of Gβγ subunit introduction on neurotransmitter release is attenuated. Finally, the demonstration that the binding site for Gβγ on SNAP-25 overlaps with that of the Ca^{2+}-sensor synaptotagmin, and that the binding of the latter is also abolished by BoNT A cleavage of the C-terminus of SNAP-25, has invoked the hypothesis that Gβγ inhibition occurs through the uncoupling of SNARE complex-synaptotagmin interaction. The effects of BoNT A-mediated cleavage of the C-terminus of SNAP-25 is even more dramatic in lamprey reticulospinal/motoneuron synapse, where inhibitory modulation by 5HT is abolished (Gerachshenko et al. 2005). Perhaps most tellingly, the 5HT-mediated modulation could be abrogated by injected peptides based on the C-terminal sequence of SNAP-25 that would be expected to scavenge the Gβγ liberated by GPCR activation. The strength of the yin-yan superposition of inhibitory Gβγ and stimulatory synaptotagmin binding to the SNAP-25 is that, while the former will bind the target in the absence of Ca^{2+}, the binding of the latter is Ca^{2+}-dependent. Gβγ competition might therefore be expected to shift the Ca^{2+}-dependence of the exocytotic event triggered by synaptotagmin. Interestingly, two recent studies suggest that the Gβγ produced by inhibitory GPCR activation at the lamprey reticulospinal synapse reduces the quantal size (Photowala et al. 2006; Schwartz et al. 2007). The implication from this is that inhibitory GPCRs may shift the fusion mode from "full-fusion" to a transient fusion-pore opening typified by a "kiss and run" mode of fusion.

2.2.2 Indirect Gα – and Gq-Mediated, Second-Messenger-Dependent Regulation of Vesicle Recruitment, Docking, Priming, and Exocytosis

As discussed above, two of the established effectors of the Gα limb of heterotrimeric G-proteins are adenylate cyclase (AC) and phospholipase C (PLCβ), which, on stimulation, lead to the generation of the second-messenger molecules, cAMP and DAG/IP_3, respectively. Through the respective activation of cAMP-dependent protein kinase (PKA) and Ca^{2+}/phospholipid-dependent protein kinase (PKC), GPCRs coupled to AC and PLCβ have the potential to effect indirect modulation of neurotransmitter release by phosphorylation of substrate proteins involved in the

recruitment of synaptic vesicles and the exocytosis process itself. In the case of AC, reciprocal regulation is mediated by stimulatory $G\alpha_s$ and inhibitory $G\alpha_{i/o}$ subunits liberated by distinct GPCRs, while $G\alpha_{q/11}$ provides a monotonic stimulation of PLCβ on the activation of facilitatory GPCRs. In addition to effects mediated by cognate protein kinases, cAMP and DAG (and PIP_2) bind other protein effectors to each produce presynaptic modulation independent of PKA or PKC activity.

Regulation of Adenylate Cyclase and Neurotransmitter Release – Protein Phosphorylation Mechanisms

Presynaptic coincidence and interaction of GPCR-mediated signaling cascades involving second messengers potentially form the basis for the modulation of neurotransmitter release underlying synaptic plasticity. Compelling evidence for the role of a GPCR regulated signaling pathways in synaptic plasticity has been obtained for GPCRs coupled to AC activation and production of the prototypic second-messenger, cyclic-3′,5′-adenosine monophosphate (cAMP). The demonstration that mouse knockouts of AC lead to impairment of synaptic potentiation (Storm et al. 1998; Villacres et al. 1998) certainly implicates $G\alpha_s/G\alpha_{i/o}$ mediated stimulation/inhibition of AC in the modulation of synaptic transmission and plasticity (Hansel et al. 2001; Nguyen and Woo 2003; Nicoll and Malenka 1995). Initial pharmacological evidence of a role for cAMP in presynaptic function arose from experiments utilizing the diterpine forskolin to directly stimulate AC, or the use of membrane-permeable analogues of cAMP. Subsequently, GPCRs such as β-adrenoreceptors, that are positively coupled to AC through Gs, have been shown to enhance synaptic transmission in slice preparations (Gereau and Conn 1994; Huang et al. 1996; Saitow et al. 2005). In direct support of a presynaptic mode of action, a β-adrenoreceptor/cAMP/PKA-dependent pathway has been ascribed a facilitatory role in the release of glutamate from cerebrocortical nerve terminals (Herrero and Sanchez-Prieto 1996; Wang et al. 2002). More recently, a potential role of the AC/PKA cascade has also been identified in the modulatory effects of facilitatory presynaptic kainate-type glutamate receptors at mossy fiber terminals in the hippocampus (Rodriguez-Moreno and Sihra 2004). The major effector for the second messenger cAMP is PKA. In addition to the AC/PKA cascade, there is evidence that cAMP, acting as a second messenger to stimulate GTP exchange factors (GEFs), can thereby activate small GTP-binding proteins like Rap1 and its incumbent signaling cascades. In either case, to elucidate the mechanisms by which a pleiotropic cAMP production leads to the specific regulation of presynaptic function, it is essential to delineate nerve terminal-specific target proteins for the respective signaling cascades.

PKA and neurotransmitter release–potential loci of action

Although many of the protein targets for PKA are evidently ion channels involved in regulating presynaptic excitability, some of the effects of the AC activator forskolin

on neurotransmitter release, under paradigms where regulation of presynaptic ion channels is obviated, point to targets for PKA downstream of Ca^{2+} entry (Trudeau et al. 1996a, b, 1998). The release process distal to Ca^{2+} entry can be nominally divided into four steps, viz. (1) synaptic vesicle recruitment/replenishment, (2) docking/tethering, (3) priming, and (4) exocytosis/fusion (Figure 5a). We will outline some of the best characterized presynaptic players (Figure 5b) and discuss the potential mechanisms by which their phosphorylation by the GPCR initiated AC/PKA signaling cascade, might regulate neurotransmitter release.

PKA-synapsins: synaptic vesicle recruitment

Functionally distinct pools of synaptic vesicles have been postulated to underpin the regulated release of neurotransmitter and presynaptic plasticity (Zucker 1993). Most proximally, Ca^{2+}-dependent excitation-secretion coupling is recognized to involve a "readily-releasable pool" (RRP) in which the synaptic vesicles are initially morphologically docked, to then subsequently be primed to instill fusion-competence, prior to the fusion event itself (Gillis et al. 1996; Parsons et al. 1995; Scheller 1995). Within the RRP, a kinetically slower component of release depends on supply from a pool of docked vesicles not yet competent for fusion (Rosenmund and Stevens 1996) and intimately linked with a large distal cluster of synaptic vesicles representing a "reserve pool" (RP) postulated to be mobilized during periods of intense stimulation (Koenig et al. 1993; Kuromi and Kidokoro 1998; Pieribone et al. 2002) (Figure 5a). Protein phosphorylation/dephosphorylation is a key regulatory influence on presynaptic function at the level of synaptic vesicle recruitment/mobilization from the RP (Greengard et al. 1993; Hilfiker et al. 1999a; Turner et al. 1999) (Figure 5a, step 1). In a logical strategy identifying presynaptic phosphoprotein substrates, synapsin I was among the first protein characterized (De Camilli et al. 1979). It is found associated with the cytoplasmic face of synaptic vesicles (De Camilli et al. 1983a, b; Hirokawa et al. 1989; Pieribone et al. 1995; Valtorta et al. 1988), and is present exclusively in the presynaptic cytomatrix (De Camilli et al. 1983b; Gotow et al. 1991; Landis et al. 1988; Mandell et al. 1990b; Takei et al. 1995), thereby invoking a role for the protein and its family members (Mandell et al. 1990a; Mandell et al. 1992; Stone et al. 1994; Südhof et al. 1989) in the regulation of synaptic vesicle pools and neurotransmitter release (De Camilli et al. 1990; De Camilli and Greengard, 1986a).

Synapsin I is a brain- and terminal-specific phosphoprotein which has long been shown to be a target for GPCR signaling (Dolphin et al. 1980; Dolphin and Greengard, 1981a, b). In vitro, it is a substrate for PKA on Ser^{9} (Site 1) (Dunkley and Robinson, 1986; Huttner and Greengard, 1979), and Ca^{2+}/calmodulin-dependent protein kinases (CaMK) I and IV also on Ser^{9}, and CaMKII and CaMKIV on Ser^{566} and Ser^{603} (site 2&3) (Burke and DeLorenzo 1981; Czernik et al. 1987; DeLorenzo et al. 1979; Dunkley et al. 1986; Gower et al. 1986; Sieghart et al. 1980). All of the canonical phosphorylation sites, 1, 2, and 3, are therefore potentially subject to regulation by GPCRs that either elevate presynaptic second messenger cAMP levels or $[Ca^{2+}]_i$ (Coffey et al. 1994b; Dunkley and Robinson 1986; Krueger et al. 1977;

Robinson and Dunkley 1985; Robinson and Dunkley 1983, 1987; Sihra et al. 1989, 1992; Sihra 1993; Walaas et al. 1983). Additional phosphosites on Ser^{62} (site 4), Ser^{67} (site 5), and Ser^{549} (site 6) respond to a noncanonical pathway instigated by neurotrophic factors (Romano et al. 1987a, b) that stimulate cognate receptor tyrosine kinase receptors to initiate signaling cascades leading to activation of proline-directed kinases mitogen-activated protein kinases (MAPK1&2; also termed extracellular signal-regulated protein kinases, ERK1&2) and cyclin-dependent protein kinase 5 (cdk5) (Jovanovic et al. 2001; Jovanovic et al. 1996; Jovanovic et al. 2000; Matsubara et al. 1996).

High concentrations of synapsin I are present in nerve terminals, about 10 μM (De Camilli et al. 1990) or 10–30 molecules synapsin I/vesicle (Ho et al. 1991), and its amphipathic properties impart phospholipid affinity along with protein binding to synaptic vesicles. These interactions are under dynamic control by phosphorylation, so that phosphorylation of site 1 and sites 2/3 (Benfenati et al. 1989a, b, 1992b; Esser et al. 1998; Hosaka et al. 1999; Schiebler et al. 1986) leads to dissociation of molecule from the vesicle membrane. Likewise, synapsin I avidity for the actin cytoskeleton (Bahler and Greengard 1987; Petrucci et al. 1991; Petrucci and Morrow 1987) is altered by phosphorylation by PKA at site 1 (decrease in the actin nucleation and bundling) and by CaMKII at sites 2 and 3 (abolition of actin-nucleation and bundling but no effect on Kd) (Bahler et al. 1989; Benfenati et al. 1992a). Consistent with these alterations of synapsin I interactions, the molecule dissociates from synaptic vesicles and the cytoskeleton following in situ phosphorylation produced by forskolin or during depolarization of isolated nerve terminals (Sihra et al. 1989), or activity-dependent phosphorylation in the frog neuromuscular junction (Torri-Tarelli et al. 1990) and hippocampal neurons (Chi et al. 2001b, 2003b). These observations have therefore collectively contributed to the working hypothesis that synapsin I controls the exocytotic availability of synaptic vesicles from the RP by reversibly tethering them to the actin-based "cytonet" of the nerve terminal, in a phosphorylation-dependent manner (Chi et al. 2001a, 2003a; De Camilli et al. 1990; De Camilli and Greengard, 1986b) (Figure 5b). Although opinions differ as to the exact consequence of synapsin phosphorylation, or even its precise function (Hosaka and Südhof 1998, 1999), given its importance in presynaptic plasticity intimated from knockout studies (Li et al. 1995; Rosahl et al. 1993; Rosahl et al. 1995), it is a irrefutable candidate for modulation of presynaptic function via by GPCR activation.

PKA – Rab3A and transport partners: vesicle recruitment/docking

Rab3A is a vesicle-associated small G-protein involved in the targeting and docking/tethering of synaptic vesicles to the plasma membrane prior to priming and eventual exocytosis (Geppert et al. 1994, 1997; Schluter et al. 2004). It associates in a GTP-dependent manner with two synaptic vesicle active zone effector proteins, rabphilin (Li et al. 1994; Shirataki et al. 1993) and Rab3A-interacting molecule (RIM) (Schoch et al. 2002; Wang et al. 1997) (Figure 5b).

Although Rab3A is not a substrate for PKA, both rabphilin and RIM are phosphorylated by PKA (Fykse et al. 1995; Lonart et al. 2003; Lonart and Südhof 1998) and hence represent potential targets for signaling initiated by presynaptic GPCRs coupled to AC. Interestingly, in knockouts of Rab3A (Castillo et al. 1997) and RIM (Castillo et al. 2002), but not rabphilin (Schluter et al. 1999), long-term potentiation (LTP) at mossy fiber-CA3 pyramidal cell synapses is abolished. LTP at this synapse has long been posited to be expressed presynaptically through an AC/cAMP/PKA signaling cascade (Huang et al. 1994, 1996; Weisskopf et al. 1994). In the cerebellar parallel fiber-Purkinje cell model, where plasticity is also underpinned by presynaptic AC/cAMP/PKA signaling (Salin et al. 1996), LTP, which is eliminated in cultures from RIM1α knockout mice, is rescued by wild-type RIM1α but not by RIM1α rendered phospho-null (S413A) at the N-terminal of its two PKA phosphorylation sites (Lonart et al. 2003) (Figure 5b). Together, these data point to RIM as a key player in the mediation of presynaptic plasticity under GPCR control. At the level of synaptic vesicle priming (Figure 5a, step 3), given that Rab3A and Munc13 possibly compete for (Betz et al. 2001), but certainly interact during (Dulubova et al. 2005) RIM binding (at nested N-terminal α-helical and zinc-finger domains, respectively), PKA phosphorylation of RIM may have the potential to regulate the equilibrium of its respective binding to these two partners (Betz et al. 2001). Functionally, this could mediate GPCR effects on release probability and plasticity, particularly as Munc13 is found so intimately involved in syntaxin function and its availability for SNARE formation in an open conformation (Betz et al. 1997, 1998) and in vesicle priming (Augustin et al. 1999b; Brose et al. 2000; see Section 2.2.2 herein). However, judging by the numerous interactions that RIM1α displays with components of the presynaptic active zone, the involvement of RIM1α in the control of neurotransmitter release is likely to manifest at multiple levels in the secretory cascade leading to exocytosis (Calakos et al. 2004), perhaps even through a major reorganization/restructuring of the active zone (Lonart 2002; Lonart et al. 2003) by virtue of interactions with proteins such as liprins (Zhen and Jin 1999).

Intriguingly, synapsin has recently been shown to bind to Rab3A (Giovedi et al. 2004b) and operate as novel Rab effector (Giovedi et al. 2004a) (Fig. 5b). As with RIM, this is another case of a Rab3A partnering with a PKA substrate, while itself not being phosphorylated. Rab3A and synapsin interaction results in reciprocal regulation of the respective functions of the two proteins. Thus synapsin stimulates the GTP-binding and GTPase activity of Rab3A, while Rab3A inhibits the actin binding/bundling and vesicle clustering abilities of synapsin I. Interestingly, the latter activity of Rab3A activity parallels that of one of its other effectors, rabphilin, in its interaction with α-actinin (Baldini et al. 2005; Kato et al. 1996) (Figure 5b), but differs in several other ways. Synapsin functions to recruit GDP-bound Rab3A to the synaptic vesicle and participates in the subsequent GTP binding of the small G-protein. Given that Rab3A would then be expected to reduce the actin-vesicle cross-linking ability of synapsin and thereby increase recruitment of vesicles into the RRP, it would be interesting to see whether Rab3A and PKA-dependent phosphorylation of synapsin synergize at this point to facilitate neurotransmitter release (Figure 5b).

PKA – SNAP-25/α-SNAP/Syntaphilin: SNARE complex formation/dissolution

At the level of SNARE complex formation and dissolution, synaptosome-associated protein (SNAP-25) (Hepp et al. 2002; Risinger and Bennett 1999), α-SNAP (Hirling and Scheller 1996) and syntaphilin (Hirling and Scheller 1996) are phosphorylated by PKA. These are therefore candidate regulators of exocytosis by GPCR/AC signaling.

Phosphorylation of SNAP-25 occurs on Thr^{138}, and in the chromaffin cell model, the maintenance of secretory vesicles in a primed state by high endogenous PKA activity is mediated by phosphorylation at this site (Nagy et al. 2004) (Figure 5b). Although pharmacological and mutational modulation of the PKA phosphorylation of SNAP-25 clearly impinges on defined kinetic components of release, it remains to be seen whether a similar phosphorylation of the t-SNARE affects the size of the releasable pool of small synaptic vesicles from nerve terminals and thereby alters synaptic efficacy following activation of GPCRs coupled to AC. Notwithstanding the lack of direct evidence of the role of SNAP-25 in the PKA-mediated modulation of exocytosis, indirect support has been forthcoming from studies using hippocampal neurons. Thus, following the cleavage of the C-terminus (containing phosphorylation site) of SNAP-25 by botulinum toxin, while the response to ruthenium red (acting as a Ca^{2+}-independent secretagogue) (Trudeau et al. 1996a) is unchanged, the facilitatory effect of forskolin seen in control conditions is abrogated (Trudeau et al. 1998).

Downstream of trans-SNARE complex formation and membrane fusion, the soluble NSF-associated protein, α-SNAP, collaborates with NSF to dissociate the resultant cis-SNARE complex. This post-fusion event promotes priming of secretory vesicles as seen by the augmentation of the RRP by overexpression of α-SNAP (Xu et al. 1999a), and begs the question as to whether the function of this protein is regulatable by GPCRs effecting increases in cAMP levels. The observation that PKA phosphorylation of α-SNAP leads to a ten-fold decrease in affinity of the protein for the 7S SNARE complex is interesting yet enigmatic (Figure 5b). On the one hand, this would imply that PKA mediates a decrease in NSF activity, a notion contrary to the recycling of the SNARES and to the stimulatory effects of PKA on neurotransmitters release. On the other hand, if, in situ, the phosphorylation-dependent decrease in association occurs following NSF function, then this would accord with the apparent docking/priming behavior of α-SNAP that has been reported (Xu et al. 1999a).

At the proximal end of the SNARE cycle, the involvement of syntaphilin as a PKA target appears less paradoxical than that of α-SNAP. Discovered as a protein that competes with SNAP-25 for syntaxin-1A binding, syntaphilin is a putative brake on SNARE complex formation and thus exocytosis (Lao et al. 2000). It is interesting therefore that PKA phosphorylation of syntaphilin at Ser^{43} and Ser^{56} dramatically decreases binding to syntaxin-1A, an effect mimicked by the phosphomimetic (S43D) mutant (Figure 5b). Thus, although overexpression of wild-type (dephospho-) syntaphilin inhibits exocytosis, the phosphomimetic mutant has no effect (Boczan et al. 2004). The implication is that dephospho-syntaphilin operates

as negative regulator of exocytosis and thus GPCRs leading to PKA activation may well operate to abrogate this control and thereby facilitate exocytosis.

PKA – Snapin and CSP: Ca^{2+} *triggering of exocytosis*

The final step in the exocytosis is the stimulation of fusion itself initiated by Ca^{2+} binding to a Ca^{2+}-sensor or trigger Figure 5a. Prior regulation of the trigger by phosphorylation would be an effective mean of controlling presynaptic function given the rate-limiting nature of this step. In this context, the facilitatory effect of PKA reported to occur via a Ca^{2+} independent stimulation of exocytosis (Trudeau et al. 1996b) is not attributable to increased vesicle docking or recruitment, or addition of terminals, but rather attests to an increase in release probability. The latter may potentially be imparted by a functional increase in the affinity of Ca^{2+} for the release apparatus or enhanced Ca^{2+} co-operativity in the release process. A mediator for this type of PKA-mediated enhancement appears to be the protein, snapin, found associated with synaptic vesicle membranes (Ilardi et al. 1999). Snapin binds directly to SNAP-25 and is thereby recruited to SNARE complex (Figure 5b). Disruption of this interaction with the C-terminal half of snapin, or the snapin-binding sequence on SNAP-25, blocks interaction of the SNARE complex with the Ca^{2+} trigger synaptotagmin, and this inhibits synaptic transmission. PKA-dependent phosphorylation of snapin on Ser^{50} results in a substantial increase in its affinity for SNAP-25 and enhances the binding of synaptotagmin, as does introduction of a phosphomimetic mutation (S50D) into the full-length protein (Chheda et al. 2001) (Figure 5b). In adrenal chromaffin cells, this increases the levels of release competent secretory granules and enhances the probability of release in response to Ca^{2+} influx. Enhancement of the probability of release also occurs in hippocampal neuron autapses with the phosphomimetic mutant of snapin, but here the increase in release is thought to contribute to a decrease in the number of vesicles in the RRP and therefore a more rapid synaptic depression (Thakur et al. 2004). Together, these studies suggest that snapin, as a SNARE complex associating protein, could well underpin a major component of the potentiation of release seen with of stimulation of GPCR/AC/PKA activity.

Cysteine string protein (CSP) is one of a family of proteins found on secretory vesicles, being distinguished by the presence of a 14-cysteine residue string sequence at the C-terminal end, responsible for membrane association through palmitoylation, and a J-domain at the N-terminal, responsible for interaction with chaperone molecules. CSP appear to have multiple functions, including roles in vesicular uptake of neurotransmitter, RabA function, G-protein modulation of voltage-dependent Ca^{2+} channels, and vesicular exocytosis (Evans and Morgan 2003). The latter effect occurs by virtue of the interaction of CSP with syntaxin and synaptotagmin. The relevance of CSP in GPCR modulation of exocytosis arises from the observation that the Ser^{10} residue, just N-terminal to the J-domain, is phosphorylated by PKA (Evans et al. 2001; Evans and Morgan 2002) (Figure 5b). Crucially, this phosphorylation reduces the affinity of CSP for both syntaxin and synaptotagmin (cf snapin above). Judging by the lack of such effect by a CSP

phosphonull (S10A) mutant, PKA-dependent phosphorylation of CSP would appear to be slowing the kinetics of release catecholamine release from chromaffin cells. In a phosphorylation-independent context, overexpression of CSP also inhibits exocytotic levels of catecholamine. While it remains to be seen whether CSP phosphorylation has a similar kinetic effect on exocytosis in neurons, GPCR/AC/PKA action on CSP would be predicted to decrease release and synaptic transmission, contrary to the generally accepted view that PKA is facilitatory in the presynaptic terminal.

Regulation of Adenylate Cyclase and Neurotransmitter Release – PKA-Independent Regulation by cAMP Through HCN Channels and cAMP-GEF

While PKA constitutes the major target for the "original" second messenger cAMP, it is increasingly apparent that the nucleotide targets many other regulatory proteins in neuronal and non-neuronal cells. Beginning with cyclic nucleotide gated channels (Kaupp and Seifert 2002), the list of partners for cAMP has expanded considerably in recent years (Seino and Shibasaki 2005; Springett et al. 2004). Two key, non-kinase cAMP effectors of note, from the point of view of GPCR-mediated exocytotic control being considered here, are the *hyperpolarization-activated cyclic nucleotide-gated* (HCN) *channels* (Biel et al. 2002) and *cAMP-regulated guanine nucleotide exchange factors* (cAMP-GEFs or Epacs; *exchange proteins directly activated by cAMP*) (Bos 2003; Springett et al. 2004).

HCN or I_h channels were originally characterized in cardiac muscle in the control of the pacemaking activity in the sinoatrial node. Subsequently, these members of the voltage-dependent K^+-channel family have been found in many cell types, including neurons. The HCN channels are nonselective to cations (Na^+/K^+), respond to negatively directed voltage steps around the resting membrane potential, and are regulated by cAMP by a C-terminus interaction site. The channels took the stage from the exocytotic viewpoint because their activation following GPCR stimulation with 5HT effects a facilitation of neurotransmitter release from motor terminals (Beaumont and Zucker 2000). Crucially, this occurs in the absence of any changes in intracellular Ca^{2+}-levels, indicating action at a distal locus in the stimulus-secretion coupling cascade. This enhancement, which lasts (tens of minutes) beyond any time-course expected of an ionotropic action of a channel, appears to arise from an increase in the mobilization of vesicle into the RRP following HCN channel activation (Wang and Zucker 1998), in the absence of any prior Ca^{2+} -influx (Zhong et al. 2004). HCN channel activation also underlies cAMP-dependent regulation of release at cerebellar basket cell terminals (Southan et al. 2000) and hippocampal mossy fiber LTP, which is contingent on presynaptic modulation by cAMP (Mellor et al. 2002). The mechanism underpinning the cAMP/HCN regulation of exocytosis remains to be fully elucidated but, interestingly, the involvement of the actin cytoskeleton in the interaction of HCN channels with the release machinery has been posited (Zhong and Zucker 2004).

Following the identification of cAMP-GEFs as cAMP-binding proteins that activate the small G-protein Rap1 (De Rooij et al. 1998; Kawasaki et al. 1998), a role

for mouse homologue of cAMP-GEFII in exocytosis was explicitly demonstrated in studies expressing the cAMP-sensor in PC12 cells, which lack the protein endogenously (Ozaki et al. 2000). cAMP-GEFII expression enhances forskolin facilitation of secretion, and this increase is insensitive to PKA inhibition by H89. All the diagnostics with mutant proteins indicate that cAMP binding is essential for the modulation. Furthermore, antisense oligonucleotides against cAMP-GEFII significantly diminish the exocytotic response to cyclic nucleotides in insulin-secreting cells, indicating that a PKA-independent pathway via cAMP-GEFII is of physiological significance. Indeed, in some cases, this PKA-independent route is exclusive. Thus in the calyx of Held synapse, the obligate involvement of cAMP-GEFs in cAMP-mediated facilitation is intimated from studies showing that PKA inhibitors are entirely ineffective (Sakaba and Neher 2001). On the other hand, cAMP analogues like 8-pCPT-2′-O-Me-cAMP, which preferentially activate cAMP-GEF/Epac, enhance the release of neurotransmitter in the same model (Kaneko and Takahashi 2004; Sakaba and Neher 2003).

How then does cAMP-GEF/Epac mediate effects on exocytosis? Some clues come from the observation that cAMP-GEFII co-precipitates with RIM (Ozaki et al. 2000), which has been discussed above (section 2.2.2) with respect to its putative roles in GPCR/AC-mediated facilitation of exocytosis. Curiously though, despite its enzymatic activity, cAMP-GEFII does not exchange nucleotides on the cognate binding partner for RIM, Rab3A (Ozaki et al. 2000). Clearly therefore, despite the molecular interaction of cAMP-GEFII with RIM/Rab3A, this is partitioned from the exchange activity of former, perhaps unsurprisingly given that cAMP-GEF/Epac was discovered as exchange factor for Rap1. One possibility is that RIM interaction with cAMP-GEF indirectly localizes Rap-1 to the active zone where RIM resides. Since activated Rap1 has been suggested to directly or indirectly feed into B-Raf (see Dumaz and Marais 2005), and B-Raf leads to the activation of ERKs, Rap-1 coordination by Rim could effectively activate the ERK signaling cascade in nerve terminals (Jovanovic et al. 1996). ERK activation has been shown to facilitate glutamate release from nerve terminals as a result of synapsin phosphorylation at ERK-specific sites (Jovanovic et al. 2000; Jovanovic et al. 2001). This reflects important intraterminal cross-talk between GPCRs linked to AC and neurotrophin Trk receptors linked to ERK signaling.

Regulation of Phospholipase C and Neurotransmitter Release – Protein Phosphorylation Mechanisms

A number of presynaptic receptors are coupled to the activation of phospholipase C (PLC) through G_q (Majewski and Iannazzo 1998). PLC activation results in the production of two second messengers, viz. diacylglycerol (DAG) and inositol triphosphate (IP_3). The former stimulates PKC together with Ca^{2+}, while the latter initiates the release of Ca^{2+} from intracellular stores. As with PKA, PKC is pleiotropic in its effects, thus the specific regulation of presynaptic function necessitates the delineation of nerve-terminal-specific target proteins. Additionally, it has

become increasingly apparent that DAG plays a fundamental role as a direct activator of some presynaptic proteins, without invoking the prior activation of PKC. Furthermore, the very same PLC activity that produces DAG and IP_3 also effectively reduces the levels of PIP_2. As with some ion channels (see Section 2.1.1), this in itself can have signaling function for membrane resident/associated proteins, as is the general norm for players involved in exocytosis. Here we will consider the role of PKC, DAG, and PIP_2 in affecting the function of specific proteins associated with the release process distal to Ca^{2+} entry, during synaptic vesicle recruitment/replenishment, docking, priming, and fusion steps (Figure 5a).

PKC and neurotransmitter release–potential loci of action

In the foregoing discussion (Sections 2.1.1 and 2.1.2), we have considered the effects of PKC activation in the regulation of neurotransmitter release by modulation of nerve terminal excitability. There are numerous instances, however, of regulation of neurotransmitter by PKC downstream of Ca^{2+} entry, evinced by studies using Ca^{2+} ionophores to bypass ion-channel involvement (see Majewski and Iannazzo 1998). In these and other studies, the usual way to stimulate PKC is through the use of phorbol esters, which mimic DAG binding to one or two cysteine-rich domains (CRDs) found near the N-terminus of conventional and novel (but not atypical) forms of PKC. Because CRDs are found in proteins other than PKCs, the use of phorbol esters alone is not sufficient to define PKC-dependent effects, as had been assumed in early studies. To distinguish de facto regulation by PKC, therefore, additional evidence from the use of PKC inhibitors targeted to the ATP-binding site (as distinct from the DAG-binding site with which some inhibitors interact), increased PKC levels (by introduction of purified protein kinase or overexpression), or introduction/expression of phosphomimetic mutants of the PKC substrate (if known), is now deemed obligatory.

Notwithstanding, but bearing in mind the technical caveats in defining PKC-dependent regulation, there are good examples of PKC-dependent facilitation of neurotransmitter release (Majewski and Iannazzo 1998), with increasing evidence of PKC-mediated phosphorylation affecting several steps leading up to synaptic vesicle exocytosis downstream of Ca^{2+} entry (Takahashi et al. 2003). These studies used bisindolylmaleimide type PKC inhibitors, which specifically target the ATP-binding site of PKC, to delineate the phosphorylation-dependent component of regulation, and verified earlier studies where the role of PKC in secretion was defined with use of pseudosubstrate peptide inhibitors of PKC (Terbush and Holz 1986, 1990) or purified PKC (Morgan and Burgoyne 1992; Naor et al. 1989) in permeablized cell systems. Indeed PKCα and calmodulin are found to be proteins essential for triggering Ca^{2+}-stimulated exocytosis in a permeablized PC12 model (Chen et al. 1999), and PKC activation underlies the increase in the RRP evinced by hypertonic sucrose-induced release in synaptosomal preparations (Khvotchev et al. 2000; Lonart and Südhof 2000).

At a proximal locus, recruitment/translocation of secretory vesicles (Figure 5a, step 1) is under the control of PKC in that its activation variously affects the RRP in

both neuronal (Berglund et al. 2002; Stevens and Sullivan 1998) and non-neuronal (Gillis et al. 1996; Smith 1999; Smith et al. 1998) models. Thus phorbol ester treatment enhanced the size of the RRP (Berglund et al. 2002; Gillis et al. 1996) as well as its recovery following depletion (Smith et al. 1998; Stevens and Sullivan 1998), indicating an increase in recruitment or refilling from the RP (Figure 5a). PKC stimulation may therefore contribute to the activity-dependent facilitation of release following Ca^{2+} entry (Smith 1999).

Several studies have noted an effect of PKC at a more distal locus. Thus, rather than an effect on the size or refilling of the RRP, phorbol esters effect an increase in Ca^{2+}-sensitivity of exocytosis in the chick ciliary ganglion (Yawo 1999a, b) (Figure 5a, step 4). So, there is a leftward shift in the exocytosis versus $[Ca^{2+}]$ relationship but without any effect on: Ca^{2+}-influx/buffering/extrusion, the number of Ca^{2+}-binding (release) site, or the size or refilling rate of the RRP. This effect on the priming of a highly Ca^{2+} sensitive pool HCSP, Figure 5a) is blocked by bisindolylmaleimide inhibition of PKC activity. The inhibitor, being designed to bind the ATP-binding site on PKC, does not affect the interaction of phorbol esters with Munc13, which invokes PKC-independent priming. PKC similarly affects the Ca^{2+}-sensitivity of exocytosis in mammalian neuronal, calyx of Held synapse (Lou et al. 2005; Wu and Wu 2001) and non-neuronal pituitary cell (Zhu et al. 2002) release models.

At a very late point in the exocytotic process (Figure 5a, step 4), a necessary stage is the formation of a fusion pore prior to the complete coalescences of the vesicle and plasma membrane to produce full vesicular fusion as posited by the quantal hypothesis of neurotransmitter release. There is, however, experimental evidence to suggest that the fusion pore formation may be transient in some circumstances, so that sub-quantal neurotransmitter release may occur as proposed by the "kiss-and-run" hypothesis (Fernandez-Peruchena et al. 2005). The dilation and contraction of the fusion pore is therefore suggested to be under dynamic control to determine the mode of exocytosis. In the calyx of Held terminal, larger fusion pore conductances are recorded prior to full-fusion events compared to kiss-and-run exocytosis (He et al. 2006). In chromaffin cells, activity-dependent dilation of the fusion pore is suggested to produce a size-exclusion filter underlying the differential release of co-packaged small (catecholamines) and large (neuropeptides) molecule transmitters; low activity (narrow pore) releasing the former, with high activity (large pore) releasing both (Fulop et al. 2005). Furthermore, this dilation of the fusion pore is controlled by PKC activity and therefore represents an important potential target for regulation by GPCRs coupled to PLC activation (Burgoyne et al. 2001; Fulop and Smith 2006; Graham et al. 2000).

There are evidently several potential loci for the action of PKC in facilitating neurotransmitter release. The question is: what are the protein targets/substrates for PKC that mediate these multiple mechanisms of regulation of exocytosis?

PKC-B50: regulation of secretory vesicle recruitment

In the chromaffin and PC12 cell models, where the cortical actin cytoskeleton restricts the availability of secretory granules (Figure 5a, step 1), PKC activation

promotes release (Shoji-Kasai et al. 2002; Tsuboi et al. 2001; Vitale et al. 1995) by the disruption of the actin cytoskeleton (Vitale et al. 1995). At one level, this occurs by the activity of scinderin, an actin-severing protein, which is competitively bound by membrane PIP_2 and PS under basal condition, but by actin when activated. Thus stimulation may be determined by PLC activation altering levels of cellular PIP_2 (Trifaro et al. 2002). Disruption of the actin cytoskeleton also occurs by de facto second messenger stimulation of PKC and phosphorylation of substrates such as the myristoylated-alanine-rich C-kinase substrate (MARCKS) (Rose et al. 2001) in response to stimulation of PLC by histamine GPCRs, for instance (see Trifaro et al. 2002). The actin cytoskeleton plays a similar role in neuronal transmitter release by multiple mechanisms (Doussau and Augustine 2000). MARCKS phosphorylation (Robinson 1991; Wang et al. 1989) and the phosphorylation of another PKC substrate, B50 (GAP-43 or neuromodulin, phosphorylated on ser^{41}) (Spencer et al. 1992), is involved in the regulation of neurotransmitter release (Dekker et al. 1991; Hens et al. 1995, 1998). In the case of B50, although a PKC-dependent modulation of the interaction with F-actin is generally acknowledged, there is no consensus as to whether B50 affects the polymerization of actin (He et al. 1997; Hens et al. 1993; Iannazzo 2001). Interestingly, B50 phosphorylation does decrease its binding of calmodulin, an effect which, in the locale of actin-bound synaptic vesicle pool, may stimulate the activation of vesicle-bound Ca^{2+}/calmodulin kinase II (Benfenati et al. 1992b). This would lead to the phosphorylation and thus dissociation of synapsin I from synaptic vesicles and the actin-cytoskeleton, and thereby mediate the mobilization of the reserve pool of synaptic vesicles (Iannazzo 2001). Given the observed facilitation of glutamate release by type I mGluRs (see Sanchez-Prieto et al. 1996), stimulation of these GPCRs may instigate PKC phosphorylation of B50 (and MARCKS) to support presynaptic plasticity processes (Coffey et al. 1994b; Ramakers et al. 1999).

PKC – $G\alpha_o$ and B50: regulation of vesicle priming

Downstream of vesicle recruitment, PKC-dependent phosphorylation may affect vesicle priming (but see PKC-independent regulation of the same in Section 2.2.2) (Figure 5a, step 3). The discovery of $G\alpha_o$ and/or $G\alpha_i$ on secretory granules and synaptic vesicles (Ahnert-Hilger et al. 1994; Ahnert-Hilger and Wiedenmann 1994; Toutant et al. 1987) begs the question as to their function therein. Functional studies using mastoparan, an activator of the $G\alpha_{o/i}$, show that that heterotrimeric G-protein activation inhibits exocytosis by inhibiting an ATP-dependent priming step (see Vitale et al. 2000 for review). One possibility is that $G\alpha_o$ prevents the dissociation of SNAP-25 from VAMP, thought to be necessary for priming (Misonou et al. 1997). Given the intracellular localization of the secretory vesicle associated pool of $G\alpha_{o/i}$, physiological stimulation by extracellular signals is necessarily indirect. One potential segue between plasma membrane signaling and vesicular G-protein is the PKC substrate B50 (GAP-43), which interacts with and stimulates $G\alpha_o$ (Strittmatter et al. 1990) (Figure 5b). Therefore as B50 (GAP-43), and PKC phosphorylation thereof, supports neurotransmitter release (Dekker et al. 1991; Hens et al. 1995, 1998), and the phosphorylated protein promotes ATP-dependent priming, one possibility is that

B50 (GAP-43) phosphorylation by PKC uncouples the inhibitory tone imposed by stimulation of $G\alpha_o$, (Misonou et al. 1998; Ohara-Imaizumi et al. 1992), and, in so doing, effects facilitation of exocytosis. Interestingly in this regard, B50 (GAP-43) has been shown to interact with components of the SNARE complex in a PKC phosphorylation-dependent manner (Haruta et al. 1997).

PKC – SNAP-25: regulation of secretory vesicle recruitment

SNAP-25 is phosphorylated in response to phorbol esters in neuronal (Genoud et al. 1999), neuroendocrine (Iwasaki et al. 2000; Kataoka et al. 2000; Nagy et al. 2002; Shimazaki et al. 1996), and non-neuronal (Gonelle-Gispert et al. 2002) cells at Ser^{187}. Phosphorylation at Thr^{138} by PKC is additionally intimated, this also being the target for PKA (Hepp et al. 2002 see above; Risinger and Bennett 1999) (Figure 5b). In vitro phosphorylation at the Ser^{187} site using purified substrate and PKC appears to reduce the interaction of the protein with syntaxin (Shimazaki et al. 1996), an effect that is not easily reconcilable with a stimulatory effect of PKC phosphorylation on exoytosis and given that it is an increase in SNAP-25-syntaxin heterodimerization that likely represents a rate-limiting step in SNARE complex formation. However, more recently, a phosphomimetic mutation of SNAP25 at Ser^{187} (S187E) was shown to cause an increase in SNAP-25 and syntaxin association (Yang et al. 2007). Overexpression of phosphomimetic (S187E) results in an increase in the kinetics of refilling (but not size) of the RRP (Nagy et al. 2002) and the size of a highly Ca^{2+}-sensitive pool (HCSP, Figure 5a) (Yang et al. 2007). This suggests causality between PKC-mediated phosphorylation of Ser^{187} on SNAP-25, increased SNARE complex formation, and facilitation of exocytosis. This neat conclusion is, however, tempered by observations in hippocampal neurons that clostridial toxin-mediated elimination of SNAP-25 is equally well rescued by expression of toxin-insensitive exogenous wild-type, phospho-null (S187A) and phospho-mimetic (S187E) SNAP-25, with phorbol esters still stimulating exocytosis in the phospho-null conditions (Finley et al. 2003). However, this study, using mild stimulation, may not have assayed refilling kinetics noted to be enhanced by S187E SNAP-25 phosphomimicry (Nagy et al. 2002), or the HCSP modulated by SNAP-25 (Yang et al. 2007) may be a specific feature of the chromaffin cell model, or at least not easily amenable in neuronal studies to date.

PKC – SNAP-25: regulation of Ca^{2+}*-sensitivity*

SNAP-25 phosphorylation by PKC may also underlie the increase in Ca^{2+}-sensitivity of exocytosis discussed above (Figure 5a, step 4). The C-terminus of SNAP-25 is known to be involved in the Ca^{2+}-triggering of exocytosis based on the essential Ca^{2+}-dependent binding of synaptotagmin to this negatively charged region (Zhang et al. 2002). Cleavage of the C-terminus 26 amino-acids of SNAP-25 with botulinum toxin E (BoNT E) abolishes binding of synaptotagmin and regulated exocytosis in PC12 cells, while removal of the C-terminus 9-amino acids by botulinum toxin A (BoNT A) increases the Ca^{2+}-concentration needed for binding of synaptotagmin

and reduces the Ca^{2+}-sensitivity of exocytosis (Gerona et al. 2000; Xu et al. 1998). A similar effect to the latter is obtained if the negatively charged acidic amino-acid tail of SNAP-25 is replaced with neutral residues (Sorensen et al. 2002). The Ser^{187} phosphorylation site which is targeted by PKC lies between the BoNT E and BoNT A cleavage sites and promotes the expression of the HSCP component of the RRP (Figure 5a). The HCSP, but not the RRP in general, is eliminated on cleavage with BoNT A (Yang et al. 2002). Thus, evidently, PKC phosphorylation in relation to SNAP-25 appears to be responsible, at least in part, for the enhancement of Ca^{2+}-sensivity of exocytosis observed following PKC activation. Clearly though, SNAP-25 phosphorylation as singular target for the DAG (phorbol ester)/PKC-induced facilitation of synaptic release is an oversimplification of the physiological situation following GPCR activation of PLC.

PKC – Munc18: regulation of the fusion pore

Members of the Sec1/Munc18 (SM) group of proteins are found in multiple membrane compartments and are responsible for providing the specificity in transport and docking underpinning the vesicular fusion process mediated by the SNARES. Although Munc-18 was prototypically defined by its ability to bind syntaxin with high affinity (Garcia et al. 1994; Hata et al. 1993; Pevsner et al. 1994a, b; Pevsner and Scheller 1994), sequestering it in a closed conformation before liberation in a primed/open-state (in tandem with Munc13 association, for instance), there is increasing evidence that Munc18 and other SM protein are involved at multiple loci during membrane trafficking and fusion events, independently of syntaxin interaction (Burgoyne and Morgan 2007; Toonen and Verhage 2003). Three mammalian homologues of Munc18 (18-1/2/3/) have been identified with Munc18-1 being involved in regulated exocytosis in contrast to the others being appropriated in intracellular membrane fusion (Halachmi and Lev 1996). Munc18-1 is uniquely phosphorylated by PKC at Ser^{306} and Ser^{313} in vitro (Fujita et al. 1996), with the latter phosphorylation, at least, also shown to occur in situ following treatment with phorbol esters (Barclay et al. 2003; Craig et al. 2003; De Vries et al. 2000) or type 1 metabotropic glutamate receptor activation (Craig et al. 2003). Crucially, phosphorylation of Munc18-1 reduces its affinity for syntaxin in vitro (Fujita et al. 1996), this effect being reproduced by phosphomimicking mutations S306E and S313E in Munc18 (Liu et al. 2004) (Figure 5b). The phosphorylation-dependent interaction of Munc18 with syntaxin could therefore represent the basis for the GPCR/PLC/PKC or phorbol ester facilitation of exocytosis. Phorbol ester stimulation and expression of phosphomimetic Munc18 mutants have mutually occlusive effects in accelerating the kinetics of exocytosis and reducing the quantal size of events (Barclay et al. 2003). The former effect is interpreted as being due to Munc18 controlling fusion pore dilation (Fisher et al. 2001; Graham et al. 2000) (Figure 5a, step 4), with the latter effect perhaps reflecting a switch to a "kiss-and-run" mode of release thought to be promoted by PKC activation among other stimuli (Cousin and Robinson 2000).

Munc18-1 likely has multiple modes of action (Burgoyne and Morgan 2007), both dependent on or independent of direct syntaxin binding, with a distal mode

involving binding to preassembled SNARE complexes (Shen et al. 2007). PKC-dependent facilitation of exocytosis could therefore occur by a relief of the inhibitory constraint of Munc18-1 bound to syntaxin together with stimulation of a late stage of fusion perhaps involving the kinase regulating fusion pore dynamics (Fulop et al. 2005; Fulop and Smith 2006). Certainly Munc18-1 appears to be an essential component of PKC-mediated facilitation of exocytosis (Wierda et al. 2007).

PKC – synaptotagmin?: regulation of the Ca^{2+}*-sensitivity of exocytosis*

Given the reports showing that phorbol esters cause an increase in the Ca^{2+} sensivity of exocytosis at some neuronal synapses, the question arises as to whether the Ca^{2+}-sensor is regulated by phosphorylation. Synaptotagmins are the established Ca^{2+}-sensors and triggers for exocytosis, functioning by virtue of two C2A and C2B domains, which each bind phospholipids in a Ca^{2+}-dependent and Ca^{2+}-independent manner respectively (Augustine 2001; Bai and Chapman 2004; Chapman 2002; Koh and Bellen 2003). Synaptotagmin 1, the trigger for rapid/fast exocytosis (Figure 5a, step 4), is phosphorylated by both PKC and Ca^{2+}/CaM kinase II at the same Thr^{112} residue, both in vitro and in situ, in response to nerve terminal stimulation (Hilfiker et al. 1999b). Interestingly, phosphorylation of synaptotagmin increases its interaction with the t-SNARES, syntaxin and SNAP-25, particularly in the presence of Ca^{2+} (Verona et al. 2000) (Figure 5b). This therefore represents a potential mechanism by which PKC might increase the Ca^{2+}-sensitivity of exocytosis. However, this hypothesis awaits confirmation in the neuronal models which display an increase in release probability in response to PKC activation (i.e. increase in release rate without an increase in RRP), because in the chromaffin cell model where PKC affects the size of the RRP, expression of phospho-mimetic or phospho-null mutants of synaptotagmin 1 is of no consequence on phorbol ester induced facilitation of exocytosis (Nagy et al. 2006).

Regulation of Phospholipase C and Neurotransmitter Release – PKC-Independent Regulation by DAG through Munc13

Phorbol esters are commonly used as pharmacological analogues of the endogenous second messenger, DAG, produced by GPCR activation of PLC. DAG activates PKCs by binding to C1 domains (cysteine, rich Zn^{2+}-finger domains) in the regulatory domain of the kinase (Silinsky and Searl 2003). There are, however, other proteins in the cell that contain C1 domains, which could act as receptors for DAG (or phorbol esters) and behave as effectors, independently of any PKC-dependent phosphorylation. Munc13-1 and its homologues represent one such receptor (Betz et al. 1998), which was originally discovered as the mutation (UNC13) in *Caenorhabditis elegans,* causing a neural defect leading to uncoordinated movements. Munc13-1 is an essential brain-specific protein (Augustin et al. 1999a, b), whose overexpression leads to an enhancement of neurotransmitter release contingent on its binding to β-phorbol esters. This suggests that, in the absence of specific inhibitors of PKC, the

faciliatory effects of phorbol esters arise from activation of Munc13-1 together with PKC-dependent pathways, with arguably a predominance of the former influence (Rhee et al. 2002; Searl and Silinsky 1998). However, recent evidence points to an interdependence of the PKC-dependent and PKC-independent effects leading to the potentiation of exocytosis in response to DAG/phorbol ester (Wierda et al. 2007).

Munc13-1 binds the N-terminus of syntaxin (Betz et al. 1997) (Figure 5b), Doc2 (Orita et al. 1997), RIM1 (Betz et al. 2001) and calmodulin (Junge et al. 2004). Munc13-1 plays a priming role in the preparation of vesicles for exocyotosis (Betz et al. 2001; Brose et al. 2000; Silinsky and Searl 2003; Brose, this volume). Augmentation of exocytosis pursuant to this may invoke GPCR/PLC synaptic plasticity (Junge et al. 2004; Wierda et al. 2007).

Regulation of Phospholipase C and Neurotransmitter Release – PKC-Independent Regulation by PIP_2 Modification of Exocytotic Protein Function

As discussed above in relation to ion channels activity, GPCR/PLC-mediated changes in PIP_2 concentrations provide potent means of regulation. Part of the ATP dependence of exocytosis certainly results from the requirement of phosphtidylinositol (PI) phosphorylation by PI 4-kinase and PI(4)P 5-kinase to produce PIP_2 necessary for vesicle priming (Klenchin and Martin 2000 for review). Alteration of the activity of these lipid kinases and consequent changes in PIP_2 levels regulate exocytosis in nerve terminals (Wiedemann et al. 1998) and neuroendocrine cell models (Hay et al. 1995; Holz et al. 2000; Milosevic et al. 2005). In mice in which the type 1 PIPγ (PI(4)P 5-kinase) is genetically ablated, levels of PIP_2 are decreased in nerve terminals and GTP-γS- or depolarization-stimulated elevation of PIP_2 is abrogated (DiPaolo et al. 2004; DiPaolo and DeCamilli 2006). Neuronal exocytosis is decreased in these mice following a decrease in the RRP. This, together with an enhancement of rapid depression following intense nerve terminal activity, indicates a lesion in vesicle priming. A similar involvement of PIP_2 in priming is indicated by experiments overexpressing PI(4)P 5-kinaseγ in chromaffin cells where the RRP is enhanced (Milosevic et al. 2005). Notably, there is no apparent compensatory change with respect to exocytosis following alterations of PIP_2 levels, suggesting that PIP_2 very much sets the gain of the exocytotic system, this therefore being potentially regulatable by GPCR activation of PLC.

Several potential candidates exist that might mediate the effects of PIP_2 on exocytosis, including rabphilin (Chung et al. 1998), Munc18-interacting proteins (Mints) (Okamoto and Südhof 1997), Ca^{2+}-dependent activator protein for exocytosis (CAPS) (Loyet et al. 1998), and synaptotagmin (Schiavo et al. 1996). Given the putative roles of these binding partners at multiple loci in the exocytotic process, regulation through PIP_2 is likely to be multifactorial, with effects on an individual step only being decernible if it is rate-limiting. Thus, for example, while the interaction of synaptotagmin with PIP_2 accelerates/aids the insertion of the protein into membranes, and might therefore be predicted to increase the rate of fusion (Bai and Chapman 2004), this is not the primary effect ob-

served in experiments upregulating PIP_2 concentrations, possibly because upstream regulation dominates (Milosevic et al. 2005).

Regulation of Phospholipase C and Neurotransmitter Release – Inositol-1,4,5-Trisphosphate(IP_3) and Intracellular Ca^{2+} Release

GPCRs coupled to G_q can, in many neurons and non-neuronal cells, induce the formation of IP_3 and a rise in intracellular Ca^{2+} through release from intracellular Ca^{2+} stores. In some neurons, presynaptic injection of IP_3 or a homologue can facilitate evoked transmitter release or enhance spontaneous release (e.g., cholinergic NG108-15 neuroblastoma hybrid cells, Higashida 1988; Aplysia neurons, Chameau et al. 2001; frog neuromuscular junction, Brailoiu et al. 2003, Brailoiu and Dun 2003; crayfish neuromuscular junction, Dropic et al. 2005). Notwithstanding, there is little direct evidence to date that IP_3 mediates the facilitatory effects of GPCRs on transmitter release from mammalian nerve endings. Thus, Higashida (1988) suggested that IP_3 might be responsible for the enhanced spontaneous release of acetylcholine from processes of NG108-15 neuroblastoma cells by bradykinin (which strongly increases both IP_3 and Ca^{2+} in these cells). However, spontaneous release was also enhanced by activating PKC, and it is this latter effect that appears to underlie the enhancement of transmitter release from homologous mammalian sympathetic neurons (Scholze et al. 2002). Probably the most convincing evidence for a role for intracellular Ca^{2+} in transducing the action of a GPCR on transmitter release is for the presynaptic auto-facilitatory action of glutamate on transmission from reticulospinal axons onto motoneurons in the lamprey spinal cord, mediated through activation of type 1 metabotropic glutamate receptors (mGluRs) (Cochilla and Alford 1998). Here, stimulation of these receptors enhanced nerve-evoked presynaptic Ca^{2+} transients and increased evoked transmitter release, and both were attenuated by ryanodine. This can be interpreted to suggest that IP_3-induced Ca^{2+} release was amplified by Ca^{2+}-induced Ca^{2+} release (CICR) from ryanodine-sensitive stores, and that this amplification was necessary for the facilitation of transmitter release, though no direct evidence for a role for IP_3 was advanced. CICR is widely present in presynaptic terminals and is known to play a role in amplifying transmitter release from several types of neuron, and in various forms of synaptic plasticity (Collin et al. 2005), but evidence for its participation in G-protein mediated events remains otherwise sparse.

3 Conclusions

3.1 Trimeric G-proteins (composed of α, β and γ subunits) intermediate between activation of G-protein coupled receptors (GPCRs) and subsequent changes in transmitter release. The most significant G-proteins in this context are G_o, G_q and G_s.

3.2 The auto-inhibitory action of most neurotransmitters on transmitter release is mediated primarily by G_o; the βγ-subunits of G_o interact directly with voltage-gated Ca^{2+} channels to produce a transient reduction of their voltage-response and hence of the Ca^{2+} influx into the terminals following the action potential. This response is facilitated by a close association between G_o, the Ca^{2+} channel and the receptor.

3.3 Downstream effects of Gβγ on the release machinery itself – specifically, the SNARE complex – also play an important part in the response to some G_o-coupled GPCRs.

3.4 Although GPCRs that activate G_o also activate G_i, and thereby open inward-rectifier (GIRK or $K_{ir}3.2$) K^+ channels, this appears to play little or no part in their effect on transmitter release – probably because of the paucity of G_i in nerve terminals.

3.5 Activation of G_q and G_s leads primarily to facilitation of transmitter release, along a slower and more prolonged time-scale than G_o-mediated inhibition. This results predominantly from downstream products of G_q and G_s enzyme activation – diacylglycerol (DAG) from G_q-activated phospholipase C (PLC) and cyclicAMP (cAMP) from G_s-activated adenylate cyclase (AC).

3.6 Subsequent activation of the kinases, protein kinase C (PKC) and protein kinase A (PKA) and consequent protein phosphorylation can enhance Ca^{2+} channel function, opposing G_o-mediated inhibition, or can modify K^+ channel activity, so indirectly altering action potential-induced Ca^{2+}-entry.

3.7 However, most of the effects of PKC and PKA activation result from phosphorylation of multiple components of the release machinery itself.

3.8 DAG can also affect release independently of PKC activation, through an interaction with Munc13. cAMP also has non-PKA-mediated actions (e.g., on hyperpolarization-activated cation channels and cAMP-GEFs), which affect release.

3.9 Since many of the proteins involved in transmitter release are also sensitive to membrane phosphatidylinositol-4,5-trisposphate (PIP_2), the reduction of PIP_2 consequent on G_q-PLC activation may also play a part in G_q-mediated changes in transmitter release.

3.10 On the other hand, the release of intracellular Ca^{2+} by the other PIP_2 product, inositol-1,4,5-trisphosphate (IP_3), seems rarely to mediate effects of G_q-coupled GPCRs.

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Presynaptic Metabotropic Receptors for Acetylcholine and Adrenaline/Noradrenaline

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Abstract Presynaptic metabotropic receptors for acetylcholine and adrenaline/noradrenaline were first described more than three decades ago. Molecular cloning has resulted in the identification of five G protein-coupled muscarinic receptors $(M_1 - M_5)$ which mediate the biological effects of acetylcholine. Nine adrenoceptors $(\alpha_{1ABD}, \alpha_{2ABC}, \beta_{123})$ transmit adrenaline/noradrenaline signals between cells. The lack of sufficiently subtype-selective ligands has prevented identification of the physiological role and therapeutic potential of these receptor subtypes for a long time. Recently, mouse lines with targeted deletions for all muscarinic and adrenoceptor genes have been generated. This review summarizes the results from these gene-targeting studies with particular emphasis on presynaptic auto- and heteroreceptor functions of muscarinic and adrenergic receptors. Specific knowledge about the function of receptor subtypes will enhance our understanding of the physiological role of the cholinergic and adrenergic nervous system and open new avenues for subtype-selective therapeutic strategies.

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1 Introduction

More than three decades ago, the first presynaptic metabotropic receptors regulating the release of acetylcholine, GABA, and noradrenaline were discovered (for reviews, see Langer 1997; Langer et al. 1985; Starke 2001; Starke et al. 1989). Presynaptic receptors are termed "autoreceptors" if they are activated by their neuron's own transmitter. In contrast, "heteroreceptors" are regulated by transmitters or modulators from neighboring cells. The present review will focus on recent studies which have investigated presynaptic metabotropic receptors for acetylcholine and adrenaline/noradrenaline in gene-targeted or transgenic mouse models.

2 Presynaptic Receptors for Acetylcholine

2.1 Acetylcholine Receptor Subtypes

Acetylcholine (ACh) is a widely distributed neurotransmitter in the central and peripheral nervous systems. As a classical neurotransmitter, acetylcholine is stored in intracellular vesicles and, after release into the extracellular space (i.e., the synaptic cleft), it may activate two classes of plasma membrane receptors: muscarinic ACh receptors (mAChR) and nicotinic ACh receptors (nAChR). While muscarinic receptors belong to the superfamily of seven transmembrane spanning G protein-coupled receptors (GPCRs), nicotinic receptors are ligand-gated ion channels. For a detailed description of nicotinic AChR and presynaptic functions, the reader is referred to the chapter by Dorostkar and Boehm in this *Handbook* and to recently published reviews focusing on insight derived from gene-targeted mouse models (Champtiaux et al. 2003; Drago et al. 2003).

2.2 Muscarinic Acetylcholine Receptors

By molecular cloning, five subtypes of G protein-coupled mAChR have been identified and are termed M_1-M_5 (Caulfield and Birdsall 1998; Wess 1996). Based on their coupling to specific groups of heterotrimeric G proteins, mAChR may be divided into two functional classes: M_2 and M_4 receptors are preferentially coupled to $G_{i/o}$ signal transduction pathways, whereas M_1, M_3, and M_5 subtypes activate G_q proteins and downstream effectors (Wess 1996).

2.2.1 Transgenic and Gene-Targeted Models

In the absence of sufficiently subtype-selective ligands, mouse lines carrying targeted deletions in the muscarinic AChR genes have been generated by several laboratories (Fisahn et al. 2002; Gerber et al. 2001; Gomeza et al. 1999a,b; Hamilton et al. 1997; Matsui et al. 2000; Miyakawa et al. 2001; Wess 1996; Yamada et al. 2001a,b). All mice deficient in single mAChR subtypes were viable and fertile and were born without any obvious malformations. With few exceptions, targeted deletion of one receptor gene in the mouse genome did not result in compensation by any of the other subtypes, allowing precise assignments of receptor subtypes with specific functions (for detailed discussion, see Wess 2004).

2.2.2 Occurrence

Muscarinic AChR are widely expressed in many organs, tissues, and cell types of the body (Table 1) (Caulfield and Birdsall 1998; Caulfield et al. 1993; Eglen 2006; Eglen and Nahorski 2000; Levey 1993; Levey et al. 1994; Wess 2004; Wolfe and Yasuda 1995; Yasuda et al. 1993; Zhang et al. 2002a). M_1 AChR are abundantly expressed in the brain, including cerebral cortex, hippocampus, and striatum. M_2 receptors have been identified in the central nervous system, heart, and organs containing smooth muscle. The M_3 subtype can be found in the brain and in peripheral organs which are innervated by parasympathetic nerves. M_4 receptors are mostly restricted

Table 1 Occurrence of Muscarinic AChR Subtypes

Organ/Tissue	Muscarinic AChR Subtype				
	M_1	M_2	M_3	M_4	M_5
Central nervous system					
Cerebral cortex	++	++	+	+	(+)
Cerebellum	+	++		+	(+)
Hippocampus	++	+	+	+	(+)
Hypohalamus			+		
Limbic sytem					(+)
Spinal cord		++		+	
Striatum	+	+	+	++	(+)
Peripheral organs					
Eye			+		(+)
Glands	+		+		
Heart	+	++	+	+	+
Lung		+	+	+	
Smooth muscle	+	+	+		
Sympathetic ganglia	+	+		(+)	
Vascular smooth muscle	+	++	+		+

For references, see text. Symbols: ++ predominant subtype, + expressed subtype, (+) low abundant subtype.

to forebrain regions, and M_5 receptors are present only at low abundance in the brain, e.g., in dopaminergic neurons of the midbrain.

2.2.3 Presynaptic Signaling

Based on studies on gene-targeted mice, M_2 and M_4 AChR have been identified as major presynaptic inhibitory receptors (Slutsky et al. 2003; Trendelenburg et al. 2003a; Zhang et al. 2002a,b; Zhou et al. 2002). These receptor subtypes are coupled to G proteins of the $G_{i/o}$ class and may affect presynaptic signaling by a variety of mechanisms (Figure 1). Activation of inhibitory $G\alpha_{i/o}$ proteins by $M_{2/4}$ AChR leads to inhibition of adenylyl cyclase, thus resulting in decreased cellular cAMP levels. In addition, $G\beta\gamma$ subunits released from activated $G_{i/o}$ proteins are important regulators of neuronal function by inhibiting neuronal Ca^{2+} channels, activating GIRK K^+ channels and stimulating mitogen-activated (MAP) kinases ERK1/2 (Wess 1996). Among these mechanisms, the $G\beta\gamma$-mediated inhibition of neuronal Ca^{2+} channels seems to be the most important mechanism for inhibition of neurotransmitter release (Figure 1) (for reviews, see Boehm and Kubista 2002; Kubista and Boehm 2006). MAP kinase activation induced by acetylcholine was almost completely abolished in neuronal cultures from newborn mice lacking M_1 receptors (Berkeley et al. 2001). Similarly, G_q protein activation and phosphoinositide hydrolysis was absent in M_1-deficient brain preparations, indicating that M_1

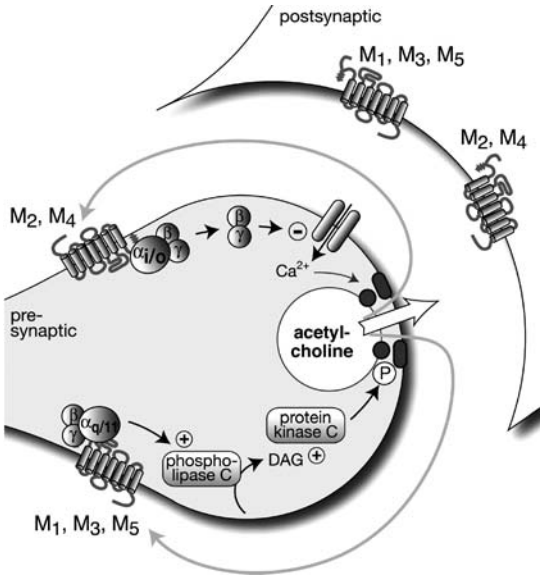

Fig. 1 Presynaptic signaling mechanisms of muscarinic receptor subtypes (M_1-M_5). Abbreviations: $\alpha_{i/o}$, β, γ, subunits of $G_{i/o}$ heterotrimeric GTP-binding proteins; DAG, diacylglycerol.

receptors are the most abundant G_q-coupled muscarinic receptors in the brain (Bymaster et al. 2003a; Porter et al. 2002). Facilitory mAChR which are coupled to $G_{q/11}$ proteins may enhance neurotransmitter release primarily via protein kinase C-mediated phosphorylation of proteins of the exocytotic release machinery (Figure 1) (Boehm and Kubista 2002; Kubista and Boehm 2006).

2.2.4 Physiological Functions

Regulation of neurotransmitter release

M_2 and M_4 AChR have been identified as the main inhibitory presynaptic muscarinic receptors in gene-targeted mouse models (Table 2). Dependent on the type of tissue, both receptors contributed to varying degrees to presynaptic autoinhibition. In cerebral cortex and hippocampus, inhibitory M_2 receptors prevailed over M_4 receptors to inhibit release of acetylcholine in vitro. In striatal preparations, M_4 receptors were functionally more important than M_2 receptors (Zhang et al. 2002b). In the periphery, M_4 receptor and to a smaller degree non-M_4 receptors, presumably M_2, inhibited acetylcholine release from atria, urinary bladder, and peripheral cholinergic motor nerves innervating the diaphragm (Slutsky et al. 2003; Zhou et al. 2002).

In addition to these autoreceptors, M_2, M_3, and M_4 receptors have also been identified as presynaptic heteroreceptors controlling noradrenaline release in atria (M_2, M_3), urinary bladder (M_2, M_4), and vas deferens (M_2, M_3, M_4) (Trendelenburg et al. 2003a; Trendelenburg et al. 2005). In the striatum, activation of mAChR facilitates dopamine release. Facilitation of potassium-mediated [^{3}H]-dopamine release by the muscarinic agonist oxotremorine was absent in M_4-deficient striatal preparations (Zhang et al. 2002b). M_4 receptors may be localized on cell bodies of striatal GABAergic neurons to increase dopamine release in basal ganglia.

M_1 AChR functions

The M_1 receptor subtype is involved in a variety of CNS functions (see Table 2). Agonist-induced seizures were absent in M_1-deficient but not affected in mouse lines lacking M_2-M_5 receptors (Bymaster et al. 2003b; Hamilton et al. 1997). Learning and memory tasks as well as nociception, motor coordination, and anxiety-associated behavioral traits were normal in M_1-deficient mice (Miyakawa et al. 2001). Mild performance deficits of M_1-deficient mice after fear conditioning were associated with significant locomotor hyperactivity of these mice (Miyakawa et al. 2001). In addition, a small reduction in hippocampal long-term potentiation was observed in M_1-deficient mice (Anagnostaras et al. 2003). Furthermore, M_1 receptors can inhibit the "M-current" (I_m) through voltage-gated K^+ channels in sympathetic ganglia (Hamilton et al. 1997), resulting in chronotropic and inotropic stimulation of the heart (Hardouin et al. 2002). Acetylcholine-mediated, slow voltage-independent

Table 2 Physiological Functions of Muscarinic AChR Subtypes

Function	Phenotype of Targeted Deletion of Muscarinic AChR Subtype				
	M_1	M_2	M_3	M_4	M_5
Central nervous system					
Autoreceptor: cortex, hippocampus		⇓⇓		⇓	
Autoreceptor: striatum		⇓		⇓⇓	
Heteroreceptor: activation of dopamine release	=	=	⇑	⇓⇓	⇓
Locomotor hyperactivity	⇑	=	=	⇑	=
Learning and memory	⇓=	⇓			⇓
Agonist-induced seizures	0	=	=	=	=
Body weight, food intake			⇓		
Locomotor coordination	=				
Agonist-induced tremor		0			
Agonist-induced akinesia		0			
Agonist-induced hypothermia		⇓			
Agonist-induced analgesia		⇓		⇓	
Anxiety-associated behavior	=				
Reward behavior to cocaine, morphine					⇓
Agonist-induced MAPK activation	⇓⇓				
Agonist-induced PI hydrolysis	0				
Peripheral nervous system					
Autoreceptor: atria, urinary bladder				⇓⇓	
Autoreceptor: phrenic nerve		⇓			
Heteroreceptor: inhibition of noradrenaline release in atria, vas deferens		⇓	⇓	⇓	
Agonist-induced bradycardia	=	⇓⇓	=	=	=
Inhibition of M current (I_m) in sympathetic ganglia	0				
Smooth muscle contraction (trachea, stomach, urinary bladder)		⇓	⇓⇓		
Pupil size			⇑		
Salivation			⇓		⇓
Pepsinogen secretion from gastric chief cells	⇓		⇓		
Cholinergic vasodilatation in cerebral vessels					0
Agonist-induced dilatation of the aorta			⇓⇓		

Assignment of functions as derived from studies in gene-targeted mice. For references, see text. Symbols: ⇑ increased function, ⇓ reduced function in receptor-deficient mice, 0 function absent, = normal function. "Agonist" refers to cholinergic agonist.

inhibition of N- and P/Q-type Ca^{2+} channels was also absent in M_1-deficient sympathetic ganglia (Shapiro et al. 1999). In the gastrointestinal tract, secretion of pepsinogen induced by cholinergic stimulation from gastric chief cells requires a mixture of M_1 and M_3 receptors (Xie et al. 2005).

M_2 AChR functions

In addition to its above mentioned functions as a presynaptic auto- and heteroreceptor, M_2 receptors are involved in a number of CNS functions. Agonist-induced whole-body tremor and akinesia were completely abolished, and hypothermia was significantly reduced in M_2-deficient mice (Gomeza et al. 1999a). M_2-deficient mice had problems in passive avoidance tests, demonstrating the role of this receptor subtype in learning and memory function (Tzavara et al. 2003). In the periphery, M_2 AChR mediate agonist-induced bradycardia (Stengel et al. 2000), contractions of smooth muscle cells in stomach, urinary bladder, and trachea (Kitazawa et al. 2007; Stengel et al. 2000; Unno et al. 2005; Unno et al. 2006), and analgesic effects (Bernardini et al. 2002; Gomeza et al. 1999a,b). Antinociception induced by the agonist oxotremorine was completely absent in mice lacking M_2 and M_4 receptors (Gomeza et al. 2001).

M_3 AChR functions

Mice with deletion of the M_3 receptor gene were lean and consumed less food than their wild-type litter-mates (Yamada et al. 2001b). Probably M_3 receptors in the hypothalamus are important to control food and energy intake. M_3-deficient mice showed an impaired glucose tolerance and greatly reduced insulin release (Gautam et al. 2006a). Their total body energy expenditure was significantly enhanced (Gautam et al. 2006b; Kitazawa et al. 2007; Unno et al. 2005; Unno et al. 2006). In the body periphery, the M_3 subtype mediates a significant part of smooth muscle contractions in peripheral organs (Matsui et al. 2000) and ACh-induced salivation (Matsui et al. 2000; Yamada et al. 2001a). The vasodilatation induced by cholinergic agonists was greatly reduced in M_3-deficient aorta (Khurana et al. 2005).

M_4 AChR functions

M_4 receptor-deficient mice showed a mild increase in locomotor activity. In the striatum, activation of M_4 AChR exerted an inhibitory effect on dopamine D_1 receptor-stimulated locomotor activity (Gomeza et al. 1999b). In striatal tissue slices from wild-type mice, the muscarinic agonist oxotremorine increased potassium-stimulated [^{3}H]-dopamine release (Zhang et al. 2002b). This effect of muscarinic agonists was completely abolished in M_4-receptor-deficient mice (Zhang et al. 2002b). In addition, M_4 receptors are involved in the antinociceptive effect of cholinergic agonists (Gomeza et al. 1999b).

M_5 AChR functions

M_5 AChR represent the predominant muscarinic subtype in dopamine-containing neurons of the pars compacta of the substantia nigra and the ventral tegmental area.

Activation of these receptors facilitates dopamine release in the striatum and in the nucleus accumbens. In M_5-deficient striatal preparations, the potency of oxotremorine to enhance potassium-stimulated release of [^{3}H]-dopamine was reduced (Yamada et al. 2001a; Zhang et al. 2002b). In the nucleus accumbens, deletion of M_5 receptors selectively blunted long-term dopamine stimulation and was thus associated with reduced rewarding effects of morphine and cocaine (Araya et al. 2006; Basile et al. 2002; Fink-Jensen et al. 2003; Thomsen et al. 2005). In cerebral arteries and arterioles of M_5-deficient mice, cholinergic vasodilatation was significantly blunted and cerebral arteries were constricted at rest (Yamada et al. 2001a). Reduced cerebral artery perfusion was associated with learning and memory deficits in M_5-deficient mice (Araya et al. 2006).

2.2.5 Relevance in Disease and Therapy

Results obtained from studies in gene-targeted mice indicate that ligands which selectively activate or inhibit single muscarinic receptor subtypes may be beneficial for the treatment of a number of diseases including, e.g., Alzheimer's and Parkinson's disease, depression, schizophrenia, and epilepsy.

In particular, M_1 AChR antagonists might be useful for the treatment of Parkinson's disease, as disruption of this receptor's gene led to increased locomotor behavior (Miyakawa et al. 2001) and facilitated dopamine release in basal ganglia (Gerber et al. 2001). M_1 agonists could be of value to treat symptoms of schizophrenia, which has been associated with enhanced dopamine release in various forebrain areas. As M_2-deficient mice displayed features of reduced memory function, including reduced hippocampal long-term potentiation (Tzavara et al. 2003), selective ligands could be tested to relieve symptoms of Alzheimer's disease. M_3 antagonists may be useful to reduce food intake, as mice lacking this subtype were lean and hypophagic (Yamada et al. 2001b). M_4-specific antagonists might be beneficial to relieve extrapyramidal motor symptoms in Parkinson's disease and M_4 agonists may represent novel analgesic compounds. Finally, antagonists which selectively bind to M_5 AChR could be helpful to treat drug addiction, as M_5-deficient mice were less susceptible to the rewarding effects of morphine and cocaine (Basile et al. 2002; Fink-Jensen et al. 2003).

3 Presynaptic Receptors for Adrenaline/Noradrenaline

3.1 Adrenoceptor Subtypes

Adrenoceptors (AR) are G protein-coupled receptors which mediate the biological effects of the endogenous catecholamines, adrenaline and noradrenaline. Nine adrenoceptor subtypes have been cloned which can be grouped into three different

functional classes (Bylund et al. 1994). α_1 adrenoceptors (α_{1A}, α_{1B}, α_{1D}) are primarily coupled to intracelluar $G_{q/11}$-dependent signals, α_2 receptors (α_{2A}, α_{2B}, α_{2C}) activate $G_{i/o}$ proteins, and the three β receptor subtypes (β_1, β_2, β_3) stimulate G_s proteins (Bylund et al. 1994). From these three receptor groups, the α_2 receptors have been identified as presynaptic inhibitory autoreceptors and will thus be discussed first.

3.2 α_2 Adrenoceptors

3.2.1 Transgenic and Gene-Targeted Models

The three genes encoding for α_2AR subtypes are located on different chromosomes in the human and mouse genome and have been targeted for deletion in murine embryonic stem cells (Altman et al. 1999; Link et al. 1995; Link et al. 1996). Using gene targeting, also a "knock-in" model inserting a point mutation in the α_{2A}AR gene (α_{2A}-D79N) has been generated (MacMillan et al. 1996). Mice lacking α_{2A}AR or α_{2C}AR were born at the expected Mendelian ratios and developed normally (Altman et al. 1999; Link et al. 1995). In contrast, deficiency of the α_{2B} subtype was associated with decreased survival due to vascular defects, which became first apparent in mice lacking all three subtypes (α_{2ABC}-/-) (Philipp et al. 2002b).

3.2.2 Occurrence

All three α_2AR subtypes have been localized in the CNS and in peripheral tissues (Table 3) (Aoki et al. 1994; Bücheler et al. 2002; Fagerholm et al. 2004; MacDonald and Scheinin 1995; Moura et al. 2006; Nicholas et al. 1993; Nicholas et al. 1996; Rosin et al. 1996; Scheinin et al. 1994; Talley et al. 1996; Uhlen et al. 1997). In brief, the α_{2A}AR subtype is widely expressed in the CNS, including brain stem nuclei, locus coeruleus, cerebral cortex, hypothalamus, and hippocampus. In the periphery, α_{2A}AR have been found in lung, kidney, and spleen. α_{2B}AR are mostly expressed in non-neuronal tissues, including heart, liver, lung, and kidney. In the CNS, the α_{2B} subtype shows most discrete expression in thalamic nuclei. The third subtype, α_{2C}, is again mostly expressed in the CNS, where it was identified in the hippocampus, cortex, striatum, and olfactory tubercle (Table 3).

3.2.3 Presynaptic Signaling

Upon activation all three α_2AR subtypes are coupled to their intracellular messenger systems via heterotrimeric $G_{i/o}$ proteins (Figure 2). Intracellular consequences of α_2AR activation are reduced cAMP levels due to inhibition of adenylyl cyclase, opening of GIRK K^+ channels, inhibition of neuronal Ca^{2+} channels, and

Table 3 Occurrence of α_2 Adrenoceptor Subtypes

Organ/Tissue	α_2 Adrenoceptor Subtype		
	α_{2A}	α_{2B}	α_{2C}
Central nervous system			
Brain stem	++		
Cerebral cortex	+		+
Hypothalamus	+		
Hippocampus	+		+
Spinal cord	+	+	+
Striatum			++
Thalamus		(+)	
Peripheral organs			
Adrenal medulla	+	+	+
Arteries		+	
Heart		+	
Kidney	+	+	(+)
Liver		+	
Lung	+	+	
Salivary glands	+		
Spleen	+		
Thymus	+		
Vessels		+	

For references, see text. Symbols: ++ predominant subtype, + expressed subtype, (+) low abundant subtype.

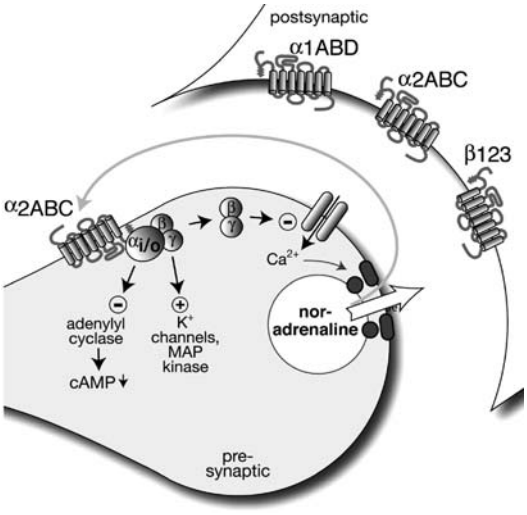

Fig. 2 Presynaptic signaling mechanisms of α_2 adrenoceptor subtypes (α_{2A}, α_{2B}, α_{2C}). Abbreviations: $\alpha_{i/o}$, β, γ, subunits of $G_{i/o}$ heterotrimeric GTP-binding proteins; α_{1ABD}, α_{2ABC}, β_{123}, adrenoceptor subtypes; MAP, mitogen-activated protein kinase.

stimulation of mitogen-activated kinase ERK1/2 (Cussac et al. 2001). Several mechanism may contribute to the inhibition of presynaptic neurotransmitter release, e.g., reduced Ca^{2+} channel opening, activation of K^+ channels, and also direct regulation of the exocytotic release machinery (for discussion see Miller 1998; Wu and Saggau 1997). Among these proposed mechanisms, the inhibitory effect of $G\beta\gamma$ subunits on presynaptic Ca^{2+} channels may be the dominant effect observed in postganglionic sympathetic neurons in culture (Figure 2) (Boehm and Kubista 2002; Kubista and Boehm 2006; Stephens and Mochida 2005).

3.2.4 Physiological Functions

Regulation of neurotransmitter release by α_2 autoreceptors

α_2AR are the main inhibitory presynaptic feedback receptors which are essential to control the release of noradrenaline from adrenergic neurons (Figure 3). Pharmacological studies using ligands with partial subtype-selectivity have led to the hypothesis that the α_{2A}AR subtype may be the major inhibitory autoreceptor in the sympathetic system (for reviews, see Starke 2001; Starke et al. 1989). This concept was later supported and extended by studies in gene-targeted mice (for detailed reviews, see Hein 2001; Philipp and Hein 2004; Philipp et al. 2002a). In brain and peripheral tissues isolated from α_{2A}-deficient mice, maximal inhibition of [^{3}H]-noradrenaline release by exogenous α_2-agonists was significantly blunted as compared with preparations from wild-type mice (Altman et al. 1999; Hein et al. 1999; Trendelenburg et al. 1999; Vonend et al. 2007). Deletion of the other α_2AR subtypes, α_{2B} or α_{2C}, did not affect maximal agonist-induced inhibition of noradrenaline release (Hein et al. 1999; Trendelenburg et al. 2001a), confirming pharmacological data that the α_{2A}AR is indeed the predominant presynaptic autoreceptor in adrenergic neurons. However, the fact that α_2 agonists still inhibited neurotransmitter release in α_{2A}-deficient tissues indicated that non-α_{2A} receptors contributed to presynaptic inhibition. In the CNS, α_{2A} and α_{2C} receptors could be identified as presynaptic regulators; in peripheral organs all three α_2AR subtypes, α_{2A}, α_{2B}, and α_{2C} operated as feedback inhibitors in vitro (Figure 3) (Trendelenburg et al. 2003b). α_2AR subtypes may not be completely redundant in their presynaptic function, but may serve different roles in the feedback inhibition. As one example, α_2AR showed differences in their frequency-activity relationship: In atria, α_{2C}AR inhibited [^{3}H]-noradrenaline release better at lower frequencies of nerve stimulation, whereas the α_{2A}AR operated at higher stimulation frequencies (Hein et al. 1999; Scheibner et al. 2001b). Furthermore, α_2AR subtypes differed in their postnatal maturation. Whereas the α_{2A}AR was functional immediately after birth, non-α_{2A}AR effects could not be observed within the first postnatal days in intact tissues or in isolated sympathetic neurons (Schelb et al. 2001; Trendelenburg et al. 2001b).

In addition to the presynaptic α_2 autoreceptor subtypes which were identified in CNS and sympathetic nerves, studies in gene-targeted mice uncovered α_{2C}AR as autoreceptors controlling catecholamine release from chromaffin cells (Brede

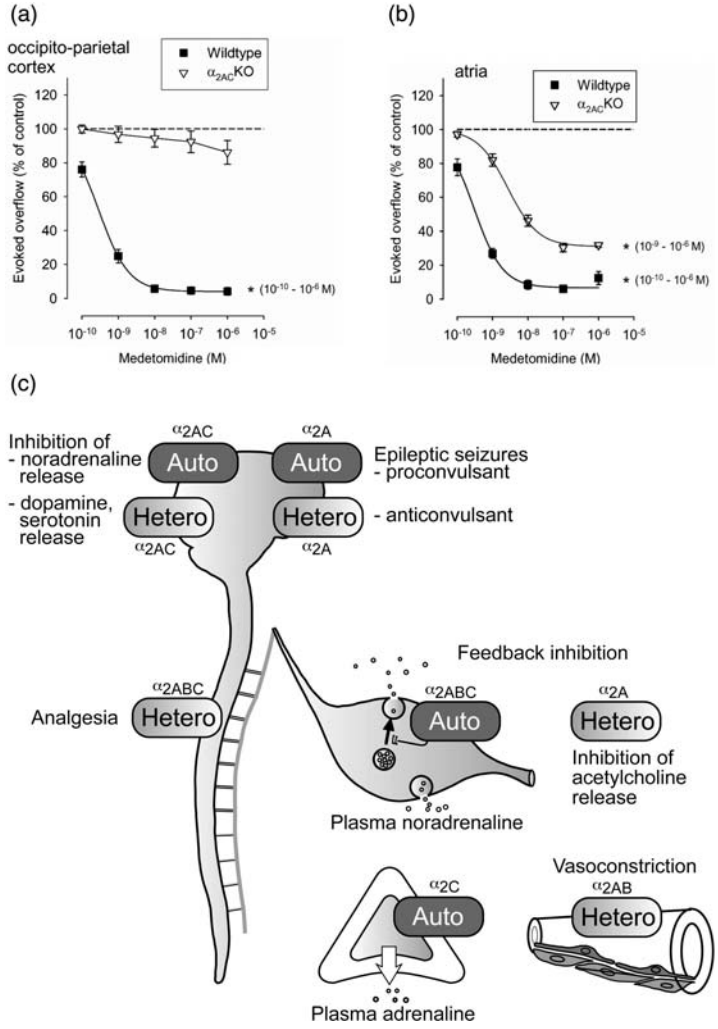

Fig. 3 Autoreceptor versus heteroreceptor functions of α_2-adrenoceptor subtypes. (**a** and **b**) Inhibition of electrically evoked [^{3}H]-adrenaline release by the α_2-agonist, medetomidine, from mouse brain cortex (**a**) or heart atria (**b**). In wild-type tissue specimens, medetomidine inhibited transmitter release by >90%. In tissues from α_{2AC}-deficient mice, the agonist effect was absent (**a**, cortex) or significantly reduced (**b**, atria). Reproduced with permission from Trendelenburg et al. 2003b. (**c**) Overview of auto- and heteroreceptor functions of α_2-adrenoceptor subtypes. For references, see text.

et al. 2002; Brede et al. 2003). Plasma and urine adrenaline levels were >2-fold higher in α_{2C}-deficient mice than in wild-type mice. Activation of α_{2C}-receptors expressed in isolated mouse chromaffin cells inhibited voltage-gated Ca^{2+} channels and reduced secretion of adrenaline. Inhibition of adrenaline secretion by this "presynaptic" α_{2C}-receptor is physiologically important, as mice deficient in this

receptor showed enhanced cardiac hypertrophy and heart failure in response to cardiac pressure overload (Brede et al. 2002). Interestingly, adrenal catecholamines are differentially regulated by α_2AR subtypes. Whereas secretion of adrenaline seems to be predominantly controlled by α_{2C}, all three α_2AR subtypes may control noradrenaline release from adrenal preparations in vitro (Moura et al. 2006).

