Mark G. Darlison
Editor

Inhibitory Regulation of Excitatory Neurotransmission

Results and Problems in Cell Differentiation

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44

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Inhibitory Regulation of Excitatory Neurotransmission

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For Annerose and Max,
and others who have made my life worthwhile.

"Learning is not attained by chance,
it must be sought for with ardor and attended to with diligence"

Abigail Adams (1744 to 1818)

Preface

That inhibitory mechanisms evolved in nervous systems after the appearance of excitatory ones seems obvious, although this is difficult to prove. And, as evidenced from studies on invertebrates, early cell-to-cell communication may have been mediated by peptides, which are abundant both in number and variety in the nerve nets of cnidarians (for example, sea anemones, corals, and jellyfish) and in the relatively simple nervous systems of mollusks. However, in mammals, the majority of excitatory and inhibitory neurotransmission is produced by just two amino acids, L-glutamate and γ-aminobutyric acid (GABA), respectively. L-glutamate binds to, and activates, a plethora of receptors that either possess an integral ion channel (ionotropic receptors) or couple to different guanine nucleotide binding proteins (G-protein coupled receptors; also called metabotropic glutamate receptors); perhaps surprisingly, this molecule can produce both excitatory and inhibitory effects. GABA also mediates its effects by binding to ionotropic GABA type A ($GABA_A$) and type C ($GABA_C$) receptors and metabotropic type B ($GABA_B$) receptors. And, at least early in development, GABA responses can be excitatory, whereas normally they are inhibitory. Since the cloning of the first of these receptors, in 1987, our knowledge of their structures, pharmacologies, locations, precise physiological functions, and down-stream signaling mechanisms has expanded enormously. As someone who has worked in this field for many years, I still find it remarkable that so many different types of receptor, and subtypes of receptor, are needed to fulfill the transmitter functions of two simple amino acids.

The aim of this book is not to cover old ground, by reviewing each type of glutamate and GABA receptor in turn, because there are many up-to-date reviews on these signaling molecules. Instead, the concept was to explore the roles played by inhibitory proteins (not just receptors), and mechanisms, in the regulation of neuronal excitation. In doing so, it was inevitable that some information would be included on $GABA_A$, $GABA_B$, and $GABA_C$ receptors; however, this has been kept to a minimum and, in the case of the ubiquitous $GABA_A$ receptors, has focused on comparatively less well-considered areas such as their extrasynaptic and presynaptic functions, their insertion and removal from the cell surface, and depolarizing actions mediated by them. Other important modes of regulation of excitation that are covered here include the activation of glycine receptors, metabotropic glutamate receptors, neuropeptide recep-

tors and potassium channels, and the role played by GABA transporters. In addition, there is detailed discussion of mutations that disrupt the regulation of excitatory neurotransmission, and their consequences, and the targeting of the GABAergic system for therapeutic benefit. It is my hope that all researchers in this field, both young and old, will find something new and of value in this Volume.

The idea for this book germinated at a Hamburg-Blankenese Conference, an international meeting that is held each year in a beautiful location on the western edge of Hamburg that overlooks the River Elbe; this Conference was organized by Prof. Dr. Gebhard Koch and Prof. Dr. Dietmar Richter. The latter is one of the Series Editors of "Results and Problems in Cell Differentiation," and I thank him for the opportunity of editing this Volume. I also wish to thank Prof. Dr. Henri Tiedge for his encouragement, Ursula Gramm and Sabine Schreck at Springer for their help, and all of the authors for their contributions, without which this book would not exist. I hope that you all agree that the end result was worth the effort.

Nottingham Mark G. Darlison

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Regulation of Excitation by $GABA_A$ Receptor Internalization

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Abstract Neuronal inhibition is of paramount importance in maintaining the delicate and dynamic balance between excitatory and inhibitory influences in the central nervous system. GABA (γ-aminobutyric acid), the primary inhibitory neurotransmitter in brain, exerts its fast inhibitory effects through ubiquitously expressed $GABA_A$ receptors. Activation of these heteropentameric receptors by GABA results in the gating of an integral chloride channel leading to membrane hyperpolarization and neuronal inhibition. To participate in neurotransmission, the receptor must reside on the cell surface. The trafficking of nascent receptors to the cell surface involves posttranslational modification and the interaction of the receptor with proteins that reside within the secretory pathway. The subsequent insertion of the receptor into specialized regions of the plasma membrane is dictated by receptor composition and other factors that guide insertion at synaptic or perisynaptic/extrasynaptic sites, where phasic and tonic inhibition are mediated, respectively. Once at the cell surface, the receptor is laterally mobile and subject to both constitutive and regulated endocytosis. Following endocytosis the receptor undergoes either recycling to the plasma membrane or degradation. These dynamic processes profoundly affect the strength of GABAergic signaling, neuronal inhibition, and presumably synaptic plasticity. Heritable channelopathies that affect receptor trafficking have been recently recognized and compelling evidence exists that mechanisms underlying acquired epilepsy involve $GABA_A$ receptor internalization. Additionally, $GABA_A$ receptor endocytosis has been identified as an early event in the ischemic response that leads to excitotoxicity and cell death. This chapter summarizes what is known regarding the regulation of receptor trafficking and cell surface expression and its impact on nervous system function from both cell biology and disease perspectives.

1 Introduction

The $GABA_A$ receptor is the predominant inhibitory receptor in brain and mediates both phasic and tonic GABAergic neurotransmission. In response to the binding of GABA, an integral chloride channel opens allowing chloride to enter the neuron which results in membrane hyperpolarization and neuronal inhibition. Deficits in $GABA_A$ receptor function have been associated with both psychiatric disease and neurological disorders. Drugs which allosterically potentiate the receptor, such as benzodiazepines, barbiturates,

and anesthetics, are widely used therapeutic agents. The $GABA_A$ receptor belongs to the Cys-loop family of ligand-gated ion channels of which the nicotinic acetylcholine receptor is prototypic (Connolly and Wafford 2004). Other members of this family include the glycine and serotonin type-3 (5-HT3) receptors. Many distinct but homologous $GABA_A$ receptor subunits (α1–6, β1–3, γ1–3, δ, ε, θ, and π) exist. Each subunit possesses a large extracellular N-terminus, four membrane spanning regions, and a short extracellular C-terminus. Between the third and fourth membrane spanning regions, a large cytoplasmic loop exists that is subject to posttranslational modification and interactions with various regulatory, chaperone, and scaffolding proteins. The various subunits are preferentially assembled to form heteropentameric receptors. Despite the vast theoretically possible number of heteropentameric assemblies, only a limited number of receptor subtypes are constructed physiologically. This preferential assembly is governed by differential expression patterns within various neuronal cell types and multiple assembly signals within the subunits themselves (recently reviewed by Bollan 2003), and receptors composed of two α1, two β2, and one γ2 subunit are expressed most abundantly. Following assembly in the endoplasmic reticulum, the receptor is trafficked to the cell surface where there is dynamic regulation of its surface expression. Such regulation profoundly affects the efficacy of GABAergic transmission and the overall excitability of the central nervous system and is the focus of this chapter.

2 Trafficking, Membrane Targeting, and Clustering of Newly Synthesized Receptors

Once assembled the oligomerized receptors are obligatorily trafficked through the trans-Golgi network on their way through the secretory pathway for exocytosis to the cell surface. Along this pathway the newly synthesized receptors are palmitoylated by the Golgi apparatus-specific protein with the DHHC zinc finger domain (GODZ) and interact with the guanine nucleotide exchange factor brefeldin A-inhibited GDP/GTP exchange factor 2 (BIG2) and the microtubule-associated protein $GABA_A$ receptor associated protein (GABARAP) to reach the cell surface by mechanisms that are just beginning to be understood. Targeting information within the receptors directs insertion at either synaptic, perisynaptic, or extrasynaptic sites where distinct receptor subtypes play unique roles in inhibitory neurotransmission. Once at the cell surface, the receptor is laterally mobile, and such movement may influence synaptic transmission.

2.1
Trafficking Through the Secretory Pathway

2.1.1
GODZ

Palmitoylation is a reversible posttranslational modification whereby palmitate, a 16-carbon saturated fatty acid, is covalently attached via a thioester bond to cysteine residues of substrate proteins. A general role for palmitoylation in the trafficking of neuronal proteins has been established (Bijlmakers and March 2003). Recently, palmitoylation of both recombinant and neuronal $\gamma 2$ subunits has been reported (Keller et al. 2004; Rathenberg et al. 2004). The GODZ (<u>Go</u>lgi apparatus-specific protein with the <u>D</u>HHC <u>z</u>inc finger domain), a palmitoyltransferase that resides in the Golgi complex, has been identified as a palmitoyltransferase that associates with a cysteine-rich region on the $\gamma 2$ subunit cytoplasmic loop (Keller et al. 2004). Mutation of cysteine residues that are substrates for palmitoylation decreases surface expression and clustering of $\gamma 2$ subunits containing receptors in neurons (Rathenberg et al. 2004), suggesting that palmitoylation of receptors in the secretory pathway is an important determinant for receptor surface expression. The influence of palmitoylation on $GABA_A$ receptor endocytosis, recycling, and stability have not been examined, although palmitoylation is an important posttranslational mechanism for regulating these processes for other neuronal proteins (Bijlmakers and March 2003).

2.1.2
BIG2

BIG2 (<u>b</u>refeldin A-<u>i</u>nhibited <u>G</u>DP/GTP exchange factor <u>2</u>) is a guanine nucleotide exchange factor, present primarily in the trans-Golgi network, that catalyzes the exchange of GDP-GTP on ADP-ribosylation factors (Jones et al. 2006). Yeast two-hybrid and coimmunoprecipitation studies using brain tissue have identified BIG2 as a $GABA_A$ receptor β subunit interacting protein (Charych et al. 2004). Overexpression of BIG2 facilitates the exit of the receptor from the endoplasmic reticulum (ER), and colocalization of BIG2 with the receptor in vesicular structures within the dendritic cytoplasm suggests that BIG2 may play a role in the transport of the receptor to the synapse. Alternatively, the dendritic presence of BIG may be related to Golgi structures in the dendroplasm that are involved in local translational events (Sutton and Schuman 2005; Ule and Darnell 2006). The association of BIG2 with recycling endosomes (Shin et al. 2005; Shen et al. 2006) may also point to a role of BIG2 in $GABA_A$ receptor recycling.

2.1.3 GABARAP

GABARAP (GABA$_A$ receptor associated protein), a 13.9-kDa microtubule-associated protein, was originally identified in a yeast two-hybrid screen for GABA$_A$ receptor γ subunit interacting proteins (Wang et al. 1999). Subsequent GST pull-down and coimmunoprecipitation experiments verified this interaction and determined that the γ subunit cytoplasmic loop binds the GABARAP N-terminal domain (Wang et al. 1999; Nymann-Anderson et al. 2002). Based on the binding of the C-terminal region of GABARAP to tubulin, GABARAP is presumed to link the receptor to microtubule networks (Wang et al. 2000). However, interactions between GABARAP and both the $\gamma 2$ subunit intracellular loop and tubulin are reported to be weak and nonspecific (Knight et al. 2002).

Despite the unresolved importance of the observed GABA$_A$ receptor–GABARAP interaction, mounting evidence supports a significant, albeit poorly understood, role for GABARAP in GABA$_A$ receptor trafficking. In this regard, GABARAP expression increases receptor cell surface levels in COS7 cells and cultured hippocampal neurons (Leil et al. 2004), an effect not observed with GABARAP constructs lacking the γ subunit binding domain. In Xenopus oocytes, coexpression of $\alpha 1\beta 2\gamma 2S$ receptors with GABARAP increases both receptor surface expression and the peak amplitude of GABA-gated chloride currents (Chen et al. 2005). This modulation requires the $\gamma 2$ subunit and the microtubule binding domain of GABARAP and is blocked by microtubule depolymerizing agents. While these findings implicate a role for GABARAP in the transportation of GABA$_A$ receptors to the plasma membrane, GABARAP is not essential for receptor surface expression since GABARAP knockout mice do not display alterations in either the total number of GABA$_A$ receptors or in their synaptic localization (O'Sullivan et al. 2005).

In addition to facilitating receptor cell surface expression, GABARAP promotes clustering of receptors (Chen et al. 2000; Everitt et al. 2004) by a mechanism that requires polymerized microtubules and both the $\gamma 2$ subunit and tubulin binding regions of GABARAP (Chen et al. 2000). GABARAP-clustered receptors expressed in nonneuronal cells display distinct functional properties, including lower sensitivity to GABA (Chen et al. 2000) and an increase in single channel conductance that closely resembles the high conductance states sometimes observed for native receptors (Everitt et al. 2004).

The mechanism(s) by which GABARAP promotes receptor cell surface expression and clustering remains to be revealed, but the preponderance of evidence does not support a role for GABARAP at the synapse. In cultured cortical neurons, GABARAP is enriched in putative ER and Golgi structures and shows very little colocalization with postsynaptic GABA$_A$ receptors (Kneussel et al. 2000). This has been confirmed in hippocampal cultures where GABARAP is found primarily within the Golgi apparatus with limited

labeling in postsynaptic cisternae (Kittler et al. 2001). Further, colocalization of GABARAP with γ2 subunit-containing receptors is observed in the perinuclear cytoplasm, with some staining in dendritic proximal regions (Leil et al. 2004). The identification of GABARAP within the Golgi (Kneussel et al. 2000; Kittler et al. 2001), the homology of GABARAP to the intra-Golgi transport factor GATE-16 (Golgi-associated transport enhancer of 16 kDa) (Sagiv et al. 2000), and the binding of GABARAP to the vesicle fusion protein *N*-ethylmaleimide-sensitive factor (NSF) (Kittler et al. 2001) point to a broad role of GABARAP in vesicular trafficking. Such a role is compatible with available data and supports the concept that GABARAP facilitates the trafficking of $GABA_A$ receptors through the secretory pathway.

2.2 Targeting to Plasma Membrane Subdomains and Clustering

2.2.1 Synaptic $GABA_A$ Receptors

A selective insertion of $GABA_A$ receptor subtypes into distinct regions of the plasma membrane is directed by receptor subunit composition (reviewed in Luscher and Keller 2004). The α1–3β1–3γ2 subunit-containing receptors are preferentially inserted into postsynaptic regions, where they form clusters and mediate the fast phasic inhibitory currents associated with miniature inhibitory postsynaptic currents (mIPSCs). While it is thought that clustering serves to concentrate the receptor postsynaptically, clustering has also been observed for certain extrasynaptic receptor populations (Petrini et al. 2004; Christie et al. 2005). Clustering appears to influence the kinetic properties of the receptor and analysis of mIPSCs and GABA-evoked currents in cultured hippocampal neurons shows that induction of receptor declustering with the microtubule depolymerizing agent nocodazole accelerates entry into the desensitized state (Petrini et al. 2003). The observations that activity deprivation decreases the number of synaptic $GABA_A$ receptor clusters and inhibitory synapses (Kilman et al. 2002), GABAergic innervation organizes receptor clustering (Christie et al. 2002), disruption of postsynaptic $GABA_A$ receptor clustering affects GABAergic innervation (Li et al. 2005), and presynaptic terminals in *Caenorhabditis elegans* induce receptor clustering (Rowland et al. 2006) indicate that an important relationship exists between clustering and synaptic activity.

Postsynaptic $GABA_A$ clustering requires a γ2 subunit (Essrich et al. 1998) and appears to be dependent on the cytoplasmic loop region of the subunit (Meier and Grantyn 2004; Christie et al. 2005). In hippocampal neuronal cultures, expression of myc-tagged chimeric γ2 subunits containing the cytoplasmic loop of the δ subunit failed to form clusters or target to synapses, despite incorporation into receptors (Christie et al. 2005). Furthermore, a GFP-

tagged γ2L cytoplasmic loop, but not GFP-tagged γ2S cytoplasmic loop, targets to inhibitory synapses in transfected spinal cord neurons (Meier and Grantyn 2004). This synaptic targeting was facilitated by PKC activation and was prevented by mutation of Ser343 (Meier and Grantyn 2004), a known PKC phosphorylation site (Moss et al. 1992), indicating that the phosphorylation state of the γ2L subunit may be a factor in determining synaptic clustering. In contrast, postsynaptic clustering has been reported to be dependent on the C-terminal domain including the fourth transmembrane region, but does not require the γ2 cytoplasmic loop (Alldred et al. 2005). Clearly, further studies will be required for a complete understanding of γ2 subunit mechanisms involved in synaptic targeting. Additional determinants of synaptic localization may involve α1–3 subunits (Brunig et al. 2002). Although it is generally believed that α and γ subunits direct receptor plasma membrane subdomain targeting, the β1 subunit can redirect α1 subunit targeting from basolateral to apical membrane in MDCK cells (Perez-Velazquez and Angelides 1993), indicating that β subunits may contain targeting information or alternatively may mask localization signals within other subunits.

Gephyrin, a 93-kDa postsynaptic inhibitory synapse tubulin-bridging protein (Kirsch et al. 1991) first identified upon copurification with glycine receptors (Pfeiffer et al. 1982), is required for the synaptic targeting and clustering of most (Essrich et al. 1998; Baer et al. 1999; Kneussel et al. 1999, 2001; Fischer et al. 2000; Levi et al. 2004), but not all (Kneussel et al. 2001), $GABA_A$ receptor subtypes where it may facilitate synaptic retention of the receptor (Jacob et al. 2005). γ2 subunits are clustered throughout development, first extrasynaptically without gephyrin and then later in development with gephyrin (Danglot et al. 2003). A poorly understood reciprocal relationship exists between γ2 subunits and gephyrin with γ2 subunit-deficient mice demonstrating a striking absence of postsynaptic gephyrin (Essrich et al. 1998). Using the Cre-loxP approach to delete the mouse γ2 subunit gene at the third postnatal week, Schweizer et al. have examined the formation of clustering at mature synapses (Schweizer et al. 2003). Similar to findings in other systems, the absence of the γ2 subunit resulted in the loss of synaptic $GABA_A$ receptors and gephyrin. Gephyrin has not been noted to directly interact with $GABA_A$ receptors and the mechanism by which it clusters the receptor remains to be elucidated; however, both the intracellular loop and C-terminal domain of the γ2 subunit are reported to be required for gephyrin recruitment to the synapse (Alldred et al. 2005).

2.2.2 Extrasynaptic and Perisynaptic $GABA_A$ Receptors

Receptors composed of α4–6β×δ subunits are predominantly targeted to extrasynaptic and perisynaptic sites, where they are activated by the spillover of synaptically released GABA (reviewed in Farrant and Nusser 2005). These

receptors mediate tonic inhibition (extrasynaptic) or prolonged phasic inhibition (perisynaptic) and contribute to the underlying inhibitory tone in the central nervous system.

In cerebellar granule cells, the δ subunit is localized to extrasynaptic sites (Nusser et al. 1998) and appears to rely on coexpression with α6 subunits (Nusser et al. 1996; Jones et al. 1997). Deletion of the α6 subunit results in an absence of tonic inhibition in cerebellar granule cells (Brickley et al. 2001). Functional data demonstrating that α6-containing receptors are highly sensitive to GABA (Saxena and Macdonald 1996) also argue for an extrasynaptic role of the α6 subunit, as extrasynaptic GABA concentrations would be low, following diffusion of synaptically released GABA. The α6 subunit is presumed to contain extracellular targeting information, since ectopically expressed α6 subunits shift the balance of tonic/phasic inhibition in hippocampal neurons to favor tonic inhibition (Wisden et al. 2002). A peri/extrasynaptic role for the α4 subunit is supported by the observation that the α4 subunit is coexpressed (Peng et al. 2002; Jia et al. 2005) and coimmunoprecipitated (Sur et al. 1999; Jia et al. 2005) with δ subunits. Similar to α4 subunits, α5 subunits are also found extrasynaptically (Crestani et al. 2002; Brunig et al. 2002). The observations that tonic (extrasynaptic) currents display sensitivity to an α5 subunit-selective ligand and that α5 knockout mice display deficits in tonic, but not phasic, currents (Caraiscos et al. 2004) provide functional support that α5 subunit-containing receptors mediate tonic inhibition. Although extrasynaptic α5 subunit-containing receptors would be expected to form heteroligomers with β*x* and δ subunits, a subpopulation of α5 subunit-containing extrasynaptic receptors may contain γ2 subunits (Fritschy et al. 1998), consistent with the extrasynaptic localization of certain γ2 subunit-containing receptors in hippocampal cultures (Scotti and Reuter 2001). Despite prevailing evidence that α5 subunits serve a tonic function, α5 subunit-containing receptors may also exist postsynaptically (Christies and de Blas 2002) and have been noted to be clustered in certain brain regions (Hutcheon et al. 2004). It is clear that receptor targeting is in part specified by the subunit composition of the receptor and, as yet ill-defined, interactions with scaffolding proteins at inhibitory synapses and extrasynaptic regions. This selective targeting has important and distinct functional consequences for tonic and phasic inhibition.

2.2.3
Lateral Receptor Diffusion

Although it is well appreciated that synaptic strength is affected by the number of postsynaptic receptors, most studies have focused on either endocytosis or membrane insertion/recycling as mechanisms for regulating receptor density at the synapse. It is becoming evident, however, that synaptic populations of ligand-gated ion channels can be augmented by the lateral diffusion

of extrasynaptic and/or perisynaptic receptors into synaptic regions as reported for glycine (Dahan et al. 2003), AMPA (Tardin et al. 2003), and NMDA (Groc et al. 2004) receptors.

Initial studies of $GABA_A$ receptors in cultured rat spinal cord neurons using fluorescent benzodiazepine derivatives for fluorescence photobleach recovery (FPR) studies revealed that $GABA_A$ receptors exist as predominantly immobile clusters on cell soma and dendrites, unlike the highly mobile voltage-gated sodium channels within the axon hillock (Velazquez et al. 1989). In contrast, FPR and single particle tracking of recombinant receptors in COS7 and PC12 cells showed that while clustered β3 homomers are mobile at the cell surface, α1β3 heteromers display greatly limited mobility (Peran et al. 2001) suggesting that α1 subunits play a role in limiting receptor mobility. To further examine the contribution of α subunits in affecting lateral mobility, α1–6 subunits were coexpressed with β2γ2S subunits in COS7 cells (Peran et al. 2004). Receptors containing α2–5 subunits were mobile while receptors containing α1 and α6 subunits were relatively immobile. The idea that α1 and α6 subunits may play a role in limiting lateral diffusion is consistent with FPR experiments in cerebellar granule cells, which express predominately α1 and α6 subunits and display relatively immobile receptor populations (Peran et al. 2004). These studies indicate that lateral receptor mobility is dictated by receptor subunit composition. An additional noteworthy observation from these studies is that receptors expressed in HEK 293 cells were highly mobile independent of subunit composition (Peran et al. 2001, 2004), suggesting that cell-type specific anchoring mechanisms or membrane composition may also be a factor in receptor diffusion.

A clever series of experiments by Thomas and colleagues (Thomas et al. 2005) suggests that extrasynaptic/perisynaptic $GABA_A$ receptors can be rapidly imported into postsynaptic regions. In these experiments, a unique property of the α1 subunit mutant V257C was exploited to indirectly examine receptor lateral mobility. In the presence of the membrane impermeant channel blocker methanethiosulfonate (MTSES), receptors containing α1(V257C) undergo rapid, irreversible inactivation upon agonist stimulation (Xu and Akabas 1996). Exogenous GABA application in the presence of MTSES to hippocampal neurons expressing α1(V257C) subunit-containing receptors resulted in a large decrease in GABA response that did not recover within 30–40 min (Thomas et al. 2005). This lack of recovery strongly suggests that membrane insertion of MTSES-naïve receptors from intracellular pools did not occur during this time period. The response of α1(V257C) subunit-containing receptors to spontaneously released or electrically evoked synaptic GABA release was then examined in the presence of MTSES. Assuming that synaptically released GABA did not activate extrasynaptic/perisynaptic $GABA_A$ receptors, this approach allows the use-dependent inactivation of α1(V257C) subunit-containing *synaptic* receptors, while not inactivating na-

tive and α1(V257C) subunit-containing extrasynaptic/perisynaptic receptors. In hippocampal neurons expressing α1(V257C) subunit-containing receptors, a decrease in spontaneous or electrically evoked IPSCs was observed in the presence of MTSES. However, this decrease was followed by a rapid recovery of the response. This result, coupled with the lack of recovery to exogenous GABA application (above), suggests that extrasynaptic/perisynaptic receptors are rapidly recruited into the postsynaptic region. To further examine the role of membrane insertion in the maintenance of the synaptic pool of receptors, IPSCs were recorded in the presence of brefeldin A, to block ER export of newly synthesized receptors, or either botulinum toxin B or *N*-ethylmaleimide to prevent exocytosis. None of these treatments affected IPSCs in hippocampal neurons, suggesting that the insertion of newly synthesized or recycled receptors does not contribute to the maintenance of the synaptic $GABA_A$ receptor pool.

While the above electrophysiological experiments support the notion that extrasynaptic receptors can be rapidly imported into postsynaptic regions, these results await confirmation by receptor visualization techniques that examine the lateral mobility of native receptor populations. Indeed, recent fluorescence recovery after photobleaching (FRAP) analysis demonstrates that extrasynaptic receptors display greater mobility than synaptic receptors, which are relatively immobile (Jacob et al. 2005). This restricted mobility appears to be dependent on gephyrin which may facilitate the retention of receptors at the synapse (Jacob et al. 2005). If, as indicated by the study of Thomas and colleagues (2005), peri/extrasynaptic receptors can replenish synaptic receptor pools, the concept that receptors of distinct subunit composition populate extrasynaptic/perisynaptic vs synaptic regions may require additional consideration. While both receptor subunit composition and gephyrin undoubtedly influence receptor surface dynamics, it is also possible that the lateral mobility may be influenced by the membrane environment. In this regard, $GABA_A$ receptors have recently been localized to lipid rafts in cerebellar granule cells (Dalskov et al. 2005) and these cholesterol–rich, specialized plasma membrane regions affect lateral diffusion of proteins within the membrane (Dietrich et al. 2002).

3 Regulation of $GABA_A$ Receptor Cell Surface Expression

Once present on the cell surface, $GABA_A$ receptors are regulated by mechanisms that (1) directly affect their functional status (agonist affinity, gating properties, inactivation/deactivation kinetics or conductance) or (2) alter receptor cell surface levels. The former occurs primarily through changes in protein phosphorylation and involves a myriad of kinases and phosphatases (Brandon et al. 2002; Song and Messing 2005). Such direct regulation of re-

ceptor function (i.e., regulation without affecting surface numbers) is highly complex and will not be considered here. It is now appreciated that alteration of receptor cell surface expression of neurotransmitter receptors is a primary mechanism for controlling synaptic efficacy (Collingridge et al. 2004). This is particularly notable for receptors that are exposed to saturating concentrations of neurotransmitters, such as synaptic $GABA_A$ receptors. Early ligand binding and photoaffinity labeling studies in neuronal cultures determined that approximately 80% of benzodiazepine receptors (Czajkowski and Farb 1986) and 60% of muscimol binding sites (Czajkowski et al. 1989) were on the cell surface. The intracellular pool of receptors was observed to be largely a steady-state pool, with only a small fraction of newly synthesized receptors being introduced. These studies offered the first evidence for the existence of a significant pool of intracellular receptors, which may participate in a dynamic process and permit the number of cell surface receptors to change in the absence of protein synthesis (Czajkowski and Farb 1989). It is now appreciated that $GABA_A$ receptors undergo both constitutive and regulated endocytosis and are then subsequently either recycled to the cell surface or degraded. Together, these processes have a defining impact on the strength of inhibitory neuronal synapses and are discussed below.