α_2 Adrenoceptors as heteroreceptors

In addition to their functions as presynaptic autoreceptors, α_2AR can also modulate release of other neurotransmitters (Figure 3). In the CNS, α_{2A} and α_{2C} receptors inhibit dopamine release in basal ganglia (Bücheler et al. 2002) as well as serotonin secretion in mouse hippocampus and brain cortex (Scheibner et al. 2001a). In the enteric nervous system, the release of acetylcholine as determined by [^{3}H]-choline overflow from tissue slices was selectively inhibited by α_{2A}AR (Scheibner et al. 2002).

α_{2A} Adrenoceptor functions

One of the main functions of the α_{2A}AR is to control sympathetic noradrenaline release. Dysfunction or deletion of this receptor leads to hypertension and tachycardia and accelerates the development of cardiac hypertrophy and heart failure in mice (Brede et al. 2002; Hein et al. 1999; Makaritsis et al. 1999b). Central α_{2A}AR are essential for the hypotensive effects of α_2 agonists, like clonidine, moxonidine, and rilmenidine (Altman et al. 1999; MacMillan et al. 1996; Zhu et al. 1999). α_{2A}-deficient mice showed greater spontaneous blood pressure variations at rest and suppressed baroreflex gain (Niederhoffer et al. 2004). The sedative and anesthetic-sparing effects of α_2 agonists were exclusively mediated by α_{2A}AR (Lakhlani et al. 1997). α_{2A}AR mediated part of the antinociceptive effect of α_2 agonists (Fairbanks and Wilcox 1999; Hunter et al. 1997; Stone et al. 1997). In mice, α_2 agonists may also produce pro- and anticonvulsant effects on seizure susceptibility (Janumpalli et al. 1998; Szot et al. 2004). Using dopamine β-hydroxylase-deficient mice, presynaptic α_{2A} autoreceptors could be identified as proconvulsant receptors, while postsynaptic α_{2A}AR mediated anticonvulsant effects of α_2 agonists (Figure 3) (Szot et al. 2004).

α_{2B} Adrenoceptor functions

Neuronal α_{2B}AR seems to play an important role in the antinociceptive pathway of nitrous oxide, which is used in anesthesia for its strong analgesic action (Sawamura et al. 2000). In the periphery, α_{2B}AR elicit vasoconstriction after rapid intravascular injection of α_2 agonists (Figure 3) (Link et al. 1996; Paris et al. 2003). Central α_{2B}AR may be important in the development of hypertension after salt-loading and nephrectomy (Kintsurashvili et al. 2001; Makaritsis et al. 1999a; Makaritsis et al. 2000).

α_{2C} Adrenoceptor functions

As mentioned above, α_{2C}AR are important feedback regulators to control adrenal adrenaline secretion (Brede et al. 2003; Moura et al. 2006). However, further studies are needed to identify the physiological relevance of the adrenal feedback mechanism. In the CNS, α_{2C}AR affect a number of behavioral functions (for a detailed review, see Scheinin et al. 2001). Activation of α_{2C}AR interferes with spatial working memory in mice, whereas stimulation of α_{2A}AR and/or α_{2B}AR may actually improve spatial working memory in mice (Bjorklund et al. 1999; Bjorklund et al. 2000; Bjorklund et al. 2001).

3.2.5 Relevance in Disease and Therapy

Agonists with sufficient selectivity for α_{2A}AR or α_{2C}AR may be helpful to prevent the detrimental cardiovascular effects of enhanced sympathetic activity (Brede et al. 2002). The failure of the α_2 agonist, moxonidine, to produce a beneficial effect in patients with chronic heart failure in the MOXCON trial has been attributed to excessive sympatholytic effects of the drug (Cohn et al. 2003). Experimental studies in mice have clearly demonstrated that dysfunction of α_{2A}AR and/or α_{2C}AR contribute to the development of cardiac hypertrophy and failure (Brum et al. 2002; Hein et al. 1999). Interestingly, sedation which has been a frequent side effect of clinical α_2 agonists may be avoided by using partial α_{2A} agonists, as hypotensive but not sedative α_2AR have a high number of spare receptors (Tan et al. 2002). Furthermore, α_{2A} activation may limit the detrimental effects of hypoxia, ischemia, and injury in the brain (Ma et al. 2004; Paris et al. 2006).

Due to the absence of major CNS phenotypes in α_{2B}-deficient mice, selective α_{2B} activation in the spinal cord may represent a novel target for the treatment of chronic pain (Sawamura et al. 2000; Zhang et al. 1999). Drugs acting via α_{2C}AR may gain therapeutic value in disorders associated with enhanced startle responses and sensorimotor gating deficits, such as schizophrenia, attention deficit disorder, posttraumatic stress disorder, and drug withdrawal (Scheinin et al. 2001).

3.3 α_1 Adrenoceptors

3.3.1 Transgenic and Gene-Targeted Models

Despite the fact that noradrenergic neurons innervate many regions of the CNS, the contribution of α_1AR to neuronal functions of catecholamines is largely unknown. Recently, gene-targeting and transgenic experiments in mice have provided novel insight in this area. Unexpectedly, transgenic expression of wild-type or constitutively active α_{1B}AR under control of their own promotor resulted in a phenotype which was characterized by severe neurological degeneration with Parkinson-like

Table 4 Occurrence of α_1 Adrenoceptor Subtypes

Organ/Tissue	α_1 Adrenoceptor Subtype		
	α_{1A}	α_{1B}	α_{1D}
Central nervous system			
Brain stem	+		+
Cerebral cortex	+	+	+
Hypothalamus	+		
Hippocampus			+
Pineal gland		+	
Spinal cord	+	+	++
Thalamus		+	+
Peripheral organs			
Bladder	+		+
Heart	++	++	(+)
Liver	+	+	
Prostate	+		
Vessels	+	+	+

For references, see text. Symbols: ++ predominant subtype, + expressed subtype, (+) low abundant subtype.

locomotor deficits and epileptic seizures (Zuscik et al. 2000). Furthermore, target deletions have been integrated into the three α_1AR genes of the mouse (Cavalli et al. 1997; Rokosh and Simpson 2002; Tanoue et al. 2002).

3.3.2 Occurrence

α_1AR are abundantly expressed in the CNS (Table 4) (Drouin et al. 2002; Michelotti et al. 2000; Piascik and Perez 2001; Xiao et al. 2006). The α_{1A} subtype has been primarily localized in the olfactory system, hypothalamus, brain stem, and spinal cord (Day et al. 1997). α_{1B}AR were identified in the pineal gland, in thalamic nuclei, the lateral nucleus of the amygdala, intermediate and deep layers of the cortex, as well as dorsal and median raphe nuclei (Day et al. 1997; McCune et al. 1993; Pieribone et al. 1994). Among the three α_1AR subtypes, the α_{1D}AR showed most discrete CNS expression in the olfactory bulb, reticular thalamic nucleus, hippocampus, layers II-V of the cerebral cortex, regions of the amygdala, motor nuclei of the brainstem, and in the spinal cord (Day et al. 1997; McCune et al. 1993; Pieribone et al. 1994). This pattern of expression was confirmed in mice with targeted expression of β-galactosidase in the α_{1D}AR gene locus. Cortex, hippocampus, olfactry bulb, dorsal geniculate, and ventral posterolateral nuclei of the thalamus were densely labeled with β-galactosidase (Sadalge et al. 2003).

3.3.3 Presynaptic Signaling

Despite their abundant expression in the CNS (see Section 3.3.2), no studies have yet been performed in gene-targeted mice to address the precise subcellular localization and signal transduction of α_1AR subtypes.

3.3.4 Physiological Functions

In the past, cardiovascular functions of α_1AR have generated the greated interest and research effort. However, cardiovascular phenotypes which have been observed in gene-targeted mouse models deficient in α_1AR are beyond the scope of this review and have been reviewed elsewhere (Chen and Minneman 2005; Koshimizu et al. 2006; Piascik and Perez 2001; Tanoue et al. 2003).

α_{1A} Adrenoceptor functions

Despite the fact that α_{1A}AR are expressed in the CNS, no studies addressing the function of this receptor in gene-targeted mice have appeared yet.

α_{1B} Adrenoceptor functions

A number of behavioral phenotypes have been reported in α_{2B}-deficient mice, including altered responses to *d*-amphetamine (Auclair et al. 2002; Drouin et al. 2002). Amphetamine and other psychostimulants cause drug addiction in humans and induce locomotor stimulant effects in rodents. Single drug doses enhance locomotor activity and repeated drug administration leads to behavioral sensitization with greatly enhanced activity. In α_{1B}-deficient mice, locomotor hyperactivity and behavioral sensitization to amphetamine, cocaine, and morphine were significantly decreased (Drouin et al. 2002). Basal and amphetamine-stimulated extracellular dopamine levels were lower in α_{1B}-deficient mice than in wild-type control animals (Auclair et al. 2004). However, basal dopaminergic transmission and locomotor activity after administration of a dopamine D1 agonist were similar in wild-type and α_{1B} mutant mice (Drouin et al. 2002). α_{1B} mutant mice showed an exaggerated biochemical and behavioral response to the serotonin releasing drug, *p*-chloroamphetamine (Salomon et al. 2006). Salomon et al. suggest that repeated amphetamine administration leads to behavioral sensitization due to reduced reciprocal inhibition of serotonin and noradrenaline signaling via 5-HT_{2A} and α_{1B} receptors, respectively. Additional studies indicate that α_{1B}AR are involved in modulation of memory consolidation and fear-motivated exploratory activity (Knauber and Muller 2000) as well as in spatial learning (Spreng et al. 2001).

α_{1D} Adrenoceptor functions

This receptor subtype has been associated with the control of pain perception (Harasawa et al. 2003), motor coordination (Mishima et al. 2004), working memory, and attention (Mishima et al. 2004), as well as exploratory behavior (Sadalge et al. 2003). Furthermore, α_{1D}-deficient mice showed altered functions of the lower urinary tract (Chen et al. 2005).

3.3.5 Relevance in Disease and Therapy

As mentioned above, transgenic expression of α_{1B}AR under control of its native promotor led to a complex phenotype with multiple system atrophy and overt neurodegeneration (Zuscik et al. 2000). This transgene expressed neurodegenerative disease that resembled Parkinson's syndrome. Neurons and oligodendocytes were filled with cytoplasmic inclusion bodies which were positive for α-synuclein and ubiquitin and therefore may be classified as a synucleinopathy (Papay et al. 2002). Other phenotypes characteristic for multiple system atrophy, such as Purkinje cell loss in the cerebellum and degeneration of the intermediolateral cell columns of the spinal cord, also existed in transgenic α_{1B}AR mice (Papay et al. 2002). Better understanding of this complex phenotype may identify the α_{1B}AR as a target for the treatment of neurodegenerative diseases.

3.4 β Adrenoceptors

3.4.1 Transgenic and Gene-Targeted Models

β-Adrenoceptors are best known for their role in the regulation of cardiovascular, airway, uterine, and peripheral metabolic functions. Mice lacking β_1, β_2 and β_3 receptors have been generated (Chruscinski et al. 1999; Rohrer et al. 1996; Susulic et al. 1995), but only few studies have used these models to identify subtype-specific functions of β-adrenoceptors in neurons.

3.4.2 Occurrence

β-Adrenoceptor subtypes are widely expressed throughout the body (Table 5) (Arch 2002; Arner 2005; Brodde and Michel 1999; Dennedy et al. 2002; Guimaraes and Moura 2001; Michel and Vrydag 2006; Ordway et al. 1987, 1988; Rohrer et al. 1999). The brain is densely innervated by noradrenaline- and adrenaline-containing neurons, most of which originate in the locus coeruleus or in the reticular formation (Foote et al. 1983). β-adrenoceptors have been identified in the target regions of brain stem catecholaminergic neurons. By *in situ* hybridization of rat brains, β_1-adrenoceptors were identified in cerebral cortex, thalamus, pineal gland, and sympathetic ganglia (Nicholas et al. 1996). In contrast, the β_2AR subtype was most densely expressed in the olfactory bulb, cerebral cortex, hippocampus, thalamus and hypothalamus, pineal gland, and spinal cord. In the pineal gland, noradrenaline released from sympathetic nerves controls the circadian rhythm of melatonin synthesis via β_1AR (for review, see Simonneaux and Ribelayga 2003).

Table 5 Occurrence of β Adrenoceptor Subtypes

Organ/Tissue	β Adrenoceptor Subtype		
	β_1	β_2	β_3
Central nervous system			
Cerebral cortex	+	+	
Cortex	+	+	
Hippocampus	+	+	
Hypothalamus		+	
Pineal gland	+	+	
Spinal cord		+	
Thalamus	+	+	
Sympathetic ganglia	+	+	
Peripheral organs			
Smooth muscle	+	++	(+)
Endothelium	+	++	(+)
Juxtaglomerular apparatus	+		
Heart	++	+	(+)
Pancreas		+	
Adipocyte	+	+	++
Lung	+	++	
Urinary bladder		+	+
Uterus		+	+
Skeletal muscle	+	++	+
Kidney	+		

For references, see text. Symbols: ++ predominant subtype, + expressed subtype, (+) low abundant subtype.

3.4.3 Presynaptic Signaling

βAR subtypes are primarily coupled to stimulatory G_s proteins, which activate adenylyl cyclase to increase intracellular cAMP levels. Based on pharmacological studies, presynaptic βAR belong mostly to the β_2AR subtype which can facilitate noradrenaline release from sympathetic nerves (Trendelenburg et al. 2000). Most likely, cAMP-dependent activation of protein kinase A resulting in phosphorylation of protein components of the vesicle exocytosis machinery is responsible for the enhanced transmitter secretion (Figure 4) (see discussion in Boehm and Kubista 2002; Kubista and Boehm 2006).

3.4.4 Physiological Functions

In addition to their peripheral functions, including presynaptic facilitation of noradrenaline release, β-adrenoceptors play a role in regulating numerous functions of the central nervous system, including sympathetic tone, learning and memory, mood, and food intake. Mice lacking dopamine β-hydroxylase or β-adrenoceptor subtypes were used to study the involvement in memory functions. Noradrenaline acting at β_1-receptors was found to be essential for retrieval of contextual and spatial memory, but it was not necessary for retrieval of emotional memories (Murchison

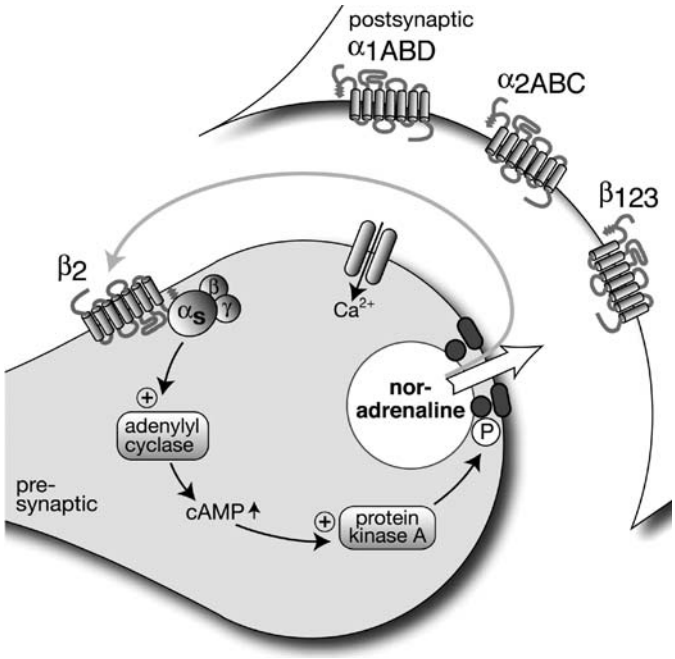

Fig. 4 Presynaptic signaling mechanisms of β_2 adrenoceptors. Abbreviations: $\alpha_{i/o}$, β, γ, subunits of $G_{i/o}$ heterotrimeric GTP-binding proteins; α_{1ABD}, α_{2ABC}, β_{123}, adrenoceptor subtypes.

et al. 2004; Winder et al. 1999). These studies suggest that memory retrieval has separate pathways from memory acquisition, consolidation, or storage and that separate signaling pathways may control these two aspects of memory function.

3.4.5 Relevance in Disease and Therapy

While the initial studies in gene-targeted mice suggest that βAR in the CNS may play an important role in memory function, further studies are required to better understand the role of central βAR and their potential role as therpeutic targets. However, due to the prominent role of β_1AR and β_2AR in the cardiovascular system, it may be difficult to identify ligands for CNS therapies which lack significant side effects.

4 Conclusions

Molecular cloning has led to the identification of a great complexity of receptor subtypes in the cholinergic and adrenergic systems. Five muscarinic GPCRs and altogether nine adrenoceptors mediate the biological effects of acetylcholine and

adrenaline/noradrenaline, respectively. Despite the fact that these receptors were the first presynaptic receptors to be described more than three decades ago, their precise physiological function and therapeutic potential has remained elusive until gene-targeted mouse models became available. These mouse models have greatly helped to identify specific roles for each of these receptors. In the context of this overview, at least two muscarinic receptors (M_2 and M_4), as well as three adrenoceptors (α_{2A}, α_{2B}, α_{2C}), have been demonstrated to serve as presynaptic inhibitory autoreceptors in their respective transmitter systems. Additional receptor subtypes act as facilitory receptors to enhance presynaptic neurotransmitter secretion. The next challenges in this field will be to identify the molecular basis of the complex intracellular signaling properties of these receptors and their differential roles in neurons and thus to validate these receptors as therapeutic target molecules.

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Presynaptic Receptors for Dopamine, Histamine, and Serotonin

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Abstract Presynaptic receptors for dopamine, histamine and serotonin that are located on dopaminergic, histaminergic and sertonergic axon terminals, respectively, function as autoreceptors. Presynaptic receptors also occur as heteroreceptors on other axon terminals. Auto- and heteroreceptors mainly affect Ca^{2+}-dependent exocytosis from the receptor-bearing nerve ending. Some additionally subserve other presynaptic functions.

Presynaptic dopamine, histamine and serotonin receptors are involved in various (patho)physiological conditions. Examples are the following:

Dopamine autoreceptors play a role in Parkinson's disease, schizophrenia and drug addiction. Dopamine heteroreceptors affecting the release of acetylcholine and of amino acid neurotransmitters in the basal ganglia are also relevant for Parkinson's disease. Peripheral dopamine heteroreceptors on postganglionic sympathetic terminals influence heart rate and vascular resistance through modulation of noradrenaline release.

Blockade of histamine autoreceptors increases histamine synthesis and release and may support higher CNS functions such as arousal, cognition and learning. Peripheral histamine heteroreceptors on C fiber and on postganglionic sympathetic fiber terminals diminish neuropeptide and noradrenaline release, respectively. Both inhibititory effects are beneficial in myocardial ischemia. The inhibition of neuropeptide release also explains the antimigraine effects of some agonists of presynaptic histamine receptors.

Upregulation of presynaptic serotonin autoreceptors is probably involved in the pathogenesis of major depression. Correspondingly, antidepressant treatments can be linked with a reduced density of 5-HT autoreceptors. 5-HT Heteroreceptor activation diminishes acetylcholine and GABA release and may therefore increase anxiety. In the periphery, presynaptic 5-HT heteroreceptor agonists shorten migraine attacks by inhibition of the release of neuropeptides from trigeminal afferents, apart from their constrictive action on meningeal vessels.

1 Introduction

Our knowledge of presynaptic dopamine and serotonin receptors dates back to the 1970s (Farnebo and Hamberger 1971). Presynaptic histamine receptors were discovered in 1983 (Arrang et al. 1983). Presynaptic dopamine receptors occur as autoreceptors, i.e., on dopaminergic axon terminals, and as heteroreceptors on nondopaminergic axon terminals. By analogy the same holds true for presynaptic histamine and serotonin receptors. The early days of the dopamine autoreceptors were stormy, but the controversies were finally solved (see Starke et al. 1989). The main function that presynaptic receptors affect is transmitter release, which in this article means Ca^{2+}-dependent exocytosis. However, some receptors discussed in

this chapter also modify other presynaptic functions, such as transmitter synthesis, using signal transduction cascades somewhat different from those leading to modulation of exocytosis.

Dopamine is one of the main neurotransmitters in the central nervous system (CNS), but it also plays a role in the periphery, where, for example, it acts as an autocrine/paracrine substance in the kidneys, the mesenteric organs, and the lungs (Goldstein et al. 1995). In the CNS, cell bodies of dopamine neurons are to be found in midbrain areas, hypothalamus, olfactory bulb, and retina. Cells responsive to dopamine express five receptor types, all G-protein-coupled and known as D_1, D_2, D_3, D_4 and D_5. Through alternative splicing the D_2 receptor gene can generate two forms of the receptor, D_{2long} and D_{2short} (Dal Toso et al. 1989).

The D_1-like receptors (D_1, D_5) couple predominantly to Gs and thus can stimulate adenylyl cyclase, yielding cAMP. The D_2-like receptors (D_2, D_3, D_4) couple to Gi/o proteins and may inhibit adenylyl cyclase or modulate many other different signalling molecules and pathways. D_3 receptors may also couple to Gs (Obadiah et al. 1999; Ilani et al. 2002).

The receptor types occurring presynaptically are mainly D_2-like. Of these, the D_2 type prevails, whereas D_3 and D_4 are uncommon. D_1-like receptors were rarely further differentiated as D_1 or D_5.

Apart from its role as a major mediator of inflammation and allergic reactions and as physiological regulator of gastric acid secretion, **histamine** is also a neurotransmitter in the CNS. Central histaminergic cell bodies are located in the posterior hypothalamus and project diffusely to almost all brain regions and to the spinal cord. There are four types of histamine receptors, all G-protein-coupled, H_1, H_2, H_3 and H_4. H_1 receptors couple to Gq/11 proteins. H_2 receptors couple to Gs. H_3 and H_4 receptors couple to Gi/o.

The histamine H_3 receptor was first identified as an autoreceptor (Arrang et al. 1983), negatively regulating the synthesis and release of histamine in the CNS. Release-inhibiting H_3 receptors have also been identified on terminals of various other neurons in the CNS and the periphery. H_1, H_2 and H_4 receptors function as presynaptic receptors rarely, if ever (Schwartz et al. 1986; Tanaka and Ichikawa 2006).

H_3 receptors are hetereogeneous for three reasons: species differences in amino acid sequence and, hence, pharmacology (Zaragoza et al. 2004); receptor oligomerization (Bakker 2004); and the existence of, so far, six splice variants, H_{3A}, H_{3B}, H_{3C}, H_{3D}, H_{3E}, and H_{3F} (Bakker et al. 2006). The latter three variants, H_{3D}, H_{3E}, and H_{3F}, are retained intracellularly and may control the cell surface expression of H_{3A}, H_{3B}, and H_{3C}. Since most reports on histamine receptor modulation of transmitter release only refer to the H_3 receptor, not to the splice variants, I do not differentiate between these in the following.

The main source of **serotonin** (5-HT) is the enterochromaffin cells, which release it as tissue hormone either into the gut lumen or the portal vein circulation. In the CNS, cell bodies of 5-HT-containing neurons are concentrated along the raphé nuclei of the brainstem and are also found in more lateral regions and in the reticular formation. The neurons terminate in almost all brain regions and in the spinal cord.

5-HT interacts with a large diversity of G-protein-coupled receptors, namely the 5-$HT_{1,2,4,5,6}$ and $_7$ families, and in addition with a ligand-gated cationic channel, 5-HT_3. The diversity has been explained by the fact that serotonin is one of the oldest neurotransmitters in evolution. The 5-HT_1 receptor family (5-$HT_{1A,1B,1D,1E\ and 1F}$) couples mainly to Gi/o. 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors couple to Gq/11. 5-HT_4, 5-HT_6, and 5-HT_7 receptors couple to Gs. The 5-HT_{5A} receptor couples principally to Gi/o. The 5-HT_{5B} receptor seems to occur only in rodents, and no transduction mechanism has been identified.

Members of all serotonin receptor families except the 5-HT_6 receptor (Hamon et al. 1999; Marcos et al. 2006) occur presynaptically. Presynaptic 5-HT_3 receptors are discussed in this *Handbook* in the chapter on ionotropic receptors by Dorostkar and Boehm.

With dopamine receptors on terminals with seven different transmitters, histamine receptors on terminals with eight different transmitters, serotonin receptors on terminals with eight different transmitters, and several of these receptors occurring in more than a single subtype, presynaptic modulation is manifold. The question always arises, of course – and I shall try to answer it in each case – whether a particular receptor receives an endogenous agonist input or lacks it and, hence, belongs to those presynaptic receptors that are "vestiges of evolution which continue to exist because they do us no harm" (Starke 1981).

2 Presynaptic Receptors for Dopamine

Presynaptic dopamine receptors modulate the release of a variety of neurotransmitters. When looking at Table 1 one may be impressed by the number of studies and the number of neuron systems studied. It is clear that, overall, D_1-like presynaptic receptors are facilitatory, whereas D_2-like presynaptic receptors are inhibitory; in the case of the few exceptions, the reason often remains unclear.

A final general observation on Table 1 is that, in several cases, both facilitation through D_1-like and inhibition by D_2-like receptors have been reported. This holds true for noradrenaline release in rat nucleus accumbens, acetylcholine release in rat striatum, GABA release in rat striatum, nucleus accumbens, and ventral tegmental area (VTA), and glutamate release in rat substantia nigra pars reticulata.

2.1 Presynaptic Dopamine Autoreceptors: Modulation of Dopamine Release

The main effect mediated by presynaptic dopamine autoreceptors is modulation of exocytotic release of dopamine (Table 1); however, the receptors in addition subserve other functions (Table 2).

Table 1 Tissues in which neurotransmitter release is increased (↑) or decreased (↓) by presynaptic dopamine receptor activation. Receptor classification (except with superscript[a]) as by the authors cited. Designation as D_1-like or D_2-like where no differentiation between D_1 and D_5, or between D_2, D_3 and D_4, was made

Release of Transmitter	Species and Tissue	Receptor Type	References
Dopamine (see 2.1)	Rat neocortex	D_2-like↓	1; Fedele et al. 1999
	striatum	D_2-like↓	1; see Goldstein et al. 1990
	nucleus accumbens	D_3↓	Joseph et al. 2002
	hypothalamus	D_2-like↓	1
	median eminence	D_2-like↓	1
	pituitary neurointermediate lobe	D_2-like↓	1
	retina	D_2↓	see Nguyen-Legros et al. 1999
	Mouse striatum	D_2-like↓	Bull et al. 1991
	cultured mesencephalic cells	D_2↓,D_3↓	Tang et al. 1994
	Guinea pig spinal cord	D_2-like↓	1
	retina	D_2-like↓	Weber et al. 2001
	Rabbit neocortex	D_2-like↓	1
	nucleus caudatus	D_2-like↓	1
	tuberculum olfactorium	D_2-like↓	1
	retina	D_2-like↓	1; see Nguyen-Legros et al. 1999
	Cat nucleus caudatus	D_2-like↓	1
	Human neocortex	D_2↓	Fedele et al. 1999; Löffler et al. 2006
Nor-adrenaline (see 2.4)	Rat medial prefrontal cortex	D_1-like↑	Pan et al. 2004
	hippocampus	D_2-like↓	Monnet 2002
	nucleus accumbens	D_1-like↑, D_2-like↓	Vanderschuren et al. 1999
	kidney	D_2-like↓	2
	portal vein	D_2-like↓	2
	Guinea pig mesenteric artery	D_2-like↓	2
	Rabbit hippocampus	D_2-like↓	Jackisch et al. 1985
	hypothalamus	D_2-like↓	Galzin et al. 1982
	ear artery	D_2-like↓	2
	heart	D_2-like↓	2
	lung	D_2-like↓	2
	kidney	D_2-like↓	2
	Cat spleen	D_2-like↓	2
	nictitating membrane	D_2-like↓	2
	atria	D_2-like↓	2
	Dog gracilis muscle	D_2-like↓	2
	saphenous vein	D_2-like↓	2
	kidney	D_2-like↓	2
	Cattle kidney	D_2-like↓	2
	Human peripheral vessels	D_2-like↓	2; Haeusler et al. 1992; Mannelli et al. 1997
	peripheral tissues	D_2-like↓	Francis 1995; Murphy 2000
	kidney	D_2-like↓	Rump et al. 1993
	right atrium	D_2↓	Rump et al. 1995
	gastric and uterine arteries	D_2-like↓	Morgadinho et al. 1999

(*Continued*)

Table 1 Continued

Release of Transmitter	Species and Tissue	Receptor Type	References
Serotonin (see 2.5)	Rat hypothalamus	D_2-like↑[a]	Cox et al. 1980
	substantia nigra	D_1-like↓	Benkirane et al. 1987
Acetyl-choline (see 2.6)	Rat striatum	D_1-like↑	Consolo et al. 1987
	striatum	D_2-like↓	1; 4; Gorell and Czarnecki 1986; Stoof et al. 1982; Enz et al. 1990
	striatum	D_2↓	Yan et al. 1997
	tuberculum olfactorium	D_2-like↓	1; 4
	nucleus acumbens	D_2-like↓	1; 4
	heart	D_2↓	3
	Mouse striatum	D_2↓	Wang et al. 2006
	Guinea pig cerebral cortex	D_2-like↓	1; 4
	caudate nucleus	D_2-like↓	1; 4
	tuberculum olfactorium	D_2-like↓	1; 4
	brain stem	D_2-like↓	1; 4
	stomach	D_2↓	3
	ileum	D_2-like↓	3
	Rabbit hippocampus	D_2-like↓	1
	striatum	D_2-like↓	1; 4; Dolezal et al. 1992
	retina	D_1-like↑	3
	Cat caudate nucleus	D_2-like↓	1
GABA (see 2.7)	Rat prefrontal cortex	D_1-like↓	Gonzalez-Islas and Hablitz 2001
	striatum	D_2-like↓	4; Delgado et al. 2000; Pisani et al. 2000; Momiyama and Koga 2001
	striatum	D_1-like↑, D_2-like↓	Guzman et al. 2003
	striatum	D_1-like↑	Arias-Montano et al. 2001; 2007; Floran et al. 1990
	nucleus accumbens	D_1-like↑	4
	nucleus accumbens	D_5↓[a]	Nicola and Malenka 1997
	cultured n. accumbens cells	D_1-like↑ or ↓[b], D_2-like↓	Geldwert et al. 2006
	dorsolateral septal nucleus	D_4↓	Asaumi et al. 2006
	supraoptic nucleus	D_4↓	Azdad et al. 2003
	paraventricular nucleus	D_4↓	Baimoukhametova et al. 2004
	substantia nigra	D_1-like↑	4
	substantia nigra pars reticulata	D_1-like↑	Floran et al. 1990; Garcia et al. 1997; Radnikow and Misgeld 1998
	entopeduncular nucleus	D_1-like↑	Floran et al. 1990
	globus pallidus	D_1-like↑	Floran et al. 1990
	globus pallidus	D_2-like↓	Cooper et al. 2001; Floran et al. 1997
	VTA	D_1↑	Cameron and Williams 1993
	VTA	D_2-like↓	Koga and Momiyama 2000

(*Continued*)

Table 1 Continued

Release of Transmitter	Species and Tissue	Receptor Type	References
Glutamate (see 2.8)	Rat striatum	D_2-like↓	4
	striatum	D_2↓	Bamford et al. 2004
	globus pallidus medialis	D_2↓, D_3↓	Hernandez et al. 2006
	substantia nigra pars reticulata	D_1-like↑, D_2-like↓	Ibanez-Sandoval et al. 2006
	olfactory bulb	D_2-like↓	Davila et al. 2003
	supraoptic nucleus	D_4↓	Price and Pittman 2001
	Mouse nucleus accumbens	D_5↓[a]	Nicola et al. 1996
	Turtle olfactory bulb	D_2-like↓	Berkowicz and Trombley 2000
Cholecysto-kinin (see 2.9)	Rat neo cortex	D_1-like↑	Brog and Beinfeld 1992
	hippocampus	D_1-like↑	Brog and Beinfeld 1992
	striatum	D_2-like↑	Meyer and Krauss 1983
	striatum	D_1-like↑	Brog and Beinfeld 1992

Table summarizes studies in which release was determined either as overflow (Starke 1977) or by means of the postsynaptic response. References to reviews: 1, Starke et al. 1989; 2, Willems et al. 1985; 3, Fuder and Muscholl 1995; 4, Starke 1981.

[a] Retrospective classification by T.J.F.

[b] Electrophysiological measurements; some neurons responded to the D_1 agonist SKF38393 with inhibition and some with enhancement of GABA release.

Table 2 Functions of presynaptic dopamine autoreceptors other than modulation of release (↑ increase, ↓ decrease in function). Receptor classification as by the authors cited. Designation as D_1-like or D_2-like where no differentiation between D_1 and D_5, or between D_2, D_3 and D_4, was made.

Modulation of	Species and Tissue	Receptor Type	References
Dopamine synthesis	Rat striatum	D_2-like↓	Booth et al. 1990; Lindgren et al. 2001; Thompson and Certain 2005; see Starke et al. 1989
	retina	D_2	see Nguyen-Legros et al. 1999
	Mouse striatum	D_2↓	Hakansson et al. 2004
	striatum	D_{2short}↓	Lindgren et al. 2003
	cultured mesencephalic cells	D_2↓, D_3↓	O'Hara et al. 1996
	Guinea pig striatum	D_2-like↓	Johnson et al. 1992a
Dopamine uptake	Rat striatum	D_2-like↑	Meiergerd et al. 1993; Wieczorek and Kruk 1994; Rothblat and Schneider 1997; Batchelor and Schenk 1998
	nucleus accumbens	D_2-like↑	Wieczorek and Kruk 1994; Rothblat and Schneider 1997
	Mouse striatum	D_2↑	Dickinson et al. 1999
	striatum	D_2↓	Schmitz et al. 2002
Neurotensin release	Rat prefrontal cortex	D_2-like↑	Bean et al. 1990; Bean and Roth 1991

The autoreceptors modulating dopamine release are exclusively D_2-like and inhibitory (Table 1). They operate in many tissues, including the retina, and in various species, including humans (Table 1). Differences between species, however, exist: [^{3}H]-dopamine release in slices of mouse neocortex was not inhibited by the D_2/D_3 preferring agonist quinpirole, whereas [^{3}H]-dopamine release evoked identically in slices of human neocortex clearly was (Löffler et al. 2006). When activated by endogenous released dopamine, the receptors mediate a physiological autoinhibition, as shown by the uniform increase in dopamine release caused by antagonists. In human neocortex slices *in vitro*, the concentration of endogenous dopamine at the autoreceptors, the so-called biophase concentration, has been estimated. At an action potential frequency of 3 Hz it was 3.6 nM, which is close to the estimated dissociation constant of the dopamine-autoreceptor complex of 5 nM (Löffler et al. 2006).

Where the D_2-like inhibitory receptor was identifed further it was mostly D_2, D_3 in rat nucleus accumbens, and never D_4 (Table 1). The D_3 receptor was thought to play a minor role also in the mouse corpus striatum (Joseph et al. 2002). However, this suggestion was refuted by the finding that no autoreceptor function remained in mouse striatum after genetic deletion of the D_2 receptor (Schmitz et al. 2002).

D_2-like receptors couple mainly to Gi/o proteins, as mentioned above. However, there is no direct evidence to support this coupling for the release-modulating autoreceptors. Moreover, the subsequent intracellular signal transduction has never been studied directly in axon terminals. Mouse AtT-20 pituitary cells, which release acetylcholine and adrenocorticotropic hormone, have been used as a model for axon terminals. When expressed in these cells, D_3 receptors mediated inhibition of P/Q-type calcium channels and activation of G protein-coupled inward rectifier potassium channels (Kuzhikandathil et al. 1998; Kuzhikandathil and Oxford 1999). Both would explain the autoreceptor-mediated inhibition of dopamine exocytosis.

Presynaptic dopamine autoreceptors are rapidly desensitized by dopamine but not other agonists auch as apomorphine or roxindole (Arbilla et al. 1985; Seyfried and Bartoszyk 1994; see also Kim et al. 2005). The reason for the difference is unknown.

A large receptor reserve – i.e., receptors in excess of those necessary to produce a maximum response – has been described for release-modulating dopamine autoreceptors in rat striatum (Yokoo et al. 1988). No reserve exists for the autoreceptors in human neocortex (Löffler et al. 2006).

2.2 Presynaptic Dopamine Autoreceptors: Additional Functions

As mentioned above, presynaptic dopamine autoreceptors subserve functions in addition to modulation of dopamine release. They are summarized in Table 2.

One such function is inhibition of tyrosine hydroxylase (TH), independently of the inhibition of release. In analogy to release, it results in a feedback inhibition of the synthesis of dopamine (Table 2). Like the release-inhibiting autoreceptors, those inhibiting dopamine synthesis are D_2-like and, where identified further, D_2 or D_3

(Table 2). In mouse cultured mesencephalic cells transfected with either D_2, D_3, or D_4 receptors, the former two but not the D_4 receptors mediated inhibition of TH (O'Hara et al. 1996). The D_2 receptor effect was maintained for at least 60 min, whereas the D_3 receptor effect desensitized rapidly (O'Hara et al. 1996). It has been suggested that D_2 autoreceptors are the D_{2short} splice variant, whereas postsynaptic D_2 receptors are D_{2long} (Usiello et al. 2000). This remains an hypothesis for the release-inhibitory, but has been borne out experimentally for the TH-inhibiting autoreceptors (Lindgren et al. 2003). Signal transduction behind the autoreceptor starts with pertussis toxin-sensitive Gi/o proteins (O'Hara et al. 1996), inhibition of adenylyl cyclase, and a fall in cAMP levels. The cAMP-dependent phosphorylation of TH at serine 40 is then diminished and enzyme activity reduced due a reinforcement of end product inhibition (see, for instance, Daubner et al. 1992).

Starting in 1993, reports have appeared that in rat striatum and nucleus accumbens D_2-like autoreceptors, when activated, enhanced removal of dopamine from the extracellular space by means of the dopamine transporter (DAT) as studied by voltammetry (Table 2). D_2 receptor activation also increased [^{3}H]-dopamine uptake in *Xenopus* oocytes transfected with both the human D_2 receptor and the human DAT (Mayfield and Zahniser 2001), as well as the uptake of a fluorescent DAT substrate in human embryonic kidney cells equally transfected with both the human D_2 receptor and the human DAT (Bolan et al. 2007). In accord with this autoreceptor-transporter connection, clearence of dopamine from the striatal extracellular space *in vivo* was reduced in D_2 receptor knockout mice (Dickinson et al. 1999). Unfortunately, however, the data in the literature are by no means consistent. No effect of D_2 receptor activation on the DAT was observed in another rat striatum study (Prasad and Amara 2001), in synaptosomes from rat and human neocortex (Feuerstein, unpublished observations), and in PC12 cells that possess D_2 receptors and the DAT (Pothos et al. 1998). In a second study with D_2 receptor knockout mice, uptake of dopamine in the striatum was increased rather than decreased (Schmitz et al. 2002). The reason for these discrepancies is not clear. It has been suggested that the clearance measurements by means of voltammetry were not appropriate for assessment of the function of the DAT (Prasad and Amara 2001).

Dopamine and neurotensin are co-transmitters in neurons projecting from the VTA to the prefrontal cortex. Dopamine release in rat prefrontal cortex, like the neocortex generally (Table 1), is inhibited through D_2-like autoreceptors. It is surprising, therefore, that prefrontal release of neurotensin was enhanced through the autoreceptors (Table 2). This is not the only example of opposite presynaptic modulation of the release of two cotransmitters. Presynaptic β-adrenoceptors increase release of noradrenaline from some postganglionic sympathetic neurons but diminish release of its cotransmitter ATP (Driessen et al. 1996). It has been suggested that in such cases some vesicles contain mainly transmitter A and others mainly transmitter B and that presynaptic receptor activation leads to inhibition of the release of one and facilitation of the release of the other vesicle population (Goncalves et al. 1996).

2.3 Dopamine Autoreceptors in Disease and Therapy

Dopamine neurons are involved in several neuropsychiatric disorders such as Parkinson's disease, schizophrenia, and psychotropic drug abuse. It seems likely that dopamine autoreceptor function is affected in these disorders as well as by their drug treatment.

Levodopa and dopamine receptor agonists improve the motor symptoms of Parkinson's disease. A severe potential side effect of the agonists but not of levodopa is impairment of cognitive functions such as learning. The reason for this difference probably is the mode of activation of dopamine receptors. Levodopa increases presynaptic dopamine availability and thereby action potential-induced dopamine release. It leaves the pattern of dopamine release (phasic versus tonic) and the pattern of presynaptic as well as postsynaptic dopamine receptor activation (phasic versus tonic) unaffected. Agonists such as pergolide, in contrast, activate pre- and postsynaptic dopamine receptors continuously, in an action potential-independent manner. Animal as well as human experiments indicate that both the continuous activation of postsynaptic receptors and the continuous autoreceptor-mediated attenuation of dopamine release contribute to the deterioration of cognitive functions during treatment with dopamine agonists (Arnsten et al. 1995; Breitenstein et al. 2006). There may be an additional component. The agonists clinically used are D_2-selective. By inhibition of dopamine release as well as their postsynaptic action they favor postsynaptic D_2 receptor activation at the expense of D_1.

Increased dopaminergic neurotransmission is a component of psychostimulant addiction, Tourette's syndrome, and schizophrenia. Chlorpromazine-like neuroleptic drugs are thought to help these patients by blockade of postsynaptic dopamine (and other) receptors. An alternative therapeutic principle would be selective activation of dopamine autoreceptors. The idea led to the development of a group of drugs (e.g., the thiazoloazepine derivative B-HT 920) allegedly displaying selective D_2 autoreceptor agonist activity. However, pharmacological differences between D_2 autoreceptors and postsynaptic D_2 receptors have remained elusive (e.g., Starke et al. 1989; Drukarch and Stoof 1990). It has been thought that a large autoreceptor reserve might make D_2 agonists more potent at the autoreceptors than at postsynaptic receptors (see Goldstein et al. 1990). However, as mentioned above, no autoreceptor reserve exists in human neocortex, an important projection field of dopamine neurons in these disorders. There is an additional reason for skepticism: dopamine autoreceptors may be nonfunctional in states of chronically increased dopaminergic transmission. DAT knockout mice are characterized by such "hyperdopaminergia." In these animals, any dopamine autoreceptor function, soma-dendritically as well as presynaptically, was almost lost (Jones et al. 1999).

As opposed to the disorders of the preceding paragraph, a *decrease* in dopaminergic transmission may be one of the neurochemical alterations in depression (Dailly et al. 2004). The selective antagonists at D_2-like receptors sulpiride and amisulpride, when given at low doses, reduce depression symptoms, presumably by blockade of D_2-autoreceptors and enhancement of dopamine release (Racagni et al. 2004). Sulpiride in fact increased the release of [^{3}H]-dopamine in human neocortex slices

(Löffler et al. 2006). Coadministration of sulpiride and fluvoxamine, a serotonin reuptake inhibitor, increased *in vivo* dopamine release in the prefrontal cortex of rats (Ago et al. 2005a). The increase probably is the neurochemical counterpart of the antidepressant-like effect of coadministration of sulpiride and fluvoxamine in mice, as measured in the tail suspension test (Ago et al. 2005b). A recent clinical trial showed superior antidepressant efficacy of the combination of sulpiride plus the serotonin reuptake inhibitor paroxetine compared with paroxetine alone and, importantly enough, an accelerated antidepressant response of the combination (Uchida et al. 2005).

A key effect of addictive psychotropic drugs is to increase the extracellular concentration of dopamine in the nucleus accumbens. Ethanol does this in part by direct excitation of the mesolimbic dopamine neurons projecting to the accumbens (Koyama et al. 2007). However, studies in alcohol-preferring rats, animals which tend to develop alcohol dependence, have indicated an additional component: a reduction of presynaptic D_2 autoreceptor function (Engleman et al. 2003; Thielen et al. 2004).

2.4 Presynaptic Dopamine Heteroreceptors on Noradrenergic Terminals

These receptors were first observed in the sympathetic nervous system: in the nictitating membrane of the cat, dopamine reduced the release of noradrenaline via a receptor distinct from the α_2-autoreceptor (Enero and Langer 1975). Most of the heteroreceptors are D_2-like and inhibitory, but in rat nucleus accumbens and medial prefrontal cortex D_1 receptors enhance noradrenaline release (Table 1). The opposite effects mediated by D_1 and D_2 receptors in rat nucleus accumbens have been explained by location of the inhibitory D_2 receptors close to, and location of the facilitatory D_1 receptors more distant from, dopaminergic varicosities (Vandschuren et al. 1999).

Although noradrenergic terminals normally contain too little dopamine for presynaptic dopamine heteroreceptors to become activated, and despite the fact that the hippocampus is only sparsely innervated by dopaminergic fibers (Bischoff et al. 1979), the release of [^{3}H]-noradrenaline in rabbit (Jackisch et al. 1985) and rat (Monnet 2002) hippocampus was inhibited by endogenous dopamine as shown by the facilitatory effect of D_2 antagonists. Voltage-sensitive calcium channels seem to play a role in the dopaminergic inhibition of noradrenaline release (Monnet 2002).

Presynaptic receptors, when located on the same terminals, may interact with one another (see Schlicker and Göthert 1998; Trendelenburg et al. 2003). Such cross-talk also occurs between presynaptic D_2-like heteroreceptor on noradrenergic neurons and the presynaptic α_2-autoreceptors: inhibition by one narrows the scope for inhibition by the other (Jackisch et al. 1985; see also 1.2.3, 1.2.5 and 1.3.3).

2.5 Presynaptic Dopamine Heteroreceptors on Serotonergic Terminals

An increase in serotonin release through a D_2-like receptor has been reported for rat hypothalamus and a decrease in serotonin release through a D_1-like receptor for rat substantia nigra (Table 1). The mechanisms of these unusual effects are not discussed in the two papers. The D_1 antagonist SCH 23390 increased the release of serotonin in the substantia nigra, thus revealing an endogenous dopamine input (Benkirane et al. 1987). No dopaminergic modulation of serotonin release was seen in rat and rabbit hippocampus (Cox et al. 1980; Jackisch et al. 1985; Matsumoto et al. 1996), rat neocortex (Baumann and Waldmeier 1981), rat nucleus accumbens (Ferre and Artigas 1995), rabbit striatum (Feuerstein et al. 1986). and rat septum (Rutz et al. 2007). It seems worth mentioning that in attempts to label serotonergic neurons with [^{3}H]-5-HT care has to be taken to avoid a false labelling of dopaminergic neurons (Feuerstein et al. 1986).

2.6 Presynaptic Dopamine Heteroreceptors on Cholinergic Terminals

Due to the dopamine-acetylcholine link in Parkinson's disease, these receptors are of particular interest, and due to this link the majority of studies have been carried out on striatal cholinergic interneurons (Table 1). They are endowed with presynaptic D_2-like – where further classified D_2 – receptors inhibiting acetylcholine release and possibly also with presynaptic D_1-like receptors increasing acetylcholine release (Table 1). They also possess soma-dendritic D_1- and D_2-like receptors that increase and decrease, respectively, their firing frequency (Aosaki et al. 1998; Centonze et al. 2003; Maurice et al. 2004). However, blockade of the soma-dendritic dopamine receptors failed to influence the tonic activity of cholinergic interneurons (Bennett and Wilson 1999), so they may not be activated by endogenous dopamine. The presynaptic receptors, in contrast, do receive an endogenous dopamine input to mediate tonic presynaptic inhibition (e.g., Hertting et al. 1980; Helmreich et al. 1982).

Single cell reverse transcription – polymerase chain reaction detected D_2 and D_5 but not $D_{3/4}$ and little D_1 receptor mRNA in rat striatal cholinergic interneurons. The D_2 receptors coupled to Gi/o and reduced somatic N-type Ca^{2+} currents (Yan et al. 1997), thus providing a cellular mechanism for the reduction of acetylcholine release by D_2 receptors located on cholinergic terminals.

A D_2 receptor reserve of 25%–30% has been estimated for the cholinergic interneurons of rat striatum, without differentiation between presynaptic and soma-dendritic receptors (Enz et al. (1990), but the reliability of the method used to assess the receptor reserve has been questioned (Agneter et al. 1997).

Transmission from cortical glutamate neurons to striatal GABAergic medium spiny neurons is subject to long-term depression (LTD), a long-lasting decrease

of transmission following repetitive activity of the glutamate neurons. Inhibitory D_2 receptors on cholinergic striatal interneurons are necessary for the expression of this LTD. The reasons have been elucidated by Wang et al. (2006). Figure 1 shows their explanation – one of the most explicit hypotheses on the physiological role of a presynaptic receptor. LTD is due to inhibition of presynaptic glutamate release by endocannabinoids synthesized in, and released from, the postsynaptic spines. One prerequisite for postsynaptic endocannabinoid synthesis is postsynaptic calcium entry through L-type calcium channels. Acetylcholine released from the spontaneously active cholinergic interneuron, by activating M_1 receptors on the spines, inhibits the L-type calcium channels and thus tends to attenuate LTD. As long as the cholinergic interneurons fire at their normal slow rate and release little

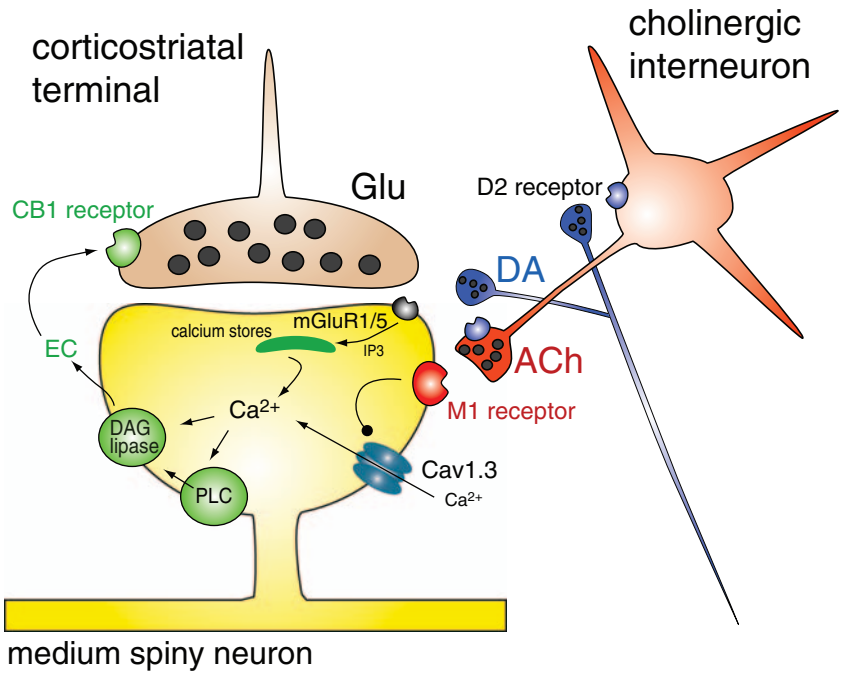

Fig. 1 Elements controlling LTD induction in the synapse between a corticostriatal glutamate terminal and the spine of a striatal medium spiny neuron. LTD is due to activation of postsynaptic metabotropic mGlu1/5 and (not shown) ionotropic AMPA receptors, promotion of postsynaptic endocannabinoid synthesis and release and subsequent CB_1 receptor-mediated inhibition of glutamate release. Calcium derived from intracellular stores as well entry through Cav1.3 L-type calcium channels is necessary for endocannabinoid synthesis. A cholinergic interneuron projects to the spine, innervating an M_1-receptor that, when activated, inhibits the L-type calcium channel. The cholinergic neuron in turn receives dopamine input from the nigro-striatal dopamine pathway. Dopamine reduces both the firing rate of the neuron and the release of acetylcholine per pulse by activation of soma-dendritic and presynaptic D_2 receptors, respectively. ACh = Acetylcholine; DA = dopamine; DAG lipase = diacylglycerol lipase; EC = endocannabinoid; PLC = phospholipase C. Reproduced, with permission, from Wang et al. (2006).

acetylcholine per impulse, LTD can be induced. When, however, the cholinergic neurons are disinhibited by sulpiride blockade of their D_2 receptors, the massive release of acetylcholine suppresses postsynaptic calcium entry to the extent that LTD disappears. In accord with this explanation, LTD in the presence of sulpiride can be rescued when postsynaptic M_1 receptors are blocked by pirenzepine (Wang et al. 2006).

2.7 Presynaptic Dopamine Heteroreceptors on GABAergic Terminals

Most of the observations of dual presynaptic effects of dopamine – inhibition of release through D_2-like receptors and facilitation (but occasionally also inhibition) of release through D_1-like receptors – refer to GABA release in rat striatum, nucleus accumbens and VTA (Table 1). It was thought for some time that the two receptor types were located on different neurons (Gerfen et al. 1990; Yung et al. 1995). However, co-localization of D_1-like and D_2-like receptors on the same GABA neuron has now been demonstrated immunohistochemically (Aizman et al. 2000; Geldwert et al. 2006) as well as functionally (Geldwert et al. 2006). Particularly noteworthy is the demonstration of different localization patterns on single varicosities (Mizuno et al. 2007). The authors measured Ca^{2+} transients in individual varicosities in mouse nucleus accumbens. In some varicosities, the transients were enhanced through D_1-like receptors but not modified through D_2-like receptors; in other varicosities, they were reduced through D_2 or D_3 receptors but not modified through D_1-like receptors; in still others, both D_1-like facilitation and $D_{2/3}$ inhibition occurred. To complete the complexity, D_3 receptors in some varicosities mediated facilitation rather than inhibition (Mizuno et al. 2007). Possibly some GABAergic neurons transport D_1-like, D_2 and D_3 receptors for various lengths and deposit them in their terminals in a target cell-specific manner (Geldwert et al. 2006). It must be emphasized, however, that despite much ingenious research, the physiological significance of the co-localization of D_1-like and D_2-like receptors remains uncertain, especially since the two receptor groups possess similar affinity for dopamine (Schoffelmeer et al. 1994).

The results of Mizuno et al. (2007) make it likely that modulation of Ca^{2+} currents underlies the various modulations of GABA release. This view is supported by other studies for both D_2-like inhibition (Pisani et al. 2000; Momiyama and Koga 2001) and D_1-like facilitation (Arias-Montano et al. 2007). The D_2-like inhibitory receptors presumably couple to Gi/o (Momiyama and Koga 2001), whereas the D_1-like facilitatory receptors presumably couple to Gs, activation of adenylyl cyclase and activation of protein kinase A (Arias-Montano et al. 2007). The riddle of the exceptional cases of inhibition through D_1-like receptors remains. For example, in rat prefrontal cortex D_1-like receptors presynaptically inhibited GABAergic transmission, an effect blocked by an inhibitor of protein kinase A (Gonzalez-Islas and Hablitz 2001) – the same enzyme involved in facilitation through D_1-like receptors

(Arias-Montano et al. 2007). The occasional facilitation by presynaptic D_3 receptors (Mizuno et al. 2007) may be due to the fact that this subtype can couple to Gs in addition to Gi/o (see Introduction).

In rat dorsolateral septal, supraoptic, and paraventricular nuclei, dopamine receptors inhibiting GABA release were pharmacologically identified as D_4 (Table 1). In the supraoptic and paraventricular nuclei the D_4 receptors reduce the GABAergic inhibition of the oxytocin- and vasopressin-secreting neurons and thus enhance neurophypophysial hormone release.

2.8 Presynaptic Dopamine Heteroreceptors on Glutamatergic Terminals

Dopamine presynaptically inhibits the release of glutamate in a number of brain nuclei through D_2-like receptors – where identified as either D_2, D_3 or D_4 (Table 1). In rat substantia nigra pars reticulata it simultaneously can facilitate the release of glutamate through D_1-like receptors – another case of co-localization of receptors mediating opposite effects. In mouse nucleus accumbens, a D_1-like receptor inhibited glutamate release. Since the inhibitory effect of dopamine persisted after genetic deletion of the D_1 receptor, the inhibitory receptor was D_5 (Table 1).

An interesting presynaptic location D_2 receptor is on corticostriatal glutamate terminals – the terminals shown in Figure 1 as targets of retrograde endocannabinoid signaling in LTD. These glutamate terminals (and not only the cholinergic interneurons as in Figure 1) are endowed with D_2 receptors. Dopamine selectively inhibits release of glutamate from terminals of low activity and thus favors transmission from the more active terminals (Bamford et al. 2004). These findings of a presynaptic D_2 receptor-mediated control of striatal glutamate release replace an older view that D_2 receptors were almost exclusively located on neurons whose somata reside in the striatum and not on terminals of corticostriatal axons (Joyce and Marshall 1987).

The glomerular layer of the olfactory bulb contains a substantial population of dopaminergic neurons. Dopamine acting at D_2-like heteroreceptors inhibits glutamate release from terminals of the olfactory sensory neurons and hence may modulate the olfactory nerve synapse (Table 1).

The neurosecretory cells of the supraoptic nucleus receive both the GABAergic inhibitory input mentioned above (Section 2.7) and an excitatory glutamate input: Whereas the D_4 heteroreceptor-mediated depression of GABA release disinhibits the neurosecretory cells (Section 2.7), the D_4 heteroreceptor-mediated depression of glutamate release (Table 1) inhibits them and, hence, the pituitary secretion of oxytocin and vasopressin.

2.9 Presynaptic Dopamine Heteroreceptors on Cholecystokinergic Terminals

Since cholecystokinin peptides show a neuroleptic-like profile in several screening tests for neuroleptics, cholecystokinin-dopamine interactions are of interest, especially the modulation of cholecystokinin release by dopamine receptors (Table 1). In the rat striatum both D_1 and D_2 receptor activation was reported to increase, and both D_1 and D_2 receptor blockade to depress, the release of the peptide.

2.10 Presynaptic Dopamine Heteroreceptors in Disease and Therapy

D_2 receptors on postganglionic sympathetic axon terminals diminish noradrenaline release and in consequence heart rate and vascular resistance (Ensinger et al. 1985). Selective D_2 agonists have therefore been studied for the treatment of hypertension. However, an unfavorable side effect profile (largely nausea and orthostatic hypotension) precluded wide use for this indication (Murphy 2000). Orthostatic hypotension is also a side effect when D_2 agonists are taken for other purposes, such as to decrease prolactin levels. Moreover, there is a therapeutic situation in which the D_2 heteroreceptors on sympathetic varicosities receive an input of endogenous dopamine. This is when parkinsonian patients are treated with L-dopa and dopamine accumulates in postganglionic sympathetic nerves (Lokhandwala and Buckley 1978). The release of noradrenaline is then reduced for two reasons: because part of the noradrenaline is replaced by the false transmitter dopamine and because exocytosis is diminished due to presynaptic D_2 heteroreceptor activation. In fact, recording of electrical sympathetic nerve activity in humans has shown that L-dopa-induced orthostatic hypotension is peripheral rather than central in origin (Takeuchi et al. 1993). Note, however, that orthostatic hypotension is also a characteristic of Parkinson's disease, independent of L-dopa treatment (Goldstein et al. 2005).

The effects of presynaptic D_1-like and D_2-like receptors on the release of amino acid neurotransmitters in the basal ganglia are important for the pathophysiology and treatment of Parkinson's disease. The fact that recent reviews on antiparkinsonian drugs highlight the old drug apomorphine with the rationale that it can stimulate both D_1-like and D_2-like receptors (e.g., Subramony 2006) may reflect the growing awareness that the benefits of pure D_2-like receptor agonists in Parkinson therapy are limited. The dopaminergic D_1-like and D_2-like modulation of amino acid release takes place mainly in the striatum and the reciprocally innervated network of the globus pallidus and the subthalamic nucleus (see Hernandez et al. 2006). It may be assumed, therefore, that apomorphine owes its superior beneficial effects in parkinsonian patients inter alia to activation of presynaptic D_1-like and D_2-like receptors located along the axons of striatal GABAergic neurons projecting to the output nuclei of the basal ganglia.

3 Presynaptic Receptors for Histamine

Presynaptic histamine receptors are more uniform than presynaptic dopamine receptors. Only one type, H_3, has been identified with certainty. Like presynaptic dopamine receptors, presynaptic H_3 receptors occur as auto- and heteroreceptors

Table 3 Tissues in which neurotransmitter release is decreased by presynaptic histamine H_3 receptor activation

Release of transmitter	Species and Tissue	References
Histamine (see 3.1)	Rat neocortex	see Starke et al. 1989; Morisset et al. 2000; Westerink et al. 2002
	hippocampus	see Starke et al. 1989
	hypothalamus	see Starke et al. 1989; Yamamoto et al. 1997
	striatum	see Starke et al. 1989
	Mouse neocortex	Morisset et al. 2000
	Guinea pig cardiac synaptosomes	Li et al. 2006
	Human neocortex	see Starke et al. 1989
Noradrenaline (see 3.3)	Rat neocortex	Schlicker et al. 1989; Smits and Mulder 1991
	hypothalamus	Smits and Mulder 1991
	heart	see Fuder and Muscholl 1995
	blood vessels	see Fuder and Muscholl 1995
	Mouse neocortex	Schlicker et al. 1993; 1994
	cardiac synaptosomes	Koyama et al. 2003
	Guinea pig mesenteric artery	Ishikawa and Sperelakis 1987
	atria	Endou et al. 1994, see Fuder and Muscholl 1995
	heart	Imamura et al. 1994; 1996b; see Fuder and Muscholl 1995
	cardiac synaptosomes	Li et al. 2006; Silver et al. 2002
	Cat iris	see Fuder and Muscholl 1995
	nictitating membrane	see Fuder and Muscholl 1995
	Human neocortex and hippocampus	Schlicker et al. 1999
	saphenous vein	see Fuder and Muscholl 1995
	atria	Imamura et al. 1995; Hatta et al. 1997
	Pig retina	see Fuder and Muscholl 1995
	Dog heart	Mazenot et al. 1999
Serotonin (see 3.4)	Rat neocortex	Schlicker et al. 1988; Smits and Mulder 1991; Fink et al. 1990
	hypothalamus	Smits and Mulder 1991
	striatum	Smits and Mulder 1991
	substantia nigra pars reticulata	Threlfell et al. 2004
Dopamine (see 3.5)	Rat nucleus accumbens	Prast et al. 1999a
	Mouse striatum	Schlicker et al. 1993
Acetylcholine (see 3.6)	Guinea pig ileum	see Fuder and Muscholl 1995
	colon	see Fuder and Muscholl 1995
	trachea	see Fuder and Muscholl 1995

(*Continued*)

Table 3 Continued

Release of transmitter	Species and Tissue	References
GABA (see 3.7)	Rat striatum	Arias-Montano et al. 2001
	substantia nigra pars reticulata	Garcia et al. 1997
	medial vestibular nucleus	Bergquist et al. 2006
Glutamate (see 3.8)	Rat dentate gyrus	Brown and Haas 1999
	thalamus	Garduno-Torres et al. 2007
	striatum	Molina-Hernandez et al. 2001; Doreulee et al. 2001
	amygdala	Jiang et al. 2005
Sensory neuro-peptides (see 3.9)	Rat dura mater	Matsubara et al. 1992; Dimitriadou et al. 1997
	lung and spleen	Dimitriadou et al. 1994
	skin	Ohkubo and Shibata 1995
	Guinea pig airways	Ichinose et al. 1990
	dura mater	Matsubara et al. 1992
	atria	Imamura et al. 1996a
	Rabbit lung	Nemmar et al. 1999

Table summarizes studies in which release was determined either as overflow or by means of the postsynaptic response.

(Table 3). They have been detected both functionally (Table 3) and, morphologically, by comparison of the distribution of H_3 receptor mRNA and H_3 receptor protein. By the latter approach it has been shown, for example, that thalamo-cortical and hippocampal pyramidal glutamate neurons and striato-nigral as well as striato-pallidal GABA neurons possess presynaptic H_3 receptors (see Pillot et al. 2002; Jin and Panula 2005; see also immunohistochemical detection in Section 3.9).

Presynaptic H_3 receptors also are uniform in their signal transduction. They couple to Gi/o proteins and decrease the depolarization-induced release of neurotransmitters by inhibiting multiple calcium channels (e.g., Arrang et al. 1985; Schlicker et al. 1994; Endou et al. 1994; Brown and Haas 1999; see Stark et al. 2004). For comparison, the signal transduction of soma-dendritic H_3 autoreceptors in histaminergic neurons also involves a pertussis toxin-sensitive G-protein with subsequent inhibition of N- and P-type Ca^{2+} channels (Takeshita et al. 1998). The few exceptions to this signal transduction pathway are discussed in the corresponding subsections below (see Sections 3.1, 3.3, and 3.9).

There are some reports on modulation of noradrenaline, acetylcholine, and glutamate release through presynaptic H_1 or H_2 receptors. The evidence is doubtful, however. For example, effects of drugs thought to be H_1- or H_2-selective may in fact have been due to an action on H_3 receptors (see page 291 of Fuder and Muscholl 1995). Morphological evidence for presynaptic H_1 or H_2 receptors similar to that mentioned for H_3 receptors is lacking.

3.1 Presynaptic Histamine Autoreceptors: Modulation of Release and Synthesis

Like presynaptic dopamine autoreceptors, presynaptic histamine autoreceptors are activated by the released endogenous transmitter to inhibit further histamine release, as shown by the increase in histamine release caused by antagonists at H_3 receptors: a definite piece of physiology. Evidence has been presented recently that cardiac postganglionic sympathetic neurons of the guinea pig synthesize and release histamine as a co-transmitter (Li et al. 2003; 2006). These noradrenaline-histamine neurons possess H_3 autoreceptors which, when activated, depress the release of both noradrenaline and histamine – unlike the D_2-like autoreceptors of dopamine-neurotensin neurons which modulate the release of the two cotransmitters in opposite direction (see Section 2.2). It would be of interest to see whether, conversely, activation of α_2-autoreceptors inhibits the release of histamine in the guinea pig heart.

Apart from exocytosis, presynaptic H_3 autoreceptors also inhibit the synthesis of histamine at the level of nerve endings, at least in part through pathways distinct from those leading to the inhibition of release. One pathway is inhibition of adenylyl cyclase (Gomez-Ramirez et al. 2002; Moreno-Delgado et al. 2006): activation of cAMP-dependent protein kinase A by cAMP stimulates histamine synthesis through phosphorylation of histidine decarboxylase, and this stimulation is diminished when adenylyl cyclase activity decreases following activation of H_3 autoreceptors and Gi/o proteins.

Presynaptic H_3 autoreceptors are among those G protein-coupled receptors now known to display constitutive activity, inducing an effect in the absence of an agonist. This has been shown, for example, for the release-inhibiting autoreceptors in rat and mouse neocortex (Morisset et al. 2000) and for the synthesis-inhibiting autoreceptors in rat neocortex (Moreno-Delgado et al. 2006). Thioperamide, previously considered as the classical H_3 receptor antagonist, is in fact an extremely potent inverse agonist. Other H_3 receptor antagonists also have been identified as inverse agonists (e.g., Leurs et al. 2005; Moreno-Delgado et al. 2006). Constitutive activity of presynaptic H_3 autoreceptors differs among splice variants and species (Coge et al. 2001). In this context it seems worth mentioning that an inverse agonist is a pure antagonist as long as the receptor under investigation is not spontaneously active, implying that the antagonist/inverse agonist character of a ligand depends on the degree of constitutive activity of the receptor under investigation.

So far, constitutive activity of presynaptic H_3 heteroreceptors has not been described.

3.2 Histamine Autoreceptors in Disease and Therapy

Wherever histamine neurons are involved in pathophysiology, their H_3 autoreceptors surely are involved as well. For example, histamine release in the prefrontal cortex is increased during handling stress, and this increase is potentiated when H_3 autoreceptors are blocked (Westerink et al. 2002).

Histaminergic neuron activity promotes arousal, attention, cognition, and learning, and H_3 antagonists exert procognitive effects in animals (Huang et al. 2004; Yu et al. 2006; see Stark et al. 2004). It seems likely that the effects are due to blockade of H_3 autoreceptors, thereby increasing histamine synthesis and release. In accord with this view, the procognitive effect of the antagonists was potentiated by histidine but attenuated by blockade of histidine decarboxylase. Released histamine acts on postsynaptic H_1 and, to a lesser extent, H_2 receptors to improve memory (above references).

Histaminergic neuron activity also seems to suppress appetite. For this reason, H_3 antagonists or inverse agonists, which promote histamine release, were expected to act similarly, whereas autoreceptor-selective agonists were expected to increase food intake. However, what was found was the opposite: the antagonists/inverse agonist thioperamide increased, whereas classical H_3 agonists decreased, food intake in diet-induced obese wild-type mice (Yoshimoto et al. 2006). To further cloud the picture, although the H_3 agonist-induced anorexigenic effect was associated with a reduction in histamine release, a comparable reduction in histamine release due to blockade of histidine decarboxylase increased appetite. Apparently the effects of H_3 receptor ligands on appetite and body weight are largely due to actions at H_3 receptors other than the autoreceptors.

Finally, histaminergic neuron activity seems to protect against convulsions (Zhang et al. 2003; Lintunen et al. 2005), epilepsy is a potential indication for H_3 receptor antagonists (Harada et al. 2004; Stark et al. 2004), and their anticonvulsive effect has been partly explained by an increase in histamine release (Yokoyama et al. 1994; Harada et al. 2004).

3.3 Presynaptic Histamine Heteroreceptors on Noradrenergic Terminals

They were the first presynaptic H_3 heteroreceptors to be discovered (in guinea pig mesenteric artery; Table 1). They mediate inhibition of transmitter release from many noradrenergic neurons, peripheral as well as central (Table 1). They may be activated by histamine from mast cells, as happens in the heart upon myocardial ischemia (see Section 3.10). In the CNS an input of endogenous histamine has not been demonstrated (see references in Table 3). For example, the release of noradrenaline in the hippocampus of freely moving rats remained unchanged upon local

administration of the H_3 receptor antagonist/inverse agonist thioperamide (Di Carlo et al. 2000).

Quantitative differences have been observed between humans and animals regarding noradrenaline release from neocortical slices: release in human tissue was inhibited by H_3 receptor activation to a much lower extent than in mouse neocortex (see Stark et al. 2004).

Like D_2-like heteroreceptors (Section 2.4), the H_3 heteroreceptors on noradrenergic terminals may interact with terminal α_2 autoreceptors: preactivation of one receptor attenuates the effect obtainable by activation of the other (see Schlicker and Göthert 1998).

As for all presynaptic H_3 receptors, the H_3 heteroreceptor-mediated inhibition of exocytotic noradrenaline release from sympathetic terminals of the heart involves Gi/o and a decrease in Ca^{2+} entry (Hatta et al. 1997; Levi and Smith 2000; Silver et al. 2002). This signal transduction is also responsible for the inhibition by histamine of exocytotic noradrenaline release in *acute* myocardial ischemia (Hatta et al. 1997). A striking change, however, takes place in *protracted* myocardial ischemia. Although noradrenaline release continues, and although histamine still inhibits release, the mechanisms are totally different. In protracted myocardial ischemia, ATP depletion and intracellular pH reduction promote axoplasmic Na^+ accumulation upon activation of the Na^+/H^+ exchanger and diminish the pH gradient between cytoplasm and vesicle interior in the sympathetic terminals, a constellation that prevents the vesicular storage of noradrenaline and engenders a massive carrier-mediated, non-exocytotic, Ca^{2+}-independent release of noradrenaline (Imamura et al. 1994; 1996b; see Levi and Smith 2000). The inhibition of this carrier-mediated release through H_3 receptors involves a depression of the Na^+/H^+ exchanger (Imamura et al. 1996b), thereby reducing the intraterminal accumulation of Na^+ with an ensuing decrease in noradrenaline outward transport. The second messengers mediating the reduction of Na^+/H^+ exchanger activity remain unclear. In addition to the depression of the Na^+/H^+ exchanger, suppression of voltage-dependent Na^+ channels may be part of the H_3 heteroreceptor signal transduction in protracted cardiac ischemia (Hatta et al. 1997).

3.4 Presynaptic Histamine Heteroreceptors on Serotonergic Terminals

H_3 receptor activation inhibits the release of serotonin in several rat brain regions (Table 3). An endogenous histamine tone has been found at the heteroreceptors in rat brain cortex (Schlicker et al. 1988; Fink et al. 1990). At high micromolar concentrations histamine may enhance the release of serotonin (and of noradrenaline) via a tyramine-like mechanism (Starke and Weitzell 1978; Young et al. 1988).

3.5 Presynaptic Histamine Heteroreceptors on Dopaminergic Terminals

The inhibitory H_3 heteroreceptors on dopaminergic nerve terminals in mouse striatal slices were not activated by endogenous histamine under the experimental conditions chosen, since their blockade did not enhance the release of dopamine (Schlicker et al. 1993). In the rat nucleus accumbens *in vivo*, however, indirect evidence (an histamine-evoked increase in acetylcholine release) suggests that dopamine release is permanently inhibited by endogenous histamine (Prast et al. 1999a; see Section 3.6).

Blockade of dopamine autoreceptors increased the extent of H_3 receptor-mediated inhibition of dopamine release in mouse striatal slices; this another example of an autoreceptor/heteroreceptor interaction (Schlicker et al. 1993; compare Sections 2.4, 3.3, 4.3).

The effect of H_3 receptor activation on release of dopamine in rat corpus striatum has not been investigated, but H_3 receptor activation reduced the synthesis of dopamine in rat striatum (Molina-Hernandez et al. 2000).

3.6 Presynaptic Histamine Heteroreceptors on Cholinergic Terminals

Presynaptic H_3 heteroreceptors, when activated, inhibit the release of transmitter from several peripheral cholinergic neuron systems, probably preganglionic as well as postganglionic parasympathetic (Table 3). The receptors may be activated by histamine secreted from mast cells.

In the CNS, the existence of presynaptic H_3 heteroreceptors on cholinergic neurons is less certain. H_3 receptor activation depressed the release of acetylcholine in slices (Clapham and Kilpatrick 1992) but not synaptosomes (Arrang et al. 1995) of rat entorhinal cortex, suggesting location of the H_3 receptors outside the cholinergic terminals. Still, Prast et al. (1999a; b) in their extensive *in vivo* push-pull superfusion studies in rat nucleus accumbens suggest a minor H_3 receptor on cholinergic terminals (Fig. 6 of Prast et al. 1999b).

Several mechanisms for modulation of acetylcholine release through cerebral H_3 receptors outside cholinergic terminals have been elucidated. First, in the parietal cortex of rats *in vivo*, local H_3 receptor activation reduced the release of acetylcholine. This was due to a primary increase in the release of GABA (at soma-dendritic H_3 receptors of GABA neurons, the authors suggest) followed by $GABA_A$ receptor-mediated depression of acetylcholine release (Giorgetti et al. 1997). Second, in the corpus amygdaloideum of rats *in vivo*, local H_3 receptor activation also reduced, whereas H_3 receptor blockade increased, acetylcholine release. Here the primary sites of action were presynaptic H_3 autoreceptors, and the ensuing change of histamine release subsequently changed the release of acetylcholine

through an H_2 receptor of unknown location (Cangioli et al. 2002; Passani et al. 2001). Third, in the nucleus acumbens of rats *in vivo*, local H_3 receptor activation *increased* the release of acetylcholine. One mechanism was primary H_3 receptor inhibition of dopamine release (see Section 3.5) and, hence, secondary attenuation of the dopaminergic inhibition of acetylcholine release (see Section 2.6). Another mechanism was primary H_3 receptor inhibition of GABA release (see Section 2.7) and secondary attenuation of a GABAergic inhibition of acetylcholine release (Prast et al. 1999a; b).

3.7 Presynaptic Histamine Heteroreceptors on GABAergic Terminals

As just mentioned, H_3 receptor-mediated inhibition of GABA release has been dissected as an intermediate step in H_3 agonist-induced enhancement of acetylcholine release in rat nucleus accumbens (Section 3.6; Prast et al. 1999a; b). While direct evidence for H_3 inhibition of GABA release in the accumbens is lacking, histamine does inhibit the release of GABA in other brain structures (Table 3). The effect in rat substantia nigra pars reticulata and corpus striatum is an interesting case of fine-tuning. In both structures, release of GABA is facilitated through dopamine D_1-like heteroreceptors (Table 1). Histamine and a selective H_3 agonist suppressed only that part of GABA release that was due to tonic D_1-like receptor activation (Garcia et al. 1997; Arias-Montano et al. 2001). The D_1 receptors increase striatal GABA release by promoting Ca^{2+} entry through P/Q-type channels. The H_3 receptors conversely inhibit release by depression of Ca^{2+} entry through these channels (Arias-Montano et al. 2007). Thus, presynaptic D_1 and H_3 heteroreceptors of striatal GABA neurons are co-localized and interact both on terminals in the target nucleus substantia nigra pars reticulata and on terminals of axon collaterals in the corpus striatum.

3.8 Presynaptic Histamine Heteroreceptors on Glutamatergic Terminals

H_3 receptor activation attenuates the release of glutmate in several brain regions (Table 3), but an endogenous input to the receptors has not been demonstrated. In the dentate gyrus adenosine, acting at A_1 receptors, also attenuated the release of glutamate and occluded further inhibition by histamine, presumably because a common signal transduction pathway (Brown and Haas 1999).

3.9 Presynaptic Histamine Heteroreceptors on Peptidergic Terminals

Sensory neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) release histamine from mast cells (Dimitriadou et al. 1994; 1997; Imamura et al. 1996a). Histamine, in turn, can inhibit the release of neuropeptides through H_3 receptors which have been detected on peripheral sensory axon terminals by immunohistochemistry (Cannon et al. 2007). In three of the studies listed in Table 3, inhibition of substance P release (Ohkubo and Shibata 1995; Nemmar et al. 1999) and CGRP release (Imamura et al. 1996a) was measured as a decrease of overflow; in two others it was measured as attenuation of plasma extravasation (Ichinose et al. 1990; Matsubara et al. 1992). The presynaptic signal transduction may involve the stimulation of ATP-sensitive K^+ channels (Ohkubo and Shibata 1995). The close proximity of mast cells and peripheral sensory axon endings makes this H_3 effect an endogenous modulation mechanism. In the guinea pig heart, for example, the H_3 antagonist thioperamide increased the release of CGRP elicited by capsaicin (Imamura et al. 1996a). It is likely that H_3 receptors are also located on the central terminals of primary sensory neurons, where their activation inhibits transmission at the first nociceptive synapse in the spinal cord or brain stem (Cannon et al. 2003; Cannon and Hough 2005).

3.10 Presynaptic Histamine Heteroreceptors in Disease and Therapy

In myocardial ischemia, several of the mechanisms presented above come into play. First, neuropeptides such as CGRP are released from cardiac sensory C fibers and subsequently release histamine from mast cells as just mentioned. Histamine then can act at least at two presynaptic H_3 heteroreceptors: on the C fibers to attenuate further neuropeptide release (Section 3.9), and on postganglionic sympathetic fibers to attenuate exocytotic as well as carrier-mediated noradrenaline release (Section 3.3). Both presynaptic effects are potentially beneficial. The H_3 receptors are unique in this pattern of effects. Presynaptic adenosine A_1 receptors, when activated, also inhibit both exocytotic and carrier-mediated noradrenaline release, but cardiac A_1 receptors in addition mediate negative chronotropic and dromotropic effects. Presynaptic α_2-adrenoceptors, when activated, reduce exocytotic noradrenaline release but *enhance* carrier-mediated noradrenaline release (due to stimulation of the Na^+/H^+ exchanger, Imamura et al. 1996b), which is the major mode of noradrenaline release and the major arrhythmogenic risk in protracted myocardial ischemia (see Levi and Smith 2000; Koyama et al. 2003).

H_3 heteroreceptors inhibiting noradrenaline release from sympathetic nerve terminals in the nasal mucosa may contribute to nasal congestion in allergic rhinitis (Varty et al. 2004).

Both the histamine neuron innervation and the density of H_3 receptors are increased in the substantia nigra of Parkinsonian patients (Anichtchik et al. 2000; 2001). The receptors probably are the H_3 heteroreceptors on striato-nigral GABA terminals, and their increase may contribute to the depression of GABA release in the substantia nigra in Parkinson's disease (Garcia-Ramirez et al. 2004).

The inhibition through H_3 receptors of the release of substance P and other neurotransmitters of primary sensory neurons may become highly important clinically: It explains the antinociceptive and antiinflammatory properties of some H_3 receptor agonists, including their antimigraine effects (McLeod et al. 1998; Cannon et al. 2003; see also Göthert et al. 1995).

4 Presynaptic Receptors for Serotonin

If presynaptic histamine receptors are more uniform than presynaptic dopamine receptors, the contrary holds true for presynaptic serotonin receptors: they are even more diverse than presynaptic dopamine receptors. As mentioned in the Introduction, presynaptic 5-HT_3 receptors, being ligand-gated ion channels, are covered in the chapter by Dorostkar and Boehm and will be mentioned here only occasionally. Presynaptic G protein-coupled 5-HT receptors inhibit the release of serotonin from serotonergic axon terminals and inhibit or enhance the release of other neurotransmitters (Table 4).

4.1 Presynaptic Serotonin Autoreceptors: Modulation of 5-HT Release

All G protein-coupled presynaptic 5-HT autoreceptors – there is no consistent evidence for 5-HT_3 autoreceptors (Dorostkar and Boehm, this book) – are inhibitory. They belong mainly to the 5-HT_1 family and where subclassified were 5-HT_{1B} or 5-HT_{1D} (Table 4). Non-5-HT_1 presynaptic autoreceptors, presumably 5-HT_5 and/or 5-HT_7, replaced the 5-HT_1-like receptors in 5-HT_{1B} knockout mice (Pineyro et al. 1995b; see page 327 of Göthert and Schlicker 1997). Note that, as opposed to dopamine and histamine autoreceptors, 5-HT autoreceptors differ in the neurons' terminal and soma-dendritic region: in the latter, they are 5-HT_{1A} (Sprouse and Aghajanian 1986).