3.1 Endocytosis

3.1.1 Constitutive Endocytosis

The identification of benzodiazepine binding sites within clathrin-coated vesicles isolated from rat brain gave the first indication that $GABA_A$ receptors may undergo constitutive clathrin-dependent endocytosis (Tehrani and Barnes 1993). Subsequent studies using bovine cerebellum revealed that approximately 10% of $GABA_A$ receptors appear within clathrin-coated vesicles (Tehrani et al. 1997). $GABA_A$ receptors are now recognized to undergo dynamin-dependent constitutive endocytosis via the clathrin pathway. In hippocampal neurons, $GABA_A$ receptor-mediated mIPSC peak amplitudes are augmented by a peptide that disrupts dynamin–amphiphysin interactions (Kittler et al. 2000), suggesting that constitutive dynamin-dependent endocytosis plays a role in synaptic signaling. Dynamin-dependent endocytosis was subsequently demonstrated in HEK 293 cells using the dominant negative dynamin construct K44A (Herring et al. 2003). Following dynamin-dependent endocytosis in hippocampal cultures, internalized $GABA_A$ receptors exist predominately in gephyrin-associated subsynaptic pools that may serve as receptor reserves (van Rijnsoever et al. 2005). Dynamin-independent receptor endocytosis has also been observed (Cinar and Barnes 2001) but may be re-

lated to unidentified factors contained in fetal bovine serum (Herring et al. 2003).

The AP2 adaptin complex is a multimeric structure that is involved in the recruitment of cargo proteins into clathrin-coated pits (Clague 1998). The intracellular loops of the $GABA_A$ receptor β and γ subunits interact with AP2 subunits in yeast two-hybrid studies, $GABA_A$ receptors coimmunoprecipitate with AP2 from brain homogenates, and the receptor is colocalized with AP2 in hippocampal neurons (Kittler et al. 2000). AP2 adaptin recruits cargo for endocytosis via dileucine (LL) and/or tyrosine-based motifs (YXXθ, where θ is a bulky hydrophobic amino acid) on target proteins (LeBorgne et al. 1998). Dileucine and tyrosine motifs are recognized by β and μ adaptin subunits of the AP2 complex, respectively. The cytoplasmic loop regions of $GABA_A$ receptor β1–3 subunits contain both LL-based and YXXθ-based (YIFF) motifs. Although the tyrosine motif does not appear to influence endocytosis, the dileucine motif on the receptor β2 subunit (L343, L344) has been identified to play a role in endocytosis (Herring et al. 2003) and a peptide corresponding to this dileucine motif is effective in increasing GABA-gated chloride currents in mouse cerebral cortical neurons (Herring et al. 2003). It is unclear whether this conserved dileucine motif also serves as an endocytic signal for β1 and β3 subunits. In this regard, the β2 subunit possesses an acidic amino acid upstream of the leucine pair, a feature that in certain proteins is required for recruitment by AP2 (Bonifacino and Traub 2003). Both β1 and β3 subunits lack such an acidic amino acid and it remains to be determined if the dileucine pair present in these subunits plays a role in endocytosis. Interestingly, an interaction between the AP2 adaptin μ subunit and receptor β3 subunit has also been reported (Kittler et al. 2005). The interaction occurs via an atypical binding site within a basic region of the β3 cytoplasmic loop and is inhibited by phosphorylation of serines 408/409. Unphosphorylated peptides corresponding to this binding site, but not their phosphorylated counterparts, increase mIPSCs and GABA-evoked currents. The observation that the unphosphorylated peptide did not display an additive increase on receptor function with a peptide that blocks dynamin-dependent endocytosis suggests that this noncanonical, AP2 adaptin μ subunit binding site on the β3 subunit may be involved in endocytosis.

Although β subunits possess molecular determinants that are important for receptor endocytosis, it is possible that γ subunits also possess trafficking signals. The γ2L subunit contains a dileucine motif within the eight amino acid alternatively spliced region of the cytoplasmic loop (Whiting et al. 1990). This motif, however, is not important for constitutive endocytosis since receptors lacking γ subunits display constitutive endocytosis (Cinar and Barnes 2001; Herring et al. 2003).

3.1.2 Regulated Endocytosis

3.1.2.1 Protein Kinase C

It has long been recognized that protein kinase C (PKC) activation leads to an inhibition of $GABA_A$ receptor function. Such inhibition is now known to result from both an effect on receptor function and an increase in $GABA_A$ receptor internalization (reviewed in Kittler and Moss 2003; Song and Messing 2005). The phenomenon that PKC activation promotes receptor internalization was initially observed in Xenopus oocytes expressing recombinant receptors (Chapell et al. 1998) but has now been extended to include recombinant receptors in mammalian cells and native receptors in primary neuronal cultures and cerebral cortical neuronal slices (Connolly et al. 1999; Cinar and Barnes 2001; Herring et al. 2005). PKC-stimulated $GABA_A$ receptor endocytosis in mammalian cells requires a γ subunit (Connolly et al. 1999; Herring et al. 2005), occurs via the dynamin pathway, and is blocked by mutation of a dileucine AP2 motif within the receptor $\beta 2$ subunit (Herring et al. 2005). This regulation occurs independently of known PKC phosphorylation sites on the receptor (Chapell et al. 1998; Connolly et al. 1999) and the PKC substrate(s) underlying this effect remains unknown. The physiological pathways by which PKC activation may promote receptor endocytosis are under-studied; however, growth factor pathways that are linked to PKC activation, such as those stimulated by brain-derived neurotrophic factor (BDNF), have been implicated in the regulation of receptor cell surface levels (see below).

3.1.2.2 Dopamine D3 Receptors/PKA

To elucidate dopamine-type 3 (D3) receptor mechanisms within the GABAergic medium spiny neurons of the nucleus accumbens (NAc), Chen et al. (2006) examined the modulation of synaptic GABAergic transmission in response to D3 receptor activation. Upon D3 receptor activation with the selective agonist PD128907, a decrease in GABA-gated chloride currents was noted in acutely dissociated neurons. In NAc slices, stimulation of D3 receptors inhibited mIPSC amplitudes. This D3 receptor-mediated decrease in $GABA_A$ receptor responses was attenuated by a protein kinase A (PKA) inhibitory peptide, as well as a peptide that blocks dynamin-dependent endocytosis. In addition, a peptide corresponding to a previously identified atypical AP2 μ subunit binding region on the receptor $\beta 3$ subunit (Kittler et al. 2005) also blocked this effect. This peptide contains not only an AP2 binding region, but also PKA phosphorylation sites that regulate AP2 μ subunit binding to the

β3 subunit (Kittler et al. 2005). The ability of D3 receptor activation to inhibit GABA responses was prevented by the nonphosphorylated, but not by the phosphorylated, peptide (Chen et al. 2006). These data suggest that D3 receptor activation suppresses GABAergic transmission by stimulating PKA phosphorylation of the receptor β3 subunit, thereby promoting AP2 binding and receptor endocytosis. Indeed, activation of D3 receptors results in internalization of β3 subunit-containing receptors, an effect blocked by a dynamin inhibitory peptide (Chen et al. 2006).

3.1.2.3
Growth Factors

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that influences the developing nervous system and is also required for the proper maintenance of synaptic function in mature neuronal circuits (for review, see McAllister 1999). Initially, BDNF was reported to induce a reduction in spontaneous, evoked, and GABA-stimulated IPSCs in rat hippocampal CA1 region (Tanaka et al. 1997; Frerking et al. 1998). This effect was blocked by the Trk receptor kinase inhibitor K252a. In another study, BDNF treatment was shown to rapidly decrease mIPSC amplitudes in a subpopulation of cultured rat hippocampal pyramidal cells without affecting mIPSC frequency or kinetics (Brunig et al. 2001). A reduction in total punctate immunostaining of α2, β2/3, and γ2 subunits was observed, suggesting that BDNF altered receptor trafficking. This mechanism was further advanced upon demonstration that BDNF decreases spontaneous and miniature IPSC amplitude in cerebellar granule cells through a reduction in β2/3 cell surface immunoreactivity (Cheng and Yeh 2003). TrkB receptor-mediated inhibitory effects of BDNF on mIPSCs and muscimol-stimulated responses have been observed in the paraventricular nucleus of the hypothalamus and are blocked by a dynamin inhibitory peptide (Hewitt and Bains 2006). BDNF−/− mice, or wild-type mice acutely treated with K252a, display an increase in evoked and spontaneous mIPSC amplitudes in mouse superior colliculus (Henneberger et al. 2001). Acute treatment of BDNF−/− mice with BDNF restores IPSC amplitudes to normal levels, an effect that is blocked by the postsynaptic application of a PKC inhibitory peptide. Taken together, these studies support the notion that BDNF promotes receptor endocytosis through the activation of PKC. Contrary to these reports on the inhibitory effects of BDNF on $GABA_A$ receptors, in rat visual cortex BDNF rapidly potentiates GABA-gated chloride currents and mIPSCs amplitudes through an increase in receptor cell surface expression (Mizoguchi et al. 2003). While it is possible that these disparate results may represent differential regulation of distinct $GABA_A$ receptor subtypes and/or a brain region/cell type-dependent phenomenon, a biphasic action of BDNF on $GABA_A$ receptor trafficking has now been reported (Jovanovic et al. 2004). In cultured neurons, BDNF produces an initial rapid

increase, followed by a decrease, in mIPSC amplitudes. These BDNF effects are blocked by the PKC inhibitor calphostin C. In agreement with the results of Mizoguchi et al. (2003) this initial effect is correlated with an increase in receptor cell surface expression.

3.1.2.4 TNF-α

The proinflammatory cytokine tumor necrosis factor alpha (TNF-α) is constitutively released from glia to maintain required levels of AMPA receptor cell surface expression (Stellwagen et al. 2005). In both neuronal cultures and hippocampal slices, treatment with exogenous TNF-α increases exocytosis of certain AMPA receptor subtypes, resulting in an increased surface expression of AMPA receptors (Stellwagen et al. 2005). Concomitant with AMPA receptor exocytosis, TNF-α promotes $GABA_A$ receptor endocytosis, thus decreasing $GABA_A$ receptor cell surface levels. This simultaneous, but opposite, regulation of cell surface levels of excitatory and inhibitory receptors demonstrates a heretofore unrecognized mechanism by which a signaling factor can alter the delicate balance between excitatory and inhibitory neurotransmission.

3.2 Postendocytic Trafficking

3.2.1 Recycling/Reinsertion

3.2.1.1 Constitutive Recycling

Basal levels of receptor insertion, presumed to be due to recycling, of recombinant α1β2γ2 receptors expressed in cultured hippocampal neurons proceed at a slow rate relative to other ligand-gated ion channels, requiring more than 1 h to completely turn over the cell surface population (Wang et al. 2003). In cultured cortical neurons, approximately 30% of the measured intracellular pool of β3 subunit-containing native receptors are demonstrated to be recycled within 5 min and greater than 70% recycled within 1 h using an indirect measure of recycling (Kittler et al. 2004). Thus, there appears to be good agreement between these two studies regarding the time course of receptor recycling of native and recombinant receptors. Furthermore, because these studies employed different β subunit isoforms, it does not appear that different β subunit isoforms differentially influence recycling kinetics.

Huntingtin-associated protein 1 (HAP1) is known to block trafficking from early to late endosomes, thereby inhibiting lysosomal degradation (Li

et al. 2002). Recently, HAP1 was identified in a yeast two-hybrid screen as a binding partner for the β1 subunit, and its interaction with β1–3 subunit isoforms was established using pulldown and coimmunoprecipitation experiments (Kittler et al. 2004). In hippocampal neuronal cultures, HAP1 is colocalized with $GABA_A$ receptors and promotes receptor recycling, thus increasing mIPSC amplitude and synaptic inhibition. Concomitant with enhanced recycling is a decrease in receptor degradation, suggesting a role for HAP1 in the postendocytic sorting of $GABA_A$ receptors.

3.2.1.2
Insulin-Facilitated Receptor Membrane Insertion/Recycling

Insulin promotes $GABA_A$ receptor cell surface expression in recombinant systems in a manner requiring the β2 subunit (Wan et al. 1997). This recruitment also occurs in neurons and serves to enhance inhibitory neurotransmission by increasing IPSC amplitudes (Wang et al. 2003). In cultured rat hippocampal neurons, insulin facilitates the membrane insertion of the receptor in a manner involving Akt (PKB) phosphorylation of β2 subunit serine 410, which lies within a conserved Akt consensus motif (Wang et al. 2003). This effect occurs through PI-3 kinase, a pathway known to be required for Akt activation by insulin.

3.2.2
Degradation/Proteolysis

The dynamic balance between protein synthesis and degradation determines protein level within the cell and presumably affects cell surface levels. Little is known regarding the proteases/systems involved in $GABA_A$ receptor degradation, the sorting mechanisms that target the receptor for degradation, the subunit motifs that serve as recognition/cleavage sites for proteases, or the regulation of these processes. In primary neuronal cultures, studies using $[^3H]$flunitrazepam photoaffinity labeling of 48- and 51-kDa subunits (presumably α subunits) showed biphasic degradation, with 42 and 58% of the receptors possessing half-lives of 3.8 and 32 h, respectively (Bordon and Farb 1988). The stability of rapidly degraded receptors is not affected by lysosomal inhibitors, indicating that they are degraded by a nonlysosomal pathway. Observations using pulse chase $[^{35}S]$methionine labeling revealed that α1 and β2 subunits expressed singly in BHK cells are degraded in ∼ 2 h, while subunits expressed together form multimeric receptors with half-lives of ∼ 40 h. Gorrie et al. (1997) indicate that unoligomerized subunits may be rapidly degraded and those forming receptors may possess longer half-lives.

Receptors that undergo clathrin/dynamin-dependent endocytosis are usually either recycled or trafficked to lysosomes for degradation (Clague 1998).

In cortical neuronal cultures, degradation of surface receptors has been assessed using a cell surface biotinylation–degradation assay. In these experiments, approximately 25% of previously biotinylated surface receptors were degraded by 6 h, an effect that was significantly attenuated by the lysosomal inhibitor leupeptin (Kittler et al. 2004), thus indicating a role for lysosomes in degrading endocytosed surface receptors. In addition to the lysosomal system, a major mechanism for protein degradation involves the 26S proteasome, which degrades polyubiquitinated substrates. Although this system is largely recognized to play a role in the degradation of short-lived cytoplasmic proteins, a more general role of the proteasome is emerging (Glickman and Ciechanover 2002). Preliminary data indicate that singly expressed β3 subunits may be degraded quickly by the proteasome (Bedford et al. 2001). It is unknown whether receptor subunits are polyubiquitinated.

Plic-1 is a ubiquitin-related protein that displays N-terminal sequence homology to ubiquitin and contains a ubiquitin-associated (UAB) domain at the C-terminus (Jentsch and Pyrowolakis 2000). Ubiquitin-like proteins are thought to negatively regulate degradation of proteasome substrates, thereby increasing protein half-life (Kleijnen et al. 2000, 2003). In a yeast two-hybrid screen, Plic-1 was identified as a $GABA_A$ receptor α1 subunit interacting protein (Bedford et al. 2001). This interaction was confirmed in pulldown assays and mapped to the UAB domain of PLIC-1. Additional yeast two-hybrid experiments revealed the association of PLIC-1 with α2, α3, α6, and β1–3 subunit intracellular domains but not with those of γ2 or δ subunits. Coimmunoprecipitation of β2/3 subunits and Plic-1 from brain tissue homogenates supports a physiological interaction of these proteins. Furthermore, coexpression of Plic-1 with β3 subunits in 293 cells increases the stability of β3 subunits, suggesting that unoligomerized β3 subunits or homomeric β3 receptors may be degraded by the proteasome. Proteasomal degradation of receptor subunits is further supported by the observation that the proteasomal inhibitor lactacystin increases subunit protein levels in cells expressing single α1 or β3 subunits. Regarding the functional significance of the PLIC-1 association, a peptide corresponding to the Plic-1 binding domain on the α1 subunit decreases GABA-gated current amplitudes in 293 cells and hippocampal brain slices by a reduction in receptor cell surface expression that was not due to an increase in endocytosis (Bedford et al. 2001). It has been proposed that Plic-1 prevents receptor ubiquitination, thus prolonging receptor half-life; however, this remains to be established.

In addition to degrading proteins, proteases also carry out limited, nondegradative proteolysis. Limited proteolysis can serve as a regulatory mechanism for controlling neurotransmitter receptor function, subunit association, subcellular mobility, and stability (Meyer et al. 2003). Nondegradative, regulatory proteolysis of $GABA_A$ receptors has not been reported.

4 Relevance to Neurological Disorders and Stroke

A relationship between $GABA_A$ receptors and epilepsy has long been recognized (reviewed in Sperk et al. 2004) and is supported by the observations that (1) drugs that are effective for the treatment of seizures, such as benzodiazepines and barbiturates, are allosteric potentiators of the $GABA_A$ receptor; (2) drugs that inhibit the receptor produce seizures; (3) changes in $GABA_A$ receptors are present in brains of epileptics; and (4) certain heritable epilepsies are associated with receptor mutations. A rapidly emerging concept favors the involvement of $GABA_A$ receptor trafficking deficiencies as important elements for certain forms of heritable epilepsy as well as for the initiation and maintenance of acquired epilepsy. This evidence is reviewed below.

4.1 Epilepsy

4.1.1 Heritable $GABA_A$ Receptor Channelopathies

Heritable disease accounts for approximately 40% of idiopathic partial and generalized epilepsies (Steinlein 2001) with a growing list of channelopathies underlying certain familial forms (Mulley et al. 2003). Originally described by Wallace et al. (2001), the $\gamma 2$ subunit R43Q mutation is a missense mutation in the N-terminal extracellular region. When expressed in HEK 293 cells, $\gamma 2$(R43Q)-containing receptors display decreased $GABA_A$ receptor peak amplitude currents (Bianchi et al. 2002). This decrease is accompanied by deficits in cell surface expression (Sancar and Czajkowski 2004), a phenomenon due to ER retention of the receptor (Kang et al. 2004; Hales et al. 2005). Similarly, a truncation mutation of the $\gamma 2$ subunit at Q351 results in the retention of the $\gamma 2$ subunit in the endoplasmic reticulum (Harkin et al. 2002) and is one of several mechanisms contributing to the syndrome of generalized epilepsy with febrile seizures plus (GEFS+) phenotype. Recently, $\gamma 2$ subunit mutations associated with febrile seizures, namely (R43Q), (K289M), and (Q351X), have been reported to display temperature-dependent trafficking deficiencies (Kang et al. 2006). In response to elevated temperature, a reduction in surface receptors is observed for these mutants relative to wild-type receptors.

In addition to trafficking defects of the $\gamma 2$ subunit, δ subunit susceptibility variants E177A and R220H are associated with complex epilepsy and display a decrease in cell surface expression when coexpressed with $\alpha 4\beta 2$ subunits (Feng et al. 2006). Furthermore, a mutation in the $\alpha 1$ subunit gene (GABRA1) results in the replacement of alanine 332 by aspar-

tate and has recently been identified to be present in autosomal dominant form juvenile myoclonic epilepsy (Cossette et al. 2002). In recombinant systems, this mutatant allele, when coexpressed with $\alpha1\beta2$ subunits, demonstrates reduced cell surface expression, increased GABA EC50s, and increased rates of deactivation (Krampfl et al. 2005). Using covalently tethered triplet $\alpha1\beta2S\gamma2S$ subunit concatamers that dictate subunit positioning of the α1A332D subunit within the pentamer, decreases in cell surface, total expression, and receptor currents were shown to be dependent on the position of the α1A332D subunit within complexes containing one copy each of wild-type $\alpha1$ and α1A332D (Gallagher et al. 2005). Total α1A332D levels were decreased due to endoplasmic reticulum-associated degradation (ERAD) prior to oligomerization with other subunits, presumably due to immature glycosylation status of the protein. These epilepsy-linked $GABA_A$ receptor channelopathies that result in reduced receptor cell surface expression underscore the critical nature of maintaining appropriate numbers of cell surface $GABA_A$ receptors.

4.1.2 In Vitro Seizure Models

Approximately half of epileptic patients exhibit acquired epilepsy, a disorder resulting from brain injury, stroke, or other environmental insults (Hauser and Hesdorffer 1990). Epileptogenesis, the processes whereby these recurrent seizures are developed and maintained, is not well understood. Low-magnesium-induced spontaneous recurrent epileptiform discharges (SREDs) in cultured neurons serve as an in vitro model of acquired epilepsy. In hippocampal neuronal cultures, SREDs are accompanied by a variety of functional alterations including a large decrease in GABA-gated chloride currents with a corresponding decrease in $GABA_A$ receptor surface expression (Blair et al. 2004). This surface decrease is apparent immediately following low magnesium exposure and is maintained in the persistent epileptic phenotype that is established in this model. Interestingly, an increased rate of receptor endocytosis is observed in this model and blockade of dynamin-dependent endocytosis by a dynamin–amphiphysin interaction blocking peptide in "epileptic" cultures restores cell surface levels of receptor and prevents SREDs. In another in vitro low magnesium model of status epilepticus, Goodkin et al. (2005) report that recurrent paroxysmal epileptiform bursting following low magnesium treatment decreased GABA mIPSCs in hippocampal pyramidal cell cultures, an effect that was accompanied by rapid intracellular accumulation of $GABA_A$ receptor $\beta2/3$ subunits. This intracellular accumulation was attenuated by hypertonic sucrose treatment, a treatment previously shown to block $GABA_A$ receptor endocytosis (Kittler et al. 2000).

4.1.3 In Vivo Seizure Models

In the lithium–pilocarpine-induced status epilepticus model, a significant internalization of synaptic $GABA_A$ receptors was observed in hippocampal sections from rats treated with lithium–pilocarpine (Naylor et al. 2005). mIPSCs from dentate gyrus granule cells displayed a decrease in current amplitude consistent with a decrease in the number of postsynaptic receptors. The authors suggest that an increase in extracellular GABA concentration during status epilepticus may promote $GABA_A$ receptor internalization. In support of this hypothesis, a 20-min application of GABA to slices from untreated animals decreases mIPSCs, similar to that observed in lithium–pilocarpine-treated animals (Naylor et al. 2005). These findings are consistent with a previous demonstration that agonist treatment induces $GABA_A$ receptor internalization (Tehrani and Barnes 1991). Thus, it is possible that GABA-mediated decreases in cell surface receptors may be the mechanism by which status epilepticus produces a rapid loss of synaptic inhibition and resistance to the antiepileptic action of benzodiazepines.

While the above in vitro and in vivo seizure models support an increase in $GABA_A$ receptor internalization following seizure induction, an increase in surface synaptic $GABA_A$ receptors has been reported in an electrical kindling model of temporal-lobe epilepsy. Following kindling, whole-cell patch-clamp recordings from rat brain hippocampal slices show an increase in the amplitude of evoked IPSCs with a concomitant increase in synaptic subunit immunoreactivity within the dentate gyrus (Nusser et al. 1998).

4.1.4 Therapeutic Strategies

Given the mounting evidence that alterations in $GABA_A$ receptor trafficking are important for the development and maintenance of epilepsy and the self-sustaining and drug-resistant nature of status epilepticus, future strategies to decrease $GABA_A$ receptor endocytosis or increase $GABA_A$ receptor recycling may be useful in the therapeutic management of epilepsy. Although approaches targeted at affecting trafficking may have consequences for the trafficking of other proteins, it is interesting to note that $GABA_A$ and AMPA receptor trafficking appear to be oppositely regulated in certain cell types (Stellwagen et al. 2005). Perhaps it is possible simultaneously, through a single effector pathway, to enhance neuronal inhibition by increasing $GABA_A$ receptor cell surface expression while decreasing surface expression of glutamate-gated ion channels. Alternatively, a more targeted approach would be to utilize gene therapy to overexpress $GABA_A$ receptors that are resistant to endocytosis, such as those receptors containing the β2 subunit dileucine motif

mutant that has recently been identified as endocytically compromised (Herring et al. 2003).

4.2
Stroke and Excitotoxicity

Convincing evidence exists that deficits in GABAergic neurotransmission, particularly related to $GABA_A$ receptors, contribute to ischemic brain damage (reviewed in Schwartz-Bloom and Sah 2001). Following transient cerebral ischemia, there is a rapid down-regulation of cell surface $GABA_A$ receptors in the hippocampus without alterations in total receptor number, suggesting that changes in receptor trafficking may be an early event that affects excitotoxicity (Alicke and Schwartz-Bloom 1995). More recent studies support this concept. Oxygen–glucose deprivation (OGD) in cultured hippocampal neurons decreases $GABA_A$ receptor cell surface expression with no effect on total cellular levels (Mielke and Wang 2005). This internalization is prevented by hypertonic sucrose, a treatment known to inhibit $GABA_A$ receptor endocytosis (Kittler et al. 2000). Interestingly, insulin, a hormone that promotes $GABA_A$ receptor insertion into the plasma membrane (Wan et al. 1997), blocks the decrease in receptor surface expression while exerting a protective effect against not only OGD, but also glutamate-induced toxicity (Mielke and Wang 2005).

5
Conclusions

It is an exciting time for $GABA_A$ receptor trafficking research. Several proteins have been identified within the secretory pathway that appear to be involved in receptor cell surface trafficking. The subunit determinants and anchoring proteins that target and cluster the receptor at defined regions of the plasma membrane are beginning to be understood. Evidence has emerged that lateral diffusion of the receptor within the membrane may contribute in a dynamic way to the efficacy of inhibitory transmission. The parameters of receptor endocytosis and recycling, and the regulation of these events by kinases, growth factors, and ubiquitination-associated proteins are emerging. Receptor degradation through lysosomal, proteasomal, and ERAD systems, as well as the possibility of regulatory proteolysis, remain largely unexplored. The potential clinical relevance of $GABA_A$ receptor trafficking is underscored by several recent reports that $GABA_A$ receptor internalization is critical to certain heritable forms of epilepsy, as well as to acquired epilepsy and the excitotoxicity subsequent to ischemic brain insult. A key challenge will be to demonstrate the in vivo and clinical significance of $GABA_A$ receptor trafficking and its regulation.

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Regulation of Excitability by Extrasynaptic $GABA_A$ Receptors

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Abstract Not only are $GABA_A$ receptors activated transiently by GABA released at synapses, but high affinity, extrasynaptic $GABA_A$ receptors are also activated by ambient, extracellular GABA as a more persistent form of signalling (often termed tonic inhibition). Over the last decade tonic $GABA_A$ receptor-mediated inhibition and the properties of $GABA_A$ receptors mediating this signalling have received increasing attention. Tonic inhibition is present throughout the central nervous system, but is expressed in a cell-type specific manner (e.g. in interneurons more so than in pyramidal cells in the hippocampus, and in thalamocortical neurons more so than in reticular thalamic neurons in the thalamus). As a consequence, tonic inhibition can have a complex effect on network activity. Tonic inhibition is not fixed but can be modulated by endogenous and exogenous modulators, such as neurosteroids, and by developmental, physiological and pathological regulation of GABA uptake and $GABA_A$ receptor expression. There is also growing evidence that tonic currents play an important role in epilepsy, sleep (also actions of anaesthetics and sedatives), memory and cognition. Therefore, drugs specifically aimed at targeting the extrasynaptic receptors involved in tonic inhibition could be a novel approach to regulating both physiological and pathological processes.