Studies, *in vitro* and *in vivo*, in which appropriate antagonists increased the release of serotonin have shown that presynaptic 5-HT autoreceptors are physiological regulators of 5-HT release in all regions of the CNS (references in Table 4; and see Table 1 of Göthert and Schlicker 1997). A study with pertussis toxin has confirmed that the 5-HT_{1B} autoreceptors in rat striatum couple to Gi/o (Sarhan and Fillion 1999). Further downstream, release is inhibited by reduction of transmembrane Ca^{2+} influx (see p. 934 of Starke et al. 1989; p. 329 of Göthert and Schlicker

Table 4 Tissues in which neurotransmitter release is increased (↑) or decreased (↓) by presynaptic5-HT receptor activation. Receptor classification (except with superscript [a]) as by the authors cited. Designation as 5-HT_1-like where no differentiation between 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} was made. Designation as 5-HT_2-like where no differentiation between 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} was made

Release of transmitter	Species and Tissue	Receptor Type	References
5-HT (see 4.1)	Rat neocortex	5-HT_{1B}↓	1; Gobert et al. 1998; Rutz et al. 2006
	neocortex	5-HT_{1B} and 5-HT_{1D}↓	2
	hippocampus	5-HT_{1B}↓	1; Birthelmer et al. 2002; 2003; Rutz et al. 2006
	diencephalon	5-HT_1-like↓	2
	septal region	5-HT_{1B}↓	Rutz et al. 2007
	striatum	5-HT_1-like↓	1
	striatum	5-HT_{1B}↓	Sarhan and Fillion 1999
	nucleus accumbens	5-HT_1-like↓	1
	hypothalamus	5-HT_{1B}↓	1
	cerebellum	5-HT_{1B}↓	1
	brain stem	5-HT_1-like↓	1
	medulla oblongata	5-HT_1-like↓	1
	spinal cord	5-HT_{1B}↓	2
	Mouse neocortex	5-HT_{1B}↓	2
	neocortex	5-HT_5 and/or 5-HT_7 ↓	2
	hippocampus	5-HT_{1B}↓	2
	hippocampus	5-HT_5 and/or 5-HT_7 ↓	2
	hypothalamus	5-HT_1-like↓	2
	cerebellum	5-HT_1-like↓	1
	Guinea pig neocortex	5-HT_{1B}↓	2
	hippocampus	5-HT_{1B}↓	2
	hypothalamus	5-HT_1-like↓	2
	substantantia nigra	5-HT_{1B}↓	2
	medulla oblongata	5-HT_1-like↓	2
	medulla oblongata	5-HT_{1D}↓	Bobker and Williams (1990)
	Rabbit neocortex	5-HT_{1B}↓	2
	hippocampus	5-HT_{1B}↓	2
	hypothalamus	5-HT_1-like↓	1
	striatum	5-HT_{1B}↓	2
	superior colliculus	5-HT_1-like↓	2
	Cat caudate nucleus	5-HT_1-like↓	1
	Pig neocortex	5-HT_{1D}↓	1
	Rhesus monkey	5-HT_{1B}↓	2
	Human neocortex	5-HT_{1B}↓	2
	hippocampus	5-HT_1-like↓	2
Noradrenaline (see 4.3)	Rat pithed *in vivo*	5-HT_1-like↓	4
	kidney	5-HT_1-like↓	4
	vena cava	5-HT_{1B}↓	4
	Guinea pig mesenteric artery	5-HT_1-like↓	4
	Rabbit ear artery	5-HT_1-like↓	4
	Cat nictitating membrane	5-HT_1-like↑	4; Park et al. 1991

(*Continued*)

Table 4 Continued

Release of transmitter	Species and Tissue	Receptor Type	References
	Dog coronary artery	5-HT_1-like↓	4
	tibial artery	5-HT_1-like↓	4
	saphenous vein	5-HT_1-like↓	4
	Cattle ovarian follicle	5-HT_1-like↓	4
	Human atria	5-HT_{1D}↓	2; Göthert et al. 1996; Göthert 2003
	saphenous vein	5-HT_{1B}↓	4; Göthert et al. 1996; 2003
	dura mater	5-HT_1-like↓[a]	Göthert et al. 1996
Dopamine (see 4.4)	Rat striatum	5-HT_2-like↓[a]	Ennis et al. 1981
	striatum	5-HT_2-like↓	Muramatsu et al. 1988a; Ng et al. 1999
	striatum	5-HT_{2C}↓	Alex et al. 2005
	striatum	5-HT_{2C}↑	Lucas and Spampinato 2000
	striatum	5-HT_{1B}↓	Sarhan et al. 1999; Sarhan and Fillion 1999; Sarhan et al. 2000
Acetyl-choline (see 4.5)	Rat neocortex	5-HT_4↑	Consolo et al. 1994
	entorhinal cortex	5-HT_{1B}↓	Feuerstein et al. 1996a
	hippocampus	5-HT_{1B}↓	3; Feuerstein et al. 1996a; Birthelmer et al. 2002; 2003; Sarhan and Fillion 1999
	hippocampus	5-HT_2-like↓	Muramatsu et al. 1988b
	hippocampus	5-HT_4↑	Siniscalchi et al. 1999
	striatum	5-HT_1- or 5-HT_2-like↓	3
	Guinea pig neocortex	5-HT_4↑	Siniscalchi et al. 1999
	n. basalis Meynert	5-HT_4↑	Siniscalchi et al. 1999
	hippocampus	5-HT_1-like↓	3
	hippocampus	5-HT_4↑	Siniscalchi et al. 1999
	striatum	5-HT_2-like↑	3
	ileum	5-HT_{1A}↓, 5-HT_4↑	4; LePard et al. 2004
	Human neocortex	5-HT_{1F}↓	Feuerstein and Seeger 1997
	stomach	5-HT_4↑	Leclere and Lefebvre 2002
	urinary bladder	5-HT_1-like↓, 5-HT_4↑	4
GABA (see 4.6)	Rat cingulate cortex	5-HT_{1B}↓	Tanaka and North 1993
	hippocampus	5-HT_{1A}↓	Katsurabayashi et al. 2003
	hippocampus	5-HT_2-like↑	Piguet and Galvan 1994
	amygdala	5-HT_{1A}↓	Koyama et al. 1999
	hypothalamus	5-HT_1-like↓[a]	Malinina et al. 2005
	suprachiasmatic nucleus	5-HT_{1B}↓	Bramley et al. 2005
	substantia nigra	5-HT_{1B}↓	Johnson et al. 1992b
	medulla oblongata	5-HT_{1B}↓	Bobker and Williams (1989)
	spinal cord	5-HT_1-like↓	Wu et al. 1991
	Mouse suprachiasmatic nucleus	5-HT_{1B}↓	Bramley et al. 2005
	ventral midbrain	5-HT_{1B}↓	Morikawa et al. 2000
	Guinea pig VTA	5-HT_{1D}↓	Cameron and Williams 1994
	Human neocortex	5-HT_{1D}↓	Feuerstein et al. 1996b

(*Continued*)

Table 4 Continued

Release of transmitter	Species and Tissue	Receptor Type	References
Glutamate (see 4.7)	Rat neocortex	5-$HT_{2A}\uparrow$	Aghajanian and Marek 1999; Arvanov et al. 1999; Marek et al. 2001
	cingulate cortex	5-$HT_{1B}\downarrow$	Tanaka and North 1993
	entorhinal cortex	5-$HT_{1A}\downarrow$	Schmitz et al. 1998
	subicular cortex	5-$HT_{1B}\downarrow$	Boeijinga and Boddeke 1996
	nucleus accumbens	5-$HT_{1B}\downarrow$	Muramatsu et al. 1998
	amygdala	5-$HT_{1A}\downarrow$	Cheng et al. 1998
	hypothalamus	5-HT_1-like$\downarrow$[a]	Malinina et al. 2005
	brainstem	5-$HT_{1B}\downarrow$	Singer et al. 1996; Mizutani et al. 2006
	cerebellum	5-HT_1-like$\downarrow$	Raiteri et al. 1986
	medulla oblongata	5-$HT_{1A}\downarrow$, 5-$HT_{1B}\downarrow$	Bobker and Williams 1989
	spinal cord	5-$HT_{1A}\downarrow$, 5-$HT_{1B}\downarrow$	Wu et al. 1991
	Mouse suprachiasmatic nucleus	5-$HT_{1B}\downarrow$	Pickard et al. 1999
	nucleus accumbens	5-$HT_{1B}\downarrow$	Morikawa et al. 2000
	Guinea pig spinal cord	5-$HT_{1D}\downarrow$	Travagli and Williams 1996
	Hamster suprachiasmatic nucleus	5-$HT_{1B}\downarrow$	Pickard et al. 1999
	superior colliculus	5-$HT_{1B}\downarrow$	Mooney et al. 1994
	Human neocortex	5-$HT_{1D}\downarrow$	see Raiteri 2006
Glycine (see 4.8)	Rat brain stem	5-$HT_{1B}\downarrow$	Umemiya and Berger 1995
Sensory neuro-peptides (see 4.9)	Rat trigeminal nerve endings	5-HT_1-like$\downarrow$	Buzzi et al. 1991

Table summarizes studies in which release was determined either as overflow or by means of the postsynaptic response. References to reviews: 1, Starke et al. 1989; 2, Göthert and Schlicker 1997; 3, Cassel and Jeltsch 1995; 4, Fuder and Muscholl 1995.
[a] Retrospective receptor classification by T.J.F.

1997). How 5-HT_7 receptors manage to inhibit release through G_s – if they couple to G_s also presynaptically – remains open.

4.2 Serotonin Autoreceptors in Disease and Therapy

Major depression has been classically explained by deficiences in the monoamine neurotransmitters 5-HT, noradrenaline, and dopamine. The importance of these monoamine systems for the vulnerablity to depression has recently been confirmed (Ruhé et al. 2007). Do presynaptic 5-HT_{1B} autoreceptors play any role? Two groups of findings tend to answer this question in the affirmative. The depression-like state

of learned helplessness in rats is associated with a decrease in cortical 5-HT release *in vivo* (Petty et al. 1992) and an increase in the density of 5-HT_{1B} binding sites – presumably including presynaptic 5-HT_{1B} autoreceptors – in the neocortex, hippocampus, and septum (Edwards et al. 1991). Conversely, chronic treatments that are clinically effective – with tricyclic antidepressants, selective serotonin reuptake inhibitors, monoamine oxidase inhibitors, and electroconvulsive shock – increased the release of 5-HT in various brain regions in animal experiments, although not in all studies (see Table 4 of Göthert and Schlicker 1997). Chronic treatment with selective serotonin reuptake inhibitors also reduced the 5-HT_{1B} mRNA in the dorsal raphé nucleus of the rat, an effect that disappeared after discontinuation of the drugs; the mRNA decrease probably reflected a decrease in the density of presynaptic autoreceptors (see Sari 2004).

5-HT_{1A} and 5-HT_{1B} receptors are probably involved in aggressive behavior (see Popova 2006). The postsynaptic 5-HT_{1A} receptor density is decreased in the frontal cortex, hypothalamus, and amygdala of highly aggressive rats. Moreover, aggressive behavior is increased in 5-HT_{1B} receptor knockout mice, and conversely 5-HT_{1B} receptor agonists dampen aggression (see Sari 2004; Popova 2006). The common denominator of these findings may be an increase in serotonin release in aggressive animals, caused by lack of 5-HT_{1B} autoreceptors in the knockout mice and leading to downregulation of postsynaptic 5-HT_{1A} receptors in the rat experiments. Importantly, a linkage of aggressive behavior with the 5-HT_{1B} receptor gene was revealed in two human populations (see Popova 2006).

4.3 Presynaptic Serotonin Heteroreceptors on Noradrenergic Terminals

5-HT heteroreceptors occur on postganglionic sympathetic (Table 4) but not central (Allgaier et al. 1995) noradrenergic terminals. All are 5-HT_1 and, where subclassified, 5-HT_{1B} or 5-HT_{1D}. Interestingly, human postganglionic sympathetic nerve terminals possess 5-HT_{1D} receptors in the heart but 5-HT_{1B} receptors in the saphenous vein (see Göthert 2003). The case is reminiscent of GABAergic neurons, which seem to transport D_1, D_2 and D_3 receptors from the cell bodies to the terminals in a target cell-specific manner (see Section 2.7). An endogenous serotonin input to the receptors has not so far been noticed and seems unlikely. The mechanism of inhibition involves a decrease of Ca^{2+} influx through N-type Ca^{2+} channels (see Göthert 2003). For a final time in this review, an heteroreceptor/autoreceptor interaction has to be mentioned: presynaptic 5-$HT_{1B/D}$ heteroreceptors and presynaptic α_2-autoreceptors mutually narrow one another's scope for inhibition (compare Sections 2.4, 3.3, and 3.5). The facilitation of noradrenaline release through 5-HT_1-like receptors in the cat nictitating membrane (Table 4; Park et al. 1991) remains a somewhat doubtful exception (see Table 2 of Fuder and Muscholl 1995).

4.4 Presynaptic Serotonin Heteroreceptors on Dopaminergic Terminals

The corpus striatum is innervated both by the nigrostriatal dopamine neurons and by serotonin neurons from the raphé nuclei, and serotonin modifies dopamine release in rat striatum (Table 4). The way it does so, however, remains uncertain. A majority of studies indicate inhibition through 5-HT_2, probably 5-HT_{2C}, receptors, but one study has reported facilitation through 5-HT_{2C} receptors, possibly due to a different region of the striatum being examined. Alex et al. (2005) showed by microdialysis *in vivo* that local administration of a 5-HT_{2C} antagonist/inverse agonist increased dopamine release in rat striatum, indicating either an endogenous serotonin input or constitutive activity of the receptors. They assumed location of the 5-HT_{2C} receptors outside dopaminergic terminals, specifically on GABA neurons. On the other hand, location of 5-HT_2-like receptors on striatal dopaminergic terminals is supported by several findings, as a decrease in 5-HT_2-like receptor binding after 6-hydroxydopamine lesioning of the nigrostriatal pathway (Ng et al. 1999), and preservation of the inhibitory effect of agonists on high potassium-evoked dopamine release in the presence of tetrodotoxin (Ennis et al. 1981).

The already mentioned false labelling of striatal dopaminergic neurons by 5-HT (see Section 2.5) also works in the opposite direction, i.e., false labelling of 5-HT terminals by dopamine (Berger and Glowinski 1978; Feuerstein et al. 1986). The question arises of whether some of the studies in the dopamine section of Table 4 in which [^{3}H]-dopamine was used (Ennis et al. 1981; Muramatsu et al. 1988a; Ng et al. 1999; Sarhan et al. 1999; Sarhan and Fillion 1999; Sarhan et al. 2000) inadvertently measured [^{3}H]-dopamine release from 5-HT neurons. In none of these studies were measures taken against false labelling. So were the presumed serotonin heteroreceptors in fact serotonin autoreceptors? This seems unlikely in the case of the 5-HT_2-like receptors, which do not occur as presynaptic 5-HT autoreceptors (Section 4.1). The three reports on 5-HT_{1B} heteroreceptors are more "suspicious" in this respect. The latter couple to Gi/o (Sarhan and Fillion 1999).

Although striatal 5-HT_4 receptors, when activated, facilitate dopamine release, the receptors probably are located on striatal GABA neurons, so the receptors have not been entered in Table 4 (see Eglen et al. 1995).

5-HT_{1A} heteroreceptors depress, whereas 5-HT_{2A} heteroreceptors enhance, the synthesis of dopamine in rat corpus striatum. The former effect (Johnson et al. 1993) occurs even in the absence of any dopamine release, is shared by endogenous serotonin, and may involve adenylyl cyclase inhibition and a diminution of TH phosphorylation as discussed above for dopamine D_2 autoreceptors (Section 2.2). The 5-HT_{2A} increase in synthesis, in contrast (Lucas and Spampinato 2000), occurs only when dopamine neurons are activated by blockade of their D_2 autoreceptors.

4.5 Presynaptic Serotonin Heteroreceptors on Cholinergic Terminals

Of all neurons, the cholinergic ones possess the greatest variety of presynaptic serotonin receptors: inhibition of acetylcholine release through 5-HT_1-like receptors, inhibition or facilitation through 5-HT_2 receptors, inhibition through 5-HT_3 receptors, and facilitation through 5-HT_4 receptors have all been reported (Table 4). I leave out the 5-HT_3 receptors here for reasons given; they are doubtful anyway (Boehm and Dorostkar, this book). As reviewed in detail by Cassel and Jeltsch (1995), the serotonergic modulation of brain cholinergic systems has cognitive implications. For this reason, a number of studies have been carried out in the hippocampus and the entorhinal cortex.

The literature agrees that, in rat and guinea pig hippocampus and rat entorhinal cortex, acetylcholine release is inhibited through 5-HT_1-like – where examined 5-HT_{1B} – receptors (Table 4). However, this is not the only serotonergic modulation. When Feuerstein et al. (1996a) applied serotonin to hippocampal or entorhinal cortex slices, it failed to change the release of [^{3}H]-acetylcholine, although the 5-HT_{1B}-selective agonist Ru24969 caused the expected inhibition. Serotonin inhibited release only when either (1) 5-HT_2 receptors were blocked, or (2) release was elicited by high K^+ concentrations in the presence of tetrodotoxin to interrupt action potential conduction, or (3) substance P was depleted by treatment of capsaicin, or (4) neurokinin$_1$ (NK_1) receptors were blocked. In the same study 5-HT_{2A} receptors were located to GABA/substance P neurons by in situ hybridization (Feuerstein et al. 1996a). The ensuing hypothesis is depicted in Figure 2. In the hippocampus and the entorhinal cortex, cholinergic terminals carrying 5-HT_{1B} inhibitory and NK_1 facilitatory presynaptic receptors are innervated by GABA/substance P interneurons. Serotonergic agonists may modulate this release by two mechanisms: (1) direct inhibition through the presynaptic 5-HT_{1B} receptor or (2) indirect facilitation through a soma-dendritic 5-HT_{2A} receptor on the GABA/substance P neuron followed by substance P release and activation of the presynaptic NK_1 receptor. In the case of serotonin itself as agonist, the two opposing mechanisms cancel each other.

A very similar twofold mechanism modulates acetylcholine release in the human neocortex, with one exception: the inhibitory presynaptic serotonin receptor is 5-HT_{1F} (Feuerstein and Seeger 1997).

5-HT_4 agonists enhance the release of acetylcholine in several peripheral tissues and several brain areas (Table 4). 5-HT_4 antagonists counteract the effect but, in the studies published so far, caused no change when given alone. The co-location of facilitatory 5-HT_4 and inhibitory 5-HT_1 receptors on hippocampal cholinergic terminals is reminiscent of the co-location of D_1 and D_2 receptors on, for example, GABAergic terminals (Section 2.7). In contrast to the two dopamine receptors,

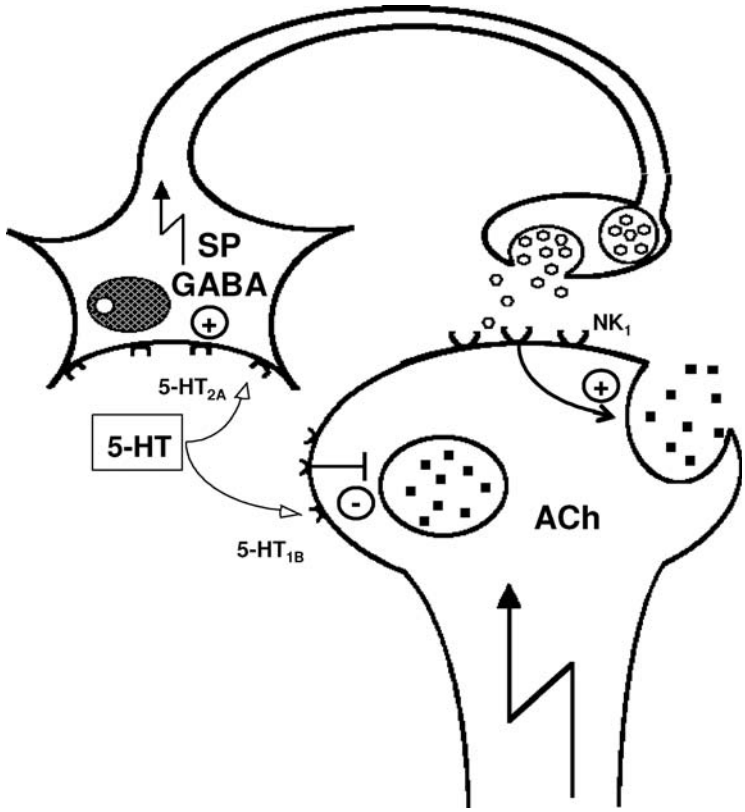

Fig. 2 Interaction between acetylcholine, 5-HT, and substance P in rat hippocampus and entorhinal cortex. ACh = Acetylcholine; SP = substance P; NK_1 = neurokinin$_1$ receptor; arrows within the neurons: filled head symbolizes action potentials, open head symbolizes a facilitatory effect, crossbar symbolizes an inhibitory effect on acetylcholine release. Reproduced, with permission, from Feuerstein et al. (1996a).

which possess similar affinity for dopamine, in the case of the two serotonin receptors the affinity of the inhibitory 5-HT_{1B} receptor for serotonin is around 10 times higher than the affinity of the facilitatory 5-HT_4 receptor.

The pertussis toxin sensitivity and, hence, Gi/o coupling of 5-HT_{1B} autoreceptors in rat striatum (Section 4.1) and of the 5-HT_{1B} heteroreceptors inhibiting dopamine release in rat striatum (Section 4.4) also holds true for the 5-HT_{1B} heteroreceptors inhibiting acetylcholine release in rat hippocampus (Sarhan and Fillion 1999). Presynaptic HT_4 receptors increase cAMP levels which in turn, by stimulating protein kinase A, may inhibit K^+ channels to enhance transmitter release (Eglen et al. 1995).

4.6 Presynaptic Serotonin Heteroreceptors on GABAergic Terminals

These are the only terminals where Boehm and Dorostkar (in this *Handbook*) accept 5-HT_3 receptors with confidence. As to G protein-coupled receptors, 5-HT_1-like inhibitory receptors – where subclassified 5-HT_{1A}, 5-HT_{1B} or 5-HT_{1D} – are common (Table 4). A comparative study detected them in human (5-HT_{1D}) but not rabbit neocortex (Feuerstein et al. 1996b).

The location of the 5-HT_2-facilitatory receptors in rat hippocampus – whether on cell bodies or terminals of GABA interneurons – is not certain, although the authors (Piguet and Galvan 1994) prefer the latter alternative.

In guinea pig hippocampal slices, low concentrations of a 5-HT_4 receptor agonist reduced, whereas high concentrations increased, the release of GABA (Bianchi et al. 2002). The modulation was not due to an action on GABA terminals but to a primary increase in acetylcholine release (Section 4.5), which then modulated the release of GABA – hence no entry in the GABA section of Table 4.

Signal transduction was studied most precisely for the 5-HT_{1A} receptor in rat amygdala (Koyama et al. 1999). The receptor operated through a N-methyl maleimide-sensitive mechanism and by adenyl cyclase inhibition rather than any change in K^+ or Ca^{2+} channels. The fall in cyclic AMP presumably was followed by decrease in the phosphorylation of synaptic vesicle proteins and, finally, in a decrease of exocytosis.

In the VTA, GABA terminals synapse onto the mesolimbic dopamine neurons. One feature of the effect of cocaine is inhibition of the release of GABA from these terminals. The effect has traditionally been attributed to a dopaminergic mechanism: blockade of the reuptake of dendritically released dopamine would be followed by reinforcement of D_2-like receptor-mediated inhibition of GABA release (see Table 1). However, in guinea pig midbrain slices the inhibition of GABA release by low concentrations of cocaine was counteracted by a 5-HT_1 antagonist and disappeared after depletion of serotonin stores. So here cocaine acted by a serotonergic mechanism: blockade of the reuptake of 5-HT released from raphé neuron terminals was followed by reinforcement of 5-HT_1 (specifically 5-HT_{1D}; Table 4) receptor-mediated inhibition of GABA release (Cameron and Williams 1994). In accord with this revised view, the inhibition by cocaine of GABA release in mouse midbrain slices disappeared after genetic deletion of the 5-HT_1 (in this case 5-HT_{1B}; Table 4) receptor (Morikawa et al. 2000).

4.7 Presynaptic Serotonin Heteroreceptors on Glutamatergic Terminals

The multitude of entries in Table 4 bears witness to an important impact of serotonin on glutamatergic neurotransmission. The predominant effect is inhibitory and

mediated by 5-HT_1-like – where identified 5-HT_{1A}, 5-HT_{1B} or 5-HT_{1D} – receptors. No evidence was found for a serotonergic modulation of [^{3}H]-glutamate release in rat hippocampal slices (Sehmisch et al. 2001).

In the calyx of Held synapse in the auditory brainstem of rats the 5-HT_{1B} receptor inhibition of glutamate release was fully explained by inhibition of Ca^{2+} entry through voltage-dependent channels (Mizutani et al. 2006). In rat nucleus accumbens, in contrast, the analogous 5-HT_{1B} effect did not involve inhibition of Ca^{2+} channels (Muramatsu et al. 1998). In rat amygdala, the 5-HT_{1A} inhibition operated via a cyclic AMP pathway and apparently inhibition of Ca^{2+} entry; an unexplained observation was the failure of pertussis toxin to abolish the 5-HT_{1A} inhibition (Cheng et al. 1998).

In the preceding section (4.6), the inhibition by cocaine of GABA release in the VTA was explained by reinforcement of serotonergic, not dopaminergic, presynaptic inhibition. An interesting parallel, but with the opposite explanation, has now to be addressed. In the nucleus accumbens, glutamate terminals synapse onto the medium spiny GABA neurons. Another feature of the effect of cocaine is inhibition of the release of glutamate from these terminals. In mouse nucleus accumbens slices, the inhibition by cocaine was the same, irrespective of whether the slices had been cut from wild-type mice or animals in which the 5-HT_{1B} receptor gene had been deleted (Morikawa et al. 2000). Cocaine operated by a dopaminergic, not a serotonergic mechanism: blockade of the reuptake of dopamine released from nigro-striatal axons followed by reinforcement of D_5 receptor-mediated inhibition of glutamate release (Nicola et al. 1996; Table 1).

In contrast to the inhibitory actions of 5-HT_1-like receptors, 5-HT_{2A} receptors facilitate glutamate release. This facilitation may be mediated through protein kinase C activation (Arvanov et al. 1999).

4.8 Presynaptic Serotonin Heteroreceptors on Glycinergic Terminals

Glycine is the major inhibitory neurotransmitter in the spinal cord and brain stem. 5-HT presynaptically inhibits glycinergic synaptic transmission to rat brain stem hypoglossal motoneurons through 5-HT_{1B} receptors (Umemiya and Berger 1995). The inhibition did not involve Ca^{2+} channel modulation.

4.9 Presynaptic Serotonin Heteroreceptors on Peptidergic Terminals

Inhibitory 5-HT receptors on sensory trigeminal fibers of blood vessels in the meninges inhibit the release of neuropeptides such as substance P and CGRP

(compare Section 3.10). This leads to inhibition of the sterile neurogenic inflammation induced by these peptides.

4.10 Presynaptic Serotonin Heteroreceptors in Disease and Therapy

An increase in serotonergic transmission in the CNS induces anxiety – hence the therapeutic effect of the 5-HT_{1A} receptor partial agonist buspirone, which probably acts by activation of the inhibitory soma-dendritic autoreceptors of serotonin neurons (see Göthert and Starke 2005). Anxiogenic effects have been obtained by activation of 5-HT_{1B} receptors in rodents (see Sari 2004). The mechanism may involve presynaptic inhibition of the release of several neurotransmitters. One is acetylcholine. In the hippocampus acetylcholine suppresses anxiety through both nicotinic and muscarinic receptors, and inhibition of its release will produce anxiety (see Sari 2004). Another is GABA. Its anxiolytic role is established (e.g., Roy-Byrne 2005), and again inhibition of its release, as through 5-HT_{1D} receptors in the human neocortex (Feuerstein et al. 1996b), will be anxiogenic. In particular, the combination of a depressed GABAergic and an enhanced serotonergic neurotransmission seems to be anxiogenic (Kahn et al. 1988; Green 1991).

As pointed out above (Section 4.2), 5-HT_{1B} agonists may dampen aggression via activation of 5-HT_{1B} autoreceptors. However, 5-HT_{1B} heteroreceptors may contribute (see Sari 2004). One hypothesis implicates inhibitory soma-dendritic 5-HT_{1B} receptors on vasopressin neurons in the anterior hypothalamus. Activity of these neurons promotes attacks and biting in rodents, hence inhibition of their activity leads to the opposite effect (Ferris et al. 1997).

Serotonin also plays an important role in the pathogenesis and therapy of migraine. The triptanes are 5-$HT_{1B/D}$ receptor agonists. They shorten migraine attacks by two mechanisms, meningeal vasoconstriction through smooth muscle 5-HT_{1B} receptors and inhibition of the release of sensory neuropeptides from trigeminal afferents through presynaptic 5-HT_{1D} receptors (see Göthert and Starke 2005).

Serotonergic/cholinergic interactions influence cognition (see Buhot et al. 1995; Cassel and Jeltsch 1995). For example, 5-HT controls subcortical cholinergic projections to the hippocampus and the neocortex, cooperating in this function with substance P-containing interneurons (Sections 4.5 and Figure 2). The cooperation has led to therapeutic speculations, as follows (Feuerstein and Seeger 1997). In Alzheimer's disease, serotonin uptake inhibitors are used to relieve depression. This may have consequences for acetylcholine release and, hence, cognition; it may detrimentally impair (through 5-HT_{1B} or 5-HT_{1F} heteroreceptors at cholinergic terminals) and beneficially enhance (through soma-dendritic 5-HT_2 receptors of the substance P interneurons) hippocampal and cortical acetylcholine release. Therapeutic improvement of cognition may be expected from a (pure) 5-HT_{1F} receptor antagonist, a 5-HT_2 receptor agonist, and an NK_1 receptor agonist or from their combination.

Apart from 5-HT and acetylcholine, glutamate is involved in most aspects of cognition, for instance in learning. 5-HT_{1B} heteroreceptors depress the release of glutamate from the terminals, in the subiculum, of glutamatergic CA1 hippocampal pyramidal cells (Boeijinga and Boddeke 1996; Table 4). 5-HT_{1B} agonists impair learning by this mechanism, whereas 5-HT_{1B} antagonists enhance learning (page 573 of Sari 2004).

Finally, serotonin contributes to the pharmacology of cocaine, although less prominently than dopamine. The 5-HT_{1B} heteroreceptor mechanism of the inhibition by cocaine of GABA release in the VTA has been explained in Section 4.6. The inhibition of GABA release in turn disinhibits VTA dopamine neurons, thus enhancing dopamine release in the terminal region of the nucleus accumbens, a key structure of addiction (compare Section 2.3).

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Presynaptic Adenosine and P2Y Receptors

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Abstract Adenine-based purines, such as adenosine and ATP, are ubiquitous molecules that, in addition to their roles in metabolism, act as modulators of neurotransmitter release through activation of presynaptic P1 purinoceptors or adenosine receptors (activated by adenosine) and P2 receptors (activated by nucleotides). Of the latter, the P2Y receptors are G protein-coupled, whereas the P2X receptors are ligand-gated ion channels and not covered in this review.

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The adenosine receptor family comprises the subtypes A_1, A_{2A}, A_{2B} and A_3; the P2Y receptor family includes eight subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$. Presynaptic A_1 receptors and the $P2Y_{1,2,4,12}$ subtypes depress neurotransmitter release, whereas presynaptic A_{2A} and A_{2B} receptors facilitate neurotransmitter release. A_3 receptors and the other P2Y subtypes do not seem to modulate neurotransmitter release. Activation of A_1 or the $P2Y_{1,2,4,12}$ receptor subtypes may inhibit calcium entry through voltage-sensitive N-type calcium channels and decrease the affinity of the release apparatus for Ca^{2+} with a subsequent inhibition of transmitter release. Facilitation of neurotransmitter release caused by activation of A_2 receptors involves an increase in cyclic AMP and/or activation of protein kinases A and C. These kinases may directly augment Ca^{2+}-currents or act directly at the exocytotic machinery, increasing the probability of vesicle-fusion and/or release or they may act indirectly, preventing an ongoing inhibition of voltage-sensitive Ca^{2+} channels.

Different types of adenosine and P2Y receptors may be present in the same nerve terminal. The effect that prevails may be determined by the functional interaction, in the receptor biophase, between purine release, uptake and metabolism.

The adenosine modulation of synaptic transmission exceeds the simple direct control of neurotransmitter release. Adenosine may have a permissive effect on responses to other presynaptic modulators, may regulate the rate of desensitization of other presynaptic receptors, or prevent an excessive influence of inhibitory receptors. In this respect it is often viewed as a "modulator of modulators" involved in the fine-tuning of neurotransmitter release.

1 The Beginning—Purines and Modulation of Neurotransmitter Release

Adenine-based purines such as adenosine and ATP are ubiquitous molecules that, in addition to their roles in metabolism, function as extracellular signalling messengers. Effects of adenosine on neurotransmitter release were first described by Ginsborg and Hirst (1972), based on a study carried out in the rat phrenic nerve-diaphragm preparation. In that study adenosine reduced the quantum content of end-plate potentials and the frequency of miniature end-plate potentials, an effect interpreted as a depression of acetylcholine release. In the same preparation, ATP and ADP also depressed release, raising the hypothesis that all these substances were acting by a common mechanism (Ribeiro and Walker 1975). The role of adenosine as inhibitory modulator of neurotransmitter release was confirmed for several other cholinergic synpases, in the peripheral nervous system (PNS) as well as in the central nervous system (CNS; Harms et al. 1979; Pedata et al. 1983; Jackisch et al. 1984), and was extended to other transmitters. At the end of the 1980s, adenosine was accepted to be a general inhibitory neuromodulator, depressing release of excitatory and inhibitory neurotransmitters in the CNS and PNS by activation of

A_1 receptors. Subsequently, the detection of release-facilitating effects of adenosine, acting at A_2 receptors, and of effects of nucleotides mediated by P2 receptors complicated the picture.

2 Adenosine Receptor-Mediated Inhibition of Neurotransmitter Release

2.1 The Receptors Involved

Effects of adenosine were initially explained by its role in intracellular signalling, as an intermediary of cyclic nucleotide production (Londos and Preston 1977). The antagonism of methylxanthines against adenosine (Ther et al. 1957) was also ascribed to an intracellular action (Sattin and Rall 1970). Later, the use of derivatives of adenosine and of theophylline covalently linked to an oligosaccharide and large enough to prevent their access to the intracellular compartment (Olsson et al. 1976) provided evidence for an involvement of cell membrane receptors in the effects of extracellular adenosine and methylxanthines. The existence of different types of membrane receptors for endogenous purines was demonstrated by differences in the order of potency of agonists, in the type of influence they exerted on cyclic AMP (cAMP) levels, and in the ability of methylxanthines to antagonize the responses (Burnstock 1978). Adenosine was the most potent endogenous agonist at receptors that were blocked by methylxanthines; therefore these were named adenosine receptors or P1 purinoceptors (Burnstock 1978). Nucleotides were more potent than adenosine on other receptors (see Section 4.1). Adenosine receptors were initially divided into A_1 and A_2 subtypes based on inhibitory and stimulatory effects on adenylate cyclase, respectively (van Calker et al. 1978). They were later further divided into A_1, A_{2A}, A_{2B} and A_3 subtypes, each presenting distinct pharmacological properties (Table 1; see Fredholm et al. 2001).

Adenosine analogues that do not easily cross the cell membrane were shown to depress neurotransmitter release either when release was measured as postsynaptic response (mechanical: Muller and Paton 1979; Paton 1981; electrophysiological: Silinsky 1980; Ribeiro and Sebastião 1985; Dunwiddie et al. 1986) or as overflow (Fredholm et al. 1983; Jackisch et al. 1984; 1985; Feuerstein et al. 1985). The target, hence, was a membrane receptor, and the order of potency of the various agonists led to the assumption that the presynaptic inhibitory adenosine receptors were of the A_1 subtype. However, differences in the order of potency of agonists raised some doubts. In the neuromuscular junction of the frog (Ribeiro and Sebastião 1985) and rat (Sebastião and Ribeiro 1988), 5′-N-ethylcarboxamidoadenosine (NECA) inhibited the release of neurotransmitter more potently than 2-chloroadenosine, while the opposite was true in the heart. A retrospective analysis of the results obtained by others was also used to claim the involvement of a xanthine-sensitive receptor distinct

Table 1 Pharmacology of Adenosine Receptor Subtypes Expressed in Mammalian Tissues

Type	Principal Agonists	Antagonists	References
A_1	CPA	DPCPX	Moos et al. 1985; Lohse et al. 1987
A_{2A}	CGS 21680	ZM 241385	Hutchison et al. 1989; Jarvis et al. 1989; Lupica et al. 1990
		SCH 58261	Poucher et al. 1995; Ongini et al. 1999
A_{2B}	(*)	Enprofylline	Auchampach et al. 1997
		MRS 1754	Feoktistov and Biaggioni 1997; Kim et al. 2000
A_3	AB-MECA	MRE-3008-F20	van Galen et al. 1994
	Cl-IB-MECA	VUF5574	van Schaick et al. 1996; Varani et al. 2000; Van Muijlwijk-Koezen et al. 2000

AB-MECA: N^6-(iodo-4-amino-benzyl)-5′-N-methyl-carboxamidoadenosine; CGS 21680: 2-p- (2-carboxyethyl) phenylamino)-5′-N-carboxamidoadenosine; Cl-IB-MECA: 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-N-methylcarboxamide; CPA: N^6-cyclopentyladenosine; DPCPX: 1,3-dipropyl-8-cyclopentylxanthine; MRE-3008-F20: 5-[[(4-methoxyphenyl)amino]carbonyl]amino-8-ethyl-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine; MRS 1754: 8-[4-[((4-cyanophenyl)carbamoylmethyl) oxy]phenyl]-1,3-di(n-propyl)-xanthine; SCH 58261: 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; VUF5574: N-(2-methoxyphenyl)-N-(2(3-pyridyl)quinazolin-4-yl)urea; ZM 241385: 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol.

(*) NECA, although nonselective, remains the most potent A_{2B} agonist so far (Brackett and Daly 1994; Feoktistov and Biaggioni 1997) and thus, characterization of A_{2B} receptor-mediated effects is based mainly on negative results, i.e. the failure of CGS 21680 or of CPA to reproduce NECA elicited responses (Feoktistov and Biaggioni 1997).

from the A_1 receptor (Ribeiro and Sebastião 1986). In addition to the discrepancies in the order of potency of the agonists, the observations obtained in some experimental models (Ribeiro et al. 1979; Dunwiddie and Fredholm 1984; Ribeiro and Sebastião 1986; Fredholm and Lindgren 1987)—that presynaptic inhibitory adenosine receptors were not negatively coupled to adenylate cyclase—were taken to support the claim that presynaptic inhibitory adenosine receptors were distinct from A_1. The controversy was later resolved by the demonstration that the same inhibitory receptor could couple to different intracellular targets to cause inhibition of neurotransmitter release (Fredholm and Dunwiddie 1988; Sebastião and Ribeiro 1988). The availability of selective A_1 adenosine receptor antagonists (Table 1) and their use in release experiments (Sebastião et al. 1990) combined with immunohistochemical and molecular biology methods (Rivkees et al. 1995; Ruiz et al. 2000; Schindler et al. 2001) provided definite confirmation that A_1 is the subtype involved in the inhibition of neurotransmitter release by adenosine.

2.2 Intracellular Events in A_1 Receptor-Mediated Presynaptic Inhibition

The proteins that regulate calcium entry seem to be the main targets of the transduction mechanism triggered by presynaptic A_1 adenosine receptors. Adenosine was shown to inhibit calcium uptake by synaptosomes (Ribeiro et al. 1979) and presynaptic calcium currents in rat hippocampal slices (Reddington et al. 1982; Schubert et al. 1986) and dorsal root ganglion cells of rat (Dolphin et al. 1986) and mouse (Gross et al. 1989). The main effector candidates are the voltage-sensitive calcium channels (VSCC), mainly of the N-type (Gross et al. 1989; Yawo and Chuhma 1993; Mynlieff and Beam 1994) but also of the P/Q-type (Ambrosio et al. 1997; Silinsky 2004).

Coupling of presynaptic A_1 adenosine receptors to VSCC occurs via $G_{i/o}$ proteins (Munshi et al. 1991), since the adenosine-induced inhibition of neurotransmitter release was blocked by N-ethylmaleimide (Fredholm et al. 1986; Allgaier et al. 1987; Fredholm and Lindgren 1987) and by pertussis toxin (PTX; Gross et al. 1989; Jeong and Ikeda 2000), two compounds that uncouple $G_{i/o}$ proteins from receptors (Hertting et al. 1990). Coupling seems to occur via a direct interaction between $G_{i/o}$ protein $\beta\gamma$ subunits and VSCC without requiring an intermediate component (Yasuda et al. 1996; Lim et al. 2001). Coupling of $\beta\gamma$ subunits to VSCC may be influenced by the composition of the heterotrimeric G protein. As far as N-type VSCC are concerned, coupling efficiency is higher when α_{i2} is the G protein α subunit (Jeong and Ikeda 2000).

The possibility that A_1 receptors influence targets downstream of calcium entry has also been proposed because, at the amphibian neuromuscular junction, adenosine inhibited acetylcholine release by a mechanism that did not depend on calcium entry into the presynaptic terminal (Hunt et al. 1994; Robitaille et al. 1999). This downstream pathway may also involve G-protein $\beta\gamma$ subunits (Blackmer et al. 2001).

Figure 1 summarizes the different transduction mechanisms. Interestingly, adenylate cyclase, a common target of $G_{i/o}$ proteins in other biological systems, does not seem to be involved in the intracellular effects triggered by activation of presynaptic A_1 adenosine receptors (Fredholm et al. 1986; Fredholm and Lindgren 1987).

2.3 Distribution of Presynaptic A_1 Receptors

The distribution of the A_1 adenosine receptor and its mRNA has been investigated by autoradiography, in situ hybridization, and immunohistochemistry in several animal species (Weber et al. 1988; Rivkees et al. 1995; Dixon et al. 1996) including humans (Fastbom et al. 1986; Glass et al. 1996; Lynge and Hellsten 2000; Schindler et al. 2001). The receptor occurs in the CNS and PNS, without marked differences between species. In the CNS, the highest densities are found in the hippocampus and dentate gyrus, cerebellum, thalamic nuclei, and neocortex (see

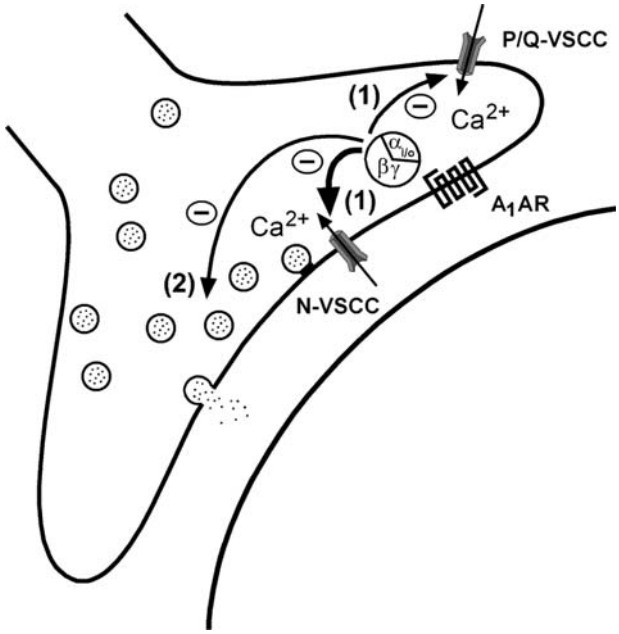

Fig. 1 Mechanisms involved in presynaptic inhibition through A_1 adenosine receptors (A_1AR). A_1AR couple to PTX-sensitive $G_{i/o}$ proteins. Two pathways are then mediated by $G_{i/o}\beta\gamma$ subunits: (1) to N-type voltage-sensitive calcium channels (VSCC) and, to a smaller degree, P/Q-type VSCC, leading to voltage -dependent inhibition, and (2) to the exocytotic machinery, leading to inhibition by a mechanism independent of calcium currents.

Fredholm et al. 2001). However, such levels of expression reflect the presence of both pre- and postsynaptic receptors. Thus, the distribution of the presynaptic A_1 receptors must be evaluated by functional approaches rather than be inferred from the total level of receptor expression.

Inhibition of neurotransmitter release mediated by A_1 adenosine receptors is a widespread phenomenon. As mentioned in Section 1, it was first described for cholinergic neurons. But presynaptic A_1 receptors also inhibit the release of several other neurotransmitters both in CNS and PNS. Table 2 summarizes early relevant studies. As to postganglionic parasympathetic neurons, there is only one study to our knowledge. In that study, carried out in guinea pig atria, no A_1 receptor-mediated modulation of acetylcholine release was found (Nakatsuka et al. 1995).

2.4 Endogenous Activation of Presynaptic $\mathbf{A_1}$ *Receptors*

The extracellular levels of adenosine have been estimated to be approximately 30 to 500 nM, but may dramatically increase during hypoxia (Decking et al. 1997; MacLean et al. 1998; see Fredholm et al. 2001; Latini and Pedata 2001). Adenosine

Table 2 Inhibition by Neurotransmitter Release through A_1 Adenosine Receptors

Neurotransmitter	Tissue	Preparation (species; receptor)	References
Acetylcholine	Striatum	Slices (rat; **)	Harms et al. 1979
	Hippocampus	Slices (rabbit; A_1)	Jackisch et al. 1984
	Brain cortex	Slices (rat; **)	Pedata et al. 1983
	Neuromuscular junction	Phrenic nerve-diaphragm preparation (rat; xanthine-sensitive receptor; A_1)	Ginsborg and Hirst 1972 Ribeiro and Walker 1975; Sebastião et al. 1990; Ribeiro and Sebastião 1985
		Phrenic nerve-diaphragm preparation (frog; xanthine-sensitive receptor)	
Noradrenaline	Striatum	Slices (rat; **)	Harms et al. 1979
	Brain cortex	Synaptosomes (guinea pig; A_1)	Ebstein and Daly 1982
	Hippocampus	Synaptosomes (guinea pig; A_1)	Ebstein and Daly 1982
		Slices (rat; A_1)	Jonzon and Fredholm 1984
		Slices (rabbit; A_1)	Jackisch et al. 1985
	Postganglionic sympathetic nerve terminals	Kidney (rabbit; **)	Hedqvist and Fredholm 1976
		Subcutaneous adipose tissue (dog; **)	Hedqvist and Fredholm 1976
		Vas deferens (guinea pig; **)	Hedqvist and Fredholm 1976
Dopamine	Striatum	Slices (rat; **)	Harms et al. 1979
		Synaptosomes (rat; **)	Michaelis et al. 1979
		Synaptosomes (guinea pig; ** A_1)	Ebstein and Daly 1982
Serotonin	Hippocampus	Slices (rabbit; A_1)	Feuerstein et al. 1985
Glutamate	Hippocampus	Slices (rat; **)	Corradetti et al. 1984; Dolphin and Archer 1983
Aspartate	Hippocampus	Slices (rat; **)	Corradetti et al. 1984; Bowker and Chapman 1986
GABA	Cerebral cortex	Slices (rat; **)	Hollins and Stone 1980

Receptor type according to authors' conclusions; ** receptor not identified.

activates A_1 receptors in concentrations ranging from 3–30 nM (Fredholm et al. 2001). Thus, the concentration of endogenous adenosine is sufficient to activate A_1 receptors either under physiological or pathophysiological conditions.

Three experimental observations prove tonic effects mediated by presynaptic A_1 adenosine receptors: effects of A_1 receptor antagonists, of exogenous adenosine deaminase (ADA; the enzyme that metabolizes adenosine), and of ADA inhibitors. A_1 receptor antagonists were shown to enhance neurotransmitter release estimated by electrophysiological responses (Dunwiddie et al. 1981; Ribeiro and Sebastião 1987) or by overflow methods (Katsuragi and Su 1982; Jackisch et al. 1984; Feuerstein et al. 1985; Gonçalves and Queiroz 1993). Incubation of tissue

preparations with exogenous ADA also enhanced neurotransmitter release, irrespectively of the method used to estimate release (electrophysiology: Sebastião and Ribeiro 1985; Haas and Green 1988; overflow: Michaelis et al. 1979; Jackisch et al. 1984; Feuerstein et al. 1985; Gonçalves and Queiroz 1996). Inhibition of ADA exerted the expected opposite effect: inhibition of transmitter release (Enero and Saidman 1977).

3 Adenosine Receptor-Mediated Facilitation of Neurotransmitter Release

3.1 The Receptors Involved and Their Distribution

The possibility that adenosine could influence neurotransmitter release by a mechanism other than the common A_1 receptor-mediated inhibition was raised by the observation that, when A_1 receptor activation was prevented, adenosine analogues enhanced release of catecholamines (brain synaptosomes: Ebstein and Daly 1982; guinea pig pulmonary artery: Wiklund et al. 1989; striatal dialysates: Zetterstrom and Fillenz 1990; rat iris: Fuder et al. 1992) and acetylcholine (rat striatal immunoaffinity-purified cholinergic synaptosomes: Brown et al. 1990). The number of studies reporting adenosine receptor-mediated facilitation increased after the discovery of the selective and high-affinity A_{2A} receptor agonist CGS 21680 (Table 1). CGS 21680 facilitated acetylcholine release at the rat neuromuscular junction without the need to block A_1 adenosine receptors (Correia-de-Sá et al. 1991) and made it possible to demonstrate the facilitation of acetylcholine, glutamate, noradrenaline, dopamine, serotonin, glycine, taurine, and GABA release (see Table 3). Interestingly, in the prostatic portion of rat vas deferens, where neuronal A_{2A} adenosine receptors seem to be absent (Diniz et al. 2003), the nonselective adenosine receptor agonist NECA, but not CGS 21680, enhanced noradrenaline release when A_1 receptors were blocked (Queiroz et al. 2002; Queiroz et al. 2004), an effect prevented by the A_{2B} adenosine receptor antagonists enprofylline and MRS 1754 (Table 1), indicating the involvement of A_{2B} receptors. This is as yet the only observation of an A_{2B} receptor-mediated facilitation of neurotransmitter release, but the possibility that it may operate in other tissues that lack A_{2A} adenosine receptors should be investigated as soon as more selective A_{2B} adenosine receptor agonists become available.

3.2 Intracellular Events in A_2 Receptor-Mediated Presynaptic Facilitation

The A_{2A} receptors modulating neurotransmitter release are known to couple to G_s, causing stimulation of adenylate cyclase, an increase in cyclic AMP formation and

Table 3 Facilitation Neurotransmitter Release Through A_{2A} Adenosine Receptors

Neurotransmitter	Tissue	Preparation (species; receptor)	References
Acetylcholine	Striatum	Synaptosomes (rat; A_{2A})	Kirk and Richardson 1994; Gubitz et al. 1996
		In vivo microdialysis (rat; A_{2A})	Kurokawa et al. 1996
	Hippocampus	Synaptosomes (rat; A_{2A})	Cunha et al. 1994a; 1994b
		Slices (rat; A_{2A})	Jin and Fredholm 1997
		Slices of CA3 region and dentate gyrus (rat; A_{2A})	Cunha et al. 1994a
	Enteric nervous system	Myenteric motoneurons (rat; A_{2A})	Duarte-Araujo et al. 2004
	Neuromuscular junction	Phrenic nerve-diaphragm preparation (rat; A_2)	Correia-de-Sá et al. 1991
		Neurons innervating transversus abdominis and lumbrical muscles (mouse; A_{2A})	Baxter et al. 2005
Noradrenaline	Brain stem	Nucleus tractus solitarii (rat; A_{2A})	Barraco et al. 1995
	Postganglionic sympathetic nerve terminals	Epididymal portion of vas deferens (rat; A_{2A})	Gonçalves and Queiroz 1993
		Tail artery (rat; A_{2A})	Gonçalves and Queiroz 1996
Dopamine	Striatum	*In vivo* microdialysis (rat; A_2)	Okada et al. 1996
	Nucleus accumbens	*In vivo* microdialysis (rat; A_{2A})	Quarta et al. 2004
Serotonin	Brain stem	Nucleus tractus solitarii (rat; A_{2A})	Helfman et al. 1996; Barraco et al. 1996
Glutamate	Cerebral cortex	*In vivo* cortical cup technique (rat; A_2)	O'Regan et al. 1992
		Synaptosomes (rat; A_{2A})	Marchi et al. 2002
	Brain stem	Nucleus tractus solitarii (rat; A_{2A})	Castillo-Melendez et al. 1994
	Striatum	*In vivo* microdialysis (rat; A_{2A})	Popoli et al. 1995
	Hippocampus	Slices (rat; A_{2A})	Lopes et al. 2002
GABA	Striatum	Sriatopallidal slices (rat; A_{2A})	Mayfield et al. 1993
		Globus pallidus (rat; A_{2A})	Shindou et al. 2001
	Hippocampus	Synaptosomes (rat; A_{2A})	Cunha and Ribeiro 2000a
Glycine	Brain stem	Brainstem slices (rat; A_2)	Umemiya and Berger 1994
Taurine	Hippocampus	*In vivo* microdialysis (rat; A_{2A})	Hada et al. 1998

Receptor type according to authors' conclusions. CGS 21680 was the agonist used.

subsequent activation of protein kinase A (PKA; Correia-de-Sá and Ribeiro 1994c; Gubitz et al. 1996; Okada et al. 2001; Rebola et al. 2002; Oliveira and Correia-de-Sá

2005). The same holds true for the A_{2B} receptors in the prostatic portion of the rat vas deferens (Queiroz et al. 2004). Possible targets of PKA are SNARE (soluble *N*-ethyl-maleimide-sensitive factor attachment protein [SNAP] receptor) proteins. PKA may enhance transmitter release by favoring the formation of the SNARE complex, increasing the readily of the releasable pool of synaptic vesicles (Nagy et al. 2004; Saitow et al. 2005). PKA may also influence Ca^{2+}-currents through P-VSCC. In rat hippocampus, both pathhways (to SNAREs and to P-VSCC) seem to operate, since the A_2 receptor-mediated enhancement of K^+-evoked serotonin release was attenuated by inhibiton of PKA, by botulinum toxin inhibition of synaptobrevin, a SNARE protein, and by inhibition of P-VSCC with ω-agatoxin IVA (Okada et al. 2001).

In addition to G_s, however, presynaptic A_{2A} adenosine receptors may couple to G_q, causing subsequent activation of a protein kinase C (PKC; Gubitz et al. 1996; Lopes et al. 1999; Cunha and Ribeiro 2000a, 2000b; Lopes et al. 2002; Queiroz et al. 2003a). In analogy to PKA, possible targets of PKC are SNAREs and VSCC, in this case N-VSCC. PKC may regulate binding of the SNARE protein syntaxin and of synaptotagmin to N-VSCC (Vaughan et al. 1998; Shoji-Kasai et al. 2002). In rat hippocampus, the A_2 receptor-mediated enhancement of basal serotonin release was attenuated by PKC inhibition, botulinum toxin inhibition of syntaxin, and inhibition of N-VSCC with ω-conotoxin GVIA (Okada et al. 2001). In the epididymal portion of rat vas deferens, A_{2A} receptor-mediated facilitation of noradrenaline release was prevented by PKC inhibition (Queiroz et al. 2003a). The PKC and the PKA pathways may interact as shown in rat tail artery, where the A_{2A} receptor-mediated facilitation of noradrenaline release required activation of both PKC and PKA, with PKA activation occurring downstream of PKC activation (Fresco et al. 2004). In some tissues, activation of PKC enhances neurotransmitter release only in the presence of an ongoing inhibition of neurotransmitter release mediated by $G_{i/o}$ proteins. A_{2A} receptor coupling to a PKC pathway seems to operate here in order to prevent excessive presynaptic inhibition (see Section 7). Figure 2 summarizes these mechanisms.

3.3 Endogenous Activation of Presynaptic A_2 Receptors

Presynaptic A_2 receptors may be activated by endogenous adenosine as revealed by the effects of A_2 receptor antagonists and exogenous ADA. For instance, in the rat tail artery, under conditions that no evidence of tonic activation of A_1 adenosine receptors was obtained, the nonselective A_2 receptor antagonist 3,7-dimethyl-1-propargylxanthine (DMPX) and exogenous ADA depressed noradrenaline release (Gonçalves and Queiroz 1996). In the rat vas deferens the effect of ADA was concentration-dependent: a low concentration depressed noradrenaline release by removing a tonic activation of adenosine A_{2A} receptors, whereas a higher concentration facilitated release likely due to removal of the remaining tonic A_1 receptor-mediated inhibition (Gonçalves and Queiroz 1993). Similar approaches

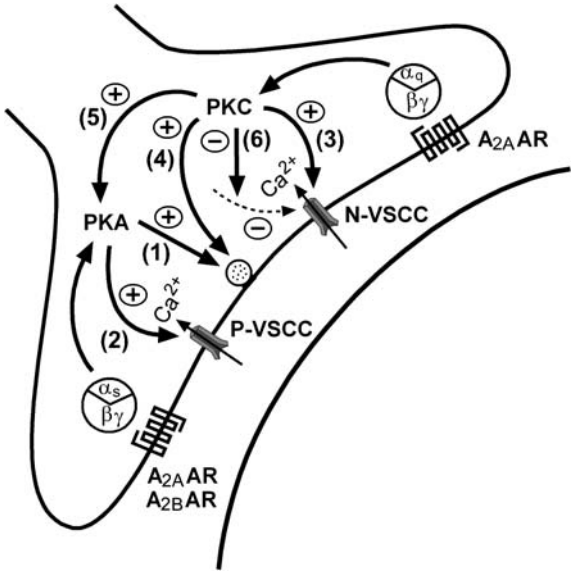

Fig. 2 Mechanisms involved in presynaptic facilitation through A_2 adenosine receptors. A_{2A} and A_{2B} adenosine receptors ($A_{2A}AR, A_{2B}AR$), by coupling to G_s, activate adenylate cyclase and protein kinase A (PKA). This may (1) influence SNARE proteins or (2) enhance calcium currents through P-type voltage-sensitive calcium channels (P-VSCC). $A_{2A}AR$ may also couple to G_q, leading to activation of a protein kinase C (PKC) pathway. This may (3) enhance calcium currents through N-VSCC, (4) influence SNARE proteins, (5) promote the PKA pathways or (6) remove an ongoing $G_{i/o}$ mediated inhibition of release.

provided evidence for an endogenous activation of facilitatory A_2 adenosine receptors in the rat neuromuscular junction (Correia-de-Sá and Ribeiro 1994a, 1994b; Correia-de-Sá et al. 1996) and hippocampus (Sebastião et al. 2000).

4 P2Y Receptor-Mediated Inhibition of Neurotransmitter Release

4.1 The Receptors Involved

The first suggestion that ATP could act as an extracellular messenger was raised by the observation that antidromic stimulation of sensory nerves elicited ATP release (Holton and Holton 1953) and that application of ATP to various regions of the brain produced biochemical or electrophysiological changes (Phillis et al. 1975). The observation that autonomic nerves could release ATP (Su et al. 1971) raised the hypothesis that it might act as neurotransmitter or as cotransmitter (Burnstock

1976), a possibility that later was confirmed for both PNS and CNS synapses (Burnstock 2004).

At some receptors for endogenous purines, nucleotides were more potent than adenosine, and responses to ATP were not antagonized by methylxanthines. These receptors were named P2 receptors (Burnstock 1978) and later subdivided in two groups, P2X and P2Y, according to pharmacological profiles, tissue distribution, and function (Burnstock and Kennedy 1985). It was also early recognized that some P2Y receptors responded to pyrimidine as well as purine nucleotides (von Kügelgen et al. 1987; von Kügelgen and Starke 1987). Studies of transduction mechanisms showed that P2X receptors were ligand-gated cationic channels, whereas P2Y receptors were coupled to G proteins. At present, the P2Y receptor subfamily includes the eight subtypes $P2Y_{1,2,4,6,11,12,13,14}$ (Abbracchio et al. 2006). According to their sensitivity to nucleotides they fall into four groups: (1) selective purinoceptors ($P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$), (2) selective pyrimidinoceptors ($P2Y_6$), (3) receptors with mixed sensitivity for purine and pyrimidine nucleotides ($P2Y_2$, $P2Y_4$, $P2Y_{11}$), and (4) receptors activated by nucleotide sugars such as UDP-glucose ($P2Y_{14}$). The $P2Y_1$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ subtypes are preferentially activated by nucleoside-diphosphates, ADP selectively activating $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, and UDP selectively activating $P2Y_6$ receptors. The $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ subtypes are preferentially activated by nucleoside-triphosphates (von Kügelgen 2006).

Effects of adenine nucleotides on neurotransmitter release were first observed at peripheral cholinergic synapses, where ATP, ADP, and adenosine were equipotent in inhibiting transmission (Ribeiro and Walker 1975; Su 1983). ATP also inhibited electrically evoked overflow of acetylcholine (Akasu et al. 1982; Silinsky and Ginsborg 1983) and noradrenaline (Clanachan et al. 1977; Enero and Saidman 1977), but since these effects were attenuated by theophylline derivatives, they were generally attributed to breakdown of ATP to adenosine and activation of presynaptic A_1 receptors. Some doubts were raised against this hypothesis, however, because ATP metabolism is not instantaneous and complete (Shinozuka et al. 1988), and yet ATP and adenosine were equipotent in inhibiting neurotransmitter release in some tissue preparations (Enero and Saidman 1977; Verhaeghe et al. 1977). These doubts eventually led to the discovery that nucleotides such as ATP inhibited transmitter release through additional, separate presynaptic P2Y receptors (Wiklund et al. 1985; Wiklund and Gustafsson 1986; von Kügelgen et al. 1989, 1993, 1994).

In contrast to presynaptic P2Y receptors, presynaptic P2X receptors mediate facilitation of neurotransmitter release. They are covered in Boehm and Dorostkar's chapter in this *Handbook*.

The major problem in the identification of P2Y receptor-mediated inhibition of ATP on neurotransmitter release lay in the difficulty to clearly exclude breakdown to adenosine and an involvement at A_1 receptors. However, the demonstration that ATP acted *per se* on P2 receptors obtained a solid support when it was shown that breakdown-resistant nucleotides such as adenosine 5′-[γ-thio]triphosphate (ATPγS) and βγ-methyleneadenosine-5′-triphosphate (β,γ-MeATP) mimicked the effects of

ATP (Wiklund et al. 1985; Shinozuka et al. 1988, von Kügelgen et al. 1989, 1994) and that the inhibitory effect of ATP was enhanced by ectonucleotidase inhibitors and not changed by exogenous ADA (Wiklund et al. 1985; Wiklund and Gustafsson 1986) or adenosine uptake inhibitors (Shinozuka et al. 1988). Furthermore, the effects of adenine nucleotides were selectively attenuated by P2 receptor antagonists: in the mouse and rat vas deferens, the P2 receptor antagonist suramin preferentially antagonized the release-inhibiting effects of nucleotides without a major influence on the effect of adenosine (von Kügelgen et al. 1989, 1994), and the P2 receptor antagonist reactive blue 2 but not the selective A_1 adenosine receptor antagonist DPCPX abolished the effect of nucleotides (von Kügelgen et al. 1994; Queiroz et al. 2003b). Similar results were obtained in rat atrium and pancreas (von Kügelgen et al. 1995; Koch et al. 1998).

In most studies in which a P2Y receptor-mediated inhibition of neurotransmitter release was demonstrated, the subtypes involved were not clearly identified. However, according to their cellular expression, involvement of $P2Y_{11}$ and $P2Y_{14}$ receptors in the presynaptic inhibition of neurotransmitter release is very unlikely (Hussl and Boehm 2006) and inhibition of neurotransmitter release by UDP has never been observed, which excludes the $P2Y_6$ receptor. Recently, the P2Y receptors inhibiting sympathetic transmitter release in rat vas deferens were identified as $P2Y_{12}$ and/or $P2Y_{13}$ (Queiroz et al. 2003b). This identification was based (a) on the rank order of potency of nucleotides, 2-methylthioadenosine 5′-triphosphate (2-MeSATP) > 2-methylthioadenosine 5′-diphosphate (2-MeSADP) > ADP ≥ ATP, which is compatible with activation of $P2Y_1$, $P2Y_{12}$ or $P2Y_{13}$ receptors, and (b) on the finding that nucleotide inhibition was prevented by the antagonist of $P2Y_{12}$ and $P2Y_{13}$ receptors, 2-methylthioadenosine 5′-monphosphate (2-MeSAMP; Hollopeter et al. 2001; Zhang et al. 2002) but not by blockade of $P2Y_1$ receptors. Similarly, in rat cultured sympathetic neurons and the ontogenetically related PC12 cell line, $P2Y_{12}$ receptors mediated an inhibition of catecholamine release triggered by K^+ depolarization (Lechner et al. 2004): 2-MeSADP was more potent than ATP or ADP and the effects of nucleotides were blocked by 2-MeSAMP and the selective and competitive $P2Y_{12}$ receptor antagonist cangrelor (AR-C69931MX; Ingall et al. 1999).

In the CNS, other P2Y receptors may be involved in presynaptic modulation. In hippocampal pyramidal neurons of rats, a combination of single-cell PCR analysis, western blot analysis of a purified presynaptic active zone fraction, and immunocytochemical analysis revealed that $P2Y_1$, $P2Y_2$ and $P2Y_4$ were present in the active zone of glutamatergic terminals (Rodrigues et al. 2005). Functional studies in hippocampal synaptosomes supported the modulation of glutamate release through these subtypes: the inhibitory effect of ATP analogues was abolished by reactive blue 2 and attenuated by the selective $P2Y_1$ receptor antagonist 2′-deoxy-N^6-methyladenosine-3′,5′-bisphosphate (MRS 2179; Boyer et al. 1998); moreover, the agonist at $P2Y_2$ and $P2Y_4$ receptors P1-(uridine 5′-),P4-(inosine 5′)tetraphosphate (INS45973; Shaver et al. 2005) inhibited glutamate release (Rodrigues et al. 2005). In the rat medial habenula, UTP caused inhibition of glutamate release by activation of a $P2Y_2$-like receptor (Price et al. 2003).

4.2 Intracellular Events in P2Y Receptor-Mediated Presynaptic Inhibition

Studies on post-receptor mechanisms activated by P2Y receptors resulted in a division into two groups that differ in G protein coupling: the $P2Y_{1,2,4,6,11}$ subtypes, which predominantly couple to $G_{q/11}$ proteins ($P2Y_{11}$ receptors may additionally couple to G_s; Communi et al. 1997); and the $P2Y_{12,13,14}$ subtypes, that almost exclusively couple to members of the $G_{i/o}$ protein family (Abbracchio et al. 2006). It should be noted, however, that presynaptically $P2Y_{1,2,4}$ receptors couple to $G_{i/o}$ in addition to $G_{q/11}$ proteins. The $P2Y_{1,2,4,6,12,13}$ subtypes were shown to modulate the activity of various classes of neuronal K^+ channels (Filippov et al. 2004), transmitter-gated ion channels (Gerevich et al. 2005; Luthardt et al. 2003) and VSCC (Brown et al. 2000).

Two main pathways lead to the modulation of neurotransmitter release by inhibitory P2Y receptors, both involving modulation of VSCC. The first pathway, mediated by $G_{i/o}$ proteins, results in PTX-sensitive, voltage-dependent inhibition of VSCC by the G-protein βγ subunits (Delmas et al. 1999; Dolphin 2003; mechanism 1 in Figure 3). Evidence for this pathway was initially obtained in adrenal chromaffin cells, where ATP and ADP reduced Ca^{2+} currents through activation of receptors

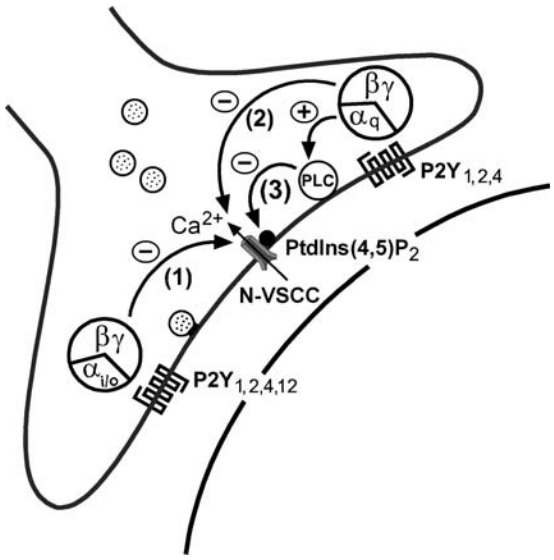

Fig. 3 Mechanisms involved in presynaptic inhibition through P2Y receptors. $P2Y_{1,2,4,12}$ receptors may couple to PTX-sensitive $G_{i/o}$ proteins and mediate voltage-dependent inhibition of calcium currents by direct interaction of the βγ subunits with the N-type VSCC (1). The $P2Y_{1,2,4}$ receptor subtypes may also couple to PTX-insensitive $G_{q/11}$ proteins and mediate voltage-independent inhibition of Ca^{2+} currents by (2) direct interaction of G protein βγ subunits with the channel or (3) activation of phospholipase C (PLC), causing depletion of membrane phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2).

coupled to PTX-sensitive $G_{i/o}$ proteins (Diverse-Pierluissi et al. 1991; Lim et al. 1997), an effect later shown to be voltage-dependent and to involve an inhibition of N- and P/Q-VSCC (Currie and Fox 1996; Lim et al. 1997; Powell et al. 2000). Subsequent studies supported the involvement of this pathway in the inhibition of VSCC mediated by the $P2Y_{1,2,4}$ receptors (Rodrigues at al. 2005) that inhibit glutamate release in hippocampal neurons (Mendoza-Fernandez et al. 2000); by $P2Y_2$ receptors in hamster submandibular neurons (Abe et al. 2003); and by $P2Y_{12}$ receptors in sympathetic superior cervical ganglion neurons (Simon et al. 2002; Lechner et al. 2004) and PC12 cells (Vartian and Boehm 2001; Lechner et al. 2004).

The second pathway, mediated by $G_{q/11}$ proteins, results in PTX-resistant and voltage-independent inhibition of VSCC. In this case, both the $G\alpha_q$ and the $\beta\gamma$ subunits carry the pathway forward, the $\beta\gamma$ subunit acting directly on the VSCC, the α_q subunit causing a PLCβ-dependent depletion of membrane phosphatidylinositol 4,5-bisphosphate as observed with other $G_{q/11}$ protein-coupled receptors (mechanisms 2 and 3 in Figure 3; Gamper et al. 2004; Gerevich et al. 2004; Lechner et al. 2005). A voltage-independent, PTX-resistant inhibition of VSCC was demonstrated in dorsal root ganglion neurons upon activation of $P2Y_1$ receptors (Gerevich et al. 2004). It was also demonstrated in sympathetic superior cervical ganglion neurons upon activation of $P2Y_{1,2,4}$ receptors, in addition to the PTX-sensitive inhibition described above (Brown et al. 2000; Filippov et al. 2003).

4.3 Distribution and Endogenous Activation of Presynaptic P2Y Receptors

Presynaptic inhibition through P2Y receptors has been detected in many tissues and several species and for cholinergic, noradrenergic, dopaminergic, sertoninergic as well as vasopressin and glutamate neurons (Table 4).

Some presynaptic P2Y receptors work as autoreceptors, mediating a negative feedback mechanism to control neurotransmitter release in synapses where ATP acts as cotransmitter, since P2 receptor antagonists, when tested alone, enhanced release (Fuder and Muth 1993; von Kügelgen et al 1993). In the CNS, P2Y receptors have been shown to tonically suppress glutamatergic transmission (Zhang et al. 2003). These P2Y receptors seem to be preferentially activated by ATP released from glial cells upon activation of glutamate receptors (Queiroz et al. 1997; Zhang et al. 2003), suggesting that modulation of synaptic transmition by presynaptic inhibitory P2Y receptors may depend on astroglial activity.

5 Presynaptic P3 Receptors?

Presynaptic P2Y receptors are well established. So are presynaptic P2X receptors, as mentioned above (see chapter by Boehm and Dorostkar in this *Handbook*). In some studies, a third class of receptors, named P3, was proposed to mediate inhibitory

Table 4 Inhibition of Neurotransmitter Release Through P2 Receptors

Neurotransmitter	Tissue	Preparation (species; receptor)	References
Acetylcholine	Brain cortex	Slices (rat; P2Y)	Cunha et al. 1994b
	Ganglion	Sympathetic ganglia (frog; P2)	Silinsky and Ginsborg 1983
	Neuromuscular junction	Sartorius muscle fibres (frog; P2)	Giniatullin and Sokolova 1998
		Phrenic nerve-diaphragm (mouse; P2, P2Y)	Hong and Chang 1998; De Lorenzo et al. 2006
	Enteric nervous system	Ileum longitudinal muscle (guinea pig; P2)	Wiklund et al. 1985; Wiklund and Gustafsson 1986
		Submucosal neurons (guinea pig; P3)	Barajas-López et al. 1995
Noradrenaline	Brain cortex	Slices (rat; P2Y)	von Kügelgen et al. 1994
	Hippocampus	Slices (rat; P2Y)	Koch et al. 1997
	Postganglionic sympathetic nerve terminals	Saphenous artery (guinea pig; P2)	Fujioka and Cheung 1987
		Tail artery (rat; P3, P2Y)	Shinozuka et al. 1988; Gonçalves and Queiroz 1996
		Mesenteric artery (rat; P3)	Shinozuka et al. 2001
		Atria (rat; P2Y)	von Kügelgen et al. 1995
		Vas deferens (mouse; P2Y)	von Kügelgen et al. 1989; Kurz et al. 1993
		Vas deferens (rat; P3, P2Y; $P2Y_{12,13}$)	Forsyth et al. 1991; von Kügelgen et al. 1993; Kurz et al. 1993; Queiroz et al. 2003b
		Vas deferens (rabbit; P3)	Todorov et al. 1994
		Iris (rat; P2Y)	Fuder and Muth 1993
		Pancreas (rat; P2Y)	Koch et al. 1998
		Kidney (rat; P2Y)	Bohmann et al. 1997
	Adrenals	Isolated chromaffin cells (bovine; P2Y)	Powell et al. 2000
Dopamine	Neostriatum	Sices (rat; P2Y)	Trendelenburg and Bültmann 2000
Serotonin	Brain cortex	Slices (rat; P2Y)	von Kügelgen et al. 1997
Vasopressin	Hypophysis	Isolated posterior lobe (rat; P2Y)	Sperlágh et al. 1999
Glutamate	Brain cortex	Sices (rat; P2Y)	Bennett and Boarder 2000
	Hippocampus	Neuronal cultures (rat; P2Y)	Koizumi and Inoue 1997; Koizumi et al. 2003
		Slices (rat; P2Y)	Inoue et al. 1999; Zhang et al. 2003
		Pyramidal neurons (rat; P2Y, P3)	Mendoza-Fernandez et al. 2000
		Synaptossomes (rat, $P2Y_{1,2,4}$)	Rodrigues et al. 2005
	Medial habenula	Slices (rat, $P2Y_2$)	Price et al. 2003

Receptor according to the authors' conclusions.

effects of nucleotides on neurotransmitter release in the CNS and PNS (see Table 4). They were described to be equally sensitive to ATP and adenosine, and blocked by xanthines. However, it is not clear whether the mixed P1/P2 pharmacology is due to a distinct molecular entity not yet cloned (Saitoh and Nakata 1996); to a particular association between ecto-nucleotidases and A_1 receptors (Sebastião et al. 1999), which might facilitate conversion of ATP into adenosine and the subsequent activation of A_1 receptors (see Section 6); or even to hetero-oligomerization that may occur between A_1 and P2Y receptors (Nakata et al. 2005).

6 The Diversity of the Purinergic Mechanisms in the Synaptic Cleft

The coexistence of inhibitory and facilitatory adenosine- and P2-receptors in the same nerve terminal raises the question of which factors influence the concentration of endogenous agonists in the vicinity of each receptor (i.e., in the receptor biophase) and, consequently, determine the net modulatory effect. Tissue differences in receptor expression and purine release/formation or inactivation systems create a diversity of possibilities that makes the net modulatory effect by endogenous purines quite variable from tissue to tissue.

6.1 Release and Inactivation of Purines

ATP may be released by exocytosis from neurons, where it is co-stored in synaptic vesicles with other neurotransmitters (Burnstock 2004), and from astrocytes (Volknandt 2002). ATP is also released upon stimulation of membrane receptors or upon mechanical stimulation by mechanisms that mainly involve transporters of the ABC protein family or ATP-permeable anion channels (Bodin and Burnstock 2001). Under pathological conditions, massive release of ATP may occur upon damage of the cell membrane or cell lysis.

ATP is converted in the extracellular space into ADP, AMP, adenosine, and eventually into inosine by the action of a set of enzymes located at cell surfaces. These enzymes include NTPDase1, NTPDase2, NTPDase3, and NTPDase8, which are members of the ecto-nucleoside 5′-triphosphate diphosphohydrolase (E-NTPDase) family (Robson et al. 2006). They further include ecto-5′-nucleotidase (CD73; Zimmermann 1992), ecto-alkaline phosphatase (Ohkubo et al. 2000) and adenosine deaminase (ADA; Franco et al. 1986). The NTPDases1, 2, and 3 are the main E-NTPDase isoforms expressed in neurons. They metabolize tri- and diphosphonucleosides, although with different kinetics and substrate preferences: NTPDase1 hydrolyses ATP and ADP equally well and very efficiently, leading to formation of AMP; NTPDase2 hydrolyses ATP rapidly but ADP poorly, leading to accumulation of ADP; NTPDase3 hydrolyses both ATP and ADP, like NTPDase1,

but less efficiently, allowing some accumulation of both ATP and ADP (Kegel et al. 1997; Kukulski et al. 2004; see also Robson et al. 2006). The chain of nucleotide degradation terminates with the hydrolysis of AMP to adenosine by ecto-5′-nucleotidase or ecto-alkaline phosphatase. Inactivation of extracellular adenosine may occur by irreversible deamination to inosine, catalyzed by ecto-ADA, a membrane-bounded form of ADA with the active site facing the extracellular space (Franco et al. 1986), or by exo-ADA, a releasable form of ADA, as recently suggested (Correia-de-Sá et al. 2006). Adenosine may also be inactivated by uptake through the concentrative nucleoside transporter family (SLC28; Baldwin et al. 1999; Gray et al. 2004) or the equilibrative nucleoside transporter family (SLC29; Baldwin et al. 1999).

The involvement of releasable enzymes in the metabolism of extracellular nucleotides has been suggested to explain an increase in the capacity to metabolize ATP observed in some tissues following nerve stimulation (Todorov et al. 1996; Mihaylova-Todorova et al. 2002). Released enzymes resembling the E-NTPDases mentioned above seem to be able to convert released ATP to AMP. In some tissues (guinea pig but not rabbit vas deferens), the released enzymes also hydrolyse AMP into adenosine (see Westfall et al. 2002).

6.2 Factors That Influence Purine Concentration in the Receptor Biophase

The concentration of nucleotides in the P2 receptor biophase – including the presynaptic P2Y receptor biophase – is determined by the type of E-NTPDases present in the vicinity of the receptors (Figure 4a–4c). The nucleotide concentration in turn determines the types of P2Y receptor activated: either the dinucleoside-preferring $P2Y_{1,2,13}$ or the trinucleotides-preferring $P_{2,4}$ receptors (see Section 4.1). Because of its high efficiency for both ATP and ADP hydrolysis, NTPDase1 minimizes any nucleotide effect at P2Yreceptors (Figure 4a). Expression of NTPDase2 favors activation of the $P2Y_{1,12,13}$ diphosphonucleoside receptors (Figure 4b). Expression of NTPDase3, allowing some accumulation of both ATP and ADP, permits activation of both the diphosphonucleoside $P2Y_{1,12,13}$ and the triphosphonucleoside $P2Y_{2,4}$ receptors (Figure 4c).

Localization of ecto-5′ectonucleotidase close to NTPDases creates a very efficient system to produce adenosine from released ATP, favoring activation of adenosine receptors (Nitahara et al. 1995; Cunha et al. 1998; Sebastião et al. 1999; Duarte-Araújo et al. 2004; see Matsuoka and Ohkubo 2004). This situation seems to prevail in the rat hippocampus and may explain the inhibition of synaptic transmission caused by ATP and ATP analogues (Cunha et al. 1998).

The concentration of adenosine in the P1 receptor biophase and the balance between A_1 and A_{2A} receptor-mediated effects is influenced by the adenosine inactivation systems (ADA and the adenosine uptake carriers; see Figure 4d). A_1 receptors are surface anchoring proteins for ecto-ADA, and binding of ecto-ADA to A_1 recep-

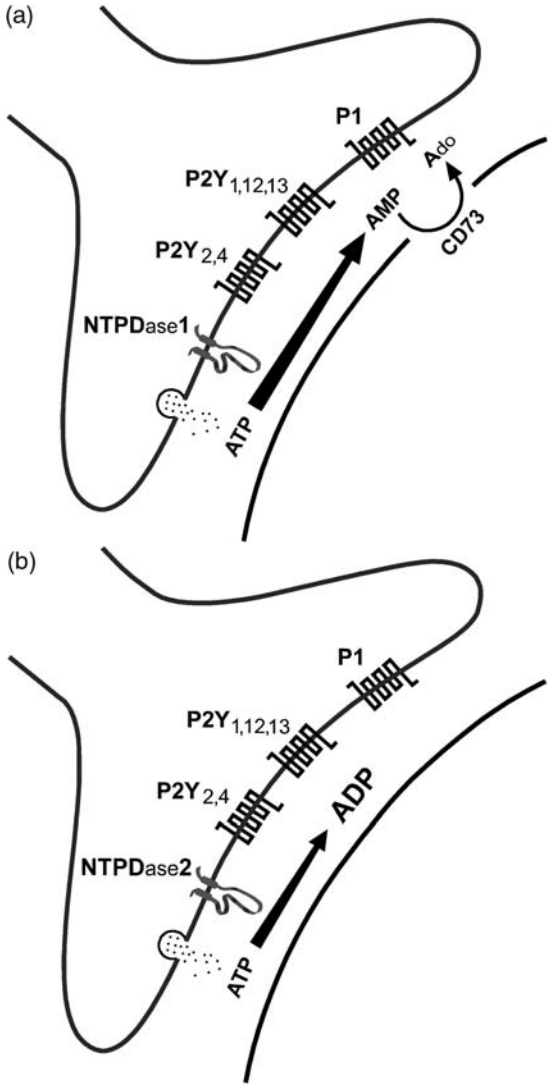

Fig. 4 Mechanisms involved in the extracellular inactivation of nucleotides (**a**, **b** and **c**) and adenosine (**d**) and their influence on purine concentration in the P2Y and P1 receptor biophases. (**a**) NTPDase1 hydrolyses ATP and ADP very efficiently, thus preventing their action on P2Y receptors; (**b**) NTPDase2 metabolizes ATP preferentially, allowing an accumulation of ADP and thus favouring activation of $P2Y_{1,12,13}$ receptors; (**c**) NTPDase3 hydrolyses both ATP and ADP slowly, giving them time to activate both $P2Y_{2,4}$ and $P2Y_{1,12,13}$ receptors. Formation of adenosine depends on the activity of ecto 5′-nucleotidase (CD73). Adenosine inactivation systems also influence adenosine concentration in the P1 receptor biophase (**d**): the nucleoside transporters take up adenosine; adenosine deaminase (ADA) regulates both the concentration of adenosine in the A_1 receptor biophase and the functionality of A_1 receptors.

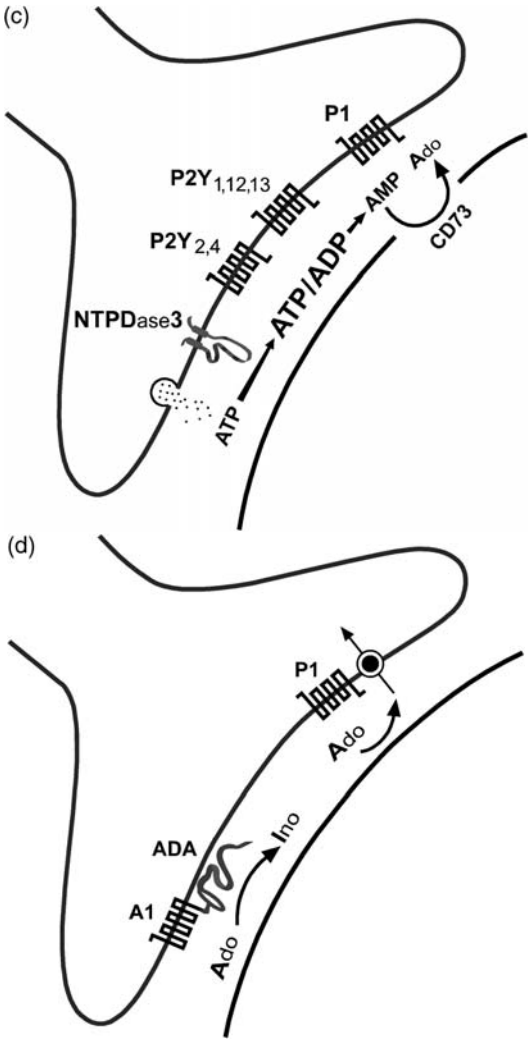

Fig. 4 Continued.

tors increases the affinity and efficacy of A_1 receptor agonists (Ciruela et al. 1996; Ruiz et al. 2000) and prevents A_1 receptor desensitization (Saura et al. 1998) and internalization (Ginés et al. 2001). Adenosine uptake carriers also influence the adenosine concentration in the receptor biophase. In rat tail artery, inhibition of adenosine uptake decreased transmitter release (Gonçalves and Queiroz 1996), suggesting that A_1 adenosine receptors are located close to the uptake carriers. However, this is not a general rule, since in other tissues inhibition of adenosine uptake facilitated neurotransmitter release (rat vas deferens: Gonçalves and Queiroz 1993; Queiroz et al. 2004; rat myenteric plexus: Duarte-Araújo et al. 2004), indicating that the functional

interaction between adenosine uptake carriers and presynaptic adenosine receptors may vary from tissue to tissue.

7 Adenosine and the Fine-Tuning of Transmitter Release

The adenosine receptor-mediated modulation of synaptic transmission exceeds the direct control of neurotransmitter release, and the concept of adenosine as a "modulator of modulators" has been developed (Sebastião and Ribeiro 2000), highlighting the potential of adenosine to fine-tune synaptic activity. Fine-tuning of transmitter release by adenosine may be exerted by (1) triggering responses to other modulators, (2) regulating the rate of desensitization of other receptors, (3) forming hetero-oligomers with other receptor types and/or (4) cross-talk of intracellular pathways they activate.

Adenosine has a permissive effect on responses to the peptides calcitonin gene-related peptide (CGRP; Correia-de-Sá and Ribeiro 1994a; Sebastião et al. 2000), vasoactive intestinal peptide (VIP; Correia-de-Sá et al. 2001), and brain derived neurotrophic factor (BDNF; Diógenes et al. 2004): the modulatory actions of these peptides depend strictly on an ongoing activation of facilitatory A_{2A} receptors. Receptors for CGRP and VIP are often positively coupled to adenylate cyclase (Rosenberg and Li 1995), and the permissive effect of A_{2A} receptors on CGRP and VIP responses has been ascribed to a synergic effect on adenylate cyclase (Sebastião et al. 2000).

A second mechanism by which adenosine receptors may indirectly influence release is by regulating the rate of desensitization of other receptors. For example, at rat phrenic motor nerve terminals, A_1 and A_{2A} receptors inhibit and facilitate, respectively, acetylcholine release (Correia-de-Sá et al. 1991; Oliveira and Correia-de-Sá 2005). Additionally, they modulate nicotinic function, with A_{2A} receptors accelerating and A_1 receptors slowing the rate of desensitization of the autofacilitatory nicotinic receptors (Correia-de-Sá and Ribeiro 1994b; Timóteo et al. 2003; Duarte-Araújo et al. 2004), which influences the global effects of adenosine on transmitter release.

Adenosine may also coactivate other release-modulatory receptors, an influence tentatively explained by receptor hetero-oligomerization and/or by a cross-talk between the intracellular pathways to which adenosine receptors and the interacting receptors are coupled. Hetero-oligomerization of adenosine receptors has been demonstrated for the pairs $A_1/P2Y_1$ (Yoshioka et al. 2002), A_1/mGluR1 (Ciruela et al. 2001), A_{2A}/mGluR5 (Ferré et al. 2002), A_1/D_1 (Ginés et al. 2000), A_{2A}/D_2 (Hillion et al. 2002), and A_1/A_{2A} receptors (Ciruela et al. 2006). The receptor complexes formed have ligand binding and signalling properties distinct from their constituent receptors, but so far no evidence supports their presynaptic localization.

In most cases, mutual influences between presynaptic receptors have been explained by a cross-talk of intracellular signalling pathways. In several synapses, presynaptic actions of A_1 receptors and other release-inhibitory receptors are mu-

tually occlusive (Schlicker and Göthert 1998). For example, in central (rabbit brain cortex: Limberger et al. 1988; rabbit hippocampus: Allgaier et al. 1991) and peripheral noradrenergic neurons (guinea pig atrium: Nakatsuka et al. 1995; rat tail artery: Bucher et al. 1992) activation of α_2-autoreceptors attenuated the A_1 receptor-mediated inhibition of noradrenaline release. This kind of interaction has been explained by competition for the same intracellular pathway (e.g., N-type VSCC and K^+-channels; Schlicker and Göthert 1998). A_1 adenosine receptors and other release-inhibitory receptors, such as the α_2-adrenoceptors, are coupled to $G_{i/o}$ proteins and the inhibition of transmitter release they cause is mainly a consequence of an inhibition of VSCC by $G_{i/o}$ protein $\beta\gamma$ subunits (Delmas et al. 1999; Lim et al. 2001; Dolphin 2003). Accordingly, the simultaneous activation of other inhibitory receptors may reduce the coupling of A_1 receptors to the $G_{i/o}$ proteins (less available) and the subsequent inhibition of neurotransmitter release.

Cross-talk between facilitatory A_2 receptors and presynaptic inhibitory receptors has also been described. In rat sympathetic neurons, facilitation of noradrenaline release mediated by A_{2A} receptors (Fresco et al. 2002; Queiroz et al. 2003a) or by A_{2B} receptors (Talaia et al. 2005) is enhanced by ongoing activation of inhibitory receptors, such as the α_2-adrenoceptors. In rat hippocampus, A_{2A} receptor-mediated facilitation of glutamate release (O'Kane and Stone 1998; Lopes et al. 1999, 2002) was shown to depend on coactivation of inhibitory A_1 receptors. The involvement of a diffusible second messenger in the cross-talk between A_1 and A_{2A} receptors has been suggested based on the observation that activation of A_{2A} receptors decreased A_1 receptor binding in hippocampal (Lopes et al. 1999) and striatal (Dixon et al. 1997) synaptosomes, but not in membrane fragments. Furthermore, in synapses where cross-talk between presynaptic A_{2A} and inhibitory receptors was observed, A_{2A} receptors were shown to couple to a PKC pathway (Dixon et al. 1997; Lopes et al. 2002; Queiroz et al. 2003a; see Figure 2) and facilitation of neurotransmitter release caused by direct activation of PKC with phorbol esters was also attenuated when activation of release-inhibitory receptors was prevented (Fredholm and Lindgren 1988; Queiroz et al. 2003a). Similar interactions have been reported in sympathetic neurons between release-inhibitory receptors and facilitatory angiotensin AT_1 (Cox et al. 2000; Trendelenburg et al. 2003; Mota and Guimarães 2003; Talaia et al. 2006) and bradykinin B_2 receptors (Cox et al. 2000), which also couple to the $G_{q/11}$-PLC-PKC pathway. A PKC-mediated disruption of the $G_{i/o}$ pathway to which the inhibitory receptors are coupled was the main mechanism proposed to explain interactions between presynaptic release-inhibitory and facilitatory receptors (Cox et al. 2000; Talaia et al. 2006), including A_{2A} receptors (Queiroz et al. 2003a). Such disruption may occur by phosphorylation of the $G_{i/o}$ proteins (Katada et al. 1985), the receptors (Liang et al. 1998; Dixon et al. 1997; Lopes et al. 1999), or the N-type of VSCC they regulate (Hamid et al. 1999; see Figure 2, mechanism 6).

Interactions between receptors do not follow a constant pattern and involve multiple mechanisms (Kubista and Boehm 2006) that depend on the cellular organization of the interacting receptors, of the proteins involved in intracellular signalling pathways, and of the molecular targets they regulate (Hur and Kim 2002). The

release-inhibitory receptors can be viewed as a local homeostatic mechanism to control the amount of neurotransmitter at the synaptic cleft. The physiological meaning of facilitatory receptors is less clear. One possibility is that they may select the more convenient cotransmitter combination (Gonçalves et al. 1996). The requirement of an ongoing activation of inhibitory receptors to observe facilitation of neurotransmitter release by A_2 receptors and other presynaptic facilitatory receptors supports the proposal that, in certain synapses, they may operate to prevent excessive inhibition, contributing to a fine-tuning of transmitter release.

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Presynaptic Metabotropic Glutamate and $GABA_B$ Receptors

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Abstract Glutamate and GABA, the two most abundant neurotransmitters in the mammalian central nervous system, can act on metabotropic receptors that are structurally quite dissimilar from those targeted by most other neurotransmitters/modulators. Accordingly, metabotropic glutamate receptors (mGluRs) and $GABA_B$ receptors ($GABA_B$Rs) are classified as members of family 3 (or family C) of G protein-coupled receptors. On the other hand, mGluRs and $GABA_B$Rs exhibit pronounced and partly unresolved differences between each other. The most intriguing difference is that mGluRs exist as multiple pharmacologically as well as structurally distinct subtypes, whereas, in the case of $GABA_B$Rs, molecular biologists have so

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far identified only one structurally distinct heterodimeric complex whose few variants seem unable to explain the pharmacological heterogeneity of $GABA_BRs$ observed in many functional studies. Both mGluRs and $GABA_BRs$ can be localized on axon terminals of different neuronal systems as presynaptic autoreceptors and heteroreceptors modulating the exocytosis of various transmitters.

1 Introduction

Glutamate and GABA mediate transmission at most central excitatory and inhibitory synapses, respectively. Besides their quick actions at ionotropic receptors, glutamate and GABA, similarly to most other chemical mediators, can activate metabotropic receptors generally endowed with modulatory functions.

The interest in mGluRs and $GABA_BRs$ has in part been stimulated by the peculiarity of their structures, which are quite different from those of all other G protein-coupled receptors (GPCRs); of note, the structure of $GABA_BRs$ could only be determined after almost two decades of unsuccessful efforts. Due to their special properties, mGluRs and $GABA_BRs$ have been included in family 3 of GPCRs (Bockaert and Pin 1999). Metabotropic receptors of family 3 possess a very large N-terminal domain in which the agonist binding sites are localized. The binding site of these GPCRs is constituted of two lobes; glutamate or GABA is supposed to bind to one lobe within the cleft between the two lobes and, according to the Venus flytrap model (O'Hara et al. 1993; Galvez et al. 1999), this leads to the closure of the two lobes.

As for other GPCRs, those of family 3 can undergo dimerization. It has been shown that mGluRs form homodimers via a disulfide bridge between the N-terminal domains (Romano et al. 1996; Pace et al. 1999). In the case of $GABA_BRs$, heterodimerization between two subunits, termed $GABA_{B1}$ and $GABA_{B2}$, seems to be the absolute requirement for an efficient coupling to G proteins and takes place in the cytoplasm via coiled-coil interaction of the subunit C-terminal domains (White et al. 1998; Kuner et al. 1999).

It is well established that mGluRs are structurally and pharmacologically heterogeneous and exist as eight subtypes (mGluR1 to mGluR8) having discrete regional, cellular, and subcellular localization (Pin and Bockaert 1995; Conn and Pin 1997; Cartmell and Schoepp 2000; Bockaert et al. 2002; Pin et al. 2003). In contrast, $GABA_BRs$ have often been reported to exhibit pharmacological heterogeneity (Bonanno and Raiteri 1993a; Mott and Lewis 1994; Bowery et al. 2002), but this has remained so far unexplained because molecular biologists tend to exclude the existence of more than one $GABA_BR$ formed by the obligatory heterodimerization of $GABA_{B1}$ and $GABA_{B2}$ subunits (see Bowery et al. 2002; Bettler et al. 2004, for reviews).

The transduction mechanisms associated with mGluR activation have been extensively reviewed (Pin and Bockaert 1995; Conn and Pin 1997; Bockaert et al. 2002). Depending on the receptor involved, the main mechanisms are activation of the phosphatidylinositol (PI) pathway with mobilization of internal Ca^{2+} ions

(group I mGluRs, including mGluR1 and 5) and inhibition of adenylyl cyclase (AC) activity (group II mGluRs, including mGluR2 and 3, and group III mGluRs, including mGluR4,6,7 and 8), although a series of mGluR-mediated cellular events do not seem to result from a simple activation of phospholipase C (PLC) or inhibition of cyclic AMP production (see Bockaert et al. 2002). Neuronal $GABA_B$Rs are negatively coupled via G proteins to their effector processes, namely, plasma membrane Ca^{2+} and K^+ channels (Dolphin et al. 1990; Gage 1992) and AC (Hill et al. 1984; Karbon et al. 1984). Activation of $GABA_B$Rs decreases Ca^{2+} conductance, increases conductance to K^+ ions, and inhibits AC activity (see Bowery et al. 2002).

PLC-coupled mGluRs are generally localized postsynaptically (Baude et al. 1993; Ottersen and Landsend 1997; Shigemoto et al. 1997), although examples of presynaptic localization have been described (see below). Conversely, mGluRs negatively coupled to AC or to voltage-sensitive Ca^{2+} channels (VSCCs) are mostly localized on presynaptic axon terminals (Shigemoto et al. 1996, 1997; Ottersen and Landsend 1997; Masugi et al. 1999; Millán et al. 2002), where they modulate neurotransmitter release. Both pre- and postsynaptically located $GABA_B$Rs have been described in the mammalian CNS (Bonanno and Raiteri 1993a; Bowery 1993; Bowery et al. 2002; Bettler et al. 2004; Raiteri 2006). $GABA_B$Rs stimulation at postsynaptic sites normally causes a long-lasting neuronal hyperpolarization mediated by an increase in K^+ conductance. The presynaptic $GABA_B$Rs mediate negative modulation of neurotransmitter exocytosis. The interested reader can find detailed information on the localization of mGluRs and $GABA_B$Rs, as determined by morphological approaches, in several of the reviews quoted in this Introduction.

Presynaptic mGluRs and $GABA_B$Rs can be both autoreceptors regulating release of glutamate or GABA, respectively, and heteroreceptors regulating release of various transmitters. Since a review article specifically devoted to the regulation of neurotransmitter release by mGluRs occurring in various *in vitro* and *in vivo* preparations was published a few years ago (Cartmell and Schoepp 2000), this chapter will mainly focus on the modulatory roles of mGluRs presynaptically located on axon terminals releasing a given transmitter in *in vitro* conditions. I will also try to update the information available on release-regulating presynaptic $GABA_B$Rs. Although modulation of neurotransmission by mGluRs and, less so, by $GABA_B$Rs, has been the object of a great number of electrophysiological studies (see for reviews Anwyl 1999; Nicoll 2004), the present chapter will emphasize neurochemical and pharmacological evidence for release-regulating presynaptic mGluRs and $GABA_B$Rs originated from direct studies of neurotransmitter overflow.

2 Metabotropic Glutamate Autoreceptors

The glutamatergic autoreceptor system is by far the most complex among the brain autoreceptor systems (compare Starke et al. 1989). Glutamatergic terminals possess several autoreceptors, both ionotropic and metabotropic, some of which coexist and may interact on the same terminal. Investigations on mGlu autoreceptors have

included multiple experimental approaches. The excellent review of Cartmell and Schoepp (2000) contains a table that summarizes the literature on release-regulating mGluRs, including autoreceptors. Interestingly, as many as 19 out of 48 reports on glutamate release had been carried out using synaptosomes, probably chosen as a relatively simple experimental approach offering easier interpretations of the results.

All three groups of mGluRs (I to III) have been implicated in the presynaptic modulation of excitatory synaptic transmission. Much of the electrophysiological evidence available (see Anwyl 1999), including that regarding PI-linked receptors, has shown inhibitory effects generally attributed to direct activation of mGluRs present on glutamate/aspartate axon terminals.

2.1 Group I Metabotropic Glutamate Autoreceptors

Many authors believe that the simplest and best established model for studying release-regulating presynaptic receptors is the isolated nerve terminal (synaptosome) preparation. This technical approach was chosen by Herrero et al. (1992) in their fundamental work on mGlu autoreceptors: synaptosomal preparations from adult rat cerebral cortex were exposed to the broad-spectrum mGluR agonist (1S,3R)-ACPD, and glutamate release was monitored by on-line fluorometry. (1S,3R)-ACPD enhanced the release of glutamate produced by 50 μM 4-aminopyridine (4-AP) in the presence of low concentrations of arachidonic acid. This facilitation of glutamate release was paralleled by a transient elevation of diacylglycerol (DAG) and by activation of protein kinase C (PKC), suggesting the involvement of PI-linked group I mGluRs (Coffey et al. 1994). In apparent contrast to these data, some electrophysiological studies reported that presynaptic group I mGluRs mediated reduction of excitatory transmission (Gereau and Conn 1995; Manzoni and Bockaert 1995).

In the meantime, 3,5-DHPG had been introduced as a novel selective group I receptor agonist (Schoepp et al. 1994), able to activate mGluR1 and mGluR5 with consequent stimulation of PI hydrolysis and mobilization of intracellular calcium (Fagni et al. 2000). Curiously, when 3,5-DHPG was added to synaptosomes, in the presence of arachidonic acid, the release of glutamate provoked by submaximal depolarization with 4-AP (50 μM) was enhanced, but the compound failed to affect glutamate exocytosis when this was induced by maximal depolarization with 1 mM 4-AP (Herrero et al. 1998). To better understand this observation, nerve terminals were pre-exposed to 3,5-DHPG for 1 min (first stimulation) and, after washings to remove the agonist, they were rechallenged with 3,5-DHPG (second stimulation). A suppression of the release-facilitatory effect of 3,5-DHPG was observed during the second stimulation. Furthermore, the agonist became concomitantly able to *inhibit* the release induced by maximal depolarization with 4-AP (1 mM). The ability of 3,5-DHPG to potentiate the release was recovered 30 min after the first stimulation, and the inhibitory action was lost. One explanation for the switch from facilitation to inhibition in the control of glutamate release might have been that, during the

desensitization of group I mGluRs, inhibitory mGluRs prevailed. However, agonists at the AC-coupled mGluRs failed to elicit release inhibition, suggesting that the receptors involved were PI-coupled group I mGluRs.

Herrero et al. (1998) then found that, while the stimulatory effect of 3,5-DHPG on the 4-AP (50 μM)-evoked glutamate release was PKC-dependent, its inhibitory effect on the 4-AP(1 mM)-evoked release was not altered by the PKC inhibitor staurosporine. PKC activity was, however, involved in the switch from release facilitation to release inhibition. To show this, nerve terminals were pre-exposed to 3,5-DHPG for 1 min to induce the switch, washed and treated with staurosporine. Under these conditions, the inhibitory effect of 3,5-DHPG on the 4-AP(1 mM)-evoked glutamate exocytosis remained unaltered. Conversely, in experiments in which the exposure to 3,5-DHPG occurred after a prior inhibition of PKC with staurosporine, the second stimulation with 3,5-DHPG failed to induce release inhibition.

The dual role of the group I mGluRs in the release of glutamate studied in nerve endings isolated from the cerebrocortex of adult rats was also identified in rat hippocampal synaptosomes (Rodriguez-Moreno et al. 1998). Moreover, to get information on the potential relevance of the mechanism in synaptic transmission, electrophysiological experiments with hippocampal slices were carried out. Excitatory postsynaptic potentials (EPSPs) were evoked by electrically stimulating the Schaffer collaterals and recorded from identified CA1 pyramidal cells. Exposure to 3,5-DHPG produced dramatic reduction (to $\sim$ 15%) in the amplitude of EPSPs, confirming previous electrophysiological findings (Gereau and Conn 1995; Manzoni and Bockaert 1995). Thus, hippocampal slices seemed to respond to 3,5-DHPG like isolated nerve terminals during the "second stimulation" with the group I mGluR agonist, suggesting that, in slices, the desensitization of the stimulatory component had already occurred, possibly due to extracellular endogenous glutamate tonically activating mGluRs. If this were the case, preincubation of the slices with an mGluR antagonist should prevent receptor desensitization. Hippocampal slices were therefore pretreated with the antagonist MCPG and then stimulated; in MCPG-treated slices, 3,5-DHPG enhanced the EPSPs amplitude. The facilitatory response was transient and was followed by an inhibition; facilitation was recovered following 30 min of renewed incubation with the antagonist (Rodriguez-Moreno et al. 1998).

A more direct approach to verify the hypothesis that, in slices, an increase in the extracellular concentrations of glutamate could promote the switch from facilitation to inhibition in response to a group I mGluR agonist was to incubate synaptosomes with a depolarizing concentration of KCl to elevate the extraterminal concentration of the natural mGluR agonist. Under basal conditions (3 mM KCl; 37° C), the extraterminal concentration of glutamate after 10 min incubation amounted to $\sim$1 μM; addition of 3,5-DHPG elicited facilitation of the 50 μM 4-AP-evoked glutamate release, while inhibition of the 1 mM 4-AP-evoked release was absent. Raising the concentration of KCl to 13 mM increased extraterminal glutamate to 3.5 μM. Under these conditions, 3,5-DHPG failed to facilitate release and became clearly inhibitory (Herrero et al. 1998; Rodriguez-Moreno et al. 1998).

It has to be noted that the electrophysiological experiments in which 3,5-DHPG exclusively caused inhibition of EPSP amplitude in hippocampal slices were carried

out at room temperature (21° C–22° C). When the temperature was raised to 31° C, 3,5-DHPG first caused some facilitation of synaptic transmission, followed by the expected inhibition. The interpretation given by Rodriguez-Moreno et al. (1998) was that, at 31° C, the extracellular concentration of glutamate was maintained lower than at 21° C by the glial/neuronal uptake systems, and this could have prevented receptor desensitization. Whatever the explanation, the work of the Spanish laboratories represents one convincing example of the usefulness of the synaptosomal preparation in investigating presynaptic mechanisms. Indeed, the dual role of group I mGluRs in the modulation of glutamate release and the switch from facilitation to inhibition of this modulation, mechanisms that may have important implications in synaptic physiology and neurotoxicity, probably have been discovered thanks to the use of isolated nerve terminal preparations. These dynamic changes had in fact not been observed in electrophysiological experiments.

The ambient concentration of glutamate appears to be critical for the function of group I mGluRs. Extrasynaptic spillover of glutamate was reported to be regulated through the function of excitatory amino acid transporters, which depends, inter alia, on the temperature (Asztely et al. 1997). When extraterminal glutamate concentrations are maintained at $\sim$1 μM in incubated synaptosomes, the dual role of group I mGluRs can be clearly observed. According to Rodriguez-Moreno et al. (1998), if, under physiological conditions, the brain extracellular concentrations of glutamate are kept low enough by the uptake systems, the facilitatory response mediated by mGluRs would not be desensitized and group I mGluRs should be ready to mediate enhancement of evoked glutamate exocytosis whenever necessary; for instance, during plasticity processes. Activity-dependent increases in extracellular glutamate will instead desensitize the group I mGluR facilitatory function, which may be particularly important in preventing undesired accumulation of extracellular excitatory amino acids consequent to repetitive activity.

Desensitization of mGluRs linked to PI hydrolysis in response to prolonged or repetitive exposure to agonists had previously been observed in cultured cerebellar neurons (Catania et al. 1991; Aronica et al. 1993). These authors reported that desensitization was reduced by inhibitors of PKC. As to presynaptic group I mGluRs autoregulating glutamate release, the functional switch from facilitation to inhibition of release is also thought to be mediated by PKC-induced phosphorylation and was found to be prolonged by inhibiting phosphatase activity. Inhibitors of phosphatase 1 and 2A indeed prevented recovery of the facilitation of evoked glutamate release lost following a first "stimulation" with group I mGluR agonists (Sistiaga and Sanchez-Prieto 2000). The targets for the phosphorylation in the PKC-mediated desensitization are likely to be the group I mGluRs themselves, based on studies performed with recombinant receptors in expression systems (see, for a review, Alagarsamy et al. 2001).

The exact identity of the autoreceptors of group I mGluR (whether mGluR1 or mGluR5 or both) undergoing the desensitization-dependent switch in glutamate exocytosis regulation has long remained elusive, mainly due to the lack of selective mGluR1 and mGluR5 ligands. Reid et al. (1999) exploited some new ligands to characterize pharmacologically these autoreceptors. Two novel group I-preferring

ligands were utilized: the mGluR5 agonist CHPG (Doherty et al. 1997) and the selective mGluR1 antagonist AIDA (Pellicciari et al. 1995; Moroni et al. 1997). When tested at a concentration reported to be mGluR5 selective, CHPG failed to potentiate 4AP-evoked [^{3}H]glutamate release from cerebrocortical synaptosomes, suggesting that presynaptic mGluR5 on glutamatergic terminals are not involved. On the other hand, the selective mGluR1 antagonist AIDA abolished the 3,5-DHPG potentiation, although at high concentration (1 mM), consistent with the possibility that the group I mGluR under study was of the mGluR1 subtype (Reid et al. 1999).

This conclusion seemed in contrast, however, with the almost concomitant findings of Sistiaga et al. (1998), according to which mGluR1 is not involved in the 3,5-DHPG-mediated facilitation of endogenous glutamate release as judged by experiments in cerebrocortical nerve terminals isolated from mGluR1-deficient mice. Whether technical problems, including insufficient selectivity of the antagonists used, or compensatory changes occurring in mGluR1 knockout animals, could explain the controversy, remained to be established. Whatever the reasons, subsequent works with selective ligands provided strong evidence that mGluR5 plays a major role as a release-enhancing group I autoreceptor.

Thomas et al. (2000) used several, and in part novel, group I mGluR ligands in experiments with rat forebrain slices preloaded with [^{3}H]D-aspartate ([^{3}H]D-ASP) and stimulated electrically. The potentiation of the evoked [^{3}H]D-ASP release produced by group I mGluR agonists was completely insensitive to a number of selective mGluR1 antagonists. In addition, CHPG, a selective mGluR5 agonist, concentration-dependently increased the evoked release of [^{3}H]D-ASP. Unfortunately, during this work, selective mGluR5 antagonists were not yet available, so that the effect of CHPG could only be counteracted by (S)-MCPG, a mixed mGluR1/mGluR5 antagonist. Subsequently, Croucher's group (Fazal et al. 2003) extended the pharmacological characterization of the mGlu autoreceptor thanks to the recent availability of novel mGluR5 selective antagonists. Rat cerebrocortical minislices were prelabeled with [^{3}H]D-ASP and depolarized with high-K^+; the evoked release of [^{3}H]D-ASP was enhanced by 3,5-DHPG. The responses to 3,5-DHPG were largely insensitive to mGluR1 antagonists, but were potently antagonized by some selective mGluR5 blockers. Fazal et al. (2003) concluded that the group I mGluRs sited on glutamatergic terminals in the cerebral cortex are autoreceptors of the mGluR5 subtype (see Figure 1, left).

Very recently, Rodrigues et al. (2005) hypothesized that the anti-Parkinsonian (Breysse et al. 2002) and neuroprotective (Battaglia et al. 2002) properties of mGluR5 antagonists could be related in part to the presence of presynaptic mGluR5 that modulate glutamate release on striatal glutamatergic terminals and control, together with dopamine D2 and adenosine A_{2a} receptors, the responsiveness of medium spiny neurons (Ferré et al. 2003). By monitoring glutamate release from superfused purified rat striatal synaptosomes, Rodrigues et al. (2005) found that the mGluR5 agonist CHPG enhanced the K^+-evoked release of the excitatory amino acid, an effect prevented by the mGluR5 antagonist MPEP. Furthermore, using a recent method of subsynaptic fractionation, Rodrigues et al. (2005) demonstrated that mGluR5 are localized (as expected) in the postsynaptic density fraction, but also

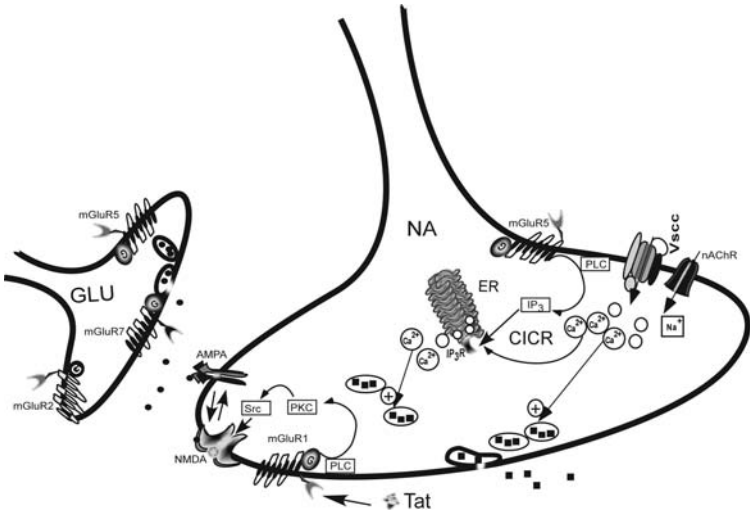

Fig. 1 Presynaptic mGluRs: metabotropic autoreceptors, metabotropic heteroreceptors on noradrenergic terminals, and their interactions with ionotropic receptors. Group I autoreceptors mediate enhancement of glutamate (solid circles) release and are likely to be mGluR5. Release inhibitory autoreceptors of group II appear to belong to the mGluR2 subtype; they exhibit high affinity for glutamate and are situated perisynaptically. Group III autoreceptors, particularly mGluR7, are localized at the active zone of presynaptic glutamatergic terminals; they exhibit very low affinity for glutamate. Presynaptic mGluR5 on noradrenaline (NA) terminals become functional, i.e., mediate release of NA (solid squares), upon application of appropriate stimuli, like activation of nicotinic cholinergic receptors (nAChRs) co-localized on the same NA terminal (right). Activation of nAChRs evokes NA exocytosis through depolarizing Na^+ entry and voltage-sensitive Ca^{2+} channel (VSCC) opening. This influx of Ca^{2+} triggers a Ca^{2+}-induced Ca^{2+} release (CICR) from the endoplasmic reticulum (ER) that allows mGluR5 to participate in the NA release by an IP_3-dependent process. Presynaptic mGluR1 on NA terminals (left) co-localize with presynaptic NMDARs. When NMDARs are activated to evoke NA exocytosis, mGluR1 contribute to the evoked release by enhancing NMDAR function through the PLC/PKC/Src pathway. The HIV-1 protein Tat is a potent agonist at mGluR1.

in the presynaptic nerve ending membrane. Moreover, by an immunocytochemistry approach, the authors showed that ∼50% of the whole nerve terminal population exhibited mGluR5 immunoreactivity, while almost 60% of the glutamatergic terminals co-stained for mGluR5 and A_{2a}. Thus, presynaptic mGluR5 are present on striatal nerve terminals. Some of them are autoreceptors on glutamatergic terminals, while others could be located on terminals of neurons whose identity remains to be determined.

Studies with cerebrocortical synaptosomes had reported high PKC activity in unstimulated conditions and in the absence of agonists (Coffey et al. 1994), suggesting the possibility of a basal, constitutive activity of group I mGluRs. If stimulatory presynaptic group I mGluRs on glutamatergic terminals were constitutively active, glutamate release should be inhibited by selective antagonists, particularly by non-competitive antagonists (also known as negative allosteric modulators) previously

reported to inhibit constitutive receptor activity occurring in heterologous expression systems (Gasparini et al. 2002).

Wang and Sihra (2004) indeed observed that SIB1893, a noncompetitive mGluR5 antagonist, inhibited glutamate exocytosis evoked by 4-AP from neocortical synaptosomes, suggesting that SIB1893 prevented an effect mediated by mGluR5 made "constitutively" active by depolarization. According to the authors, a presynaptic mGluR5/DAG/PKC pathway mediates facilitation of glutamate release. Moreover, the reactions that follow the activation of PKC mediated by mGluR5 take place at the Ca^{2+} entry step of the stimulus-exocytosis coupling cascade. Vazquez and Sanchez-Prieto (1997) had previously reported that presynaptic modulation of glutamate release targeted different VSCCs in cerebrocortical synaptosomes. In the work of Wang and Sihra (2004), the inhibition of glutamate release induced by SIB1893 was abrogated by the combined blockade of N- and P/Q-type channels brought about by ω-conotoxin GVIA and ω-agatoxin IVA, consistent with the hypothesis that mGluR5 activation leads to potentiation of glutamate release through a facilitation of N- and P/Q-type Ca^{2+} channel activities. SIB1893 inhibited both the 4AP- and the high-K^+-evoked release of glutamate, but did not affect the exocytotic release evoked by the Ca^{2+} ionophore ionomycin, which directly introduces Ca^{2+} into the cytosol without VSCC activation, a finding that, according to Wang and Sihra (2004), rules out any significant effect of mGluR5 activation at steps downstream of Ca^{2+} entry and at the level of the coupling of Ca^{2+} to the exocytotic release machinery itself.

2.2 Group II and Group III Metabotropic Glutamate Autoreceptors

Apart from the studies on the dual action of group I mGluRs on glutamate release and the switch from facilitatory to inhibitory that occurs upon receptor desensitization, there is sufficient functional evidence supporting an inhibitory role on glutamate exocytosis of group II and III mGluRs localized at presynaptic glutamatergic terminals.

Studies by Attwell et al. (1995; 1998a,b) with cerebrocortical synaptosomes prelabeled with [^{3}H]D-ASP and depolarized in superfusion with veratridine, showed that the group II selective mGluR2/3 agonists DCG-IV and 2R,4R-APDC blocked the neuronal release of the glutamate analogue, possibly through inhibition of VSCCs. A number of reports had described modulations of [^{3}H]D-ASP release from *slices* by mGluRs. Of interest, the broad-spectrum agonist 1S,3R-ACPD was found to inhibit the output of [^{3}H]D-ASP evoked by high-K^+ from rat striatal slices (Lombardi et al. 1993), whereas it potentiated the evoked release in cortical slices (Lombardi et al. 1994). These different effects of 1S,3R-ACPD probably reflect activation of group I mGluRs in the cortex, but not in the striatum, where inhibitory group II mGluRs might prevail. It should be considered that brain slices include abundant glial components. Astrocytes are known to express mGluR3 and mGluR5 (Winder and Conn 1996), and Bezzi et al. (1998) reported that 1S,3R-ACPD and

3,5-DHPG elicited glutamate exocytotic release from cultured astrocytes, under some particular conditions. Purified synaptosomes contain very modest "gliosome" contaminations (Stigliani et al. 2006) and seem therefore more appropriate as a model to investigate presynaptic auto- and heteroreceptors regulating glutamate release.

Most of the information available on group II mGluRs (see, for instance, Shigemoto et al. 1997) indicates that mGluR2, more than mGluR3, are localized to presynaptic nerve endings of glutamatergic neurons (Figure 1). Experiments with mGluR2 knockout mice are consistent with the view that mGluR2 is a presynaptic receptor that modulates glutamate release in the hippocampus (Yokoi et al. 1996). As determined with cloned mGluR2, the affinity for glutamate is low micromolar (Schoepp et al. 1999); following release, glutamate is thought to reach much higher concentrations in the synaptic cleft, so that mGluR2, if they were localized in the immediate vicinity of the synapse, would be almost saturated by the endogenous ligand. Indeed, although experiments on glutamate release with exogenous mGluR2/3 agonists support a location of mGluR2 on presynaptic terminals, immunocytochemical studies could not show mGluR2 near the active zone of the glutamatergic synapse. Synaptic spillover of glutamate seems therefore to be necessary for activation of presynaptic mGluR2 (see Scanziani et al. 1997). It is likely that mGluR2 plays a role in a neuronal mechanism endowed with essentially protective functions.

The selective group III agonist L-AP4 exhibits high affinity ($\leq 1\,\mu M$) at mGluR4, mGluR6 (almost exclusively expressed in retina) and mGluR8, but low affinity ($>100\,\mu M$) at mGluR7 (see Cartmell and Schoepp 2000). Inhibitory effects of L-AP4 on glutamate release from synaptosomes suggest the involvement of different subtypes of group III receptors depending on the age of the animals and on the brain region examined (East et al. 1995; Vazquez et al. 1995; Herrero et al. 1996; Rodriguez-Moreno et al. 1998; Reid et al. 1999). A recent article by Millán et al. (2002) reported that, in cerebrocortical synaptosomes from adult rats, L-AP4 inhibited 4-AP(1 mM)- or KCl(30 mM)-evoked glutamate release with an IC_{50} of $\simeq 300\,\mu M$. The localization of mGluR7 within presynaptic active zones (Shigemoto et al. 1996; see Figure 1, left), together with the low affinity for glutamate, are consistent with their role as autoreceptors that, in certain synapses, mediate feedback inhibition of glutamate release under normal physiological conditions. Indeed, the low affinity of mGluR7 for glutamate appears as an appropriate characteristic if these receptors are localized within synapses in which glutamate concentrations following release likely reach hundreds of micromolar. It has to be noted that autoreceptors of the mGluR7 subtype do not seem to be present on all glutamatergic terminals in the CNS; their expression may be more concentrated in certain regions and their function could be limited to certain synapses (see below in this section).

The mechanisms underlying the inhibition of glutamate release by mGluR7 have been studied in detail by Millán et al. (2002). The L-AP4 inhibition was abolished by the group III mGluR antagonist CPPG (see Cartmell and Schoepp 2000) and largely prevented by pertussis toxin, suggesting involvement of $G_{i/o}$ proteins. Surprisingly enough, the inhibition of glutamate release by L-AP4 seemed to be unrelated to

decreases in intrasynaptosomal cyclic AMP, although group III mGluRs are known to reduce AC activity.

Immunolabeling with mGluR7 antibodies and Ca^{2+}-imaging of single nerve terminals revealed that the population of mGluR7-expressing nerve terminals largely responded to L-AP4 by reducing their cytoplasmic Ca^{2+}-concentration, consistent with the idea that mGluR7 is the principal presynaptic release-inhibiting autoreceptor in the cerebral cortex of adult rats. Assuming that synaptophysin is a reliable marker for axon terminals, Millán et al. (2002) performed double immunolabeling experiments and found that, in their purified synaptosomal preparation, 25%–35% of the synaptophysin-positive particles also contained mGluR7. These results correlate with those obtained in Ca^{2+}-imaging experiments showing that 28% of nerve terminals responded to L-AP4 which, in turn, inhibited by 25% the Ca^{2+}-dependent glutamate exocytosis. According to Millán et al. (2002), the majority of the axon terminals expressing mGluR7 may be glutamatergic, other nonglutamatergic nerve endings that possess heteroreceptors of the mGluR7 subtype, i.e., GABAergic nerve endings, representing relatively minor subpopulations of nerve terminals. The apparently limited inhibition of glutamate exocytosis by L-AP4 (25% maximal effect) is probably due to the expression of mGluR7 in only a subpopulation of glutamatergic terminals.

Given that mGluR7 activation reduces the depolarization-evoked rise in cytosolic $[Ca^{2+}]$ and release of glutamate, and considering that the effect of L-AP4 on glutamate release seemed unrelated to changes in cyclic AMP levels, Millán et al. (2002) investigated the involvement of Ca^{2+} channels. The results obtained suggest that presynaptic mGluR7 attenuate the function of N-type VSCCs but not that of P/Q-type channels. The effects of L-AP4 were insensitive to PKA and PKC inhibitors, suggesting a direct interaction between the $G_{i/o}$ proteins linked to mGluR7 and N-type channels.

The finding that the inhibition of the evoked glutamate release by mGluR7 activation occurred without changes in synaptosomal cyclic AMP levels was unexpected, as mentioned, considering that activation of group III mGluRs either in heterologous expression systems or in neuronal preparations had been shown to decrease cyclic AMP levels. However, Millán et al. (2002) found that mGluR7 activation could reduce intrasynaptosomal cyclic AMP levels provided that they were previously increased by forskolin. To conclude, mGluR7 mediates inhibition of glutamate release by signaling through two pathways. In the first, depression of glutamate release is consequent to reduction in the activity of N-type VSCCs and no detectable change in cyclic AMP. In the second pathway, if cyclic AMP levels are high, mGluR7 mediates decrease in the nucleotide levels, thereby counteracting the facilitation of glutamate release by PKA activation.

The presynaptic mGluR7 shows the highest evolutionary conservation within mGluRs; its characterization has however been hampered because no selective ligand was available until very recently. Mitsukawa et al. (2005) have just characterized an mGluR7-selective agonist, AMN082, which is able to activate the mGluR7 signaling via an allosteric site located in the receptor transmembrane domain. Its effects on glutamate release remains to be determined.

3 Metabotropic Glutamate Heteroreceptors

The review by Cartmell and Schoepp (2000) covered almost exhaustively the field of mGluRs that are localized on nonglutamatergic terminals and regulate as presynaptic heteroreceptors the release of various transmitters; this section of my chapter will therefore be essentially restricted to recent aspects of the functional pharmacology of presynaptic metabotropic glutamate heteroreceptors.

3.1 Modulation of Acetylcholine Release

Marti et al. (2001) studied effects of mGluR activation on the release of acetylcholine (ACh) in corpus striatum. The authors monitored release of endogenous ACh from rat striatal synaptosomes depolarized in superfusion with high-K^+. The group I agonist (S)-3,5-DHPG potentiated the K^+-evoked exocytosis of ACh. The effect of (S)-3,5-DHPG was counteracted by a mixture of CPCCOEt, a mGluR1 selective antagonist, and MPEP, a mGluR5 selective antagonist (Gasparini et al. 1999), but not by either antagonist alone. Thus group I mGluRs (mGluR1 or mGluR5 or both) able to enhance the depolarization-evoked release of ACh are present on nerve terminals of striatal cholinergic interneurons. On the other hand, the K^+-evoked release of ACh from striatal synaptosomes was inhibited by the group II agonist DCG-IV, an effect prevented by the group II selective antagonist EGLU. To conclude, the study by Marti et al. (2001) supports the view that the terminals of striatal cholinergic interneurons are endowed with release-stimulating mGluRs of group I and release-inhibiting mGluRs of group II. The availability of novel ligands and knockout animals should permit to verify the possible coexistence of mGluR1 and mGluR5 in striatal cholinergic interneurons, as proposed by Pisani et al. (2001), and to identify the group II mGluR subtype involved in the inhibition of ACh release.

Changes in the function of presynaptic group II mGluRs inhibiting ACh release in experimental parkinsonism were then investigated by the Ferrara laboratory (Marti et al. 2003). The effects on ACh release of group II mGluR and NMDA receptor ligands were examined in striatal slices and synaptosomes from naive rats, 6-hydroxydopamine hemilesioned rats, and 6-hydroxydopamine hemilesioned rats chronically treated with L-DOPA plus benserazide. The inhibition of ACh release caused by the group II mGluR agonist LY354740 was reduced on the lesioned side, whereas the potentiating effect of NMDA (Morari et al. 1998) was enhanced. Interestingly, chronic L-DOPA normalized the function of both group II mGluRs and NMDA receptors. According to Marti et al. (2003), it can be predicted that the loss of presynaptic mGluR-mediated inhibition of ACh release and the enhancement of the NMDA-evoked release seen in hemiparkinsonian rats will shift the glutamatergic input to cholinergic interneurons towards enhanced excitability.

Assuming that the above group II mGluRs and NMDARs coexist on the same striatal cholinergic terminals, possible receptor-receptor interactions were investigated (Mela et al. 2006). The group II mGluR agonist LY354740 and NMDA were

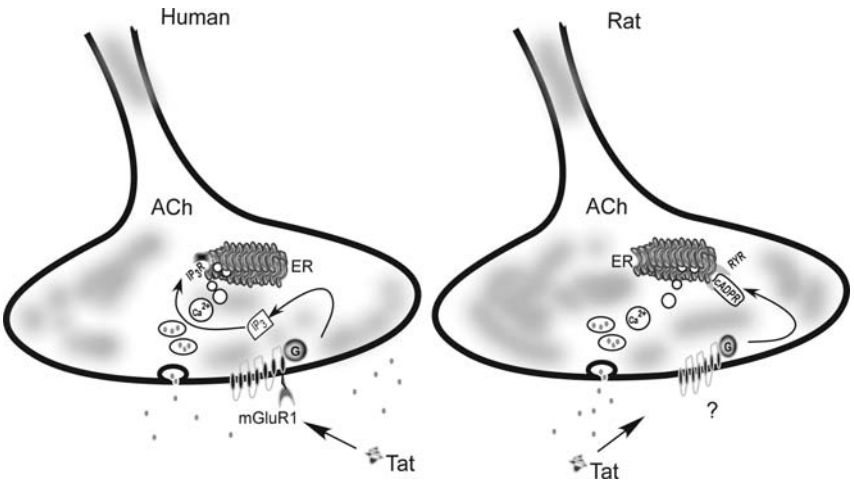

Fig. 2 Presynaptic mGluRs on human and rat neocortical cholinergic (ACh) nerve endings and effect of the HIV-1 protein Tat. In human neocortex, Tat activates mGluR1 leading to inositol trisphosphate (IP_3) production, IP_3 receptor (IP_3R) activation, mobilization of Ca^{2+} from the endoplasmic reticulum (ER), and vesicular ACh release. In rat neocortex ACh terminals Tat binds to an unidentified receptor whose activation also leads to ACh release. This release again is dependent on intraterminal Ca^{2+}, but this is mobilized by ryanodine receptor (RYR) activation via the endogenous agonist cyclic adenosine diphosphoribose (cADPR).

co-applied to striatal slices or synaptosomes. LY354740 inhibited the NMDA-evoked ACh release from both synaptosomes and slices. VSCCs of the P/Q-type were involved, as the modulation was prevented by ω-agatoxin IVA. Co-immunoprecipitation experiments excluded direct interaction between the receptors, whose coexistence is however indicated by the experiments with synaptosomes.

Group I mGluRs were found to exist also on cholinergic terminals of human neocortex (Feligioni et al. 2003). The selective group I mGluRs agonist 3,5-DHPG released [^{3}H]ACh from human neocortical synaptosomes. The effect was prevented by CPCCOEt, suggesting activation of mGluR1 (see Figure 2). Differently from the effect observed by Marti et al. (2001) in striatal cholinergic terminals, activation of the mGluR1 on human neocortical cholinergic terminals caused ACh release in absence of depolarization.

Our laboratory has long been interested in studying effects of the human immunodeficiency virus (HIV-1) proteins gp120 and Tat on neurotransmitter release and on receptors modulating neurotransmitter release (Pittaluga and Raiteri 1994; Pittaluga et al. 1996; Pattarini et al., 1998; Gemignani et al. 2000; Feligioni et al. 2003; Longordo et al. 2006). In fact, these proteins may play important roles in the neuropsychiatric symptoms (termed AIDS dementia) that infected patients frequently develop (Lawrence and Major 2002). When human neocortical synaptosomes prelabeled with [^{3}H]choline were exposed in superfusion to Tat (1 nM), a release of [^{3}H]ACh partly dependent on external Ca^{2+} ions and partly on mobilization of Ca^{2+} from intraterminal stores was observed (Feligioni et al. 2003). Xestospongin

C, a membrane-permeant IP_3R antagonist, inhibited the Tat-induced release of [^{3}H]ACh indicating involvement of IP_3-sensitive internal Ca^{2+} stores. Among the various possibilities, Tat might have activated group I mGluRs, possibly of the mGluR1 subtype (Figure 2). Accordingly, the effect of 1 nM Tat on the release of [^{3}H]ACh from human neocortex synaptosomes was prevented by the selective mGluR1 antagonists CPCCOEt and LY367385. Of note, the concentrations of Tat ($\leq$1 nM) able to significantly enhance the release of ACh approach those found extracellularly during HIV-1 infection (Gurwell et al. 2001). Tat may represent a potent pathological agonist at mGluR1 possibly involved in the neurotoxic events characteristic of HIV-1 infection.

Tat also caused release of [^{3}H]ACh in synaptosomes from rat neocortex (Feligioni et al. 2003). However, the mechanisms involved were different from those in human brain. In rats, the release was independent of external Ca^{2+}; it was largely dependent on intraterminal Ca^{2+}, but insensitive to xestospongin C, excluding involvement of IP_3Rs. The Tat effect in rat synaptosomes was blocked by dantrolene, indicating ryanodine-sensitive stores as the origin of intraterminal Ca^{2+} (Figure 2). Ryanodine receptors are thought to be endogenously activated by cADP-ribose (Galione et al. 1993; Lee 2001). When rat cerebrocortical synaptosomes were exposed to Br-cADP-ribose, a competitive antagonist of the cADP-ribose binding site (Walseth and Lee 1993), the Tat-induced release of [^{3}H]ACh was prevented, an effect not observed in human neocortex synaptosomes (Feligioni et al. 2003). The differences between the mechanisms of Tat-evoked ACh release in human and rat cholinergic terminals are further supported by the observation that, in rats, Tat did not act through group I mGluRs since 3,5-DHPG was unable to elicit [^{3}H]ACh release.

In conclusion, Tat can evoke exocytotic release of ACh from human and rat cerebrocortex cholinergic axon terminals, by different mechanisms. In human brain Tat behaves as an extremely potent mGluR1 agonist mediating VSCCs activation and mobilizing intraterminal Ca^{2+} through IP_3. Binding of Tat to rat cholinergic terminals leads to ACh release that is also dependent on intraterminal Ca^{2+}, which is, however, mobilized by ryanodine receptor activation, probably via cADP-ribose. In rat brain Tat appears to be a potent activator of an as yet unidentified "receptor"' linked to the second messenger cADP-ribose (Figure 2).

3.2 Modulation of Noradrenaline Release

Presynaptic group I mGluRs on noradrenergic axon terminals were identified only very recently (Longordo et al. 2006; Parodi et al. 2006), because the low density of noradrenergic terminals in CNS makes immunocytochemical approaches quite difficult. Moreover, some receptors (and this is one case) may be silent under basal conditions and exhibit function only in presence of appropriate stimuli.

Several laboratories have reported that nicotinic cholinergic receptors (nAChRs) are situated on central noradrenergic terminals where they mediate enhancement of

NA release (see review by Wonnacott 1997). Parodi et al. (2006) found that the external Ca^{2+}-dependent overflow of [^{3}H]NA provoked by (−)-nicotine in rat hippocampal synaptosomes was significantly enhanced when the group I mGluR agonist 3,5-DHPG, inactive on its own, was added to the superfusion medium together with (−)-nicotine. This effect was reduced down to the release evoked by (−)-nicotine alone when MPEP, a selective mGluR5 antagonist was also present. Addition of CPCCOEt, a selective mGluR1 antagonist, was instead unable to affect the overflow caused by 3,5-DHPG. The nAChR antagonist mecamylamine abrogated the release of [^{3}H]NA evoked by (−)-nicotine plus 3,5-DHPG. Thus release-enhancing presynaptic mGluR5 exist on noradrenergic terminals of rat hippocampus. Exposure to a group I mGluR agonist alone is insufficient to cause release of NA; a mGluR5-mediated release can however be observed when (−)-nicotine and a group I mGluR agonist are added together, suggesting coexistence on the same noradrenergic terminal of nAChRs and mGluR5 with nAChRs exerting a permissive role on the activation of mGluR5 (Figure 1, right). The 3,5-DHPG-mediated component of the release provoked by (−)-nicotine plus 3,5-DHPG was sensitive to the IP_3 receptor antagonist xestospongin C (Parodi et al. 2006).

As to the mechanism of this receptor-receptor interaction, it has to be considered that nAChRs mediating NA release are non-α7 receptors highly permeable to Na^+. Influx of Na^+ can cause membrane depolarization and consequent activation of VSCCs (Kulak et al. 2001). Ca^{2+} influx through VSCCs may trigger a calcium-induced calcium release (CICR) from Ca^{2+} stores primed by IP_3 (Berridge 1998); indeed group I mGluRs are positioned close to IP_3 receptors on the endoplasmic reticulum by the Homer family proteins (Brakeman et al. 1997; Fagni et al. 2000). In other words, nicotine could first trigger influx of Na^+ into noradrenergic terminals and activation of VSCCs; entry of Ca^{2+} would then (1) directly induce NA exocytosis and (2) permit, perhaps through a CICR process, an additional release of NA mediated by mGluR5 (Figure 1, right).

The noradrenergic system is involved in stress-related and anxiety disorders (Bremner et al. 1996). *In vivo* microdialysis studies show that exposure of animals to stressful stimuli leads to increase in the release of noradrenaline in corticolimbic areas including prefrontal cortex and hippocampus (Kawahara et al. 2000; Dazzi et al. 2002). Moreover, there is increasing evidence that excessive glutamatergic transmission also plays a role in the pathophysiology of stress and anxiety conditions (see Moghaddam and Jackson 2004). Interactions between glutamatergic and noradrenergic systems are therefore likely to be enhanced during stressful stimuli. Accordingly, blockade of mGluR5 by MPEP has recently been reported to decrease stress-induced cortical noradrenaline release in rodents (Page et al. 2005) and morphine withdrawal-induced activation of locus coeruleus neurons (Rasmussen et al. 2005). Interestingly, Grilli et al. (2005) have found that nAChRs mediating enhancement of NA release from hippocampal nerve terminals are functionally upregulated soon after withdrawal from chronic nicotine, a condition that, according to the findings of Parodi et al. (2006), may favor mGluR5 activation. In rodents, MPEP was reported to decrease nicotine self-administration (Paterson et al. 2003; Tessari et al. 2004). Considering that some of the behavioral modifications experienced

by smokers acutely withdrawn from nicotine represent stress symptoms, possibly related to increased noradrenergic transmission mediated by nAChR upregulation and mGluR5 activation, mGluR5 antagonists may turn out to be useful to quit smoking also by reducing withdrawal stress.

In addition to the interaction of group I mGluRs with nAChRs, an interaction with NMDA receptors on noradrenergic terminals also seems to occur. In particular, noradrenergic terminals in human and rat neocortex (Fink et al. 1992; Pittaluga et al. 1996) and in rat hippocampus (Fink et al. 1990; Pittaluga and Raiteri 1990) are endowed with release-enhancing NMDA receptors which might interact with mGluRs coexisting on the same terminals. The HIV-1 protein Tat played a role in the discovery of the interaction. In the absence of external Mg^{2+}, concomitant addition of NMDA and glycine to the superfusion medium elicited release of [^{3}H]NA from human neocortex synaptosomes. Interestingly, when this preparation was exposed to NMDA plus Tat (1 nM), without adding glycine, a release of [^{3}H]NA quantitatively identical to that provoked by NMDA plus 10 μM glycine was observed. This effect was entirely dependent on NMDA receptor activation since it was almost eliminated by the NMDA receptor antagonist MK-801 (Longordo et al. 2006). Results qualitatively similar to those in human neocortex were obtained with rat cortical or hippocampal synaptosomes. Based on these data, the effect of Tat would appear compatible with an extremely potent action at the glycine site of the NMDA receptors. However, differently from the HIV-1 coat protein gp120, which does mimic glycine at NMDARs (Pittaluga and Raiteri 1994; Pattarini et al. 1998), Tat failed to revert that antagonism of the NMDA/Tat effects brought about by the glycine site blocker 7-Cl-kynurenate both in human and rat synaptosomes, a result inconsistent with an action of Tat at the NMDA receptor glycine site (Longordo et al. 2006). Tat and glycine (or gp120) act therefore at different sites on NA terminals, a view also supported by the ability of Tat to further enhance the maximal effect of glycine or gp120 (see Pittaluga and Raiteri 1994; Pittaluga et al. 1996; Longordo et al. 2006).

The possibility existed that Tat could bind to a "receptor," possibly a mGluR, coexisting with NMDA receptors on noradrenergic terminals and able to interact with the ionotropic receptor. The release of [^{3}H]NA caused by NMDA/Tat or NMDA/glycine/Tat from human or rat synaptosomes was pertussis-toxin sensitive suggesting that Tat acts through $G_{i/o}$ protein-coupled receptors. The Tat-mediated component of the NMDA/glycine/Tat-evoked release was completely blocked by the mGluR1 antagonist CPCCOEt, whereas the mGluR5 antagonist MPEP only exerted a slight nonsignificant inhibition. To confirm the involvement of an mGluR1 subtype, LY367385, a selective mGluR1 antagonist, was tested both in human neocortical and rat neocortical or hippocampal synaptosomes: LY367385 abrogated the Tat-mediated component of the NMDA/glycine/Tat-evoked [^{3}H]noradrenaline release (Longordo et al. 2006). Thus, mGluR1 seems to be localized on human and rat noradrenergic axon terminals where they interact with coexisting NMDA receptors and Tat can activate mGluR1 with extremely high potency (Figure 1). Whether, given the absence of selective mGluR1 agonists, Tat could represent a useful tool in studying mGluR1 remains to be established.

The mechanisms underlying the enhancement of NMDA receptor function by Tat acting at presynaptic mGluR1 have been in part clarified by Longordo et al. (2006). The release of [^{3}H]NA evoked by NMDA/glycine/Tat from human or rat noradrenergic terminals was significantly inhibited by the PLC inhibitor U73122, by the PKC blocker GF 109203X, and by the inhibitor of cytosolic tyrosine kinase (Src) PP2. On the contrary, PKA and Ca^{2+}/calmodulin-dependent protein kinase II seemed not to be involved. The inhibitions observed likely concerned the Tat-mediated component of the NMDA/glycine/Tat-evoked release since the inhibitors did not affect the release evoked by NMDA/glycine. The involvement of Src in the NMDAR activation would imply phosphorylation of tyrosine residues. Src is associated with NMDA receptors, and phosphorylation by Src was reported to enhance NMDA receptor currents (Yu et al. 1997). Notably, activation of Src family kinase can be triggered by PKC (Salter and Kalia 2004). Thus, the PLC/PKC/Src pathway seems to couple presynaptic mGluR1 activation and NMDA receptor function in brain noradrenergic terminals. NMDA receptor function is usually studied in the absence of external Mg^{2+}. However, NMDA was found to elicit NA release in the presence of physiological concentrations of Mg^{2+} during concomitant activation of AMPA receptors, which coexist with NMDA receptors on noradrenergic terminals (Raiteri et al. 1992). The following scenario could be envisaged *in vivo* (see Figure 1, left): glutamate reaching noradrenergic terminals activates depolarizing AMPA receptors, which in turn permit Mg^{2+} removal and NMDA receptor activation; if Tat mimics glutamate and binds at mGluR1, the NMDA receptor-mediated response could be strongly enhanced. In addition, Tat might also contribute by releasing ACh (Feligioni et al. 2003) onto α7 nicotinic receptors localized on glutamatergic terminals and eliciting glutamate release (Marchi et al. 2002), as well as onto non-α7 nicotinic receptors on noradrenergic terminals able to exert a permissive role on NMDA receptor (Risso et al. 2004) and mGluR5 (Parodi et al. 2006) function.

The inability of Tat (or of the group I agonist 3,5-DHPG; Parodi et al. 2006) to release NA when added alone suggests that the mere activation of mGluR1/5 stimulates the above enzymatic pathway insufficiently to trigger NA release although, alternatively, group I mGluRs might be in a desensitized state (that can be reverted by NMDA) under basal conditions (see Alagarsamy et al. 2001). Nonetheless, presynaptic mGluR1 activation can dramatically promote the functions of NMDA receptors if one considers that these receptors, almost silent when glycine was not added (only the unavoidable glycine contamination present in the solutions at $\sim$50 nM was available), became fully responsive in the presence of a concentration of Tat as low as 1 nM. If glycine is an obligatory glutamate coagonist, a reasonable explanation for the Tat effect could be that the protein causes, through mGluR1 activation and the above phosphorylation pathway, a several-fold increase in the affinity of the glycine site for its natural ligand.

The concentrations of Tat able to activate mGluR1 and modulate directly ACh release (Feligioni et al. 2003) or, through interactions with NMDA receptors, NA release (Longordo et al. 2006) are lower than those reported to cause overt neurotoxicity (Nath and Geiger 1998). It is therefore possible that mGluR1 represents a

major target through which Tat produces early impairments in neurotransmission independent of cell death. Since Tat or its active fragments cannot be removed as easily as glutamate in the CNS, mGluR1 could therefore be "pathologically" activated during HIV-1 infection. The persistent effects of Tat may provoke transmitter release outside of the physiological range and with abnormal kinetic characteristics, potentially contributing to cognitive impairments occurring in AIDS-dementia. The situation could become quite serious because gp120 also could be present: the HIV-1 coat protein is a potent agonist at the glycine site of NMDA receptors and its effects can be additive to those of Tat (Nath et al. 2000; Longordo et al. 2006). Assuming that Tat and some of its fragments can behave as pathological mGluR1 agonists, AIDS-dementia symptoms might be controlled, in part, by mGluR1 antagonists or mGluR1 negative allosteric modulators.

4 Presynaptic $GABA_B$ Receptors

In studies on modulation of transmitter release from CNS slices, Bowery et al. (1980) found that (-)baclofen and GABA decreased the evoked release of noradrenaline, dopamine, and serotonin by acting at a novel site. These inhibitory actions of (-)baclofen and GABA were not blocked by bicuculline, were not mimicked by the GABA analogue isoguvacine, and were not mediated by Cl^- influx, all of which are characteristics of the classical ionotropic GABA receptor. The classical GABA receptor was insensitive to baclofen which, in ligand-binding studies, showed distinct attachment sites on CNS membranes. For these reasons, the term $GABA_B$ receptor was proposed for the baclofen-sensitive site to distinguish it from the bicuculline-sensitive receptor, which was, in turn, designated $GABA_A$ (Hill and Bowery 1981).

Differently from the ionotropic $GABA_A$ receptors, $GABA_B$ receptors ($GABA_B$Rs) are metabotropic GPCRs (Karbon and Enna 1985) endowed with peculiar structural and pharmacological properties. Contained in the present article is an overview of recent reports on release-modulating $GABA_B$Rs, concentrating on the mystery of their unexplained pharmacological heterogeneity. Readers desiring additional or more detailed information on particular aspects of this topic may consult other sources (e.g., Bowery 1993, 2006; Bonanno and Raiteri 1993a; Bowery and Enna 2000; Bowery et al. 2002; Bettler et al. 2004; Couve et al. 2004; Raiteri 2006).

4.1 Structural Characteristics and Distribution of $GABA_B$ *Rs*

Some of the structural properties of $GABA_B$Rs appear unique. It is widely accepted that GPCRs can be expressed as a single protein or form homodimers (Bouvier 2001). Differently, $GABA_B$Rs exist as heterodimers with the subunits designated $GABA_{B1}$ and $GABA_{B2}$ (Jones et al. 1998; Kaupmann et al. 1998a; White et al.

1998; Kuner et al. 1999; Ng et al. 1999). The $GABA_{B1}$ subunit was first cloned (Kaupmann et al. 1997); however, this seven-transmembrane-spanning protein with homology to mGluRs exhibited poor binding affinities for agonists and inefficient coupling to presumed $GABA_B$ effector systems in heterologous cells. It was subsequently shown (Couve et al. 1998) that $GABA_{B1}$ needs to be transported from the endoplasmic reticulum to the plasma membrane by the $GABA_{B2}$ subunit, which not only serves to escort $GABA_{B1}$ to the cell surface, but is likely to include the receptor portion that links to the G protein, while $GABA_{B1}$ is critical for agonist activation (Margeta-Mitrovic et al. 2000; Pagano et al. 2001). It is now generally accepted that the coupling of $GABA_{B2}$ with $GABA_{B1}$ yields a full functional $GABA_BR$ in which the $GABA_{B1}$ subunit, now expressed as a part of the heterodimer, exhibits agonist affinity similar to that of the wild-type $GABA_B$ receptor (Kaupmann et al. 1998a; White et al. 1998). In other words, the binding of GABA or $GABA_BR$ agonists to $GABA_{B1}$ causes activation of G protein via $GABA_{B2}$. $GABA_BRs$ are located in the CNS both pre- and postsynaptically, where they are coupled to Ca^{2+} and K^+ channels which mediate inhibition of transmitter release and neuronal hyperpolarization upon receptor activation.

Some splice variants of the $GABA_{B1}$ subunit have been identified (see Bettler et al. 2004 for a review). Most studies concern the two variants of the $GABA_{B1}$ gene originally identified, $GABA_{B1(a)}$ and $GABA_{B1(b)}$, which originate molecules that differ at the extreme amino terminal domain (Kaupmann et al. 1997; see Sections 4.3 and 4.4).

In the CNS, $GABA_B$ binding sites are widely distributed and particularly abundant in the thalamic nuclei, the molecular layer of the cerebellum, the cerebral cortex, and the dorsal horn of the spinal cord (Bowery et al. 1987). Based on in situ hybridization studies of mRNA for the two major splice variants of $GABA_{B1}$, $GABA_{B1(a)}$ exhibits association with presynaptic receptors in rat and human cerebellum and spinal cord, whereas $GABA_{B1(b)}$ is predominantly postsynaptic (Kaupmann et al. 1998b; Billinton et al. 1999; Bischoff et al. 1999; Princivalle et al. 2000; Towers et al. 2000). In other CNS regions, however, $GABA_{B1(b)}$ appears to be on presynaptic terminals and $GABA_{B1(a)}$ is postsynaptically localized (Benke et al. 1999; Princivalle et al. 2001), suggesting that a cellular location or function cannot be generally attributed to a given $GABA_{B1}$ splice variant.

4.2 Presynaptic $GABA_B$ *Autoreceptors and Heteroreceptors*

Compelling evidence indicates that a major function of $GABA_BRs$ is to mediate inhibition of neurotransmitter release from nerve terminals where they are localized as presynaptic auto- and heteroreceptors (see Bonanno and Raiteri 1993a; Bowery et al. 2002; Raiteri 2006, for reviews).

Electrophysiological studies in mammalian CNS suggested subtle distinctions between pre- and postsynaptic $GABA_BRs$ (see Nicoll 2004 for a review). Stronger evidence that pharmacological differences may exist between $GABA_B$ presynaptic

Table 1 Drug Potencies at $GABA_B$ Receptor Subtypes Regulating Transmitter Release in Human and Rat Neocortex

Drug	Neurotransmitter			
	GABA	Glutamate	Somatostatin	Cholecystokinin
		Rat Neocortex		
Phaclofen	79.2	>300	62.6	66.1
CGP35348	>300	4.2	3.6	3.5
CGP52432	0.08	9.3	3.4	0.11
CGP47656	3.1	Partial agonist	Agonist	>300
CGP36742	>100	>100	0.14	>100
		Human Neocortex		
Phaclofen	≈100	>300	Not determined	Not determined
CGP35348	>100	≈10	24.4	13.9
CGP52432	≪1	>1; <30	0.06	0.08
CGP47656	<10	Not determined	Agonist	Agonist
CGP36742	>100	>100	≈5	>100

Reported are IC_{50} values (μM) of $GABA_B$ receptor antagonists tested against (-)baclofen. Reproduced from Bowery et al. (2002)

autoreceptors and presynaptic heteroreceptors was obtained in transmitter release studies with native rat and human $GABA_B$Rs (see, for reviews, Bonanno and Raiteri 1993a; Mott and Lewis 1994; Bowery et al. 2002; Raiteri 2006). Following an experimental paradigm that had been fruitful in studying several other receptor systems, we tested comparatively a number of $GABA_B$R antagonists on different $GABA_B$R-sensitive release systems. The existence of pharmacologically distinct subtypes of a native receptor in the peripheral or central nervous system has traditionally been considered likely when the affinities of one antagonist for the hypothetical subtypes differed by at least one order of magnitude. As shown in Table 1, in the case of $GABA_B$Rs sited on rat cerebrocortical axon terminals releasing GABA, glutamate, cholecystokinin, or somatostatin, not only did the antagonist affinities differ in some cases by more than two orders of magnitude, but the orders of potency of some antagonists differed between receptors, and qualitatively similar results were obtained in nerve endings isolated from human cerebrocortex (Fassio et al. 1994; Bonanno et al. 1996, 1997, 1999; Raiteri et al. 1996; see, for a recent review, Raiteri 2006). In other studies we found that, surprisingly enough, $GABA_B$ autoreceptors inhibiting GABA release in rat cerebral cortex differ pharmacologically from $GABA_B$ autoreceptors in the rat spinal cord (Bonanno and Raiteri 1993b; Bonanno et al. 1998). While GABA and the $GABA_B$ agonist 3-APPA were almost equipotent inhibitors of GABA release in the two areas (EC_{50} for GABA $\simeq 1\,\mu M$; EC_{50} for 3-APPA $\simeq 0.1\,\mu M$), (-)baclofen was a very weak agonist at the spinal cord autoreceptor ($EC_{50} = 425\,\mu M$), although it behaved like GABA ($EC_{50} \simeq 1\,\mu M$) in the cortex. Moreover, the $GABA_B$R antagonists, phaclofen and CGP35348, exhibited opposite behavior at the two $GABA_B$ autoreceptors: phaclofen blocked cortical autoreceptors ($IC_{50} \simeq 50\,\mu M$) but not spinal autoreceptors ($IC_{50} > 1000\,\mu M$); conversely, CGP35348 blocked spinal autoreceptors ($IC_{50} \simeq 1\,\mu M$), being ineffective

($IC_{50} > 300\,\mu M$) at cortical autoreceptors. The clear selectivity of (-)baclofen in inhibiting evoked glutamate release with respect to evoked GABA release observed in our study (Bonanno and Raiteri 1993b; Bonanno et al. 1998) might contribute to its therapeutic efficacy as antispastic agent.

Of particular interest appear to be the $GABA_B$Rs that regulate the release of somatostatin (SRIF). Human and rat neocortex synaptosomes released SRIF in a Ca^{2+}-dependent manner when depolarized in superfusion with high-K^+. The SRIF overflow was inhibited by (-)baclofen, an effect blocked by CGP35348 or CGP52432 (Gemignani et al. 1994; Bonanno et al. 1996), indicating the presence of presynaptic $GABA_B$Rs on SRIF-releasing terminals. SRIF and GABA coexist in a large percentage of neocortical neurons (Hendry et al. 1984). Based on the cotransmitter idea, axon terminals costoring SRIF and GABA are expected to release both compounds. However, the observation that SRIF release from rat cortical slices could only be elicited by electrical stimulation frequencies 10 to 30 times higher than those used to release GABA (Bonanno et al. 1988; Waldmeier et al. 1988) suggests that GABA and SRIF are not costored in the same vesicles and are released differentially. In consequence, the release of SRIF and GABA could both be under the control of $GABA_B$, "autoreceptors," but the $GABA_B$Rs inhibiting GABA release could differ from those inhibiting SRIF release (see Table 1 and see Figure 3). It was reported that reduced release of SRIF may play a role in cognitive impairments typical of Alzheimer's disease (Gabriel et al. 1993) or accompanying other pathologies; accordingly, selective $GABA_B$R antagonists could lead to increased SRIF release and consequently to cognitive improvement. A $GABA_B$R antagonist, CGP36742, had been found to ameliorate the cognitive performances of mice, rats, and monkeys in tests covering diverse manifestations of learning and memory (Mondadori et al. 1993; Froestl et al. 1995). We examined the ability of CGP36742 to block release-regulating $GABA_B$ receptors. In particular, CGP36742 was tested against the inhibition by (-)baclofen of the depolarization-evoked release of GABA, glutamate, cholecystokinin, and SRIF in rat and human neocortex axon terminals (Bonanno et al. 1999). It was found (see Table 1) that CGP36742 antagonized with high affinity the inhibition by (-)baclofen of the release of SRIF from rat ($IC_{50} = 0.14\,\mu M$) and human ($IC_{50} \sim 5\,\mu M$) neocortex synaptosomes. In contrast, the effects of (-)baclofen on GABA, glutamate, and cholecystokinin release was insensitive to CGP36742 up to $100\,\mu M$, indicating that the antagonist has low affinity for the $GABA_B$ autoreceptor regulating GABA release. The compound is the first $GABA_B$R antagonist exhibiting great selectivity for the $GABA_B$ presynaptic receptors regulating SRIF release. Considering the implication of SRIF in cognitive mechanisms, disinhibition of SRIF release through $GABA_B$R blockade and consequent activation of SRIF receptors might represent one of the processes involved in the behavioral activities of CGP 36742. Of note, one of the SRIF receptors (sst5) possibly activated during CGP36742 treatment was found to facilitate the function of NMDA receptors coexisting on the same membrane (Pittaluga et al. 2000, 2001), which are known to play a primary role in cognitive processes. If humans respond as rats, CGP36742 (now termed SGS742) could act according to the schematic model

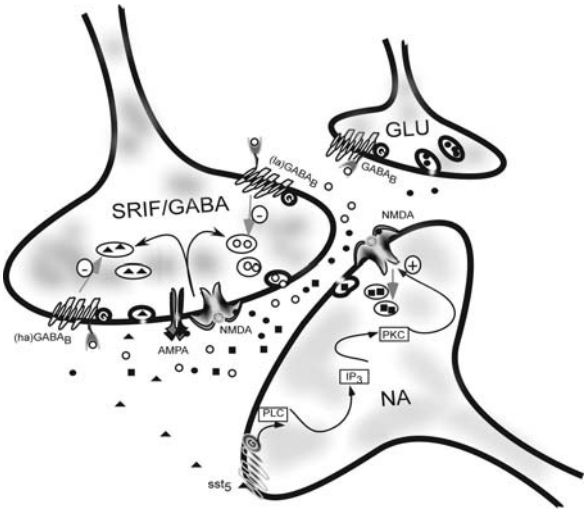

Fig. 3 Mechanisms that may underlie the cognitive enhancing properties of the $GABA_BR$ antagonist CGP36742 (now termed SGS742). GABA and somatostatin (SRIF) are often co-stored in and co-released from interneurons. Glutamate (GLU; black dots) evokes release of noradrenaline (NA; solid squares), GABA (white dots) and SRIF (solid triangles) by activating ionotropic NMDA/AMPA receptors located on the respective releasing axon terminals. GABA can inhibit its own release and SRIF release through pharmacologically distinct $GABA_BR$ subtypes (see Table 1). CGP36742 (SGS742) binds with high affinity to the $GABA_B$ receptor (ha, high affinity, $GABA_B$) modulating SRIF release but with very low affinity to the $GABA_B$ receptor (la, low affinity $GABA_B$) modulating GABA release (Bonanno et al. 1999). SRIF enhances NA release by activating sst_5 receptors colocalized with NMDAR on noradrenergic terminals. Upon activation of NMDA receptors on GABAergic terminals, GABA is released and inhibits the release of SRIF. CGP36742 (SGS742) prevents this GABAergic inhibition, allowing the neuropeptide to reach the sst_5 receptors on NA terminals. These SRIF receptors trigger potentiation of coexisting NMDAR receptors through the phospholipase C (PLC)-inositol trisphosphate (IP_3)-protein kinase C (PKC) pathway (see Pittaluga et al. 2005). Figure redrawn from Raiteri (2006).

illustrated in Figure 3 in which SRIF and GABA are shown as cotransmitters whose release can be inhibited through $GABA_B$ autoreceptors exhibiting high affinity (ha) and low affinity (la) for CGP36742/SGS742, respectively. It has to be noted that CGP36742/SGS742 is the first $GABA_BR$ antagonist entered into clinical trials as a possible treatment of mild cognitive impairments and, more recently, of more severe conditions such as Alzheimer's disease (Froestl et al. 2004).

Thus the evidence for pharmacologically distinct subtypes of the presynaptic $GABA_BR$ derived from release studies with superfused synaptosomes appears to at least equate with other receptor systems which can boast the chrism of molecular biology evidence. No doubt, from the data reported in Table 1, the existence of subtypes of the $GABA_BR$ seems quite obvious to a pharmacologist, with strong similarities between human and rat receptors.

4.3 Structural Heterogeneity Does not Reflect Pharmacological Heterogeneity

As a rule, pharmacological heterogeneity is based on structural diversities between the proteins representing receptor subtypes. This seems not to be the case for $GABA_B$ receptors, however, whose pharmacological subtypes are likely to originate through novel, unconventional, and yet unexplained strategies. The mystery of $GABA_BR$ subtypes lies in the so far unsuccessful attempts to identify subunits other than $GABA_{B1}$ and $GABA_{B2}$ and the increasing belief that $GABA_{B1}$ and $GABA_{B2}$ exclusively form all $GABA_B$ receptors. Having assumed such a peculiar structural uniqueness of the heterodimeric $GABA_B$ receptor, the origin of the observed striking pharmacological heterogeneity of release-regulating presynaptic $GABA_BRs$ remains a matter of speculation.

When it became clear that probably all $GABA_BRs$ in the CNS brain are the sole products of the $GABA_{B1}$ and $GABA_{B2}$ genes, much attention focused on subunit variants as a potential source of the pharmacological heterogeneity observed with native $GABA_BRs$. In general, mixing and matching of isoforms did not lead to $GABA_BRs$ with distinct pharmacological profiles; only in some cases, differences were reported that remain, however, highly controversial (see Bettler et al. 2004, for references).

The most investigated $GABA_BR$ isoforms are $GABA_{B1(a)}$ and $GABA_{B1(b)}$, which differ in their extracellular domain (Kaupmann et al. 1998a). These two isoforms are the most abundant variants and the only variants highly conserved among different species (see Bettler et al. 2004). At the NH_2 terminus, the first 147 amino acids of the $GABA_{B1(a)}$ isoform are replaced in $GABA_{B1(b)}$ with a sequence of 18 amino acid residues. Alternative NH_2 termini are rather exceptional and do not occur in other families of GPCRs. One major difference between $GABA_{B1(a)}$ and $GABA_{B1(b)}$ consists in the presence of a pair of sushi repeats (see Figure 4) in the $GABA_{B1(a)}$-specific domain (Bettler et al. 1998; Hawrot et al. 1998). Sushi repeats, also known as short consensus repeats, were originally observed in complement proteins and proposed to take part in protein-protein interactions (Blein et al. 2004). Of note, sushi repeats have been rarely, if any, observed in neurotransmitter receptors, adding one more peculiarity to the $GABA_B$ receptor.

The review article of Bettler et al. (2004) reports numerous studies indicating that $GABA_{B1(a)}$ and $GABA_{B1(b)}$ exhibit differences in their spatial and temporal expression patterns. Although these spatial and temporal differences in the expression of the $GABA_{B1(a)}$ and $GABA_{B1(b)}$ subunits are consistent with their separate transcriptional regulation and suggest distinct functional roles, they have so far not been of help in explaining the heterogeneous pharmacology of $GABA_BRs$. Several additional $GABA_{B1}$ isoforms $(GABA_{B1(c\text{-}g)})$ were reported; however, in contrast to the $GABA_{B1(a)}$ and $GABA_{B1(b)}$ variants, the existence of a protein *in vivo* has not yet been demonstrated for any of the $GABA_{B1(c\text{-}g)}$ splice variants. Finally $GABA_{B2}$ splice variants do not seem to occur (Bettler et al. 2004).

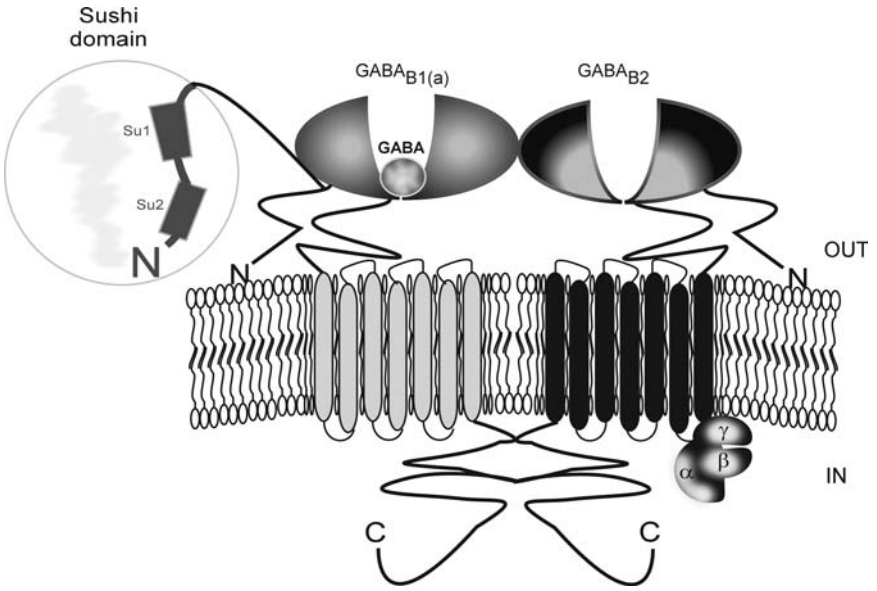

Fig. 4 Schematic representation of the $GABA_{B1(a)}$-$GABA_{B2}$ heterodimer. The receptor represented contains the 1a variant of the $GABA_{B1}$ subunit. The 1a variant differs from the 1b variant because it contains in the N terminus an additional 120-residue-long sequence consisting of two sushi domains (Su1 and Su2). The sushi domains can interact with a variety of proteins, not only extracellular but also present in the plasma membrane. Such interactions might be neuron-specific and induce conformational changes in the GABA binding site of the $GABA_{B1}$ subunit able to determine binding selectivity towards ligand antagonists and, consequently, "pharmacological" heterogeneity of $GABA_B$ receptors.

If the only prominent molecular diversity in the $GABA_BR$ system resides in the two variants $GABA_{B1(a)}$ and $GABA_{B1(b)}$, it seems justified to concentrate on these variants in efforts aimed to find out if these structural diversities could originate functional and pharmacological $GABA_BR$ heterogeneity. Accordingly, it was recently thought that a genetic approach, i.e., producing mice with selective ablations of the $GABA_{B1(a)}$ or $GABA_{B1(b)}$ subunit to dissociate the native functions of the two subunits, could have been fruitful.

4.4 Structural and Functional Diversity Between Presynaptic and Postsynaptic $GABA_B$ *Receptors*

Two elegant studies (Pérez-Garci et al. 2006; Vigot et al. 2006) have demonstrated for the first time that $GABA_{B1(a)}$ and $GABA_{B1(b)}$ show strategically distinct subcellular localization and physiological function and may represent the starting point on which robust working hypothesis aimed to understand $GABA_BR$ diversity can be constructed.

Bettler's group (Vigot et al. 2006) used a combined genetic, physiological, and morphological approach to demonstrate that $GABA_{B1}$ isoforms localize to distinct synaptic sites and convey separate functions in the hippocampus *in vivo*. These authors generated homozygous mice with mutations in the $GABA_{B1(a)}$ (referred to as $1a^{-/-}$ mice) or $GABA_{B1(b)}$ ($1b^{-/-}$ mice). Using whole-cell patch-clamp recording in slice preparations, Vigot et al. (2006) first checked for the presence of $GABA_B$ heteroreceptors on excitatory terminals. Baclofen was effective in reducing the excitatory postsynaptic currents in CA1 pyramidal neurons induced by stimulation of the Schaffer collateral-commissural fibers, both in wild-type and $1b^{-/-}$ mice, but not in $1a^{-/-}$ mice. This indicates that $1a^{-/-}$ mice, in contrast to $1b^{-/-}$ mice, lack $GABA_B$ presynaptic heteroreceptors on Schaffer collateral terminals, consistent with the idea that heteroreceptors on glutamate terminals in wild-type animals are $GABA_{B1(a)}$-$GABA_{B2}$ receptors.

The study of Vigot et al. (2006) was then extended to the $GABA_B$ autoreceptors on GABAergic terminals by recording inhibitory postsynaptic currents in CA1 pyramidal neurons. Baclofen reduced the amplitude of the currents in the neurons of all genotypes, suggesting that both $GABA_{B1(a)}$ and $GABA_{B1(b)}$ can efficiently participate in autoreceptor function. Whether GABAergic terminals possess autoreceptors assembled with $GABA_{B1(a)}$ or $GABA_{B1(b)}$ or with both subunits remains to be clarified.

Postsynaptic $GABA_B$Rs induce a late inhibitory postsynaptic current by activating Kir3-type K^+ channels (Lüscher et al. 1997). In CA1 pyramidal cells, baclofen induced similar outward currents in $1a^{-/-}$ and wild-type mice; however, in cells of $1b^{-/-}$ mice, the effect of baclofen was reduced by $\sim 60\%$ compared to wild-type or $1a^{-/-}$ mice, indicating that postsynaptic inhibition is predominantly mediated by the $GABA_{B1(b)}$ isoform. Electron microscopy observation in the CA1 stratum radiatum revealed a synaptic distribution of $GABA_{B1}$ isoforms in agreement with the observed functional differences, with $GABA_{B1(b)}$ mostly found on dendritic spines in $1a^{-/-}$ mice, whereas, in $1b^{-/-}$ mice, $GABA_{B1(a)}$ was predominantly localized to axon terminals. Finally, Vigot et al. (2006) showed that transfected CA3 neurons selectively express $GABA_{B1(a)}$ in distal axons, suggesting that the sushi repeats specify heteroreceptor localization of $GABA_B$Rs on presynaptic nerve terminals.

In an accompanying report, Pérez-Garci et al. (2006) used the knockout mice generated according to Vigot et al. (2006) to perform experiments with L5 pyramidal neurons in slices of the primary somatosensory cortex. The authors conclude that postsynaptic GABA-mediated inhibition of Ca^{2+} spikes involves direct blockade of dendritic Ca^{2+} channels and is mediated by $GABA_{B1(b)}$ containing $GABA_B$Rs. On the other hand, presynaptic inhibition of GABA release was absent in $1a^{-/-}$ but not $1b^{-/-}$ mice, suggesting that the $GABA_{B1(a)}$ subunit exclusively makes up the presynaptic autoreceptors in the inhibitory terminals of the circuit studied by Pérez-Garci et al. (2006).

The findings that the $GABA_{B1(a)}$ isoform, which possesses the pair of sushi repeats, a conserved protein interaction motif, assembles presynaptic auto- and heteroreceptors, may have important implications. In fact, the pair of sushi repeats in

$GABA_{B1(a)}$ exhibit different structural properties that would favor interactions with multiple auxiliary proteins (Blein et al. 2004), thus generating $GABA_B$ receptor heterogeneity.