1 Introduction

The activation of synaptic $GABA_A$ receptors through the transient vesicular release of GABA at synapses leads to brief inhibitory post-synaptic currents/potentials (IPSC/Ps) (Mohler et al. 1996). IPSPs shunt excitatory inputs, and can hyperpolarise (or early in development depolarise) the neuron. IPSPs may also play a part in neuronal synchronisation and oscillatory behaviour. When groups of inhibitory synapses synchronously release GABA, it can spill outside the synapse to activate extrasynaptic $GABA_A$ receptors, thus prolonging the inhibitory post-synaptic potential (Isaacson et al. 1993; Rossi and Hamann 1998). This may act as a homeostatic mechanism increasing inhibition during periods of synchronous network activity. Just over 10 years ago, it was recognised, however, that these extrasynaptic $GABA_A$ receptors could be activated by ambient GABA (Farrant and Nusser 2005; Semyanov

et al. 2004). This results in persistent $GABA_A$ receptor activation, a form of signalling termed tonic inhibition. The properties of receptors that mediate these currents are very different from those of the synaptic receptors—they have a greater affinity for GABA and desensitise more slowly. Here, we will review the extent of the expression of such currents, the receptor subtypes mediating them, possible sources of extracellular GABA, and the physiological effects and regulation of tonic inhibition.

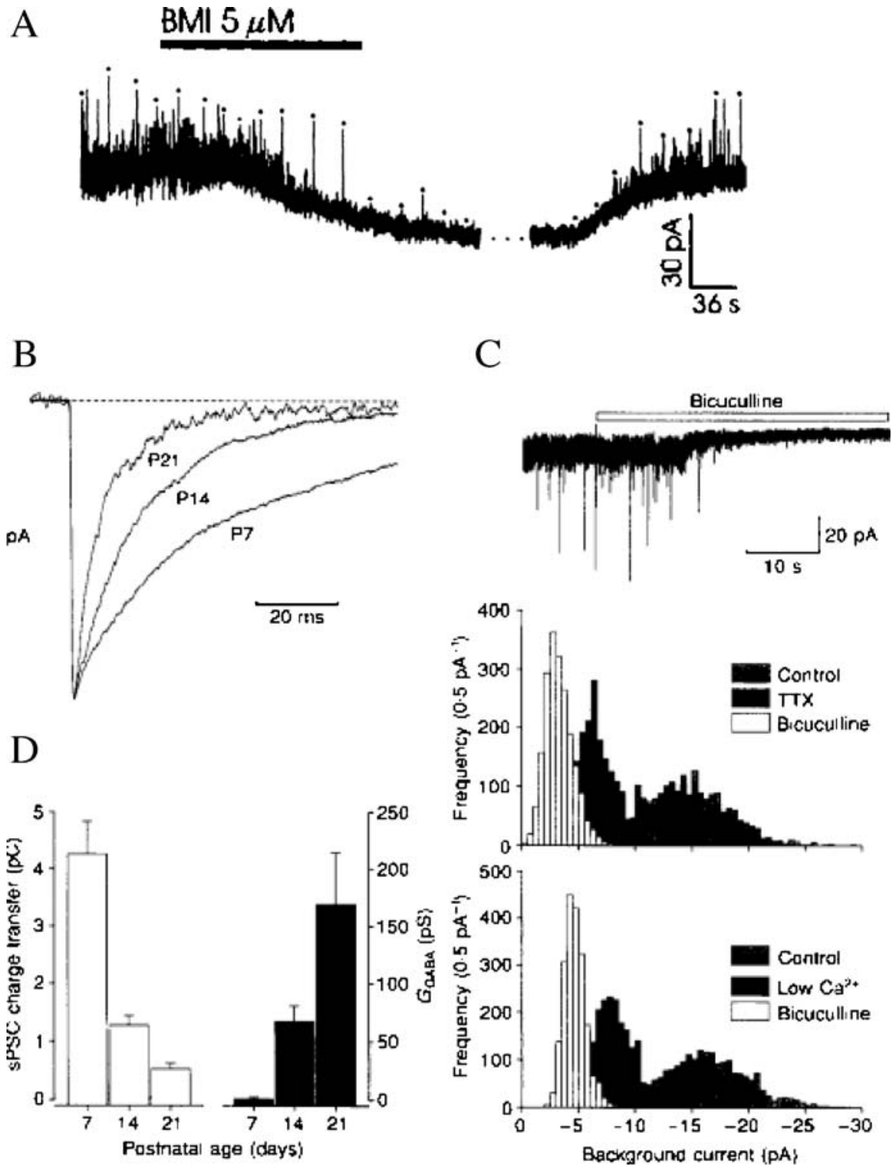
2
The Extent of Expression of Tonic Currents

Tonic $GABA_A$ currents were first described by Salin and Prince (1996) (Fig. 1a) in layer III cells of somatosensory cortical slices. They demonstrated that application of the $GABA_A$ receptor antagonist bicuculline reduced resting membrane conductance and produced an inward shift in baseline current. Shortly afterwards, $GABA_A$ receptor-mediated tonic currents were described in cerebellar granule cells (Brickley et al. 1996; Wall and Usowicz 1997) (Fig. 1b–d). Similar to neocortical slices, application of $GABA_A$ receptor antagonists to voltage-clamped cerebellar granule cells resulted in a shift in the baseline current and a decrease in background noise, consistent with a block of stochastic channel openings. This tonic current is developmentally regulated and becomes more evident with age (Brickley et al. 1996) (Fig. 1). Indeed, in the adult, the tonic current represents a greater proportion of the total $GABA_A$ receptor-mediated current than that mediated by spontaneous synaptic activity. This development is mostly due to the developmental regulation of $GABA_A$ receptor expression, but is also partly due to changes in morphology, GABA release and uptake.

There were later descriptions of tonic $GABA_A$ receptor currents in dentate granule cells (Nusser and Mody 2002; Overstreet and Westbrook 2001) and hippocampal neurons (Bai et al. 2001; Semyanov et al. 2003). In the hippocampus, tonic currents demonstrate cell-type specificity, being expressed

Fig. 1 **A** Chart recording showing spontaneous and evoked IPSCs (*marked by dots*) generated in a layer III pyramidal cell before, during and after bath perfusion of bicuculline methiodide (BMI; 5 μM). BMI reversibly blocks spontaneous and evoked outward currents, resulting in a prolonged inward current. Holding voltage: 0 mV; E_{cl}: – 59 mV. From Salin & Prince (1996) with permission. **B** Superimposed averaged IPSCs at P7, P14 and P21. Traces are normalized to the peak and show the first 80 ms of the decay. **C** Upper trace shows the effect of bicuculline on spontaneous post-synaptic currents (sPSCs) and noise (10 μM; P14, – 70 mV). The all-point histograms illustrate the effect of tetrodotoxin (TTX) (300 nM), low Ca^{2+} (0.5 mM Ca^{2+}, 5 μM Mg^{2+}) and bicuculline (10 μM). **D** Histograms showing the developmental decrease in charge transfer by discrete IPSCs (*open bars*) and the increase in the background (tonic) conductance (*closed bars*). From Brickley et al. (1996) with permission ▶

to a greater degree in interneurons than pyramidal cells in the same hippocampal subfields (Semyanov et al. 2003); this has important implications for network excitability (see below). In the hippocampus, tonic GABA$_A$ receptor-mediated currents appear to be expressed prior to synapse formation and play a part in development (Demarque et al. 2002). In contrast to the developmental regulation of tonic currents in the cerebellum, the tonic cur-



rent is larger in the hippocampus early in development, perhaps secondary to less GABA transporter expression. Thus, tonic currents are not the preserve of established neurons, but have also been described in developing neurons, where their primary role may be to depolarise neurons (Ge et al. 2006). Tonic $GABA_A$ currents have also been described in embryonic neocortical cells in situ in the ventricular zone (Loturco et al. 1995). These $GABA_A$ receptor currents can modulate neuronal development (discussed below).

More recently, neurons in a number of subcortical structures have been shown to express tonic currents, including thalamocortical neurons (Belelli et al. 2005; Cope et al. 2005) and hypothalamic neurons (Sergeeva et al. 2005). Evidence is also accruing that tonic currents may also be expressed in the dorsal horn of the spinal cord (Cronin et al. 2004). Thus tonic currents are ubiquitous in the central nervous system. They are developmentally regulated and demonstrate cell-type specificity that has important implications for network excitability.

3
Receptor Subtypes Expressing Tonic Currents

$GABA_A$ receptors are pentameric structures composed of five subunits from a possible 16 (Mehta and Ticku 1999; Mohler et al. 1996). Although not all combinations are equally expressed, there is a myriad of possible subunit combinations. Different subunit combinations have different conductances, affinities for GABA and kinetic characteristics. Although gamma subunits are generally required for synaptic expression, gamma subunit containing receptors are also found extrasynaptically (Luscher and Keller 2004). Different pharmacological properties support the proposition that the receptors mediating tonic current are different from those contributing to the peak synaptic current. Nevertheless, during substantial synaptic activity GABA can escape beyond the synaptic cleft to activate peri- and extrasynaptic receptors. Recent work has demonstrated that modifying alpha1–3 subunits to make them insensitive to benzodiazepines renders the synaptic currents benzodiazepine insensitive, but leaves the tonic current in CA1 pyramidal cells benzodiazepine sensitive (Prenosil et al. 2006). Because alpha1, 2, 3 or 5 and gamma subunits are required for benzodiazepine sensitivity, this strongly indicates that the alpha5 subunit mediates tonic current in CA1 pyramidal cells, but plays no part in synaptic currents. Interestingly, in this study they found no effect of an alpha5 specific inverse agonist on the tonic current under baseline conditions. They thus suggested that at low GABA concentrations different $GABA_A$ receptor subtypes mediate tonic inhibition in pyramidal cells, but that at higher GABA concentrations or when receptor affinity is increased by benzodiazepines, then alpha5 containing GABA receptors come into play. This is consistent with findings in other work (see below) (Scimemi et al. 2005).

The receptors mediating tonic currents need to be able to detect low ambient concentrations of GABA and should be slowly desensitising. Amongst the receptor subtypes that demonstrate these properties are the alpha4delta receptors that have been proposed to mediate the tonic current in dentate granule cells (Nusser and Mody 2002; Stell et al. 2003; Stell and Mody 2002) and thalamocortical neurons (Belelli et al. 2005; Cope et al. 2005) and the alpha6delta containing receptors that mediate the tonic current in cerebellar granule cells (Brickley et al. 2001; Hamann et al. 2002; Stell et al. 2003). Other receptor subtypes may also mediate tonic currents including alpha5gamma containing receptors in CA1 pyramidal cells (Caraiscos et al. 2004) and possibly epsilon containing receptors in hypothalamic neurons (Wagner et al. 2005). The epsilon containing $GABA_A$ receptors, expressed in the hypothalamus, amygdala and locus coereleus, are of particular interest in this regard, as these receptors can spontaneously open in the absence of GABA, but desensitise with high concentrations of GABA (Wagner et al. 2005). This leads to the intriguing possibility of a tonic $GABA_A$ receptor current in these brain structures that is "turned off" by increases in extracellular GABA.

It is likely that other $GABA_A$ receptor subunit combinations are also expressed extrasynaptically and are able to mediate a tonic current. There are single channel data and in situ patch clamp data that support the presence of a zolpidem sensitive $GABA_A$ receptor that can mediate a tonic current in the hippocampus, indicating the presence of alpha1, 2 or 3 with the gamma subunit (Lindquist and Birnir 2006; Semyanov et al. 2003). These subunit combinations are not usually associated with high affinity, extrasynaptic receptors, but rather low affinity, synaptic receptors. These findings serve to illustrate the multifarious $GABA_A$ receptor subtypes that may mediate tonic current, and investigations of the specific $GABA_A$ receptor involved are hampered by the relative paucity of subunit or $GABA_A$ receptor subtype specific agonists and antagonists. The situation is even more complicated as there is growing evidence that within one cell type (e.g. CA1 pyramidal cells), more than one receptor subtype may contribute to the tonic current (Glykys and Mody 2006; Prenosil et al. 2006; Scimemi et al. 2005; Semyanov et al. 2003). Indeed, extrasynaptic $GABA_A$ receptors with differing affinities for GABA can extend the dynamic range over which extracellular GABA can be detected and will modify the biophysical and pharmacological properties of the tonic current depending on ambient GABA concentration (Scimemi et al. 2005).

4
What is the Source of Ambient GABA?

GABA can escape the synaptic cleft during synaptic transmission to activate peri- and extrasynaptic receptors. This raises the question of whether this is the main source of GABA mediating the tonic current. This view has

been challenged in a number of studies. Tonic currents in adult cerebellum were unaffected by tetrodotoxin to block action-potential-dependent release and removal of external calcium to inhibit synaptic transmission (Rossi et al. 2003). However, in both these situations there is still some synaptic release. In order to prevent any synaptic release, concanamycin was applied to the slice (Rossi et al. 2003). This blocks the uptake of GABA into synaptic vesicles, but has no effect on the magnitude of tonic currents, suggesting that tonic GABA currents persist in the absence of GABA release (Fig. 2). Before synapse development, a substantial proportion of the GABA is released in a calcium-independent, SNARE-independent fashion indicating a non-vesicular mechanism (Demarque et al. 2002). Thus extracellular GABA could have both a vesicular and non-vesicular source.

Astrocytes can also release GABA in a calcium-dependent manner that depends upon the activation of P2X7 receptors, but is independent of vesicular release (Papp et al. 2004; Sperlagh et al. 2002). An intriguing finding has been

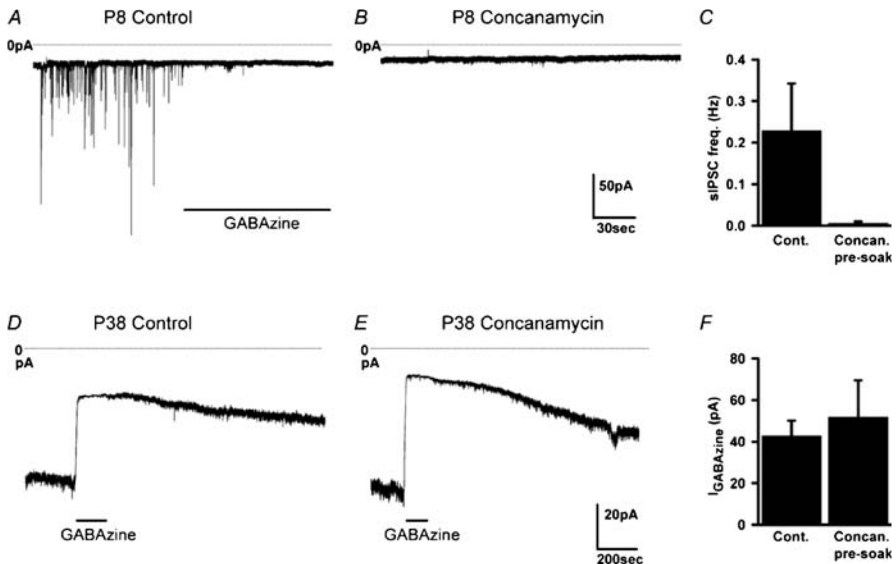


Fig. 2 The vesicular H^+-ATPase blocker concanamycin blocks exocytosis but does not affect the tonic current. **a,b** Membrane current in P8 granule cells in slices pre-soaked in solution lacking (**a**) or containing (**b**) 0.5 μM concanamycin. Control cells show spontaneous IPSCs that are blocked by GABAzine (10 μM), which are absent after concanamycin soaking. **c** Mean frequency of spontaneous IPSCs in P7–8 slices pre-soaked in control (seven cells) or concanamycin (six cells) solution. **d,e** Membrane current in adult granule cells in slices pre-soaked in solution lacking (**d**) or containing (**e**) 0.5 μM concanamycin. Control and concanamycin cells show a tonic current that is blocked by GABAzine (10 μM). **f** Magnitude of tonic current in cells pre-soaked in control (seven cells) or concanamycin (seven cells) solution. From Rossi et al. (2003) with permission

the large GABA release that occurs on activation of acetylcholine receptors (Rossi et al. 2003); the precise mechanisms underlying this phenomenon remain uncertain.

Is GABA necessary for the tonic activation of $GABA_A$ receptors? One study used an extracellular GABA scavenger system, yet still detected a tonic current (Wall 2002). Although, the efficacy of the scavenger system is debatable, it raises the possibility that other amino acids are involved in the generation of tonic currents. Taurine, for example, is a weak agonist at $GABA_A$ receptors (Albrecht and Schousboe 2005). There is also the possibility that certain $GABA_A$ receptors can open spontaneously without the presence of GABA (Birnir et al. 2000). Such behaviour is seen with epsilon containing receptors (Wagner et al. 2005); whether it can occur with other $GABA_A$ receptors is a matter of debate.

In addition to GABA release, there is also GABA uptake (Richerson and Wu 2003). GABA uptake efficiency varies from brain region to brain region and even within specific regions (e.g. hippocampus) (Semyanov et al. 2003). GABA released into the extracellular space is transported into neurones and glial cells via Na^+/Cl^- coupled GABA transporters (GATs) that can transport GABA against its chemical gradient (Richerson and Wu 2003). In human and rat, four GAT proteins have been identified and cloned: GAT-1, GAT-2, GAT-3 and BGT-1 (Borden 1996). These transporters have different regional and interregional localisations. GAT-1 is predominantly present on pre-synaptic GABAeregic terminals and glia, and is the most prevalent GABA transporter in the rat forebrain. In contrast, GAT-3 is localised exclusively to astrocytes and glia, and GAT-2 has a more diffuse distribution. GABA uptake and GAT expression change during development, and are also regulated by protein kinase C, a direct effect of GABA and tyrosine phosphatase (Beckman et al. 1999; Bernstein and Quick 1999; Law et al. 2000; Quick et al. 1997). There is evidence for differing roles of GAT1 and GAT3 transporters in regulating extracellular GABA, and thus tonic current in different brain areas (Keros and Hablitz 2005). In the supraoptic nucleus, tonic currents were independent of the degree of synaptic activity but were strongly modulated by GAT3, whilst in hippocampal pyramidal cells GAT1 plays an important role in regulating the tonic current (Semyanov et al. 2003).

One phenomenon that has been observed is that inhibition of both GAT1 and GAT3 transporters in the neocortex leads to a much greater rise in tonic currents than would be predicted from the results of specific inhibition of each transporter (Keros and Hablitz 2005). This suggests that the two transporters operate in tandem with one being able to compensate for inhibition of the other. An alternative explanation is that when both transporters are blocked, ambient GABA reaches a level which recruits lower affinity $GABA_A$ receptors, resulting in non-linear summation.

GABA transporters are easily reversed, as it has been estimated that their equilibrium potential may be around the resting membrane potential; trans-

porter reversal may occur with rises in intracellular sodium, rises in intracellular GABA and neuronal depolarisation, precisely the conditions that occur in pathological conditions such as epilepsy (Richerson and Wu 2003). The role of reverse uptake in physiological conditions is a matter of speculation; most studies demonstrate an increase in tonic currents when vesicular release is intact and GABA transporters are inhibited. Nevertheless, the magnitude of tonic currents is very dependent upon the expression of transporters. Indeed, the difference in the expression of tonic currents in interneurons and principal cells in the hippocampus may be due to a difference in the efficacy of GABA uptake in the vicinity of these cell types (Semyanov et al. 2003).

It remains unclear what part each of these potential mechanisms plays in regulating tonic GABA. Extracellular GABA is not a fixed quantity. Microdialysis studies in vivo have demonstrated that extracellular GABA can vary threefold with exposure to new environments (Bianchi et al. 2003) and fivefold during pathological conditions such as epilepsy (Pena and Tapia 1999). Given the importance of tonic currents in physiological and pathological processes, identifying methods of modifying tonic currents will undoubtedly have far-reaching implications.

5 The Physiological Effects of Tonic Current

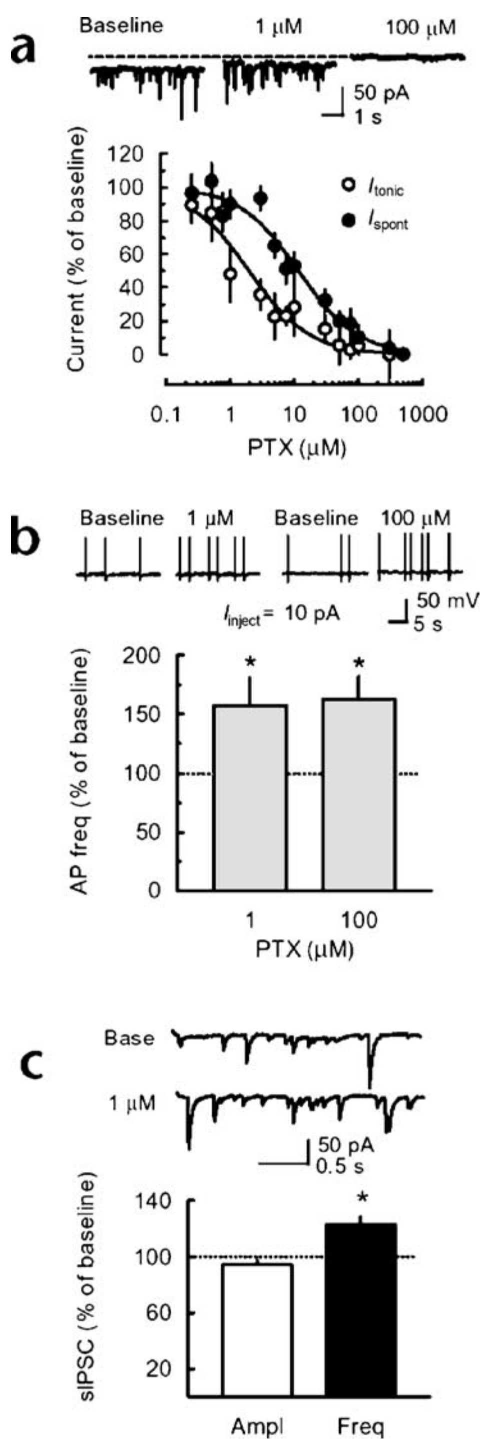
During development, prior to the expression of the chloride extrusion mechanism, GABA is depolarising (Represa and Ben-Ari 2005; Rivera et al. 2005). Depolarising tonic currents plays an important role in migration and in dendritic arborisation (Represa and Ben-Ari 2005). A similar role is also apparent in newly born dentate granule cells in the adult dentate gyrus, where the tonic $GABA_A$ receptor-mediated current occurs prior to synapse formation, is depolarising and plays a critical role in dendritic arborisation and the formation of synapses (Ge et al. 2006).

Tonic current is involved in physiological effects at the level of individual neurons and at the level of the local neuronal network. The effects of tonic activation of $GABA_A$ receptors depends upon the cellular compartment. Tonic activation of pre-synaptic $GABA_A$ receptors can regulate release of neurotransmitter, and axonal $GABA_A$ receptors may change action potential threshold (Kullmann et al. 2005; Ruiz et al. 2003). Somatic tonic currents can regulate signal processing. It has been hypothesised that a combination of noise and shunting inhibition that occurs with synaptic inhibition contributes to a change in the gain of the input-neuronal firing function (Chance et al. 2002). Tonic current increases the shunt, but also decreases the membrane time constant (so increasing the noise) (Mitchell and Silver 2003). The net effect of this is also a change in gain of the input-firing rate function (Fig. 3). By changing the membrane time constant, tonic currents expressed in the den-

drite will change the integration time window. The role or occurrence of tonic currents in the dendrite have, however, not yet been investigated.

Modulation of neuronal excitability is important for maintaining the firing rate within an operational range over a wide range of excitatory drive (Mitchell and Silver 2003). This permits cells that have a limited dynamic range to operate over a wide range of network conditions without saturating. Thus if the extracellular GABA concentration (and so tonic inhibition) increases as excitatory drive increases, then this will in turn result in a decrease in neuronal excitability and so prevent neuronal saturation (Mitchell and Silver 2003). It will also limit the number of neurons that are active during the excitatory inputs, leading to increasing sparseness of the neuronal code (Hamann et al. 2002). Sparseness has specific advantages for memory, as computational models have indicated that decreasing the number of neurons that are simultaneously active increases the amount of information that can be stored (Marr 1969). In addition, inhibiting the firing of those neurons that do not receive adequate input will also decrease noise. Thus tonic inhibition may contribute to development of a low-noise sparse coding system. It is thus perhaps not surprising that the cerebellum (necessary for motor memory) and hippocampus (necessary for declarative memory) have a mechanism to maintain sparse coding of excitatory inputs.

Further insight into the significance of tonic $GABA_A$ receptor-mediated conductances comes from examining their cell-type-dependent expression in the context of network behaviour (Semyanov et al. 2004). By adjusting the excitability of the network, tonic inhibition regulates the magnitude and frequency of network oscillations, which may be important for temporal signal processing (i.e. providing a temporal context for signals) (Towers et al. 2004). Under baseline conditions, the $GABA_A$ receptor-mediated tonic current in interneurons is considerably larger than the current mediated by spontaneous IPSCs, and thus is an important determinant of interneuronal excitability and firing. In hippocampal pyramidal cells, tonic $GABA_A$ receptor-mediated currents are small and inhibition is predominantly mediated by synaptic IPSCs. In keeping with this, a low concentration of picrotoxin that relatively selectively inhibits tonic current significantly increased the frequency of spontaneous IPSCs in hippocampal pyramidal cells (Fig. 4). These findings suggest that a possible role of tonic conductance in interneurons is to act as a homeostatic regulator of synaptic inhibition of principal cells: if the ambient GABA concentration decreases, this renders interneurons more excitable, resulting in a compensatory increase in the frequency of GABA receptor-mediated IPSCs in pyramidal cells (Semyanov et al. 2003). Conversely (although this remains to be tested), an increase in ambient GABA concentration would be expected to render interneurons relatively unexcitable, leading to a decrease in synaptic inhibition of pyramidal neurons. On the other hand, an increase in GABA will lead to increased tonic inhibition of pyramidal cells. This suggests that with increasing extracellular GABA, such as occurs with exposure


a
Baseline
1 μM
100 μM
50 pA
1 s
Current (% of baseline)
120
100
80
60
40
20
0
I_{tonic}
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0.1
1
10
100
1000
PTX (μM)
b
Baseline
1 μM
Baseline
100 μM
I_{inject} = 10 pA
50 mV
5 s
AP freq (% of baseline)
200
150
100
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1 μM
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0.5 s
sIPSC (% of baseline)
140
100
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◀ **Fig. 3** Inhibition of the interneuron-specific tonic current increases the excitability of interneurons and the frequency of spontaneous IPSCs (sIPSCs) in pyramidal cells. **a** Representative traces taken from one interneuron showing effects of increasing concentrations of picrotoxin on sIPSCs and *I* hold. Below: summary concentration–response curves obtained for I_{tonic} and I_{spont} for seven interneurons. The points were fitted with Hill equations (Levenburg–Marquart algorithm). I_{tonic} shows a greater sensitivity to picrotoxin than I_{spont}. **b** Representative traces from one neuron in current clamp with 10 pA injected current, showing that inhibiting the tonic current with picrotoxin (1 or 100 μM) results in an increase in action potential firing rate. Below: summary data for firing rate with 10 pA current injection with the addition of picrotoxin (1 μM ($n = 5$) or 100 μM ($n = 5$)) compared to baseline firing rate with no picrotoxin present. **c** Traces taken from one pyramidal cell showing the effects of 1 μM picrotoxin on sIPSCs. Below: summary data for the effect of picrotoxin (1 μM) on sIPSC amplitude and frequency in four pyramidal cells. Picrotoxin resulted in a significant increase in sIPSC frequency in pyramidal cells, but no significant effect on sIPSC amplitude. * $P < 0.05$. From Semyanov et al. (2003)

to a new environment (see below), there would be a shift in pyramidal cells from synaptic inhibition to tonic inhibition. This prediction is supported by experimental work which indicates that on exposure to a new environment, there is a decrease in interneuronal firing without an increase in pyramidal cell firing (Wilson and Mcnaughton 1993). What is the purpose of this? This remains uncertain, but synaptic inhibition may have a greater effect on gain than tonic inhibition (Fig. 3) which may also affect offset, and so could be

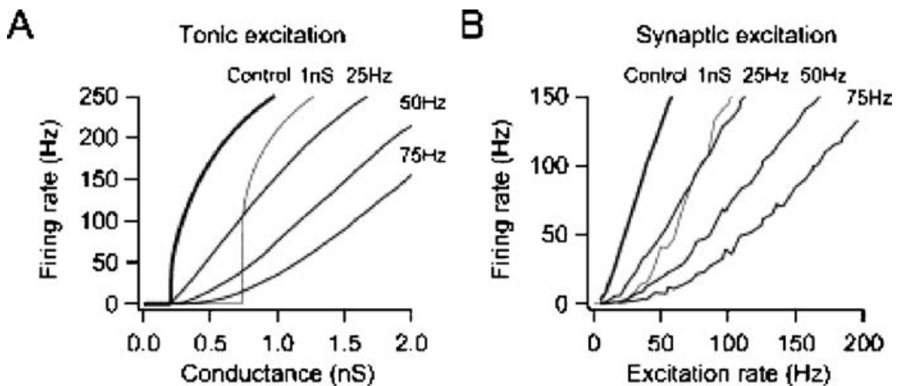

Fig. 4 Random trains of synaptic inhibition produce larger gain changes and smaller offset shifts than tonic inhibition. **A** Output firing frequency–conductance relationship for an integrate and fire (I&F) model driven by tonic excitation for control conditions (*thickest line*), with 1 nS tonic inhibition (1 nS; *thinnest line*), and inhibition mediated by Poisson trains of synaptic conductance waveforms (synaptic inhibition) applied at 25, 50 and 75 Hz. Mean conductance during 25-Hz inhibition was 1 nS. **B** Output firing frequency–input frequency relationships for an I&F model driven by synaptic excitation for control conditions (*thickest line*), with 1 nS tonic inhibition (1 nS; *thinnest line*), and synaptic inhibition at 25, 50 and 75 Hz. From Mitchell and Silver (2003) with permission

a mechanism by which the network increases gain without leading to excessive pyramidal cell firing (this at present remains, however, speculative).

The potential importance of tonic currents in regulating cognition and memory is illustrated by the expression and role of the alpha5 subunit. This subunit is highly expressed in pyramidal cells in the hippocampus, where it is present extrasynaptically (Brunig et al. 2002; Crestani et al. 2002; Fritschy et al. 1998). It has been shown to contribute to the tonic current in CA1 pyramidal cells, but to play no part in spontaneous synaptic currents (Caraiscos et al. 2004; Prenosil et al. 2006). Slices from alpha5 knockout mice demonstrate enhanced gamma rhythm generation (Towers et al. 2004). In accord with this, deletion and point mutations of the gene encoding for alpha5 improve cognitive performance (Collinson et al. 2002; Crestani et al. 2002), an effect that is mimicked by alpha5-selective benzodiazepine inverse agonists (Dawson et al. 2005).

Cell-type specificity is also present in the thalamus where tonic $GABA_A$ receptor currents, mediated by alpha4delta containing receptors, are present in the thalamocortical neurons but not in the reticular thalamic neurons (Belelli et al. 2005; Cope et al. 2005). The thalamocortical neurons gate sensory input into the neocortex, whilst the reticular thalamic neurons are inhibitory and synapse onto thalamocortical neurons and receive afferents from thalamocortical neurons and the neocortex. The reticular thalamic neurons are an integral part of thalamic and thalamocortical loops involved in the generation of oscillatory behaviour. Hyperpolarisation of thalamocortical neurons by tonic $GABA_A$ receptor activation changes the firing pattern of thalamocortical neurons from regular firing, in which sensory information can be transmitted to the cortex, to a burst firing pattern typical of slow-wave sleep and associated with gating of sensory inputs (Cope et al. 2005). Drugs that prolong slow-wave sleep enhance the tonic current in thalamocortical neurons (Belelli et al. 2005). Thus cell-type specificity is critical for an understanding of the network effects of tonic currents, and such currents may play a critical role in information processing.

6 Regulation of Tonic Inhibition and Plasticity

There are several ways in which the magnitude of the tonic current can be regulated. The magnitude of tonic currents depends upon the concentration of extracellular GABA, the expression of extrasynaptic receptors, the subtype of $GABA_A$ receptors expressed and the presence or absence of various modulators of $GABA_A$ receptors.

There are several mechanisms that may regulate extracellular GABA and it can rise in response to a number of stimuli. For example, kainate application to hippocampal slices produces a robust firing of interneurons (and

thus GABA release), which results in an increase in tonic $GABA_A$ receptor-mediated currents both in hippocampal pyramidal cells and in interneurons (Cossart et al. 1998; Kullmann and Semyanov 2002). Similarly, application of acetylcholine to cerebellar slices produces a fourfold increase in the tonic conductance via a calcium-dependent, action-potential-independent GABA release mechanism (Rossi et al. 2003). Short-term changes in GABA uptake can also occur with depolarisation and changes in electrochemical gradients for transportable substances (e.g. GABA and sodium ions) (Richerson and Wu 2003). Structural change and transporter expression may play an important part in the expression of tonic current. For example, glial cell coverage (and therefore neurotransmitter uptake) changes with hormonal status in the rat supraoptic nucleus (Oliet et al. 2001). Extracellular GABA rises during physiological and pathological states have also been measured in vivo using microdialysis. Extracellular GABA rises at least threefold in the hippocampus when an animal is exposed to a new environment (Bianchi et al. 2003). The mechanisms underlying such a rise are a matter of speculation, but the observation that acetylcholine can mediate a significant increase in tonic currents in vitro (Rossi et al. 2003) and that acetylcholine is released in the hippocampus during exploration (Bianchi et al. 2003) gives rise to the hypothesis that such rises may be acetylcholine dependent. This state-dependent increase in tonic current may be advantageous for signal processing (see above). Whether GABA accumulation occurs in other structures during physiological perturbations or states (such as sleep) is largely an unexplored question. GABA also increases during pathological states, and large elevations (four- to fivefold) of hippocampal GABA have been observed during seizures (Pena and Tapia 1999). This would result in an increase in tonic currents, perhaps as an adaptive mechanism during network hyperexcitability. The source of the GABA during seizures is again a matter of debate, but reverse transport would be an ideal candidate (see above).

Delta subunit containing $GABA_A$ receptors are highly sensitive to endogenous neurosteroids that are synthesised from progestagens (Belelli and Lambert 2005); the neurosteroid, *allo*-tetrahydrodeoxycorticosterone (THDOC), at physiological concentrations almost doubles the magnitude of the tonic current in cerebellar and dentate granule cells (Stell et al. 2003). It has been suggested that the fall in progesterone levels that accompanies menstruation would result in a decrease in neurosteroid concentrations and consequently a decrease in tonic inhibition. This may explain the clustering of seizures around menstruation (catamenial epilepsy) and even affective symptoms associated with the menstrual cycle. Importantly, the modulation of tonic $GABA_A$ receptor-mediated currents by neurosteroids is influenced by local neurosteroid metabolism, inhibition of which can greatly enhance the response of the tonic current in dentate granule cells to endogenous neurosteroids (Borghese et al. 2005). Hormonal fluctuations of neurosteroids during the oestrous cycle in rats can also regulate the expression of the delta subunit,

such that there is increased delta subunit expression during the dioestrous phase when progestagen levels are high and this drops during the oestrous phase when progestagens are low (Maguire et al. 2005). This is unlikely to be due to a direct effect of progestagens on delta subunit expression, as other studies have demonstrated increased expression of delta subunits on progestagen withdrawal (Lovick 2006; Mostallino et al. 2006). The effect of the decreased delta subunit expression is a decrease in tonic currents in dentate granule cells associated with an increase in seizure susceptibility and an increase in measures of anxiety (Maguire et al. 2005).

Another potentially important modulator of high affinity $GABA_A$ receptors is ethanol, which has been reported to potentiate currents mediated by delta containing receptors at anxiolytic concentrations (Wallner et al. 2003; Wei et al. 2004). In addition, there are rats with a specific polymorphism in the alpha6 subunit that makes the alpha6beta3delta $GABA_A$ receptors, expressed in cerebellar granule cells, more sensitive to alcohol (Hanchar et al. 2005). These rats are more sensitive to the ataxic effects of alcohol. Much of the conclusions of this work have, however, recently been challenged by studies that find no effects of alcohol on delta subunit containing $GABA_A$ receptors (Borghese et al. 2005). At present it is difficult to reconcile these opposing findings.

Longer-term plastic changes in $GABA_A$ receptors and GABA uptake can occur during the development of epilepsy or alcohol withdrawal. Decreases in delta subunit expression in the dentate gyrus during epileptogenesis (Peng et al. 2004) or with alcohol withdrawal (Liang et al. 2006) have been proposed to contribute to decreased tonic currents and possibly hyperexcitability. In addition, a decrease in the expression of the alpha5 subunit with epileptogenesis in the hippocampus has been hypothesised to be a pro-epileptogenic phenomenon (Fritschy et al. 1999; Houser and Esclapez 2003; Sperk et al. 1998). However, the downregulation of the alpha5 subunit in epileptogenesis is associated with an upregulation of other $GABA_A$ receptors leading to enhanced rather than decreased tonic currents (Scimemi et al. 2005) (Fig. 5). This may be an adaptive mechanism that increases tonic current in the face of increased network excitability, perhaps at the expense of memory and cognition (see above).

Tonic currents also exhibit adaptive change following knockout of specific $GABA_A$ receptor subunits. Thus knockout of the alpha5 subunit leads to a compensatory upregulation of alpha4delta-mediated tonic currents in CA1 pyramidal cells (Glykys and Mody 2006). Knocking out the alpha6 subunits, which are solely responsible for tonic current in cerebellar granular cells, results in a compensatory upregulation of two pore potassium conductances. This adaptive change of other conductances in the face of decreased tonic $GABA_A$ receptor currents raises two important points: first, the danger of over-interpreting data from knockout animals in which there may be compensatory changes with complex effects, and second, such compensation emphasises the important physiological role of these tonic currents.

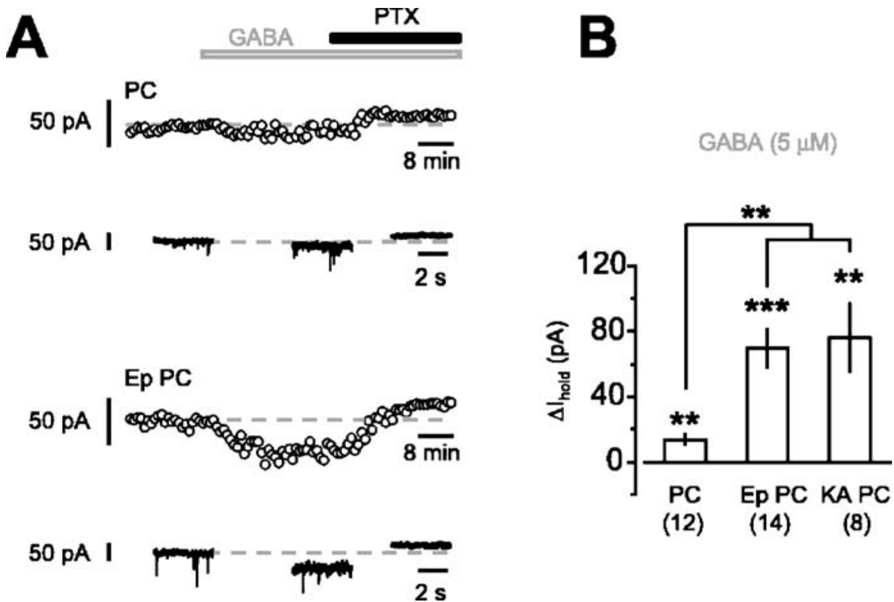

Fig. 5 The effect of increasing $[GABA]_o$ on the tonic current in pyramidal cells from epileptic and control animals. **A** Time course of I_{hold} in a pyramidal cell from a control rat and from an epileptic rat after perfusion with 5 µM GABA. Insets show example traces obtained at the steady state of each drug application. PTX, picrotoxin. **B** Histogram summarising the increase in I_{hold} in pyramidal cells induced by adding GABA (5 µM) to the perfusing solution in pyramidal cells from control, epileptic animals induced with pilocarpine (Ep PC), and epileptic animals induced with kainic acid (KA PC). The increase was significantly greater in pyramidal cells from epileptic animals (whether generated with pilocarpine or kainic acid) compared with those from control animals. ** $p < 0.01$; *** $p < 0.001$. Error bars represent SEM. From Scimemi et al. (2005) with permission

7 Conclusion

Tonic inhibition is present throughout the central nervous system, but is expressed in a cell-type specific manner. These tonic currents can have a profound effect on neuronal excitability and complex effects on network activity. They can be modulated by regulation of extracellular GABA, by endogenous and exogenous modulators and by developmental, physiological and pathological regulation of GABA uptake and GABA$_A$ receptor expression. The growth in specific GABA$_A$ receptor subtype agonists and antagonists in order to target such extrasynaptic receptors may be an important method of regulating physiological processes such as sleep, cognition and memory, and pathological processes such as anxiety and epilepsy.

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GABA$_C$ Receptors in Retina and Brain

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Abstract The expression of GABA$_C$ receptors has long been regarded as a specific property of bipolar cells in the inner retina where they control the information transfer from bipolar to retinal ganglion cells. A number of recent anatomical and physiological studies, however, have provided evidence that GABA$_C$ receptors are also expressed in many brain structures apart from the retina. The presence of GABA$_C$ receptors in many GABAergic neurons suggests that this receptor type may be involved in the regulation of local inhibition. This chapter focuses on the distribution of GABA$_C$ receptors and their possible function in various brain areas.

1 Introduction

The fundamental importance of GABA as an inhibitory neurotransmitter is reflected in the ubiquitous presence of GABA receptors in the mammalian CNS. Because most GABAergic neurotransmission is mediated by GABA$_A$ receptors it seems reasonable to assume that every mammalian CNS neuron expresses GABA$_A$ receptors. In addition, in a large number of neurons GABA$_B$ receptors are co-expressed with GABA$_A$ receptors throughout the mammalian brain. Compared to the ubiquitous expression of these two GABA receptor types, however, the occurrence of the third identified GABA receptor type, the GABA$_C$ receptor, seems to be topographically much more restricted. Furthermore, because GABA$_C$ receptors have been characterized more recently than GABA$_A$ and GABA$_B$ receptors their functional significance is also less well understood. Although less abundant than the two other GABA receptor types, GABA$_C$ receptors have been localized in a variety of CNS structures, and it seems that the number of areas in which GABA$_C$ receptors are found is still increasing as more sensitive methods to discover their expression are used.

The first description of a GABA receptor that did not resemble characteristics of the classical GABA$_A$ and GABA$_B$ receptor origins from the spinal cord (Johnston et al. 1975). Later, neurons in other brain areas were shown to also exhibit GABA receptors with "unusual" pharmacological properties. To date, the brain areas where the presence of GABA$_C$ receptors has been demonstrated include both structures of generalized function, like for example the amygdala (Delaney and Sah 1999), the hippocampus (Hartmann et al. 2004),

the cerebellum (Boué-Grabot et al. 1998), and the spinal cord (Johnston et al. 1975), as well as functionally more specialized structures such as different components of the subcortical visual system, like the dorsal lateral geniculate nucleus (Zhu and Lo 1999), the superior colliculus (Arakawa and Okada 1988; Pasternack et al. 1999; Schmidt et al. 2001; Boller and Schmidt 2003; Kirischuk et al. 2003), and the pretectum (Boller and Schmidt 2003). However, the strongest expression of $GABA_C$ receptors seems to be found in the vertebrate retina and this chapter will therefore first concentrate on functional aspects of $GABA_C$ receptors in this particular part of the CNS, before it depicts $GABA_C$ receptor functions in extraretinal structures.

2 Retina

Numerous anatomical and physiological studies in different species from almost all vertebrate classes have been carried out in order to clarify the functional significance of $GABA_C$ receptors in the retina. Because it has been that part of the brain in which $GABA_C$ receptors have been most extensively studied, functional aspects of retinal $GABA_C$ receptors will be the focus of interest here.

2.1 Outer Plexiform Layer

$GABA_C$ receptors have been reported to occur in the outer plexiform layer (OPL), particularly in the teleost fish retina (Qian and Dowling 1994; Dong et al. 1994). Thus, cone- but not rod-driven horizontal cells in the catfish retina exhibit a GABA receptor-gated picrotoxin-sensitive chloride current, which can be divided into two components based on its sensitivity to the specific $GABA_A$ receptor antagonist bicuculline (Fig. 1). While a bicuculline-sensitive component was found only in some cells, a bicuculline-resistant, picrotoxin-sensitive component was found in all cells tested (Dong et al. 1994). In goldfish, $GABA_C$ receptors are found in luminosity-type (H1) cone horizontal cells and axon terminals, but not in chromaticity-type (H2 and H3) horizontal cells (Paik et al. 2003).

In contrast, in the white perch OPL, $GABA_C$ receptors have been reported to mediate GABA currents exclusively in isolated rod-driven horizontal cells (Qian and Dowling 1993, 1994). Because horizontal cells release GABA, this suggests that $GABA_C$ receptors participate in a GABA-mediated autofeedback in horizontal cells. However, the chloride equilibrium potential in horizontal cells is more positive than the resting membrane potential, therefore such a pathway will provide a positive feedback pathway and slow down the kinetics of the light response (Kamermans and Werblin 1992).

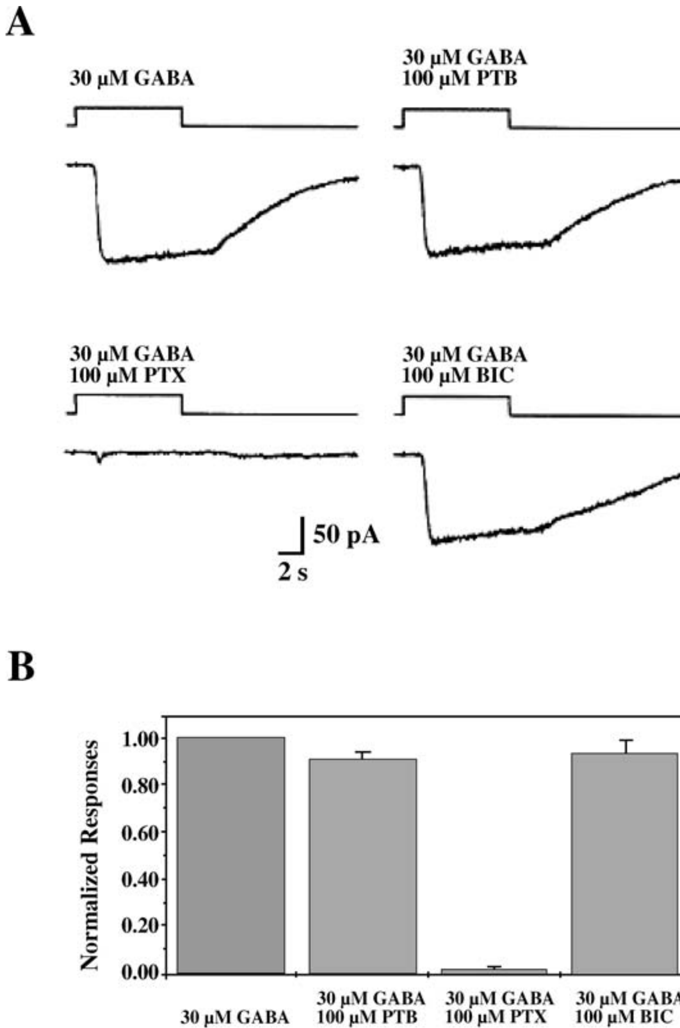

Fig. 1 GABA$_C$ receptor-mediated currents recorded in a catfish cone-driven horizontal cell. **A** GABA-induced currents were mostly insensitive to blockade by bicuculline methiodide (*BIC*) and to enhancement by pentobarbital (*PTB*), but were almost completely blocked by picotoxin (*PTX*). Periods of drug application are indicated by the upward deflections of the horizontal lines above each current trace. **B** Quantification of the effects of GABA$_A$ and GABA$_C$ receptor-related drugs on GABA-induced currents. Averaged responses were normalized to those elicited by GABA alone. Reproduced with permission from Dong et al. (1994)

In mammals, GABA$_C$ receptor expression in the OPL is much less obvious (Enz et al. 1996; Lukasiewicz 1996; Koulen et al. 1997). However, adult mammalian cone photoreceptors have been reported to generate large GABA-

elicited currents mediated by both $GABA_A$ and $GABA_C$ receptors. In contrast, rods had no GABA-elicited responses (Picaud et al. 1998). These results support the notion that mammalian cones but not rods receive a GABA-mediated feedback from horizontal cells.

2.2 Inner Plexiform Layer

While their presence in the OPL might differ between different vertebrate classes, the expression of $GABA_C$ receptors in the inner plexiform layer (IPL) seems common to all vertebrates, from white perch (Qian and Dowling 1994) to primates, including humans (Cutting et al. 1991). Results from immunohistochemical studies at the light microscopic level suggested that $GABA_C$ receptors in the IPL are concentrated at synaptic sites (Enz et al. 1996) and immunocytochemistry at the electron microscopic level confirmed this earlier assumption (Fletcher et al. 1998; Koulen et al. 1998). The IPL contains processes from three different retinal cell classes: bipolar cells, amacrine cells, and ganglion cells. Of these, only bipolar cells have been shown to express $GABA_C$ receptors (Feigenspan et al. 1993; Lukasiewicz et al. 1994; Qian and Dowling 1995). Their occurrence is confined to the bipolar cell axonal terminal (Lukasiewicz et al. 1994) at synaptic sites where bipolar cells receive input from GABAergic amacrine cells, which in turn are postsynaptic to the bipolar cells (Koulen et al. 1998). Because of this arrangement, upon bipolar cell activation glutamate released from the bipolar cell terminal activates GABAergic amacrine cells. These amacrine cells will release GABA back onto the activated bipolar cell terminal and, thereby, lead to feedback inhibition. It has been demonstrated that the amount of glutamate released from a single bipolar cell is sufficient to activate the GABAergic amacrine cell feedback (Hartveit 1999).

Both $GABA_A$ and $GABA_C$ receptors contribute to GABA responses in bipolar cells (Fig. 2) (Lukasiewicz et al. 1994; Qian and Dowling 1995; Euler and Wässle 1998; Du and Yang 2000; Shields et al. 2000). However, the proportion of the $GABA_C$ receptor-mediated component varies considerably between different bipolar cell classes (Fig. 3). Thus, in rat and rabbit retina, the highest proportion of $GABA_C$ receptor-mediated currents is found in rod bipolar cells ($\sim$ 70% of the total GABA current) while in cone bipolar cells, on average, only up to $\sim$ 20% of the total GABA current is mediated by $GABA_C$ receptors, with differences between different cone bipolar cell types (Euler and Wässle 1998; McGillem et al. 2000). Similarly, bipolar cells in the ferret retina show a general trend for $GABA_C$ receptor-mediated current components to increase from OFF cone to ON cone to rod bipolar cells. Concomitantly, the $GABA_A$ receptor-mediated component shows the opposite behavior, increasing from rod to OFF cone to ON cone bipolar cells (Shields et al. 2000). As a consequence, $GABA_C$ receptor activation preferentially influences ON responses in

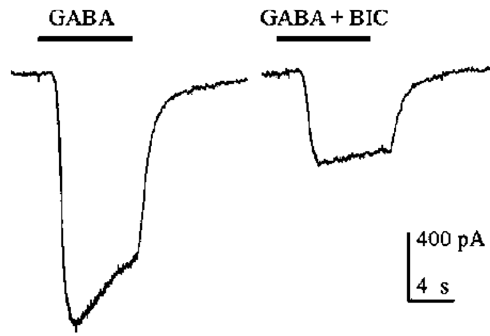

Fig. 2 GABA responses from isolated white perch bipolar cells. Currents elicited by GABA application (*left*) contain both transient and sustained components, indicating that both GABA$_A$ and GABA$_C$ receptors are present. In the presence of bicuculline (*BIC*) GABA$_A$ receptor activity is inhibited, revealing sustained GABA responses mediated by GABA$_C$ receptors (*right*). Reproduced with permission from Qian (2000)

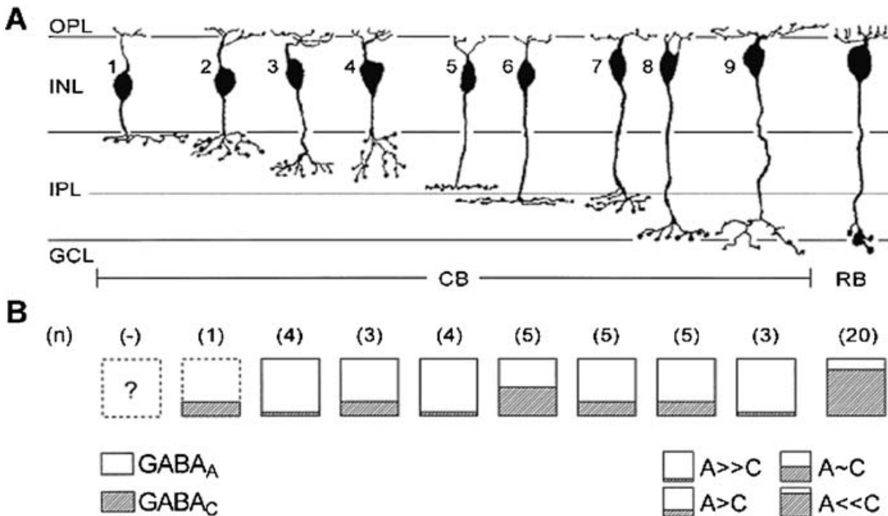

Fig. 3 GABA receptors in bipolar cells in the rat retina. **A** Summary of the different bipolar cell types. Cone bipolar (*CB*) cell types (*1* to *9*) are arranged according to the stratification levels of their axons within the inner plexiform layer (*IPL*). A rod bipolar (*RB*) cell is indicated on the *right*. **B** Contribution of GABA$_A$ and GABA$_C$ receptors to total GABA currents for the different bipolar cell types. *Boxes* symbolize GABA$_A$: GABA$_C$ ratio (■, relative contributions of GABA$_C$ receptors; □, relative contributions of GABA$_A$ receptors). The number of cells recorded is given in *brackets*. Reproduced with permission from Euler and Wässle (1998)

third order retinal neurons (Zhang and Slaughter 1995), which indicates a differential role for this receptor class in ON and OFF retinal pathways. Also, light-evoked responses of ON amacrine cells in the mouse retina are greatly

enhanced by a blockade of $GABA_C$ receptors. The proportion of the light-evoked response, which is NMDA receptor-mediated, also increases, probably because glutamate release by the bipolar cell is enhanced (Matsui et al. 2001).

The cell-type specific expression of $GABA_C$ receptors in bipolar cells calculated from the contribution of $GABA_A$ and $GABA_C$ receptors to the total GABA currents is in good agreement with results from immunocytochemical studies, which revealed that rod bipolar cell axon terminals express higher amounts of $GABA_C$ receptors than cone bipolar cell terminals (Enz et al. 1996; Shields et al. 2000).