4.5 Is Pharmacological Heterogeneity Limited to Presynaptic $GABA_B$ *Receptors?*

The two recent studies by Vigot et al. (2006) and Pérez-Garci et al. (2006) provide convincing evidence that $GABA_{B1(a)}$ and $GABA_{B1(b)}$ isoforms localize to distinct synaptic sites, i.e., presynaptic and postsynaptic, respectively, and convey separate functions, i.e., $GABA_B$ autoreceptor and heteroreceptor-mediated inhibition of GABA and glutamate exocytosis and $GABA_B$R-mediated postsynaptic inhibition. However, there is no mention in these reports of $GABA_B$R pharmacological diversity, which remains therefore an unresolved question.

The literature on $GABA_B$Rs includes several reports regarding the existence of pharmacological receptor subtypes (see reviews by Bonanno and Raiteri 1993a; Bowery 1993, 2006; Mott and Lewis 1994; Kerr and Ong 1995; Malcangio and Bowery 1996; Marshall et al. 1999; Bowery and Enna 2000; Bowery et al. 2002; Calver et al. 2002; Bettler et al. 2004; Couve et al. 2004; Nicoll 2004; Raiteri 2006). The results obtained with various experimental approaches (ligand binding with brain homogenates, electrophysiology, tests with cloned transfected receptors, transmitter release from brain preparations) are either negative or far from conclusive and are often contradictory. In my opinion, one observation deserves particular attention: the only way to clearly show the existence of pharmacologically distinct functional $GABA_B$Rs seems to be monitoring release from superfused synaptosomes (Raiteri et al. 1974; Raiteri and Raiteri 2000), a technique best suited to study presynaptic events and that has been successful in identifying pharmacological subtypes of a variety of receptors for which structural diversity has also been demonstrated by molecular biology.

According to Vigot et al. (2006) and Pérez-Garci et al. (2006), $GABA_{B1(a)}$ assembles release-regulating presynaptic receptors at axon terminals; since only $GABA_{B1(a)}$ possesses the sushi repeats, synaptosomes would represent a subcellular fraction in which $GABA_B$Rs with sushi repeats are particularly concentrated. On the other hand, nerve terminals of different neuronal families are likely to express on their plasma membrane neuron-specific proteins (transporters and so on) which might interact differentially at the sushi repeat sites of the unique $GABA_{B1(a)}$-$GABA_{B2}$ heterodimer expressed on the terminal axons. Such interactions might induce in the cleft of the $GABA_{B1(a)}$ Venus Flytrap module neuron-specific conformational changes able to affect the binding characteristics of $GABA_B$R ligands, especially of antagonists, although changes in the heptahelical domains leading to differential binding of $GABA_B$R allosteric modulators (Chen et al. 2006) can not be ruled out. Such a scenario would be compatible with the existence of $GABA_B$R subtypes with distinct location (terminals of different neurons), function (inhibition of release of different

transmitters), and pharmacology (selective blockade by different antagonists) as summarized in some review articles (Bonanno and Raiteri 1993a; Bowery et al. 2002; Raiteri 2006).

If this were the case, the $GABA_BR$ heterogeneity could be largely, though not exclusively, a presynaptic phenomenon compatible with the idea that presynaptic regulation of transmitter release is a major function of $GABA_BRs$. Postsynaptic $GABA_BRs$ mostly contain the $GABA_{B1(b)}$ isoform, which lacks the sushi repeats necessary to bind the neuron-specific proteins that would make the receptors pharmacologically distinct. Considering that, in general, the density of receptors sited on soma and dendrites is much higher than that of the receptors sited on terminal boutons, the presence of pharmacologically distinct presynaptic $GABA_BRs$ could be obscured by the abundant population of identical postsynaptic $GABA_BRs$ during experiments of binding with brain tissue homogenates, which in fact rarely showed signs of pharmacological heterogeneity. Similarly, significant pharmacological differences should not be expected from experiments with cloned heterodimeric receptors expressed in cells unable to produce the neuron-specific proteins able to bind at the sushi repeats (see for references, Bettler et al. 2004) and generate pharmacologically distinct $GABA_BRs$.

5 Conclusions and Future Implications

The autoreceptor modulation of glutamate release from presynaptic terminals is very complex, but since glutamate is the major excitatory transmitter in the mammalian CNS, a thorough understanding of how glutamate exocytosis is regulated is of fundamental importance. While group III mGluR7 appear to be autoreceptors relatively well characterized, group I autoreceptors, although the most studied, remain less well understood. Group I mGluRs localized on glutamatergic terminals may on the one side participate in physiological processes of synaptic plasticity; on the other side, inappropriate facilitation of autoreceptor-mediated glutamate release may contribute to a number of pathological states, including ischemia, epilepsy, and neurodegenerative disorders. Given the relevance of these implications, the definition of the relative contributions of the two group I mGluR subtypes to the facilitation of glutamate release, still a matter of debate (Moroni et al. 1998; Sistiaga et al. 1998; Reid et al. 1999; Thomas et al. 2000; Fazal et al. 2003; Rodrigues et al. 2005), is of critical importance.

The investigation on the mGluR subtypes present as presynaptic heteroreceptors on quantitatively minor families of nerve endings, but able to modulate the exocytosis of transmitters involved in important physiopathological conditions, is still in its infancy. Due to the limitations imposed to the immunocytochemical approaches by the low density of most nerve ending families, major advances regarding presynaptic heteroreceptors regulating exocytosis release of classical transmitters and neuropeptides will continue to originate, in my opinion, from functional studies of release.

Opposite to glutamate, the autoreceptor system of the other major CNS transmitter, GABA, appears surprisingly simple: one, and only one, heterodimeric metabotropic receptor, termed $GABA_B$, would subserve the function of inhibiting GABA exocytosis throughout the CNS. Even more impressive, this very same $GABA_B$ heterodimer would regulate the release of tens of transmitters as an heteroreceptor located on the corresponding families of releasing nerve terminals. In the next few years we will probably show that this cannot be the case, after having unveiled the tricks used by the $GABA_B$ receptor to play as an extraordinary quick-change actor.

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Presynaptic Neuropeptide Receptors

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Abstract Presynaptic receptors for four families of neuropeptides will be discussed: opioids, neuropeptide Y, adrenocorticotropic hormone (ACTH), and orexins. Presynaptic receptors for the opioids (μ, δ, κ, and ORL_1) and neuropeptide Y (Y_2) inhibit transmitter release from a variety of neurones, both in the peripheral and central nervous systems. These receptors, which were also identified in human tissue, are coupled to $G_{i/o}$ proteins and block voltage-dependent Ca^{2+} channels, activate voltage-dependent K^+ channels, and/or interfere with the vesicle release machinery. Presynaptic receptors for ACTH (MC_2 receptors) have so far been identified almost exclusively in cardiovascular tissues from rabbits, where they facilitate noradrenaline release; they are coupled to G_s protein and act via stimulation of adenylyl cyclase. Presynaptic receptors for orexins (most probably OX_2 receptors) have so far almost exclusively been identified in the rat and mouse brain, where they facilitate the release of glutamate and γ-aminobutyric acid (GABA); they are most probably linked to G_q and directly activate the vesicle release machinery or act via a transduction mechanism upstream of the release process. Agonists and antagonists at opioid receptors owe at least part of their therapeutic effects to actions on presynaptic receptors. Therapeutic drugs targeting neuropeptide Y and orexin receptors and presynaptic ACTH receptors so far are not available.

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1 Introduction

One reason to describe presynaptic neuropeptide receptors in a separate chapter is the chemistry of their endogenous ligands – peptides with up to about 50 amino acids. However, peptides have more properties that discriminate them from endogenous ligands at other types of presynaptic receptors. First, peptide transmitters, unlike transmitters of low molecular weight (e.g., noradrenaline, acetylcholine, GABA, or glutamate), are not formed in the axon terminals but are synthetized in the perikaryon of the neurone and are transported to the axon terminals; they are stored there in separate vesicles characterized by a large diameter and a dense core. Second, peptide transmitters frequently are cotransmitters, which are released in addition to one or even two low-molecular-weight transmitter(s). Third, endogenous peptides do not act via ionotropic receptors as opposed to many small transmitters, e.g., GABA (via $GABA_{A/C}$ receptors), glutamate (via NMDA or AMPA receptors), acetylcholine (via nicotinic receptors), or serotonin (via 5-HT_3 receptors). In other words, peptide transmitters are slower to act than some transmitters of low molecular weight.

There are dozens of presynaptic receptors for endogenous peptides. This review will focus on receptors for four peptides or peptide families: the opioid peptides, neuropeptide Y and related peptides, adrenocorticotropic hormone (corticotropin, ACTH), and the orexins. This choice is representative, since part of the receptors inhibit and part of them facilitate transmitter release; moreover, the receptors under consideration are coupled to the major G proteins, namely $G_{i/o}$, G_s and G_q (Table 1).

Table 1 Synopsis of Receptors Activated by the Four Peptide Families

Peptide Family	Endogenous Ligands	Receptors		G Protein
		Total	With Presynaptic Location	
Opioid	Endorphins, enkephalins, dynorphins, nociceptin	μ, δ, κ, ORL_1	μ, δ, κ, ORL_1	$G_{i/o}$
Neuropeptide Y	Neuropeptide Y, peptide YY, pancreatic polypeptide	Y_1, Y_2, Y_3, Y_5, Y_6[1]	Y_2	$G_{i/o}$
Melanocortin	ACTH, α-, β-, γ-melanocyte-stimulating hormone	MC_1, MC_2, MC_3, MC_4, MC_5	MC_2	G_s
Orexin	Orexin A and B	OX_1, OX_2	OX_2?	G_q

[1] This subtype is functional in the mouse only.

This review is based mainly on four types of experiments in which exocytotic, vesicular, ATP-dependent transmitter release was determined directly or indirectly. Other types of transmitter release including "basal" release or carrier-mediated release will not be considered. (1) In superfusion studies transmitter release is determined directly (as "overflow"). (2) In other studies on isolated tissues transmitter release is not determined directly but via the endorgan response. (3) In electrophysiological studies, spontaneously occurring or electrically evoked currents or potentials elicited by the transmitter are studied at the postsynaptic membrane. (4) A small part of the experiments stem from pithed animals in which the noradrenaline spillover or the increase in blood pressure or heart rate elicited by electrical sympathetic nerve stimulation is studied. Apart from experiments on pithed animals, in vivo studies were usually not considered in this review since their interpretation bears difficulties. In the studies under (2), (3) and (4), control experiments were carried out in which direct effects of the drug under study at the postsynaptic receptor were excluded. In the electrophysiological studies a special type of experiment was performed to demonstrate presynaptic receptor location: spontaneously occurring currents or potentials were determined in the presence of tetrodotoxin in order to block impulse flow along the axon. If the drug affects the frequency of the remaining currents or potentials without affecting their amplitude, a presynaptic site of action can be assumed. Although transmitter release was not determined directly in numerous of the studies considered here, the term "transmitter release" will be used for this type of investigation as well.

For each of the four presynaptic receptors or receptor families, the occurrence in the autonomic and/or central nervous system will be described. Next, the signal transduction will be considered. Subsequently we will discuss their physiological role and their possible role in disease and therapy.

2 Presynaptic Opioid Receptors

Although an example of a presynaptic opioid receptor, activated by morphine, was discovered 90 years ago (Trendelenburg 1917), the endogenous peptides acting on opioid receptors have been described only since 1975 (Figure 1). There are four types of opioid receptors, termed μ, δ, and κ opioid and opioid receptor-like$_1$ (ORL_1). Although other nomenclatures have been proposed (e.g., OP_3, OP_1, OP_2 and OP_4, respectively; Alexander et al. 2006), the traditional designations will be used in the present review. The term "opioid receptors" will be used in a broad sense covering not only the opioid receptors *sensu stricto* (μ, δ, and κ) but also the ORL_1 receptor. There are at least four types of endogenous peptides acting on opioid receptors: endorphins, enkephalins, dynorphins, and nociceptin. These peptides are not synthetized as such but are cleaved from precursors (e.g., proopiomelanocortin), which in turn have been processed from pre-precursors (e.g., pre-proopiomelanocortin). ß-Endorphin activates μ, the enkephalins activate δ, and the dynorphins activate κ opioid receptors, whereas nociceptin activates

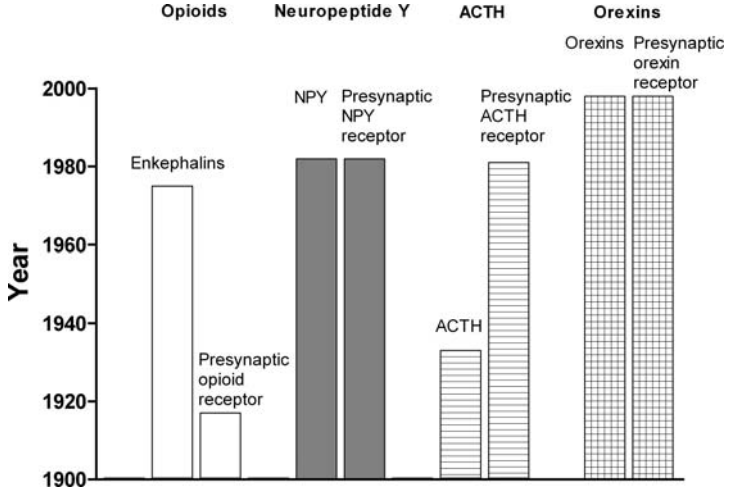

Fig. 1 First description of the four peptides (or peptide families) and their respective presynaptic receptors. The papers are (from left to right): Trendelenburg (1917); Hughes et al. (1975); Tatemoto et al. (1982); Allen et al. (1982); Collip et al. (1933); Göthert (1981); de Lecea et al. (1998); van den Pol et al. (1998).

ORL_1 receptors. Although nociceptin is selective for ORL_1 receptors, the other three peptides show a marked degree of promiscuity with regard to the classical opioid receptors (μ, δ, and κ). Endorphins occur in the brain (almost exclusively in neurones which have their somata in the infundibular nucleus) and in the adenohypophysis. Enkephalins are found on many sites in the CNS but also occur in the adrenal medulla and in the gut wall. Dynorphins are also found in the CNS and in the gut (for review, see Gutstein and Akil 2006).

Figure 2 gives an example how presynaptic opioid receptors can be identified in a superfusion model. We examined the effect of subtype-selective agonists on noradrenaline release in mouse brain cortex slices. Figure 2a shows that noradrenaline release was inhibited by a μ and an ORL_1 receptor agonist but was not affected by high concentrations of a δ and κ opioid receptor agonist. In further experiments we showed that the effects of the μ agonist (Figure 2b) and of the ORL_1 agonist (Figure 2c) indeed involve μ and ORL_1 receptors since the concentration-response curves were shifted to the right by antagonists with apparent pA_2 values close to their potency values in other functional studies for each receptor subtype (Guerrini et al. 1998; Berger et al. 2006).

Using superfusion experiments, electrophysiological techniques, pithed animal preparations, and experiments in which transmitter release was determined indirectly via the end-organ response (e.g., twitch response of vas deferens preparations), numerous presynaptic opioid receptors have been identified (Table 2). For the identification of the receptors, classical drug tools were used; for future studies, knockout mice (now available for each of the four opioid receptor subtypes) and special nucleotides (e.g., antisense oligodeoxynucleotides or short interfering

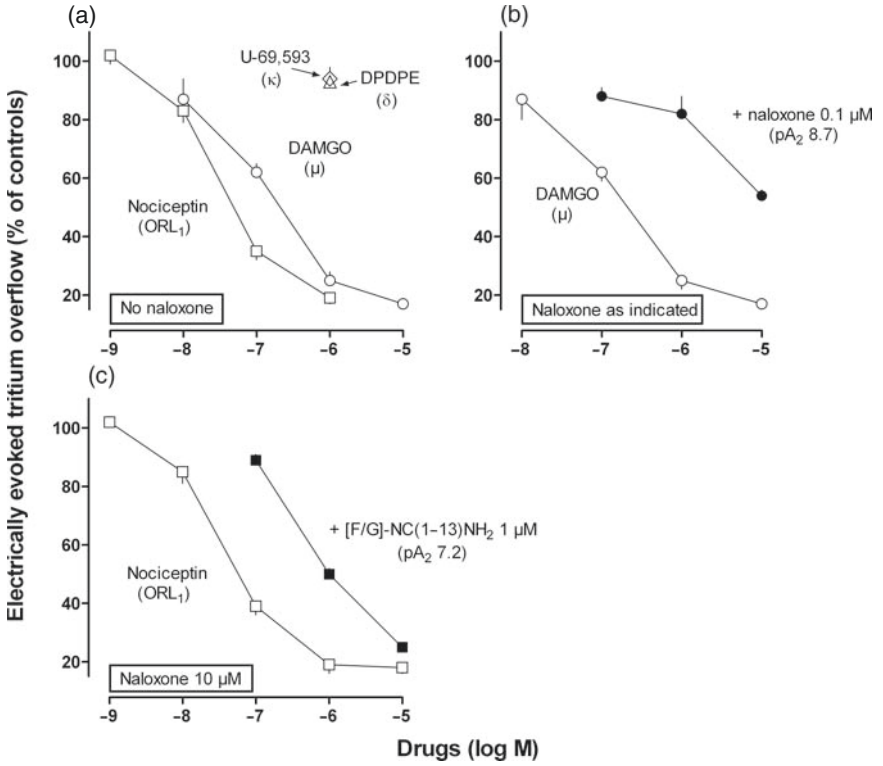

Fig. 2 Effect of opioid receptor agonists on the electrically (0.3 Hz) evoked tritium overflow from superfused mouse brain cortex slices preincubated with ^{3}H-noradrenaline. The evoked overflow represents quasi-physiological exocytotic noradrenaline release. (**a**) shows that noradrenaline release was inhibited by agonists at the μ and the ORL_1 receptor but not affected by high concentrations of a δ and κ receptor agonist. The effect of DAMGO in fact involved μ receptors since it was antagonized by a low concentration of naloxone, which possesses some preference for μ over δ, κ and ORL_1 receptors (**b**). On the other hand, the effect of nociceptin was indeed related to the activation of ORL_1 receptors since it was antagonized by the ORL_1 antagonist [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]-nociceptin(1–13)$NH_2$ ([F/G]-NC(1–13)NH_2) ([F/G]-NC(1–13)NH_2) (**c**). Data with nociceptin and its antagonist from Schlicker et al. (1998; redrawn); other data published in abstract form only (Schlicker and Kathmann 2000). Similar data were published by Trendelenburg et al. (2000).

RNA) are promising new approaches. In all examples listed in Table 2 transmitter release was inhibited. Each of the four opioid receptor subtypes can serve as an inhibitory presynaptic receptor (Table 1), and each has been identified also in human tissue (Table 2). In a few papers (not further considered here) a facilitatory rather than inhibitory effect of opioids on transmitter release was reported. In such cases, the possibility has to be considered that the opioid receptor was located presynaptically at an inhibitory interneurone projecting to the neurone under study (and not at the latter itself).

Table 2 Synopsis of presynaptic inhibitory opioid receptors

	μ opioid receptors			
	Transmitter	Tissue	Species	References
Sympathetic nervous system	*preganglionic* Acetylcholine	Superior cervical ganglion	Cat, rabbit	2
		Hypogastric ganglion	Mouse	Rogers and Henderson 1990
		Superior and inferior mesenteric ganglion	Guinea-pig	2
	postganglionic Noradrenaline	Tail artery	Rat	1
		Nictitating membrane	Cat	1
		Colon	Guinea-pig	1
		Vas deferens	Rat, mouse	1
Parasympathetic/ enteric nervous system	Acetylcholine	Heart	Rabbit	2
		Lung	Rat	Yu et al. 2006
		Ciliary ganglion	Chick	Endo and Yawo 2000
		Small intestine myenteric plexus	Guinea pig	Cherubini and North 1985
		Ileum	Guinea-pig, rat	2, Storr et al. 2002
		Colon	Cat	2
		Gall bladder	Guinea-pig	Guarraci et al. 2002
Central nervous system	Noradrenaline	Cerebral cortex, hippocampus, cerebellum	Guinea-pig	3
		Cerebral cortex, hippocampus, amygdala, nucleus tractus solitarii, periaqueductal grey, cerebellum	Rat	3
		Cerebral cortex	Mouse	see Fig. 2
	Dopamine	Striatum	Rat	Schlosser et al. 1995
	Serotonin	Cerebral cortex	Human	Berger et al. 2006
		Cerebral cortex, hippocampus	Rat	3, Berger et al. 2006
	Acetylcholine	Nucleus accumbens, olfactory tubercle, hippocampus, amygdala	Rat	3
	Glutamate	Cerebral cortex	Rat	Ostermeier et al. 2000
		Amygdala	Rat	*Zhu and Pan 2005**
		Arcuate nucleus, ventromedial hypothalamus	Rat	Emmerson and Miller 1999
		Subthalamic nucleus	Rat	Shen and Johnson 2002
		Hypothalamus (supraoptic nucleus)	Rat, mouse	*Liu et al. 1999*; *Honda et al. 2004*
		Periaqueductal grey	Rat	Vaughan and Christie 1997
		Raphe pallidus	Rat	Bouryi and Lewis 2004
		Dorsal motor nucleus	Rat	Browning et al. 2002
		Nucleus tractus solitarii	Rat	Glatzer and Smith 2005
		Rostral ventrolateral medulla	Rat	*Hayar and Guyenet 1998*
		Spinal cord	Rat	Glaum et al. 1994

(*Continued*)

Table 2 Continued

	μ opioid receptors			
	Transmitter	Tissue	Species	References
	GABA	Hippocampus	Rat	Capogna et al. 1993
		Amygdala	Rat	*Finnegan et al. 2005; 2006*
		Globus pallidus	Rat	Stanford and Cooper 1999
		Hypothalamus (supraoptic nucleus)	Mouse	*Honda et al. 2004*
		Subthalamic nucleus	Rat	Shen and Johnson 2002
		Ventral tegmental area	Rat	Bergevin et al. 2002
		Locus coeruleus	Rat	*Pan et al. 2004*
		Periaqueductal gray	Rat	Kishimoto et al. 2001
		Rostral ventrolateral medulla	Rat	Hayar and Guyenet 1998
		Spinal cord (substantia gelatinosa)	Rat	Grudt and Henderson 1998
		Spinal cord (dorsal horn)	Rat	*Kerchner and Zhuo 2002*
	Glycine	Spinal cord (substantia gelatinosa)	Rat	*Grudt and Henderson 1998*
	Substance P	Hypothalamus, spinal cord	Cat	3, 4
		Spinal cord	Rat	Aimone and Yaksh 1989
	ß-Endorphin, dynorphin	Hypothalamus	Rat	Nikolarakis et al. 1989
	Cholecystokinin	Hypothalamus	Cat	3, 4
	δ opioid receptors			
	Transmitter	Tissue	Species	References
Sympathetic nervous system	*preganglionic* Acetylcholine	Hypogastric ganglion	Mouse	Rogers and Henderson 1990
	postganglionic Noradrenaline	Atrium	Guinea-pig, mouse	1
		Arteries and veins	Rabbit	1
		Colon	Guinea-pig	1
		Spleen	Cat	1
		Vas deferens	Hamster, rat, mouse	1
		Cultured sympathetic neurones	Chicken	1
Parasympathetic nervous system	Acetylcholine	Heart	Rabbit	2
		Ciliary ganglion	Chick	Endo and Yawo 2000
		Ileum	Mouse	2
		Colon	Cat	2
		Gall bladder	Guinea-pig	Guarraci et al. 2002
		Sacral colonic ganglia	Cat	2

(*Continued*)

Table 2 Continued

	δ opioid receptors			
	Transmitter	Tissue	Species	References
Central nervous system	Noradrenaline	Cerebral cortex	Human	Berger et al. 2006
	Dopamine	Striatum	Rat	Schlosser et al. 1995
	Serotonin	Hippocampus	Rat	3
	Acetylcholine	Striatum, nucleus accumbens, olfactory tubercle	Rat	3
	Glutamate	Cerebral cortex	Rat	Ostermeier et al. 2000
		Raphe pallidus	Rat	Bouryi and Lewis 2004
		Spinal cord	Rat	*Glaum et al. 1994*
	GABA	Globus pallidus	Rat	*Stanford and Cooper 1999*
		Subthalamic nucleus	Rat	Shen and Johnson 2002
		Locus coeruleus	Rat	Pan et al. 2002
	Substance P	Hypothalamus, spinal cord	Cat	3, 4
		Spinal cord	Rat	3, 4, Aimone and Yaksh 1989
	Metenkephalin, ß-endorphin, dynorphin	Hypothalamus	Rat	Nikolarakis et al. 1989
	κ opioid receptors			
	Transmitter	Tissue	Species	References
Sympathetic nervous system	Noradrenaline	Heart	Rabbit, guinea-pig	1
		Vasculature	Rabbit	1
		Colon	Guinea-pig	1
		Vas deferens	Rabbit, mouse	1
		Cultured sympathetic neurones	Chicken	1
Parasympathetic nervous system	Acetylcholine	Small intestine (myenteric plexus)	Guinea-pig	Cherubini and North 1985
		Ileum	Guinea-pig, mouse	2
		Colon	Cat	2
		Gall bladder	Guinea-pig	Guarraci et al. 2002
Sensory neurones	Calcitonin gene-related peptide	Skin	Rat	Averbeck et al. 2001
Central nervous system	Noradrenaline	Cerebral cortex, hippocampus, cerebellum	Guinea-pig	3

(*Continued*)

Table 2 Continued

	κ opioid receptors			
	Transmitter	Tissue	Species	References
	Dopamine	Striatum, frontal cortex, olfactory tubercle, nucleus accumbens, mediobasal hypothalamus, amygdala	Rat	3
		Striatum	Cat, guinea-pig	3
	Serotonin	Cerebral cortex	Human, rat	Berger et al. 2006
		Superior colliculus	Rabbit	3
	Histamine	Cerebral cortex	Rat	3
	Acetylcholine	Cerebral cortex	Human	Feuerstein et al. 1996
	Glutamate	Hippocampus	Guinea-pig	Gannon and Terrian 1991; Simmons et al. 1994
		Hypothalamus (arcuate nucleus)	Rat	*Emmerson and Miller 1999*
		Nucleus accumbens (shell)	Rat	*Hjelmstadt and Fields 2001*
		Rostral ventromedial medulla	Rat	Ackley et al. 2001
	GABA	Globus pallidus	Rat	Ogura and Kita 2000
	Glycine	Nucleus ambiguus	Rat	*Wang et al. 2004*
	Metenkephalin	Brainstem	Rat	Ueda et al. 1987
	Dynorphin	Hippocampus	Guinea-pig	Gannon and Terrian 1991
		Hypothalamus	Rat	Nikolarakis et al. 1989
	ORL_1 receptors			
	Transmitter	Tissue	Species	References
Sympathetic nervous system	Noradrenaline	Heart	Rat	1
		Atrium	Mouse	1
		Tail artery	Rat	1
		Resistance vessels	Rat	1
		Anococcygeus muscle	Rat	1
		Vas deferens	Rabbit, rat mouse	1
Parasympathetic/enteric nervous system	Acetylcholine	Small intestine (myenteric plexus)	Guinea-pig	Nicholson et al. 1998; Liu et al. 2001
	Non-adrenergic-non-cholinergic transmitter	Small intestine (myenteric plexus)	Guinea-pig	Nicholson et al. 1998

(*Continued*)

Table 2 Continued

	ORL_1 receptors			
	Transmitter	Tissue	Species	References
Central nervous system	Noradrenaline	Cerebral cortex, cerebellum, hippocampus, hypothalamus	Mouse	5
		Cerebral cortex	Human	Rominger et al. 2002
	Serotonin	Cerebral cortex	Human, rat, mouse	5; Berger et al. 2006
	Glutamate	Hippocampus	Rat	Yu et al. 1997
		Hypothalamus (arcuate nucleus)	Rat	Emmerson and Miller 1999
		Periaqueductal grey	Rat	*Vaughan et al. 1997*
	GABA	Periaqueductal grey	Rat	*Vaughan et al. 1997*

1 Reviewed by Boehm and Kubista (2002)
2 Reviewed by Illes (1989)
3 Reviewed by Mulder and Schoffelmeer (1993)
4 Reviewed by Jackisch (1991)
5 Reviewed by Schlicker and Morari (2000)
* In the studies given in *italics* opioids decreased postsynaptic currents or potentials also in the presence of tetrodotoxin.

Presynaptic opioid receptors were found in all parts of the autonomic nervous system (sympathetic, parasympathetic, and enteric) and on many sites of the central nervous system. The release of more than 10 transmitters can be inhibited via opioid receptors. The latter serve as presynaptic heteroreceptors in most instances, i.e., the opioid modulating release differs from the transmitter subject to modulation. However, there are also examples of presynaptic opioid autoreceptors. For example, in the study of Nikolarakis et al. (1989) on rat hypothalamus slices, antagonists of the μ, δ, and κ opioid receptor increased the release of endogenous opioids, suggesting that the latter decreased their own release. Another example of an autoreceptor has been shown in the guinea pig hippocampus, where the release of dynorphin (which mainly activates κ opioid receptors) was inhibited by the κ opioid receptor agonist U-69,593 (Gannon and Terrian 1991). With respect to the distribution of presynaptic opioid receptors, marked species differences exist. For example, noradrenaline release in the cerebral cortex is inhibited via δ and ORL_1 receptors in humans, via κ and ORL_1 receptors in the guinea pig, and via μ and ORL_1 receptors in the rat and mouse (Table 2, Figure 2). These examples show that there are nerve terminals in which more than one opioid receptor can be identified. Transmitter release from the postganglionic sympathetic neurone in the mouse vas deferens is even subject to inhibition via each of the four opioid subtypes (Table 2).

Which mechanisms are involved in the opioid receptor-mediated inhibition of transmitter release? In general, the four types of opioid receptor are $G_{i/o}$ protein-coupled receptors (Alexander et al. 2006), but theoretically another transduction pathway might exist for presynaptic opioid receptors. However, the coupling of presynaptic opioid receptors to $G_{i/o}$ (labelled with **1** in Figure 3) has been

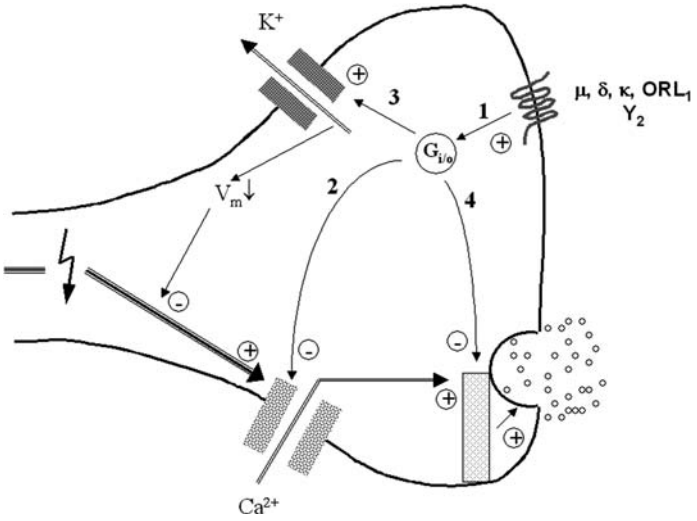

Fig. 3 Mechanisms involved in the opioid (μ, δ, κ, ORL_1) and neuropeptide Y (Y_2) receptor-mediated inhibition of exocytotic transmitter release. Following activation of the respective receptor and $G_{i/o}$ (**1**), three signal transduction pathways are possible, namely inhibition of voltage-dependent Ca^{2+} channels (**2**), opening of K^+ channels (**3**), and a direct inhibitory effect on the vesicle release machinery (**4**). *Glossary*: $->$ - leading to; $=>$ - ion flux; $\equiv>$ - action potential; V_m - membrane potential; "(+)" and "(−)" mean stimulatory and inhibitory effect, respectively.

demonstrated in several studies, using pertussis toxin or the somewhat less selective agent N-ethylmaleimide. In this manner, coupling to $G_{i/o}$ has been shown (1) for presynaptic μ opioid receptors inhibiting the release of noradrenaline in rat and guinea pig hippocampus (Werling et al. 1989) and of GABA in rat hippocampus (Capogna et al. 1993) and periaqueductal grey (Kishimoto et al. 2001; Hahm et al. 2004) and (2) for presynaptic κ opioid receptors inhibiting noradrenaline release in rabbit hippocampus (Allgaier et al. 1989).

In order to better understand how the events downstream of G protein activation are influenced by opioid receptors, one should briefly recall the major steps of the electrosecretory coupling. When an action potential invades the axon terminal, voltage-dependent Ca^{2+} channels open. The Ca^{2+} ions entering the axoplasma finally lead to fusion of vesicles with the plasma membrane and release of the transmitter into the synaptic cleft. Ca^{2+} influx is crucially dependent on the membrane potential; e.g., hyperpolarization will impair Ca^{2+} influx. Hyperpolarization can be elicited by the efflux of K^+ ions. In general, $G_{i/o}$-coupled receptors can decrease Ca^{2+} influx via voltage-dependent Ca^{2+} channels (labelled with **2** in Figure 3), increase K^+ efflux via voltage-dependent K^+ channels (**3** in Figure 3), and/or directly interfere with the release process (**4** in Figure 3) (Miller 1998). Each of the three mechanisms, alone or in combination, has been reported to play a role in opioid receptor-mediated inhibition of transmitter release. To show the involvement of ion channels, (more or less) selective drug tools have been used. The direct influence on the release machinery has been shown in electrophysiological studies in

which impulse flow was abolished by tetrodotoxin or Ca^{2+} influx through voltage-dependent Ca^{2+} channels was blocked by appropriate agents. If a drug inhibits the frequency of postsynaptic currents or potentials under this scenario, a direct interference with the release process can be assumed (Miller 1998).

Voltage-dependent *Ca^{2+} channels* play a role in the μ receptor-mediated inhibition of noradrenaline release in the rat cerebral cortex (Mulder and Schoffelmeer 1993), of glutamate release in the chick ciliary ganglion (Endo and Yawo 2000), and of GABA release in the rat striatum (Barral et al. 2003). Voltage-dependent *K^+ channels* are involved in the μ receptor-mediated inhibition of the release of glutamate in the rat subthalamic nucleus (Shen and Johnson 2002) and periaqueductal grey (Vaughan and Christie 1997) and of GABA in the rat striatum (Barral et al. 2003), amygdala (Finnegan et al. 2006), subthalamic nucleus (Shen and Johnson 2002), ventral tegmental area (Bergevin et al. 2002), and periaqueductal grey (Vaughan and Christie 1997). They are also involved in the κ receptor-mediated inhibition of glutamate release in the guinea pig dentate gyrus (Simmons et al. 1994). A *direct effect on the release machinery* has been shown for each of the four opioid receptors in at least one region of the central nervous system, and both for fast excitatory (glutamate) and inhibitory transmission (GABA, glycine) (references given in italics in Table 2).

Are the presynaptic opioid receptors described here activated by endogenous opioids? If so, one would expect that an opioid receptor antagonist like naloxone would alter transmitter release in the direction opposite to that elicited by opioid receptor agonists, i.e., would increase transmitter release. In most experimental models, an effect of naloxone per se has not been found, suggesting that the corresponding opioid receptors are not subject to an endogenous tone (listed in the reviews by Starke 1977; Jackisch 1991; Boehm and Kubista 2002). In a series of models, however, naloxone did increase transmitter release. This has been shown for the opioid receptors (most probably μ, see Table 2) modulating noradrenaline release in the rat cerebral cortex (Taube et al. 1976) and for the opioid receptors modulating acetylcholine release in the guinea pig thalamus (Beani et al. 1982). It has also been shown for the μ and δ opioid receptors inhibiting substance P release in the cat spinal cord (Go and Yaksh 1987) and the δ opioid receptors inhibiting acetylcholine release in the cat parasympathetic colonic ganglia (Kennedy and Krier 1987) and the rat striatum (Sándor et al. 1991). Using subtype-selective opioid receptor antagonists, an endogenous tone has furthermore been shown for the μ, δ, and κ opioid autoreceptors causing inhibition of ß-endorphin, metenkephalin, and dynorphin release in the rat hypothalamus (Nikolarakis et al. 1989), for the μ and κ opioid receptors inhibiting noradrenaline and acetylcholine release in the guinea pig myenteric plexus (Cosentino et al. 1995), for the κ opioid autoreceptors inhibiting metenkephalin in the rat brainstem (Ueda et al. 1987), and for the ORL_1 receptors inhibiting noradrenaline release in the rat anococcygeus muscle (Ho et al. 2000) and serotonin release in the human cerebral cortex (Berger et al. 2006). In a final study on human cerebral cortex (Feuerstein et al. 1996), an endogenous tone at δ opioid receptors was shown to be likely. This is suggested by the fact that the selective δ opioid receptor antagonist naltrindole facilitated acetylcholine release in the presence (but

not absence) of a cocktail of peptidase inhibitors and that the cocktail per se inhibited acetylcholine release. The simplest explanation is that the concentration of endogenous opioids in the synaptic cleft normally is relatively low but can reach a critical level if degradation is blocked. (The situation in the study is complicated since the δ opioid receptor is not located at the cholinergic axon terminals but on an unidentified interneurone; Feuerstein et al. 1996.)

The observation that an endogenous tone at presynaptic opioid receptors is rarely detectable fits well to the fact that an endogenous tone is rare in opioid receptor models in general (see Table 21-2 in the review by Gutstein and Akil 2006). Endogenous opioid systems come into play under stressful situations. For example, the slight euphoria occurring after physical exercise or the suppression of pain that sometimes occurs in severely injured persons is believed to be caused by a marked increase in the release of endogenous opioids (Gutstein and Akil 2006).

Drugs acting at opioid receptors are among the oldest and continue to be among the most important medicines. There is no doubt that at least some targets for their desired, as well as undesired, effects are presynaptic receptors. This holds true for analgesia. Presynaptic μ receptors involved in the analgesic effect of, e.g., morphine are located both spinally and supraspinally. Spinal presynaptic μ opioid receptors inhibit the release of transmitters like glutamate, substance P, and calcitonin gene-related peptide from the terminals of the primary afferent neurones conveying pain from the periphery to the central nervous system (Zöllner and Stein 2007). One supraspinal site is in the periaqueductal grey, from where the descending pain-suppressing tract originates. This tract is under the control of tonically active GABAergic neurones, the axon terminals of which are equipped with presynaptic μ receptors. Activation of these receptors increases the pain-suppressing effect of the descending pathway (Vaughan and Christie 1997). Finally, presynaptic μ opioid receptors on tonically active GABAergic interneurones in the ventral tegmental area are associated with the rewarding properties of opioids. These GABAergic interneurones synapse with dopaminergic neurones projecting to the nucleus accumbens; activation of the presynaptic μ receptors leads to an increase in dopamine release and, hence, euphoria (Bergevin et al. 2002).

3 Presynaptic Neuropeptide Y Receptors

Neuropeptide Y and its presynaptic receptors were discovered 25 years ago (Figure 1). There are four subtypes of neuropeptideY receptors, Y_1, Y_2, Y_4 and Y_5 (Table 1, Alexander et al. 2006); a fifth subtype, Y_6, is functional in the mouse, whereas in primates the related gene is nonfunctional due to a frame-shift mutation (Alexander et al. 2006). Neuropeptide Y receptors are also activated by another two peptides, peptide YY (PYY) and pancreatic polypeptide (PP). The three peptides are not synthetized as such but are processed from larger precursors via two steps, i.e., an initial cleavage of the N-terminal signal peptide and the subsequent cleavage of the C-terminal part (Tatemoto 2004). The affinity of PYY is very similar to that

of neuropeptide Y at each of the five receptor subtypes; PP has about the same affinity as neuropeptide Y (or PYY) at Y_5 receptors, a lower affinity at Y_1, Y_2 and Y_6 receptors and a higher affinity at Y_4 receptors (Alexander et al. 2006). Neuropeptide Y is a neurotransmitter in sympathetic, parasympathetic, enteric, and sensory neurones and in neurones of the CNS. PYY and PP are hormones; the former is stored in the pancreas and the latter in the terminal intestine (Brain and Cox 2006).

Figure 4 shows how a presynaptic neuropeptide Y receptor was identified in a superfusion model. In mouse brain cortex slices, serotonin release was concentration-dependently inhibited by neuropeptide Y and this effect was potently mimicked by neuropeptide Y-(13-36). Since the latter is selective for Y_2 over Y_1 and Y_5 receptors (Alexander et al. 2006) one may conclude that neuropeptide Y acts via Y_2 receptors.

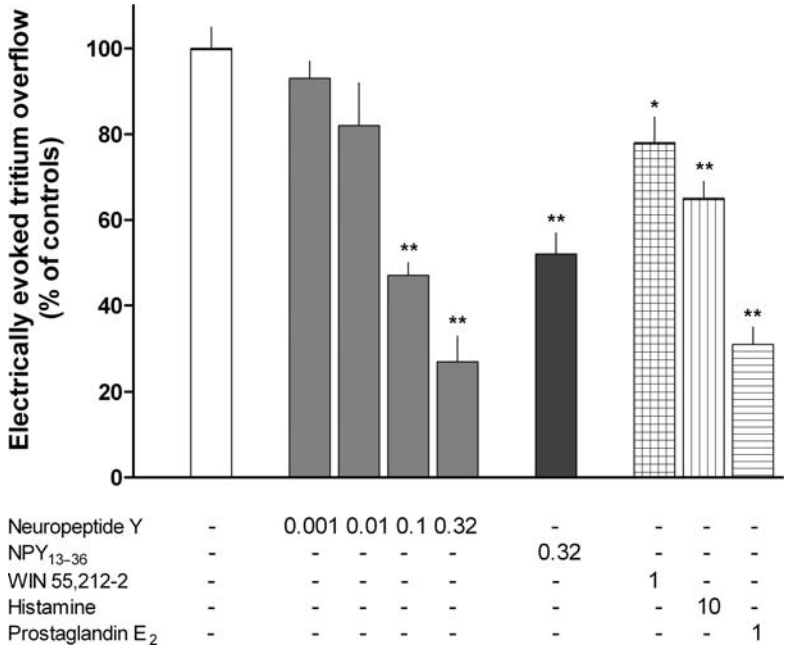

Fig. 4 Effect of various peptides and nonpeptides on the electrically (3 Hz) evoked tritium overflow from superfused mouse brain cortex slices preincubated with ^{3}H-serotonin. The evoked overflow represents quasi-physiological exocytotic serotonin release. In all experiments, serotonin autoreceptors were blocked by metitepine. The figure shows that human neuropeptide Y concentration-dependently inhibited serotonin release and that this effect was mimicked by human neuropeptide Y (13–36) ($NPY_{13–36}$), which has a high affinity for Y_2 but a very low affinity for Y_1 receptors. These results are compatible with the view that neuropeptide Y acts via Y_2 receptors in the present model. For the sake of comparison, the figure also shows the inhibitory effects of another three agonists, acting via cannabinoid CB_1, histamine H_3 and prostaglandin EP_3 receptors and used at concentrations causing the maximum or near-maximum effect at their respective receptors. Drug concentrations in μM. $^*P < 0.05$, $^{**}P < 0.003$, compared to the control (from Nakazi et al. 2000 and Nakazi 2001; redrawn).

Figure 4 also shows that the extent of inhibition obtained with neuropeptide Y was very marked and comparable to that obtained with prostaglandin E_2 (acting via EP_3 receptors), which was examined for the sake of comparison. The maximum inhibition obtained with WIN 55,212-2 (acting via cannabinoid CB_1 receptors) and histamine (acting via H_3 receptors) was far lower.

Many examples of inhibitory presynaptic neuropeptide Y receptors have been found in tissues from experimental animals and humans (Table 3), based on experiments with each of the four techniques described in the Introduction (overflow experiments, electrophysiological techniques, pithed animals, electrically induced twitches in isolated tissues). For the identification of the receptors, the three peptides and modified (e.g., truncated) congeners (Figure 4) were the only tools for many years. More recently, selective antagonists (e.g., in the study by Schwertfeger et al. [2004] on the human heart) and knockout mice (e.g., in the study by Smith-White et al. [2002] on the mouse heart) have been used. According to most studies, only Y_2 receptors serve as presynaptic neuropeptide Y receptors.

Presynaptic neuropeptide Y receptors have been identified in the sympathetic, parasympathetic and enteric nervous system, on sensory neurones, and on many sites in the CNS; they inhibit the release of 11 different transmitters (Table 3). Most presynaptic neuropeptide Y receptors are heteroreceptors, but some serve as autoreceptors, both in the sympathetic nervous system (pig spleen and kidney) and the brain (rat hypothalamus) (Table 3). Marked species differences occur. For example, Y_2 receptors inhibit the sympathetically mediated tachycardia, the vagally mediated bradycardia, and the vagally mediated bronchoconstriction in the guinea pig but not in the rabbit (Serone et al. 1999; Abrahamsson 2000). Moreover, Y_2 receptors inhibit noradrenaline release in the guinea pig carotid but not femoral artery, and conversely inhibit noradrenaline release in the rat femoral but not carotid artery (Potter and Tripovic 2006).

Like opioid receptors, neuropeptide Y receptors are $G_{i/o}$ protein-coupled, and for this reason the marked similarities in signal transduction are not surprising. The coupling to $G_{i/o}$ was proven for the presynaptic Y_2 receptors inhibiting the substance P-mediated plasma extravasation in the rat dura mater (Yu and Moskowitz 1996) and the release of noradrenaline in the rat pineal gland (Simmoneaux et al. 1994) (step **1** in Figure 3). With respect to the downstream pathways, a coupling to voltage-dependent Ca^{2+} channels (**2** in Figure 3) has been shown for the presynaptic neuropeptide Y receptors inhibiting noradrenaline release in the dog splenic artery (Yang and Chiba 2002), glutamate release in the rat hippocampus (Klapstein and Colmers 1992; Qian et al. 1997), and GABA release in the rat thalamus (Sun et al. 2001). Relatively little information is available on whether presynaptic neuropeptide Y receptors can couple to voltage-dependent K^+ channels (step **3** in Figure 3). This may hold true for the neuropeptide Y receptor involved in inhibition of ATP release in the mouse vas deferens (Stjärne et al. 1989). For three presynaptic neuropeptide Y receptors in the rat spinal cord a direct effect on the vesicle release machinery (step **4** in Figure 3) can be assumed, since agonists inhibited the frequency of the miniature inhibitory or excitatory postsynaptic currents (Moran et al. 2004; italics in Table 3).

Table 3 Synopsis of presynaptic neuropeptide Y receptors

	Transmitter	Tissue	Species	References
Sympathetic nervous system	Noradrenaline	Heart	Human, guinea-pig	1
		Submandibular artery	Human	1
		Splenic artery	Dog	Yang and Chiba 2002
		Gracilis muscle vasculature	Dog	1
		Ear artery	Rabbit	1
		Carotid artery	Guinea-pig	Potter and Tripovic 2006
		Mesenteric, renal, femoral, basilar artery, resistance vessels	Rat	1; Potter and Tripovic 2006
		Saphenous vein	Dog	1
		Portal vein	Rat	1
		Kidney	Human, pig, rabbit	1
		Spleen	Pig	1
		Urinary bladder	Guinea-pig	1
		Vas deferens	Rat, mouse	1
		Uterus	Rat	1
		Oviduct	Rabbit	1
	ATP	Vas deferens	Guinea-pig, mouse	1
	Neuropeptide Y	Spleen	Pig	Modin et al. 1994
		Kidney	Pig	1
Para-sympathetic nervous system	Acetylcholine	Heart	Human	Schwertfeger et al. 2004
		Heart	Dog, guinea-pig, rat, mouse	1; Abrahamsson 2000
		Bronchial arterioles	Dog	Mahns et al. 1998
		Nasal vessels	Dog, cat	1
		Trachea, bronchial muscle	Guinea-pig	Abrahamsson 2000; 1
		Uterus	Rat	1
Sensory nerves	Substance P	Dura mater vasculature	Rat	Yu and Moskowitz 1996
	Calcitonin-gene-related peptide	Mesenteric artery	Rat	1
		Small intestine	Guinea-pig	1
CNS	Noradrenaline	Cerebral cortex, hippo-campus, hypothalamus, pineal gland, medulla oblongata	Rat	1
	Dopamine	Striatum	Rat	1
	Serotonin	Cerebral cortex	Rat, mouse	1; Nakazi et al. 2000; Nakazi 2001
	Glutamate	Cerebral cortex	Rat	Wang 2005
		Olfactory bulb	Rat	Blakemore et al. 2006
		Hippocampus	Rat	1
		Striatum	Rat	1

(*Continued*)

Table 3 Continued

	Transmitter	Tissue	Species	References
		Hypothalamus	Rat, mouse	Rhim et al. 1997; Fu et al. 2004
		Spinal cord	Rat	*Moran et al. 2004*[*]
	GABA	Thalamus	Rat	1
		Hypothalamus	Rat	1
		Spinal cord	Rat	*Moran et al. 2004*
	Glycine	Spinal cord	Rat	*Moran et al. 2004*
	Neuropeptide Y	Hypothalamus	Rat	1

1 Reviewed by Westfall (2004)
[*]In this study neuropeptide Y decreased the frequency of postsynaptic currents also in the presence of tetrodotoxin.

To address the question of whether presynaptic neuropeptide Y receptors are also activated by endogenous neuropeptide Y or related peptides, the selective Y_2 receptor antagonist BIIE 0246 was used. Based on these experiments, an endogenous tone can be assumed for the presynaptic Y_2 receptors inhibiting noradrenaline release in the perfused mesenteric arterial bed of the rat (Westfall 2004), noradrenaline and ATP release in the dog splenic artery (Yang and Chiba 2002), and the release of neuropeptide Y itself in the rat hypothalamus (King et al. 2000). The number of tonically activated presynaptic Y_2 receptors may be much higher since many sites were examined earlier than 1999 when BIIE 0246 became available.

There is now good evidence for a role of presynaptic neuropeptide Y receptors in disease states. As to the cardiovascular system, plasma neuropeptide Y levels are slightly increased in hypertensive patients and to a greater extent in cardiac failure (Morris 2004). An increase in neuropeptide Y levels is even an important prognostic marker for cardiovascular death in hemodialysis patients (Odar-Cederlof et al. 2003). The exact role played by the presynaptic neuropeptide Y receptor, however, cannot easily be pinpointed for at least two reasons. First, presynaptic inhibitory Y_2 receptors occur both on sympathetic and parasympathetic nerve endings of the human heart (Table 3); i.e., they inhibit two opposing systems. Second, within the sympathetic nervous system postsynaptic neuropeptide Y receptors (Y_1) lead to vasoconstriction and increase the effect elicited by other vasoconstrictors (Morris 2004), whereas presynaptic Y_2 receptors inhibit the release of noradrenaline, ATP, and neuropeptide and thereby impair vasoconstriction.

The pathophysiological role played by another presynaptic Y_2 receptor is better understood. In humans suffering from temporal lobe epilepsy (with Ammon's horn sclerosis) and in rats with chemically induced epilepsy, the density of Y_2 receptors on hippocampal glutamatergic mossy fibers is greatly increased. The increase is believed to be a protective mechanism against epileptic seizures since the Y_2 receptor-mediated inhibition of the release of the excitatory transmitter glutamate is also enhanced (Vezzani and Sperk 2004). In an electrophysiological study glutamate release from the mossy fibers was inhibited by exogenous neuropeptide Y in epileptic but not in control rats. In the epileptic animals there was even a tonic inhibition by

endogenous neuropeptide Y since the Y_2 antagonist BIIE 0246 increased glutamate release (Tu et al. 2005).

Clinical trials with neuropeptide Y receptor ligands have so far not been successful (Brain and Cox 2006).

4 Presynaptic ACTH Receptors

Although ACTH has been known since the 1930s, presynaptic ACTH receptors were identified much later (Figure 1) and have attracted the attention of investigators for only a few years. ACTH consists of 39 amino acid residues and is processed from proopiomelanocortin, i.e., the same precursor molecule giving rise to ß-endorphin (see section 2). Besides ACTH and endogenous opioids, a series of melanocyte-stimulating hormones are formed from proopiomelanocortin (Table 1). The latter activate MC_1, MC_3, MC_4 and MC_5 receptors. ACTH activates the MC_2 receptor (i.e., the receptor involved in its corticotropic effect) but also has a high affinity for some of the other melanocortin receptors (Gantz and Fong 2003; Alexander et al. 2006). ACTH is not only one of the hormones of the adenohypophysis but serves also as a neurotransmitter in hypothalamic neurones (Bloch et al. 1979; Bugnon et al. 1979).

Unlike the opioids and neuropeptide Y, ACTH increases transmitter release. As shown in superfusion studies and in pithed and anesthetized animal preparations, ACTH increases noradrenaline release in cardiovascular tissues from the rabbit; in an electrophysiological study it increased acetylcholine release in frog skeletal muscle (Table 4). Thus, according to our present knowledge, presynaptic ACTH receptors occur as heteroreceptors only. No effect of ACTH on noradrenaline release was found in rat atrium and rat and guinea pig pulmonary artery (Costa and Majewski 1988). Human tissues have not been examined. The studies summarized in Table 4 are based on experiments with the full-length ACTH molecule and its active sequence $ACTH_{1–24}$. The effect of ACTH was not mimicked by $ACTH_{4–10}$, a

Table 4 Synopsis of presynaptic facilitatory ACTH receptors

	Transmitter	Tissue	Species	References
Sympathetic nervous system	Noradrenaline	Heart	Rabbit	Costa and Majewski 1988; Szabo et al. 1988
		Aorta	Rabbit	Göthert 1984
		Pulmonary artery	Rabbit	Göthert 1981; 1984; Göthert and Hentrich 1984; Costa and Majewski 1988
		Various sympathetically innervated tissues	Rabbit	Szabo et al. 1987; 1989
Motor nerves	Acetylcholine	Cutaneous pectoris and sartorius muscle	Frog	Johnston et al. 1983

behaviorally active peptide devoid of a corticotropic effect, and was antagonized by an appropriate concentration of the ACTH receptor antagonist $ACTH_{7–38}$ (Göthert 1981). The data suggest that the facilitatory effect is mediated via the MC_2 receptor.

In general, the MC_2 receptor is G_s protein-coupled (Table 1), and two studies in the rabbit pulmonary artery indicate that this is also true for the presynaptic receptor. The evidence is, indirect, however, in that it suggests activation of adenylyl cyclase, the typical transduction step downstream from G_s. In the study by Göthert and Hentrich (1984), the facilitatory effect of ACTH was increased by simultaneous administration of forskolin, an activator of the catalytic subunit of adenylyl cyclase, and AH 21–132, a phosphodiesterase inhibitor. In the study by Costa and Majewski (1988), the facilitatory effect of ACTH was occluded when the vessel was superfused with a lipid-soluble cAMP analogue.

The presynaptic MC_2 receptor plays no physiological role, since the concentration of ACTH found in the blood is far too low for its activation. This view is supported by the finding that the antagonist $ACTH_{7–38}$, when given alone, did not affect noradrenaline release in the rabbit heart (Szabo et al. 1989) or pulmonary artery (Göthert 1981). The situation may change under pathophysiological conditions when ACTH concentrations are increased. ACTH may then increase noradrenaline release in cardiovascular tissues and help to maintain cardiovascular function (Szabo et al. 1989). However, at presynaptically effective concentrations ACTH has additional postsynaptic cardiovascular effects: vasodilation and an increase in heart rate (Szabo et al. 1987; 1989).

Although $ACTH_{1–24}$ is used for diagnostic purposes and for the treatment of epilepsies in babies and infants, drugs specifically targeting *presynaptic* MC_2 receptors are not available.

5 Presynaptic Orexin Receptors

Orexins (also known as hypocretins) and presynaptic receptors activated by orexins were first described in 1998 (Figure 1). Orexin-A (hypocretin-1) and orexin-B (hypocretin-2) consist of 33 and 28 amino acid residues, respectively, and are derived from a common precursor molecule (prepro-orexin). They act on two receptors, OX_1 and OX_2. Orexinergic neurones have their perikarya in the lateral and posterior part of the hypothalamus and project to many parts of the brain, including the cerebral cortex, thalamus, limbic system, locus coeruleus and raphe nuclei, and to the spinal cord. Orexin-like immunoreactive neurones also occur in the small intestine (for review, see Smart and Jerman 2002).

Orexins increase transmitter release. This has been shown in electrophysiological studies for acetylcholine release in the myenteric plexus of the ileum and for GABA and glutamate release in a series of locations of the central nervous system (Table 5); in other words, presynaptic orexin receptors serve as heteroreceptors. Experiments on human tissue are so far lacking. In some of the experimental models listed in Table 5 orexin-A and orexin-B were studied and orexin-B was at least as

Table 5 Synopsis of presynaptic facilitatory orexin receptors

	Transmitter	Tissue	Species	References
Parasympathetic nervous system	Acetylcholine	Ileum	Guinea-pig	Katayama et al. 2003; 2005
Central nervous system	GABA	Medial hypothalamus	Rat	*van den Pol et al. 1998**
		Dorsal vagal complex	Rat	*Davis et al. 2003*
	Glutamate	Prefrontal cortex	Rat	Lambe and Aghajanian 2003
		Medial hypothalamus	Rat	*van den Pol et al. 1998*
		Lateral hypothalamus	Mouse	*Li et al. 2002*
		Laterodorsal tegmentum	Mouse	Burlet et al. 2002
		Caudal nucleus tractus solitarii	Rat	*Smith et al. 2002*

*In the studies given in *italics* orexins facilitated postsynaptic currents or potentials also in the presence of tetrodotoxin.

potent as orexin-A (Burlet et al. 2002; Li et al. 2002; Davis et al. 2003; Lambe and Aghajanian 2003; Katayama et al. 2005). Since both orexins are equally potent at OX_2 receptors, whereas orexin-A is 10 times more potent than orexin-B at OX_1 receptors (Alexander et al. 2006), the presynaptic effects of orexins may involve OX_2 receptors or perhaps a mixture of OX_1 and OX_2. For final proof, selective antagonists would be necessary, but OX_2 receptor antagonists are so far not available and the OX_1 receptor antagonists have a relatively weak potency; the agonist peptide [Ala^{11}, D-Leu^{15}] orexin-B, which possesses a high preference for OX_2 receptors, may be helpful (Alexander et al. 2006). Experiments with knockout mice would be valuable as well.

Since the orexin receptors are G_q protein-coupled (Alexander et al. 2006), one may assume that this also holds true for the presynaptic orexin receptor(s), but so far no data are available. Nonetheless, the six studies carried out in central nervous preparations permit some conclusions on the post-G protein mechanisms. In all instances, the orexins increased the frequency of spontaneous inhibitory or excitatory postsynaptic potentials or currents. The results differed, however, with respect to the influence of tetrodotoxin. In the medial and lateral hypothalamus (van den Pol et al. 1998; Li et al. 2002), dorsal vagal complex (Davis et al. 2003), and caudal nucleus tractus solitarii (Smith et al. 2002), orexins increased the frequency of the miniature potentials or currents also in the presence of tetrodotoxin, suggesting that they directly influenced the vesicle release machinery (references in italics in Table 5). On the other hand, in the prefrontal cortex (Lambe and Aghajanian 2003) and laterodorsal tegmentum (Burlet et al. 2002), the orexins did not retain their facilitatory effect in the presence of tetrodotoxin, suggesting an effect further upstream e.g., on Ca^{2+} and/or K^+ channels.

The physiological and pathophysiological role of presynaptic orexin receptors is incompletely understood, and drugs targeting orexin receptors so far have not been developed. Orexins play an important role in the control of sleep and wakefulness, as highlighted by the findings that the knockout of preproorexin in mice (Chemelli

et al. 1999) or a mutation of the OX_2 gene in dogs (Lin et al. 1999) produces a disturbance resembling narcolepsy in humans. Indeed a decreased number of orexin neurones was found in humans suffering from that disease (Thannickal et al. 2000). There are two presynaptic facilitatory sites that may be involved in the control of sleep and wakefulness by the orexins. The first one is on glutamatergic neurones in the laterodorsal tegmentum, and the second one is on glutamatergic neurones in the prefrontal cortex (i.e., at the final synapse in the ascending arousal pathway). One may assume that the increase in glutamate release excites ascending cholinergic neurones involved in the arousal reaction in the first instance (Burlet et al. 2002; Table 5) and glutamatergic output neurones in the second model (Lambe and Aghajanian 2003; Table 5).

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Presynaptic Modulation by Endocannabinoids

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Abstract Modulation of neurotransmitter release by G-protein-coupled receptors (GPCRs) is a prominent presynaptic mechanism for regulation of synaptic transmission. Activation of GPCRs located at the presynaptic terminal can decrease the probability of neurotransmitter release. This presynaptic depression involves activation

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of Gi/o-type G-proteins that mediate different inhibitory mechanisms, including inhibition of voltage-gated calcium channels, activation of potassium channels, and direct inhibition of the vesicle fusion process. A variety of neurotransmitters and modulatory agents can activate GPCRs that produce presynaptic depression. Among these are lipid metabolites that serve as agonists for GPCRs. The discovery of endocannabinoids and their cognate receptors, including the CB1 receptor, has stimulated intense investigation into the neurophysiological roles of these lipid metabolites. It is now clear that presynaptic depression is the major physiological role for the CB1 receptor. Endocannabinoids activate this receptor mainly via a retrograde signaling process in which these compounds are synthesized in and released from postsynaptic neuronal elements, and travel back to the presynaptic terminal to act on the CB1 receptor. This retrograde endocannabinoid modulation has been implicated in short-term synaptic depression, including suppression of excitatory or inhibitory transmission induced by postsynaptic depolarization and transient synaptic depression induced by activation of postsynaptic GPCRs during agonist treatment or synaptic activation. Endocannabinoids and the CB1 receptor also play a key role in one form of long-term synaptic depression (LTD) that involves a long-lasting decrease in neurotransmitter release. Endocannabinoid-dependent synaptic modulation and plasticity have now been implicated in a variety of brain processes, including learning and memory. The present chapter discusses our current knowledge about the cellular and molecular mechanisms involved in endocannabinoid-mediated presynaptic modulation and endocannabinoid-dependent LTD.

1 Introduction

Presynaptic neurotransmitter release is crucial to the function of chemical synapses, as it provides a rapid surge of the chemical needed to activate postsynaptic processes that insure effective intercellular communication. Neurotransmitter release is subject to control from a variety of molecules, as the chapters in this volume will attest. One prominent source of such control is modulation by presynaptic G protein-coupled receptors (GPCRs) that inhibit transmitter release. A variety of receptors for a host of neuromodulatory agents have been identified (see Dunwiddie and Lovinger 1993; Miller 1998; Wu and Saggau 1997 for review). Such inhibition can be either homosynaptic, inhibition of release of a neurotransmitter by receptors for that same transmitter, or heterosynaptic, inhibition of release of a neurotransmitter by receptors for a second neurotransmitter. These neuromodulatory mechanisms allow for control of the amount and timing of neurotransmitter release. Ultimately this form of presynaptic modulation can regulate the pattern and timing of activity in neural circuits. Presynaptic GPCRs are also targets for therapeutic agents. For example, morphine and other opioid drugs produce their analgesic actions, at least in part, through activation of presynaptic opiate receptors (see Reisine and Pasternak 1996). Baclofen, an agonist at presynaptic GABAB receptors, is effective in treatment of spasticity and other neuromuscular disorders (c.f. Orsnes et al. 2000). The therapeutic efficacy of these compounds likely stems from the fact that they

can produce subtle adjustments in neurotransmitter release without fully blocking synaptic transmission.

There are several common features of GPCR-mediated presynaptic depression. First, the receptors mediating such effects are coupled to Gi/o-type G-proteins that are known to engage intracellular signaling pathways, including inhibition of adenylyl cyclase, inhibition of voltage-gated calcium channels, and activation of potassium channels (Dunwiddie and Lovinger 1993; Miller 1998). Second, receptors and downstream signaling can be activated on a subsecond timescale, providing rapid feedback for intercellular alterations in secretion (Mintz and Bean 1993; Heinbockel et al. 2005). Third, regulation of intracellular calcium, due mainly to inhibition of the aforementioned calcium channels, appears to play a prominent role in receptor-mediated decreases in neurotransmitter release (Wu and Saggau 1997). Given the strong calcium sensitivity of excitation/secretion coupling, regulation of presynaptic calcium levels is certainly an attractive mechanism for presynaptic modulation. Fourth, presynaptic receptors have been shown to inhibit neurotransmitter release via mechanisms downstream from calcium channel activity, and likely independent of ion channel activity (see Dunwiddie and Lovinger 1993; Wu and Saggau 1997 for review). Receptor-mediated alterations in vesicle exocytosis have been proposed to account for these effects. The preponderance of data suggests that inhibition of voltage-gated calcium channels and inhibition of exocytosis are the major mechanisms through which GPCRs inhibit transmitter release (Wu and Saggau 1997).

The many neurotransmitters and modulatory agents involved in GPCR-mediated presynaptic depression appear to come from a variety of cellular sources. Traditional neurotransmitters and many neuropeptides are released from presynaptic terminals to act on GPCRs on their own terminals or on those of nearby neurons (Dunwiddie and Lovinger 1993; Miller 1998). Neuromodulators such as adenosine and glutamate might be released from glia as well as neurons (Volterra and Meldolesi 2005), and the constant presence of low levels of adenosine is thought to have a "tonic" modulatory effect at brain presynaptic terminals (Dunwiddie and Masino 2001). Yet other substances appear to be made in, or released from, postsynaptic neuronal elements and can act on presynaptic terminals via signaling in a "retrograde" fashion (Stanton et al. 2004). Among the molecules thought to serve such retrograde signaling roles are adenosine (Brundege and Dunwiddie 1996), integrin proteins (Sargent Jones 2005), small gaseous molecules (Kyrozis et al. 2004), neurotrophins (Bramham and Messaoudi 2004), and various lipid metabolites (Feinmark et al. 2003; Chevaleyre et al. 2006). Signaling by certain members of this latter class of compounds is the subject of this chapter.

Long-lasting decreases in the efficacy of synaptic transmission (so-called long-term depression, or LTD) are observed at synapses throughout the brain (Malenka and Bear 2004). The initiation of LTD often begins with repetitive activation of a given set of synapses, and LTD is expressed as decreased synaptic function at the same set of synapses or another synaptic input impinging on the same postsynaptic neuron (homosynaptic versus heterosynaptic LTD, respectively). The maintenance of certain subtypes of LTD appears to involve decreased presynaptic

neurotransmitter release (Choi and Lovinger 1997a,b; Feinmark et al. 2003; Castellucci et al. 1978; Mato et al. 2005). Presynaptic GPCRs, including receptors for glutamate and opioid peptides, have been implicated in presynaptically expressed LTD (Kahn et al. 2001; Kobayashi et al. 1996; Weisskopf et al. 1993).

In this chapter, the roles of cannabinoid-type GPCRs in regulating neurotransmitter release wiil be discussed. In addition, the role of retrograde endocannabinoid signaling and cannabinoid receptors in short-term and long-term synaptic depression will be discussed. This latter line of research has its origins in the discovery of the phenomena termed depolarization-induced suppression of excitation and inhibition (DSE and DSI, respectively). These forms of short-lasting synaptic depression were first discovered in the early 1990s at synapses in the cerebellum and hippocampus (Llano et al. 1991; Pitler and Alger 1992). The experimental paradigm for eliciting DSE/I starts with depolarization of a postsynaptic neuron. This depolarization leads to a decrease in excitatory glutamatergic or inhibitory GABAergic transmission onto that same neuron. The subsequent discovery that retrograde signaling involving endocannabinoids and their cognate receptors is necessary for DSE and DSI will be discussed in detail later in the chapter. A short time after the characterization of DSE and DSI, investigators studying long-term synaptic plasticity in the striatum observed a form of LTD that was later found to involve postsynaptic induction and presynaptic expression mechanisms (Calabresi et al. 1992a; Lovinger et al. 1993; Walsh 1993; Choi et al. 1997a,b). Endocannabinoid retrograde signaling and cannabinoid receptor activation play key roles in this and related forms of LTD, and this long-lasting endocannabinoid-dependent plasticity will be discussed and compared with DSE/I and other shorter-lasting endocannabinoid actions. Prior to discussing endocannabinoid-dependent synaptic plasticity it is necessary to review our current knowledge of endocannabinoids and their receptors.

2 Endocannabinoids and their Receptors

2.1 Endocannabinoid Synthesis and Degradation

The so-called endocannabinoids are metabolites of membrane lipids that are part of a host of modified lipid and fatty acid molecules that have now been implicated in intercellular signaling across a wide spectrum of biological organisms. Within the mammalian body, paracrine effects of lipid metabolites are widespread (see Bazan 2005 for review). The endocannabinoids were so named because of their ability to activate the GPCRs known to be targets for the active ingredient, Δ9-tetrahydrocannabinol, in marijuana, hashish, and other drugs generated from *Cannabis sativa*. Two metabolites of arachidonic acid-containing phospholipids, arachidonoyl ethanolamide (AEA or anandamide) and 2-arachidonoyl glycerol (2-AG) are the most prominent endocannabinoids in the brain and periphery.

Anandamide was the first endocannabinoid to be discovered (Devane et al. 1992). This compound can be formed by transacylation of phosphatidylinositol and subsequent degradation by the so-called N-acetyl-phospatidylethanolamine phospholipase D (NAPE-PLD) (Di Marzo et al. 1994; Schmid et al. 1990), as well as via a newly characterized pathway involving phospholipase C (PLC) and dephosphorylation mediated by protein tyrosine phosphatase N22 (PTPN22) and possibly other phosphatases (Liu et al. 2006). It should be noted that AEA is only one of a class of fatty acid ethanolamides, including oleaoyl ethanolamide (OEA) and palmitoylethanolamide (PEA) made from lipids containing other fatty acid moieties (Bachur et al. 1965). These other FA ethanolamides do not act on the CB1 receptor, and the molecular targets of these compounds are just beginning to be elucidated (LoVerme et al. 2005a,b). Both OEA and PEA are substrates for the AEA catabolic enzyme fatty acid amide hydrolase (FAAH), and it is possible that these compounds could slow AEA degradation competing for FAAH activity, an action that has been dubbed the entourage effect (Ben-Shabat et al. 1998). However, on balance it is inaccurate to refer to this class of compounds as endocannabinoids, as they form a group of juxtacrine/autocrine-acting FA ethanolamides with several molecular targets. Anandamide can be released into the extracellular environment (Di Marzo et al. 1994; Giuffrida et al. 1999) and can participate in cell-cell signaling (Chevaleyre et al. 2006; Ralevic 2003). The FAAH enzyme is widely distributed throughout the body (Cravatt et al. 1996). Two FAAH isoforms have been cloned, but the subtype identified more recently is not expressed in mouse and rat (Wei et al. 2006). FAAH activity appears to be a key step in termination of AEA signaling (Glaser et al. 2003).

The endocannabinoid 2-AG is produced via a two-step process: degradation of arachidonate-containing phospholipids to diacylglycerol (DAG) by PLC followed by DAG lipase-catalyzed degradation to 2-AG (Mechoulam et al. 1995; Sugiura et al. 1995), although an alternative synthesis pathway has been proposed (Bisogno et al. 1999). Release of 2-AG from cells has also been measured (Bisogno et al. 1997), and in general brain tissue levels of 2-AG are higher than those of AEA (Jung et al. 2005, but see Giuffrida et al. 1999). This may reflect the presence of a metabolic as well as a signaling pool of 2-AG, and therefore some refinement will be needed to obtain accurate measures of the 2-AG involved in synaptic modulation. Inactivation of 2-AG proceeds through monoglycerol lipase (MGL) (Dinh et al. 2002), although cyclooxygenase-2 (Cox-2) may also participate in 2-AG catabolism (Kozak et al. 2002). Studies examining the synaptic localization of enzymes involved in 2-AG synthesis and degradation have generally concluded that DAG lipase is mainly present in postsynaptic elements, while MGL is localized to presynaptic terminals (Bisogno et al. 2003; Gulyas et al. 2004; Katona et al. 2006). Interestingly, the AEA-degrading enzyme FAAH is often found in a postsynaptic neuronal location, where it has been associated with endoplasmic reticulum and other intracellular membranous structures (Gulyas et al. 2004). Thus, the steps involved in AEA degradation could be quite complex (Mckinney and Cravatt 2005). Other compounds have been suggested to act as endocannabinoids, including virodhamine (Porter et al. 2002) and arachidonoyl dopamine (Bisogno et al. 2000), but little evidence has accumulated

for actions of these compounds in vivo. Table 1 lists the compounds that inhibit endocannabinoid synthetic and degradative enzymes and known nonspecific targets of these drugs.

2.2 The CB1 Receptor

The neural actions of endocannabinoids are mediated mainly through activation of the CB1 class of cannabinoid receptors (Freund et al. 2003; Mackie 2005; Matsuda et al. 1990), although recent reports indicate the presence of another major CB receptor, CB2, in glial cells as well as in neurons in some brain regions (Gong et al. 2006; Van Sickle et al. 2005). Cannabinoid receptors are classical Gi/o-coupled GPCRs (Howlett et al. 1986; Matsuda et al. 1990). As such, activation of CBs by their cognate ligands produces typical responses such as inhibition of adenylyl cyclase, phosphorylation/activation of extracellular signal-related kinase (ERK), activation of G-protein-activated inwardly rectifying potassium channels (GIRKs), and inhibition of voltage-gated calcium channels (VGCCs) (Clapham and Neer 1997; Freund et al. 2003; Davis et al. 2003; Wartmann et al. 1995). Additional signaling pathways for CBs have been noted (e.g., Glass and Felder 1997), but it is not clear if these responses are widespread. Table 1 lists the most commonly used CB1 receptor ligands, and compares their actions at CB2 receptors.

Within the central nervous system CB1 receptors are found mainly on presynaptic terminals (Freund et al. 2003; Mackie 2005), and within a given brain region selected terminals express the receptor (Figure 1). Postsynaptic CB1 receptors and CB1 signaling has been seen as well (Tsou et al. 1997; Bacci et al. 2004), but appear to be much rarer than presynaptic receptors. Activation of CB1 has been demonstrated to decrease release of a wide variety of neurotransmitters, including ACh, GABA, glutamate, NE, and others at synapses throughout the CNS and periphery (Alger 2002; Mackie 2006; Szabo and Schlicker 2005). These presynaptic inhibitory effects proceed through the sorts of mechanisms described above, namely Gi/o-mediated alterations in ion channel function and other mechanisms involved in neurotransmitter release. More will be said about the role of particular mechanisms later in this chapter.

2.3 Endocannabinoid Release and Reuptake

Endocannabinoids are lipophilic biomolecules, which complicates our understanding of mechanisms of release of these substances. Most neurotransmitters are released from cells via vesicular mechanisms, and such a mechanism has even been suggested to underlie dopamine release from postsynaptic elements in ventral tegmental area (VTA) dopaminergic neurons (Beckstead et al. 2004). Endocannabinoids would not be packaged into the lumen of a vesicle like hydrophilic molecules.

Table 1 Drugs that Act on the Endocannabinoid System

Target	Compound	Action	Affinity/Potency
CB1 Receptor	ACEA	Agonist (AEA analog)	1.4 nM (Ki)
	ACPA	Agonist (AEA analog)	2.2 nM (Ki)
	(*R*)-(+)-Methanandamide	Agonist (AEA analog)	20 nM (Ki)
	Noladin ether	Agonist (endogenous)	21.2 nM (Ki)
	NADA	Agonist (endogenous)	250 nm (Ki)
	DEA	Agonist (endogenous)	34.4 nM (Ki)
	Leelamine	Agonist	2.9 μM (Ki)
	AM 251	Antagonist/inverse agonist	7.5 nM (Ki)
	AM 281	Antagonist/Inverse Agonist	14 nM (Ki)
	LY 320135	Antagonist/Inverse Agonist	141 nM (Ki)
	O-2050	Antagonist	2.5 nM (Ki)
	SR 141716A	Antagonist/Inverse Agonist	1.2 nM (Kd)
CB2 Receptor	JWH 133	Agonist	34 nM (Ki)
	JWH 015	Agonist	13.8 nM (Ki)
	L-759,633	Agonist	6.4 nM (Ki)
	L-759,656	Agonist	11.8 nM (Ki)
	AM 630	Antagonist/Inverse Agonist	31.2 nM (Ki)
	JTE 907	Antagonist/Inverse Agonist	0.38–35.9 nM (Ki)
CB1 and CB2 Receptors	Anandamide (AEA)	Agonist (endogenous)	89, 371 nM; CB1, CB2 (Ki)
	2-AG	Agonist (endogenous)	0.47, 1.4 μM; CB1, CB2 (Ki)
	Virodhamine	Agonist (endogenous)	1.9 , 1.4 μM; CB1, CB2 (EC_{50})
	CP 55,940	Agonist	0.6, 0.7 nM; CB1, CB2 (Ki)
	HU 210	Agonist	0.06, 0.52 nM; CB1, CB2 (Ki)
	WIN 55,212-2	Agonist	62, 3.3 nM; CB1, CB2 (K_i)
	Bay 59–3074	Partial Agonist	48.3, 45, 5 nM; CB1, CB2 (Ki)
	CAY10508	Antagonist/Inverse Agonist	243 nM CB1 (Ki)
	Δ9-THC	Agonist/Partial Agonist	37-199 nM (Ki)
Endocannabinoid Uptake	AM 404	Transport/FAAH inhibitor	1 μM at transporter (IC_{50})
	LY 2183240	Transport inhibitor	270 pM at transporter (IC_{50})
	O-2093	Transport inhibitor	17.3 μM(IC_{50})
	OMDM-2	Transport inhibitor	3 μM (Ki)
	UCM 707	Transport inhibitor	0.8 μM(IC_{50})
	VDM 11	Transport/FAAH inhibitor	4–11 μM at transporter (IC_{50})
AEA and 2-AG Hydolyzing Enzymes (FAAH and MGL)	Palmitoyliso-propylamide	FAAH Inhibitor	∼10 μM(IC_{50})
	MAFP	Inhibitor (FAAH and MGL)	2.5 nM at FAAH (IC_{50})
	ATFMK	Inhibitor (FAAH and MGL)	low μM(IC_{50})
	URB 597	FAAH Inhibitor	4.6 nM (IC_{50})
Diacylglycerol Lipase (2-AG Synthetic Enzyme)	Tetrahydrolip-statin (THL)	Inhibitor	2–6 nM (IC50)
	RHC 80267	Inhibitor	1.1–4 μM(IC_{50})

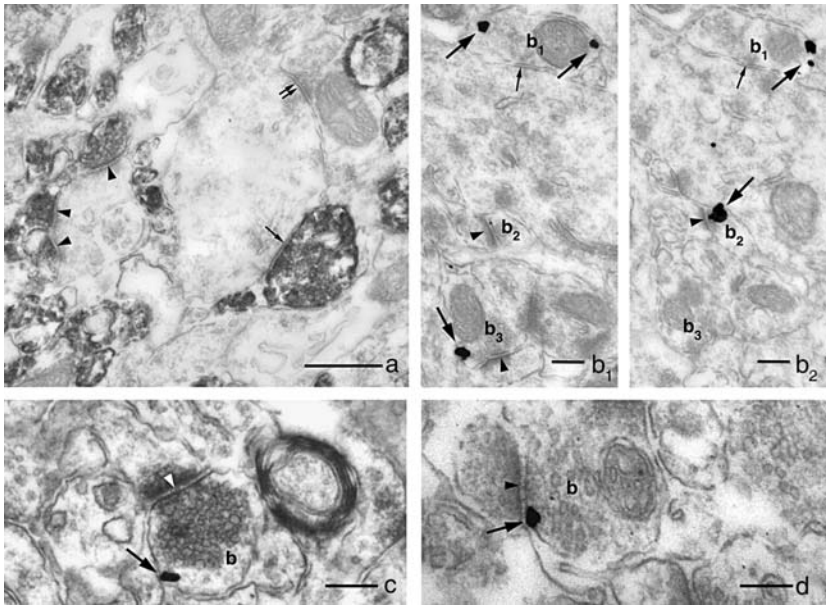

Fig. 1 Presynaptic expression of CB1 receptors. (**a**) Electron micrograph showing CB1 labeling on both asymmetrical, presumed glutamatergic, excitatory synaptic terminals (arrowheads), and symmetrical, presumed GABAergic, inhibitory terminals (single arrow). Note that some terminals at symmetrical, presumed GABAergic, synapses do not show CB1 labeling (double arrows). No evidence of postsynaptic labeling is observed. These images are from the molecular layer of the dentate gyrus. (**b1**, **b2**) Labeling of CB1 receptors (denoted by large arrows) in both types of axon terminals (b1-b3) in the molecular layer of the dentate gyrus using the EM-immunogold technique. Note that receptors are localized to the intracellular surface of the plasma membrane in both excitatory (arrowheads) and inhibitory (small arrows) synaptic terminals. Similar CB1 labeling is also seen in stratum oriens of the CA3 hippocampal subfield (**c**), and stratum oriens of the CA1 subfield (**d**) (terminals and receptors identified as in **b1** and **b2**). Scale bars: **a**, 0,5 µM, **b**-**d**, 0.2 µM. (Reprinted with permission from Katona et al. 2006, copyright 2006, by the Society for Neuroscience).

Although ECs might reside in vesicular membranes, there is no reason to believe that this would constitute a better delivery system than having the molecules exit the cell via the plasma membrane itself. Furthermore, evidence from studies of EC signaling in the hippocampus suggests that botulinum toxin E light chain does not block postsynaptic EC release (Wilson and Nicoll 2001). These findings support the idea that vesicular fusion is not necessary for EC release. Two potential EC release mechanisms have been suggested. The first is simple diffusion through the plasma membrane followed by liberation into the extracellular space. This is a simple and elegant model, and of course there is ample biophysical evidence that ECs can move through lipid membranes (Bojesen and Hansen 2005; Glaser et al. 2003; Alexander and Cravatt 2006; Makriyannis et al. 2005b; McFarland and Barker 2004). The second idea involves a transporter or carrier-mediated system that facilitates the diffusion of ECs from one side of the membrane to the other (Fegley et al. 2004; Hillard

and Jarrahian 2000; Maccarrone et al. 2002; Moore et al. 2005; Ronesi et al. 2004). At first glance, it would appear that an EC transport system is needlessly complex for a lipophilic molecule. However, a system for increasing the rate of transmembrane EC movement might help to localize the most rapid influx and efflux of these molecules near synthetic and degradative enzymes, thus ensuring rapid handling of the largest EC pools.

Evidence for and against the existence of an EC transporter (called the anandamide membrane transporter or endocannabinoid membrane transporter, AMT or EMT alternatively) has accumulated, and this has been a topic of fierce debate in recent papers (Bojesen and Hansen 2005; Glaser et al. 2003; Alexander and Cravatt 2006; Makriyannis et al. 2005b; McFarland and Barker 2004; Fegley et al. 2004; Hillard and Jarrahian 2000; Maccarrone et al. 2002; Mechoulam and Deutsch 2005; Moore et al. 2005). This topic can only be summarized briefly at present. Pharmacological experiments have indicated that cellular uptake and release of AEA and 2-AG can be disrupted by various agents that appear to act independently of other EC-interacting proteins (Hillard and Jarrahian 2000; Fegley et al. 2004; Moore et al. 2005). However, the protein or other molecules involved in EC transport has yet to be identified. Furthermore, the specificity of the putative AMT blockers has been called into question, and in particular it appears that many of these compounds also inhibit the FAAH enzyme (Glaser et al. 2003, Alexander and Cravatt 2006). Thus, it is possible that the slowing of EC uptake produced by these enzymes is secondary to disruption of an EC gradient maintained by FAAH activity (although it is not so clear how this would disrupt 2-AG uptake if this compound is not metabolized by FAAH; Lichtman et al. 2002; Saario et al. 2004). It is clear that FAAH plays a large role in regulating AEA uptake, as disruption of this enzyme greatly slows the uptake process (Glaser et al. 2003). Indeed, FAAH itself may even serve as a conduit for entry of fatty acids into the cell (Mckinney and Cravatt 2005). Thus, the field awaits either creation of a cleaner AMT blocker (Moore et al. 2005; Alexander and Cravatt 2006) or definitive molecular identification of a transporter molecule(s). Table 1 lists the drugs that are currently used as AMT inhibitors along with their known nonspecific targets.