Although both $GABA_A$ and $GABA_C$ receptors are present in bipolar cells, they show a rather distinct subcellular distribution. $GABA_A$ receptors are preferentially located at the bipolar cell dendrites, which seem to lack $GABA_C$ receptors. In contrast, $GABA_C$ receptors are expressed predominantly at the bipolar cell terminals where they mediate feedback inhibition from amacrine cells (Lukasiewicz et al. 1994; Vaquero & de la Villa 1999; Euler and Masland 2000; Shields et al. 2000). Given their presynaptic localization at bipolar cell terminals, $GABA_C$ receptor activation will strongly modulate glutamate release to both amacrine and ganglion cells by suppressing the depolarization-induced Ca^{2+} influx into the bipolar cell terminal (Pan and Lipton 1995; Wellis and Werblin 1995). Thus, GABA released from the amacrine cell will truncate glutamate release from the bipolar cell terminal and make the bipolar cell output to amacrine and ganglion cells more transient. This has been shown in the tiger salamander, where picrotoxin (a blocker of both $GABA_A$ and $GABA_C$ receptors) but not bicuculline (the $GABA_A$ receptor antagonist) converted light-evoked responses of amacrine and ganglion cells from transient to more sustained (Zhang et al. 1997; Dong and Werblin 1998; Roska et al. 1998; Shen and Slaughter 2001).

The significance of $GABA_C$ receptor function has also been demonstrated in knock-out mice that lack the ρ1 subunit (McCall et al. 2002), which is one of the three specific subunits of $GABA_C$ receptors (Cutting et al. 1991; Bormann and Feigenspan 1995). Rod bipolar cells in these ρ1 knock-out mice do not express $GABA_C$ receptor-mediated currents, which indicates that no functional $GABA_C$ receptors are expressed. As a consequence, under mesopic stimulus conditions the b-wave of the electroretinogram, which reflects the activation of bipolar cells, is increased and prolonged in comparison to normal control animals. This demonstrates that the bipolar cell responses are more sustained in the knock-out mice since they lack the $GABA_C$ receptor-mediated inhibitory feedback from GABAergic amacrine cells (McCall et al. 2002). As a consequence of the increased bipolar cell activity, both spontaneous as well as light-evoked activities of ganglion cells are higher in the ρ1 knock-out mice than in wild-type mice (Lukasiewicz et al. 2004).

$GABA_C$ receptors have a tenfold higher affinity for GABA than $GABA_A$ receptors (Bormann and Feigenspan 1995), therefore they are activated at lower amounts of GABA released from the amacrine cells. $GABA_C$ receptors also

activate and deactivate at slower rates than $GABA_A$ receptors. Therefore, the presence of both receptor types in bipolar cell axon terminals leads to a much larger dynamic range in the overall response to GABA than either subtype alone. Because bipolar cells respond with graduated depolarizations to visual stimuli, glutamate release can be relatively slow and sustained compared with that from a spiking neuron. Inhibition mediated by $GABA_C$ receptors is temporally matched to the kinetics of this glutamate release. In contrast, $GABA_A$ receptors, which induce much faster responses to GABA, suppress glutamate release more rapidly and transiently (Pan and Lipton 1995).

Furthermore, the specific distribution of $GABA_C$ receptors in different bipolar cell classes nicely matches the kinetics of visual responses in the rod and cone pathways. Rod light responses are ten times slower than cone responses, and the kinetics of synaptic transfer are ten times longer at rod synapses than at cone synapses (Schnapf and Copenhagen 1982). In the cone pathway, synaptic events in ON bipolar cells are longer than those in OFF bipolar cells (Ashmore and Copenhagen 1980), consistent with the notion of slower synaptic transfer in the ON cone pathway than in the OFF cone pathway. These differences are most likely attributable to the presence of metabotropic glutamate receptors on ON bipolar cell dendrites (Slaughter and Miller 1981; Nawy and Jahr 1990) and kainate receptors on OFF bipolar cell dendrites (Devries and Schwartz 1999).

Recently, it has been shown that bipolar cell $GABA_C$ receptors shape ganglion cell responses by affecting the timing of transmitter release from the bipolar cell terminal through the amacrine to bipolar cell feedback loop (Freed et al. 2003). Spontaneous EPSCs that occurred in ganglion cells were temporally correlated in bursts of 10–55 ms. A blockade of $GABA_C$ receptors with the selective antagonist TPMPA abolished this correlation. Using intact retina, similar effects were observed to light-evoked EPSCs. As expected from the timing of the bursts, this $GABA_C$ receptor-mediated effect was restricted to ganglion cells that show "sluggish" responses to light stimulation.

$GABA_C$ receptors are in the perfect position to modulate the information flow from bipolar to amacrine and to ganglion cells, not only in the temporal domain. Instead, there is multiple evidence that $GABA_C$ receptors also modulate spatial characteristics of ganglion and amacrine cell responses. Thus, surround inhibition in ganglion cell responses, which is mediated by GABAergic amacrine cell input, is also influenced by $GABA_C$ receptor activation. However, because ganglion cells do not express $GABA_C$ receptors, their influence on surround inhibition is only indirect. As stated above, an activation of $GABA_C$ receptors on bipolar cell terminals makes the bipolar cell response more transient. Because amacrine cell activity depends on excitatory bipolar cell input, the activity of amacrine cells is also truncated. A blockade of $GABA_C$ receptors thus will prolong amacrine cell activation and thereby also increase the surround inhibition in the ganglion cell response. Thus, in the tiger salamander dim surround stimuli reduced the sensitivity of the gan-

glion cells to central illumination, and $GABA_C$ receptor antagonists blocked the reduction in light sensitivity (Ichinose and Lukasiewicz 2005). Similarly, GABA blockers eliminated mesopic surround inhibition to rabbit ganglion cells (Flores-Herr et al. 2001) and to rat bipolar cell terminals (Euler and Masland 2000).

In the spatio-temporal domain, $GABA_C$ receptors have been shown to also convey inhibition laterally to compress the spatial representation of a visual stimulus across an array of bipolar cell terminals (Roska et al. 2000). Light-evoked activity patterns at the ON bipolar terminal were both spatially and temporally restricted. These patterns expanded in both space and time when $GABA_C$ receptors were blocked. Blocking $GABA_C$ receptors caused a massive spatial blur in the activity patterns at bipolar terminals. Increases during $GABA_C$ receptor blockade were observed in the maximum space constant, the duration, the time-to-peak, and the charge transfer (Roska et al. 2000).

Spatio-temporal modulation of ganglion cell responses mediated by $GABA_C$ receptors has also been observed in the tiger salamander retina (Jacobs and Werblin 1998). Single ganglion cell responses to a flashed square were recorded and replayed in a spatially aligned array of a uniform cell layer with identical response properties. In the control situation, the achieved ganglion cell activity pattern first nicely represented the shape of the stimulus. However, shortly after that the activity in the center of the stimulus representation collapsed, leaving a representation of only the edges of the square. Application of picrotoxin, but not bicuculline, blocked the collapse of the center representation leaving the activity pattern temporally unchanged. This edge-enhancing effect may be attributed to a spatially expanding delayed inhibition. Because picrotoxin prevented the "hole" in the expanding representation from developing, it was assumed that the inhibitory interaction that suppresses the interior of the square is mediated by $GABA_C$ receptors (Jacobs and Werblin 1998).

Although $GABA_C$ receptors can modulate many different aspects of information processing in the inner retina, only few studies have been able to show behavioral consequences of $GABA_C$ receptor activation or inactivation. One example, however, has been reported recently in the frog (Ishikane et al. 2005). When stimulated with an expanding dark spot, which mimics the approach of a putative predator, these animals responded with escape behavior as soon as the increase in stimulus diameter reached a certain threshold. Intraocular injections of $GABA_A$ receptor antagonists reduced this response by a shift of the threshold towards larger spot diameters. In contrast, intraocular injections of the $GABA_C$ receptor antagonist TPMPA potentiated the escape response by shifting the threshold towards smaller spots. At the ganglion cell level, responses of ganglion cells that are specially tuned to expanding dark spots, the so-called dimming detectors, show increased responses to stimulation and more synchronized oscillatory activity in the presence of TPMPA. A blockade of $GABA_C$ receptors increases glutamate release from bipolar cells

and enhances the direct excitatory input to dimming detectors and/or the inhibitory activity of amacrine cells, thereby enhancing oscillatory activities of dimming detectors. This activity increase in dimming detectors finally results in enhanced escape behavior. In contrast, activation of $GABA_C$ receptors in bipolar cell terminals attenuates the oscillatory activity of dimming detectors and thus optimizes the stimulus size tuning for eliciting escape behavior. Such adjustment may be useful to avoid unnecessary escape from tiny prey or a too distant predator (Ishikane et al. 2005).

3
Superior Colliculus

Although the retina is not the only CNS structure in which $GABA_C$ receptors are expressed, functional studies in all other areas in which $GABA_C$ receptors have been found are much less numerous and hypotheses about possible $GABA_C$ receptor functions are therefore less elaborated. Among $GABA_C$ receptor-expressing extraretinal visual structures, most studies have concentrated on the superior colliculus (SC). Studies using in situ hybridization and immunohistochemistry revealed that expression of $GABA_C$ receptors in SC is restricted to the superficial gray layer, the stratum griseum superficiale (SGS). Other SC layers appeared to be void of $GABA_C$ receptors (Boué-Grabot et al. 1998; Wegelius et al. 1998; Pasternack et al. 1999; Clark et al. 2001).

Physiologically, a first indication of GABA effects neither mediated through $GABA_A$ nor through $GABA_B$ receptors emerged from local field potential recordings in SGS in guinea pig slices. There, field potential amplitudes selectively increased when low concentrations of GABA were applied, while higher GABA concentrations decreased field potential amplitudes (Arakawa and Okada 1988). In the rat SC, this effect could later be related to $GABA_C$ receptors because the field potential amplitude enhancement by low GABA concentrations could be selectively blocked by $GABA_C$ receptor, but not by $GABA_A$ receptors antagonists. From these results it was proposed that $GABA_C$ receptors may be predominantly expressed by local GABAergic interneurons (Pasternack et al. 1999). Because GABAergic interneurons provide feedforward inhibition to SGS projection neurons, a selective activation of $GABA_C$ receptors by low levels of GABA inhibits GABAergic interneurons and thereby increases the response of projection neurons, as reflected in the field potential amplitude increases. Results obtained at the single cell level also showed $GABA_C$ receptor-specific actions of low GABA concentrations on SGS interneurons (Fig. 4) and thus supported a disinhibitory function of $GABA_C$ receptors in the rat SGS (Schmidt et al. 2001; Boller and Schmidt 2001).

Thus, as for the retina, $GABA_C$ receptors in SGS are in a position to modulate the visual information transfer through SGS projection neurons. However, the exact location of $GABA_C$ receptors in SGS interneurons remains

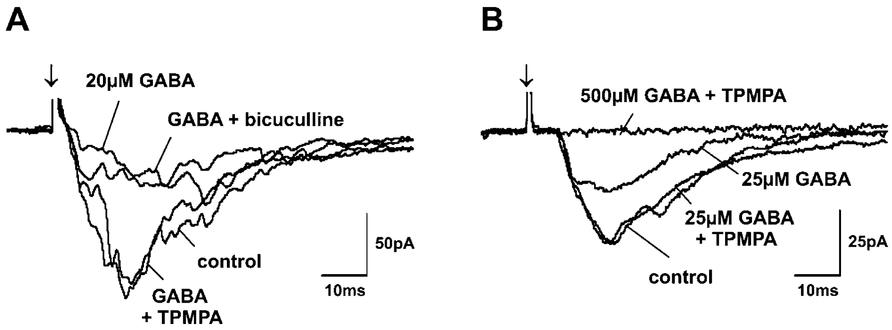

Fig. 4 Interaction of $GABA_A$ and $GABA_C$ receptor antagonists with GABA-induced effects on postsynaptic currents to afferent stimulations in rat SGS interneurons recorded in vitro. **A** EPSC amplitude reductions induced by low GABA concentrations are completely blocked by coapplication of 50 μM TPMPA. In contrast, coapplication of 20 μM bicuculline is unable to change the depressive effects of low GABA concentrations. **B** In contrast, EPSC reductions induced by high GABA concentrations are unaffected by TPMPA. *Arrows* indicate SO stimulus onset. Reproduced with permission from Schmidt et al. (2001)

to be elucidated. One possibility is that $GABA_C$ receptors are located presynaptically at or close to the interneuron terminals. GABA released by the interneurons would activate them and truncate GABA release. As in retinal bipolar cells, $GABA_C$ receptors in this case would modulate the temporal profile of the postsynaptic current, leading to a more transient postsynaptic response. Alternatively, $GABA_C$ receptors could be located postsynaptically at synapses through which, possibly from some unidentified external GABAergic SGS input, SGS interneurons activity is modulated. Because evidence exists for both a pre- and a postsynaptic localization (Boller and Schmidt 2003; Kirischuk et al. 2003) both expression patterns could coexist.

Expression of $GABA_C$ receptors by GABAergic SGS interneurons does not exclude the possibility that SGS projection neurons might also express this receptor type (Kirischuk et al. 2003). As probably all projection neurons possess $GABA_A$ receptors, a co-localization of $GABA_C$ together with $GABA_A$ receptors could increase the dynamic range of neurotransmission at GABAergic synapses. However, $GABA_C$ receptors could also be restricted to specific synapses, which upon GABA release would lead to postsynaptic inhibition with specific temporal properties. As in the case of SGS local GABAergic interneurons, the exact subcellular localization of $GABA_C$ receptors is needed to differentiate between the two possibilities.

$GABA_C$ receptors in SGS have been reported to be involved in mediating GABA-induced synaptic plasticity. Thus, long-term potentiation (LTP) of field potentials evoked by afferent stimulation seems to occur after bath application of GABA. This GABA-induced LTP can be blocked by addition of $GABA_C$, but not $GABA_A$ or $GABA_B$, receptor antagonists (Platt and Withington 1998;

White and Platt 2001). However, a possible functional role for GABA$_C$ receptors in LTP generation has not been followed further. Thus, whether or not GABA$_C$ receptors are involved in specific functions apart from regulating the temporal profile of SGS interneuron activity and SGS output remains to be analyzed.

4 Lateral Geniculate Nucleus

As already noted earlier, subcortical visual structures seem to be selectively rich in GABA$_C$ receptors. Thus, in addition to SC, there is also multiple evidence for an expression of GABA$_C$ receptors in the dLGN. Results from anatomical studies using either in situ hybridization (Wegelius et al. 1998)

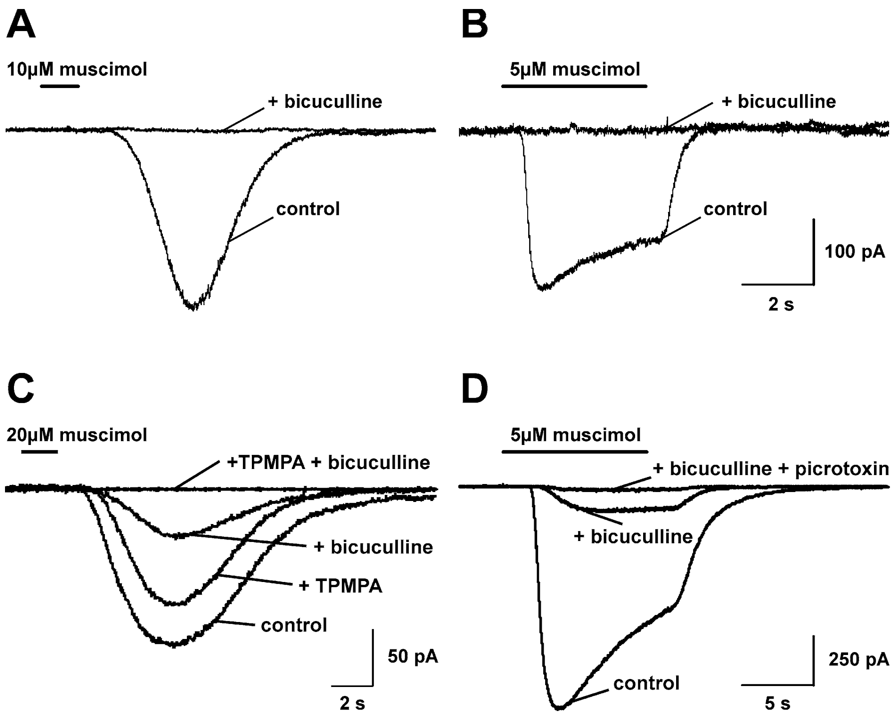

Fig. 5 Effects of GABA$_A$ and GABA$_C$ receptor antagonists on muscimol-induced currents in isolated rat dLGN cells in vitro. **A,B** Complete block of currents by bicuculline indicating the presence of only GABA$_A$ receptors. **C** Current reductions by both bicuculline and TPMPA indicating that both GABA$_A$ and GABA$_C$ receptors were present. **D** Currents insensitive to bicuculline can also be blocked by picrotoxin, an antagonist at both GABA$_A$ and GABA$_C$ receptors. *Bars* above current traces indicate muscimol application periods. *Scale bar* in **B** also applies to **A**. Reproduced with permission from Schlicker et al. (2003)

or immunocytochemistry (Schmidt 1997) indicate the presence of ρ subunits in dLGN, although the expression level seems lower than in SGS. This could, however, just reflect differences in the densities of $GABA_C$ receptor-expressing neurons in the two structures.

Physiological and pharmacological evidence for $GABA_C$ receptor expression by dLGN neurons has been obtained both in recordings from thalamic slices (Zhu and Lo 1999) and from acutely isolated single dLGN cells (Fig. 5). Results from both studies strongly suggest that $GABA_C$ receptors in dLGN most likely are specifically expressed by local GABAergic interneurons while thalamocortical projection cells seem void of this receptor type. Thus, GABA-induced currents that are resistant to the $GABA_A$ receptor antagonist bicuculline, but can be blocked by $GABA_C$ receptor antagonists, have been observed in isolated neurons that show dendritic morphologies of GABAergic interneurons but not in cells that show morphologies of thalamocortical neurons (Schlicker et al. 2004). Furthermore, bicuculline-resistant IPSCs evoked by afferent fiber stimulation also only appear in morphologically identified GABAergic interneurons and not in thalamocortical cells (Zhu and Lo 1999). As in the case of GABAergic interneurons in SGS, $GABA_C$ receptors in dLGN could be involved in the regulation of local inhibition and, through a reduction of feedforward inhibition to thalamocortical cells, modulate the dLGN output to the visual cortex. Unfortunately, as in the case of SGS, the subcellular localization of $GABA_C$ receptors in dLGN is also unclear so that more elaborated functional hypotheses for $GABA_C$ receptor function in dLGN are still missing.

5
Other Visual Structures

Reports on $GABA_C$ receptor expression in other subcortical visual nuclei as well as in the visual cortex are even more sparse. In the pretectum, $GABA_C$ receptor expression has been observed using immunohistochemistry (Schmidt 1997), and bicuculline-resistant currents have also been observed in pretectal cells in vitro (Boller and Schmidt 2003). However, most pretectal nuclei consist of multiple neuronal populations that serve very different visual and oculomotor functions. Because neurons from these different populations seem not to differ significantly in their dendritic morphologies they can only be identified by retrograde labeling from their specific projection targets. An identification of possible $GABA_C$ receptor functions in pretectal neurons therefore awaits more selective recording from identified neuronal populations.

$GABA_C$ receptors have also been identified in neurons in accessory optic nuclei, particularly in the medial terminal nucleus (MTN) of the accessory optic system, by immunohistochemistry (Schmidt 1997) and in situ

hybridization (Wegelius & Pasternack, unpublished). Because local inhibition is particularly important for information processing in the MTN, GABA$_C$ receptors could be involved in the fine-tuning of GABAergic neurotransmission and preliminary data from MTN neurons support this assumption (Schlicker and Schmidt 2005).

Finally, although GABA$_C$ receptor-specific ρ subunits have been reportedly expressed in the visual cortex (Enz et al. 1995; Wegelius et al. 1998), a physiological confirmation of this observation has not been published.

6 Hippocampus

Although GABA$_C$ receptors seem to be most strongly expressed in structures of the visual system, they also occur in non-visual brain centers at considerable density. Within the telencephalon, in situ hybridization and immunohistochemical data suggest that the strongest expression of GABA$_C$ receptors is found in the hippocampus (Enz et al. 1995; Wegelius et al. 1998; Ogurusu et al. 1999; Didelon et al. 2002; Rozzo et al. 2002; Alakuijala et al. 2005). In rat, the ρ subunit density changes during development, reaching peak expression in the CA1 and dentate gyrus subfields at the end of the first postnatal week. In adult rats, GABA$_C$ receptor expression seems to decline as compared to earlier in development, leaving interneurons throughout the entire hippocampus as the main cell type in which GABA$_C$ receptors can be found (Rozzo et al. 2002; Alakuijala et al. 2005).

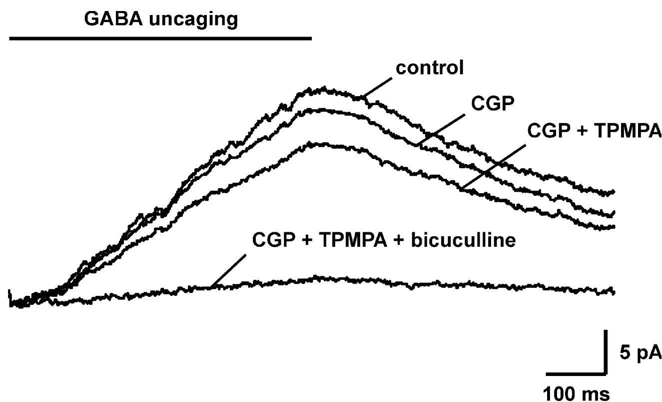

Fig. 6 Effects of GABA receptor blockers on GABA-induced currents in cerebellar Purkinje cells. Currents mediated by GABA receptors were elicited by UV light-induced uncaging of caged GABA. Application of the selective blockers CGP 52432 (for GABA$_B$ receptors), TPMPA (for GABA$_C$ receptors), and bicuculline (for GABA$_A$ receptors) revealed that all three receptor types are present in Purkinje cells. Reproduced with permission from Lemos and Schmidt, unpublished results

While the presence of $GABA_C$ receptor ρ subunits seems certain, their possible functional role, again, remains unclear. A first report provided evidence for $GABA_C$ receptor expression in the CA3 region of the rat hippocampus during the first and second postnatal week but not in the adult hippocampus (Strata and Cherubini 1994). Later studies indicated the presence of GABA receptors in the CA1 region that are characterized by a mixed $GABA_A$ and $GABA_C$ receptor pharmacology (Semyanov and Kullmann 2002; Hartmann et al. 2004). This is mainly based on the observation that currents induced by *cis*-aminocrotonic acid (CACA), a $GABA_C$ receptor agonist, were sensitive to bicuculline, the classical $GABA_A$ receptor antagonist. However, at the concentration used CACA might also activate $GABA_A$ receptors (Wall 2001) and, thus, the results might indicate that both $GABA_A$ and $GABA_C$ receptors are present at individual synapses in the hippocampal CA1 region.

7 Cerebellum

Because most of the cell types in the cerebellar cortex are GABAergic, it is not surprising that $GABA_C$ receptors have been found there. Most prominent, cerebellar Purkinje cells are heavily labeled for ρ subunit mRNA and protein (Enz et al. 1995; Boué-Grabot et al. 1998; Rozzo et al. 2002). Concomitantly, spontaneous activity observed in Purkinje cells in vitro is strongly reduced after $GABA_C$ receptor activation by the agonists GABA or muscimol at concentrations that do not co-activate $GABA_A$ receptors. Furthermore, IPSCs in Purkinje cells evoked by stimulation of afferents in the molecular layer can only partly be blocked by simultaneous application of the $GABA_A$ receptor antagonist bicuculline and the $GABA_B$ receptor antagonist CGP52432, but completely disappear if $GABA_C$ receptor antagonists are also added (Fig. 6) (Lemos and Schmidt, unpublished results). Thus, in the cerebellar cortex, where multiple GABAergic feedforward and feedback circuits exist, $GABA_C$ receptors seem perfectly situated to be involved in mediating synaptic neurotransmission of selective local GABAergic mechanisms to Purkinje cells. More detailed analyses are needed to uncover the local cerebellar circuits in which $GABA_C$ receptors participate.

8 Spinal Cord

Although the spinal cord has been the structure where the scientific history of $GABA_C$ receptors began, studies on $GABA_C$ receptor expression and function are astonishingly sparse. Expression of ρ subunit mRNA has been demonstrated in both motor neurons and interneurons of the rat spinal cord

(Enz et al. 1995; Rozzo et al. 2002). Consistently, in the neonatal spinal cord, blockade of $GABA_C$ receptors decreases the inhibitory action of exogenously applied GABA, indicating the presence of this receptor in interneurons. Furthermore, $GABA_C$ receptors slow down rhythmic bursting and thus appear to contribute to the excitation of spinal interneurons that support rhythmic bursting activity (Rozzo et al. 1999).

9
Conclusion

Although most extensively studied in the mammalian retina, $GABA_C$ receptors have been demonstrated to be present in a variety of central nervous structures. Most strikingly, in almost all nuclei of subcortical visual pathways, at least in those which receive direct retinal input, neurons can be found that express $GABA_C$ receptors. Because the number of functional studies on non-retinal $GABA_C$ receptors is still restricted, a generalized functional hypothesis cannot be proposed. From the data available to date, it seems possible that outside retina GABAergic cells, mostly local GABAergic interneurons are the predominant cell type to express $GABA_C$ receptors. In this case, $GABA_C$ receptors would be perfectly suited to control GABA release. To serve such a function, both a presynaptic or a postsynaptic localization of $GABA_C$ receptors would be conceivable.

In the case of a presynaptic localization, $GABA_C$ receptors could act as autoreceptors regulating the temporal profile of GABA release. Comparable to $GABA_C$ receptor function in the retina, activation of presynaptic $GABA_C$ receptors would terminate further GABA release and thereby make postsynaptic inhibition more transient. $GABA_C$ autoreceptors may be expected in particular in structures that are characterized by strong feedforward inhibitory circuitry, like SC and dLGN where excitatory afferent input synchronously activates projection cells and local GABAergic interneurons. Following a strong excitatory input, a $GABA_C$ receptor-mediated autoregulation of GABA release could prevent the build-up of strong inhibition and allow a faster functional restoration of postsynaptic excitability. However, presynaptic $GABA_C$ receptors could also be targets of specific GABAergic input systems that control local GABA release in a distinct functional context.

Postsynaptic $GABA_C$ receptors, on the other hand, could be useful to extend the operational range at GABAergic synapses. High-affinity $GABA_C$ receptors would mediate moderate inhibition at GABA levels too low to activate $GABA_A$ receptors. As more GABA is released, activation of $GABA_A$ receptors would lead to stronger inhibition. Due to differences in receptor activation threshold and dynamics, co-localization of $GABA_A$ and $GABA_C$ receptors can create nonlinearities, possibly task-related, at GABAergic synapses. Clearly,

an important goal for future functional studies on $GABA_C$ receptors outside the retina is to unravel its subcellular localization.

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Presynaptic Ionotropic GABA Receptors

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Abstract Following the classical work on presynaptic inhibition in the spinal cord, recent work has revealed an astonishing abundance and diversity of presynaptic ionotropic GABA receptors. While modern techniques allow for detailed studies at the cellular and molecular level in almost all regions of the CNS, our understanding of the function of such receptors is still far from complete. One major shortcoming is the lack of knowledge regarding chloride concentration inside axons or axon terminals. Therefore, the voltage change upon activation of presynaptic GABA receptors is difficult to predict. Moreover, even if the presynaptic potential transient was known, it turns out difficult to predict the effects on presynaptic function, which may be differentially influenced by various mechanisms, including activation or inactivation of voltage-gated ion channels and shunt effects. This review summarizes several key examples of presynaptic ionotropic GABA receptors and outlines the possible mechanisms that have to be kept in mind when unravelling this potentially important mechanism of synaptic signalling and plasticity.