Regardless of the mechanism of transmembrane endocannabinoid movement, investigators interested in the mechanisms of intercellular communication by ECs must explain how these lipophilic compounds are induced to leave the plasma membrane of one cell and travel to receptors on another cell. Fatty acids and their derivatives are known to interact with carrier proteins such as albumin, and anandamide interacts with such proteins as well (Hsu and Storch 1996; Sacchettini and Gordon 1993; Bojesen and Hansen 2003; Makriyannis et al. 2005a). Fatty acid binding proteins also mediate movement of these lipophilic molecules within the cell (Bass 1998). Thus, the idea of an FA-binding carrier protein mediating the extraction from the cell membrane and the intercellular movement of ECs is attractive (Makriyannis et al. 2005a). However, no such carrier protein has been identified to date. It is possible that EC movement across the synapse involves a lipophilic component of

the molecular matrix within the synaptic cleft itself, but that hypothesis has not yet been fully explored. Thus, there is still much to learn about how ECs participate in cell-cell communication.

3 Cannabinoids, Endocannabinoids, and Presynaptic Depression

3.1 Actions of Synthetic Cannabinoids

The most widely reported effect of CB1 agonists is reduction in synaptic transmission or the extracellular concentration of neurotransmitters (Alger 2002; Chevaleyre et al. 2006; Freund et al. 2003; Mackie 2006; Szabo and Schlicker 2005). This synaptic depression appears to result predominantly from reductions in neurotransmitter release produced by presynaptic CB1 receptors. The presynaptic depressant effect of CB1 agonists is most often blocked by CB1 antagonists and is absent in gene-targeted CB1-/- mice (Domenici et al. 2006; Gerdeman et al. 2002; Hajos et al. 2001; Katona et al. 2006; Kawamura et al. 2006; Takahashi and Castillo 2006; Wilson et al. 2001; Yoshida et al. 2002), with notable exceptions (Hajos et al. 2001; Hoffman et al. 2005).

The mechanisms underlying CB1-mediated presynaptic depression are similar to those implicated in presynaptic depression by other presynaptic GPCRs. Direct demonstrations of CB1-mediated inhibition of VGCCs and activation of GIRK and A-type potassium channels have provided convincing evidence that this receptor can activate molecular pathways implicated in presynaptic inhibition (Bacci et al. 2004; Henry and Chavkin 1995; Mackie and Hille 1992; Mackie et al. 1995, Pan et al. 1996; Guo and Ikeda 2004). Inhibition of VGCCs almost certainly plays a role in presynaptic CB1 actions (Brown et al. 2004; Foldy et al. 2006; Kushmerick et al. 2004). The study by Kushmerick et al. (2004) is particularly convincing, as the authors took advantage of their ability to perform electrophysiological recordings in the large presynaptic terminals at the calyx of Held to provide direct evidence for decreased presynaptic calcium current induced by CB1 activation. The study by Brown et al. (2004) also provides compelling evidence for a calcium channel role from experiments using presynaptic calcium imaging. Activation of potassium channels may also be involved in CB1-mediated presynaptic depression (Daniel and Crepel 2001; Diana and Marty 2003; Varma et al. 2002), but it is often difficult to disentangle potassium and calcium channel function when examining presynaptic modulation with indirect methods (Brown et al. 2004). At synapses in the amygdala and nucleus accumbens CB1 activation does not appear to alter the frequency or other properties of miniature synaptic responses, indicating that the receptor has little effect on mechanisms downstream from VGCCs (Hoffman and Lupica 2001; Katona et al. 2001; Zhu and Lovinger 2005). However, agonist-induced decreases in miniature frequency have been detected, suggesting a more proximal action on neurotransmitter release mechanisms at some synapses (Chevaleyre et al. 2006; Gerdeman and Lovinger 2001).

Cannabinoid and endocannabinoid-induced synaptic depression is observed in both the peripheral nervous system and the CNS. Indeed, Δ9-THC inhibition of transmitter release was first demonstrated in mouse vas deferens (Graham et al. 1974), and further evidence for presynaptic inhibition has been obtained using this preparation (Ishac et al. 1996; Pertwee and Fernando 1996) and in the myenteric plexus (Coutts and Pertwee 1997; Kulkarni-Narla and Brown 2000). In addition, anandamide was first characterized as an EC based on its actions in the mouse vas deferens (Devane et al. 1992). Subsequently, CB1 receptor-mediated inhibition of release of several neurotransmitters has been documented in various regions of the PNS (see Szabo and Schlicker 2005 for review). Cannabinoids also inhibit neural effects on contraction in the ileum (Croci et al. 1998; Lopez-Redondo et al. 1997), although it is not clear that this is effect involves direct inhibition of neurotransmitter release (Croci et al. 1998). The CB1 receptor has been localized to enteric neurons, and thus the effect on ileum certainly involves actions on these presynaptic neurons. In addition, anandamide produces ileal relaxation via a non-CB1, non-CB2-mediated mechanism (Mang et al. 2001).

In general the effects of synthetic cannabinoids are reversible when rigorous steps are taken to test for reversibility (Auclair et al. 2000; Hajos et al. 2001; Kreitzer and Regehr 2001; Levenes et al. 1998; Maejima et al. 2001; Ronesi et al. 2004; Yin et al. 2006; Zhu and Lovinger 2005, but see Kreitzer and Malenka 2005 and Robbe et al. 2001). However, the cannabinoid drugs and endocannabinoids have molecular properties that make it difficult to demonstrate reversibility in many experimental preparations. The highly lipophilic nature of these compounds yields appreciable affinity for lipid-rich white matter, and for plastics used in experimental settings. This is important to bear in mind when designing and interpreting experiments to examine the effects of CB1 activation or inhibition in neural tissue, particularly in brain slices that contain white matter. Long-lasting effects of the compounds cannot always be attributed to biological actions as they may result from the inability to remove compounds from the experimental preparation. Application of a CB1 antagonist following the cessation of agonist application is the most effective way to determine if the agonist action is reversible in the brain slice setting (Kreitzer and Regehr 2001; Kreitzer and Malenka 2005; Maejima et al. 2001; Ronesi et al. 2004; Yin et al. 2006).

3.2 Endocannabinoid Actions

Application of ECs produces presynaptic depression, but this action has not been demonstrated as consistently as that of synthetic CB1 agonists (see Ameri et al. 1999; Di et al. 2005; Stella et al. 1997; Straiker and Mackie 2005; Szabo et al. 2006, for examples). Two factors likely underlie the relative scarcity of data on EC effects. First, ECs are subject to both cellular uptake and degradation, as mentioned above. Thus, convincing demonstration of effects at some synapses might require blocking either or both of these mechanisms, especially in multicellular preparations such as

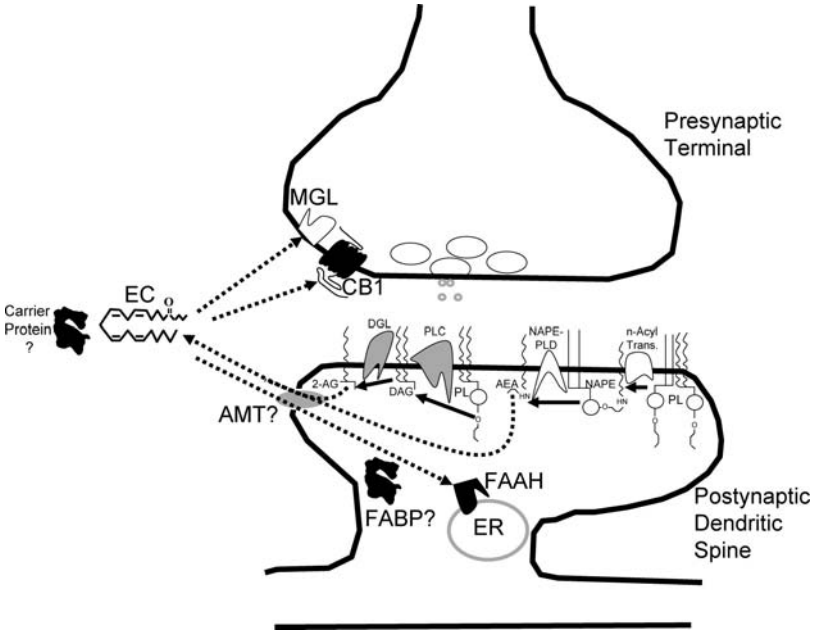

Fig. 2 Identity and presumed location of endocannabinoid synthetic and degradative enzymes at the synapse. A major synthetic pathway for AEA involves transacylation followed by PLD-catalyzed degradation of the NAPE intermediate. However an alternative pathway involving PLC has been proposed, as discused in the text. Synthesis of 2-AG starts with PLC-catalyzed degradation to DAG, followed by degration to 2-AG involving DGL. EC release from the postsynaptic neuron may involve simple diffusion or an AMT-like transporter. Upon release the EC is thought to traverse the synapse and act on presynaptic CB1 receptors. The lipophilic EC may require some sort of carrier protein to move across the hydrophilic environment of the synaptic cleft. After reuptake that may involve the AMT, ECs are degraded by specific enzymes. FAAH catalyzes AEA breakdown, and there is evidence that this enzyme is located in intracellular membranous organelles such as the ER. If this is the case, then an FABP-like protein may be required to shuttle AEA from the plasma membrane to the ER. The 2-AG catabolic enzyme MGL is thought to reside in the presynaptic plasma membrane, and appears to have a role in termination of 2-AG signaling during DSE and DSI. Abbreviations: 2-AG = 2-arachidonoyl glycerol, AEA = arachidonoylethanolamid (anandamide), AMT = anandamide membrane transporter, CB1 = type 1 cannabinoid receptor, DAG = diacylglycerol, DGL = diacylglycerol lipase, EC = endocannabinoid, ER = endoplasmic reticulum, FAAH = fatty acid amide hydrolase, FABP = fatty acid binding protein, MGL = monoglyceride lipase, NAPE = n-acylphosphatidylethanolamine, NAPE-PLD = NAPE-specific phospholipase D, PL = phospholipid, PLC = phospholipase C.

brain slices. Second, AEA acts as a partial agonist in most experimental settings when CB1 modulation of channels or other effector pathways with little amplification are examined (Breivogel et al. 1998; Burkey et al. 1997; Guo and Ikeda 2004; McAllister et al. 1999; Mackie et al. 1993; but see Glass and Northup 1999). Thus, there is some doubt as to whether AEA-induced short-term synaptic depression can be readily demonstrated. However, synaptic depression induced by application of AEA or 2-AG has been reported several times, and thus it is clear that ECs can initiate presynaptic depression. Interestingly, Straiker and Mackie (2005) examined

EC-induced synaptic depression in an autaptic hippocampal culture preparation, and reported that AEA produces synaptic depression that is poorly reversible, while 2-AG produced larger depression that was rapidly reversible. These findings may indicate additional differences in synaptic depression produced by these two ECs that cannot be explained by a simple difference in relative efficacy at CB1 receptors.

Investigators have made interesting use of AEA in characterizing presynaptic CB1-mediated depression. For example, a compelling recent study used a "caged" form of AEA to demonstrate the action and time course of EC and CB1-mediated presynaptic inhibition at a GABAergic synapse in hippocampal slices (Heinbockel et al. 2005). This tool allowed the investigators to photolytically release the AEA with precise timing using an argon-ion laser for UV chemical uncaging. The AEA uncaging produced presynaptic depression that was CB1 mediated. From these studies, the authors concluded that the mechanisms linking CB1 activation to depression of neurotransmitter release occur on a timescale of 50–175 msec, and these effects were reversed within tens of seconds. Two other investigators have used patch pipettes to fill postsynaptic neurons with AEA and provided evidence that this EC can reach its presynaptic receptor target via a retrograde signaling pathway (Ronesi et al. 2004; Jo et al. 2005). The retrograde signaling function of ECs will now be considered in greater detail. Figure 2 provides a schematic diagram of the likely synaptic arrangement of molecules involved in retrograde endocannabinoid signaling.

4 Retrograde Endocannabinoid Signaling in Short-Term Synaptic Depression

The presynaptic location of CB1 receptors, and their implication in agonist and EC-induced presynaptic depression, provided strong clues as to the neuromodulatory roles that might be played by the endocannabinoids. It was also becoming evident that increased intracellular calcium had an important role in endocannabinoid synthesis (Bisogno et al. 1999; Di Marzo et al. 1994). Investigators in a few different laboratories deduced from this information that endocannabinoids could participate in retrograde signaling that was initiated by postsynaptic calcium increases and expressed via endocannabinoid actions on presynaptic CB1 receptors (Kreitzer and Regehr 2001; Maejima et al. 2001; Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001). This provided a crucial breakthrough in our understanding of a major mechanism of endocannabinoid action in the nervous system.

4.1 Depolarization-Induced Suppression of Excitatory and Inhibitory Transmission

As mentioned in the introduction, DSE and DSI involve presynaptic depression of neurotransmitter release brought about by postsynaptic depolarization. The postsynaptic-to-presynaptic signaling involved in DSE and DSI indicated the involve-

ment of a retrograde signal in these forms of synaptic plasticity. Furthermore, it was known that activation of voltage-gated calcium channels and increased postsynaptic calcium levels are necessary and sufficient for DSE/I induction (Llano et al. 1991; Pitler and Alger 1992; Wang and Zucker 2001; Wilson and Nicoll 2001), providing a clue that formation of the retrograde signal depended on a rise in postsynaptic calcium. The idea of retrograde signaling at synapses was not new, as retrograde messengers had been invoked to explain other forms of synaptic plasticity (most notably hippocampal LTP; Madison and Schuman 1995). In the case of DSE/I the retrograde signal hypothesis has held up well under intense scrutiny (see Alger 2002; Chevaleyre et al. 2006 for review). Still, nearly a decade passed between the discovery of the basic phenomena and the discovery of the molecular identity of the messenger.

Several groups nearly simultaneously reported evidence that an EC was the retrograde messenger in DSE and DSI(Kreitzer and Regehr 2001; Maejima et al. 2001; Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001). In at least one laboratory this appeared to be the result of a realization that mechanisms for endocannabinoid production matched those known to be involved in DSI induction (see discussion in Chevaleyre et al. 2006). The main evidence for an EC retrograde signal included (1) the finding that CB1 agonists and/or ECs inhibit transmission at synapses that show DSE/I, and this inhibition involves presynaptic mechanisms (Kreitzer and Regehr 2001; Maejima et al. 2001; Wilson and Nicoll 2001); (2) the demonstration that CB1 antagonists prevent DSE/I (Kreitzer and Regehr 2001; Maejima et al. 2001; Wilson and Nicoll 2001) (see Figure 3 for an example of DSI and blockade by the CB1 antagonist SR141716A); and (3) the demonstration that inhibition of the putative EC transporter mimics and occludes DSI (Wilson and Nicoll 2001). Subsequent studies confirmed and extended these findings (reviewed in Alger 2002; Chevaleyre et al. 2006). It has now been demonstrated that CB1 receptors are present on presynaptic terminals of synapses that show DSE/I, and EC synthetic enzymes are present in postsynaptic elements at these synapses (Freund et al. 2003; Katona et al. 2006; Mackie 2005). Manipulations such as the AEA uncaging and postsynaptic loading of AEA mentioned above have also generated findings consistent with the retrograde signaling idea (Heinbockel et al. 2005; Ronesi et al. 2004).

Many of the mechanisms involved in induction and expression of DSE/I have been elucidated, although some points remain unclear. The depolarization-induced increase in postsynaptic calcium appears to play a role in EC production and/or release (Brenowitz and Regehr 2005; Heibockel et al. 2005; Wang and Zucker 2001; Wilson and Nicoll 2001). However, there is as yet no definitive evidence that loss of any EC synthetic enzyme activity prevents DSE or DSI. Some reports have claimed that DSE/I are unaffected by blockade of PLC or DAG lipase activity, and are not lost following knockout of the PLCβ1 or β4 enzymes (Edwards et al. 2006; Hashimotodani et al. 2005; Maejima et al. 2005; Safo and Regehr 2005). However, a more recent study indicates that blockade of DAG lipase prevents DSI of synapses onto cerebellar Purkinje neurons and neurons in the VTA, but not at inhibitory synapses in the hippocampal CA1 region (Szabo et al. 2006). Inhibition of PLC and DAG lipase does alter other EC-dependent forms of plasticity, as does

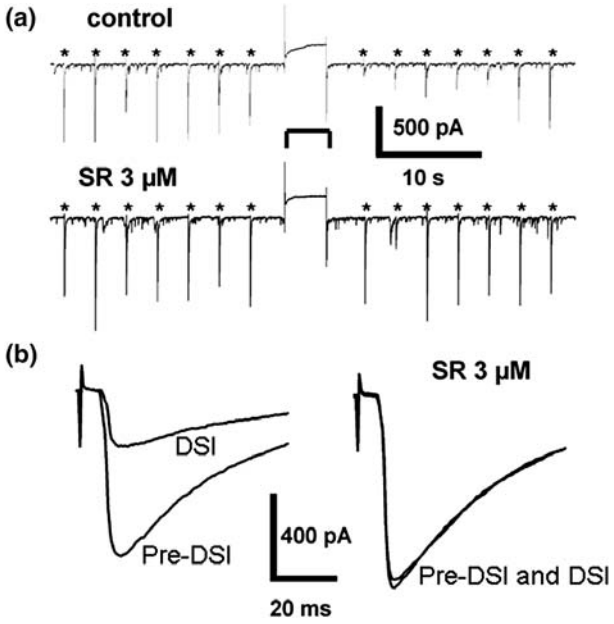

Fig. 3 Depolarization-induced supression of Inhibition (DSI) is endocannabinoid and CB1 receptor-mediated. (**a**) Current traces showing evoked and spontaneous IPSCs before and after a 2 sec depolarization from −60 to 0 mV in the absence (top) and presence (bottom) of the CB1 antagonist SR141716A (SR). Asterisks denote the evoked IPSCs; bracket indicates the period of depolarization. (**b**) Individual evoked IPSCs evoked before and just after depolarization shown at higher temporal resolution in the absence (left) and presence (right) of SR.

knockout of PLCβ1, and these data will be discussed later in this chapter (see Sections 4.2, 4.3, 6.1). The lack of inhibitor effects at hippocampal synapses has led to speculation that increased postsynaptic calcium plays a role in EC release or "mobilization" during induction of DSE/I (Edwards et al. 2006). However, the mechanisms involved in these processes have yet to be fully elucidated. As mentioned above, Wilson and Nicoll (2001) provided evidence that interference with postsynaptic vesicle fusion steps does not alter hippocampal DSI.

Following their postsynaptic release, ECs are thought to traverse the synaptic cleft and act on presynaptic CB1 receptors (Alger 2002; Chevaleyre et al. 2006). This receptor activation leads to presynaptic depression involving mechanisms described above. Several groups have attempted to determine the spatial specificity of the endocannabinoid signal. The bulk of evidence indicates that ECs released at a single set of synapses have an effective range of 10s of µm, indicating that ECs will mainly influence inputs to the cells and synapses from which they are released during DSE and DSI (Brown et al. 2003; Maejima et al. 2001). However, synaptic inhibition induced by DSE/I has been shown to spread to other neurons (Vincent and Marty 1993; Wilson and Nicoll 2001), and supression of interneuron firing facilitates this spread in cerebellum (Kreitzer et al. 2002).

The temporal characteristics of DSE/I have also been examined in greater detail in recent studies. Estimates from the experiments using AEA uncaging indicate that the time from the postsynaptic depolarization/calcium increase to the onset of synaptic depression is on the order of 400 msec (Heinbockel et al. 2005). The duration of DSE/I ranges from a few sec to 10s of sec (see Jo et al. 2005, Heinbockel et al. 2005 for examples). The duration of these short-lasting forms of plasticity appear to be directly related to the lifetime of the EC messenger in the synaptic cleft. The effects of transport blockers mentioned above support this idea (Wilson and Nicoll 2001). In addition, drugs that inhibit EC catabolic enzymes, MGL inhibitors in particular, prolong DSI lifetime (Kim and Alger 2004; Makara et al. 2005; Szabo et al. 2006). DSE duration decreases as temperature increases (Kreitzer and Regehr 2001), consistent with the fact that EC lifetime in the cleft and related signaling events should proceed faster at higher temperatures. The elegant study by Kreitzer and Regehr (2001) in cerebellum also indicated that the duration of DSE is well correlated with the time course of an associated decrease in the presynaptic calcium transient. This finding supports the idea that CB1 activation inhibits presynaptic VGCC production, and this inhibition is directly linked to DSI expression.

It is still not clear which endocannabinoid mediates DSI/E. As the experiments utilizing PLC and DAG lipase inhibitors have produced ambiguous results, the evidence supporting a role for 2-AG in DSI/E comes mainly from experiments in which Cox-2 or MGL inhibitors prolong DSI (Kim and Alger 2004; Makara et al. 2005; Szabo et al. 2006). Complementary experiments in these same studies indicate that inhibition of FAAH does not alter the time course of DSI, and thus the evidence at present indicates that AEA is not the EC retrograde messenger involved in this type of plasticity, despite the fact that AEA can clearly produce a DSI-like effect (Heinbockel et al. 2005). Full elucidation of the EC responsible for DSE and DSI will require the development of better molecular tools for examination of EC release, reuptake, and degradation.

Interestingly, DSE/I are not observed at all synapses where presynaptic CB1 receptors are found. For example, in the dorsal striatum EC-dependent DSE is not observed at glutamatergic synapses known to express other forms of EC-dependent plasticity (Gerdeman et al. 2002; Kreitzer and Malenka 2005; Yin et al. 2006). Perhaps postsynaptic activation by depolarization alone is not sufficient to induce EC production or release from the postsynaptic elements of these synapses.

The physiological roles of DSE/I are the subject of debate and continued investigation (Alger 2002; Chevaleyre et al. 2006; Hampson and Deadwyler 1998). Regulation of the pattern and timing of neuronal activity in discrete circuits is one attractive possibility. Depression at a subset of synapses onto a given postsynaptic neuron might also boost the relative efficacy or signal/noise ratio of other inputs to those same cells. In this regard, it has been proposed that depression may be one mechanism that helps to equalize synaptic efficacy across extensive dendritic arbors (Ramsey and Abbott 2006). For example, decreasing efficacy at synapses proximal to the soma would yield more equal weighting relative to distal synapses in influencing action potential generation. Evidence has accumulated that the disinhibition produced by DSI in hippocampus enhances neuronal excitability (Wagner

and Alger 1996), excitatory transmission and synaptic excitation via an increase in the efficacy of EPSP-spike coupling during disinhibition (Chevaleyre and Castillo 2003). Disinhibition during DSI can also enhance induction of long-term potentiation (LTP), a long-lasting increase in synaptic efficacy at excitatory synapses (Carlson et al. 2002). To date there is little evidence that DSE/I-like mechanisms take place in vivo. Indeed, the depolarization needed to trigger these types of plasticity is strong and prolonged, and perhaps beyond the physiological range of neuronal activity (although see Brown et al. 2004). Nonetheless, endocannabinoid actions in the intact brain are becoming increasingly apparent, and these functions may well involve DSE, DSI or the other EC-dependent forms of synaptic plasticity that will be discussed in the following sections of this chapter (see Sections. 4.2, 6.1).

DSI and DSE have been observed at synapses in several brain regions in addition to the cerebellum and the hippocampal CA1 subregion. These include inhibitory synapses in the basolateral amygdala (Zhu and Lovinger 2005), dentate gyrus of the hippocampus (Isokawa and Alger 2005); hypoglossal interneurons (where DSI alters glycine release) (Mukhtarov et al. 2005), hypothalamus (Di et al. 2005; Hentges et al. 2005; Jo et al. 2005), neocortex (Bodor et al. 2005; Trettel and Levine 2003), substantia nigra (Yanovsky et al. 2003), and the ventral tegmental area (VTA) (Melis et al. 2004a). It is possible that DSE/I and similar EC-dependent transient decreases in synaptic transmission could play roles in brain functions as diverse as addiction, feeding, motor control, and spatial learning. However, much more work is needed to distinguish possible roles of DSE/I from those of the other EC-dependent forms of synaptic plasticity, which will now be discussed.

4.2 Synaptically Driven EC-Dependent Short-Term Depression

Neural production and release of ECs is likely to be stimulated by repetitive firing of neurons or repetitive synaptic input to a neuron rather than the prolonged depolarization used to initiate DSE/I. Investigators have now discovered a number of patterns of neuronal activity that generate short-lasting EC-dependent synaptic depression in different brain regions. The majority of these involve activation of neurotransmitter receptors or brief periods of repetitive synaptic activation (see Chevaleyre et al. 2006 for review).

One motif that has emerged from these investigations is stimulation of EC-dependent plasticity by metabotropic receptors that couple to Gq-like G-proteins. In particular, metabotropic glutamate receptors (mGluRs) of the group I subtypes have been strongly linked to EC production at synapses in several brain regions. Direct activation of mGluRs enhances DSI (Martin and Alger 1999; Morishita et al. 1998; Ohno-Shosaku et al. 2002; Varma et al. 2001) and also directly produces EC-dependent synaptic plasticity (Edwards et al. 2006; Galante and Diana 2004; Kushmerick et al. 2004; Maejima et al. 2001; Martin and Alger 1999; Morishita et al. 1998; Ohno-Shosaku et al. 2002; Varma et al. 2001). In the hippocampal CA1 region, activation of muscarinic acetylcholine receptors produces a similar action

(Edwards et al. 2006; Fukudome et al. 2004; Martin and Alger 1999; Morishita et al. 1998). In many cases, these metabotropic receptor-induced forms of synaptic depression persist only as long as the receptors are activated. However, in some cases depression persists after agonist has been removed (Azad et al. 2004; Chevaleyre and Castillo 2003; Edwards et al. 2006; Kreitzer and Malenka 2005; Robbe et al. 2002; Yin et al. 2006). This long-lasting effect will be discussed in more detail in the section on long-term depression (see Section 6.1).

The idea that Gq activation leads to stimulation of EC formation is logical given that PLC is known to be activated by this class of G-proteins (Sternweiss et al. 1992). Activation of mGluRs has been shown to increase EC levels measured using mass spectrometric analysis of brain tissue (Jung et al. 2005), and activation of PLC and DAG lipase were implicated in this mGluR action.

Short-term EC-dependent synaptic plasticity (eSTD) follows afferent stimulation leading to synaptic activation at synapses in cerebellum (Brown et al. 2003; Galante and Diana 2004; Maejima et al. 2005; Rancillac and Barbara 2005), neocortex (Trettel et al. 2004) and VTA (Melis et al. 2004b). This phenomenon has been observed at excitatory glutamatergic and inhibitory GABAergic synapses. The eSTD at excitatory synapses is homosynaptic. In other words depression is observed at the same synapses whose activation leads to STD induction. In the majority of cases, this eSTD requires activation of a group I mGluR (Brown et al. 2003; Galante and Diana 2004; Maejima et al. 2005; Melis et al. 2004b). At these synapses, the mechanism for eSTD induction involves repetitive activation of glutamate release following afferent activation, glutamate activation of the mGluR, stimulation of PLC or other enzymes implicated in EC formation, retrograde EC signaling to presynaptic CB1 receptors, and ultimately a decrease in glutamate release such as that observed during DSE (Hashimotodani et al. 2005). Activation of mAChR activation can enhance eSTD in neocortex (Trettel et al. 2004).

The eSTD observed at GABAergic inhibitory synapses is heterosynaptic, or at least has a heterosynaptic component (Galante and Diana 2004; Martin and Alger 1999; Morishita et al. 1998; Varma et al. 2001). In this case, activation of glutamatergic synapses appears to be required for STD induction. This type of plasticity usually begins with brief periods of moderate-high frequency activation of glutamatergic synapses or application of a group I mGluR agonist. This leads to mGluR activation and a subsequent increase in postsynaptic EC production and EC release. The EC then acts on presynaptic CB1 receptors on what are presumed to be nearby GABAergic terminals, suppressing GABA release. The reduction in GABA release is thought to persist for the duration of the rise in synaptic EC concentration. However, certain aspects of this scenario are as yet unclear. For example, it is not known if activation of GABAergic synapses plays any role in eSTD initiation. Also, there is little evidence to date concerning the relative locations of the glutamatergic and GABAergic synapses involved in this plasticity. Indeed, it is not yet clear if the EC itself is released from postsynaptic elements associated with the glutamatergic or the GABAergic synapse.

4.3 Comparison of DSE/I and eSTD

There are intriguing differences in the mechanisms of induction of DSE/I in comparison to eSTD. Induction of DSE/I does not require activation of metabotropic receptors, such as mGluRs (Kreitzer and Regehr 2001; Maejima et al. 2001; Wilson and Nicoll 2001), while receptor activation does appear to be required for most forms of eSTD (Brown et al. 2003; Edwards et al. 2006; Galante and Diana 2004; Kushmerick et al. 2004; Maejima et al. 2001,2005; Martin and Alger 1999; Melis et al. 2004b; Morishita et al. 1998; Ohno-Shosaku et al. 2002; Varma et al. 2001). In general, blockade of STD with PLC and DAG lipase inhibitors is more consistent than inhibitor effects on DSE or DSI (Edwards et al. 2006; Hashimotodani et al. 2005; Maejima et al. 2005; Safo and Regehr 2005; but see Szabo et al. 2006). This dissociation has been observed even at a single population of inhibitory inputs to hippocampal pyramidal neurons (Edwards et al. 2006). Similar results have also been obtained using gene-targeted mice that lack the PLCβ1 enzyme. In hippocampal neurons from these mice, eSTD induced by receptor activation was lost while DSI persisted (Hashimotodani et al. 2005). In cerebellum, the PLCβ4 enzyme appears to serve a similar role, being necessary for receptor- and synaptically-driven eSTD, but not for DSE (Maejima et al. 2005). The necessary role for increased postsynaptic calcium levels in DSE/I induction is clear. However, the role of this type of signaling in eSTD is less certain. In the cerebellum eSTD can be induced even under conditions of strong postsynaptic calcium chelation (Galante and Diana 2004; Maejima et al. 2001), indicating a lack of necessity for a calcium increase. These findings support a model in which mGluR activation leading to phospholipase-mediated EC formation is necessary for eSTD. In contrast, only activation of voltage-gated calcium channels and a postsynaptic rise in calcium appear to be necessary for induction of DSE/I. Thus, the roles of calcium, EC production, and EC signaling in DSE/I and eSTD differ. The insensitivity of DSI to lipase blockers has led Alger and coworkers to suggest that the ECs involved in DSE/I are part of a pre-existing pool that is "mobilized" by the postsynaptic calcium increase, as mentioned above (Edwards et al. 2006). It is possible that some sort of EC release mechanism, perhaps the transport processes discussed in Section 2.3, plays a role in postsynaptic EC availability for DSE/I, but as yet there is no evidence to support this idea. Evidence from one study suggests that a postsynaptically loaded AMT inhibitor prevents induction of eSTD at synapses onto dopaminergic neurons in the ventral tegmental area (VTA)(Melis et al. 2004b). Thus, some sort of EC mobilization or release mechanism is implicated in this form of synaptic depression.

Synergism between DSE and eSTD has been observed in elegant studies by Brenowitz and Regehr (2005). Parallel fiber synapses onto cerebellar Purkinje neurons exhibited both types of synaptic depression, and stimuli that are subthreshold for induction of either type of depression alone can be co-activated to yield depression. This synergism is dependent on both postsynaptic calcium levels and mGluR activation, but the mGluRs do not appear to participate in the calcium rises crucial for induction of depression.

The evidence available at present implicates 2-AG as the EC involved in agonist and stimulation-induced eSTD. In cerebellar, hippocampal, and VTA neurons eSTD is prevented by blockers of PLC and DAG lipase (Edwards et al. 2006; Galante and Diana et al. 2004; Melis et al. 2004b). The loss of STD in PLC knockout mice is also consistent with a role for 2-AG (Hashimotodani et al. 2005; Maejima et al. 2005). However, the recent evidence for an AEA synthesis mechanism involving PLC (Liu et al. 2006) makes this conclusion less certain. Activation of mGluRs stimluates production of 2-AG in preference to AEA in the hippocampus and striatum (Jung et al. 2005), and both PLC and DAG lipase are involved in this mGluR-stimulated EC production. There may well be a role for AEA in eSTD at some synapses, but the bulk of evidence available at this time favors involvement of 2-AG. Figure 4 summarizes the presynaptic mechanisms thought to participate in transient synaptic depression during DSE/I and eSTD.

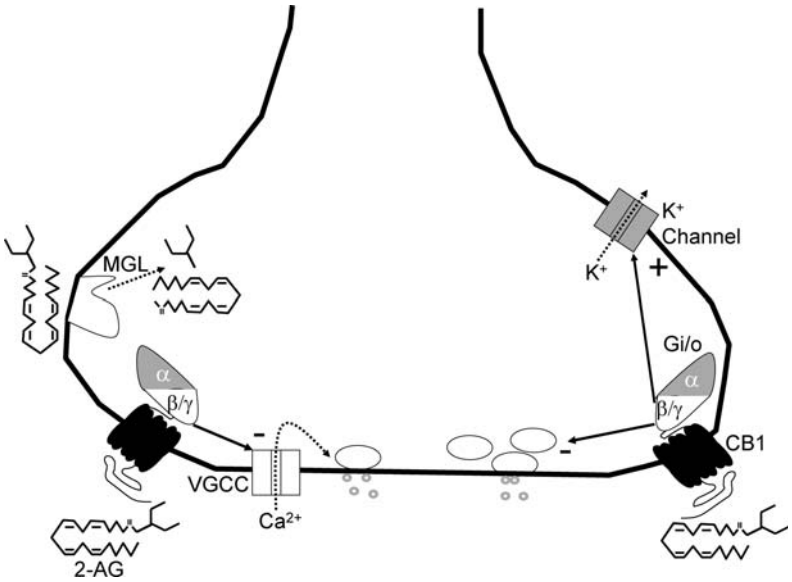

Fig. 4 Mechanisms underlying presynaptic expression of transient endocannabinoid-mediated synaptic depression. Activation of the CB1 receptor by an EC (most likely 2-AG) stimulates dissociation of Gi/o-type G-proteins. The liberated $G\beta/\gamma$ subunit inhibits voltage-gated calcium channels (VGCCs), activates potassium channels, and may also directly inhibit the vesicle fusion process. Inhibition of the VGCC reduces the calcium signal involved in excitation-secretion coupling, thereby reducing vesicle fusion and lowering the probability of neurotransmitter release. Activation of potassium channels may hyperpolarize the presynaptic membrane potential, shunt presynaptic current, and/or increase the rate of repolarization of the terminal during excitation. All these effects would reduce calcium entry and decrease the probability of neurotransmitter release. Disruption of vesicle fusion directly reduces the probability of release. EC signaling is terminated, most likely via MGL-catalyzed degradation of 2-AG, and this is a key step in termination of transient EC-mediated synaptic depression such as DSE/I and eSTD.

Endocannabinoid-dependent STD and DSE/I are modulated by other neurotransmitters in a brain region-dependent manner. In the striatum the neuromodulatory transmitter dopamine is abundant (Carlsson 1993). Dopamine activation of D2 receptors produces synaptic depression, especially when paired with activation of glutamatergic synaptic transmission (Calabresi et al. 1992a; Cepeda et al. 1993; Flores-Hernandez et al. 1997; Tang et al. 2001; Bamford et al. 2004). A recent study has implicated retrograde endocannabinoid signaling in this form of STD (Yin and Lovinger 2006). Synaptic activation of mGluRs also appears to play a role in this D2-mediated eSTD. In the lateral hypothalamus DSI is observed at synapses onto neurons that express melanin-concentrating hormone (Jo et al. 2005). As in most forms of DSI, activation of a postsynaptic VGCC is necessary for the induction of plasticity. These lateral hypothalamic neurons express postsynaptic receptors for the neuropeptide leptin. Activation of the leptin receptors suppresses calcium channel function and prevents EC retrograde signaling, presumably by inhibiting EC formation or release. This type of mechanism is likely to be widespread given the large number of GPCRs that can inhibit VGCCs (Dolphin 2003; Elmslie 2003). However, it remains to be determined if there are particular receptors that preferentially inhibit the channels involved in EC production/release during induction of synaptic plasticity.

5 Endocannabinoid Tone and Intrinsic CB1 Activity

Given the relationship between neuronal activity, postsynaptic calcium increases, and EC production, it is possible that neurons with especially high levels of activity or periodic or sustained increases in intracellular calcium could tonically produce and/or release EC messengers. This could lead to sustained CB1 activation and a "tone" of decreased transmission. Several reports have now been published that indicate such a tone at inhibitory synapses in neurons from basolateral amygdala (Zhu and Lovinger 2005), hippocampus (Foldy et al. 2006), and hypothalamus (Jo et al. 2005). The main evidence for tonic endocannabinoid suppression of transmission comes from experiments in which CB1 antagonists enhance synaptic transmission. It has been argued that these effects might reflect inverse agonist actions of the drugs, and indeed there is evidence that the most commonly used CB1 antagonists, AM251 and SR131716A, can act as inverse agonists (Bouaboula et al. 1997; Pan et al. 1998). The argument that antagonist actions are due to inhibition of EC effects and not inverse agonist actions is bolstered by experiments involving postsynaptic manipulations designed to prevent EC production/release. Filling the postsynaptic neuron with a calcium chelator, such as BAPTA, prevents the CB1 antagonist action (Foldy et al. 2006; Zhu and Lovinger 2005). It is difficult to see how such a result could reflect interference with an inverse agonist action, as the chelator effects are confined to the interior of the postsynaptic neuron while the antagonist effects appear to be presynaptic. It is known that postsynaptic calcium chelation can block retrograde EC signaling in DSE/I and some forms of EC-STD, as mentioned

above. Thus, the finding that chelation blocks antagonist effects is most consistent with prevention of postsynaptic EC generation/release that would normally induce presynaptic CB1 activation. Extracellular application of AMT inhibitors can produce a CB1-mediated decrease in transmission at some synapses (Wilson and Nicoll 2001), as previously discussed in Section 4.1. This finding is also consistent with the idea that ECs can be tonically produced and released, albeit in many cases without accumulation to levels that produce detectable CB1 activation.

At present it is difficult to evaluate the possible contribution of intrinsic CB1 receptor activity to synaptic modulation. It is well known that GPCRs have the intrinsic ability to stimulate their cognate G-proteins in the absence of exogenous or endogenous ligand (see Lefkowitz et al. 1993 for review). Indeed studies of heterologously expressed CB1 indicated that the receptor was tonically active, as measured by inhibition of VGCCs (Pan et al. 1998). However, most, if not all, neurons contain the molecular machinery to synthesize ECs, and thus it is possible that tonic receptor activation might result from actions of an EC produced even in a single cell during a patch-clamp experiment. Pan et al. (1998) examined this possibility using postsynaptic calcium chelation to prevent EC formation and a mutant receptor that is insensitive to AEA activation. The findings in these experiments suggested that the receptor tone is due to intrinsic receptor activity rather than activation by native ECs. More recent studies of tonic GPCR inhibition of calcium channels indicate evidence of tonic receptor activation/channel modulation when receptors are overexpressed at high levels, but not at lower levels of receptor expression (Guo and Ikeda, 2004). Antagonist-induced increase in calcium current was observed in peripheral neurons that express native CB1 receptors (Pan et al. 1998). Nonetheless, the role of intrinsic CB1 activity in altering neuronal function at physiological levels of receptor expression remains unclear, particularly at CNS synapses.

6 Endocannabinoid-Dependent Long-Term Synaptic Depression

6.1 EC-LTD Induction Mechanisms

Retrograde signaling involving ECs and the CB1 receptor has now been implicated in long-lasting decreases in synaptic transmission at synapses in many brain regions (see Gerdeman and Lovinger 2003; Chevaleyre et al. 2006 for review). This long-term synaptic plasticity is generally referred to as endocannabinoid-dependent long-term synaptic depression, or EC-LTD. Long-lasting changes in the efficacy of synapses such as long-term potentiation (LTP) and LTD have been postulated to play roles in synaptic development, information storage, homeostatic plasticity, nervous system development, and even pathological phenomena like epilepsy (McEachern and Shaw 1996; Malenka and Bear 2004).

Induction of LTD and other forms of long-lasting synaptic plasticity usually begin with repetitive activation of synapses, or in some cases, deliberately timed

coactivation of pre- and postsynaptic neurons (so-called Spike-timing-dependent plasticity, or STDP). Repetitive synaptic activation can initiate several mechanisms that induce changes in synaptic efficacy. At glutamatergic synapses activation of NMDA receptors and mGluRs are widely recognized mechanisms for initiation of plasticity (Malenka and Bear 2004). Most NMDAR-dependent forms of LTP or LTD appear to be both initiated and expressed postsynaptically, with an expression mechanism involving alterations in postsynaptic AMPAR number or function (Malenka and Bear 2004). However, several forms of LTD that involve postsynaptic induction but presynaptic expression have been discovered over the years (Bolshakov and Siegelbaum 1994; Chevaleyre and Castillo 2003; Choi and Lovinger 1997a,b; Marsicano et al. 2002; Robbe et al. 2002; Sjostrom et al. 2003). Activation of mGluRs is a key step in many of these forms of LTD (Boshakov and Siegelbaum 1994; Gubellini et al. 2001; Robbe et al. 2002; Sung et al. 2001). Over the last several years it has become apparent that most forms of LTD that involve this postsynaptic induction-presynaptic expression mechanism are, in fact, EC-LTD.

Initial evidence for EC involvement in LTD came from experiments in which synaptic depression was blocked by CB1 receptor antagonists or lost in CB1-/- mice (Gerdeman et al. 2002, Robbe et al. 2002; Marsicano et al. 2002; Chevaleyre and Castillo 2003; Sjostrom et al. 2003). The LTD expressed at these synapses was associated with changes in transmission indicative of a decrease in probability of neurotransmitter release involved in presynaptic depression (Chevaleyre and Castillo 2003; Choi et al. 1997a,b; Gerdeman et al. 2002; Marsicano et al. 2002; Robbe et al. 2002; Sjostrom et al. 2003). This evidence included increases in PPR and the coefficient of variation of transmission, decreased frequency but not amplitude of miniature synaptic responses, and increases in synaptic failures during paired pre- and postsynaptic recordings. The predominant presynaptic expression of CB1 receptors supported the idea that EC-LTD is expressed presynaptically. Evidence for postsynaptic induction of EC-LTD has come from experiments utilizing postsynaptic manipulations to enhance or block plasticity. Postsynaptic calcium chelation blocks most forms of EC-LTD, presumably by preventing the increase in intracellular calcium needed for EC production/release (Bender et al. 2006; Calabresi et al. 1992b; Choi et al. 1997a; Robbe et al. 2002; Sjostrom et al. 2003; Soler-Llavina and Sabatini 2006). At corticostriatal synapses this blockade can be overcome by extracellular application of an AMT blocker which would enhance the increase in EC levels produced during synaptic activation (Gerdeman et al. 2002). This finding suggests that ECs released from cells other than the postsynaptic neuron can rescue LTD that would normally be blocked by the postsynaptic chelator, lending additional support to the hypothesis that ECs mediate this form of LTD. In layer 3 of visual cortex postsynaptic blockade of NMDA receptors prevents EC-LTD induction (Crozier et al. 2007). Thus, postsynaptic NMDA receptors can participate in some forms of EC-LTD, although this is the exception rather than the rule. Interestingly, EC-LTD in barrel-field cortex is resistant to postsynaptic NMDAR blockade (Bender et al. 2006) indicating that there is heterogeneity in EC-LTD induction mechanisms even within sensory cortex.

The EC-LTD observed at GABAergic synapses in hippocampus and BLA is not blocked by postsynaptic calcium chelation even at levels that prevent EC-mediated DSI (Azad et al. 2004; Chevaleyre and Castillo 2003; Zhu and Lovinger 2005). It is probable that mGluR activation of PLC leading to EC formation bypasses any need for increased intracellular calcium during induction of these forms of plasticity.

Subsequent studies have reinforced the idea that the ECs involved in EC-LTD are released from the postsynaptic cell. Blockers of DAG lipase prevent EC-LTD induction at GABAergic synapses in hippocampus (Chevaleyre and Castillo 2003), and this enzyme is thought to reside postsynaptically at these synapses (Bisogno et al. 2003; Gulyas et al. 2004; Katona et al. 2006). In neocortex, loading the DAG lipase inhibitor directly into the patch pipette prevented LTD induction (Bender et al. 2006). Direct intracellular application of GDPβS, a compound that locks G-proteins in an inactive form, prevents LTD induction (Chevaleyre and Castillo 2003). Postsynaptic G-protein activation is likely necessary to couple mGluRs to EC production during LTD induction. Intracellular postsynaptic application of AMT inhibitors has also been found to prevent LTD induction at synapses in the sensory neocortex and striatum (Bender et al. 2006; Ronesi et al. 2004). These findings support the idea that some sort of postsynaptic transporter or carrier-mediated EC release process is involved in EC-LTD induction. In the striatum blockade of L-type calcium channels prevents EC-LTD induction (Calabresi et al. 1992b, Tang et al. 2001, Wang et al., 2006). A recent study implicates the low-threshold, CaV1.3 form of the L-type channel in this process (Wang et al. 2006). This channel is the likely source of much of the postsynaptic calcium involved in LTD induction given that the channel is expressed postsynaptically in striatal medium spiny neurons (Olson et al. 2005), and L-type channels have little or no direct influence on presynaptic function at these synapses (Lovinger et al. 1994). Compounds that alter intracellular calcium release also prevent EC-LTD in nucleus accumbens (Robbe et al. 2002). This finding is consistent with a scenario in which activation of postsynaptic mGluRs stimulates calcium release from intracellular stores, and this calcium increase contributes to the EC production and release necessary for EC-LTD induction.

Little is known as yet about the molecular mechanisms involved in EC-LTD expression. If CB1 receptor activation is crucial for EC-LTD induction, then it is possible that application of a CB1 agonist might be sufficient to induce LTD. However, the synaptic depression produced by application of CB1 agonists is reversible when transmission is evoked by low-frequency stimulation of afferents or when spontaneous transmission is measured (Auclair et al. 2000; Hajos et al. 2001; Huang et al. 2001; Kreitzer and Regehr 2001; Levenes et al. 1998; Ronesi et al. 2004; Yin et al., 2006; Zhu and Lovinger 2005). Notably, these observations were made at synapses that are known to express EC-LTD. Thus, for the most part experimental evidence suggests that an additional factor acts together with CB1 receptor activation to induce the long-lasting decrease in neurotransmitter release during EC-LTD expression. In the case of EC-LTD within the visual cortex one such factor has been identified, namely presynaptic NMDA receptors (Sjostrom et al. 2003). Receptors that appear to contain the NR2B subunit have presynaptic actions at this synapse.

Antagonists of these receptors prevent EC-LTD, as do CB1 antagonists. The authors suggest that a mechanism involving synergism of the CB1 and NMDAR mechanisms is needed for EC-LTD expression. However, it is not yet clear what molecular mechanisms are involved in this synergism. It must be noted that application of CB1 agonists and ECs appear to induce LTD at some synapses (Bender et al. 2006; Kreitzer and Malenka 2005; Robbe et al. 2001; Sjostrom et al., 2003, Huang et al., 2003). However, Sjostrom et al. (2003) showed that this effect required a certain level of afferent activation. It is possible that during induction of EC-LTD CB1 activation synergizes with molecular mechanisms induced by activation of afferents themselves that are independent of postsynaptic signaling. To date it has been difficult to test this hypothesis because intact glutamatergic transmission is needed to measure EC-LTD expression. However, a prelminary report from Heifets et al. (2006) suggests that both presynaptic activation and CB1 activation are necessary for LTD induction at GABAergic synapses in hippocampus. Application of more direct measures of presynaptic function to the study of EC-LTD would be helpful in this context.

Endocannabinoid-dependent LTD induced by various pharmacological manipulations has been observed in some brain regions. Activation of group I mGluRs with DHPG induces an endocannabinoid-dependent long-lasting decrease in synaptic efficacy that resembles LTD (Azad et al. 2004; Chevaleyre and Castillo 2003; Edwards et al. 2006; Robbe et al. 2002, Kreitzer and Malenka 2005; Yin et al. 2006). This mGluR-induced EC-LTD occludes LTD produced by afferent stimulation, and thus appears to share common mechanisms with EC-LTD (Chevaleyre and Castillo 2003; Robbe et al. 2002; Kreitzer and Malenka 2005). In the dorsal striatum, mGluRs only effectively elicit EC-LTD when the postsynaptic membrane potential is slightly depolarized relative to the resting potential (Kreitzer and Malenka, 2005). This finding suggests the involvement of a voltage-gated channel in mGluR-induced EC-LTD, and indeed a dihydropyridine L-type channel antagonist blocks this form of LTD. It appears that mGluRs can induce LTD by activating endocannabinoid synthesis and that they act in concert with VGCCs , but this has not been explicitly explored at all synapses that show mGluR-induced EC-LTD. Furthermore, the role of presynaptic activation/synaptic transmission in mGluR-EC-LTD has not been fully examined.

In the lateral amygdala, application of amphetamine produces LTD that is dependent on CB1 receptor activation (Huang et al. 2003). The ability of amphetamine to induce LTD is enhanced by extracellular application of an AMT blocker, supporting an EC role. This form of EC-LTD is mimicked and occluded by a CB1 agonist. However, no attempt to reverse the agonist-induced depression was made in this study, and thus it is not entirely clear that the agonist induces true LTD. Postsynaptic calcium chelation prevents the amphetamine-induced EC-LTD, suggesting a postsynaptic locus of EC production/release. Measurement of mEPSCs suggests a presynaptic site of amphetamine-induced LTD expression, consistent with other forms of EC-LTD. This study suggests one possible link between EC-LTD and actions of drugs of abuse.

6.2 Mechanisms Involved in Maintained Expression of EC-LTD

Information about the molecular mechanisms involved in the presynaptic expression of EC-LTD is starting to appear. Amphetamine-induced EC-LTD in amygdala is accompanied by a change in the frequency, but not amplitude, of miniature EPSCs (Huang et al. 2003). Thus, expression of this form of LTD is independent of presynaptic action potential firing, and likely involves mechanisms downstream from calcium entry into the terminal. Similar findings were obtained for stimulation-induced EC-LTD in the nucleus accumbens (Robbe et al. 2002). However, in the Robbe et al. (2002) study only spontaneous EPSCs and not true miniature EPSCs were examined, making it difficult to determine if alterations in presynaptic calcium influx have a role in EC-LTD expression at this synapse. Protein kinase inhibitors have been shown to prevent EC-LTD at synapses in striatum (Calabresi et al. 1994), but it is unclear if protein phosphorylation contributes to EC production, as proposed for EC-LTD in amygdala (Azad et al. 2004), or if these signaling mechanisms are involved in presynaptic EC-LTD maintenance. Inhibition of protein translation, but not transcription, prevents EC-LTD at excitatory synapses in striatum (Yin et al. 2006). This finding implicates new protein synthesis in this form of plasticity. Experiments in this study were performed in a preparation lacking the somata of the presynaptic glutamatergic neurons, effectively ruling out contributions of presynaptic nuclei. Inhibition is not effective when applied to the postsynaptic neuron alone, and these inhibitors do not appear to be act on receptors or channels necessary for EC-LTD induction. These findings narrow the potential sites of plasticity-related protein translation to the presynaptic axon or terminal and/or other intact somata in the slice, including glia and interneurons. The possibility of a contribution of presyaptic translation to EC-LTD is intriguing, but further evidence is required to pinpoint the important site of protein expression. Figure 5 presents potential mechanisms involved in the presynaptic expression of EC-LTD.

6.3 Endocannabinoids and Cerebellar LTD

The first type of LTD ever described was that which occurs at synapses between parallel fibers and Purkinje neurons in cerebellum (so-called Cerebellar LTD) (Ito and Kano 1982; Ito 2001). Much is known about cerebellar LTD, including the fact that both the mechanisms involved in the initial LTD induction and the ultimate expression of synaptic depression are most likely postsynaptic (see Ito 2001 for review). Thus, it was surprising when Safo and Regehr (2005) recently reported a role for EC retrograde signaling in cerebellar LTD. These investigators demonstrated that cerebellar LTD was blocked by CB1 antagonists and was lost in the CB1 knockout mouse. Application of DAG lipase inhibitors either to the whole preparation, or just to the interior of the postsynaptic neuron, also blocked LTD induction. Activation of CB1 receptors alone produced synaptic depression that was reversed by subsequent antagonist application, indicating that CB1 activation alone is not sufficient

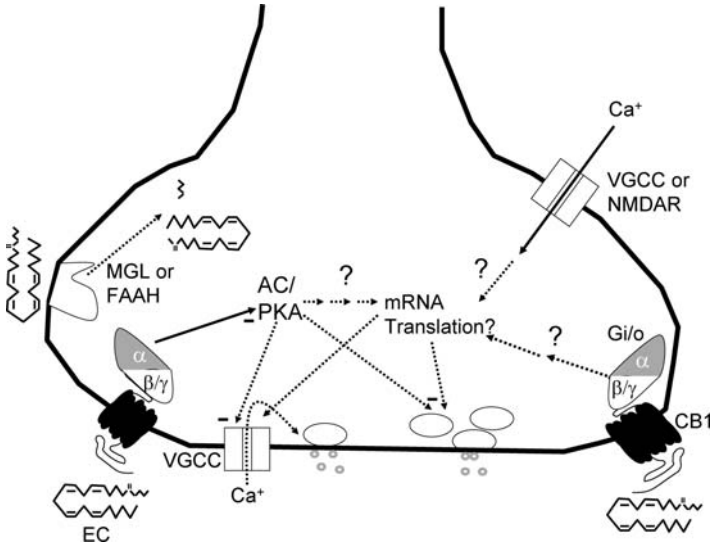

Fig. 5 Mechanisms that potentially contribute to presynaptic expression of endocannabinoid-mediated LTD. Activation of the presynaptic CB1 receptor may snyergize with other signals in the presynaptic terminal to induce a long-lasting decrease in probability of neurotransmitter release. The CB1-mediated stimulation of Gi/o dissociation likely inhibits adenylate cyclase (AC) which would, in turn, inhibits protein kinase A (PKA). Disruption of PKA-mediated phosphorylation may alter calcium channel function or proteins involved in vesicle fusion. Protein translation has recently been implicated in EC-LTD expression, and it is possible that CB1 signaling may impact this process in the presynaptic terminal. Depolarization of the presynaptic terminal during repetitive afferent activation will stimulate calcium entry via VGCCs, and it is possible that the increase in intraterminal calcium stimulates signaling pathways that add to or synergize with the CB1-stimulated signals. In the neocortex, activation of presynaptic NMDARs due to homosynaptic glutamate release provides a signal that interacts with CB1 activation in LTD induction. Once again, calcium entry is likely to mediate this action, as NMDAR channels are highly calcium permeable. The EC responsible for LTD induction is likely removed from the synapse via uptake and ezymatic breakdown within the first few minutes of EC-LTD expression. However, the decrease in probability of release outlasts the EC signal. The mechanisms directly involved in decreased release probability during EC-LTD expression are not yet known, but long-lasting alterations in calcium channel function and/or vesicle fusion are logical candidate mechanisms.

to induce LTD at this synapse. Evidence supporting the prevailing hypothesis of a postsynaptic locus of cerebellar LTD expression was presented in this paper. Thus, the authors suggested a new model for induction of this most widely studied form of LTD. In this model, the retrograde EC signal stimulates production of another anterograde signal that then helps to produce the final postsynaptic mechanism of LTD. These intriguing findings appear to conflict with earlier studies suggesting that induction and expression of cerebellar LTD do not require any presynaptic participation (Narasimhan and Linden 1996). It is possible that endocannabinoids and CB1 receptors have a modulatory role that can impact LTD under certain experimental conditions (see van Beugen et al. 2006 for discussion of one possibility).

Further studies are clearly needed to sort out the endocannabinoid and CB1 roles in cerebellar LTD, as well as in cerebellar learning and memory.

6.4 Physiological Roles of EC-LTD

There are many potential roles for EC-LTD in the modulation of physiology at the cellular and circuit levels. Long-lasting reduction of inhibitory transmission can increase the relative effectiveness of excitatory drive, as we will consider in discussing endocannabinoids and long-term potentiation (Chevaleyre and Castillo 2004). In this manner, EC-LTD may increase excitatory throughput in circuits like those found in the basolateral amygdala, leading to increased net circuit and behavioral output (e.g., as suggested in Marsicano et al. 2002). Within the visual and barrel field areas of neocortex, EC-LTD may participate in deprivation-induced decreases in neuronal responsiveness, indicating a potential role in sensory plasticity during development (Bender et al. 2006; Crozier et al. 2007; Sjostrom et al. 2003). Decreasing the efficacy of excitatory synapses may also serve as a mechanism for extinction or erasure of memory when LTD takes place at previously potentiated synapses (Marsicano et al. 2002). However, EC-LTD likely has more subtle effects that are important for circuit function and information storage. For example, decreasing efficacy of excitatory inputs may increase the need for spatial or temporal summation in order to reach action potential threshold. This could be important in the maturation of sensory-motor integration in brain regions such as the striatum where such a mechanism might prevent unwanted movements (Choi et al. 1997a; Tang et al. 2001). Long-lasting synaptic depression has also been suggested to be an effective mechanism for equalizing the relative efficacy of synapses on different parts of a large dendritic arbor (Rumsey and Abbott 2006). For example, an EC-LTD-like decrease in synaptic strength at proximal synapses might render them equieffective with distal synapses in terms of their ability to drive the neuron to threshold. Alterations in the efficacy of inhibitory synapses made by local-circuit interneurons may also regulate the timing of circuit input-output relationships. Additional studies of the in vivo impact of EC-dependent synaptic plasticity will be needed to evaluate these possibilities.

7 Endocannabinoids and Long-Term Potentiation

Endocannabinoids have also been suggested to participate in long-term synaptic potentiation (LTP), a widely recognized model of synaptic changes underlying learning and memory (Malenka and Bear 2004). Prior to discovery of the CB1 receptor, one study indicated that Δ9-THC alters hippocampal LTP (Nowicky et al. 1987). Activation of CB1 receptors by 2-AG was shown to prevent LTP induction at Schaffer collateral-CA1 pyramidal cell synapses in hippocampus (Stella et al.

1997; Slanina et al. 2005). This inhibition most likely results from suppression of glutamate release at these synapses that decreases the excitatory drive necessary to induce LTP (Misner and Sullivan 1999). Thus, it is presently thought that ECs can modulate LTP in this manner, but are not strictly necessary for this form of plasticity.

Subsequent studies demonstrated another mechanism through which ECs modulate LTP. It has long been known that reducing synaptic inhibition enhances the likelihood of LTP induction (Wigstrom and Gustafsson 1986). The observation that EC retrograde signaling reduces GABAergic inhibition of CA1 pyramidal neurons via DSI and EC-LTD stimulated investigators to explore the possibility that this disinhibition could promote LTP induction. Indeed, Carlson et al. (2002) demonstrated that LTP was more easily induced while DSI was ongoing, and that this effect was blocked by a CB1 antagonist. Subsequently, Chevaleyre and Castillo (2004) showed that EC-LTD of GABAergic synapses could promote LTP induction in a "metaplastic" manner in the hippocampal CA1 region. The term metaplasticity refers to a change in the threshold for plasticity based on the past history of plasticity at that same synapse (Abraham and Bear 1996). In this case, the long-lasting decrease in inhibitory transmission during EC-LTD allowed for stronger excitation during repetitive synaptic activation, thus lowering the stimulus threshold needed for LTP induction. These findings have been somewhat controversial because other investigators report that CB1 antagonists promote LTP (Slanina et al. 2005), rather than having the LTP-blocking effect associated with metaplastic disinhibition (Carlson et al. 2002; Chevaleyre and Castillo 2004). Endocannabinoids and CB1 receptors may participate differently in LTP depending on the relative level of EC release at excitatory versus inhibitory synapses during repetitive synaptic activation. The relative activity of different afferent inputs likely determines which synapses produce ECs. Clearly, there is much work to be done in sorting out the modulatory and metaplastic effects of ECs.

8 Endocannabinoids, CB1 Receptors, and Behavior

Endocannabinoids have now been implicated in a variety of brain functions. For example, EC levels increase with exposure to stressful stimuli, and may play a role in neuroadaptations to stress and well as in neural function and behaviors related to anxiety and affective states (Bortolato et al. 2006; Connell et al. 2006; Gobbi et al. 2005; Hohmann et al. 2005).

8.1 Endocannabinoid and CB1 Roles in Learning and Memory

In light of the evidence presented above that endocannabinoids and CB1 play crucial roles in synaptic plasticity, it is interesting to examine their roles in learning and memory. It has long been known that THC and other exogenous CB1 agonists

impair learning and memory (Hampson and Deadwyler, 1998; Miller and Branconnier, 1983; Sullivan, 2000), particularly on tasks that involve the hippocampus. However, CB1 agonists will activate all receptors when given peripherally, and all receptors within a given brain region when applied intracerebrally. In contrast, endocannabinoids will likely act on only a subset of synapses within a given brain region during the learning and memory process. Thus, it is important to examine the role of endocannabinoids in learning and memory using experimental tools such as selective CB1 antagonists, CB1 knockout animals, and drugs that prolong endocannabinoid actions at the synapse. This work has been undertaken in several laboratories with mixed results.

CB1 antagonists impair learning in an aversive conditioning paradigm when injected into the medial prefrontal cortex, and CB1 agonists have the opposite effect (Laviolette and Grace 2005). In addition, CB1 receptors have been implicated in extinction of fear conditioning, which is believed to be an active unlearning process (Marsicano et al. 2002). Extinction is blocked by peripheral injection of CB1 antagonist, and is impaired in the CB1-/- mouse. Subsequent studies have also implicated CB1 receptors in extinction in both aversively motivated and appetitively motivated tasks (Suzuki et al. 2004; Varvel and Lichtman 2002; Varvel et al. 2005). In general, these studies suggest the endocannabinoids and CB1 receptors participate in formation of certain types of memories.

Considerable evidence that endocannabinoid actions in the brain impair learning and memory has also been collected. Most of the evidence comes from studies in which CB1 antagonists improve learning or memory (Lichtman 2000; Shiflett et al. 2004; Terranova et al. 2005; Wolf and Leander 2003). These antagonists can also reverse the amnestic effects of agents such as β-amyloid and scopolamine (Mazzola et al. 2003; Takahashi et al. 2005). Thus, it appears that activation of CB1 receptors, presumably following release of brain endocannabinoids, has actions that counteract mechanisms involved in learning and memory.

Little is currently known about the mechanisms that underlie the memory-promoting and memory-impairing functions of endocannabinoids and CB1 receptors. Marsicano and coworkers have attempted to link extinction of fear conditioning to EC-LTD at inhibitory synapses in the basolateral amygdala. However, it is still not clear if activation of CB1 receptors in this brain region is crucial for extinction, and other brain regions could be involved. Facilitation of LTP induction by DSI or EC-LTD in the hippocampal CA1 region has been suggested as a mechanism by which endocannabinoids and CB1 receptors participate in spatial learning and other forms of hippocampal-based memory (Carlson et al. 2003; Chevaleyre and Castillo 2004). It has also been demonstrated that CB1 antagonists block LTD induction in vivo (de Oliveira Alvares et al. 2006). Clearly, behavioral studies are needed to determine if CB1 receptors participate in hippocampal memory formation.

It is tempting to speculate that disinhibitory effects of endocannabinoids would promote learning, while endocannabinoid-mediated reductions in excitatory tranmission underly amnestic effects. Thus, the role of the endocannabinoid system in learning and memory would depend on which synaptic inputs within a given brain region contain the receptor, and which inputs are activated during a given phase of

learning and memory. However, this disinhibition = memory, inhibition = impaired memory idea is almost certainly too simple. Within the complex circuitry of brain regions such as the cerebellum, hippocampus, neocortex, and striatum both excitatory and inhibitory synapses are likely to participate in learning as well as in supression of learning and memory. Clearly a systematic investigation of the endocannabinoid and CB1 receptor actions within different brain regions during the many varieties of learning and memory is needed to sort out their roles in brain information storage.

8.2 The Neural Basis of Cannabinoid Intoxication

The CB1 agonist Δ9-THC is of course the major psychoactive ingredient in cannabinoid drugs such as marijuana and hashish, and thus discussion of the neural role of CB1 receptors brings to mind questions about acute THC intoxication. The mechanisms underlying the cannabis high and the reinforcing effects of cannabis are not fully understood. Marijuana use produces feelings of stress relief and relaxation in regular users, although the drug can also be anxiogenic, especially in first time users (Parolaro et al. 2005). Blockade of acute marijuana intoxication by SR141716A indicates that these effects are mediated by CB1 receptors (Huestis et al. 2001). Likewise, drug discrimination studies in rodents indicate that the subjective effects of acute THC are mediated by CB1 receptors (Tanda et al. 2003; Rinaldi-Carmona et al. 1994; Mansbach et al. 1996; Perio et al. 1996). From these findings it seems clear that activation of CB1 is crucial to the marijuana high.

There has been some debate as to whether cannabinoid drugs are truly reinforcing, but recent studies indicate that animals will self-administer THC, and will even perform tasks in order to receive self-administration into the VTA and shell of the nucleus accumbens (Tanda et al., 2000; Justinova et al., 2003 refs, Zangen et al. 2006). Adminstration of THC also lowers the threshold for intracranial self-stimulation (Gardner et al., 1988), and can be used as a stimulus for conditioned place preference (Valjent and Maldonado 2000). These findings indicate that cannabinoid drugs are indeed reinforcing. However, negative findings have been reported in both self-administration and conditioned place preference studies (reviewed by Parolaro et al. 2005), and thus it is worth considering what factors may affect THC action in these paradigms.

Most discussion of the neuronal mechanisms underlying acute intoxication and reinforcement has centered on the activation of dopamine release by THC and other CB1 agonists. Indeed, these agonists stimulate the activity of neurons in the VTA and increase extracellular dopamine levels in nucleus accumbens (Ng Cheong Ton et al., 1988; French, 1997; Tanda et al., 1997). It is reasonable to speculate that the increase in dopamine contributes to the reinforcing effects of THC, but the subjective high feeling is more difficult to ascribe to this mechanism. The stress-relieving effects of the drug may in and of themselves enhance relaxation and feelings of well-being, and these mechanisms may involve interactions with GABAergic and glutamatergic transmission in brain regions like the BLA. Interestingly, the

opiate receptor antagonist naloxone blocks THC-induced increases in extracellular dopamine (Tanda et al. 1997). Opiate receptor agonists certainly produce intoxication involving strong feelings of well-being, and thus it is possibly that enkephalin or endorphin actions contribute to the cannabinoid high. Ultimately, it will be interesting to determine which brain regions and neurotransmitters participate in the subjective effects of THC. This question could be approached experimentally using the drug discrimination procedure. Indeed, one clue as to the role of GABA in acute intoxication comes from the finding that benzodiazepines produce partial substitution in animals trained to discriminate THC (Wiley and Martin 1999). The tools now seem to be in place to settle this, and other important issues concerning the neural effects of cannabinoids and endocannabinoids in the very near future.

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Presynaptic Ionotropic Receptors

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Abstract The release of transmitters through vesicle exocytosis from nerve terminals is not constant but is subject to modulation by various mechanisms, including prior activity at the synapse and the presence of neurotransmitters or neuromodulators in the synapse. Instantaneous responses of postsynaptic cells to released transmitters are mediated by ionotropic receptors. In contrast to metabotropic receptors, ionotropic receptors mediate the actions of agonists in a transient manner within milliseconds to seconds. Nevertheless, transmitters can control vesicle exocytosis not only via slowly acting metabotropic, but also via fast acting ionotropic receptors located at the presynaptic nerve terminals. In fact, members of the following subfamilies of ionotropic receptors have been found to control transmitter release: ATP P2X, nicotinic acetylcholine, $GABA_A$, ionotropic glutamate, glycine, 5-HT_3, and

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vanilloid receptors. As these receptors display greatly diverging structural and functional features, a variety of different mechanisms are involved in the regulation of transmitter release via presynaptic ionotropic receptors. This text gives an overview of presynaptic ionotropic receptors and briefly summarizes the events involved in transmitter release to finally delineate the most important signaling mechanisms that mediate the effects of presynaptic ionotropic receptor activation. Finally, a few examples are presented to exemplify the physiological and pharmacological relevance of presynaptic ionotropic receptors.

1 Introduction

Information transfer between two neurons or between neurons and effector cells involves the release of chemical substances, which then act on the target cell by binding to appropriate receptors embedded in the plasma membrane. This process, as originally described by Otto Loewi (Loewi 1921), is termed chemical neurotransmission and occurs at contact sites known as synapses. Neurotransmitters exert their effects via members of two major families of receptors: ionotropic and metabotropic neurotransmitter receptors. Activation of ionotropic receptors leads to an increase in the ion conductance of the membrane within a time scale of milliseconds or even less, whereas activation of metabotropic receptors results in slow effects (within seconds or even minutes) which involve more or less complex signaling cascades. Accordingly, information transfer via ionotropic receptors is called fast synaptic transmission, whereas the slow counterpart is called neuromodulation (Kaczmarek and Levitan 1987).

Alterations in the strength of neurotransmission at various synapses underlie complex functions of the nervous system such as learning and memory (Kandel 2001). There are two major types of changes in neurotransmission that occur at single synapses: one depends on the prior activity in the synapse, and the other one is caused by mediators released from neurons or glial cells (Zucker and Regehr 2002). In both cases, alterations in the strength of synaptic transmission may be caused either by changes in the release of the chemical transmitters or by changes in the sensitivity of the according receptors. As these alterations arise at either side of the synapse, they are subsumed under pre- and postsynaptic modulation of synaptic transmission, respectively. Typically, the phenomena of pre- and postsynaptic modulation are experimentally observed on a time scale of seconds or minutes rather than milliseconds and thus represent one prominent aspect of neuromodulation. In support of this categorization, the mechanisms involved are in most instances metabotropic ones.

Presynaptic modulation by neurotransmitters is mediated via receptors, which are accordingly named “presynaptic receptors.”. Is, therefore, a presynaptic receptor only a receptor activation of which modulates transmitter release? The answer

is no, because activation of a presynaptic receptor may also affect transmitter reuptake via the appropriate carrier proteins (Zahniser and Doolen 2001). This latter effect does not necessarily impinge on transmitter release, unless the carriers are involved in the release process, which is then nonvesicular (Attwell et al. 1993). In this review, however, we will only consider transmitter release mediated by vesicle exocytosis. Hence, by functional means a presynaptic receptor is one, activation of which modulates one or more of the various functions of a presynapse. which also include endocytosis and neurotransmitter synthesis or metabolism. Nevertheless, here we will only deal with receptors that influence the amount of transmitter being released from a nerve terminal.

In morphological terms, presynaptic receptors are those located at the presynapse, i.e., at the neuronal structures from which the neurotransmitters are released. There, one can find a large number of neurotransmitter-containing vesicles which are clustered at specialized regions of the presynapse, called "active zones." At these active zones, vesicle exocytosis takes place, but the active zones occupy only a small part of an entire axon terminal (Südhof 2004). Accordingly, one has to clarify whether a presynaptic receptor is necessarily located at or close to the active zone, or whether it can also be located somewhere else at the nerve terminal. In addition, one has to consider receptors that are found at the axon in close proximity to the nerve terminal. Finally, on a micromorphological level one also has to certify that a receptor within a synapse is indeed embedded at the presynaptic, but not the postsynaptic, side. Thus, it is not easy to define a presynaptic receptor by morphological means. As a consequence, various techniques have been employed to functionally verify whether a receptor is indeed a presynaptic one: (1) the use of synaptosomes (Whittaker 1993); (2) the destruction of nerve terminals by lesioning of the corresponding neuronal cell bodies and the subsequent demonstration of a loss of function or loss of ligand binding; (3) the use of dissociated neurons in cell cultures in which postsynaptic structures are lacking; (4) finally, the blockade of voltage-activated Na^+ and/or Ca^{2+} channels. This latter procedure is particularly important when characterizing presynaptic ionotropic receptors. The Na^+ channels mediate the propagation of action potentials down the axons into the presynaptic nerve terminals, and the Ca^{2+} channels link the depolarization of the nerve terminal caused by an invading action potential to transmembrane Ca^{2+} entry, which then triggers exocytosis. Vesicle exocytosis typically requires intracellular Ca^{2+} concentrations in the high micromolar range; due to limited diffusion of Ca^{2+} ions in neurons, such concentrations are restricted to the sites of transmembrane Ca^{2+} entry (Augustine 2001). Accordingly, physical interactions between the SNARE proteins syntaxin and SNAP 25, which are involved in vesicle exocytosis and Ca^{2+} channels of the Ca_V2 family, provide the proximity between the sites of Ca^{2+} entry and vesicle exocytosis (Zamponi 2003). If this interaction is disrupted, action potential-dependent vesicle exocytosis is greatly reduced (Mochida et al. 1996). Hence, if transmitter release is triggered by the activation of a transmitter-gated ion channel and this effect is insensitive towards a blockade of voltage-gated Ca^{2+} channels (e.g., by Cd^{2+}), the ionotropic receptor involved must be Ca^{2+}-permeable and in close proximity

to the sites of vesicle exocytosis. If this effect is sensitive to a blockade of Ca^{2+} channels but insensitive towards a blockade of voltage-gated Na^{+} channels (e.g., by tetrodotoxin), the receptor must also be near to the sites of exocytosis because the local depolarization due to the activation of that ligand-gated ion channel suffices to activate the Ca^{2+} channels that mediate the depolarization-dependent exocytosis. If, however, the effect is sensitive to a blockade of voltage-gated Na^{+} channels, the ionotropic receptor is too far away from the sites of exocytosis, and the propagation of action potentials is required. In this latter case, it is questionable whether these ionotropic receptors are truly presynaptic ones.

In the light of this latter possibility, one also has to consider the term 'preterminal', which means pre-presynaptic. As specified by this latter expression, this designates a receptor that is located at regions of the axons which are close to the terminals, but not at the terminals themselves. In parallel with the above considerations, the distinction between presynaptic and preterminal receptors cannot be made by morphological criteria. Again, the use of synaptosomes and the blockade of Na^{+}-dependent action potentials can help to prevent the contribution of preterminal receptors to presynaptic modulation via ionotropic receptors (Figure 2a).

Although evidence for effects of agonists at ionotropic receptors on nerve terminals was reported more than 80 years ago (Dixon 1924), the first mention of the specific term "presynaptic receptor" was apparently provided by Riker et al (1957), who detected stimulatory effects of quarternary ammonium compounds at the neuromuscular junction. In the same year, Frank and Fuortes (1957) furthered the concept of functional presynaptic ionotropic receptors by implicating γ-aminobutyric acid (GABA) in the reduction of transmitter release from primary afferent nerve endings in the spinal cord. Simultaneously, Trendelenburg (1957) demonstrated that morphine reduced contractions of the nictitating mebrane through a presynaptic site of action, thereby setting the scene for presynaptic metabotropic receptors. Hence, the presynaptic modulation of transmitter release via both ionotropic and metabotropic receptors has been a focus of research for five decades. During the first half of this time period, reports concerning presynaptic G protein-coupled receptors prevailed (see, e.g., Langer 1977; Starke 1981), but more recently the importance of presynaptic transmitter-gated ion channels has been appreciated. Accordingly, several excellent reviews describing presynaptic ionotropic receptors have been published during the last decade (McGehee and Role 1996; MacDermott et al. 1999; Engelman and MacDermott 2004). In particular, the detailed review by MacDermott and colleagues (1999) gives a full account of all the presynaptic ionotropic receptors known at that time, and a shorter update has appeared more recently (Engelman and MacDermott 2004). Therefore, this review does not aim at listing every report that has described some kind of presynaptic ionotropic receptor. We rather try to highlight recent developments in the field of presynaptic transmitter-gated ion channels as a counterbalance to the five preceding chapters of this book which deal with presynaptic metabotropic receptors.

2 Types of Presynaptic Ionotropic Receptors

The superfamily of ionotropic receptors can be subclassified according to several principles: by structural means, by functional criteria, and according to the neurotransmitters by which they are activated. Transmitter- and ligand-gated ion channels are the prototypic examples of oligomeric receptors (Figure 1). They are composed of several subunits, and depending on the number of subunits contained within one functional receptor one can discern between three groups: $GABA_A$ receptors, glycine receptors (GlyR), nicotinic acetylcholine receptors (nAChRs), and 5-HT_3 serotonin receptors are composed of 5 subunits; the ionotropic glutamate receptors and TRP channels display tetrameric structures; and ATP-gated P2X receptors contain only 3 subunits each. The single subunits contributing to the build-up of members of each of these different receptor classes also show diverging structural characteristics. The pentameric receptors are also called Cys-loop receptors, as each subunit contains a sequence of 13 residues flanked by cysteines which covalently bind to each other and thereby form a closed loop that is situated between the ligand-binding domain and the receptor ionophore. These subunits have a large N-terminal extracellular domain responsible for ligand binding, four transmembrane regions, a large intracellular loop between transmembrane segments 3 and 4, and a short C-terminus. The second transmembrane domain of each of the five subunits contributes to the formation of the ionophore (Lester et al. 2004). The subunits of ionotropic glutamate receptors, in contrast, possess only three transmembrane regions and one re-entrant loop each. The re-entrant loop contributes to the formation of the ionophore and thus determines the functional properties of the receptor (Madden 2002). Subunits for TRPV channels also contain one re-entrant loop contributing to the pore and six transmembrane segments (Liedtke and Kim 2005). Finally, ATP P2X receptors are characterized by two putative transmembrane

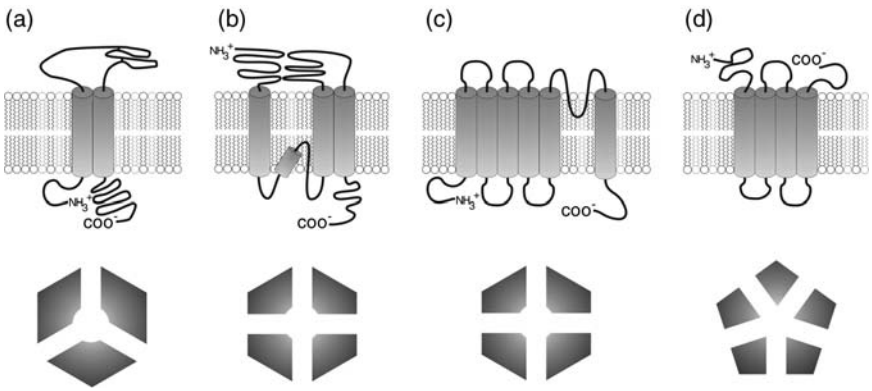

Fig. 1 Structures of subunits (top) and subunit oligomerization (bottom) in ligand-gated ion channels. (**a**) P2X receptor family. (**b**) Ionotropic glutamate receptor family. (**c**) TRPV channel family. (**d**) Cys-loop superfamily comprising nAChRs, 5-HT_3, $GABA_A$, and glycine receptors.

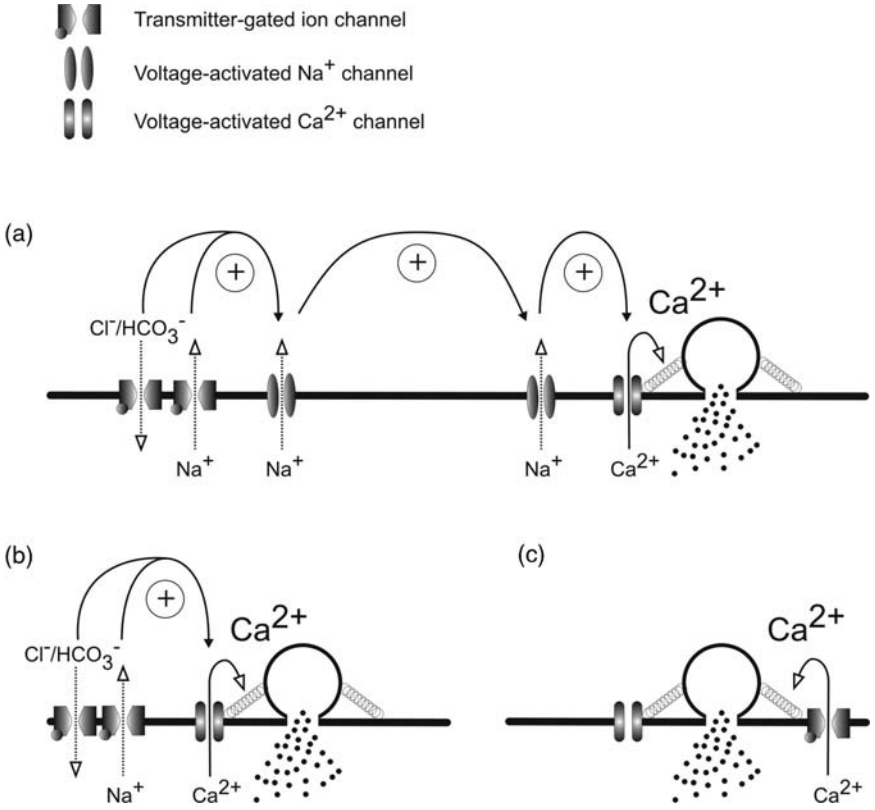

Fig. 2 Increase in spontaneous transmitter release caused by the activation of ionotropic receptors. (**a**) Activation of a **preterminal** transmitter-gated anion or cation channel depolarizes the membrane either through influx of cations or efflux of anions. The depolarization gates voltage-activated Na^+ channels and thereby triggers action potentials which then gate voltage-activated Ca^{2+} channels at the active zone, and the rise in intraterminal Ca^{2+} leads to enhanced vesicle exocytosis. (**b**) Activation of **presynaptic** transmitter-gated anion or cation channels depolarizes the presynaptic membrane as described above and thereby gates voltage-activated Ca^{2+} channels at the active zone. (**c**) Activation of a **presynaptic** transmitter-gated cation channel with a high Ca^{2+} permeability directly raises intraterminal Ca^{2+} at the active zone and thus leads to Ca^{2+}-dependent vesicle exocytosis.

segments, which are believed to form the ionophore and which are linked by a large extracellular loop with 10 conserved cysteines that may build disulfide bridges (Khakh and North 2006).

When comparing transmitter-gated ion channels on a functional level, one can discern between anion and cation channels. The former ones comprise the $GABA_A$ and glycine receptors, which display a rank order of anion selectivity of $I^- > Br^- > Cl^-$ and which are also permeable to HCO_3^-. All other ionotropic receptors mentioned here are cation channels, which discriminate rather poorly between various monovalent cations, at least when compared with voltage-gated ion channels. Some

transmitter-gated cation channels are not only highly permeable to divalent cations (Rogers and Dani 1995), but also to anions (Khakh and North 2006). As the functional characteristics, in particular the ion selectivities of the ionotropic receptors, are of utmost importance for the presynaptic modulation of transmitter release, the receptors will be first categorized by this parameter and subsequently according to their activating neurotransmitter.

2.1 Presynaptic Anion Channels

2.1.1 $GABA_A$ Receptors

γ-Aminobutyric acid is viewed as the major inhibitory transmitter in the brain, and a large part of its action is mediated by ionotropic GABA receptors. As mentioned above, these receptors are composed of five out of a repertoire of at least 19 different subunits which are named by greek letters (α_1 to α_6, β_1 to β_3, γ_1 to γ_3, δ, ε, θ, and ρ_1 to ρ_3). Most $GABA_A$ receptors are made up of α, β, and γ subunits. These subunits are differentially targeted to synaptic and extrasynaptic sites, and depending on the composition of the receptors, they display diverging pharmacological properties (Korpi and Sinkkonen 2006). Thus, synaptic and extrasynaptic $GABA_A$ receptors may not only mediate phasic and tonic inhibition, respectively, they can also be affected by specific agents. Receptors containing ρ subunits are designated $GABA_C$ receptors and are pharmacologically different from $GABA_A$ receptors in terms of agonist and antagonist sensitivity (Zhang et al. 2001). Presynaptic inhibition via $GABA_A$ receptors has been demonstrated for the first time more than 40 years ago at the crayfish neuromuscular junction (Dudel and Kuffler 1961), but unequivocal evidence in support of presynaptic modulation via $GABA_C$ receptors has been missing for a long time (Kirischuk et al. 2003). Although a large number of reports have described the various functions of presynaptic $GABA_A$ receptors (MacDermott et al. 1999), the molecular composition and the pharmacological characteristics of such presynaptic receptors remained largely obscure.

In accordance with the general notion that GABA is an inhibitory transmitter, activation of presynaptic $GABA_A$ receptors has been found to reduce depolarization-evoked release of various transmitters (Table 1). This inhibitory action was mainly observed in conjunction with action potential-evoked release (Cerrito et al. 1998; Jang et al. 2002; Belenky et al. 2003; Axmacher and Draguhn 2004; Axmacher et al. 2004). However, in the case of presynaptic $GABA_A$ autoreceptors at hippocampal synapses, their activation did not only reduce action potential-evoked, but also spontaneous, GABA release (Axmacher and Draguhn 2004), and the underlying mechanism was proposed to be a hyperpolarization.

With monoamine (Bonanno and Raitreri 1987; Cerrito et al. 1998) and glycine (Jang et al. 2002) release, however, activation of presynaptic $GABA_A$ receptors was found to enhance spontaneous, but to inhibit stimulated, release. The effect on monoamine release was accompanied by $GABA_A$ receptor-mediated increases

Table 1 Examples of Presynaptic $GABA_A$ and Glycine Receptors

Receptor	Spont.	Na^+	Ca^{2+}	Stim.	Transmitter	Location	References
$GABA_A$	↑	no	yes	n.d.	glutamate	hippocampus	(Jang et al. 2006)
$GABA_A$	↑	yes	yes	↑ / ↓	glutamate	hippocampus	(Jang et al. 2005)
$GABA_A$	↑	yes	yes	n.d.	glutamate	locus coeruleus	(Koga et al. 2005)
$GABA_A$	n.d.			↑	glutamate	brain stem	(Turecek and Trussell 2002)
$GABA_A$	↓	no	n.d.	↓	GABA	hippocampus	(Axmacher and Draguhn 2004; Axmacher et al. 2004)
$GABA_A$	n.d.			↓	GABA	suprachiasmatic n.	(Belenky et al. 2003)
$GABA_A$	↑	no	yes	↓	glycine	spinal cord	(Jang et al. 2002)
$GABA_A$	↑	n.d.	n.d.	↓	NA, DA, 5-HT	cerebellum	(Cerrito et al. 1998)
GlyR	↑	no	yes	↑	glutamate	brain stem	(Turecek and Trussell 2001)
GlyR	↑	yes	yes	↑	GABA	VTA	(Zheng and Johnson 2001; Ye et al. 2004)
GlyR	↓			↓	GABA	VTA	(Ye et al. 2004)
GlyR	↑	yes	yes	↑	glycine	spinal cord	(Jeong et al. 2003)

Na^+, are voltage-activated Na^+ channels involved?; Ca^{2+}, are voltage-activated Ca^{2+} channels involved?; ↑, increase; ↓, decrease; n.d., not determined; NA, noradrenaline; DA, dopamine; VTA, ventral tegmental area.

in Ca^{2+} entry, which were mimicked by depolarizing K^+ concentrations (Cerrito et al. 1998). The increase in spontaneous glycine release from spinal cord neurons triggered by $GABA_A$ agonists was abolished by the addition of Cd^{2+} and by the removal of extracellular Ca^{2+}, thus indicating that Ca^{2+} entry via voltage-gated Ca^{2+} channels was involved. However, blockade of action potentials by tetrodotoxin had no such effect (Jang et al. 2002). Together, these results indicate that the $GABA_A$ receptor mediated a depolarization of the nerve terminals. $GABA_A$ receptor activation was also found to enhance depolarization-evoked noradrenaline release, but this latter effect may not only involve an activation of $GABA_A$ receptors, but also an action at neurotransmitter transporters (Bonanno et al. 1989; Rosenstein et al. 1990). The functions of $GABA_A$ receptors are modulated by various agents, such as benzodiazepines and barbiturates (Rudolph and Möhler 2004). Accordingly, the facilitation of noradrenaline release via presynaptic $GABA_A$ receptors was reported to be enhanced by diazepam (Martire et al, 2002).

In hippocampal mossy fibers, activation of presynaptic $GABA_A$ receptors augmented basal, but decreased depolarization-evoked, increases in intraaxonal Ca^{2+} concentrations. An antagonist at these receptors left basal Ca^{2+} unchanged, but also reduced depolarization-dependent rises in Ca^{2+} (Ruiz et al. 2003). These results suggested (1) that these presynaptic $GABA_A$ receptors are tonically activated, (2) that they mediate a depolarizing action, and (3) that activation of presynaptic $GABA_A$ receptors may exert opposite effects on transmitter release depending on the concentration of the agonist, as discussed in Section 2.2.3, for presynaptic kainate receptors (see Schmitz et al. 2001).

In the locus coeruleus (Koga et al. 2005) and the hippocampus (Jang et al. 2006), presynaptic $GABA_A$ receptor activation facilitated spontaneous glutamate release via Ca^{2+} influx through voltage-gated Ca^{2+} channels. At Schaffer collaterals, presynaptic $GABA_A$ receptors were also found to mediate a facilitation of spontaneous glutamate release in a tetrodotoxin-sensitive and Ca^{2+}-dependent manner, and in that case a $GABA_A$ receptor-mediated increase in action potential-evoked release was also observed (Jang et al. 2005). An enhancement of stimulated glutamate release via $GABA_A$ receptors was also observed at the giant presynaptic nerve terminals of the calyx of Held (Turecek and Trussell 2002). Most recently, evidence has been presented that presynaptic $GABA_C$ receptors control synaptic transmission in the retina and thereby contribute to the fine-tuning of the light response (Sagdullaev et al. 2006).

2.1.2 Glycine Receptors

Glycine receptors are ligand-gated anion channels closely related to $GABA_A$ receptors. They are composed of α and β subunits. Currently, at least four different α subunits (α1 to α4) and one β subunit are known, and alternative splicing of these may contribute to additional heterogeneity. α And β subunits form hetero-oligomers with a stoichiometry of $2\alpha : 3\beta$, but α subunits can also form homomeric receptors. As with $GABA_A$ receptors, glycine receptor subunits are targeted to synaptic and extrasynaptic sites in order to mediate phasic and tonic effects, respectively (Betz and Laube 2006).

Presynaptic glycine receptors were found to operate at several synapses (Table 1). In the spinal cord, for instance, presynaptic glycine autoreceptors mediated a facilitation of spontaneous glycine release in a tetrodotoxin- and Cd^{2+}-sensitive manner, which is indicative of presynaptic depolarization. An enhancing effect was also observed with action potential-evoked glycine release (Jeong et al. 2003).

At the calyx of Held in the medial nucleus of the trapezoid body, activation of presynaptic glycine receptors was also shown to facilitate spontaneous glutamate release and to enhance amplitudes of evoked excitatory postsynaptic currents (Turecek and Trussell 2001). The action on spontaneous postsynaptic currents were insensitive towards a blockade of Na^+ channels by tetrodotoxin, but abolished by the Ca^{2+} channel blocker Cd^{2+}. This indicated that glycine acted by causing a depolarization, which was also corroborated by direct voltage measurements.

In the ventral tegmentum of adult rats, glycine was originally found to reduce the frequency of spontaneous GABAergic inhibitory postynaptic potentials (Zheng and Johnson 2001). In more recent experiments on dopaminergic ventral tegmental neurons of newborn rats, however, activation of presynaptic glycine receptors by either glycine or taurine augmented spontaneous as well as evoked GABA release. These effects were antagonized by strychnine, which on its own caused effects opposite to those of the agonists. The glycine-induced increase in spontaneous GABA release was tetrodotoxin- and Cd^{2+}-sensitive, thus indicating a role for glycine receptor-mediated depolarizations. In older animals, glycine inhibited spontaneous

as well as evoked GABA release, and strychnine exerted reverse actions (Ye et al. 2004). Hence, the actions of presynaptic glycine receptors may be developmentally regulated.

2.2 Presynaptic Cation Channels

2.2.1 Nicotinic Acetycholine Receptors

Nicotinic acetylcholine receptors (nAChRs) are acetylcholine-gated cation channels composed of five subunits, each having four membrane spanning domains. Currently, at least 17 different nicotinic receptor subunits are known which are named by Greek letters (α_1 to α_{10}, β_1 to β_4, γ, δ, ε). The α_1, β_1, γ, δ, ε subunits are found in the musculature, whereas all other subunits are present in the nervous system. The acetylcholine binding sites of the receptors involve the N-termini of α subunits, and they can either assemble to form functional homomers or coassemble with β subunits at a 2:3 ratio to build heteromeric receptors (Lukas et al. 1999; Gotti et al. 2006). The most widely expressed nAChR subunits in the brain are α_4, α_7, and β_2 and the predominating native nicotinic receptor in the central nervous system is an α_4/β_2 containing heteropentamer.

In the peripheral (Wessler 1989) as well as central (Wonnacott 1997) nervous system, presynaptic nicotinic autoreceptors were reported to control the release of acetylcholine. In both locations, the consequence of presynaptic nAChR activation most commonly is an increase in both spontaneous and evoked acetylcholine release (MacDermott et al. 1999), whereas presynaptic muscarinic receptors mediate the opposite effect, an autoinhibition. Recent studies have focused on the composition of presynaptic nAChRs (Table 2). In the hippocampus, nicotinic autoreceptors were suggested to be α_3/β_4 receptors (Tani et al. 1998), but a role of β_2 subunits has also been implicated (Lloyd et al. 1998). Likewise, in the neocortex, presynaptic nicotinic autoreceptors are likely to be α_4/β_2 receptors (Marchi et al. 2002). In contrast, in the interpeduncular nucleus the autoreceptors were suggested to mainly contain α_3 and β_4 subunits (Grady et al. 2001).

In addition to the autoreceptor function as described above, presynaptic nAChRs were reported to operate as heteroreceptors, which control the release of various transmitters including glutamate and GABA (Table 2). Early evidence for a presynaptic enhancement of glutamatergic neurotransmission by nicotine acting at nAChRs had been obtained in the medial habenula (McGehee et al. 1995) and in the hippocampus (Gray et al. 1996). There, short applications of nicotine enhanced spontaneous as well as action potential-evoked excitatory postsynaptic currents in a transient Ca^{2+}-dependent and tetrodotoxin-insensitive manner, and the receptors involved were suggested to be α_7 (Radcliffe and Dani 1998). Subsequently, presynaptic nAChRs containing α_4/β_3 subunits were found to enhance spontaneous glutamate release in the hippocampal CA1 region (Alkondon and Albuquerque 2002). A role of α_7 nAChRs in the presynaptic regulation of glutamate release was

Table 2 Examples of Presynaptic nAChRs

Receptor	Spont.	Na^+	Ca^{2+}	Stim.	Transmitter	Location	References
α2β4	↑	n.d.	n.d.	n.d.	ACh	hippocampus	(Lloyd et al. 1998)
α3β4	↑	n.d.	n.d.	n.d.	ACh	interpeduncular n.	(Grady et al. 2001)
α4β2	↑	n.d.	n.d.	n.d.	ACh	hippocampus	(Tani et al. 1998)
α4β2	↑	n.d.	n.d.	n.d.	ACh	cortex	(Marchi et al. 2002)
nAChR	↑	no	n.d.	n.d.	glutamate	area postrema	(Funahashi et al. 2004b)
α4β3	↑	no	n.d.	n.d.	glutamate	hippocampus	(Alkondon and Albuquerque 2002)
α7	↑	yes	n.d.	n.d.	glutamate	cerebellum	(Reno et al. 2004)
α7	↑	no	n.d.	↑	glutamate	amygdala	(Alkondon et al. 1996; Barazangi and Role 2001)
α7	↑	no	n.d.	↑	glutamate	hippocampus	(Radcliffe and Dani 1998)
α7	↑	yes	n.d.	n.d.	glutamate	hippocampus	(Barik and Wonnacott 2006)
α7	↑	n.d.	n.d.	↑	aspartate	cortex	(Marchi et al. 2002; Rousseau et al. 2005)
α?β2	↑	no	yes/no	↑	GABA	thalamus	(Lena and Changeux 1997)
non-α7	↑	no	yes	↑	GABA	locus coeruleus	(Seddik et al. 2006)
non-α7	↑	yes	yes	n.d.	GABA	interpeduncular n.	(Lena et al. 1993)
α4β2	↑	no	n.d.	n.d.	GABA	subst. gelatinosa	(Rashid et al. 2006)
α4β2	↑	yes	n.d.	n.d.	GABA	hippocampus	(Alkondon and Albuquerque 2001)
α4β2	↑	no	no	n.d.	glycine	subst. gelatinosa	(Kiyosawa et al. 2001)
α3β4	↑	yes	n.d.	n.d.	NA	hippocampus	(Sacaan et al. 1995; Luo et al. 1998)
α?β2	↑	n.d.	n.d.	n.d.	DA	striatum, olfactory tubercle, n. accumbens, cortex	(Grady et al. 2002)
α3β2	↑	no	n.d.	n.d.	DA	striatum	(Sacaan et al. 1995; Luo et al. 1998)
α3β4*	↑	n.d.	n.d.	n.d.	DA	hippocampus	(Cao et al. 2005b)
α4β2*	↑	no	n.d.	n.d.	DA	prefrontal cortex	(Cao et al. 2005a)
α6β2β3 α4α6β2β3 α4β2 α4α5β2	↑	n.d.	n.d.	n.d.	DA	striatum	(Luetje 2004; Salminen et al. 2004; Cao et al. 2005a)
α7	↑	n.d.	n.d.	n.d.	DA	striatum	(Kaiser and Wonnacott 2000)
α4β2*	↑	no	n.d.	n.d.	5-HT	striatum	(Reuben and Clarke 2000)

*additional unidentified subunits are involved; Na^+, are voltage-activated Na^+ channels involved?; Ca^{2+}, are voltage-activated Ca^{2+} channels involved?; ↑, increase; ↓, decrease; n.d., not determined; ACh, acetylcholine; NA, noradrenaline; DA, dopamine.

confirmed by indirect means in experiments originally determining noradrenaline release (Barik and Wonnacott 2006). Presynaptic facilitation of glutamate release via nAChRs has also been observed in the area postrema (Funahashi et al. 2004b), in the amygdala (Barazangi and Role 2001), in the cerebellum (Reno et al. 2004), and in olfactory bulb neurons (Alkondon et al. 1996).

In the hippocampus, activation of presynaptic nAChRs was also reported to enhance spontaneous GABAergic synaptic currents, and depending on the nature of the postsynaptic neuron, the receptors involved were either α_7 or α_4/β_2 (Alkondon and Albuquerque 2001). Before, nAChRs that did not contain α_7 subunits were found to facilitate spontaneous GABA release in a tetrodotoxin-sensitive manner in the interpeduncular nucleus (Lena et al. 1993). In the thalamus, nAChRs containing β_2 subunits enhanced spontaneous and stimulation-evoked GABA release. The former effect was tetrodotoxin-insensitive, and depending on the types of neurons investigated, it was either Cd^{2+}-sensitive or not (Lena and Changeux 1997). At synapses between olfactory bulb and amygdala neurons, presynaptic nAChRs also mediated an increase in spontaneous as well as action potential-evoked GABA release (Barazangi and Role 2001). In the spinal cord, spontaneous GABA release onto preganglionic sympathetic neurons was augmented by acetylcholine in a Cd^{2+}-sensitive, but not tetrodotoxin-sensitive, manner. The receptor subtype involved was devoid of α_7 subunits and also mediated an increase in evoked synaptic GABA responses (Seddik et al. 2006). In substantia gelatinosa neurons of the lumbar spinal cord, activation of α_4/β_2 receptors facilitated not only spontaneous GABA (Rashid et al. 2006), but also glycine (Kiyosawa et al. 2001) release in a tetrodotoxin-insensitive manner.

A facilitation of release via presynaptic nAChRs was also observed for catecholamines. About 40 years ago, Loffelholz (1970) showed that acetylcholine and nicotinic agonists applied to sympathetically innervated organs caused noradrenaline release (see, e.g., Starke 1977; Fuder and Muscholl 1995). More recently, activation of nAChRs was found to stimulate noradrenaline release in the hippocampus (Sacaan et al. 1995). The noradrenergic input of the hippocampus originates in the locus coeruleus, and locus coeruleus neurons with large somata projecting to the hippocampus were reported to express nAChRs mainly comprised of $\alpha6\beta3\beta2$ subunits. The remaining small-diameter locus coeruleus neurons, in contrast, contain predominantly $\alpha3\beta4$ subunits (Lena et al. 1999). However, evidence has been presented that these latter receptor types were also mediating noradrenaline release in the hippocampus (Luo et al. 1998). Finally, presynaptic nAChRs that regulate noradrenaline release in the hippocampus were found to be composed of differing subunits in different species (Azam and McIntosh 2006).

The control of dopamine release via presynaptic nAChRs has been investigated in great detail. Originally, modulation of dopamine release via nAChRs has been revealed in striatal synaptosomes (Rapier et al. 1990). More recently, the receptors involved have been documented to contain α_4, α_5, α_6, β_2, and β_3 subunits (Luetje 2004; Salminen et al. 2004). Although α_7 containing receptors were found to contribute to the facilitation of dopamine release, this was due to an indirect effect mediated by increases in glutamate release (Kaiser and Wonnacott 2000). In other

brain areas, including the prefrontal cortex (Cao et al. 2005a), the hippocampus (Cao et al. 2005b), the olfactory tubercle, and the thalamus (Grady et al. 2002), presynaptic nAChRs were also reported to control dopamine release. In addition, presynaptic regulation of 5-HT release via nAChRs has also been observed (Reuben and Clarke 2000).

2.2.2 5-HT_3 Serotonin Receptors

Among the seven families of serotonin receptors, only the 5-HT_3 receptors are ligand-gated ion channels, whereas all others are G protein-coupled receptors (Barnes and Sharp 1999). 5-HT_3 receptors are rather unselective cation channels with a high permeability for Ca^{2+} (Ronde and Nichols 1998). In the central nervous system, they are expressed abundantly in the neocortex, the anterior olfactory nucleus, the hippocampus, and the amygdala. In addition, 5-HT_3 receptors are present in the caudate putamen and the nucleus accumbens, and, at high levels, in the brain stem (Parker et al. 1996; Morales et al. 1998; Chameau and van Hooft 2006).

In rodents, two different isoforms of the 5-HT_3 receptor exist, termed 5-HT_{3A} and 5-HT_{3B}. While the majority of 5-HT_3 receptors, in particular in the central nervous system, seem to be homomers of 5-HT_{3A} subunits, heteromers may exist as well, particularly in the peripheral nervous system. Nevertheless, the expression and functional relevance of 5-HT_{3B} subunits remain to be resolved (Morales and Wang 2002; van Hooft and Yakel 2003). In addition, several novel subunits have been cloned from other species, including humans, but their functional relevance still needs to be established (Karnovsky et al. 2003; Niesler et al. 2003).

In the hippocampus and the amygdala, 5-HT_3 receptors are expressed in a subset of GABAergic neurons (Kawa 1994; Morales et al. 1996; Koyama et al. 2000; Koyama et al. 2002; Katsurabayashi et al. 2003; Turner et al. 2004; Somogyi and Klausberger 2005; Dorostkar and Boehm 2007) and were found to control GABA release (Table 3). Receptor activation transiently increased the frequency of miniature inhibitory postsynaptic currents reflecting a presynaptic facilitation of spontaneous GABA release (Kawa 1994; McMahon and Kauer 1997; Koyama et al. 2000; Koyama et al. 2002; Turner et al. 2004; Dorostkar and Boehm 2007). In the hippocampus (Turner et al. 2004; Dorostkar and Boehm 2007), but not in the amygdala (Koyama et al. 2000), this effect was sensitive to a blockade of voltage-gated Ca^{2+} channels by Cd^{2+}. In contrast to spontaneous release, presynaptic 5-HT_3 receptor activation was reported to inhibit action-potential-dependent GABA release (Dorostkar and Boehm 2007). In cortical neurons, activation presynaptic 5-HT_3 receptors also facilitated spontaneous GABA release (Zhou and Hablitz 1999; Ferezou et al. 2002; Puig et al. 2004). A Cd^{2+}-insensitive facilitation of glutamate release due to presynaptic 5-HT_3 receptor activation has been observed in the area postrema (Funahashi et al. 2004a).

In the cortex, 5-HT_3 receptor activation was shown to inhibit stimulation-evoked acetylcholine release (Barnes et al. 1989; Crespi et al. 1997), thus corroborating

Table 3 Examples of Presynaptic 5-HT_3 Feceptors

Receptor	Spont.	Na^+	Ca^{2+}	Stim.	Transmitter	Location	References
5-HT_3	↑	no	no	n.d.	glutamate	area postrema	(Funahashi et al. 2004a)
5-HT_3	↑	no	yes	n.d.	GABA	hippocampus	(Kawa 1994; McMahon and Kauer 1997; Koyama et al. 2000)
5-HT_3	↑	no	no	n.d.	GABA	hippocampus	(Turner et al. 2004)
5-HT_3	↑	no	yes	↓	GABA	hippocampus	(Dorostkar and Boehm 2007)
5-HT_3	↑	no	no	↓	GABA	amygdala	(Koyama et al. 2002)
5-HT_3	↑	n.d.	n.d.	n.d.	GABA	cortex	(Zhou and Hablitz 1999)
5-HT_3	n.d.			↓	ACh	cortex	(Crespi et al. 1997)
5-HT_3	↑	no	n.d.	↑	NA	hippocampus hypothalamus	(Mongeau et al. 1994)
5-HT_3	↑	partly	n.d.	n.d.	DA	striatum	(Blandina et al. 1989)

Na^+, are voltage-activated Na^+ channels involved?; Ca^{2+}, are voltage-activated Ca^{2+} channels involved?; ↑, increase; ↓, decrease; n.d., not determined; ACh, acetylcholine; NA, noradrenaline; DA, dopamine.

inhibitory effects of presynaptic 5-HT_3 receptor activation on evoked release. However, in subsequent experiments an effect of 5-HT_3 receptor ligands could not be confirmed (Johnson et al. 1993).

With respect to catecholamines, 5-HT_3 receptor activation was found to mediate an enhancement of dopamine release in the striatum (Blandina et al. 1989), but contradictory results have also been presented (Crespi et al. 1997). The question of whether presynaptic 5-HT_3 receptors control noradrenaline release also remains controversial: 5-HT_3 ligands were reported to either stimulate or inhibit noradrenaline release in perfused hearts (Fozard and Ali 1978; Göthert and Dührsen 1979) and to increase evoked noradrenaline release in brain slices (Mongeau et al. 1994). However, these results must be interpreted with caution as 5-HT_3 ligands may interfere with presynaptic α_2 autoreceptors (Allgaier et al. 1995).

Similarly, presynaptic 5-HT_3 receptors were suggested to control 5-HT release in various brain areas (Blier and Bouchard 1993), but this has also been disputed afterwards (Crespi et al. 1997). Taken together, with the exception of consistent reports concerning the GABAergic system, the possible roles and functions of presynaptic 5-HT_3 receptors remains somewhat controversial (van Hooft and Vijverberg 2000).

2.2.3 Ionotropic Glutamate Receptors

As mentioned above, members of the superfamily of ionotropic glutamate receptors are tetramers composed out of a repertoire of at least 18 subunits. They can be categorized into three families: N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropioinic acid (AMPA) receptors, and kainate receptors, the two latter forming the group of non-NMDA receptors. While NMDA receptors are composed of NR1, −2 (A, B, C, and D), and −3 (A and B)

subunits, AMPA receptors are made up of GlurA to GlurD (or Glur1 to Glur4) and kainate receptors of Glur5 to Glur7 and KA-1 and −2 subunits (Dingledine et al. 1999; Madden 2002). In addition, there are glutamate receptor δ1 and δ2 subunits, the roles of which in the function of ionotropic glutamate receptors are only beginning to be elucidated (Hirai et al. 2003). Subsequently, we will separately deal with presynaptic ionotropic glutamate receptors: NMDA, AMPA, and kainate. Thereafter, a role of presynaptic glutamate transporters will be discussed in brief.

NMDA Receptors

Among the ionotropic glutamate receptors, the NMDA receptors are characterized by co-activation by glutamate and glycine, slow gating, weak desensitization, a voltage-dependent block of the ionophore by Mg^{2+}, and a high Ca^{2+} permeability (Dingledine et al. 1999). This latter feature renders this receptor subtype particularly interesting with respect to the presynaptic modulation of transmitter release (Table 4). In fact, NMDA receptors were reported to act as presynaptic autoreceptors mediating a tonic facilitation of spontaneous glutamate release in the entorhinal cortex (Berretta and Jones 1996). This effect was tetrodotoxin-insensitive and involved receptors containing the NR2B subunit. Evoked glutamate release is also controlled by presynaptic NMDA receptors. They appear to mediate a frequency-dependent facilitation: evoked excitatory postsynaptic currents are enhanced when the stimulation frequency is raised from 0.5 Hz to 3 Hz, and this enhancement is blocked by NMDA receptor antagonists (Berretta and Jones 1996; Woodhall et al. 2001). In cortical neurons, NR2B containing presynaptic NMDA receptors mediate akin actions on glutamate release (Sjostrom et al. 2003). Interestingly, the function of presynaptic NMDA autoreceptors declines with increasing age (Yang et al. 2006). At Schaffer collaterals, presynaptic NMDA receptors containing NR2A subunits were also found to positively modulate excitatory postsynaptic potentials (Suarez et al. 2005; Suarez and Solis 2006). Moreover, a facilitation of glutamate release via presynaptic NMDA autoreceptors was confirmed in hippocampal synaptosomes (Breukel et al. 1998).

However, presynaptic NMDA autoreceptors may also mediate opposite effects, i.e., a reduction of glutamate release. At parallel fiber Purkinje cell synapses, activation of presynaptic NMDA receptors caused significant reductions in excitatory postsynaptic currents (Casado et al. 2002). Likewise, spontaneous and evoked glutamate release from the nerve endings of primary afferents in the spinal cord was reduced when presynaptic NMDA receptors were activated (Bardoni et al. 2004). In contrast to this, substance P release at such synapses was shown to be enhanced by the activation of presynaptic NMDA receptors (Liu et al. 1997).

Presynaptic NMDA receptors have also been reported to control GABA release. At interneuron synapses onto Purkinje neurons, activation of NMDA receptors raised spontaneous GABA release, and this effect lasted for more than 10 minutes; voltage-gated Ca^{2+} channels were not involved, but a blockade of ryanodine receptors attenuated the enhancement of GABA release and restricted the effect by time

Table 4 Examples of Presynaptic NMDA and AMPA Receptors

Receptor	Spont.	Na^+	Ca^{2+}	Stim.	Transmitter	Location	References
NR2A	n.d.		no	↑	glutamate	hippocampus	(Suarez et al. 2005; Suarez and Solis 2006)
NMDA				↑	glutamate	hippocampus	(Breukel et al. 1998)
NR1/NR2B	↑	no	yes	↑	glutamate	entorhinal cortex	(Berretta and Jones 1996; Woodhall et al. 2001)
NR2B	↑	no	n.d.	↑	glutamate	cortex	(Sjostrom et al. 2003)
NMDA	n.d.			↓	glutamate	cerebellum	(Casado et al. 2002)
NMDA	↓	no	n.d.	↓	glutamate	dorsal horn	(Bardoni et al. 2004)
NMDA	↑	no	no	n.d.	GABA	cerebellum	(Duguid and Smart 2004)
NMDA	↑	no		↓	GABA	*Xenopus* retinotectum	(Lien et al. 2006)
NMDA	↑	yes	n.d.	n.d.	NA	cortex	(Fink et al. 1989)
NMDA	↑	n.d.	n.d.	n.d.	DA	striatum	(Petrov et al. 2001)
NR2A	↑	no	n.d.	n.d.	ACh	zebrafish endplate	(Todd et al. 2004)
KA							
NMDA	↑	n.d.	n.d.	n.d.	substance P	dorsal horn	(Liu et al. 1997)
AMPA	n.d.			↓	glutamate	calyx of Held	(Takago et al. 2005)
AMPA	n.d.			↓	glutamate	dorsal horn	(Lee et al. 2002a)
AMPA	↑	yes	n.d.	n.d.	GABA	hippocampus	(Vignes 2001)
GluR5							
AMPA	↑	no	n.d.	↓	GABA	cerebellum	(Satake et al. 2004; Rusakov et al. 2005)
AMPA	↑	no	no	↓	GABA, glycine	dorsal horn	(Engelman et al. 2006)
AMPA, NMDA	↑	no	n.d.	↑	NA	spinal cord	(Sundström et al. 1998)

Na^+, are voltage-activated Na^+ channels involved?; Ca^{2+}, are voltage-activated Ca^{2+} channels involved?; ↑, increase; ↓, decrease; n.d., not determined; ACh, acetylcholine; NA, noradrenaline; DA, dopamine.

to last for less than 2 minutes only (Duguid and Smart 2004). Interestingly, a depolarization of the postsynaptic Purkinje neurons also led to the activation of the presynaptic NMDA receptors, indicating that these can be activated by glutamate as a retrograde messenger. This latter phenomenon was called depolarization-induced potentiation of inhibition (Duguid and Smart 2004). In the retinotectal system of *Xenopus* tadpoles, presynaptic NMDA receptor activation enhanced spontaneous GABA release, as reflected by an increase in the frequency of miniature inhibitory postsynaptic currents; simultaneously, amplitudes of action potential-dependent inhibitory postsynaptic currents were reduced (Lien et al. 2006).

NMDA receptor ligands were also observed to control the release of catecholamines via a presynaptic site of action. Noradrenaline release in the cortex and hippocampus, for instance, was enhanced by NMDA agonists, and this effect was augmented by glycine (Fink et al. 1989; Pittaluga and Raiteri 1990). In the spinal cord, NMDA also facilitated noradrenaline release in a partially tetrodotoxin-sensitive manner (Sundström et al. 1998). In the striatum, NMDA agonists were found to increase dopamine release (Petrov et al. 2001).

AMPA Receptors

In contrast to NMDA receptors, AMPA receptors gate instantaneously, desensitize rapidly, and are much less permeable to Ca^{2+} (Dingledine et al. 1999). Despite these characteristics, which are rather unfavorable for mediating a presynaptic modulation, various actions of presynaptic AMPA receptors have been described (Table 4). Activation of AMPA autoreceptors has been detected to cause a presynaptic inhibition of excitatory postsynaptic currents at the calyx of Held synapse in the brainstem (Takago et al. 2005). In cultured hippocampal neurons, AMPA receptor activation tonically facilitated spontaneous GABA release in the absence of tetrodotoxin (Vignes 2001). In contrast, GABA release in the cerebellum was reduced when presynaptic AMPA heteroreceptors were activated (Satake et al. 2004; Rusakov et al. 2005). At synapses on dorsal horn neurons of the spinal cord, however, AMPA receptor activation increased the spontaneous release of GABA and glycine in a tetrodotoxin- and Cd^{2+}-insensitive manner. Evoked inhibitory postsynaptic currents were simultaneously inhibited by AMPA receptor activation (Engelman et al. 2006). At the very same location, presynaptic AMPA receptor activation was reported to also inhibit evoked glutamate release (Lee et al. 2002a).

Release of transmitters other than glutamate or GABA has also been found to be subject to a regulation via presynaptic AMPA receptors. Noradrenaline release, as one example, was reported to be enhanced when presynaptic AMPA receptors were activated (Sundström et al. 1998), and evidence has been presented that the release of other amines is also regulated by AMPA receptors (Ohta et al. 1994).

Kainate Receptors

In terms of functional characteristics, kainate receptors are similar to AMPA receptors, displaying rapid gating and desensitization. The major difference lies in the recovery from desensitization, which takes about 10 times longer for kainate receptors (Dingledine et al. 1999). For kainate receptors, a large variety of presynaptic functions have been described (Table 5).

Initial evidence for presynaptic kainate autoreceptors was obtained 25 years ago (Ferkany et al. 1982). Although they were originally found to stimulate glutamate release in the hippocampus, later evidence suggested that these presynaptic receptors were inhibitory rather than stimulatory (Chittajallu et al. 1996). It is now clear that hippocampal presynaptic kainate autoreceptors work in a sophisticated manner: at the very same synapse, nanomolar concentrations of kainate receptor agonists enhanced glutamate release, whereas micromolar concentrations elicited an inhibition (Schmitz et al. 2001). Furthermore, within the hippocampus the consequences of presynaptic kainate receptor activation depended on the synapse under investigation: Evoked glutamate release from mossy fibers onto CA3 neurons was inhibited via receptors containing GluR6 subunits, whereas glutamate release from the perforant path onto these neurons was facilitated via receptors with GluR5 and GluR6 subunits. Spontaneous glutamate release onto CA3 neurons was facilitated via receptors

Table 5 Examples of Presynaptic Kainate Receptors

Receptor	Spont.	Na^+	Ca^{2+}	Stim.	Transmitte	Location	References
GluR5	n.d.			↓	glutamate	hippocampus	(Partovi and Frerking 2006)
GluR5, GluR6/(5)	↓	n.d.	n.d.	↓	glutamate	dorsal horn	(Kerchner et al. 2001b; Kerchner et al. 2002)
KA2	n.d.	n.d.	n.d.	↑ / ↓	glutamate	hippocampus	(Schmitz et al. 2001; Contractor et al. 2003)
GluR6	↔			↓ / ↑	glutamate	hippocampus	(Contractor et al. 2000)
GluR5	↑	no	n.d.	↑			
GluR6	n.d.			↓	glutamate	hippocamus	(Partovi and Frerking 2006)
GluR5/GluR6 or GluR5/KA2	↓	no		↓	GABA	hippocampus	(Clarke et al. 1997; Rodriguez-Moreno et al. 1997; Rodriguez-Moreno and Lerma 1998; Christensen et al. 2004)
non-GluR5	↑	no	no	↑	GABA	hippocampus	(Cossart et al. 2001)
GluR5, AMPA	↑	yes	n.d.	n.d.	GABA	hippocampus	(Vignes 2001)
KA	↑	no	n.d.	↑	GABA	hypothalamus	(Liu et al. 1999)
GluR5 KA	↑	no	yes	↑ / ↓	GABA, glycine	dorsal horn	(Kerchner et al. 2001a; Xu et al. 2006)
GluR5	↑ / ↓	no	no	↑ / ↓	GABA	basolat. amygdala	(Braga et al. 2003)

Na^+, are voltage-activated Na^+ channels involved?; Ca^{2+}, are voltage-activated Ca^{2+} channels involved?; ↑, increase; ↓, decrease; n.d., not determined.

containing GluR5 subunits (Contractor et al. 2000). Additional investigations also tried to identify the receptor subunits involved in the presynaptic modulation of glutamate release via kainate receptors, and evidence has been presented for a role of both GluR5 (Lauri et al. 2003; Partovi and Frerking 2006) and GluR6 (Perkinton and Sihra 1999) subunits. In cortical synaptosomes, activation of kainate receptors with GluR6 subunits facilitated evoked glutamate release (Perkinton and Sihra 1999). In the cerebellum, presynaptic kainate receptors located on parallel fibers mediate differing effects depending on the postsynaptic neuron: with Purkinje cells only a facilitation of evoked glutamate release was observed; with stellate cells, moderate receptor activation caused an enhancement and strong receptor activation an inhibition (Delaney and Jahr 2002). In the spinal cord, spontaneous and evoked glutamate release from primary afferent fibers was decreased by presynaptic kainate receptor activation, and the receptors involved were suggested to contain GluR5 subunits (Kerchner et al. 2001b; Kerchner et al. 2002).

Presynaptic kainate receptors have also been reported to control GABA release and to mediate opposing effects. In the hippocampus, spontaneous and evoked

release of GABA was originally found to be inhibited by kainate (Clarke et al. 1997; Cunha et al. 1997; Rodriguez-Moreno et al. 1997; Rodriguez-Moreno and Lerma 1998; Clarke and Collingridge 2004). Later on, however, low kainate receptor agonist concentrations were reported to facilitate inhibitory postsynaptic currents, while high concentrations caused the opposite effect (Jiang et al. 2001). The presynaptic receptor subtypes involved were suggested to be heteromers of either GluR6 or KA2 subunits together with GluR5 subunits (Christensen et al. 2004). A facilitation of GABA release via presynaptic kainate receptors was also observed at synapses between different interneurons in the hippocampus, and both spontaneous and evoked GABA release were affected (Cossart et al. 2001). The effect of activated kainate receptors on spontaneous inhibitory synaptic currents in the hippocampus depended on the functions of voltage-gated sodium channels: the frequency of spontaneous inhibitory postsynaptic currents in the absence of tetrodotoxin was increased by kainate receptor activation, while the frequency of miniature inhibitory postsynaptic currents was decreased (Maingret et al. 2005). Apparently contradictory effects of kainate receptor agonists on GABA release can be explained by two distinct receptor subtypes being involved: an inhibitory receptor with high affinity for glutamate and a stimulatory receptor with lower affinity (Rodriguez-Moreno et al. 2000).

In dorsal horn neurons, spontaneous release of GABA and glycine was facilitated by kainate in a tetrodotoxin-insensitive but Cd^{2+}-sensitive manner. Evoked inhibitory postsynaptic currents were also modulated biphasically: low concentrations of kainate caused a facilitation, whereas higher concentrations caused an inhibition. This inhibition, however, was suggested to involve $GABA_B$ receptors (Kerchner et al. 2001a). The facilitatory mechanism has been shown to be mediated by GluR5 containing receptors (Xu et al. 2006).

In the basolateral amygdala, the effect of presynaptic GluR5 receptor activation also depended on the agonist concentration: at low concentrations, spontaneous and evoked GABA release was facilitated, the first in a tetrodotoxin- and Cd^{2+}-insensitive manner; at high agonist concentrations, both effects were reversed to inhibition. The receptors involved were suggested to be various GluR5-containing heteromers (Braga et al. 2003). In hypothalamic neurons, presynaptic kainate receptor activation facilitated spontaneous and evoked GABA release (Liu et al. 1999).

Presynaptic kainate receptors control not only the release of glutamate and GABA.. In the striatum, for instance, GluR6 activation facilitated adenosine release, which then acted onto A_{2A} receptors to inhibit evoked GABA release, whereas spontaneous GABA release was directly facilitated via kainate receptors (Chergui et al. 2000). At neuromuscular junctions of zebrafish larvae, activation of NR2A and kainate receptors increased spontaneous acetylcholine release (Todd et al. 2004).

Excitatory Amino Acid Transporters

The postsynaptic actions of transmitters being released from presynaptic nerve terminals depend not only on the kinetics and the extent of vesicle exocytosis, but also on the functions of reuptake mechanisms, which are provided inter alia by

transporter proteins embedded in the presynaptic plasma membrane. At glutamatergic synapses, reuptake is mediated by members of the family of excitatory amino acid transporters (Amara and Fontana 2002). Interestingly enough, all neurotransmitter transporters mediate not only the reuptake of previously released transmitters, but simultaneously provide a significant ion conductance. Accordingly, currents through these proteins can be measured, and therefore these transporters can also be viewed as transmitter-gated ion channels (Gerstbrein and Sitte 2006). Excitatory amino acid transporters 4 and 5 provide particularly large Cl^- conductances (Amara and Fontana 2002), and the latter protein is found in the retina. There, the excitatory amino acid transporter 5 was found to be activated by endogenously released glutamate and to mediate Cl^--dependent changes in the electrical properties of rod bipolar cells that led to a reduction in transmitter release (Veruki et al. 2006; Wersinger et al. 2006). Hence, the presynaptic excitatory amino acid transporter can be included in the list of ionotropic "receptors."

2.2.4 ATP P2X Receptors

P2X receptors are transmitter-gated ion channels activated by micromolar concentrations of ATP that consist of three out of a repertoire of seven subunits. The P2X subunits may form homomeric and/or heteromeric receptors, which are characterized by their ligand specificity and current kinetics (North 2002) and which all display a high Ca^{2+}-permeability (Khakh and North 2006), thus providing these receptors with a function important for presynaptic modulation (Table 6). First evidence for a role of presynaptic P2X receptors in the regulation of transmitter release was obtained in the spinal cord (Gu and MacDermott 1997) and brain stem (Khakh and Henderson 1998), respectively. In both locations, application of ATP raised the frequency of spontaneous excitatory postsynaptic currents, and this effect was not prevented when Na^+-carried action potentials were blocked. In the brain stem, the activation of presynaptic P2X receptors enhanced spontaneous glutamate release even when voltage-activated Ca^{2+} channels were blocked (Khakh and Henderson 1998), and these results were later corroborated in the spinal cord (Nakatsuka and Gu 2001). There, evoked glutamate release onto dorsal horn neurons was also facilitated by presynaptic P2X receptor activation, and the blockade of ATPases which degrade endogenously released ATP was sufficient achieve receptor activation (Nakatsuka and Gu 2001).

In the brain stem, the ATP facilitation of spontaneous glutamate release in a tetrodotoxin- and Cd^{2+}-insensitive, but Ca^{2+}-dependent, manner was mediated by presynaptic $P2X_3$ homomers (Jin et al. 2004; Shigetomi and Kato 2004). In addition, ATP reduced evoked glutamate release, but this was due to its degradation towards adenosine and subsequent activation of inhibitory A_1 receptors (Kato and Shigetomi 2001). In hypoglossal motoneurons (Ireland et al. 2004) and cortical synaptosomes (Patti et al. 2006), activation of P2X7 receptors was reported to facilitate glutamate release.

Table 6 Examples of Presynaptic P2X Receptors

Receptor	Spont.	Na^+	Ca^{2+}	Stim.	Transmitter	Location	References
P2X	↑	no	yes	n.d.	glutamate	trigeminal mesencephalic n.	(Khakh and Henderson 1998)
P2X	↑	no	no	↑	glutamate	dorsal horn	(Nakatsuka and Gu 2001)
P2X	↑	yes	n.d.	n.d.	glutamate	spinal cord	(Gu and MacDermott 1997)
P2X2	↑	no	n.d.	n.d.	glutamate	hippocampus	(Khakh et al. 2003)
P2X3	↑	no	no	↔	glutamate	n. tractus solitarius	(Kato and Shigetomi 2001; Jin et al. 2004)
P2X7	n.d.			↓	glutamate	hippocampus	(Armstrong et al. 2002)
P2X7	↑	n.d.	n.d.	n.d.	glutamate, aspartate	neocortex	(Patti et al. 2006)
P2X7	↔		n.d.	↑	glutamate	hypoglossus motoneurons	(Ireland et al. 2004)
P2X2	↑	no	no	n.d.	glycine	subst. gelatinosa	(Rhee et al. 2000)
P2X2	↑	partially	partially	n.d.	NA	SCG	(Boehm 1999)
P2X7	↑	n.d.	yes	n.d.	ACh	neuromuscular junction	(Fu and Huang 1994; Moores et al. 2005)

Na^+, are voltage-activated Na^+ channels involved?; Ca^{2+}, are voltage-activated Ca^{2+} channels involved?; ↑, increase; ↓, decrease; n.d., not determined; NA, noradrenaline; ACh, acetylcholine; SCG, superior cervical ganglion.

In the hippocampus, presynaptic $P2X_2$ receptors mediated increases in the frequencies of miniature excitatory postsynaptic currents in stratum radiatum interneurons, but not in pyramidal neurons (Khakh et al. 2003). Glutamate release from hippocampal mossy fibers, in contrast, was found to be inhibited by $P2X_7$ receptor activation (Armstrong et al. 2002).

Evidence for the regulation of the release of inhibitory transmitters via presynaptic P2X receptors has also been obtained. In the spinal cord, $P2X_2$ receptor activation facilitated spontaneous glycine release in a tetrodotoxin- and Cd^{2+}-insensitive, but Ca^{2+}-dependent, manner (Rhee et al. 2000).

ATP is a well-established co-transmitter to noradrenaline in the central (Poelchen et al. 2001) as well as peripheral (von Kugelgen and Starke 1991) nervous system. Therefore, the functions of presynaptic P2X receptors have been investigated in various preparations containing noradrenergic nerve terminals (Cunha and Ribeiro 2000). Early evidence for a stimulation and positive feedback modulation of noradrenaline via presynaptic P2X receptors has been obtained in sympathetic neurons (Boehm 1999). At the neuromuscular junction, ATP stimulated the release of acetylcholine (1994), and this effect was suggested to be mediated by presynaptic $P2X_7$ receptors (Moores et al. 2005).

2.2.5 Vanilloid Receptors (TRPV)

The family of mammalian vanilloid receptors (TRPV) has at least six members and is part of the superfamily of transient receptor potential (TRP) ion channels. TRPV1 is a Ca^{2+}-permeable ion channel that is gated by capsaicin and other vanilloids, by protons, and by heat (Liedtke and Kim 2005). It is thus involved in transmitting sensory information. Accordingly, initial evidence for presynaptic vanilloid receptors has been obtained in sensory neurons (Table 7). The spontaneous release of glutamate from axon terminals of these neurons in the spinal cord was enhanced by capsaicin in a tetrodotoxin-insensitive way (Yang et al. 1998), whereas evoked release was inhibited (Yang et al. 1999). The increase in spontaneous glutamate release was also observed when other activators of TRPV1 were used instead of capsaicin (Yue et al. 2004). After peripheral inflammation, but not in naïve tissue, a TRPV1 antagonist exerted effects opposite to those mentioned above, thus indicating that the presynaptic vanilloid receptors were activated by some endogenous mechanism (Lappin et al. 2006).

In the brain stem, TRPV1 receptor activation, similarly to $P2X_3$ receptor activation (see above), facilitated spontaneous glutamate release in a tetrodotoxin- and Cd^{2+}-insensitive manner, even though the two presynaptic receptors were located on different primary afferent fibres (Jin et al. 2004). Action potential-dependent glutamate release, in contrast, was reduced by TRPV1 activation (Doyle et al. 2002). In paraventricular neurons, TRPV1 receptor activation facilitated spontaneous glutamate release in a tetrodotoxin- and Cd^{2+}-insensitive way, but in that case evoked glutamate release was enhanced too (Li et al. 2004).

According to the above reports, only glutamate release is subject to a modulation by presynaptic vanilloid receptors, as GABA release, for instance, was not affected (Yang et al. 1998). In apparent contradiction, activation of vanilloid receptors was

Table 7 Examples of Presynaptic Vannilloid Receptors

Receptor	Spont.	Na^+	Ca^{2+}	Stim.	Transmitter	Location	References
TRPV1	↑	no	n.d.	↑	glutamate	subst. gelatinosa	(Lappin et al. 2006)
TRPV1	↑	no	n.d.	↓	glutamate	subst. gelatinosa	(Yang et al. 1998; Yang et al. 1999; Yue et al. 2004)
TRPV1	↑	no	no	↑	glutamate	paraventricular nucleus	(Li et al. 2004)
TRPV1	↑	no	no	↓	glutamate	nucleus tractus solitarius	(Doyle et al. 2002; Jin et al. 2004)
TRPV1	↑	no	n.d.	↓	GABA	dorsal motor nucleus of vagus	(Derbenev et al. 2006)
TRPV1	↑	n.d.	n.d.	n.d.	CGRP	trigeminal sensory neurons	(Price et al. 2004)
TRPV1	↑	n.d.	n.d.	n.d.	CGRP	DRG	(Ahluwalia et al. 2003)

Na^+, are voltage-activated Na^+ channels involved?; Ca^{2+}, are voltage-activated Ca^{2+} channels involved?; ↑, increase; ↓, decrease; n.d., not determined; CGRP, calcitonin gene related peptide; DRG, dorsal root ganglion.

reported to control the spontaneous release of GABA in the dorsal motor nucleus of the vagus. However, these effects were blocked by glutamate receptor antagonists and were thus mediated by an enhancement of glutamate release (Derbenev et al. 2006). In cultures of sensory neurons, however, TRPV1 receptor activation was shown to facilitate the release of a transmitter other than glutamate, namely calcitonin gene-related peptide (Ahluwalia et al. 2003).

3 Mechanisms of Action

3.1 Spontaneous and Stimulated Release

In principle, the activation of a presynaptic ionotropic receptor may either increase or decrease the amount of transmitter being released. However, the effect may depend on the situation prior to the activation of the receptor. In this context one has to mention that transmitter release occurs either due to spontaneous fusion of vesicles with the plasma membrane or as a consequence of an event that triggers increases in the intracellular Ca^{2+} concentration at the active zone where vesicle exocytosis takes place. The Ca^{2+} concentrations required to promote exocytosis towards maximal rates lie between approximately 10 and 100 μM (Augustine 2001; Schneggenburger and Neher 2005), depending on the synapse being investigated. These two types of transmitter release are usually named spontaneous and stimulated (or stimulation-evoked) release, respectively.

Several stimulation paradigms are used to experimentally promote transmitter release: (1) the triggering of action potentials by short (micro- to millisecond) depolarizations evoked via intra- or extracellular electrodes; (2) the application of depolarizing K^+ concentrations: this usually takes several seconds, but can be optimized to also determine K^+-evoked release on a subsecond time scale (Turner and Dunlap 1995); (3) the application of drugs that cause depolarization through an opening of Na^+ channels (Bouron and Reuter 1996) or via a blockade of K^+ channels (Raffaelli et al. 2004); (4) flash photolysis of intracellular caged Ca^{2+} (Heidelberger et al. 1994). With the former three types of manipulations, the stimulation will promote exocytotic transmitter release only in the presence of extracellular Ca^{2+} and thus depends on transmembrane Ca^{2+} entry via voltage-gated Ca^{2+} channels (Heidelberger et al. 1994). In the latter case, however, stimulated exocytosis is independent of transmembrane Ca^{2+} entry pathways and thus of the local arrangement of ion channels. Likewise, spontaneous vesicle exocytosis is independent of extracellular Ca^{2+} and transmembrane Ca^{2+} entry (Scanziani et al. 1992; Scholz and Miller 1992). Hence, the difference between spontaneous and stimulated release is the Ca^{2+} dependence of the latter.

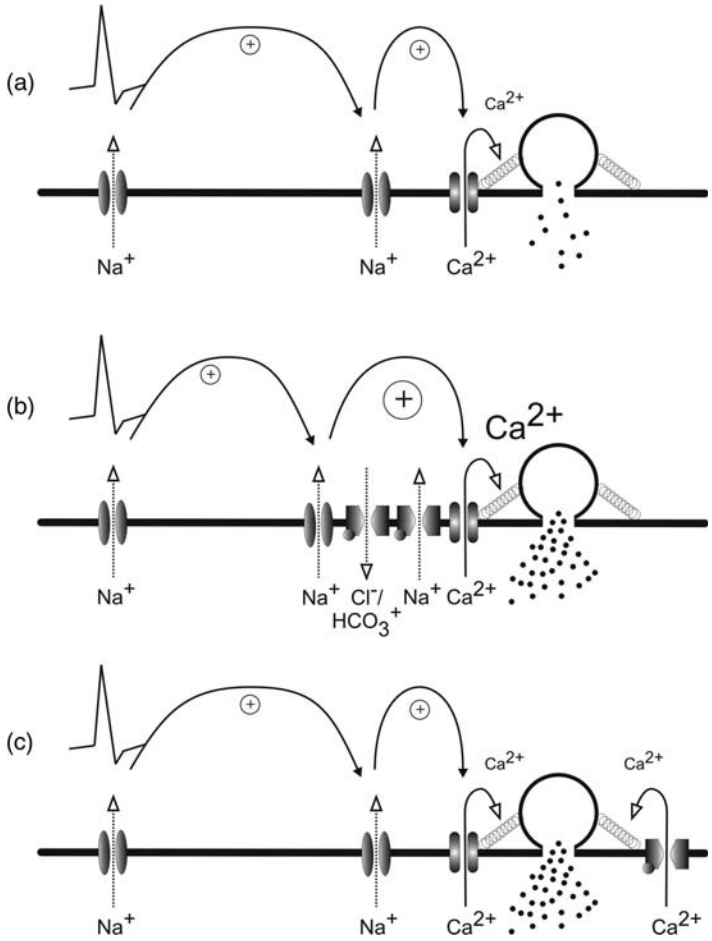

Fig. 3 Facilitation of stimulated transmitter release caused by the activation of ionotropic receptors. (**a**) Action potentials are propagated via voltage-activated Na^+ channels to the presynaptic active zone. There, voltage-activated Ca^{2+} channels are gated, and the rise in intraterminal Ca^{2+} leads to Ca^{2+}-dependent vesicle exocytosis. (**b**) Concurrent activation of presynaptic transmitter-gated anion or cation channels, together with an invading action potential, enhances the depolarization of the presynaptic membrane, which leads to the increased gating of voltage-activated Ca^{2+} channels. The additional rise in intraterminal Ca^{2+} at the active zone may cause further increases in Ca^{2+}-dependent vesicle exocytosis. (**c**) Concurrent activation of presynaptic transmitter-gated cation channels with high Ca^{2+} permeabilities together with invading action potentials leads to an additional rise in intraterminal Ca^{2+} at the active zone which may cause further increases in Ca^{2+}-dependent vesicle exocytosis.

3.2 Excitation-Secretion Coupling

Under physiological conditions, release of neurotransmitters is initiated by a propagating action potential that invades the nerve terminal and triggers vesicle exocytosis

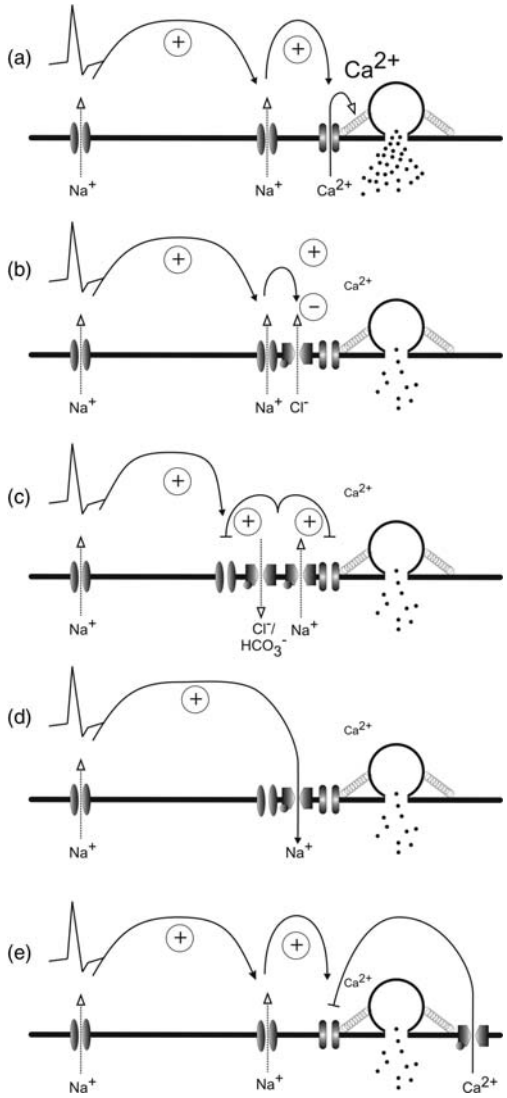

Fig. 4 Inhibition of stimulated transmitter release caused by the activation of ionotropic receptors. (**a**) Action potentials are propagated via voltage-activated Na^+ channels to the presynaptic active zone. There, voltage-activated Ca^{2+} channels are gated, and the rise in intraterminal Ca^{2+} leads to Ca^{2+}-dependent vesicle exocytosis. (**b**) Activation of presynaptic transmitter-gated anion channels hyperpolarizes the presynaptic membrane through anion influx thereby counteracting the depolarization of the invading action potential. This may reduce or even prevent the gating of voltage-activated Ca^{2+} channels at the active zone. (**c**) Activation of presynaptic transmitter-gated anion or cation channels depolarizes the presynaptic membrane and thereby inactivates voltage-activated Na^+ and/or Ca^{2+} channels. Consequently, action potential propagation and/or Ca^{2+} influx at the active zone is prevented. (**d**) Activation of presynaptic transmitter-gated anion or cation channels shunt an invading action potential as the membrane resistance is too low. (**e**) Activation of presynaptic transmitter-gated cation channels with high Ca^{2+} permeabilities raise the intraterminal Ca^{2+} levels and thereby inactivate voltage-activated Ca^{2+} channels, which reduces or prevents action potential-evoked Ca^{2+} entry at the active zone.

within less than 1 millisecond. There are three crucial steps involved in this excitation-secretion coupling (Figures 3a and 4A): (1) the transient depolarization of the nerve terminal by a Na^+-carried action potential; (2) the concomitant transmembrane Ca^{2+} entry due to the opening of voltage-gated Ca^{2+} channels; (3) the rise of free intracellular Ca^{2+} up to high micromolar concentrations which trigger vesicle exocytosis (Augustine 2001; Schneggenburger and Neher 2005). These Ca^{2+} channels are clustered at active zones, and after opening, high Ca^{2+} concentrations accumulate in the proximity of the vesicle fusion machinery (Zhai and Bellen 2004; Schneggenburger and Neher 2005). However, Ca^{2+} elevations due to individual action potentials do not saturate the Ca^{2+} sensor of the vesicle fusion machinery, as sustained micromolar Ca^{2+} concentrations may trigger transmitter release more effectively than Ca^{2+} rises produced by presynaptic action potentials. This and the high cooperativity of Ca^{2+} ions in eliciting vesicle exocytosis suggest that a minor inhibition of voltage-gated Ca^{2+} channels may have pronounced effects on transmitter release (Schneggenburger and Neher 2005). In addition, during a millisecond action potential the presynaptic membrane is just briefly depolarized. Therefore, an only slight inhibition of the gating of Ca^{2+} channels may profoundly affect the presynaptic Ca^{2+} elevation (Garcia et al. 1998). Accordingly, appropriate Ca^{2+} channel blockers reduce or abolish stimulation-evoked release (e.g., Hirning et al. 1988; Scanziani et al. 1992), and so do all other mechanisms that hamper the gating of voltage-gated Ca^{2+} channels involved in excitation-secretion coupling.

Various subtype-specific Ca^{2+} channel blockers were found to be efficient inhibitors of stimulation-evoked transmitter release. For example, the release of monoamines in the hippocampus is greatly reduced by ω-conotoxin GVIA (Dooley et al. 1987), which selectively blocks Ca_V 2.2 Ca^{2+} channels, whereas the release of glutamate involves both, Ca_V 2.2 and Ca_V 2.1 channels (Wheeler et al. 1994). In contrast, the release of GABA at hippocampal synapses is either abolished by a Ca_V 2.2 channel blocking conotoxin, or by a Ca_V 2.1 channel blocking agatoxin, depending on the synapse (Poncer et al. 1997). In addition, there is increasing evidence to suggest that transmitter release may also involve Ca_V 2.3 channels (Kamp et al. 2005). At any rate, Ca_V 2, but not Ca_V 1 or Ca_V 3 Ca^{2+} channels are involved in stimulated transmitter release, and all the members of the Ca_V 2 family interact directly with proteins involved in vesicle exocytosis (Spafford and Zamponi 2003; Kamp et al. 2005). Disruption of the interaction between Ca_V 2 channels and SNARE proteins reduces depolarization-evoked transmitter release (Mochida et al. 1996; Mochida et al. 2003). This suggests that a physical link between the route of transmembrane Ca^{2+} entry and the vesicle exocytosis machinery is important for action potential-dependent transmitter release.

K^+ channels are responsible for setting the resting membrane potential and for the repolarizing phase of action potentials. In addition, K^+ channels mediate afterhyperpolarizations to terminate periods of high neuronal activity and to modulate firing rates. When located in presynaptic nerve terminals, these actions of K^+ channels will contribute to the regulation of transmitter release. In fact, most types of the huge superfamily of K^+ channels, in particular delayed rectifier, fast transient, and Ca^{2+}-sensitive K^+ channels, have been found in a variety of nerve terminals and

there contribute to transmitter release (Meir et al. 1999). In contrast, G protein-gated inward rectifier K^+ channels (GIRK) were reported to mediate hyperpolarizations at somatodendritic regions, but not in presynaptic axon terminals. More recently, transmitter release was suggested to be under the control of presynaptic KCNQ K^+ channels (Martire et al. 2004), although contradictory evidence has also been presented (Kristufek et al. 1999a; Lechner et al. 2003). Interestingly, certain K^+ channel subtypes have also been found to interact with proteins involved in vesicle exocytosis (Michaelevski et al. 2002).

The actions of voltage-activated ion channels in excitation-secretion coupling as described in Section 3.2, will help to reason about signaling mechanisms employed by presynaptic ionotropic receptors. Receptors, the activation of which mimics one of the functions of voltage-activated channels, will mediate the same effect as that channel. In contrast, if receptor activation interferes with one of the actions of the voltage-sensitive channels, the result will be the opposite.

3.3 Ionotropic Mechanisms

Generally, opening of an ion channel located at axon terminals, i.e., directly at or near the sites of vesicle exocytosis, provides an increase in the membrane conductance and may thus cause changes in the membrane potential, i.e., hyper- or depolarization. Depolarization may exert multiple actions on spontaneous as well as stimulated release. With respect to spontaneous release, one has to consider that increasing depolarization will bring the membrane potential closer to and then beyond the activation threshold of voltage-activated Na^+ and Ca^{2+} channels (Figure 2a). Accordingly, increases in depolarizing K^+ concentrations lead to increases in the rate of vesicle exocytosis and transmitter release, whether Na^+ channels are blocked or not (Kristufek et al. 1999a; Dorostkar and Boehm 2007). Only at moderate (25 mM), but not at high (40 mM), K^+ concentrations, the blockade of voltage-gated Na^+ channels by, e.g., tetrodotoxin may cause a reduction in K^+-evoked transmitter release (Kristufek et al. 1999a). This indicates that larger depolarizations per se block action potential propagation ("depolarization block"), but nevertheless permit ongoing Ca^{2+}-triggered vesicle exocytosis which can be abolished by Ca^{2+} channel inhibitors or by the removal of extracellular Ca^{2+}. Hence, if activation of presynaptic ionotropic receptors depolarizes nerve terminals to an extent that is sufficient to open voltage-dependent Na^+ and/or Ca^{2+} channels, this will finally cause transmembrane Ca^{2+} entry and thereby elicit Ca^{2+}-dependent exocytosis (Figure 2b). For presynaptic ligand-gated cation channels, there is an additional possibility of how to raise spontaneous release. If the ionophores of the receptors are sufficiently Ca^{2+}-permeable (Rogers and Dani 1995), their activation will lead to Ca^{2+} influx independently of voltage-activated Ca^{2+} channels (Figure 2c), and the rise in interterminal Ca^{2+} promotes Ca^{2+}-dependent exocytosis.

The mechanisms by which activation of presynaptic ligand-gated cation channels elicits depolarization are obvious: at negative membrane potentials, a more or

less unselective cation conductance leads to influx of positive charges, since permeant cations are equally distributed at both sides of the membrane and thus have an equilibrium potential of about 0 mV. The situation is more complex with regard to ligand-gated anion channels. Whether the activation of an anion, more precisely a Cl^-, conductance results in a hyper- or depolarization depends on the intracellular Cl^- concentration and the resulting Cl^- equilibrium potential. With low intracellular Cl^-, the equilibrium potential is negative to the resting membrane potential. Accordingly, a Cl^- conductance hyperpolarizes the membrane. With high intracellular Cl^-, however, the equilibrium potential is positive to the resting membrane potential. Then, the Cl-conductance depolarizes the membrane (e.g., Staley 1992; Owens et al. 1996). If the Cl^- equilibrium potential is more positive than the threshold for action potential firing, activation of a Cl^- conductance leads to the generation of action potentials (Staley 1992). In spinal cord neurons, for example, inhibition of intracellular Cl^- accumulation (by the $Na^+/K^+/2Cl^-$ cotransporter blocker bumetanide) reduced the enhancement of spontaneous glycine release via $GABA_A$ receptors (Jang et al. 2002). This corroborates the importance of high intracellular Cl^- for stimulatory actions of presynaptic ligand-gated anion channels. In this study, however, evoked glycine release was reduced by presynaptic $GABA_A$ receptor activation. Hence, activation of a Cl^- conductance may have diverging effects on the resting membrane potential and on action potentials, respectively. This might be explained by the fact that an active Cl^- conductance, even with high intracellular Cl^-, provides a Cl^- influx as soon as the membrane voltage during the action potential exceeds the reversal potential for Cl^-. Such a transient Cl^- influx counteracts the full discharge of action potentials and may thereby provide an inhibitory effect. In addition, a depolarization may per se cause opposing effects on spontaneous and action potential-evoked transmitter release, as described further below.

Considering that high intracellular Cl^- concentrations are a prerequisite for the depolarizing action mediated by ligand-gated anion channels, one needs to clarify how intracellular Cl^- is controlled. During the postnatal development of the nervous system, various growth factors drive the expression of the Cl^--extruding Cl^-/K^+ co-transporter KCC2. About two weeks after birth, this switches the depolarizing action of $GABA_A$ receptor activation to a hyperpolarizing one (Rivera et al. 1999; Rivera et al. 2005). Nevertheless, even at later developmental stages, depolarizing $GABA_A$ receptor-mediated effects have been observed. However, in these cases the permeant anion determining the nature of the $GABA_A$ response is HCO_3^- rather than Cl^-, and the intracellular supply of HCO_3^- is mediated by cytosolic carbonic anhydrases (Rivera et al. 2005).

Alternatively, opening of ligand-gated anion channels can elicit membrane hyperpolarizations, and these may lead to an inhibition of transmitter release (Figure 4b). In theory, such an action increases the difference between the resting membrane voltage and the activation threshold of voltage-gated Na^+ or Ca^{2+} channels. In accordance, 20 mV hyperpolarizations rendered single rat sympathetic neurons less excitable, and they fired less action potentials in response to scaled current injections. Nevertheless, despite such hyperpolarizations the overall amount of action potential-mediated transmitter release from these neurons remained unchanged (Lechner et al. 2003).

With respect to spontaneous transmitter release, neither increases in the membrane conductance nor hyerpolarizations can be expected to cause significant changes, as the rate of vesicle exocytosis is only determined by the intracellular Ca^{2+} concentration but not by the membrane potential (Felmy et al. 2003).

Opening of an ion channel located at axon terminals provides an increase in the membrane conductance independently of the charge of the permeating ions. If the increase in the membrane conductance is sufficiently large, action potentials might become shunted and the propagation of an action potential could be hampered or even prevented (Figure 4d). This mechanism could provide an inhibitory effect on action potential-dependent transmitter release, whether the transmitter-gated ion channel is anionic or cationic. However, the conductance changes caused by the activation of $GABA_A$ receptors were found to exert only a minor impact on action potential propagation (Zhang and Jackson 1995). Likewise, simulations of action potentials in synaptic boutons suggested that mere conductance increases are unlikely to shunt action potentials but likely to mediate changes in transmitter release by other mechanisms (Graham and Redman 1994). Nevertheless, there is also evidence in support of the notion that shunting of action potentials may represent the predominant mechanism in the inhibition of action potential-dependent transmitter release (Cattaert and El Manira 1999).

One issue remains to be discussed: how does depolarization affect action potential-evoked transmitter release? As the membrane is increasingly depolarized from -80 to -70, -60, and -50 mV, Ca_V2 channels become slightly activated and permit tiny Ca^{2+} currents and small increases in intraterminal Ca^{2+} concentrations (Figure 3b). This phenomenon does not require activation of Na^+ channels. As a consequence of the intraterminal Ca^{2+} increase, action potential-dependent transmitter release is enhanced with increasing depolarizations (Awatramani et al. 2005). However, if the depolarization exceeds a certain threshold, this facilitatory effect is converted into an inhibition. This becomes evident from the fact that the addition of up to 5 mM K^+ caused an increase in action potential-evoked glutamate release at hippocampal synapses, whereas higher K^+ concentrations (8 to 12 mM) caused an inhibition (Schmitz et al. 2001). Likewise, action potential-evoked GABA release from hippocampal neurons remained largely unaltered when 10 mM K^+ were added, but was almost abolished by the addition of 20 mM K^+ (Dorostkar and Boehm 2007). Thus, depolarizations by up to about 30 mV do not prevent action potential propagation and may lead to an increase in elicited transmitter release due to small increases in intraterminal Ca^{2+} (Figure 3b). Larger depolarizations, however, lead to a voltage-dependent inactivation of Na^+ channels and/or to a voltage- and Ca^{2+}-dependent inactivation of Ca^{2+} channels, and both mechanisms result in an inhibition of action potential-evoked transmitter release (Figure 4c).

In addition to causing presynaptic depolarization, activation of presynaptic ionotropic receptors may provide another way of transmembrane Ca^{2+} entry in order to promote Ca^{2+}-dependent vesicle exocytosis: the Ca^{2+}-permeable ionophores of transmitter-gated cation channels (Rogers and Dani 1995). In this latter case, Ca^{2+} entering the nerve terminal via an ionotropic receptor may bypass voltage-gated Ca^{2+} channels and directly stimulate Ca^{2+}-dependent vesicle exocytosis. If

action potentials invade while such Ca^{2+} permeable receptors are activated, at least two different scenarios can be envisaged: (1) the Ca^{2+} entering the axon terminal via an ionotropic receptor can sum up with the Ca^{2+} entering via voltage-gated Ca^{2+} channels; if the Ca^{2+} sensor for vesicle exocytosis is not saturated (see Section 3.3), this results in an enhancement of action potential-evoked transmitter release (Figure 3c). (2) the Ca^{2+} entering via the ionotropic receptor can lead to Ca^{2+}-dependent inactivation of the voltage-gated Ca^{2+} channels, and the combined Ca^{2+} influx may then be less than the Ca^{2+} entry provided by only the voltage-gated channels; such a mechanism results in an inhibition of action potential-evoked release (Figure 4e). (3) A similar effect can be achieved if the depolarization caused by the activation of the ligand-gated ion channel causes voltage-dependent inactivation of either Na^{+} or Ca^{2+} channels (Figure 4c).

3.4 Metabotropic Mechanisms

Although this review deals with ionotropic receptors, we need to consider some signaling mechanisms associated with G protein-coupled receptors (GPCRs), as these may also be employed by some so-called ionotropic receptors, such as AMPA and kainate receptors. Presynaptic inhibition via GPCRs frequently operates via changes in excitation-secretion coupling. It has been recognized quite some time ago that voltage-gated Ca^{2+} and K^{+} channels are regulated by a variety of G proteins (Brown and Birnbaumer 1990). A large number of neurotransmitters and agonists at GPCRs have been found to inhibit voltage-gated Ca^{2+} channels in various types of neurons (Hille 1994; Elmslie 2003), and many GPCRs that were found to mediate an inhibition of voltage-dependent Ca^{2+} currents were also reported to cause presynaptic inhibition (Lipscombe et al. 1989; Boehm et al. 1991, 1992; Koh and Hille 1997; Wu and Saggau 1997). The G protein-mediated modulation relevant for presynaptic inhibition targets primarily the channels involved in excitation-secretion coupling, i.e., Ca_V 2 family channels; members of the Ca_V 1 channel family are affected to only a minor extent (Hille 1994; Elmslie 2003). There are two major G protein-linked signaling pathways that control Ca^{2+} channels: one is membrane-delimited, the other is not and rather involves diffusible second messengers. The membrane-delimited pathway involves pertussis toxin-sensitive inhibitory G proteins, achieves full inhibition within one second, slows the activation kinetics of the currents, and is characterized by voltage-dependence (the more the membrane is depolarized, the less the G proteins can inhibit Ca^{2+} channel gating). The underlying mechanism is a direct binding of G protein $\beta\gamma$ subunits to the α_1 subunits of voltage-gated Ca^{2+} channels at a 1:1 stoichiometry. Through this interaction, Ca^{2+} channels become unwilling to open, and the current-voltage dependence of the channels is shifted to more positive values (Elmslie 2003; De Waard et al. 2005). The diffusible second messenger pathway involves pertussis toxin-insensitive G proteins of the Gq family, is much slower, does not affect current kinetics, and controls, in addition to Ca_V 2 family channels, the opening of Ca_V 1 channels (Hille 1994). This pathway involves

activation of phospholipase C and depletion of membrane phosphatidylinositol 4,5-bisphosphate (Gamper et al. 2004; Lechner et al. 2005; Liu et al. 2006).

The first evidence for metabotropic signaling of a priori ionotropic receptors has been obtained with kainate receptors involved in the inhibition of GABA release in the hippocampus. This action was pertussis toxin-sensitive and appeared to involve phospholipase C and proteinkinase C (Rodriguez-Moreno and Lerma 1998). A role for G proteins in the presynaptic inhibition via kainate receptors has also been described for glutamate release from hippocampal neurons (Frerking et al. 2001), and the signaling cascade was found to be abrogated by pertussis toxin and proteinkinase C inhibitors (Lauri et al. 2005). In independent experiments, GluR5 containing kainate receptors were found to mediate a pertussis toxin-sensitive rise in intracellular Ca^{2+} and an inhibition of voltage-gated Ca^{2+} channels (Rozas et al. 2003). This latter effect involved the diffusible second messenger rather than the membrane-delimited pathway. The kainate receptor-mediated inhibition of evoked glutamate release from hippocampal neurons was shown to be occluded when presynaptic $GABA_B$ and adenosine A_1 receptors were activated (Partovi and Frerking 2006). This is in line with the frequently observed phenomenon that the effects of two inhibitory presynaptic receptors are not additive if they share common signaling mechanisms, such as the inhibition of voltage-gated Ca^{2+} channels (Schlicker and Göthert 1998; Kubista and Boehm 2006).

In cortical neurons, activation of AMPA receptors was found to activate inhibitory G proteins, which led to the reduction of forskolin-stimulated cyclic AMP formation (Wang et al. 1997). Later on, AMPA receptor activation in the cerebellum was reported to limit evoked GABA release through an inhibition of voltage-activated Ca^{2+} channels of the Ca_V 2.1 subfamily via N-ethylmaleimide sensitive G proteins (Satake et al. 2004; Rusakov et al. 2005), thus suggesting that the membrane-delimited pathway was involved. Likewise, the inhibition of evoked glutamate release via presynaptic AMPA receptors at the calyx of Held has been shown to involve a G protein-dependent and voltage-sensitive inhibition of voltage-gated calcium channels (Takago et al. 2005), which again favors a role of the fast membrane pathway.

Another example of metabotropic mechanisms involved in the presynaptic inhibition via ionotropic receptors was revealed when the reduction of glutamate release via $P2X_7$ receptors was investigated (Armstrong et al. 2002). The inhibition via this ATP-gated ion channel required an activation of p38 MAP kinase and was suggested to involve an inhibition of voltage-gated Ca^{2+} channels.

Not only the inhibition, but also the facilitation, of transmitter release via presynaptic ionotropic receptors occasionally involves metabotropic mechanisms. The enhancement of evoked glutamate release at mossy fiber-CA3 synapses via presynaptic kainate receptors was shown to involve adenylyl cyclase and proteinkinase A (Rodriguez-Moreno and Sihra 2004). This corroborates that increases in cyclic AMP and concomitant activation of proteinkinase A leads to an enhancement of transmitter release A and raises the question concerning the underlying mechanisms. Since transmembrane Ca^{2+} influx is decisive in excitation-secretion coupling, activation of PKA might operate through an enhancement of voltage-dependent Ca^{2+}

currents (e.g., Artalejo et al. 1990). In addition, proteinkinase A may act downstream of Ca^{2+} entry at the level of exocytosis. This enzyme is known to mediate the phosphorylation of some of the SNARE proteins and interaction partners involved in vesicle exocytosis. α-SNAP, for instance, was identified as a good substrate for phosphorylation by protein kinase A, and phosphorylated α-SNAP displayed reduced binding to the SNARE core complex (Hirling and Scheller 1996). Snapin, in contrast, shows increased binding to SNAP-25 when phosphorylated by protein kinase A (Chheda et al. 2001). And the interaction between syntaphilin and syntaxin-1A is inhibited by protein kinase A-dependent phosphorylation (Boczan et al. 2004). All these effects may contribute to the direct modulation of vesicle exocytosis by the cyclic AMP – protein kinase A pathway, which was found to enhance the Ca^{2+} sensitivity of the release machinery and to increase the readily releasable pool of vesicles (Saitow et al. 2005).

Taken together, there is ample evidence that presynaptic ionotropic receptors, in particular kainate receptors, may control transmitter release via signaling mechanisms that are usually employed by GPCRs and involve heterotrimeric GTP binding proteins. The big question as to how ionotropic receptors are coupled to G proteins remained unanswered (Lerma, 2006).

4 Interactions Between Ionotropic Receptors

It has been demonstrated for a variety of transmitter-gated ion channels that their functions are regulated by a plethora of other membrane proteins, in most instances GPCRs. Most frequently, this modulation via G proteins involves one or more phosphorylation reactions (Swope et al. 1999). In addition, transmitter-gated ion channels can be controlled by G protein subunits that directly bind to the channel protein (Yevenes et al. 2003). However, the functions of ligand-gated ion channels can also be influenced by membrane receptors independently of cytosolic or membrane-associated signaling cascades: GPCRs have been found to interfere with ionotropic receptors by direct protein-protein interactions (Liu et al. 2000; Lee et al. 2002b). In addition, it has been shown for various ligand-gated ion channels that their gating is directly influenced by other ionotropic receptors.

The latter type of interaction has been demonstrated for the following pairs of ionotropic receptors: P2X and nAChRs (Nakazawa 1994), P2X and $GABA_A$ (Boue-Grabot et al. 2004), ATP and $5HT_3$ (Barajas-Lopez et al. 2002), and $GABA_A$ and glycine receptors (Li and Xu 2002). In general, the opening of one ionotropic receptor was found to impede the gating of the other and vice versa. Such a cross-inhibition was not only found with recombinant receptors (Khakh et al. 2000; Boue-Grabot et al. 2004), but also with receptors natively expressed in neurons (Nakazawa 1994), and even with postsynaptic receptors activated by endogenously released transmitters (Khakh et al. 2000). These interactions can be revealed on a functional level by measuring currents through the receptor ionophores (Nakazawa 1994), but also by biochemical methods (Boue-Grabot et al. 2004) and fluorescence resonance

energy transfer (Khakh et al. 2005), which both indicate direct physical interactions. Taken together, it is well established that several ionotropic receptors co-localize in neuronal membranes and at postsynaptic sites and cause mutual inhibition.

It is well known that nerve terminals harbor more than one type of presynaptic receptor. As a consequence, there are multiple interactions between various presynaptic receptors, and the activation of one can positively or negatively interfere with the function of another one. Such interactions were not only described for two different types of presynaptic GPCRs, but also for GPCRs and ionotropic receptors (Kubista and Boehm 2006). Nerve terminals may also possess more than one type of ionotropic receptor. The axon endings of primary sensory afferents in the dorsal horn of the spinal cord, for instance, are known to be equipped with $GABA_A$ receptors and with members of each of the subfamilies of ionotropic glutamate receptors (Rustioni 2005). Nevertheless, reports on functional interactions between these receptors are scarce. The interaction between two different ionotropic receptors that was detected first, between P2X receptors and nAChRs, has also been investigated at the presynaptic level. In contrast to what has been observed with postsynaptic receptors (Khakh and Henderson 2000), activation of presynaptic P2X receptors on neocortical glutamatergic nerve terminals potentiated glutamate release due to the activation of nAChRs and vice versa (Patti et al. 2006). The mechanisms underlying both the inhibitory postsynaptic and the facilitatory presynaptic, interactions remained unknown, and it will be interesting to learn more about the interactions of ionotropic receptors in presynaptic nerve terminals in the future.

5 Physiological and Pharmacological Relevance of Presynaptic Ionotropic Receptors

As evident from the large variety of presynaptic ionotropic receptors, one can expect that these impinge on various aspects of higher-order functions in the nervous system. However, it is beyond the scope of this review to give a full account of all the possible implications. We will rather select a few examples to highlight how presynaptic transmitter-gated ion channels may contribute to the integrated organization of the nervous system.

As monosynaptic reflexes were among the first experimental systems in which presynaptic inhibition has been observed (Frank and Fuortes 1957), it is not astonishing that considerable progress has been made in that field. In the spinal cord, the presynaptic nerve terminals of primary afferent fibers that are in direct contact with motoneurons receive themselves a synaptic input from GABAergic interneurons: an axo-axonic synapse (Conradi 1969). There, the opening of $GABA_A$ receptors causes primary afferent depolarization (Eccles et al. 1962) due to Cl^- efflux and concomitant presynaptic inhibition. Tonic presynaptic inhibition via such axo-axonic synapses is reduced at the onset of voluntary movements in humans at the afferents from the muscle-spindles of the muscle that is to be contracted. In contrast, there is a simultaneous increase in presynaptic inhibition at the afferents innervating

the muscles that are to remain relaxed (Hultborn et al. 1987). Evidence has been presented that similar presynaptic mechanisms are operating when we walk (Faist et al. 1996). Thus, presynaptic $GABA_A$ receptors are well established as most important entities in daily human life. After spinal cord injuries, a disturbance in the presynaptic inhibition via these receptors is believed to contribute to the clinical symptoms of spasticity (Morita et al. 2001). Accordingly, an increase in the function of presynaptic $GABA_A$ receptors due to the application of benzodiazepines contributes to the well-known muscle relaxant properties of these anxiolytics (Rudolph and Möhler 2004).

Similarly to $GABA_A$ receptors, activation of presynaptic kainate receptors was found to reduce glutamate release from primary afferents in the dorsal horn of the spinal cord (Youn and Randic 2004), where nociceptive stimuli are processed. Although this effect involves GluR5 containing receptors, the nociceptive thresholds in GluR5-deficient mice were not significantly different from those in wild-type animals (Sailer et al. 1999). Nevertheless, responses to inflammatory pain were significantly reduced in these transgenic mice (Ko et al. 2005). In agreement with these latter results, a GluR5-selective antagonist was found to attenuate experimental hyperalgesia in humans (Sang et al. 1998) and proved to be efficacious in acute migraine (Sang et al. 2004).

As indicated above, presynaptic kainate receptors operate not only in the spinal cord but also in the brain, for instance, the hippocampus. The hippocampal receptors are decisive in two different aspects, namely development and synaptic plasticity. In immature hippocampal neurons, the filopodia of axons move rapidly to "search" for their targets, and this movement is terminated by synapse formation. Activation of presynaptic kainate receptors by low glutamate concentrations enhances the filopodia mobility, whereas high concentrations have the opposite effect. As the perisynaptic space becomes tighter and tighter when synapses are formed, the concentration of released glutamate will increase as synapse formation proceeds. Thus, the bidirectional modulation of filopodia mobility via presynaptic kainate receptor was suggested to be essential for synapse formation in the hippocampus (Tashiro et al. 2003). In addition, the opposite effects of presynaptic kainate receptor activation on glutamate release at various hippocampal synapses, together with the modulation of GABA release, determine the network activity in the neonatal hippocampus (Lauri et al. 2005). As such network oscillations are important for the development of proper network circuitry (Zhang and Poo 2001), these actions of presynaptic kainate receptors also determine the coordinated hippocampal development.

In the hippocampus, the mossy fiber synapses onto CA3 pyramidal neurons are characterized by a particularly high degree of synaptic plasticity. On a short-term time scale in the range of seconds, one can observe pronounced paired pulse as well as frequency facilitation. The latter phenomenon reflects a multifold increase in synaptic strength when the stimulation frequency is enhanced and involves activation of presynaptic kainate autoreceptors (Schmitz et al. 2001). On a longer time scale in the range of minutes, one can observe long-term potentiation (LTP). At the mossy fiber synapses, LTP is independent of NMDA receptors, but there is sufficient

experimental evidence to suggest that presynaptic kainate recptors do participate (Nicoll and Schmitz 2005).

It is well established that presynaptic nAChRs can function as auto- as well as heteroreceptors. As presynaptic autoreceptors controlling the release of acetylcholine at the neuromuscular junction, nAChRs are believed to be presynaptic amplifiers of neuromuscular transmission (Wessler 1989): by activating these autoreceptors, endogenously released acetylcholine mediates a positive feedback, in particular during pronounced muscle activity. Blockade of the presynaptic nicotinic autoreceptors was also inferred in the phenomenon of titanic fade or train-of-four-fade of neuromuscular transmission which is observed when nAChR blocking agents, such as tubocurarin, are applied (Bowman et al. 1986): when tetanic stimuli or trains of four stimuli are applied to motor nerves, muscle contractions remain rather constant, but decrease in the presence of nAChR antagonists. This is explained by the interruption of the positive feedback which then results in an increasing decline of acetylcholine release (Bowman et al. 1986), but there also are experimental data that are incompatible with this explanation (Wessler 1989).