1 Introduction

Most synapses of the central nervous system share some common basic mechanisms. “Standard” elements of synaptic signalling include small synaptic vesicles and their release machinery, postsynaptically clustered transmitter receptors, and transmitter (re)-uptake mechanisms. In addition, most synapses contain receptors for feedback signals, which usually limit vesicular release by G-protein coupled autoreceptors that are activated upon release of the respective transmitter. This negative feedback should not only be viewed as a homeostatic mechanism, protecting against pathologically strong release. It might also constitute a mechanism of synaptic plasticity, i.e. activity-dependent adaptive changes in synaptic efficacy. Presynaptic receptors meet this definition because, by definition, synaptic plasticity requires that past events exert an effect on subsequent pre- or postsynaptic function. Meanwhile it has become clear that G-protein coupled autoreceptors are just one example of multiple presynaptic receptors for neurotransmitters, or neuromodulators, which should be understood as a large repertoire of regulatory

mechanisms of different direction, efficacy and duration. Recent evidence shows that ligand-gated ion channels are expressed at axon terminals of various central neurons (see, e.g., MacDermott et al. 1999). Their functional effects are, though, far less well understood than those of G-protein coupled receptors. This review will focus on presynaptic actions of GABA which are mediated by GABA-gated ion channels.

The classical presynaptic autoreceptor for GABA (γ-aminobutyric acid) is called the $GABA_B$ receptor ($GABA_BR$). Activation of $GABA_BR$ results in a long-lasting K^+-mediated hyperpolarization and in reduced probability of transmitter release from presynaptic terminals, partly mediated by reduced calcium influx (Misgeld et al. 1995). The typical experimental paradigm to demonstrate the presynaptic activation of $GABA_BR$ is paired-pulse inhibition. Stimulation of axons from GABAergic neurons results in the release of GABA and activation of presynaptic $GABA_BR$. This will decrease the probability of release of synaptic vesicles and, hence, diminish the response to a second stimulus at intervals between some 10 ms to about 1 s (Davies and Collingridge 1990). Meanwhile, it has become clear that $GABA_BR$ occur at a large variety of GABAergic synapses. In addition, glutamatergic synapses can be hit by synaptically released GABA from neighbouring inhibitory terminals ("spillover"). By this mechanism, GABA can exert a negative feedback at these excitatory synapses, as has been shown in the rodent hippocampus (Isaacson et al. 1993) or the cerebellum (Dittman and Regehr 1997). Indeed, $GABA_BR$ are abundantly expressed at excitatory synapses as well as in extrasynaptic membrane regions (Kulik et al. 2003). Recently, the underlying proteins were identified as members of the GPCR superfamily (Kaupmann et al. 1997) and it was shown that $GABA_BR$ must form dimers in order to achieve their activated state (White et al. 1998; Kuner et al. 1999).

Like many other neurotransmitters, GABA can activate two distinct types of receptors – metabotropic $GABA_BR$ and ligand-gated ion channels, grouped into $GABA_AR$ and $GABA_CR$. In the following, we use the term "iGABAR" (ionotropic GABA receptors) for both groups of receptors. This review will focus on the function of iGABAR at presynaptic terminals. We will include examples of axo-axonic synapses at, or close to, axon endings where the "presynaptic" iGABAR are "postsynaptic" as viewed from the modulatory terminal. We will also take into account specific examples of iGABAR that are expressed along axonal fibres. Such receptors most likely modulate the efficacy of action potential propagation along these axons. All these receptors do not contribute to the usual mechanisms of synaptic signal integration, which require a spatial and temporal interplay between excitatory and inhibitory inputs in order to generate a "decision" about the generation of an action potential. We will not include iGABAR at axon initial segments that do form a layer-specific target for axons of GABAergic neurons (e.g. chandelier cells; Soriano et al. 1990). Such synapses should be discussed in the context of cortical or hippocampal network functions where interneurons serve specific and

differential roles in the temporal and spatial organization of neuronal activity (see Whittington and Traub 2003; Klausberger et al. 2003).

2 Possible Modes of Action

Before going into specific examples within defined networks, some basic features of GABAergic signalling shall be briefly summarized:

The subunits that constitute $GABA_AR$ and $GABA_CR$ are members of the superfamily of ligand-gated ion channels, sharing homology with nicotinic acetylcholine receptors and glycine receptors. Molecular cloning of the underlying cDNA has revealed a large family of subunits which can form thousands of different pentamers, comprising functionally distinct molecular isoforms of GABA receptors. While $GABA_AR$ are heteromultimers, $GABA_C$ receptors are homomeric assemblies of ρ subunits which exhibit different pharmacological properties and an unusually high affinity for GABA. The latter property is also typical for those molecular subtypes of $GABA_AR$ that participate in tonic inhibition (Semyanov et al. 2004; Farrant and Nusser 2005).

Once opened, iGABAR are permeable for Cl^- and, to a lesser extent, for HCO^{3-}. Therefore, GABA causes increased local chloride permeability and the induced currents reverse close to the chloride equilibrium potential. A local increase in chloride permeability can have several different effects at neuronal membranes. In each case, the increased membrane conductance will shorten the membrane's length constant, thus attenuating the electrotonic propagation of other transient (synaptic or action) potentials. This effect is called "shunting" and can exert powerful inhibition at different subcellular compartments, including axons (see below). In most cases, activation of iGABAR does change the membrane potential. The direction and extent of this potential change depends on the amplitude of the GABA-induced conductance (a function of the number and opening time of the iGABAR), the difference between membrane and chloride equilibrium potential (mainly a function of intracellular chloride content) and passive membrane properties (resistance and capacity in the complex spatial structure of the neuron). In many instances, GABA may hyperpolarize the membrane, at others it can have a depolarizing effect or can even leave the membrane potential unchanged (if $E_{Cl^-} = E_M$). Depolarizing GABAergic potentials, in turn, can have inhibitory or excitatory effects on neurons, depending on their relation to action potential threshold and on the temporal and spatial relationship of excitatory postsynaptic potentials (Williams and Stuart 2003; Chavas and Marty 2003). In summary, it is very difficult to predict the effects of GABA at any given synapse. Possibly, the most important variable parameter governing the local response is E_{Cl^-}. Unfortunately, the chloride equilibrium potential of presynaptic terminals is, in most cases, unknown.

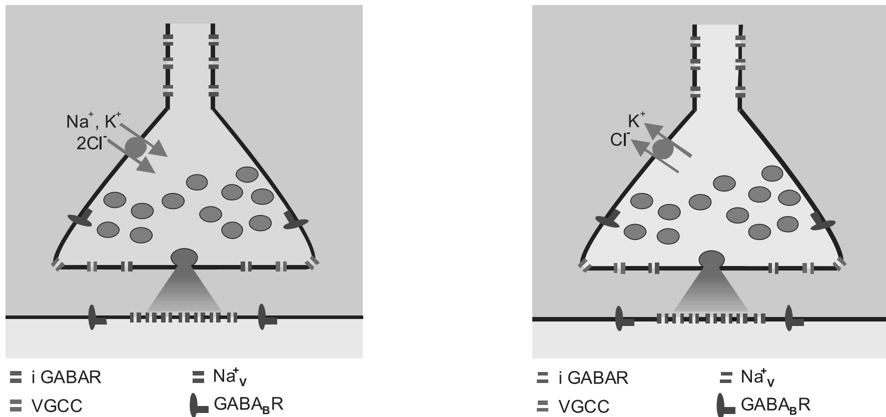

Fig. 1 Schematic representation of an inhibitory presynaptic terminal. GABA is enriched in presynaptic vesicles, which are released upon activation of presynaptic calcium channels (VGCC). The transmitter activates ionotropic GABA receptors at the postsynaptic site and (as reviewed here) at the presynaptic terminal. It also activates $GABA_BR$ on both sides of the synapse. The effect of presynaptic iGABAR on membrane potential depends on the chloride gradient across the presynaptic membrane. This is determined by NKCC (yielding high intraterminal Cl^-; *left panel*) or by KCC (yielding low intraterminal Cl^-; *right panel*)

Effects of iGABAR at axons and axon terminals are also influenced by another mechanism, i.e. the effect of membrane potential on the biophysical behaviour of voltage-gated ion channels (VGIC), which are highly abundant in these structures. Hyperpolarizing potential shifts can impair activation of VGIC and can, thereby, depress the action potential-induced release of transmitter. They can, however, also support recovery from inactivation and thereby enhance excitability of the presynaptic membrane. Depolarizing currents can, in principle, increase basal calcium influx, a powerful mechanism enhancing the probability of release. They can, however, also drive VGIC into inactivation, thereby diminishing their availability for action potential-induced release. In addition to these effects, there is some evidence for a direct effect of the presynaptic membrane potential on the release machinery (Hochner et al. 1989; Parnas et al. 2000). In summary, there is no easy way to predict the action of GABA at any give axon terminal from basal rules of GABAergic transmission. In accordance with this statement, presynaptic iGABAR have very different effects in different neuronal circuits and, in some places, even at different developmental stages of the same synapse.

Before we continue to summarize the effects of presynaptic iGABAR at specific synapses, it should be mentioned that there might be different, non-conventional effects of GABA on presynaptic terminals. Recently, it has been shown that vesicle endocytosis is dependent upon the action of $GABA_AR$ at goldfish retinal bipolar cell terminals (Hull and von Gersdorff 2004). More-

over, it should be emphasized that chloride and bicarbonate are both involved in volume regulation. Activation of iGABAR may alter (local) osmotic pressure and cause cells or subcellular compartments to swell or shrink (Chavas et al. 2004). Such changes in osmotic pressure can massively alter the probability of vesicular transmitter release. Although potentially important, these actions of GABA at presynaptic terminals have not yet been systematically analysed. We will now focus on several well-studied examples of presynaptic iGABAR.

3 Presynaptic iGABAR in Defined Systems

3.1 Presynaptic Inhibition in the Spinal Cord

The presence and modulating function of presynaptic $GABA_AR$ was first established in the mammalian spinal cord, following the initial observation by Frank and Fuortes (1957) that excitatory postsynaptic potentials in spinal motoneurons could be depressed by a "remote" mechanism, i.e. without visibly altering the postsynaptic cell. This effect can by elicited by stimulating afferent fibres from muscle spindles and tendon organs and causes an extracellularly detectable depolarization of the dorsal root fibres, called primary afferent depolarization (PAD). It was very much due to the pioneering work by Eccles and coworkers (e.g. Eccles et al. 1962, 1963, 1964) that the underlying mechanisms could be clarified, although a surprisingly large number of questions still remain open. The major effect of PAD is a chloride-mediated depolarization of the primary afferent fibre or terminal, reducing action potential-evoked release of glutamate onto spinal motoneurons. The structural basis for this effect has been firmly established by electron microscopy, showing GABA-containing axo-axonic synapses on group I and group II muscle afferents (originally described by Gray 1962) as well as on primary cutaneous afferents in the dorsal horn and in the gracilis and cuneate nuclei (reviewed in Rudomin and Schmidt 1999).

Pharmacological evidence shows that PAD is due to the activation of $GABA_A$ receptors. It should be noted, however, that in addition activity-dependent potassium increases may play a role in certain situations (Kremer and Lev-Tov 1998) and that $GABA_BR$ are also present on primary spinal afferent fibres, though they do not seem to contribute much to PAD (Stuart and Redman 1992). The chloride-mediated potentials at primary spinal afferents are generally depolarizing and may reach an amplitude of > 20 mV. The high intraterminal chloride concentration needed for this depolarization is, likely, due to the presence of the sodium–potassium–chloride co-transporter NKCC in these fibres. This secondarily active transporter increases intraterminal

chloride concentration and moves E_{Cl^-} to values positive from equilibrium. Besides setting the chloride equilibrium, NKCC may also serve a homeostatic role: the efflux of chloride (and, possibly, subsequently of potassium) may result in a reduced osmotic load and shrinkage. This effect may be counteracted by ion influx via NKCC. It should be added that most studies on postsynaptic mechanisms of PAD (i.e. chloride equilibrium potential and chloride transport) have been performed at the somata of sensory neurons from the dorsal horn, assuming similar conditions at the central afferent fibre. While this seems to be a pragmatic and fruitful approach (Alvarez-Leefmans 1988), it should be kept in mind that the different compartment geometry and selective molecular sorting mechanisms may well cause functional differences between both sites (see below for the calyx of Held).

Surprisingly, the precise mechanisms underlying EPSP suppression by PAD have not yet been established and several factors may act together. The main effects of the increased chloride conductance seem to be shunting of the afferent action potential and inactivation of voltage-activated Na^+- or Ca^{2+}-channels. If positioned distally from branching points, axo-axonic synapses may even selectively inhibit axonal branches and thereby redirect the flow of information (Eguibar et al. 1994, 1997). Similar mechanisms appear to be present in the posterior pituitary, where GABA suppresses the release of peptides (Zhang and Jackson 1993). Here, shunting does not suffice to explain the inhibitory effect of GABA. Rather, membrane depolarization is necessary indicating that at these terminals inactivation of voltage-gated ion channels is more important than increasing membrane conductance.

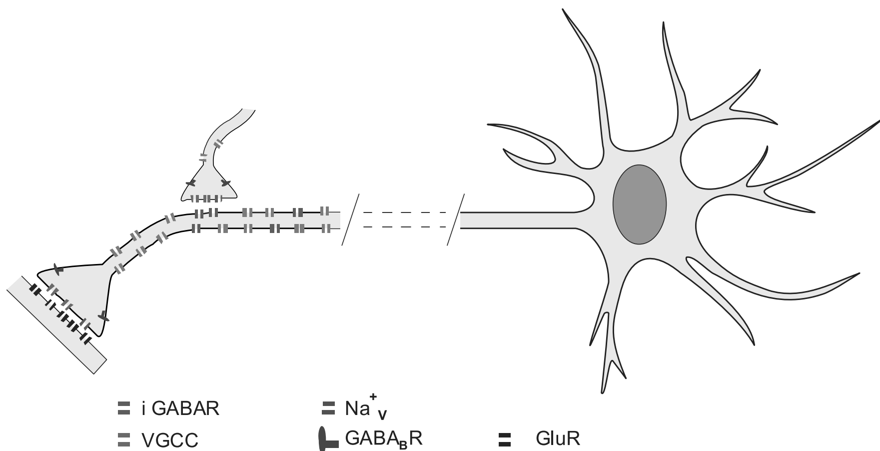

Fig. 2 Presynaptic inhibition. An afferent terminal forms a glutamatergic synapse on a dendrite (e.g. an α-motoneuron). A GABAergic synapse terminating on the afferent axon may shunt the membrane or inactivate voltage-gated sodium channels ($Na^+{}_V$). Depending on the position of the axo-axonal synapse, voltage-gated calcium channels may also be affected. Similar rules may apply to axo-axonal synapses on inhibitory neurons

PAD seems to be important in the regulation of sensory information flow and motor behaviour. At sensory afferents from muscles and tendon organs, PAD can modulate the efficacy of polysynaptic spinal reflexes. Primary afferent depolarization is regulated itself in a cyclic fashion during locomotion, indicating a specific function in this rhythmic network behaviour (Duenas and Rudomin 1988). In the sensory system, PAD may contribute to enhanced signal-to-noise levels of incoming information, a mechanism similar to lateral inhibition in other sensory systems (reviewed in Rudomin and Schmidt 1999). An open question of key importance is the role of presynaptic GABAergic synapses in the regulation of pain sensations, which are mediated by afferent information from nociceptive C- and (possibly) Aδ fibres (reviewed in Rudomin and Schmidt 1999). Interestingly, the depolarization of afferent fibres or terminals can trigger action potentials that propagate antidromically into the dorsal root ganglion cells and into the periphery. This effect has been termed "dorsal root reflex" (Barron and Mathews 1938; Toennies et al. 1938; see also Nicoll and Alger 1979). If present in nociceptive fibres, such antidromic action potentials may be of great importance for the release of vasoactive and pro-inflammatory substances from nociceptive fibre endings, i.e. neurogenic inflammation, peripheral hyperalgesia and allodynia (Willis 1999).

Presynaptic terminals on inhibitory (GABAergic or glycinergic) axon endings in the spinal cord have been less extensively studied. However, besides anatomical evidence for their existence (Todd et al. 1996; Maxwell et al. 1997), a recent physiological study on dissociated neurons of the sacral dorsal commissural nucleus of rats has revealed interesting results (Jang et al. 2002). The preparation maintains functional GABAergic and glycinergic terminals that release transmitter spontaneously as well as upon focal electrical stimulation. Spontaneous glycinergic IPSCs were increased by ~fivefold in frequency upon application of the $GABA_AR$ agonist muscimol whereas electrically evoked IPSCs were actually suppressed. As for the synapses undergoing classical PAD, intraterminal chloride concentration was elevated by NKCC. The authors showed that depolarizing GABAergic potentials can activate voltage-dependent calcium channels (both directly and mediated via prior activation of sodium channels), which enhance spontaneous release. Action potentials, however, seem to be diminished or blocked by the inactivation of (some) Na^+ and/or Ca^{2+} channels. These results demonstrate the high complexity of presynaptic modulatory mechanisms. They also show that spinal interneurons that are involved in nociception possess presynaptic ionotropic GABA receptors, opening potential routes for new strategies in pain research.

It should be noted that PAD and GABA-mediated presynaptic inhibition are not restricted to the mammalian nervous system. Many pioneering studies have been performed in the crayfish, where presynaptic inhibition is present at the neuromuscular junction and appears to act mainly

via shunting of action potentials (Cattaert et al. 2001). A recent study by Parnas and coworkers (Parnas et al. 2000) has revealed an interesting new effect: presynaptic inhibition at the crayfish neuromuscular junction can be induced by activation of the inhibitory fibre after activation of the excitatory fibre. This modulation, allowing for delays of up to 2 ms, cannot be explained by shunting or inactivation of voltage-gated ion channels and may, therefore, constitute a new pathway of presynaptic GABAergic signalling.

3.2 Ionotropic GABA Receptors at Excitatory Fibre Terminals in the Hippocampus

Excitatory synaptic transmission in the mammalian hippocampus belongs to the most extensively studied topics in cellular neurosciences, mostly due to their pronounced plasticity, which may serve functions in declarative memory formation. Therefore, the modulation of these synapses by neurotransmitters or neuromodulators is of great importance. With respect to presynaptic ionotropic receptors, recent studies have focussed on presynaptic ionotropic glutamate receptors, especially AMPA receptors at mossy fibre terminals (Schmitz et al. 2000). However, there is also evidence for presynaptically or axonally expressed iGABAR in the hippocampus. Stasheff and coworkers (1993a,b) have provided evidence for the generation of antidromic spikes in CA3 pyramidal cells upon activation of $GABA_AR$ at Schaffer collaterals. This finding hints towards an axonal depolarization by GABA-induced chloride currents, similar to the situation in spinal cord afferents. More recently, the mossy fibre connection between dentate granule cells and CA3 pyramidal cells has been studied in detail (Ruiz et al. 2003). Again, these fibres are glutamatergic, although they might release GABA under certain conditions (Gutierrez 2005). Ruiz and coworkers found that the $GABA_AR$ agonist muscimol and GABA strongly inhibit antidromically recorded sodium currents in dentate granule cells. This effect can also be induced by synaptically released GABA and it seems to be present in the absence of additional electrical stimulation, indicating tonic baseline activation of such axonal $GABA_AR$. At mossy fibres, E_{Cl^-} may be between the (very negative) resting membrane potential of granule cells and the action potential threshold. Therefore, depolarizing inhibition is likely due to shunting of the membrane. Increasing internal $[Cl^-]$ does, indeed, convert the effect of $GABA_AR$-induced suppression of action potential propagation to an increased excitability (Ruiz et al. 2003). Interestingly, an ultrastructural analysis of the distribution of $\alpha 2$-subunits of $GABA_AR$ revealed that the iGABAR occur at mossy fibre terminals as well as along the axon cylinder. While the presence and efficacy of these axonal $GABA_AR$ has been convincingly shown, their relevance to physiological functions of the hippocampal circuitry remains largely unclear (Kullmann et al. 2005). Possibly, this question can not be solved without tak-

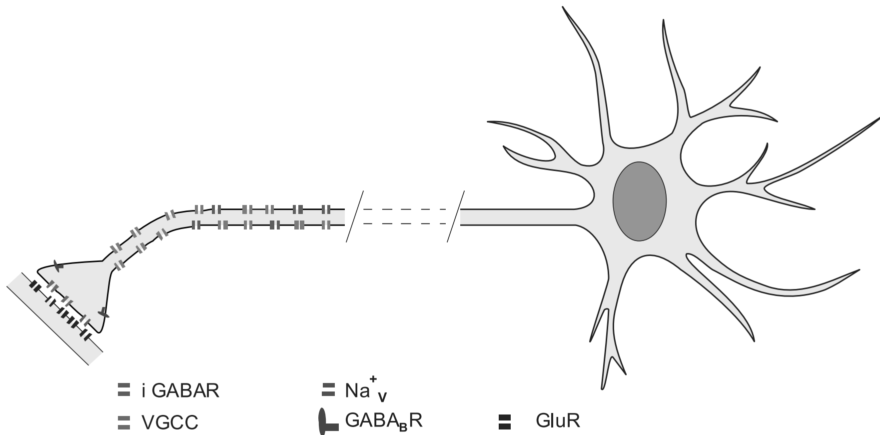

Fig. 3 Presynaptic axonal GABA receptors at mossy fibres. $GABA_AR$ are present at the axonal membrane as well as at axon terminals. Propagation of the action potential is suppressed upon activation of these receptors

ing into account the activity-dependent GABAergic phenotype of mossy fibres (Gutierrez 2005).

3.3 Presynaptic $GABA_AR$ at Inhibitory Terminals in the Hippocampus

Evidence for presynaptic ionotropic GABA receptors at inhibitory terminals came first from paired recordings of synaptically connected rat hippocampal neurons in culture (Vautrin et al. 1994). The authors observed coincident currents in pre- and postsynaptic neurons that were sensitive to $GABA_AR$ antagonists and reversed at chloride equilibrium. Thus, hippocampal inhibitory interneurons appear to express a mechanism for rapid autoinhibition of GABA release. It remained open, however, whether such receptors are also present and operational in GABAergic neurons in situ, i.e. in normally differentiated hippocampal circuits.

In a recent series of experiments we have studied the effect of $GABA_AR$ activation on the release of GABA from inhibitory synapses on CA3 pyramidal cells. We first found that application of the $GABA_AR$ agonist muscimol (1 μM) reduces both electrically evoked IPSCs as well as the frequency of TTX-resistant miniature IPSCs (Axmacher and Draguhn 2004). A methodological difficulty of such studies is, however, that GABAergic agonists or antagonists also alter the postsynaptic response to GABA release, which is used as a “reporter” or “readout” of presynaptic function. In our study, the effect of muscimol on synaptic release did last longer than washout of the substance, outlasting postsynaptic effects (measured as increased membrane noise and reduced input resistance) by several minutes. At this time, post-

synaptic GABA sensitivity had fully recovered while the effects on mIPSC frequency were still present. Amplitudes of miniature IPSCs were not affected, again pointing towards the presynaptic origin of the effect. Therefore, we analysed the effect of muscimol on presynaptic vesicular dynamics without interference with postsynaptic GABA responses (Axmacher et al. 2004a), using optical methods (Stanton et al. 2001). We stained rat hippocampal slices with the styryl dye FM 1–43 and observed spontaneous destaining in the presence of TTX, using two-photon confocal microscopy. Destaining of bright puncta (putative synaptic terminals) is a measure for the fusion of vesicles and accompanying transmitter release (Betz et al. 1992; Ryan et al. 1993). Application of muscimol did indeed slow down the time course of destaining in those putative synaptic terminals that were located in the proximal somatodendritic region of the CA3 pyramidal layer. Distal (possibly glutmatergic) synapses with significantly slower fluorescence decay did not show this effect. These results are compatible with an inhibiting action of GABA on GABA release at inhibitory terminals in CA3, mediated via iGABAR. Again, the functional consequences of this action are not known. It is very feasible that negative feedback of synaptically released GABA leads to a fast decrease in efficacy of inhibition upon repetitive presyaptic activation. Fast repetitive spiking patterns are indeed observed in hippocampal interneurons (e.g. Csicsvari et al. 1999) and presynaptic $GABA_A R$ may dynamically down-regulate inhibitory efficacy under these conditions, reducing temporal summation of the relatively long-lasting postsynaptic inhibitory potentials. This feedback mechanism would operate in the millisecond time-range and precede the slower, $GABA_B R$-mediated negative feedback. Furthermore, changes in the presynaptic GABA concentration leading to a variable amount of GABA released from individual synaptic vesicles may be balanced by a feedback of GABA on further release via presynaptic $GABA_A R$ (Engel et al. 2001; Overstreet and Westbrook 2001; Axmacher and Draguhn 2004). Other functions, like detection of exceedingly high levels of ambient GABA or activation of presynaptic iGABAR by GABA spillover from other synapses, may also be relevant and await further studies.

While direct structural evidence for $GABA_A R$ at the presynaptic membrane of inhibitory synapses in the hippocampus is still missing, specific sorting mechanisms have been clearly demonstrated indicating, for example, that $\alpha 2$-subunit-containing GABA receptors are preferentially expressed at axo-axonic synapses (Nusser et al. 1996). While these synapses form part of the highly laminated synaptic innervation pattern of hippocampal principal cells, the findings by Ruiz et al. (2003) show that $\alpha 2$-subunits can also be found at more remote axonal locations.

3.4 Giant Synapses (Held)

The calyx of Held is the excitatory synapse between bushy cells in the cochlear nucleus and inhibitory neurons in the medial nucleus of the trapezoid body. This nucleus is a fast and reliable relay between the first central nucleus of the auditory system and the superior olivary complex. It may be due to the extremely fast and precise timing of auditory inputs that the calyx of Held has evolved into the largest synapse in the mammalian CNS, with one afferent terminal ensheating a large portion of the postsynaptic cell body (Satzler et al. 2002). This peculiar morphological property has turned the calyx into one of the most important preparations for the study of synaptic transmission, not least because its size allows for paired simultaneous recordings of a presynaptic terminal and its postsynaptic target.

In two elegant studies, Turecek and Trussel (2001, 2002) have shown that transmission at the rat calyx of Held is regulated by presynaptic anion channels. While the authors first reported on presynaptic glycine receptors, the subsequent study showed that there is a developmental switch from the expression of presynaptic $GABA_A$ receptors to glycine receptors. The transition occurs around postnatal day 11, just before the onset of hearing. At the same time, the postsynaptic cells express their full glycinergic phenotype, opening the possibility of glycine leakage or spillover as an activation mechanism for the presynaptic glycine receptors. Both ligand-gated anion channels mediate presynaptic depolarizations and lead to an enhanced release of glutamate upon presynaptic stimulation. It is likely that this action is due to the activation of voltage-dependent calcium channels at the synaptic terminal. Thus, the chloride equilibrium potential at this presynaptic terminal does not appear to follow the general developmental shift to more negative potentials, in contrast to E_{Cl^-} in the somatodendritic region of the presynaptic bushy cells.

In addition to the peculiar developmental time course, several features of presynaptic ionotropic modulation of this synaptic model system are remarkable: First, single channel recordings from presynaptically derived outside–out patches revealed four different conductance states of $GABA_AR$, indicating receptor heterogeneity at this presynaptic location. This makes the mechanisms of receptor sorting between different compartments even more puzzling. Second, somatic GABA and glycine receptors at the same cells are not developmentally regulated in parallel with the presynaptic receptors. Again, the signal leading to the switch in the axonal sorting between GABA and glycine receptors remains unknown. Third, besides positive regulation of glutamate release by $GABA_AR$ (early) or GlyR (later), there is a constant negative regulation by presynaptic $GABA_BR$. Thus, GABA has opposite effects via a fast, ionotropic and a slow, metabotropic pathway, respectively.