A major source for nicotine in humans is smoking, and first- as well as second-hand smoke are major causes for a multitude of cardiovascular diseases (Barnoya and Glantz 2005). However, only a few of the various aspects of cardiovasular pathology caused by smoking are believed to be mediated by nicotine (Benowitz 1997). Accordingly, various types of nicotine replacement therapies have been devised which aim at keeping smokers away from cigarettes by delivering nicotine via other routes of administration instead (Henningfield 1995). Nevertheless, nasal and transdermal nicotine still exert powerful effects on the cardiovascular system, and raise, for instance, systolic as well as diastolic blood pressure and the heart rate (Benowitz et al. 2002). As nicotine itself mediates detrimental cardiovascular effects, one has to pose the question as to how they arise: the effects of nicotine are known to be mediated by the sympathetic nervous system (Haass and Kubler 1997), but nAChRs are located in the sympathetic ganglia and at the axon terminals within the innervated tissues (Löffelholz 1970), such as the heart. Hence, nicotine might either enhance the firing rate of the postganglionic sympathetic neurons (by a central effect or by ganglionic stimulation) or directly cause noradrenaline release from the nerve endings. In early investigations related to this topic, smoking was found to raise the blood pressure and the heart rate, but to simultaneously reduce sympathetic nerve activity (Grassi et al. 1994). This appeared to indicate that the effects of nicotine were rather mediated by direct effects on the nerve terminals, i.e., by presynaptic nAChRs. However, subsequent studies revealed that smoking does increase sympathetic nerve activity as long as this phenomenon is not counteracted by the baroreflex (Narkiewicz et al. 1998). Moreover, nicotine uptake by routes other than smoking was also found to stimulate the sympathetic outflow (Wolk et al. 2005). Thus, the effects of nicotine involve central or ganglionic stimulation rather than activation of presynaptic nAChRs on sympathetic axons. This is also indicated by experiments on postganglionic sympathetic neurons, where nicotine was found to first stimulate noradrenaline release via action potential propagation, and to cause a direct presynaptic effect at higher concentrations only (Kristufek et al. 1999b).

6 Conclusion

It is now 50 years since first evidence for presynaptic modulation via ligand-gated ion channels was obtained. During the last decade, the list of ionotropic receptors has been growing rapidly, and it does not appear too speculative to predict that many more examples will be added in the future. Members of each of the known families of the transmitter-agetd ion channels have been detected at various types of presynaptic nerve terminals. Their activation may cause an increase in a priori spontaneous vesicle exocytosis and/or a facilitation or inhibition of activity-dependent transmitter release. Thus, the superfamily of ionotropic receptors provides presynaptic regulators, which are as multifunctional and versatile as members of the superfamily of presynaptic GPCRs. Considering that each ionotropic receptor is composed of at least three subunits, and that there is a selection of more than 70 diverging subunits, one can easily imagine that an unpredictably huge number of different presynaptic ionotropic receptors exist which by far outnumber the various presynaptic GPCRs. In light of the numerous GPCRs involved in the presynaptic modulation of transmitter release as summarized in the previous five chapters of this handbook, we can expect that the number of reports concerning presynaptic ionotropic receptors will continue to grow in the future.

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NO/cGMP-Dependent Modulation of Synaptic Transmission

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Abstract Nitric oxide (NO) is a multifunctional messenger in the CNS that can signal both in antero- and retrograde directions across synapses. Many effects of NO are mediated through its canonical receptor, the soluble guanylyl cyclase, and the second messenger cyclic guanosine-3′,5′-monophosphate (cGMP). An increase of cGMP can also arise independently of NO via activation of membrane-bound particulate guanylyl cyclases by natriuretic peptides. The classical targets of cGMP are cGMP-dependent protein kinases (cGKs), cyclic nucleotide hydrolysing phosphodiesterases, and cyclic nucleotide-gated (CNG) cation channels. The NO/cGMP/cGK signalling cascade has been linked to the modulation of transmitter release and synaptic plasticity by numerous pharmacological and genetic studies. This review focuses on the role of NO as a retrograde messenger in long-term potentiation of transmitter release in the hippocampus. Presynaptic mechanisms of NO/cGMP/cGK signalling will be discussed with recently identified potential downstream components such as CaMKII, the vasodilator-stimulated phosphoprotein, and regulators of

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G protein signalling. NO has further been suggested to increase transmitter release through presynaptic clustering of α-synuclein. Alternative modes of NO/cGMP signalling resulting in inhibition of transmitter release and long-term depression of synaptic activity will also be addressed, as well as anterograde NO signalling in the cerebellum. Finally, emerging evidence for cGMP signalling through CNG channels and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels will be discussed.

1 Introduction

The gas nitric oxide (NO) is well suited to serve as a messenger molecule because of its capacity for rapid diffusion in both aqueous and lipid environments. A myriad of reports document a broad range of NO effects in the CNS, such as the modulation of neuronal development, nociception, apoptosis, synaptic plasticity, and complex behavioral responses (for review see Prast and Philippu 2001; Hofmann et al. 2003). Nearly 20 years ago, Garthwaite and co-workers (1988) recognized for the first time the potential of NO as a neuromodulator. They observed that activation of Ca^{2+}-permeable ionotropic glutamate receptors in cultured cerebellar granule cells triggers the release of a messenger similar to endothelium-derived relaxing factor, which is identical to NO (Ignarro et al. 1987; Furchgott 1996). Based on these results, they put forward the truly inspiring hypothesis that this factor may provide a universal link from postsynaptic activity to functional modifications of neighbouring presynaptic terminals and glial cells. Ironically, the concept of retrograde signalling via NO has been proven in many regions of the brain, but does not hold true for glutamatergic parallel fiber synapses in the cerebellum (see Section 3.2.2). This chapter aims to summarize the current knowledge about NO-dependent mechanisms implicated in the modulation of synaptic transmission. First, the cellular and molecular components involved in NO signalling in the brain will be described. Then, we will provide a synopsis of NO/cGMP-dependent effects on synaptic transmission and corresponding signal transduction based on observations from electrophysiological studies and studies with radioactive or fluorescent markers for presynaptic function. With regard to the general topic of this volume, which is the control of transmitter release, the focus will be set on presynaptic signalling mechanisms of NO and its intracellular second messenger cyclic guanosine-3′,5′-monophosphate (cGMP).

2 Overview of the NO/cGMP Signalling System and Its Expression in the CNS

2.1 Sources of NO and cGMP

NO is produced by a complex reaction via oxidative release from L-arginine giving rise to L-citrulline. Three mammalian isozymes catalyzing this reaction have been identified in various cell types, the constitutively expressed neuronal and endothelial NO synthase (nNOS/NOS1 and eNOS/NOS3), and the inducible NO synthase (iNOS /NOS2). The nNOS is ubiquitously and abundantly expressed throughout the CNS and represents the principal source of NO in many neuronal populations (Bredt and Snyder 1990; Dawson and Dawson 1996; Prast and Philippu 2001). It is a Ca^{2+}/calmodulin-regulated enzyme, which can be activated by Ca^{2+} influx via N-methyl-D-aspartate (NMDA) receptors (Garthwaite et al. 1988). This functional relationship is thought to be especially effective, because the scaffolding molecule PSD-95 keeps the nNOS protein in close proximity to NMDA receptors (Christopherson et al. 1999; Valtschanoff and Weinberg 2001). The eNOS, another Ca^{2+}/calmodulin-dependent NOS initially detected in endothelial cells, has also been reported to be expressed in hippocampal pyramidal cells and neurons of other brain regions (Dinerman et al. 1994; O'Dell et al. 1994). However, this observation has been challenged by others (Stanarius et al. 1997; Demas et al. 1999; Blackshaw et al. 2003). It is now suggested that eNOS expression is confined to endothelial cells, and that NO released within the cerebral vasculature is able to signal to axons located in close proximity (Garthwaite et al. 2006). In contrast to the two constitutive forms of NOS, the iNOS is normally not detectable in the CNS, but upregulated following toxic or inflammatory stimuli.

In many cases, NO acts in the brain by increasing the concentration of cGMP, which is achieved through activation of the canonical NO receptor, the soluble guanylyl cyclase (sGC) (Figure 1) (Friebe and Koesling 2003). The NO-sensitive sGC is a heme-containing enzyme consisting of two different subunits, α and β. Heterodimers of the composition $\alpha_1\beta_1$ and $\alpha_2\beta_1$ have been shown to be functional enzymes, while the role of a putative β_2 subunit remains unclear (for review see Koesling et al. 2005). The $\alpha_1\beta_1$ isoform is ubiquitously expressed in the CNS, whereas expression of $\alpha_2\beta_1$ may be restricted to specific areas in the brain (Mergia et al. 2003; Ding et al. 2004; Russwurm and Koesling 2004). Taken together, it appears that principally every neuron in the CNS can generate NO and/or cGMP. Note that, due to the diffusible nature of NO, the sites of NO and cGMP synthesis must not necessarily overlap. A useful tool to analyze and compare the expression patterns of components of the NO/cGMP signalling pathway in the brain is the recently released Allen Brain Atlas (http://www.brainatlas.org/aba/), which contains the mRNA expression profiles of virtually all mouse genes in the brain (Lein et al. 2006).

It is important to note that many cell types possess alternative NO-independent mechanisms to generate cGMP (Figure 1), namely through activation of membrane-

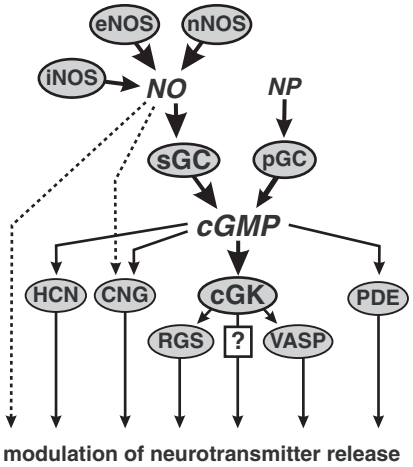

Fig. 1 NO signalling pathways. Nitric oxide (NO) can be generated by endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). NO synthase and activates the cytosolic soluble guanylyl cyclase (sGC), leading to a rise of intracellular cGMP, which in turn activates cGMP-dependent protein kinase (cGK). An increase of cGMP can also be induced by natriuretic peptides (NP) through activation of membrane-bound particulate guanylyl cyclases (pGC). cGK substrates that may be functionally important in the brain include the vasodilator-stimulated phosphoprotein (VASP protein) and regulators of G protein signalling (RGS proteins). The question mark indicates unknown cGK substrates. cGMP can also signal via cGK-independent mechanisms, e.g., by activating cyclic nucleotide-gated (CNG) channels, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, or by modulating various phosphodiesterases (PDE). Note that the mechanisms that modulate transmitter release downstream of cGMP receptors are not well understood. There have been proposed signalling mechanisms of NO not related to the cGMP pathway (dotted lines). For example, NO may trigger Ca^{2+} entry by S-nitrosylation of a CNG channel subunit.

bound particulate guanylyl cyclases (pGCs), e.g., GC-A (NPR-A) and GC-B (NPR-B), by natriuretic peptides (NPs) (for review see Kuhn 2004). NPs comprise a family of three homologous members, atrial (ANP), brain (BNP), and C-type (CNP) natriuretic peptide. Despite the fact that BNP and CNP as well as their receptors (GC-A and GC-B) are found in the brain (Sudoh et al. 1988, 1990; Komatsu et al. 1991; Herman et al. 1993; DiCicco-Bloom et al. 2004), their function in the CNS remains largely elusive and, therefore, NPs and pGCs will not be discussed further in the following sections. Recent findings suggest a modulation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors and ionotropic γ-amino butyric acid ($GABA_A$) receptors by NPs in retinal neurons (Tian and Yang 2006; Yu et al. 2006).

2.2 cGMP Receptors

Extensive analysis of the cGMP system has led to the identification of several effectors for this intracellular second messenger. The classical cGMP receptors include

cGMP-dependent protein kinases (cGKs), cyclic nucleotide-gated (CNG) cation channels, members of the family of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, and of the family of cyclic nucleotide hydrolysing phosphodiesterases (PDEs). We will not discuss mechanisms related to cGMP-mediated cross-activation of cAMP-dependent protein kinase.

2.2.1 cGMP-Dependent Protein Kinases (cGKs)

A major role in the signal transduction of NO and cGMP in the nervous system has been assigned to cGKs (for review see Feil R et al. 2005b; Hofmann et al. 2006). cGKs belong to a family of serine/threonine kinases found in diverse eukaryotic organisms and various tissues, including the nervous system (Muller 1997; Wang and Robinson 1997; Francis and Corbin 1999; Pfeifer et al. 1999). Mammals possess two cGK genes, *prkg1* and *prkg2*, that encode cGKI and cGKII, respectively. The cGKI gene encodes two isoforms, cGKIα and cGKIβ. These isoforms are identical except for their N-termini (≈90–100 amino acid residues), which are encoded by two alternatively used 5′-exons of the cGKI gene. In vitro, cGKs are activated at submicromolar to micromolar concentrations of cGMP, the cGKIα isoform being ≈10-fold more sensitive to activation by cGMP than cGKIβ (Ruth et al. 1991). Both the cGKI and cGKII are homodimers of subunits with a molecular weight of ≈75 kDa and ≈85 kDa, respectively. Each subunit is composed of three functional domains. The *N-terminal domain* comprises regions that control dimerization, targeting, and the activity of the catalytic center. In contrast to the cytosolic cGKI, the N-terminus of the cGKII is myristoylated, thereby anchoring the enzyme to the plasma membrane. The *regulatory domain* contains two tandem cGMP-binding sites that bind cGMP with high and low affinity and interact allosterically. Occupation of both binding sites induces a large change in secondary structure (Landgraf et al. 1990) to yield a more elongated protein molecule (Wall et al. 2003). The *catalytic domain* bears the kinase activity and contains the MgATP- and peptide-binding pockets. Binding of cGMP to both sites in the regulatory domain releases the inhibition of the catalytic center by an N-terminal autoinhibitory/pseudosubstrate site and allows the phosphorylation of serine/threonine residues in target proteins and in the N-terminal autophosphorylation site.

Both cGKs are expressed in the nervous system, while the cGKI appears to be the dominating isoform (http://www.brainatlas.org/aba/). Correlating with the maximum of enzymatic cGK activity in the brain, the cGKIα isoform is highly expressed in cerebellar Purkinje cells (Hofmann and Sold 1972; Lohmann et al. 1981; Geiselhoringer et al. 2004; Feil S et al. 2005). Significant levels of cGKIα have also been observed in nociceptive neurons of the dorsal root ganglia (Qian et al. 1996; Schmidt et al. 2002; Tegeder et al. 2004). Other brain regions with cGKI expression include the dorsomedial nucleus of the hypothalamus, the suprachiasmatic nucleus, the hippocampus, the amygdala, the olfactory bulb, and the cerebral cortex (El-Husseini et al. 1999; Kleppisch et al. 1999; Revermann et al. 2002; Feil S et al. 2005). Noteworthy, cGKIβ appears to be the prevailing isozyme in these

latter regions (Geiselhoringer et al. 2004; Feil S et al. 2005). For the cGKII, low to moderate levels of expression have been reported in the hippocampus, the olfactory tubercle, the suprachiasmatic nucleus, and the thalamus (El-Husseini et al. 1995, 1999; Kleppisch et al. 1999; De Vente et al. 2001; Oster et al. 2003).

Several substrates of cGKs have been identified unequivocally in non-neuronal tissues (Hofmann et al. 2006), while evidence for functional cGK substrates in the nervous system is lagging behind. Table 1 summarizes proteins phosphorylated by

Table 1 cGK Substrates with Possible **Funtion** in the Nervous System

Substrate	Possible Function of Phosphorylation in the CNS	Brain Region Experimental System	References
G-substrate	Protein phosphatase inhibition Initiation of cerebellar LTD	Cerebellum	Aswad and Greengard 1981; Ajima and Ito 1995; Hall et al. 1999; Endo et al. 2003
DARPP-32	Protein phosphatase inhibition Modulation of signallingr pathways	Substantia nigra	Tsou et al. 1993; Nishi et al. 2005
IP_3 receptor type I	Stimulation of Ca^{2+} release from IP_3 sensitive stores	Cerebellum	Haug et al. 1999; Wagner et al. 2003
VASP	Regulation of actin dynamics Vesicle trafficking Aggregation of vesicle proteins	Cerebellum, Hippocampus	Hauser et al. 1999; Arancio et al. 2001; Wang et al. 2005
RhoA	Regulation of actin dynamics	Hippocampus	Wang et al. 2005[#]
	Vesicle trafficking	Rat C6 glioma cells	Zhuang et al. 2004
	Aggregation of vesicle proteins	Vascular smooth muscle	Sauzeau et al. 2000
		NIH 3T3 cells	Ellerbroek et al. 2003
Septin-3	Vesicle trafficking	Hippocampus	Xue et al. 2000, 2004
		COS7 cells	
PDE5	Enhanced cGMP degradation	Cerebellar Purkinje cells	Shimizu-Albergine et al. 2003
	Accelerated termination of cGMP signal	Smooth muscle	Rybalkin et al. 2002

(*Continued*)

Table 1 Continued

Substrate	Possible Function of Phosphorylation in the CNS	Brain Region Experimental System	References
RGS3 and RGS4	Termination of GPCR activation	Astrocytes	Pedram et al. 2000
RGS2	Termination of GPCR activation	Hippocampus	Oliveira-Dos-Santos et al. 2000; Han et al. 2006[§]
	Downregulation of G protein-mediated presynaptic inhibition	Vascular smooth muscle	Tang et al. 2003
	Increase in transmitter release		
ADP ribosyl cyclase	Decreased transmitter release	Hippocampus	Fossier et al. 1999; Reyes-Harde et al. 1999a,b
	Initiation of hippocampal LTD		
BK_{Ca}	Increased open probability	Pituitary nerve terminals	Klyachko et al. 2001
	Enhances afterhyperpolarisation	Smooth muscle	Sausbier et al. 2000

Substrates were suggested according to *in vivo* or *in vitro* phosphorylation studies or based on functional studies. Note that most of the proteins were described as substrates of cGKI; cGKII substrates in the brain are poorly characterized.
[#] Wang et al. (2005) suggest that RhoA, though involved in synaptic plasticity, is not a downstream target of cGK. RhoA has been identified as a cGK substrate in other tissues.
[§] Studies in the hippocampus solely support a possible function of RGS2 in synaptic transmission, but do not prove its phosphorylation by cGK. RGS2 has been identified as a cGK substrate in other tissues.

cGKs in neurons and known substrates of cGKs that are expressed in the brain and known to modulate transmitter release. In general, cGK substrates identified so far include ion channels, G proteins and associated regulators, and cytoskeleton-associated proteins.

2.2.2 Cyclic Nucleotide-Gated (CNG) Channels

Cytosolic cGMP can activate CNG channels. These ion channels are not only an important part of the signal transduction pathway in the visual and olfactory system (Biel et al. 1999; Hofmann et al. 2003), but are also expressed in other neurons where they may be involved in the modulation of various functions by NO. The CNG channel family comprises six homologous subunits. Based on phylogenetic relationship, these proteins are classified as A subunits (CNGA1-A4) and B subunits (CNGB1 and CNGB3) (Biel et al. 1999; Bradley et al. 2001). Native CNG channels

are heterotetramers composed of A and B subunits, and their sensitivity for cGMP largely depends on the individual subunit composition. For instance, the cGMP-gated rod channel consists of CNGA1 (Kaupp et al. 1989) and a long splice variant of CNGB1 (CNGB1a) (Korschen et al. 1995). Activation of CNG channels causes depolarization and, thus, facilitates excitation of neurons. In addition, CNG channels have been suggested to permit Ca^{2+} influx relevant for synaptic function (Broillet and Firestein 1997; Parent et al. 1998).

2.2.3 Phosphodiesterases (PDEs)

Cyclic nucleotide signalling is controlled not only at the level of cAMP and cGMP synthesis, but also by the rate of cyclic nucleotide degradation via PDEs (Sonnenburg and Beavo 1994). The PDE superfamily comprises 11 subfamilies with 21 genes that are transcribed into perhaps more than 50 enzyme species by alternative splicing, and virtually all PDEs are expressed in the CNS (Menniti et al. 2006). The families are classified according to substrate specificity, mechanism of regulation, sensitivity to inhibitors, and amino-acid sequence. Cyclic GMP is degraded by a number of cGMP-specific PDEs as well as by dual-substrate PDEs that hydrolyse both cAMP and cGMP. Importantly, cGMP can also inhibit or activate specific PDE subtypes via binding to their regulatory domains. Thereby, cGMP can modulate its own level and/or that of cAMP. For instance, cGMP can increase cAMP levels via binding to the cGMP-inhibited cAMP-PDE, PDE3A; it can lower cyclic nucleotide levels by binding to the cGMP-stimulated cAMP/cGMP-PDE, PDE2A, or by binding to the cGMP-specific PDE5A, the target of sildenafil. Thus, the cGMP-regulated PDEs are important cGMP receptors helping to shape the spatiotemporal profile of cyclic nucleotide signals. Particularly, they might represent “switches” that transform cGMP signals into cAMP signals, allowing cyclic nucleotide cross-talk in the brain. Another interesting mode of PDE regulation is represented by the dual-substrate PDE1 family. This family has the unique feature of being activated by the binding of Ca^{2+}/calmodulin, providing a mechanism for cross-talk between the Ca^{2+} and cyclic nucleotide signalling pathways.

2.3 cGMP-Independent NO Signalling

It is important to note that not all effects of NO on synaptic transmission can be accounted for by cGMP-dependent signalling, i.e., NO can also signal through alternative mechanisms independent of cGMP. Owing to the limited space and the general scope of this chapter, we will not cover these mechanisms in detail but just discuss them briefly. First, NO can give rise to free radical species that, in turn, may affect cellular functions through various mechanisms (for review see Stamler et al. 1997). Second, NO can modulate proteins controlling neuronal functions by stimulating their ADP ribosylation (Schuman et al. 1994; Zhang et al. 1994; Sullivan et al.

1997). This mechanism may account partially for the stimulatory action of NO on transmitter release in hippocampal synapses (Schuman et al. 1994). Last but not least, NO may modulate cellular functions by S-nitrosylation of various proteins. For example, S-nitrosylation of nuclear proteins associated with cAMP response element (CRE) binding protein is involved in regulation of its DNA binding and, hence, CRE-mediated gene expression (Riccio et al. 2006). S-nitrosylation has also been reported to modify the function of various ion channels (Broillet and Firestein 1996, 1997; Xu et al. 1998; Jaffrey et al. 2001).

3 NO and cGMP as Modulators of Synaptic Transmission

3.1 Effects of NO/cGMP on Transmitter Release

The proposal that NO might act presynaptically to modify transmitter release (Garthwaite et al. 1988) prompted a plethora of investigations into the effects of NO donors and inhibitors of NOS and sGC on transmitter release. Studies were performed in vitro, e.g., on brain slices, synaptosomes, or cultured neurons, or in vivo using microdialysis or the push-pull superfusion technique. The effects of NO donors suggest that the release of several transmitters, including acetylcholine, catecholamines, and excitatory and inhibitory amino acids, may be influenced by endogenous NO. NO donors also inhibit the uptake of various radiolabeled transmitters (Pogun et al. 1994). However, these NO donor studies usually lacked important controls for potential unspecific effects of NO carrier molecules (Guevara-Guzman et al. 1994). Other experiments more convincingly show that authentic NO (Guevara-Guzman et al. 1994) as well as endogenous NO that is "tonically" present in unstimulated tissue (Prast and Philippu 1992; Bugnon et al. 1994) or "phasically" generated by NMDA receptor activation (Dickie et al. 1992; Hanbauer et al. 1992; Sorkin 1993; Montague et al. 1994) modulates transmitter release. Recent in vitro studies suggest that tonic NO might come from eNOS in blood vessels, while the phasic NO signals are produced by nNOS in neurons (Garthwaite et al. 2006; Hopper and Garthwaite 2006). The concept of vessel-to-neuron signalling by NO is supported by the finding that mice lacking eNOS in the cerebral vasculature exhibit various neuronal phenotypes, including altered transmitter release or turnover (Kano et al. 1998; Frisch et al. 2000).

It appears that NO, predominantly via cGMP-dependent mechanisms, regulates primarily the release of glutamate, which then modulates the release of various other transmitters in several brain regions, such as the hippocampus, the striatum, the hypothalamus, and the locus coeruleus (Prast and Philippu 2001). Depending on the brain region and NO concentration, NO can both stimulate and inhibit the release of a particular transmitter. Based on the observations of many groups, Prast and Philippu (2001) have proposed the following model for the modulation of acetylcholine release by endogenous NO: NO stimulates cGMP synthesis within

glutamatergic neurons. Depending on the NO concentration, glutamate release is either enhanced (high NO) or decreased (low NO), resulting in either enhanced or decreased activation of postsynaptic NMDA or AMPA/kainate receptors on cholinergic neurons followed by enhanced or decreased acetylcholine release. NO may influence cholinergic transmission not only via excitatory glutamatergic neurons, but also via effects on inhibitory GABAergic neurons. The release of catecholamines, histamine, and serotonin may be modulated in a similar manner.

Basically, then, NO seems to be able to modulate vesicular release of transmitter in either direction, or not at all, depending on the coincident level of presynaptic activity and NO concentration. The concept of an activity-dependent retrograde NO signal that is generated in the postsynapse and then diffuses into the presynapse to regulate transmitter release has been investigated extensively, due to its possible involvement in neuronal excitability and memory processes (see Section 3.2.1).

Although many studies have indicated that NO/cGMP can modulate transmitter release (Arancio et al. 1995; Meffert et al. 1996; Sporns and Jenkinson 1997; Klyachko et al. 2001), the molecular mechanisms and functional relevance of presynaptic NO/cGMP signalling are not well understood. It is generally accepted that activity-dependent changes in synaptic strength, such as long-term potentiation (LTP) and long-term depression (LTD), underlie learning and memory (Pastalkova et al. 2006; Whitlock et al. 2006). However, there are ongoing debates whether a given form of synaptic plasticity in a given brain region results from presynaptic and/or postsynaptic alterations, as will be exemplified below for the hippocampus. Conflicting evidence for presynaptic changes has been obtained largely from the classical electrophysiological approach of quantal analysis of pairs of monosynaptically connected neurons, vesicular antibody uptake, and postsynaptic drug-infusion studies (Stanton et al. 2003 and refs. therein). A more direct visualization of presynaptic vesicle release has been achieved with the fluorescent styryl dye FM1-43 (Betz and Bewick 1992; Ryan et al. 1993). FM1-43 is taken up into synaptic vesicles in an activity-dependent manner as a result of endocytosis after transmitter release. Subsequent synaptic stimulation evokes the release of the dye by exocytosis, which is visualized as a destaining whose rate is a direct measure of presynaptic release efficacy. FM1-43 imaging of cultured neurons and acute brain slices demonstrated that certain forms of LTP or LTD in the hippocampus depend, at least in part, on increased or decreased transmitter release from the presynaptic neuron. Interestingly, retrograde NO signalling appears to contribute to both presynaptic LTP and LTD of transmitter release.

A model of retrograde NO signalling in a glutamatergic synapse is presented in Figure 2. The pathway comprises (1) an action potential-driven release of glutamate from presynaptic axon terminals, (2) activation of postsynaptic NMDA receptors, which trigger the synthesis of NO, (3) diffusion of NO to a presynaptic site, where (4) it stimulates cGMP production and activation of cGK leading to (5) increase (as illustrated in Figure 2) or decrease (not shown in Figure 2) of transmitter release (Sporns and Jenkinson 1997; Stanton et al. 2001, 2003, 2005). In line with such a model, increases in cGMP have been implicated in the induction of both hippocampal LTP (Arancio et al. 2001) and LTD (Gage et al. 1997; Reyes-Harde et al. 1999a).

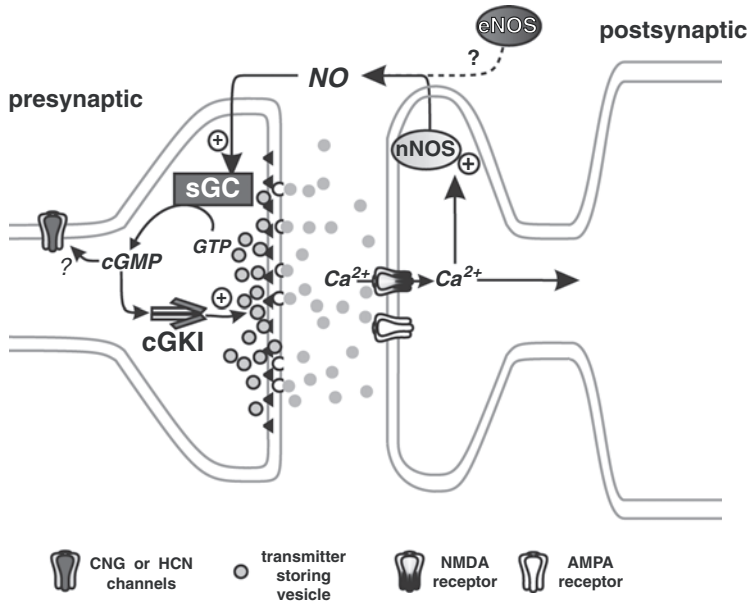

Fig. 2 Retrograde NO signalling in a glutamatergic synapse. Nitric oxide (NO) generated postsynaptically by Ca^{2+}/calmodulin-activated neuronal NO synthase (nNOS) and/or derived from endothelial NO synthase (eNOS) in nearby vessels diffuses to the presynaptic terminal, where it activates the soluble guanylyl cyclase (sGC). The resulting increase of the intracellular second messenger cGMP activates various receptors, including the cGMP-dependent protein kinase I (cGKI). Through phosphorylation of its substrates, cGKI leads to an increase in presynaptic transmitter release. This action is thought to involve clustering of vesicular proteins and other proteins of the docking/fusion machinery (black triangles) at the release sites. In addition, cGMP may modulate transmitter release by activating presynaptic ion channels regulated by cyclic nucleotides (CNG and HCN channels).

However, the mechanisms or mediators controlling the switch from one to the other effect dominating the net change remain to be identified (see also Section 3.2.1). The dual role of the NO/cGMP/cGK pathway in both LTP and LTD may also explain some contradictory reports on the role of this pathway in synaptic plasticity.

What are the molecular mechanisms of presynaptic NO/cGMP signalling that alter transmitter release? In some systems, NO-induced transmitter release occurs independently of an increase in presynaptic Ca^{2+} (Schuman et al. 1994; Stewart et al. 1996), perhaps by modulating the interaction of components of the vesicle docking/fusion machinery (Meffert et al. 1996). On the other hand, it has been reported that one action of presynaptic cGMP required for LTD induction is the stimulation of presynaptic ryanodine receptor-mediated Ca^{2+} release (Reyes and Stanton 1996; Reyes-Harde et al. 1999a), but how an *increase* in presynaptic Ca^{2+} results in long-term *reduction* of release probability is not clear. An alternative mechanism to increase Ca^{2+} is the stimulation of presynaptic Ca^{2+} channels. In the brain stem, for

instance, the NO/cGMP/cGK pathway might facilitate glutamate release through an enhancement of presynaptic N-type Ca^{2+} channel activity (Huang et al. 2003).

In posterior pituitary nerve terminals, NO stimulates Ca^{2+}-activated K^+ (BK) channel activity via a cGMP/cGK-dependent mechanism (Klyachko et al. 2001). Opening of BK channels results in K^+ efflux and membrane hyperpolarization. The enhancement of BK channel activity by cGMP was greatest at depolarized potentials, so it will manifest during and immediately after action potentials and, thus, increase the magnitude of the spike afterhyperpolarization, which in turn promotes Na^+ channel recovery from inactivation. This mechanism would reduce action potential failures and allow more Ca^{2+} to enter followed by increased transmitter release. The stimulation of presynaptic K^+ channels by the NO/cGMP/cGK cascade could generate a short-term, use-dependent enhancement of release at nerve terminals and may also operate at a certain stage of LTP induction in the hippocampus (Klyachko et al. 2001).

After exocytosis, synaptic vesicles must be retrieved and refilled with transmitters to supply the needs of an active neuron. Work by Micheva and colleagues (2001, 2003) showed that retrograde signalling via NO promotes synaptic vesicle recycling in CNS neurons, especially at synapses with high levels of recycling, i.e., in an activity-dependent manner. The authors monitored exo- and endocytosis in cultured hippocampal neurons using a pH-sensitive green fluorescent protein (GFP) reporter protein and the fluorescent dye FM4-64. Based on fluorescence imaging and pharmacological analysis they proposed that NMDA receptor-dependent postsynaptic production of NO accelerates vesicle endocytosis by increasing the level of presynaptic phosphatidylinositol 4,5-bisphosphate (PIP_2) via a cGMP-dependent mechanism. PIP_2 has been extensively implicated in vesicle cycling (Martin 2001; Osborne et al. 2001), but how NO/cGMP leads to an increase in PIP_2 at the synapse is not known. Studies in non-neuronal cells suggest that the NO/cGMP/cGK pathway can inhibit the PIP_2-hydrolysing phospholipase C (Clementi et al. 1995; Xia et al. 2001).

Recent results support the view that microstructural changes occur already at early stages of long-lasting synaptic plasticity (Engert and Bonhoeffer 1999; Maletic-Savatic et al. 1999). For instance, glutamate-induced potentiation of synaptic transmission in cultured hippocampal neurons is accompanied by a rapid increase in clusters of presynaptic synaptophysin and postsynaptic GluR1 protein (Antonova et al. 2001). A recent study by Wang and colleagues (2005) suggests that NO/cGMP/cGKI signalling, actin, and the small GTPase RhoA play important roles in potentiation in cultured hippocampal neurons. Surprisingly, cGMP and cGKI appear to act directly in both the presynaptic and postsynaptic neurons, where they contribute to an increased aggregation of synaptic proteins, perhaps via phosphorylation of the cGKI substrate vasodilator-stimulated phosphoprotein (VASP) and regulation of the actin cytoskeleton. The structural changes promoted by the NO/cGMP/cGKI pathway may not only affect transmitter release and strengthen existing synapses, but may also trigger the activity-dependent formation of new synapses (cf. Wang et al. 2005). These mechanisms will be discussed in more detail later in the context of hippocampal LTP (see Section 3.2.1).

3.2 Long-term Potentiation (LTP) and Long-Term Depression (LTD) as Model Systems to Study NO/cGMP Signalling in Central Synapses

Long-lasting activity-dependent changes of synaptic transmission in a neural network are thought to serve as cellular mechanisms for learning and memory (Hebb 1949). Two prominent forms of synaptic plasticity, LTP and LTD, are indeed associated with different types of learning (Rogan et al. 1997; Manahan-Vaughan and Braunewell 1999; Ito 2002; Whitlock et al. 2006) and have become well-established model systems for studying the modulation of synaptic transmission in various brain regions (Malenka and Bear 2004). It is now generally acknowledged that both pre- and postsynaptic processes can contribute to the changes in glutamatergic synaptic transmission seen after induction of LTP and LTD (for review see Bear and Malenka 1994; Larkman and Jack 1995; Lisman and Raghavachari 2006). Postsynaptic mechanisms ultimately lead to changes in the density and/or function of AMPA type glutamate receptors. For example, LTP is associated with the insertion of AMPA receptors directly into the synaptic region and/or into the extrasynaptic membrane with subsequent diffusional redistribution to the synaptic region. Conversely, internalization of AMPA receptors from these sites plays an important role in LTD. Presynaptic mechanisms lead to a modification of glutamate release, e.g., by changing the mode of vesicle fusion and/or the number of vesicles released in response to afferent stimulation. In addition, aligning presynaptic release sites with postsynaptic receptors may enhance synaptic transmission (Antonova et al. 2001; Wang et al. 2005). NO and cGMP have been reported to signal through many of these mechanisms (see also Section 3.1). The subsequent section is dedicated to the function of NO as a retrograde messenger, i.e., its presynaptic signalling.

3.2.1 NO as a Retrograde Messenger in Hippocampal LTP

Considerable efforts have been made to elucidate the role of NO for synaptic plasticity in various brain regions (for review see Larkman and Jack 1995; Huang 1997; Daniel et al. 1998; Hawkins et al. 1998; Feil R et al. 2005b). The following discussion focuses on findings about the role of NO for LTP in Schaffer collateral/CA1 synapses of the hippocampus, a NMDA receptor-dependent form of synaptic plasticity characterized in great detail (for review see Malenka and Bear 2004; Lisman and Raghavachari 2006). In spite of some controversial findings, it is accepted that NO (1) is involved in LTP in the Schaffer collateral pathway and (2) acts as a retrograde messenger (Garthwaite and Boulton 1995; Arancio et al. 1996; Huang 1997; Hawkins et al. 1998). Both nNOS and eNOS have been detected in postsynaptic pyramidal neurons (Dinerman et al. 1994; O'Dell et al. 1994; Brenman et al. 1996) and various NOS inhibitors suppress LTP in the CA1 region (Schuman and Madison 1991; Arancio et al. 1996). Analysis of mice lacking nNOS and/or eNOS revealed the functional contribution of either NOS isoform to

hippocampal LTP (Table 2). Mice deficient in either nNOS or eNOS appear to be capable of normal LTP in the CA1 region (O'Dell et al. 1994; Son et al. 1996), while a strong impairment was observed in compound nNOS/eNOS knockout mice (Son et al. 1996). Other groups have shown that the lack of eNOS alone can result in reduced LTP in the Schaffer collateral pathway (Wilson et al. 1999) and other regions (Haul et al. 1999; Doreulee et al. 2001). A major role for this isoform has also been suggested by Kantor et al. (1996), who reported that disrupting the localization of eNOS to the membrane causes impairment of LTP, and that this effect could be overcome by expressing a chimeric form of eNOS constitutively targeted to the membrane. On the other hand, the function of nNOS has been underscored by a recent report that selective inhibitors of this isoform reduce hippocampal LTP (Hopper and Garthwaite 2006). It was further suggested that phasic and tonic NO signals are needed for LTP, and that these signals are derived from nNOS in hippocampal pyramidal cells in response to neural activity and eNOS expressed in nearby vascular endothelial cells, respectively (Garthwaite et al. 2006; Hopper and Garthwaite 2006). The findings discussed so far demonstrate the ability of NO to support LTP, but they do not discriminate between possible sites of its action. The observation that bath application of hemoglobin, a membrane-impermeable scavenger of NO, blocks LTP (O'Dell et al. 1991) suggests that NO travels extracellularly through the synaptic cleft. Additional evidence for a presynaptic action of NO comes from studies of miniature excitatory postsynaptic currents (EPSC) (O'Dell et al. 1991) and NMDA receptor-dependent LTP in cultured hippocampal neurons (Arancio et al. 1995, 1996, 2001; Wang et al. 2005). NO increases the frequency of spontaneous miniature EPSCs. Oxymyoglobin, another membrane-impermeable NO scavenger, is able to suppress LTP between pairs of cultured neurons following a tetanus, but its extracellular application has no effect on LTP induced by a weak tetanus in conjunction with photolytic uncaging of NO in the presynaptic cell. These data can be compiled into a scheme with NO as a retrograde messenger in hippocampal LTP (Figure 2). The following section will review efforts made to unravel presynaptic signalling mechanisms of NO involved in the strengthening of synaptic transmission.

Role of the Canonical NO/cGMP/cGK Pathway in Retrograde Signalling

There are some reports that NO can support LTP through cGMP-independent mechanisms, e.g., by stimulating the ADP ribosylation of proteins regulating synaptic transmission (Schuman et al. 1994; Zhang et al. 1994; Sullivan et al. 1997). Also, direct modulation of a CNG channel subunit by NO may cause a Ca^{2+} influx with functional impact on synaptic function (Broillet and Firestein 1997; Parent et al. 1998). But it is no big surprise that the bulk of pharmacological studies as well as the phenotypes of transgenic mouse models (Table 2) support the conclusion that NO signals through its canonical pathway via the sGC, cGMP, and cGKs. LTP-inducing stimuli elicit an increase of cGMP in the hippocampus that is sensitive to NOS inhibitors and NO scavengers (Chetkovich et al. 1993). Moreover,

Table 2 Phenotypes Related to Synaptic Plasticity in Transgenic Mice with Genetic Alterations of NO/cGMP Signalling

Gene	Mouse Model	Phenotypes	References
nNOSα	Null mutation*	Reduced LTD in cerebellar parallel fiber synapses	Lev-Ram et al. 1997b
		Normal LTP in Schaffer collateral pathway	O'Dell et al. 1994
eNOS	Null mutation	Defective LTP in Schaffer collateral pathway	Wilson et al. 1999
		Defective LTP mossy fiber pathway	Doreulee et al. 2001
		Defective LTP in cerebral cortex	Haul et al. 1999
eNOS + nNOSα	Null mutation#	Defective LTP in Schaffer collateral pathway Normal LTP in stratum oriens	Son et al. 1996
sGC α1	Null mutation	Reduced LTP in visual cortex	Mergia et al. 2006
sGC α2	Null mutation	Reduced LTP in visual cortex	Mergia et al. 2006
cGKI	Null mutation	Normal LTP in Schaffer collateral pathway of young mice (4 weeks old)	Kleppisch et al. 1999
cGKII	Null mutation	Normal LTP in Schaffer collateral pathway of adult mice (12 weeks old)	Kleppisch et al. 1999
cGKI + cGKII	Null mutation	Normal LTP in Schaffer collateral pathway of young mice (4 weeks old)	Kleppisch et al. 1999
cGKI	Hippocampus-specific mutation	Reduced L-LTP in Schaffer collateral pathway of adult mice (12 weeks old)	Kleppisch et al. 2003
cGKI	Purkinje cell-specific mutation	Reduced LTD at the parallel fiber-Purkinje cell synapse	Feil R et al. 2003

*The nNOS knockout mice reported were created by a deletion of exon 2 eliminating the splice variant nNOSα, which accounts for about 95% of the catalytic activity in the whole brain. nNOS mutants retain significant NOS activity in the brain, perhaps reflecting the function of the remaining isoform, eNOS. However, this interpretation is complicated by the expression of two alternative splice variants, nNOSβ and nNOSγ, lacking exon 2 in these mice (Eliasson et al. 1997).

the membrane-permeable cGMP analogue dibutyryl cGMP partially restores LTP blocked by a NOS inhibitor (Haley et al. 1992), and various sGC inhibitors suppress LTP (Zhuo et al. 1994; Arancio et al. 1995; Boulton et al. 1995). Evidence for the presynaptic localization of sGC came from studies of LTP between pairs of cultured hippocampal neurons (Arancio et al. 1995). Importantly, cGMP produces activity-dependent LTP when it is injected into the presynaptic neuron, but not when it is injected into the postsynaptic neuron. Results from quantal analysis together with the observation that 8-Br-cGMP mimicks the NO effect on the frequency of spontaneous miniature EPSCs support the conclusion that cGMP-dependent potentiation of synaptic transmission is due to an increase in presynaptic transmitter release.

As described above, the cGKs are major receptors of cGMP in the CNS. The first pharmacological evidence for a role of cGK in hippocampal LTP came from a study of Zhuo and coworkers (1994). These authors observed an enhancement of LTP following a weak tetanic stimulation in the presence of cGK activators, and, conversely, a suppression of LTP in the presence of cGK inhibitors. However, due to the inadequate selectivity of the pharmacological tools, the functional relevance of individual cGK isoforms was not clear. Two pieces of evidence hint at a predominant function of cGKI. First, the expression level of cGKII in the hippocampus appears substantially lower than that of cGKI (El-Husseini et al. 1995; Kleppisch et al. 1999; De Vente et al. 2001). Second, in line with the idea of NO as a retrograde messenger, cGKI has been detected in presynaptic terminals colocalized with the vesicle protein synaptophysin (Arancio et al. 2001). The concept of presynaptic NO/cGMP signalling via cGKI was further supported by studies of NMDA receptor-dependent LTP between pairs of cultured hippocampal neurons (Arancio et al. 2001): infusion of purified cGKI protein into the presynaptic neuron facilitated LTP in response to a weak tetanus, while a cGK inhibitor injected into the presynaptic neuron blocked LTP. Both substances failed to affect LTP when applied postsynaptically, underlining that cGK functions as a presynaptic target of NO/cGMP (Arancio et al. 2001). Apparently at odds with these findings, conventional knockout mice lacking cGKI, cGKII, or both (double knockout mice) are capable of normal LTP in the Schaffer collateral pathway (Kleppisch et al. 1999; Table 2). This may be due to the fact that null mutants lack the cGKs during their entire ontogenesis and in all tissues, so that other phenotypes or functional compensation could occlude potential defects of LTP. Notably, the analysis of synaptic plasticity in the conventional cGKI knockout was limited to juvenile mice (up to $\sim$4 weeks of age) due to their premature death caused by cardiovascular and gastrointestinal defects (Pfeifer et al. 1998). Hippocampus-specific cGKI knockout ($cGKI^{hko}$) mice, which exhibit a normal life expectancy, also show normal hippocampal LTP in response to a single theta burst stimulation (Kleppisch et al. 2003). NO and cGMP have been shown to support late phase LTP (L-LTP) in the CA1 region (Lu et al. 1999; Lu and Hawkins 2002), a protein synthesis-dependent form of LTP that requires multiple strong tetanic stimulation of Schaffer collaterals (Frey et al. 1988; Barco et al. 2002). The following findings in $cGKI^{hko}$ mice support the view that cGKI may act as a downstream target of NO/cGMP in L-LTP (Kleppisch et al. 2003). First, LTP following multiple episodes of strong theta burst stimulation is decreased in adult $cGKI^{hko}$ mice ($\sim$12

weeks of age). Second, the protein synthesis inhibitor anisomycin has no effect on LTP in $cGKI^{hko}$ mice and decreases LTP in control mice to the level observed in the mutants. It remains unresolved whether this cGKI-dependent form of L-LTP is related to presynpatic and/or postsynaptic changes in protein synthesis.

Signal Transduction Mechanisms Downstream of cGK

What are the functionally relevant substrates and downstream effectors of cGK involved in LTP? Studies in cultured hippocampal neurons have shed light on potential presynaptic effector molecules of the NO/cGMP/cGK signalling cascade, such as the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Ninan and Arancio 2004), the VASP protein (Wang et al. 2005), and α-synuclein (Liu et al. 2004). Some regulators of G protein signalling (RGS proteins) and a few other synaptic proteins thought to modulate transmitter release are candidate substrates for cGKs in the brain (see Table 1). Differently from the VASP protein and RGS proteins, CaMKII and α-synuclein have not been shown to be phosphorylated directly by cGK.

The CaMKII is expressed in hippocampal neurons and plays an important role in synaptic plasticity. Primary interest was focussed on the postsynaptic function of the enzyme (for review see Lisman et al. 2002). A recent study with cultured hippocampal neurons has elucidated the presynaptic function of CaMKII in synaptic plasticity in the context of NO/cGMP/cGK signalling (Ninan and Arancio 2004). Presynaptic injection of the membrane-impermeable CaMKII inhibitor peptide 281-309 blocks long-lasting potentiation of synaptic transmission induced by combining a weak tetanus with brief superfusion of either the NO donor DEA/NO or the cGK activator 8-pCPT-cGMP. The CaMKII inhibitor peptide also suppresses the increase in the number of active presynaptic boutons occurring normally under these conditions. Thus, CaMKII appears to be essential for potentiation of synaptic transmission induced by retrograde NO/cGMP/cGK signalling. Additional findings show that the enzyme can also be activated independent of NO/cGMP/cGK signalling, e.g., by increased presynaptic Ca^{2+} entry, and that this is sufficient to induce long-lasting potentiation. However, few data support a function of CaMKII in transmitter release. A study in conditional knockout mice lacking the CaMKII α subunit selectively in presynaptic CA3 neurons of the Schaffer collateral pathway even suggests that the enzyme *inhibits* the activity-dependent increase of transmitter release (Hinds et al. 2003). Future studies are needed to improve our understanding of the functional relationship between NO/cGMP/cGK signalling, CaMKII activation, and presynaptic transmitter release.

Two additional putative downstream components of presynaptic NO/cGMP/cGK signalling in the course of synaptic plasticity are VASP and the small GTP binding protein RhoA (Wang et al. 2005). VASP has been detected in the brain and shown to serve as an endogenous substrate of cGK in hippocampal neurons (Hauser et al. 1999; Arancio et al. 2001). It is also well known that VASP and related proteins are crucial in the regulation of actin dynamics, which have been linked to synaptic plasticity (for review see Lisman 2003; Lisman and Raghavachari 2006).

Activity patterns leading to LTP are associated with actin filament reorganization in dendritic spines (Fukazawa et al. 2003), and actin filament reorganization is essential to enhance AMPA receptor-mediated transmission during LTP (for review see Lisman and Raghavachari 2006). How is this related to presynaptic NO/cGMP/cGK signalling? It has emerged that similar cytoskeletal changes occur in an activity-dependent manner at the presynaptic site and support a rapid increase in clusters of the vesicular proteins synaptophysin and synapsin I (Antonova et al. 2001; Wang et al. 2005). Evidence from studies in cultured hippocampal neurons further suggests that this presynaptic process can be induced through retrograde NO/cGMP/cGK signalling with cGK-dependent VASP phosphorylation as a critical step (Wang et al. 2005). Elements of the NO signalling cascade, including sGC, cGKI, and VASP, are expressed in presynaptic terminals, where cGKI colocalizes with synaptophysin. Finally, VASP is phosphorylated at the site preferentially phosphorylated by cGK (Ser-239) during synaptic potentiation (Arancio et al. 2001). Hence, cGK, via phosphorylation of VASP, appears to modulate presynaptic cytoskeletal structures linked to vesicle function and transmitter release. Unexpectedly, mice lacking VASP or related proteins do not show severe phenotypes related to synaptic plasticity (Hauser et al. 1999; Reinhard et al. 2001).

Another interesting substrate of cGK is the small GTP binding protein RhoA (Sauzeau et al. 2000; Ellerbroek et al. 2003). Its cGK-dependent phosphorylation, similar to that of VASP, may contribute to the regulation of actin filament dynamics in presynaptic terminals. The finding that the general inhibitor of Rho GTPases, *Clostridium difficile* toxin B, and the inhibitor of RhoA-dependent kinase Y27632 reduce glutamate-induced synaptic potentiation in cultured hippocampal neurons (Wang et al. 2005) would indeed support this idea. However, toxin B has no effect in the presence of the cGK activator 8p-CPT-cGMP, which argues against a role of RhoA as a downstream target of cGK.

It has been reported recently that α-synuclein, a protein linked to neurodegeneration, contributes to synaptic potentiation by increasing transmitter release and that activation of the NO signalling cascade may promote this effect (Liu et al. 2004). Long-lasting-potentiation of synaptic transmission between cultured hippocampal neurons is accompanied by an increase in the number of presynaptic α-synuclein clusters, while turning down the expression of α-synuclein expression (e.g., by antisense nucleotides or knockout techniques) blocks potentiation and the increase in the presynaptic number of functional boutons normally associated with it. Moreover, presynaptic injection of α-synuclein causes rapid and long-lasting enhancement of synaptic transmission and rescues potentiation in cultures from mice carrying a α-synuclein null mutation (Liu et al. 2004). A link between NO signalling and α-synuclein has been suggested based on findings that exogenous NO can also increase the number of α-synuclein clusters and that NOS inhibitors can block glutamate-induced increase in their density. In line with these data, α-synuclein may function as a downstream effector linking NO/cGMP/cGK signalling to increased transmitter release from presynaptic terminals. However, without doubt more experiments are needed to substantiate this view. The discussion above underlines that mechanisms of synaptic potentiation involve presynaptic changes. Retrograde

NO-dependent signalling mechanisms promote coordinate changes in the distribution of proteins in the presynaptic terminal which, ultimately, increase presynaptic transmitter release, e.g., by altering vesicle function. In addition, these mechanisms may support the alignment of presynaptic release sites with postsynaptic receptors to increase the efficacy of synaptic transmission.

G protein-mediated signalling pathways are also critically involved in the regulation of transmitter release, e.g., by a negative feedback via presynaptic autoreceptors (cf. Starke 1981). Besides the signal-receiving G protein-coupled receptor (GPCR), a linked heterotrimeric G protein and its downstream effectors, this signal transduction machinery includes modulatory RGS proteins. The more than 30 currently known RGS proteins, which have been classified into seven subfamilies (Hollinger and Hepler 2002), promote the termination of GPCR-mediated signalling by interacting with Gα–GTP and accelerating GTP hydrolysis. Studies in astrocytes and vascular myocytes indicate that members of subfamilies 2, 3, and 4 are cGK substrates (Pedram et al. 2000; Tang et al. 2003). Moreover, cGKI-dependent RGS2 phosphorylation might play a functional role for NO-dependent effects in the vasculature (Tang et al. 2003; Sun et al. 2005). Morphological and electrophysiological analyses support the idea that RGS2 is an important regulator of synaptic plasticity in hippocampal CA1 neurons (Ingi et al. 1998; Oliveira-Dos-Santos et al. 2000; Han et al. 2006). Given these findings, it is tempting to speculate that the NO/cGMP/cGK signalling cascade can modulate presynaptic transmitter release by phosphorylating the RGS2 protein.

Autoinhibition of transmitter release has been largely attributed to GPCR-dependent suppression of Ca^{2+} influx via presynaptic voltage-dependent Ca^{2+} channels (for review see Reid et al. 2003). The intracellular Ca^{2+} concentration and, hence, transmitter release are also controlled by Ca^{2+} discharged from intracellular stores (Fossier et al. 1999; Collin et al. 2005). Ryanodine-sensitive stores in presynaptic terminals have been reported to mobilize Ca^{2+} needed to induce LTD at Schaffer collateral/CA1 synapses (Reyes and Stanton 1996; Reyes-Harde et al. 1999a,b). Ca^{2+} release causing LTD can be induced by an endogenous activator of ryanodine receptors, cyclic ADP ribose (cADPR). This messenger is synthesized by ADP-ribosyl cyclase, which is expressed in various types of neurons (for review see Higashida et al. 2001). Importantly, the activity of the enzyme is regulated through the NO/cGMP/cGK signalling cascade (Galione et al. 1993; Galione 1994; Reyes-Harde et al. 1999a,b; Higashida et al. 2001). A rise in cGMP leads to an increase of the cADPR concentration in hippocampal slices. It is assumed that ADP-ribosyl cyclase is a presynaptic target of cGK. Its phosphorylation boosts cADPR synthesis, which leads to enhanced Ca^{2+} release from ryanodine-sensitive stores. This store-dependent presynaptic Ca^{2+} pool, ultimately, causes a decrease in transmitter release through unknown mechanisms. Collectively, the observations discussed above indicate that presynaptic target molecules with opposite functional impact on transmitter release, e.g., VASP (increase in release) and ADP ribosyl cyclase (decrease in release), may become activated through the NO/cGMP/cGK signalling cascade. Differential expression of these signal transduction elements may provide a mechanism enabling discrete recruitment of effectors that either facilitate or inhibit transmitter release.

cGK-Independent Presynaptic cGMP Signalling

In most of the aforementioned studies the involvement of cGKs as mediators of NO/cGMP effects has been analyzed by using pharmacological "cGK inhibitors." However, it is emerging that the presently available "cGK inhibitors" might be less specific than previously thought and, therefore, produce effects not related to cGK inhibition (Burkhardt et al. 2000; Gambaryan et al. 2004; Marshall et al. 2004; Feil R et al. 2005a). In the future, the analysis of cGK-deficient mouse models should help to dissect presynaptic NO/cGMP signalling without the use of uncertain pharmacological inhibitors. It is likely that not all effects of NO/cGMP are mediated by cGKs. Cytosolic cGMP can activate members of two ion channel families, CNG and HCN channels (Hofmann et al. 2005) leading to membrane depolarization and excitation. Moreover, both CNG and HCN channels have been reported to permit Ca^{2+} influx that may contribute to modulation of neuronal excitability and synaptic function (Koutalos and Yau 1996; Broillet and Firestein 1997; Parent et al. 1998; Yu et al. 2004). This section shortly summarizes evidence supporting the view that cGMP can indeed modulate transmitter release through these ion channels clustered in presynaptic terminals.

Recordings of membrane currents and capacitance changes from cones in the salamander retina have demonstrated that exocytosis can be triggered by a Ca^{2+} influx mediated through a cGMP-gated channel in the inner segment and in synaptic processes of cones (Rieke and Schwartz 1994). These CNG channels are clustered at high-density in cone terminals and can be activated by endogenous NO through stimulation of sGC-dependent cGMP synthesis (Savchenko et al. 1997). Furthermore, the sGC inhibitors, ODQ and LY-83583, suppress NO-induced glutamate release from cones. CNG channels are expressed in a variety of brain regions, including the hippocampus and, accordingly, have been suggested to serve as universal presynaptic transducers linking activity-dependent generation of NO and cGMP to enhanced transmitter release (Kingston et al. 1996; Bradley et al. 1997).

Garthwaite and co-workers (2006) have recently shown that rat optic nerve axons express various members of the HCN family and that HCN channels can serve as presynaptic transducers for the NO/cGMP signalling cascade. Actually, NO can cause a massive rise in cGMP levels sufficient to saturate the cyclic nucleotide binding site of HCN channels (Garthwaite 2005). The HCN channel blocker ZD7288 diminishes depolarization induced by the NO donor PAP/NO or by 8-Br-cGMP in optic nerve axons. NO-induced effects in this preparation are associated with an increase in cytosolic cGMP but not cAMP. There are a few reports suggesting that NO/cGMP can activate HCN channels also in other neurons (Pape and Mager 1992; Ingram and Williams 1996; Abudara et al. 2002; Pose et al. 2003; Kim et al. 2005). Within the HCN family, the HCN2 and HCN4 isoforms exhibit the most prominent regulation by cyclic nucleotides. The HCN2 subtype is expressed at considerable levels throughout the brain, while substantial expression of HCN4 channels is limited to a few areas, including the thalamus (Moosmang et al. 1999; Bender et al. 2001; Notomi and Shigemoto 2004). This hints at the HCN2 isoform as a possible general effector of NO signalling in the brain. As for CNG channels,

NO/cGMP-dependent activation of HCN channels leads to depolarization, increased excitability, and increased ability to conduct action potential bursts (for review see Robinson and Siegelbaum 2003).

3.2.2 NO as an Anterograde Messenger in Cerebellar LTD

LTD of the parallel fiber (PF)-Purkinje cell (PC) synapse in the cerebellum is a cellular model system, which has been suggested to underlie certain forms of motor learning. Induction of cerebellar LTD requires a postsynaptic kinase limb and a complementary phosphatase limb. The latter involves NMDA receptor-dependent generation of NO and postsynaptic cGMP synthesis followed by inhibition of protein phosphatases. Activation of both limbs promotes AMPA receptor phosphorylation and internalization resulting in LTD of synaptic transmission (Ito 2002). A postsynaptic source of NO is highly unlikely, since neither NMDA receptors nor nNOS were detected in postsynaptic PCs. Instead, and in contrast to the hippocampus, it is thought that NO acts in the cerebellum as an anterograde messenger that is produced in PF terminals or interneurons (Shin and Linden 2005) and then diffuses to the postsynaptic PC to induce LTD via a cGMP/cGKI-dependent mechanism. These postsynaptic actions will be discussed only briefly, because the focus of this review is presynaptic NO/cGMP signalling. A comprehensive review of the role of the NO/cGMP/cGKI cascade in cerebellar LTD and learning can be found elsewhere (Feil R et al. 2005b).

NO or cGMP present coincidently with a postsynaptic Ca^{2+} signal are sufficient to induce LTD (Lev-Ram et al. 1995, 1997a). LTD is blocked by inhibitors of NO synthase or guanylyl cyclase, can be restored by exogenous NO and cGMP, and is abolished in nNOS mutant mice (Hartell 1994; Boxall and Garthwaite 1996; Lev-Ram et al. 1997a,b). Among the multiple receptors for cGMP, the cGKI might serve a key function in cerebellar LTD. It is highly expressed in PCs (Hofmann and Sold 1972; Lohmann et al. 1981; Feil S et al. 2005), while cGKII has not been detected (El-Husseini et al. 1995; Geiselhoringer et al. 2004). A role for cGKI was shown by inhibitor studies (Hartell 1994; Lev-Ram et al. 1997a) and, more recently, by the analysis of PC-specific cGKI knockout mice (Feil R et al. 2003). These conditional mouse mutants are fully viable and show no generalized structural or physiological abnormalities in the cerebellum. However, they exhibit a near complete loss of cerebellar LTD. Interestingly, this LTD defect does not affect general motor performance of the mutants, but results in a rather specific defect in the adaptation of the vestibulo-ocular reflex (VOR), a simple form of motor learning (Feil R et al. 2003). Similar phenotypes, defective cerebellar LTD but relatively mild and specific defects in motor learning, have also been reported for other transgenic mouse models, including nNOS-deficient mice (Lev-Ram et al. 1997b) and mice overexpressing a protein kinase C inhibitor peptide selectively in PCs (De Zeeuw et al. 1998). On the other hand, it appears that the impaired general motor performance observed in many conventional knockout mouse models is usually associated with structural alterations of the cerebellum caused by the gene knockout (Ito 2001). Together, these

results unequivocally identify cGKI as a critical component in cerebellar LTD and, moreover, suggest that this type of synaptic plasticity is involved in specific forms of motor learning, such as VOR adaptation, rather than in general motor performance. This concept has been recently supported by the observation that pharmacological prevention of cerebellar LTD in rodents does not affect motor learning in several standard tests (Welsh et al. 2005). Indeed, there is little doubt that synaptic plasticity in other brain regions, such as the striatum, plays also an important role in motor learning (Dang et al. 2006).

How could activation of cGKI in PCs contribute to LTD and cerebellum-dependent learning? As described above, the induction of LTD is thought to depend on the balance between protein kinases and phosphatases that regulates the level of postsynaptic AMPA receptor phosphorylation and internalization. The NO/cGMP/cGKI cascade has been linked to the phosphatase limb (Ito 2002). LTD can be facilitated by inhibiting the protein phosphatase 1/2A (Ajima and Ito 1995). Intriguingly, the phosphorylated form of G-substrate, a well-characterized cGK substrate expressed in PCs, reduces the activity of protein phosphatase 1/2A (Hall et al. 1999). Decreased phosphatase activity should result in increased levels of AMPA receptor phosphorylation, which is assumed to facilitate its clathrin-mediated endocytotic removal from the postsynaptic membrane (Wang and Linden 2000; Chung et al. 2003). Thus, a likely signalling pathway is as follows: NO/cGMP-dependent activation of cGKI in PCs results in phosphorylation of G-substrate followed by inhibition of protein phosphatases 1/2A and increased phosphorylation and endocytosis of AMPA receptors. The removal of AMPA receptors from the postsynaptic site induces LTD associated with specific forms of motor learning.

4 Conclusion

Presynaptic NO/cGMP signalling modulates transmitter release and thereby supports synaptic plasticity important for learning and memory. Dysfunction of this signal transduction cascade may also contribute to various neurological disorders that involve an altered release of transmitters. Thus, components of the presynaptic NO/cGMP signalling pathway represent interesting targets for the development of new drugs improving cognition and drugs effective in neurodegenerative and psychiatric diseases, e.g., Morbus Alzheimer and schizophrenia. Indeed, initial promising findings in this direction have been reported for PDE inhibitors as well as for sGC stimulators (Chien et al. 2005; Feil R and Kemp-Harper 2006; Hebb and Robertson 2006; Menniti et al. 2006). Certainly, future therapeutic efforts will include further elements of NO/cGMP signalling involved in the modulation of transmitter release.

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Therapeutic Use of Release-Modifying Drugs

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Abstract Presynaptic inhibitory or facilitatory autoreceptors are targets for the endogenous neurotransmitter of the respective neuron, and also for exogenous agonists, partial agonists and antagonists which can produce pharmacological actions through changes in transmitter release. In addition, presynaptic inhibitory or facilitatory heteroreceptors can also be acted upon by exogenous agonists, partial agonists or antagonists to induce changes in transmitter release with useful therapeutic effects. This article summarizes drugs that are known or likely to produce their therapeutic effects through presynaptic modulation of neurotransmitter release. Included are drugs acting on α and β adrenoceptors, dopamine receptors,

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angiotensin, opioid, cannabinoid, and nicotinic acetylcholine receptors. Also discussed are changes in presynaptic receptor mechanisms produced by drugs that inhibit transmitter re-uptake.

1 Introduction

A considerable number of inhibitory and facilitatory presynaptic autoreceptors were characterized in the peripheral and central nervous systems. The available evidence indicates that these autoreceptors correspond to a specific subtype of transmitter receptor (Table 1). Since presynaptic autoreceptors play a physiological role in the regulation of transmitter release, it follows that their selective activation by agonists will lead to a decrease (inhibitory autoreceptors) or increase (facilitatory autoreceptors) in transmitter output, while blockade by antagonists will produce the opposite change. These presynaptic effects on neurotransmitter release can result in mild pharmacological effects possessing useful therapeutic properties. It can be argued that the side effect profile of drugs acting presynaptically on transmitter release may be different and probably milder than that obtained with the classical approach of agonists or antagonists acting at postsynaptic receptors and therefore modifying effector organ responses in every tissue where such postsynaptic receptor subtype is located.

The number of presynaptic inhibitory or facilitatory heteroreceptors is larger than that of autoreceptors (Table 2), but these presynaptic receptors are mainly the target of action for exogenous agonists or partial agonists, unless the presynaptic heteroreceptor is activated under physiological or pathological conditions by a locally formed autacoid or by the transmitter released from neighboring nerve terminals.

An additional mechanism whereby presynaptic autoreceptors can be acted upon corresponds to drug–induced increases in the synaptic concentration of the neurotransmitter, as observed with antidepressants which inhibit the neuronal uptake of

Table 1 Presynaptic autoreceptors. Shown are the transmitters for which a negative or positive feedback mechanism mediated by presynaptic autoreceptors has been established. Note that in each case a specific receptor subtype is involved in the presynaptic modulation of transmitter release

Transmitter	Inhibitory Receptor Subtype	Facilitatory Receptor Subtype
NA	α_{2A}-Adrenoceptors (mainly)	β_2-Adrenoceptors (periphery)
DA	D_2 / D_3	
ACh	M_2	Nicotinic
5-HT	5-HT_{1D}	
Histamine	H_3	
GABA	$GABA_B$	
Glutamate	mGluR3 – mGluR5	

Table 2 Presynaptic heteroreceptors. The list is not exhaustive. Agonists acting on the presynaptic heteroreceptor subtypes inhibit or facilitate transmitter release. Selective antagonists block the effect of the agonist. Given alone, antagonists modify transmitter release only if there is activation of the heteroreceptors by an endogenous agonist from a neighboring synapse or a locally produced or circulating endocoid

Transmitter	Inhibitory Receptor Subtype	Facilitatory Receptor Subtype
NA	Opioid, M_2, H_3, CB_1, PGE, D_2	ANG AT-1, Nicotinic, NMDA
DA	M_2, CB_1	Nicotinic, NMDA
ACh	α_2, D_2, Opioid, CB_1	
5-HT	α_2	
Glutamate	5-HT_{1D}, $GABA_B$, CB_1	Nicotinic, NMDA
CCK	$GABA_B$	
GABA	CB_1	
CGRP	5-HT_{1D}	
Somatostatin	$GABA_B$	

ANG: angiotensin; CCK: cholecystokinin; CGRP: calcitonin-gene-related peptide; NMDA: N-methyl–D-aspartate; PGE: prostaglandin E; CB: cannabinoid.

noradrenaline or serotonin. Such antidepressant drugs initially induce a decrease in transmitter release by overactivation of presynaptic inhibitory autoreceptors. Chronic activation of inhibitory autoreceptors by the endogenous agonist, however, leads to downregulation and desensitization, and the net result then is a facilitation of noradrenergic or serotonergic neurotransmission.

In some cases, the discovery and characterization of presynaptic receptors established the existence of a new receptor subtype, like the α_2 adrenoceptor (Dubocovich and Langer 1974; Langer 1974) and histamine H_3 receptor (Arrang et al. 1987). It should be noted, however, that the receptor subtypes located presynaptically are also located postsynaptically. The latter should be taken into account in the strategy of discovery and development of new drugs with preferential presynaptic effects.

2 Presynaptic α_2 Autoreceptor Agonists and Antagonists

The α_2 subtype of adrenoceptors was first discovered on peripheral noradrenergic nerve endings (Dubocovich and Langer 1974; Langer 1974; Cubeddu et al. 1974) and on cell bodies and dendrites of central noradrenergic neurons (Svensson et al. 1975). Following the classification of adrenoceptors into α and β subtypes by Alquist (1948) it was generally accepted for many years that the α adrenoceptors represented a homogeneous population. In 1974, however, pharmacological differences were established between α_1 and α_2 adrenoceptors, with the potency of the antagonist phenoxybenzamine differing by 100-fold in blocking postsynaptic α_1 adrenoceptors when compared to the presynaptic α_2 adrenoceptors in the perfused cat spleen (Dubocovich and Langer 1974 ; Cubbedu et al. 1974). Subsequently, the

pharmacological differences between α_1 and α_2 adrenoceptors were further confirmed by establishing differences in the relative order of potencies of many agonists as well of antagonists. More than 10 years later three α_1 and three α_2 adrenoceptor subtypes were cloned and expressed: α_{1A}, α_{1B} and α_{1D} on the one hand, α_{2A}, α_{2B} and α_{2C} on the other hand (for review see Bylund et al. 1994). It is of interest to note that the majority of the presynaptic inhibitory α_2 autoreceptors belong to the α_{2A} subtype (Table 1; Starke 2001), although α_{2B} and α_{2C} autoreceptors also exist (Trendelenburg et al. 2003; Gilsbach and Hein, this volume).

2.1 Agonists: Clonidine and Analogs

Clonidine is a classical reference compound, discovered by serendipity (Hoefke and Kobinger, 1966) and developed for its antihypertensive and bradycardic properties. Subsequently, clonidine was shown to possess α_2 adrenoceptor agonist properties, both in the periphery and in the central nervous system (Kobinger 1978). The therapeutic effects of clonidine in hypertension are accompanied by sedation and dry mouth as side effects, which limit its wide clinical use. The sympathoinhibitory effects of clonidine and clonidine–like drugs are due to activation of postsynaptic α_2 adrenoceptors in the cardiovascular centers of the medulla oblongata. Peripheral inhibition of neurotransmitter release from postganglionic sympathetic neurons by clonidine contributes to the bradychardic and hypotensive effects, through activation of presynaptic α_2 autoreceptors (Langer et al. 1980; Urban et al. 1995). It is of interest that dry mouth is mainly due to the activation by clonidine of presynaptic α_2 heteroreceptors on the cholinergic nerves innervating the salivary gland (Izumi et al. 1995).

A second hypothesis to explain the cardiovascular effects of clonidine involves an interaction with imidazoline-1 receptors (Bousquet and Feldman 1999). However, the available evidence at present does not prove conclusively the imidazoline hypothesis for the mechanism of action of clonidine and analog drugs, like rilmenidine and moxonidine (Szabo 2002).

2.2 Antagonists: Mianserin, Mirtazapine, Idazoxan, Efaroxan

Both mianserin and mirtazapine are antidepressant drugs which possess central α_2 adrenoceptor blocking properties (pA_2 7.3). However, mirtazapine is much more potent at histamine H_1 receptors (pA_2 9.1) and at 5-HT_2 and 5-HT_3 receptors (pA_2 8.2). Blocking of H_1 receptors explains the main side effects of mirtazapine, which produces marked sedation and weight gain. Blockade of presynaptic inhibitory α_2 autoreceptors increases the release of NA, while blockade of presynaptic α_2 inhibitory heteroreceptors on serotonin nerve terminals (Table 2) is likely to increase the release of serotonin.

These facilitatory effects on NA and serotonin are likely to contribute to the antidepressant actions of these two drugs, since a decrease in monoaminergic neurotransmission involving both NA and serotonin in the brain is associated with the physiopathology of depression (Svensson 2000; Mongeau et al. 1997).

Based on the enhancement of noradrenergic and serotonergic transmission induced by central α_2 adrenoceptor antagonists, it was proposed that this class of compounds may possess antidepressant properties (Langer 1978). Idazoxan is a potent and selective α_2 adrenoceptor antagonist that penetrates readily the blood-brain barrier (De Boer et al. 1996), yet only a few studies exist in the literature where idazoxan was administered in monotherapy in monopolar depressed patients, and the antidepressant effects were present only in some of the patients. There are no published double-blind studies of idazoxan against placebo in monopolar depression. There is one double-blind study comparing idaxozan and bupropion during six weeks in bipolar depressed patients but without a placebo group (Grossman et al. 1999). In this study, both idaxozan and bupropion demonstrated significant improvement over time with reductions in the Hamilton Depression Rating Scale of 50% (Grossman et al. 1999).

Use of idaxozan as add-on therapy to antipsychotics in the treatment of schizophrenia is supported by preclinical data (Hertel et al. 1999; Wadenberg et al. 2007) and clinical results (Litman et al. 1996).

It should be noted that idazoxan has a short duration of action in humans and has to be administered three times a day. In addition, only racemic idazoxan has been administered to humans. The two enantiomers of idazoxan were studied on both α_1 and α_2 adrenoceptors (Dabire et al. 1986). It was shown that $(+)$ idazoxan was more potent than $(-)$ idazoxan. Furthermore, $(+)$ idazoxan antagonizes both α_1 and α_2 adrenoceptors, while $(-)$ idazoxan possesses both α_1 and α_2 adrenoceptor agonist effects (Dabire et al. 1986).

It is of interest that idazoxan was tested in Parkinson's disease, although this indication was abandoned for lack of efficacy (F. Colpaert, personal communication). Efaroxan and its active dextro enantiomer dexefaroxan are also potent and selective centrally acting α_2 adrenoceptor antagonists (Bauer et al. 2003; Rizk et al. 2006). Efaroxan was developed for the treatment of chronic neurodegenerative diseases but abandoned because of side effects (P. Sokoloff, personal communication).

Activation of α_2-adrenoceptors in pancreatic β-cells inhibits insulin release, and this effect is blocked by α_2 adrenoceptor antagonists (Angel and Langer. 1988; Lorrain et al. 1992). A peripherally active α_2 adrenoceptor antagonist, SL 84.0148, was developed for the treatment of type ll diabetes (Angel et al. 1992;1996) but was abandoned in Phase ll (S.Z. Langer, unpublished).

α_2 Adrenoceptor antagonists may increase heart rate as well as blood pressure. These side effects are due to increased release of NA resulting from the blockade of presynaptic terminal α_2 autoreceptors on peripheral sympathetic neurons (Langer 1981). These cardiovascular side effects are minimal or absent with idazoxan (Schmidt et al. 1997).

3 Presynaptic Central Dopamine Autoreceptor Partial Agonist: Aripiprazole

Aripiprazole is a new drug for the treatment of schizophrenia and was approved by the Food and Drug Administration in November, 2002. Aripiprazole possesses presynaptic D_2 autoreceptor partial agonist activity and postsynaptic D_2 receptor antagonist properties (Kane et al. 2002; Kasper et al. 2003; Potkin et al. 2003). In contrast to most antipsychotic drugs, aripiprazole does not elevate the circulating prolactin levels, one of the undesirable side effects of this class of drugs. Like many of the presynaptically acting compounds, aripiprazole acts also on other receptors, possessing 5-HT_{1A} partial agonist properties and 5-HT_{2A} antagonist actions.

4 Presynaptic Peripheral Dopamine Heteroreceptor Agonist: Lergotrile

Presynaptic inhibitory dopamine heteroreceptors on noradrenergic nerve endings were first reported in in vitro studies and shown to be of the D_2 receptor subtype (Table 2; Enero and Langer 1975; Dubocovich and Langer 1980a). In vivo studies in the dog showed that the hypotensive and bradycardic effects of lergotrile, a D_2 receptor agonist, were due to the stimulation of presynaptic DA receptors on noradrenergic nerve terminals (Barret and Lockandwala 1981). In support of this view, lergotrile inhibited the positive chronotropic and the renal vasoconstrictor effects elicited by postganglionic sympathetic stimulation. These effects were prevented by prior treatment with sulpiride, a selective D_2 receptor antagonist (Barret and Lockandwala 1981). Additional in vivo evidence confirmed that compounds with D_2 receptor agonist effects like pergolide and N-n-propyl-N-n-butyl dopamine possess bradycardic and antihypertensive actions due to a reduction in NA output from peripheral sympathetic nerve fibers (Hamed et al. 1981 ; Cavero et al. 1985). However, the main side effect of D_2 agonists, nausea and vomiting, represents an important obstacle in the development of compounds in this category for the treatment of hypertension.

5 Presynaptic 5-HT_{1D} Heteroreceptor Agonists: Sumatriptan and Analogs

Presynaptic 5-HT_{1D} inhitory heteroreceptors exist on substance P- and CGRP-containing terminals (Smith et al. 2002; Hou et al. 2001; Table 2). Activation of these receptors by agonists like sumatriptan and related analogs leads to inhibition of the release of these two neurotransmitters (Williamson et al. 1997, 2001). The therapeutic efficacy of sumatriptan and analogous drugs in the treatment of

migraine is associated at least partially with these inhibitory presynaptic effects on substance P and CGRP release and reflects a significant therapeutic improvement when compared with other available medications – an important recent example of the suitability of pharmacological presynaptic modulation for therapeutic purposes.

6 Presynaptic Angiotensin Heteroreceptors: Angiotensin Receptor Antagonists

Blockade of presynaptic facilitatory angiotensin AT_1 receptors on noradrenergic nerve endings in the cardiovascular system (Table 2) may contribute to the antihypertensive effects of angiotensin receptor antagonists. By reducing the enhanced release of NA induced by locally produced or circulating angiotensin, the antagonists reduce sympathetic tone in the cardiovascular system.

7 Presynaptic β Autoreceptors: β Adrenoceptor Antagonists

Peripheral but not central noradrenergic nerve endings possess facilitatory β_2 in addition to the inhibitory α_2 autoreceptors (Adler-Graschinsky and Langer 1975; Table 1). The presynaptic facilitatory β adrenoceptors in noradrenergic nerve endings of peripheral blood vessels play a physiopathological role in Raynaud's disease. It was reported that infusion of a very low dose of isoprenaline (1 μg/minute) in patients with Raynaud's disease produced a highly significant reduction in blood flow when compared to healthy controls (Giovanni et al. 1984). These low doses of isoprenaline enhance NA release but are not high enough to produce β_2 adrenoceptor-mediated vasodilation postsynaptically. Similar conclusions were reached by Brotzu et al. (1989), who administered atenolol and/or flunnarizine to 40 patients with Raynaud's disease.

Increased responsiveness to presynaptic facilitatory β adrenoceptors on peripheral noradrenergic nerve endings has been suggested to contribute to the development, rather than the maintenance, of hypertension (Borkowski, 1990). Accordingly, enhanced vasoconstriction would result from the increased release of NA through activation of presynaptic facilitatory β-receptors by the transmitter NA and by circulating adrenaline. The antihypertensive effect of β adrenoceptor antagonists is well established. It was suggested that upon chronic administration of β adrenoceptor antagonist drugs there is a reduction in NA release due to blockade of the presynaptic facilitatory β adrenoceptors.

8 Presynaptic Opioid Heteroreceptor Agonists: Morphine and Related Drugs

The inhibition by opioid receptor agonists of peripheral noradrenergic neurotransmission was reported as early as 1957 (Trendelenburg 1957). Subsequently, it was established that presynaptic inhibitory opioid heteroreceptors exist in many peripheral postganglionic sympathetic neurons (Dubocovich and Langer 1980b). It is of interest that the presynaptic inhibitory opioid receptors in the vas deferens were used successfully as a bioassay in the discovery of the endogenous pentapeptides leu- and met-enkephalin. Presynaptic opioid inhibitory receptors also exist in the central nervous system (Arbilla and Langer 1978). One therapeutically important effect is inhibition of the release of substance P from primary afferent pain fibers (Jessel and Iversen 1977). The possible role of presynaptic opioid receptors in the development of addiction or in the symptomatology of withdrawal remains an area of interest. Such a role is supported by the finding that in opioid withdrawal, contrary to the acute effect of opioids, release of noradrenaline from locus coeruleus neurons is increased, both in situ (Montel et al. 1975) and when the neurons are kept in culture (Ronken et al. 1993).

9 Presynaptic Cannabinoid Heteroreceptor Agonists: Cannabis and Related Drugs

Presynaptic inhibition through cannabinoid subtype-1 (CB_1) receptors is the most prominent neuronal effect of cannabinoids. Inhibitory CB_1 occur on terminals of noradrenergic, dopaminergic, cholinergic, GABAergic, and glutamatergic neurons (Katona et al. 1999; Gobel et al. 2000; Schlicker and Kathmann 2001; Szabo and Schlicker 2005; Domenici et al. 2006; Table 2). Endocannabinoids regulate the function of many synapses as retrograde messengers (Harkany et al. 2007; Lovinger, this book). It is very likely, therefore, that presynaptic inhibition also underlies the proposed therapeutic effects of cannabinoids – as well as the effects sought by cannabis abusers. For example, cannabinoids, like opioids, inhibit transmitter release from primary nociceptive afferents (Liang et al. 2003) – a basis for their analgesic properties. Sativex®, a whole cannabis plant extract, has been approved in Canada for neuropathic pain associated with multiple sclerosis.

10 Presynaptic Nicotinic Heteroreceptor Agonists: Nicotine as a Drug of Addiction

Nicotine enhances dopamine release by acting on presynaptic facilitatory heteroreceptors located on the terminal regions of dopaminergic neurons (Marshall et al. 1997). It is tempting to associate these effects of nicotine and the fact that tobacco dependence, the most common substance abuse disorder, is due to nicotine. Nicotine

is thought to interact with the mesocorticolimbic DA system in a manner similar to that of other drugs of abuse, such as cocaine (Corrigal et al. 1992). This may explain the tolerance, dependence, and withdrawal syndrome in both animals and humans (Stein et al. 1998).

11 Changes in Sensitivity of Presynaptic Autoreceptors Induced by Antidepressant Drugs

It is generally accepted that a decrease in monoaminergic neurotransmission involving NA and serotonin is central to the pathophysiology of depression. This view is the rationale for the clinical use of inhibitors of neuronal uptake of NA and/or serotonin, which augment and prolong the synaptic actions of these transmitters in the central nervous system. However, while the inhibition of neuronal uptake of NA or serotonin is established within 24 hours of starting the treatment, the clinical improvement takes three to four weeks to develop – a paradoxical phenomenon, which can be explained through effects on somadendritic and presynaptic autoreceptors. The acute administration of drugs that inhibit neuronal uptake (NA or serotonin) increases the synaptic concentration of the corresponding transmitter, which produces overactivation of somatodendritic and terminal autoreceptors. Such effects result in suppression of the firing rate of NA or serotonin neurons or both, and in a decrease in transmitter release from the nerve terminals. Chronic activation of inhibitory autoreceptors by endogenous transmitter in turn leads to their downregulation; after a few weeks, the firing rate and nerve terminal release of the transmitter recover, and the result is a facilitation of noradrenergic or serotonergic neurotransmission (Svensson and Usdin 1978; Langer 1997).

On this basis, the potential antidepressant action of central α_2 adrenoceptor antagonists was predicted in 1978 (Langer 1978). However, as mentioned above, idazoxan, a selective antagonist on central α_2 adrenoceptors, does not possess antidepressant effects in monotherapy in unipolar depression. The antidepressant effects of idazoxan alone in bipolar depression remain to be confirmed by additional double blind studies against placebo.

It is possible that the therapeutic indication for idazoxan in depression may be as add-on therapy in patients who are non-responders to inhibitors of neuronal uptake of serotonin or of NA. In addition, drugs which block presynaptic autoreceptors on serotonergic neurons may possess antidepressant properties in monotherapy or as add-on therapy to serotonin uptake inhibitors.

12 Conclusions and Future Perspectives

The original observations made in the early 1970s led to the hypothesis that neurotransmitter release was regulated at the level of the nerve terminals by presynaptic auto- and heteroreceptors. These discoveries were confirmed and extended

during the following 30 years. Presynaptic release-modulating receptors represent suitable targets for pharmacological intervention by exogenous compounds acting as agonists, partial agonists, or antagonists, with therapeutic value by influencing transmitter release. Such drugs may have useful therapeutic properties and, in some cases, like depression, address unmet medical needs. In addition, by influencing transmitter release presynaptically, this novel family of drugs may possess fewer side effects than drugs which stimulate or block the classical postsynaptic receptors. In addition to the development of increasingly selective drugs acting on presynaptic release-modulating receptors, it is important to consider the relevance of changes in sensitivity of presynaptic autoreceptors induced by antidepressant drugs which inhibit the neuronal transporters in noradrenergic and serotonergic neurons.

Several families of drugs of addiction (opioid receptor agonists, cannabinoid receptor agonists, and nicotine) possess important presynaptic effects which result in inhibition (opioids and cannabinoids) or facilitation (nicotine) of the release of several neurotransmitters in the periphery and the central nervous system. These effects are mediated through specific receptor subtypes and may be relevant to tolerance, dependence, or withdrawal syndromes. It is tempting to speculate that presynaptic effects of these three families of drugs of addiction may provide useful insights in understanding neurochemical mechanisms in addiction and developing novel drug treatments for these patients.

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