The function of these presynaptic receptors remains largely unknown. It has been suggested that the early expression of $GABA_AR$ serves some trophic

role in synapse formation or maturation, while glycine receptors might be directly involved in the functional regulation of the mature calyx synapse (Turecek and Trussel 2002).

3.5 Cerebellum

The cerebellar cortex contains various types of inhibitory interneurons that contribute to the precisely regulated temporal signal processing in this network. Using patch clamp recordings from stellate and basket cells, Pouzat and Marty (1999) identified a transient current that could be elicited by brief depolarizations. Such short somatic depolarizations caused "action currents" in the axon, i.e. fast and large inward currents. At more remote localizations these currents induce depolarizations similar to action potentials, due to the fading efficacy of voltage clamp in such electrotonically remote structures. Following such stimuli, Pouzat and Marty observed slowly rising transient currents of smaller amplitude, reminiscent of postsynaptic currents. They were sensitive to bicuculline and dependent on intact presynaptic calcium signalling. Several lines of evidence suggest that these currents are not autaptic, i.e. not due to the activation of synapses terminating on the very same neuron from which they were elicited. Computer simulations support the notion that the recorded signals are indeed distorted waveforms of synaptic currents that are elicited at multiple axon terminals and altered by the cable properties and active conductances of the axon cylinder. While the functional significance of this finding for cerebellar signal processing is still not clear, it is likely that the iGABAR regulate efficacy of transmission upon repetitive activation of the interneurons. It remains unclear, however, whether they boost or depress synaptic efficacy – an open question which again depends on the presently unknown chloride reversal potential at these axonal boutons. It is also important to note that the presynaptic autoreceptor current decreases with age, indicating some role in the developing circuitry of the cerebellum.

3.6 Retina

Retinal bipolar cells connect cones with ganglion cells and rods with amacrine (type II) cells, where they form large glutamatergic ribbon-synapses. These synapses are reciprocal, i.e. they serve simultaneously as presynaptic and postsynaptic elements. An early study in the carp retina (Kondo and Toyoda 1983) revealed that application of GABA in the inner plexiform layer (where synaptic interaction with amacrine cells takes place) exerts a hyperpolarizing response in bipolar cells. Later studies in isolated goldfish rod-activated on-center bipolar cells confirmed that the terminals are indeed highly sensitive to GABA and mediate a hyperpolarization via

$GABA_AR$ (Tachibana and Kaneko 1987). More recent experiments in the mammalian (ferret) retina revealed that iGABAR undergo some sorting in bipolar cells with an increased contribution of $GABA_C$ receptors at the terminals, as compared to the dendrites (Shields et al. 2000). Similar mixed responses of $GABA_AR$ and $GABA_CR$ have been found in rat bipolar cells. $GABA_C$ receptors are homooligomeric ion channels composed of ρ-subunits, which (despite their defining lack of bicuculline-sensitivity) have slow kinetics and high agonist affinity (for review see Bormann and Feigenspan 1995). These properties might be of importance for the time course and efficacy of amacrine-to-bipolar-cell signalling.

Similarly to bipolar cells, the inhibitory endings of horizontal cells on photoreceptor cells mediate inhibition at the site of the presynaptic terminal. Recordings from turtle retina have shown that this type of presynaptic inhibition is mainly present in red- and green-sensitive cones, but only marginally in blue-sensitive cones or rods (Tachibana and Kaneko 1984). In the salamander retina, GABA seems to exert a positive feedback on GABA release from horizontal cells (Kamermans and Werblin 1992), in accordance with the rather positive chloride equilibrium potential (– 20 mV).

Recent evidence indicates that presynaptic iGABAR at goldfish bipolar terminals may not only affect membrane potential, conductance and glutamate release but also vesicular endocytosis (Hull and von Gerstorff 2004). These data point towards new, non-conventional effects of GABA at axon endings and indicate that the full presynaptic vesicle cycle should be taken into account.

4 Summary and Outlook

The examples given above illustrate different presynaptic effects of GABA, which are all mediated by ionotropic GABA receptors. It becomes obvious that the structural correlates of these signals are highly diverse. There are classical synaptic boutons at or close to axon terminals, iGABAR along axonal cylinders and ionotropic GABA receptors at synaptic boutons. The latter are in the right position to sense GABA released from the very same bouton, conferring a "spillback effect". Likewise, the molecular diversity is impressive, including developmental regulation of the expressed iGABAR subtype and very precise subcellular sorting, which causes differences between somatodendritic and axonal receptors. Presynaptic iGABAR have been found in many different circuits and this review is far from complete (see, e.g., the recent discovery of presynaptic iGABAR in the suprachiasmatic nucleus; Belenky et al. 2003). A proper analysis will have to take into account the enormous diversity of these circuits at the network, cellular and molecular level. We have to assess the effects of presynaptic GABAergic signalling within each

of these different situations separately. The most important issue to clarify, however, is the chloride gradient at presynaptic membranes. Only with this information (or the corresponding E_{Cl^-}) will it be possible to predict the effects of GABA on synaptic efficacy. New methods are emerging in the field of chloride regulation and chloride measurements and may help to tackle this issue in the future (Kuner and Augustine 2000; Ebihara et al. 1995).

While most studies have focussed on the electrophysiological effects of GABA, it should be noted that other parameters may be of similar importance, especially osmotic tension. Recently, it has been shown that chloride influx increases intracellular calcium concentration by a mechanism different from depolarization (Chavas et al. 2004). Chloride-induced swelling of dendrites appears to induce volume regulatory responses, which, in turn, result in elevated $[Ca^{2+}]$. Similar mechanisms at axon terminals, if present, might be very efficient in regulating transmitter release and should be addressed in appropriate experiments. Other non-canonic effects of GABA have been mentioned above and include effects on vesicle endocytosis (Hull and von Gersdorff 2004) or presently unknown interferences with vesicle release that are not mediated by changes in intraterminal calcium concentration (discussed in Axmacher et al. 2004b).

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The Role of $GABA_B$ Receptors in the Regulation of Excitatory Neurotransmission

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Abstract $GABA_B$ receptors are the metabotrophic receptors for GABA. They are members of the G-protein coupled superfamily of receptors but are highly unusual as they are made up of a dimer of 7-transmembrane spanning subunits. The receptors are widely distributed throughout the central nervous system where they act post-synaptically to cause a long-lasting hyperpolarisation through the activation of a potassium conductance. They are also present pre-synaptically where they act as auto and heteroreceptors to inhibit neurotransmitter release. $GABA_B$ receptors play a complex role in the regulation of excitatory transmission and their activation can have both inhibitory and dis-inhibitory effects. This has profound physiological and behavioural consequences including modification of LTP and memory, regulation of seizure activity and nociception.

1 Introduction

In addition to the ionotropic $GABA_{A/C}$ receptors GABA also mediates its effects through a slower acting metabotropic receptor known as the $GABA_B$ receptor. This receptor was first identified in 1980 by Bowery et al. (1980) who demonstrated bicuculline insensitive responses to GABA in a number of isolated tissue preparations and went on to show that this receptor had the characteristics of a G protein coupled receptor (GPCR) and that baclofen (4-amino 3-(4-cholorphenyl)-butanoic acid) (Lioresal) was a specific agonist. It was not until 30 years later that the molecular identify of the $GABA_B$ receptor was determined (Marshall et al. 1999) and found to be an unusual heterodimeric structure made up of two heptahelical spanning membrane proteins. In the intervening years much work was done to characterise the function of $GABA_B$ receptors in the central nervous system. This was greatly facilitated by the availability of a wide range of potent and selective agonists and antagonists synthesised by pharmaceutical companies in particular at Novartis (Bowery et al. 2002; Bittiger 1993; Froestl and Michel 1997). $GABA_B$ receptors are widely distributed in the central and peripheral nervous system where they modulate neurotransmission at both pre- and post-synaptic sites of action (Bowery 2002). The important role for $GABA_B$ receptors in the regulation of neurotransmission is suggested by the behavioural effects ob-

served with $GABA_B$ agonists and antagonists in normal and disease states. Baclofen is used clinically in the treatment of spasticity and some types of pain, although when given orally its efficacy is limited by side effects including sedation, headaches, hypotension and gastrointestinal disturbances. In animal models $GABA_B$ agonists also have effects on seizures, anxiety and drug dependence. $GABA_B$ antagonists have effects on cognition (Mondadori 1993) and depression (Nakagawa 1999) as well as an anticonvulsant activity on absence seizures (Marescaux 1992; Hosford 1992).

2 The Molecular Structure of $GABA_B$ Receptors

The $GABA_B$ receptor has a unique structure among the superfamily of GPCRs. The receptor consists of two subunits, $GABA_{B1}$ and $GABA_{B2}$, each consisting of a seven-transmembrane spanning unit with a large extracellular domain (ECD) containing an amino-acid binding domain. The subunits are a member of the Family C sub-division of GPCRs which also contains the metabotropic glutamate receptors and the Ca^{2+} sensing receptor (Pin 2003). The $GABA_{B2}$ subunit interacts with the $GABA_{B1}$ subunit both through the transmembrane domains and through a coiled-coil interaction in the C-terminal tails (Pagano 2001; Margeta-Mitrovic 2000). This association causes an unmasking of an endoplasmic reticulum retention sequence, RXRR, in the $GABA_{B1}$ subunit C-terminal tail by the $GABA_{B2}$ subunit and enables trafficking of correctly assembled functional heterodimeric receptor complexes to the cell surface. Thus, the $GABA_{B2}$ subunit plays a critical role in regulating the levels of functional receptors at the cell surface. This is not the only function of the $GABA_{B2}$ subunit. Mutations of the $GABA_{B1}$ subunit which allow surface expression of the receptor still do not result in functional receptors (Pagano 2001; Margeta-Mitrovic 2000; Calver et al. 2001). Whilst the $GABA_{B1}$ subunit contains the ligand binding site for GABA and all known competitive ligands of the receptor, the $GABA_{B2}$ subunit is primarily responsible for recognition and activation of G-proteins. However, for a fully effective coupling to effectors there is a requirement for a co-operative interaction between the transmembrane domains of both subunits (Galvez 2001; Lui 2004). Furthermore, a co-operative association also occurs at the level of the ECD. The ligand binding site of the receptor comprises of a bi-lobed structure which closes upon agonist binding. It has been suggested that this change in conformation and an association with the ECD of $GABA_{B2}$ results in the activation of the receptor.

2.1 Coupling to Second Messenger Effectors

Activation of the $GABA_B$ receptor heterodimer leads to coupling to G proteins of the $G_i\alpha/G_o\alpha$ subtype. Through this coupling $GABA_B$ receptor agonists cause an inhibition of basal and forskolin stimulated adenylate cyclase (Knight and Bowery 1996). In some cells $GABA_B$ receptor activation can also cause a potentiation of cAMP elevation by $G_s\alpha$ coupled GPCRs (Enna 2000).

Within the CNS the major effector pathways of $GABA_B$ receptor activation involve coupling to ion channels through $G_o\alpha$. Pre-synaptically $GABA_B$ receptors cause auto or heteroreceptor inhibition of neurotransmitter release through modulation of pre-synaptic Ca^{2+} channels. Inhibition of calcium currents by agonists such as baclofen is blocked by agatoxin IVB or in some cases by ω-conotoxin suggesting the involvement of either P/Q or N-type calcium channels (Huston et al. 1995; Chen and van den Pol 1998; Lambert and Wilson 1996; Barral et al. 2000). This modulation of calcium channels is primarily mediated by an action of $G_{\beta\gamma}$ binding to calcium channel subunits (Ikeda and Dunlap 1999). Recently, $GABA_B$ receptors have been shown to modulate N-type Ca^{2+} channels via an indirect mechanism involving Src and MAP kinases (Richman et al. 2004). Pre-synaptic $GABA_B$ receptors also regulate a background K^+ conductance through activation of TASK channels (Fearon et al. 2003).

At post-synaptic sites $GABA_B$ receptors are responsible for a long-lasting hyperpolarisation due to an increase in K^+ conductance. Several types of K^+ channels have been shown to be modulated by $GABA_B$ receptors. In transfected mammalian cells $GABA_B$ receptors can modulate heteromeric Kir3.1/3.2 and Kir3.1/3.4 channels (Kaupmann et al. 1998). Similar results have been reported in vivo, for example in mouse cerebellar granule neurones $GABA_B$ receptors activate Kir3.1/3.2 channels (Slesinger et al. 1997).

2.2 Receptor Sub-Types

The majority of neurotransmitters mediate their effects through activation of multiple ionotropic and/or metabotropic (G-protein coupled) receptors. For example, glutamate activates three classes of ionotropic receptors and eight different G-protein coupled receptors (Pin 2002). In the case of GABA it was assumed that in addition to activating the ionotropic $GABA_A$ receptors there would be multiple subtypes of $GABA_B$ receptors. Prior to the cloning of the receptor there were several lines of evidence which pointed to the possible existence of receptor subtypes, however no truly subtype selective compounds were ever identified. These early studies included extensive neurotransmitter release experiments which suggested that the pre-synaptic auto-receptors on GABAergic terminals differed in pharmacology with respect to a num-

ber of antagonists compared to the $GABA_B$ hetero-receptors on the terminals of neurones releasing 5-HT, glutamate or neuropeptides (Bonanno 1993; Cunningham 1996). In addition electrophysiological experiments pointed to differences in agonist potency between pre- and post-synaptic receptors (Yamada et al. 1999).

Molecular studies have identified the existence of a single $GABA_B$ receptor made up of the $GABA_{B1}$ and $GABA_{B2}$ subunits. Heterogeneity does exist in that these subunits have a number of splice variants. The most widely expressed of these includes the two N-terminal splice variants of $GABA_{B1}$. $GABA_{B1(a)}$ has 147 amino acids at the extreme N-terminal which are replaced by 18 different residues in $GABA_{B1(b)}$. These two proteins show a different pattern of expression and can both form heterodimers with $GABA_{B2}$ (Kaupmann 1997). The extreme N-terminus of the receptor is not involved in ligand binding and therefore is not likely to affect the pharmacology. So far no differences in pharmacology, between the splice variants, have been found with any $GABA_B$ compounds.

3
Distribution of $GABA_B$ Receptors in the Central Nervous System

The distribution of $GABA_B$ receptors within the central nervous system of human and rodents has been characterised using radioligand binding, in situ hybridisation and immunohistochemistry. Data from these various studies give broad agreement on the localisation of $GABA_B$ receptors. Areas with high levels of receptors include the thalamic nuclei, cerebellum, cortex, hippocampus, basal ganglia and the dorsal horn of the spinal cord (Bowery 1987; Billington 2000). Although both associated with $GABA_{B2}$, the splice variants of $GABA_{B1}$ are differentially expressed both across different brain areas and during development (Fritschy 1999). $GABA_{B1a}$ appears preferentially at post-synaptic densities, whereas $GABA_{B1b}$ may be mainly attributed to presynaptic or extrasynaptic sites (Benke 1999). For example, cell bodies of the deep cerebellar nuclei stain for $GABA_{B1a}$, whilst the terminals surrounding the cell bodies strongly label with antibodies to $GABA_{B1b}$ (Poorkhalkali 2000). However, in the thalamo-cortical pathway $GABA_{B1b}$ appears to be the presynaptic isoform whilst the $GABA_{B1a}$ subunit is present at post-synaptic sites on cell bodies (Princivalle 2001). Thus, the two splice variants do not subserve a common function but rather their function will differ depending on the particular brain region in which they are expressed. Within the spinal cord $GABA_B$ receptors mRNA is found at high levels in the dorsal root ganglia (DRG) and over the neuron cell bodies, consistent with $GABA_B$ receptor protein being formed in the sensory neurons and transported to the primary afferent terminals. $GABA_{B2}$ mRNA is also evenly distributed across the spinal cord laminae and DRG.

In the majority of cases $GABA_{B1}$ is co-localised with $GABA_{B2}$, however there are exceptions. For example during development there is a notably selective $GABA_{B2}$ staining of axonal tracts, such as the corticothalamic projection, and a prominent $GABA_{B1}$ expression in astrocytes (Fritschy 2004). In addition a number of studies have shown a mis-match between the two subunits with higher levels of $GABA_{B1}$ in the caudate and hypothalamus compared to $GABA_{B2}$ (Clark et al. 2000).

An interesting feature of $GABA_B$ receptor localisation has been revealed by electron microscopy. The subcellular localisation of receptors is un-related to GABAergic inputs (Fritschy et al. 1999). This means that to activate these receptors a high enough concentration of GABA must be generated to allow overflow from the synaptic cleft. This can occur as a result of repetitive stimulation or through simultaneous release of GABA from multiple sites such as during synchronous rhythmic activity (Scanziani 2000).

4
Regulation of Excitatory Transmission in Vitro

As we have seen $GABA_B$ receptors are widespread throughout the CNS being expressed both pre- and post-synaptically on excitatory and inhibitory neurones. The effects of $GABA_B$ agonists on excitatory neurotransmission are complex and varied depending on the exact site or sites of action and can be both inhibitory and dis-inhibitory. Iontophoretic application of baclofen to brain slices will usually result in a depression of neuronal activity. Post-synaptically baclofen activates a potassium conductance with a delayed onset of 20 ms which lasts for several hundred ms (Nicoll 2004). This results in a large, long lasting hyperpolarisation which can be blocked by both pertussis toxin and GTPβS confirming the involvement of G proteins (Andrade et al. 1986). These effects are similar to both adenosine and 5-HT which also act through receptors coupling to pertussis sensitive G proteins and inwardly rectifying potassium channels (Andrade et al. 1986). In some neuronal systems a strong or repetitive stimulus is required to evoke a $GABA_B$ mediated inhibitory post-synaptic potential (IPSP), this includes the hippocampus and thalamus (Scanziani et al. 2000; Kim et al. 1997). In these cases $GABA_B$ receptors may be recruited during rhythmic or synchronous activity.

$GABA_B$ receptors can also regulate excitatory transmission via alternative post-synaptic mechanisms. In developing hypothalamic and cortical neurones baclofen causes a potent depression of calcium transients in response to exogenous glutamate. This effect is also sensitive to pertussis toxin and may involve activation of second messenger pathways leading to changes in phosphorylation of proteins involved in calcium responses (Obrietan and Van den Pol 1999). Recently, a novel activity of $GABA_B$ receptors has been identified which does not involve GABA. In cerebellar Purkinje cells $GABA_B$ receptors

are able to increase the sensitivity of metabotropic gluatamate receptor 1 (mGluR1) to glutamate possibly by a direct interaction of $GABA_B$ receptors with mGluR1. This effect requires extracellular calcium but not the presence of GABA (Tabata et al. 2004).

Pre-synaptic $GABA_B$ receptors are present on both inhibitory and excitatory terminals. These receptors cause an inhibition in neurotransmitter release primarily through an inhibition of calcium conductance (Doze et al. 1995; Isaacson 1998). Under conditions where the post-synaptic actions of baclofen are blocked it is possible to isolate the effect of presynaptic receptors. Application of baclofen under such conditions can lead to decreases in both IPSPs and EPSPs. For example in recordings from deep cerebellar nuclei, baclofen reduces the amplitude and frequency of spontaneous IPSPs evoked by stimulation of Purkinje cells (Mouginot and Gahwiler 1996). In whole cell patch clamp recordings from rat supraoptic magnocellular neurones baclofen reduced the frequency, but not amplitude of both EPSPs and IPSPs from glutamatergic and GABAergic neurones respectively (Kabashima et al. 1997). In adult rat spinal cord slices baclofen inhibited the C-fibre EPSC and to a lesser extent the A-delta EPSC through an inhibition of glutamate release (Ataka et al. 2000).

As might be expected from its multiple sites of action $GABA_B$ receptors have a complex effect on long-term potentiation causing both facilitation and suppression depending on the method of LTP induction. Blockade of $GABA_B$ receptors by antagonists facilitates LTP in response to long tetanic trains of high-frequency stimulation (Olpe and Karlsson 1990; Olpe et al. 1993). Under these conditions antagonists are blocking post-synaptic $GABA_B$ receptors which contribute to a GABAergic post-synaptic hyperpolarisation which prevents NMDA receptor activation. In contrast, brief high frequency burst theta pattern stimulation enables activation of $GABA_B$ autoreceptors, which inhibit GABA release and permits sufficient NMDA receptor activation for the induction of LTP. Under theta pattern stimulation $GABA_B$ antagonists cause a suppression of LTP (Davies et al. 1991; Mott and Lewis 1991; Staubli et al. 1999).

5
Behavioural Consequences of Regulating Excitatory Transmission

Modulation of LTP in the hippocampus by $GABA_B$ receptor agonists and antagonists provides a molecular mechanism to behavioural effects seen in a variety of cognitive tests. However, as might be expected from the complex dose dependent profile seen on LTP different effects of agonists and in particular antagonists have been reported on learning and memory paradigms. In general $GABA_B$ antagonists enhance learning and memory consistent with their post-synaptic block of $GABA_B$ receptors and enhancement of NMDA re-

ceptor activation. For example, CGP36742 has cognitive enhancing effects in mice, rats and rhesus monkeys in tests of active and passive avoidance, in an eight-arm radial maze, the Morris water maze and in a social recognition test (Froestl et al. 2004; Mondadori et al. 1996). These effects are observed over a wide dose range. Others have reported a narrower dose range as well as bell-shaped dose response curves. Staubli et al. (1999) found CGP36742 to facilitate spatial memory at doses between 25–200 mg/kg whilst higher doses were in-effective. In contrast, Brucato found that the more potent antagonist CGP35348 suppressed learning in the Morris water maze at 100 mg/kg and also inhibited LTP at these doses presumably via a pre-synaptic autoreceptor mechanism.

Another well-characterised behavioural action of $GABA_B$ antagonists is their ability to inhibit absence seizures. The exact mechanisms involved in the formation of spike and wave discharges (SWDs) within cortical and thalamic neurones, the main electroencephalographic (EEG) feature of absence seizures is not well understood. $GABA_B$ antagonists reduce spike and discharges as well as absence seizures in genetic absence epilepsy rats (GAERS), lethargic mice or in rats treated with γ-hydroxybutyrolactone (GHB) (Hosford 1999; Marescaux 1992; Snead 1992). CGP35348 also suppresses absence seizures in a mutant mouse which has a deficiency in succinic semialdehyde dehydrogenase, resulting in elevated levels of GHB and absence seizures (Cortez et al. 2004). In vivo recordings from thalamo-cortical neurones of GAERS indicate rhythmic sequences of synaptic potentials consisting of an EPSP closely followed by several IPSPs superimposed on a tonic hyperpolarisation that might represent a long-lasting $GABA_B$ IPSP occurring during spike wave discharge (Charpier 1999).

Although $GABA_B$ antagonists inhibit absence seizures some also have proconvulsant activity. For example CGP 56999A (Badran 1997) produced myoclonic seizure-like episodes in mice and tonic-clonic convulsions in rats. Furthermore, both $GABA_{B1}$ and $GABA_{B2}$ deficient mice have a pronounced epileptic phenotype, with ${GABA_{B2}}^{-/-}$ having several episodes of spontaneous clonic seizures per day, whilst ${GABA_{B1}}^{-/-}$ mice had absence-type and spontaneous tonic-clonic seizures (Gassmann 2004, Schuler et al. 2001). This proconvulsive activity observed in the ${GABA_{B1}}^{-/-}$ mice is likely due to a general loss of post-synaptic inhibition leading to an increase in N-methyl-d-aspartate (NMDA) receptor activity as well as a possible increase in developmental depolarising IPSPs due to the removal of $GABA_B$ autoreceptor function (Brown et al. 2003).

Inhibition of the release of glutamate and other transmitters in the dorsal horn of the spinal cord (Malcangio and Bowery 1996) and brain areas such as the medial thalamus (Ipponi et al. 1999), by $GABA_B$ receptor activation, results in anti-nociception. In animal models baclofen is anti-nociceptive in models of acute thermal and mechanical pain (Hammond and Washington 1993), inflammatory pain (Dirig and Yaksh 1995) and neuropathic pain (Pa-

tel 2001). In the hot-plate, tail flick and paw pressure tests of acute pain $GABA_{B1}^{-/-}$ and $GABA_{B2}^{-/-}$ mice both show hyperalgesia, probably due to hyperexcitability in spinal nociceptive neurones (Gassmann et al. 2004; Schuler et al. 2001; Vacher and Bettler 2003).

The use of baclofen in clinical pain however, is of limited use primarily due to CNS-mediated side effects and the development of tolerance. Intrathecal administration of baclofen is used effectively to treat pain associated with spasticity (Plassat et al. 2004) and is also given orally in severe cases of neuropathic pain (Fromm 1984).

Activation or inhibition of $GABA_B$ receptors also leads to many other physiological and behavioural effects in addition to those on memory, seizures and pain which are described here. These effects include muscle relaxation which is utilised in the treatment of spasticity by baclofen (e.g. Penn et al. 1989), inhibition of craving associated with drugs of abuse including cocaine (Ling et al. 1998), alcohol (Addolorato et al. 2000) and nicotine (Corrigall et al. 2000) as well as anti-depressant effects (Nakagawa et al. 1999). The exact mechanisms of action underlying these effects are complex and not well understood but most likely involves pre- and post-synaptic regulation of both excitatory and inhibitory neurotransmission.

$GABA_B$ receptors play an important role in regulating excitatory neurotransmission within all areas of the central nervous system. Although our understanding of these unusual G-protein coupled receptors has greatly increased since the cloning of the receptors to date baclofen remains the only approved drug acting through these receptors. The lack of receptor subtypes which could separate pre- and post-synaptic activities allowing separation of therapeutic benefit from side effects has impeded drug development. However, alternative approaches, such as the development of allosteric ligands (Cryan et al. 2004) offers the promise that new drugs may be developed in the future which target $GABA_B$ receptors (Vacher et al. 2003).

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GABAergic Control of CA3-driven Network Events in the Developing Hippocampus

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Abstract Endogenous activity is a characteristic feature of developing neuronal networks. In the neonatal rat hippocampus, spontaneously occurring network events known as "Giant Depolarizing Potentials" (GDPs) are seen *in vitro* at a stage when GABAergic transmission is depolarizing. GDPs are triggered by the CA3 region and they are seen as brief recurrent events in field-potential recordings, paralleled by depolarization and spiking of pyramidal neurons. In the light of current data, GDPs are triggered by the glutamatergic pyramidal neurons which act as conditional pacemakers, while the depolarizing action of GABA plays a permissive role for the generation of these events in *in vitro* preparations. From an *in vivo* perspective, GDPs appear to be an immature form of hippocampal sharp waves.

Abbreviations

AE	Na^+-independent anion exchanger that mediates an exchange of Cl^- for HCO_3^-
CCC	Cation–chloride cotransporter
$[Cl^-]_i$	Intracellular chloride concentration
CNS	Central nervous system
E_{Cl}	Cl^- Equilibrium potential
E_{GABA}	Reversal potential of $GABA_A$ receptor-mediated responses
fp	Field potential
GABA-PSC	$GABA_A$ receptor-mediated postsynaptic current (used in the case of immature neurons with depolarizing $GABA_A$ receptor-mediated responses)
GDP	Giant depolarizing potential
IPSP	Inhibitory postsynaptic potential
$[K^+]_o$	Extracellular K^+ concentration
KCC2	Neuron-specific K-Cl cotransporter isoform 2
NDAE	Na^+-dependent anion exchanger that mediates an exchange of Cl^- for HCO_3^-
NKCC1	Na-K-2Cl cotransporter isoform 1
P	Postnatal day
SPW	Sharp positive wave
TTX	Tetrodotoxin

1 Introduction

1.1 Background

In the immature central nervous system at early stages of development where sensory systems are not yet functional, all activity must have an endogenous origin. The first systematic studies of this kind of spontaneous activity date back to the work on chick embryos by William Preyer (1885), who showed that motor activity is in operation several days before reflex circuits are formed. The pioneers in this field of research also noticed that a salient characteristic of the early spontaneous motility is rhythmicity, which was attributable to the activity of distributed neuronal pacemakers within the developing spinal cord (Hamburger 1963). During the last decades it has become clear that early endogenous activity is not a specific property of developing motor systems (O'Donovan 1999). Extensive studies carried out on the hippocampus (Ben-Ari 2001; Ben-Ari et al. 1989), cortex (Garaschuk et al. 2000; Vanhatalo et al. 2005; Vanhatalo et al. 2002; Yuste et al. 1992), brainstem (Kandler 2004; Gummer and Mark 1994; Ho and Waite 1999) and retina (Maffei and Galli-Resta 1990; Meister et al. 1991) have shown that self-organized population are a common feature of immature neuronal networks in the central nervous system (CNS). However, it should be emphasized that endogenous activity is also a salient feature of the mature CNS, as already inferred by the pioneers in the field of ethology on the basis of behavioral observations (Hinde 1970). Historically, this conclusion was a major scientific and philosophical victory of the Darwinian (ethological) tradition over the Watson-Skinner-type behaviorism in the study of animal and human behavior.

Given the universal presence of rhythmic activity in developing neuronal networks, it is not surprising that a vast number of studies have been carried out to find out the potential roles of the early activity in shaping the structural and functional properties of neuronal connections. There is a fair level of consensus that the immensely complex connectivity in the CNS cannot be based on a blueprint that is fully governed by a genetic program. Here, one should immediately ask what (if anything) a "pure" genetic program would imply at the molecular, cellular, and systems level. The way genes are read in any given neuron is influenced by the cell's own activity (Borodinsky et al. 2004; Owens and Kriegstein 2002). Since the developing neuron is a part of a more complicated and continuously evolving system, a corollary is that a neuron's genes and other subcellular signaling devices are both a part and a target of the overall systems-level activity (Fig. 1). On the other hand, recent work on motor neuron identity and connectivity has revealed a surprisingly high degree of "genetic determination" that is achieved by networks of transcription factors endowed by self-organizing features (Dasen et al. 2005).

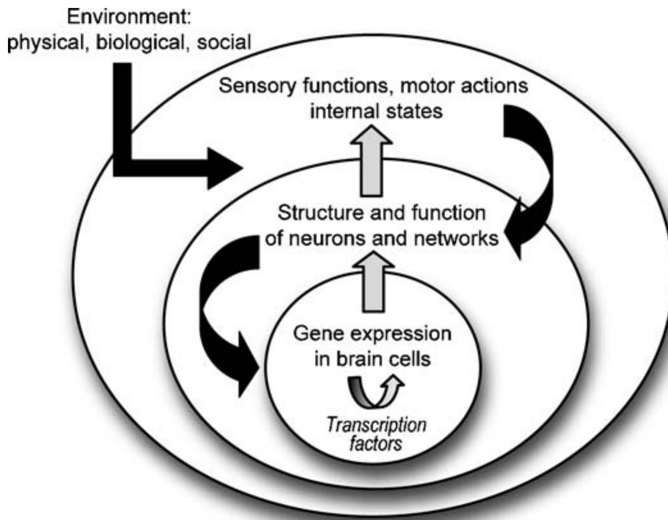

Fig. 1 Brain development is influenced by feed-forward and feedback loops that span all hierarchical levels of organization, from genes to neurons and to neuronal networks. In this scheme, the meaning of the word "environment" includes factors ranging from intrauterine conditions at early stages of development to social interactions of a mature individual. For further details, see text

In the case of the chick embryo, Preyer's work showed that by the time of hatching the elaboration of the early motor programs enables the chick not only to emerge from the egg but also to have a repertoire of behaviors (such as walking and pecking) that are essential for survival when the individual is for the first time exposed to the external environment. An unresolved problem was, and still is, to what degree the spontaneous early activity is merely a reflection of the emerging anatomical connectivity; and to what degree it affects the ontogeny of motor networks and patterns. The absence of solid information on the role of early activity in the generation of neuronal connectivity is also true with respect to the early hippocampal network events that are the focus of this review. Nevertheless, the influence of activity on the generation and maintenance of the hippocampal neuronal connectivity cannot be examined without detailed knowledge of the mechanisms underlying the endogenous events.

1.2
Aims and Scope of the Review

The depolarizing action mediated by $GABA_A$ receptors is thought to play an important role in shaping spontaneous activity patterns within specific time windows during the development of various kinds of neuronal networks, in-

cluding those in the spinal cord (Milner and Landmesser 1999; Nishimaru et al. 1996), hippocampus (Ben-Ari et al. 1989), neocortex (Fukuda et al. 1998; Owens et al. 1996; Sernagor et al. 2003; Yuste and Katz 1991), hypothalamus (Gao and van den Pol 2001), brainstem (Kandler and Gillespie 2005) and retina (Fischer et al. 1998). However, the exact mechanisms whereby depolarizing GABAergic transmission affects network events have not been fully identified. Moreover, these mechanisms are likely to differ in various structures and at distinct maturational stages in any given network.

In this article, our focus is on the role of depolarizing $GABA_A$ receptor-mediated actions in network events in the immature hippocampus. These events were originally termed "giant depolarizing potentials" (GDPs) in work based on intracellular recordings from CA3 pyramidal cells in rat hippocampal slices (Ben-Ari et al. 1989). In most of the pertinent literature, GDPs are considered GABAergic hippocampal events that are expressed only during a restricted time window of development. More specifically, their occurrence is thought to correspond to the period when postsynaptic $GABA_A$ receptor-mediated actions undergo a developmental maturation from depolarizing to hyperpolarizing (Khazipov et al. 2004). However, it has been known for several decades that *in vivo* the adult hippocampus generates endogenous network events known as sharp positive waves (SPWs) (Buzsaki et al. 1983; Jouvet et al. 1959), which reflect synchronous discharges of CA3 pyramidal neurons (Buzsaki 1986; Suzuki and Smith 1987). Interestingly, recent evidence shows that SPWs are already seen in the early postnatal period in rats and, furthermore, GDPs have been proposed to be the *in vitro* counterpart of early SPWs *in vivo* (Leinekugel et al. 2002). However, at the mechanistic level, the relationship between GDPs (thought to be driven by GABAergic transmission) and SPWs (driven by glutamatergic pyramidal neurons) has remained enigmatic. Hence, we will concentrate on the developmental aspects of GABAergic control over the bursting activity of CA3 pyramidal neurons.

In the existing literature, the term "GDP" is ambiguous since it refers to both the network event and the associated intracellular depolarization seen in individual neurons. Below, GDPs are, unless stated otherwise, network events seen *in vitro*, whereas "SPW" refers to network events seen in *in vivo* recordings (Buzsaki et al. 1983).

2 Development of the Ionic Mechanisms Underlying $GABA_A$ Receptor-mediated Responses

In a seminal paper published almost 30 years ago, Obata et al. (1978) showed that GABA has an excitatory action in co-cultures of muscle and spinal neurons taken from 6 to 8-day-old chick embryos, while an inhibitory effect was seen when culturing was started on day 10. The excitatory effect of GABA

(and glycine) was unequivocally shown to take place in intact neurons, since the experiments were based on recordings on muscle cells where the GABA-induced end-plate potentials were found to be TTX-sensitive, indicative of motoneuronal spiking. In addition, direct intracellular recordings from the neurons showed that there was a developmental change from depolarizing to hyperpolarizing $GABA_A$ receptor-mediated action. At present, it is evident that this kind of "ontogenetic switch" or rather *ontogenetic shift* from depolarizing to more negative $GABA_A$ receptor-mediated responses is a universal feature of central neurons (Payne et al. 2003).

Depolarizing GABAergic responses have been shown to activate voltage-gated Ca^{2+} channels which leads to intracellular Ca^{2+} transients and to the activation of various kinds of intracellular signaling cascades in immature neurons (Fukuda et al. 1998; Khazipov et al. 1997; Leinekugel et al. 1997; Leinekugel et al. 1995; Marty et al. 1996; Yuste and Katz 1991). Such mechanisms are central to the well-known trophic actions of GABA that influence neuronal DNA synthesis (Haydar et al. 2000; LoTurco et al. 1995), migration (Owens and Kriegstein 2002), morphological maturation of individual neurons (Marty et al. 1996; Wolff et al. 1978) and synaptogenesis (Marty et al. 2000). Before the functional maturation of synapses, GABA exerts its effects via non-synaptic (tonic) $GABA_A$-receptor activation (Owens and Kriegstein 2002). An important role in the mediation of the trophic effects of GABA has been attributed to brain-derived neurotrophic factor (Marty et al. 1996), which is released in an activity-dependent manner. In addition, GABAergic depolarization may induce plastic changes via removal of the Mg^{2+} block of NMDA receptors (Leinekugel et al. 1997).

$GABA_A$ receptors are permeable to two physiologically relevant anions, Cl^- and HCO_3^- (Fig. 2A), with a bicarbonate/chloride permeability ratio of about 0.2–0.4 (Kaila 1994). The intraneuronal pH is maintained at a more alkaline level than what would be predicted on the basis of a passive distribution of H^+ and HCO_3^- ions. Therefore the equilibrium potential for HCO_3^- is much more positive (typically about – 10 to – 15 mV) than the resting membrane potential, and bicarbonate mediates an inward current across $GABA_A$ receptors (Kaila and Voipio 1987). During a large channel-mediated net efflux of HCO_3^-, the intracellular bicarbonate is quickly replenished by the activity of intracellular carbonic anhydrase (Kaila et al. 1993; Pasternack et al. 1993). In neonatal pyramidal neurons, CA activity is absent until around postnatal day 10 (P10), and thereafter a steep increase in the expression of the isoform CAVII takes place, which acts as a second developmental switch (Ruusuvuori et al. 2004) at the ion-regulatory level to modulate postsynaptic GABAergic responses (Rivera et al. 2005). In some mature neurons, such as neocortical pyramids in slice preparations, the magnitude of the postsynaptic HCO_3^- current can exceed the Cl^- current, which leads to depolarizing $GABA_A$ receptor-mediated GABA-PSPs evoked by single stimuli in spite of a Cl^- equilibrium potential (E_{Cl}) that is more negative than the resting mem-

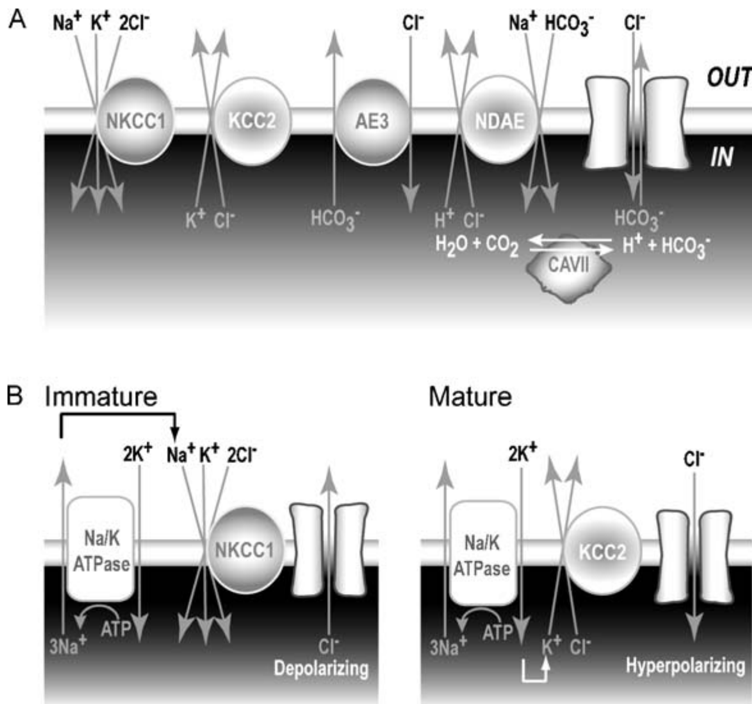

Fig. 2 Anion transport mechanisms control E_{GABA}. **A** The Na-K-2Cl cotransporter isoform 1, NKCC1, mediates electrically neutral Cl^- uptake fuelled by Na^+, and the K-Cl cotransporter isoform KCC2 mediates Cl^- extrusion fuelled by K^+. In addition, the Na^+-dependent and Na^+-independent anion exchangers (NDAE and AE3, respectively) have an influence on neuronal Cl^- homeostasis and provide a link between neuronal HCO_3^-/pH and Cl^- regulation. In several types of neurons, the carbonic anhydrase isoform VII (CAVII) catalyzes the formation of HCO_3^- from CO_2. The different molecules depicted here show distinct temporal patterns of expression, thereby controlling the developmental changes in E_{GABA}. **B** In immature pyramidal neurons, depolarizing $\mathrm{GABA_A}$ receptor-mediated responses are largely attributable to neuronal Cl^- accumulation via NKCC1. During maturation, the developmental expression of KCC2 produces a decrease in intraneuronal Cl^- thereby providing the basis for classical hyperpolarizing IPSPs

brane potential (Kaila et al. 1993). However, the much more positive reversal potential of $\mathrm{GABA_A}$ receptor-mediated responses (E_{GABA}) in immature pyramidal neurons is caused by a high intracellular Cl^- concentration. Under these conditions, simple considerations based on the Goldman–Hodgkin–Katz equation show that the contribution of bicarbonate to E_{GABA} is not significant (Kaila 1994; Kaila et al. 1993). Hence, in this review, we will focus on Cl^- when discussing the early postnatal maturation of the ionic basis of GABAergic inhibition, which involves a progressive shift in the capacity of neuronal Cl^- extrusion leading to a negative shift in E_{Cl} (Luhmann and Prince 1991; Zhang et al. 1991).

Cl^- homeostasis in neurons is controlled by cation–chloride cotransporters, CCCs (Fig. 2A). The CCCs are secondary active transporters that do not directly consume ATP, but derive the energy for Cl^- translocation from the Na^+ and K^+ gradients generated by the Na-K-ATPase (Payne et al. 2003). Two members of the CCC family are of particular significance in the present context: the Na-K-2Cl cotransporter NKCC1, which mediates Cl^- uptake (Fukuda et al. 1998; Ge et al. 2006; Li et al. 2002; Rohrbough and Spitzer 1996; Sipilä et al. 2006; Sun and Murali 1999; Yamada et al. 2004); and the neuron-specific K-Cl extruder KCC2, which is responsible for the generation of a hyperpolarizing E_{Cl} (Rivera et al. 1999). In addition to the CCCs, E_{Cl} is also influenced by the Na^+-dependent and Na^+-independent anion exchangers (NDAE and AE, respectively) that mediate an exchange of Cl^- for HCO_3^- (Payne et al. 2003).

While the Cl^- uptake mechanisms may vary among different kinds of neurons, there is much evidence in favor of the view that accumulation of Cl^- in immature pyramidal neurons is mediated by NKCC1 (Fig. 2B) (Marty et al. 2002; Sipilä et al. 2006; Yamada et al. 2004). In agreement with this, the depolarizing GABAergic responses in immature pyramidal neurons can be blocked by the specific NKCC1 antagonist, bumetanide (Isenring et al. 1998; Payne et al. 2003; Sipilä et al. 2006). During maturation, a progressive increase in the expression of KCC2 takes place, and a wealth of data indicate that the developmental expression of KCC2 is responsible for the gradual emergence of a negative E_{Cl} in pyramidal neurons (Fig. 2B) (Balakrishnan et al. 2003; Hubner et al. 2001; Rivera et al. 1999; Zhu et al. 2005). Here, one should recall that the central role of K-Cl cotransport in the generation of classical, hyperpolarizing IPSPs was demonstrated already in the 1980's (Deisz and Lux 1982; Misgeld et al. 1986; Thompson and Gahwiler 1989). At the whole-animal level, a full KCC2 "knock-out" is lethal, and mice with such a gene disruption die at birth due to malfunction of respiration (Hubner et al. 2001). In contrast, while $GABA_A$ receptor-mediated depolarizations are absent in NKCC1 knockout mice (Sung et al. 2000), no drastic phenotype has been observed in the central nervous system.

Co-expression of KCC2 and NKCC1 has been demonstrated in several kinds of mature neurons (Martina et al. 2001; Marty et al. 2002; Payne et al. 2003; Vardi et al. 2000). In fact, it is not unusual for a given cell to have parallel transport mechanisms, where the principal ionic substrate is transported in opposite directions. While this looks like a mere waste of energy, it permits a very precise control of the "set-point" of the particular intracellular ion under various physiological conditions (Roos and Boron 1981). In addition, a discrete subcellular distribution of NKCC1 and KCC2 may produce intraneuronal Cl^- gradients that shape GABAergic responses in a cell-region dependent manner (Duebel et al. 2006). Intriguingly, KCC2 is highly expressed in dendritic spines (Gulyas et al. 2001), which raises the question whether KCC2 has functions other than those related to the

maintenance of the postsynaptic Cl^- gradient underlying hyperpolarizing inhibition. It should also be pointed out that in addition to the spatiotemporal expression patterns of the CCCs and other chloride transporters, neuronal Cl^- homeostasis is also affected by posttranslational modifications including trafficking and kinetic regulation of these molecules. In general, Na-K-2Cl cotransport is activated by phosphorylation while the opposite is true for K-Cl cotransport (Payne et al. 2003). However, the literature on kinetic regulation of CCCs in neurons is scarce and much of the current ideas in this field are derived from work on non-neuronal cells. A recent study points to oligomerization of KCC2 as a mechanism associated with functional activation (Blaesse et al. 2006).

What are the cellular signaling cascades that trigger the expression of KCC2 and the postsynaptic shift from depolarizing to hyperpolarizing GABA action? At present, it is clear that (at least in neuronal cultures) neither spiking nor transmitter actions mediated by GABA or glutamate are absolute requirements for the induction of KCC2 expression (Ludwig et al. 2003) (but see (Ganguly et al. 2001)). Not unexpectedly, neurotrophins are heavily implicated in the developmental shift (Aguado et al. 2003; Rivera et al. 2004), and there is evidence from *in vitro* work that selective activation of the SHC pathway downstream TrkB receptors (i.e., in the absence of the PLCγ pathway) leads to an increase in KCC2 expression (Rivera et al. 2004). Whether this mechanism is important during brain development remains to be seen.

In rodent hippocampal pyramids, the increase in the efficacy of Cl^- extrusion and expression of KCC2 take place during the first two postnatal weeks (Khazipov et al. 2004; Khirug et al. 2005; Rivera et al. 1999). In contrast to cultures of cortical neurons as well as to neurons in the brain stem where KCC2 is initially (for several days) expressed in an inactive form (Balakrishnan et al. 2003), KCC2 in native cortical pyramidal neurons becomes functionally active at the time of its expression (Khirug et al. 2005). Hence, in CA3 pyramids that have a major role in early hippocampal network events, KCC2 expression is rate-limiting for the ontogenetic shift in E_{GABA}, and for the emergence of classical hyperpolarizing IPSPs.

3 Giant Depolarizing Potentials (GDPs) in the Immature Hippocampus

3.1 Early Views on GABAergic Mechanisms in GDP Generation

Early experiments on the mechanisms of GABAergic inhibition in hippocampal and cortical slice preparations pointed to significant qualitative differences between immature vs. mature neurons (Ben-Ari et al. 1989; Luhmann

and Prince 1991; Mueller et al. 1984; Zhang et al. 1991). GABAergic inhibition was sometimes not detected at all, while in some experiments the postsynaptic responses were found to be depolarizing rather than hyperpolarizing. In a milestone work on immature CA3 pyramidal neurons, as already mentioned, Ben-Ari et al. (1989) detected spontaneous GDPs in intracellular recordings. GDPs could be evoked by electrical stimuli in an all-or-none manner, and the authors concluded that both the spontaneous and evoked GDPs were generated by recurrent network events within the CA3 field. The GDPs were inhibited by $GABA_A$-receptor antagonists and the main component of the intracellular responses had its reversal potential at a rather depolarized level (even in recordings done with Cl^--free microelectrodes), which was similar to the reversal potential of voltage responses evoked by exogenous $GABA_A$-receptor agonists. Furthermore, the GDPs and the effects of GABAergic drugs showed a developmental profile pointing to a progressive negative shift in E_{GABA} during postnatal maturation, and GDPs were reported to disappear at around P12. In recent work, a somewhat more protracted time course has become evident, and functionally excitatory GABAergic events based on Cl^- currents have been reported until ~P14–17 (Khazipov et al. 2004). More importantly, it has become clear that the presence of GDPs is a general feature of the immature hippocampus *in vitro*, as evidenced by experiments on rats (Ben-Ari et al. 1989), mice (Aguado et al. 2003) rabbits (Menendez de la Prida et al. 1996), and primates (Khazipov et al. 2001).

3.2 Neurotransmitter Mechanisms in GDP Generation

Originally, the basic mechanism responsible for the endogenous triggering ("pacing") of GDPs was assumed to be primarily GABAergic, residing in oscillations in the interneuronal network. This was an obvious idea in the light of the depolarizing actions that $GABA_A$ receptor-mediated transmission has in immature neurons with a high $[Cl^-]_i$. However, recent evidence has led to an alternative hypothesis, where glutamatergic transmission has an "instructive role" in the generation of GDPs (Sipilä et al. 2005). This is based on (1) the intrinsic bursting properties of immature CA3 neurons and (2) the recurrent glutamatergic connections among the pyramids. In this scenario, depolarizing GABAergic transmission facilitates GDP generation. However, GABAergic transmission is not responsible for the pacing of the GDPs and a temporally non-patterned mode of GABA transmission is sufficient for this kind of "permissive action". Below, these ideas are examined in the light of the available data.

With respect to the mechanisms underlying GDP generation, it is important to note that during hippocampal development the emergence of GDPs in the CA3 region coincides with that of functional ionotropic glutamatergic

transmission (Khazipov et al. 2001). In voltage-clamp recordings, GDPs are associated with a burst of postsynaptic glutamatergic and $GABA_A$ receptor-mediated currents, GABA-PSCs (Khazipov et al. 1997; Lamsa et al. 2000; Leinekugel et al. 2002). Furthermore, a pronounced postsynaptic glutamatergic response is unmasked during GDPs in experiments with intracellular application of fluoride that blocks $GABA_A$ receptor-mediated conductances (Bolea et al. 1999; Khazipov et al. 1997). The pyramidal firing peaks rapidly and decays at the beginning of GDPs, whereas the GABA-PSCs occur within a longer time window (Sipilä et al. 2005). While the different regions of the hippocampus (CA1, CA3 and dentate gyrus) can support GDPs even in isolation (Garaschuk et al. 1998; Khazipov et al. 1997; Menendez de la Prida et al. 1998), the CA3 area has the highest propensity for their generation and most likely acts as the pacemaking region *in vivo* (Ben-Ari 2001).

A combined application of AMPA-kainate antagonists (e.g., CNQX, NBQX) and an NMDA antagonist fully abolishes GDPs, while NMDA-receptor antagonists as such have no effect or merely cause a decrease in GDP frequency (Ben-Ari et al. 1989; Bolea et al. 1999; Gaiarsa et al. 1991; Hollrigel et al. 1998; Khazipov et al. 2001; Lamsa et al. 2000). An obligatory role for AMPA transmission in the generation of GDPs was demonstrated in experiments where a non-competitive, selective AMPA-receptor antagonist, GYKI 53655, completely blocked spontaneous and evoked GDPs (Bolea et al. 1999). Selective activation of kainate receptors has recently been shown to have a modulatory action on GDPs by affecting glutamate release (Lauri et al. 2005). Hence, all the available information indicates that postsynaptic glutamatergic transmission mediated by AMPA receptors is mandatory for the generation of GDPs. Moreover, upon application of glutamate blockers, the rhythmic activity of the interneuronal network is blurred into irregular firing patterns and the robust cross-correlation between interneuronal activity and field-recorded GDPs is abolished (Sipilä et al. 2005). Hence, the rhythmic activity of the interneuronal network during GDPs (Khazipov et al. 1997) is a consequence of (not a cause of) the rhythmic activity of the pyramidal population.

3.3 Role of Bursting Properties of Immature CA3 Pyramidal Neurons in GDP Generation

As stated above, glutamatergic transmission is obligatory for GDP generation. Immature CA3 pyramidal neurons have at least three important properties with respect to GDP generation: 1) they generate voltage-dependent intrinsic bursts when depolarized (Sipilä et al. 2005) [see also (Menendez de la Prida and Sanchez-Andres 2000)], 2) they exhibit a significant endogenous tonic and phasic $GABA_A$ receptor-mediated conductance, which leads to a depolarizing current (Ben-Ari et al. 1989; Sipilä et al. 2005), and 3) they are interconnected via excitatory recurrent collaterals (Bolea et al. 1999). Each

burst consists of ~2-8 action potentials with an intraburst frequency of ~ 10–50 Hz. The main channel mechanism driving the spontaneous action potentials during bursts is a persistent Na^+ current (Sipilä et al. 2006).

In recent work (Sipilä et al. 2005) we have found that the temporal patterns of GDP occurrence with a peak at ~ 0.3 Hz are strongly influenced by the intrinsic bursting properties of the immature CA3 pyramids. As a general rule, the rate of occurrence of both CA3 pyramidal bursts and GDPs is enhanced in a similar manner by experimental maneuvers that depolarize the cell: by elevation of $[K^+]_o$, or by application of a low concentration of $GABA_A$-receptor agonists. On the other hand, factors that lead to hyperpolarization, such as blocking tonic $GABA_A$ receptor-mediated actions and a decrease in $[K^+]_o$, lead to opposite effects. Hence, the intrinsic bursting properties of the immature CA3 pyramids provide a parsimonious explanation for the finding that $GABA_A$-receptor antagonists inhibit GDPs (see above): these drugs inhibit the voltage-dependent bursting by suppressing the endogenous synaptic and tonic depolarizing GABAergic drive to the CA3 neurons. As could be expected, a subsequent elevation of $[K^+]_o$ in the continuous presence of the $GABA_A$-receptor blockers leads to a recovery of the intrinsic bursting of individual pyramidal neurons and of the GDPs monitored by field-potential recordings. Furthermore, a selective enhancement of the tonic $GABA_A$ receptor-mediated current in the complete absence of synaptic $GABA_A$ receptor-mediated transmission promotes field GDPs (Sipilä et al. 2005).

At first sight, the enhancement of field GDP occurrence by depolarizing maneuvers seems to conflict with findings based on intracellular recordings, where $GABA_A$-receptor antagonists often completely block both spontaneous and evoked cellular responses reflecting GDPs (Ben-Ari et al. 1989; Gaiarsa et al. 1991; Menendez de la Prida et al. 1998). One should note, however, that a complete block of intracellular responses by $GABA_A$-receptor antagonists during network GDPs must take place in a subpopulation of the immature CA3 pyramidal neurons, since a number of them have only functional GABAergic synapses but no glutamatergic ones (Danglot et al. 2006; Hennou et al. 2002; Tyzio et al. 1999). Hence, data of the above kind, based on single-cell responses, merely indicate that the network events are *transmitted* to some individual neurons exclusively via GABAergic synapses.

The intrinsic properties of immature CA3 pyramidal neurons also influence the duration of GDPs. The bursts of the immature CA3 pyramids are terminated by a slow afterhyperpolarization, which largely accounts for the silent ("refractory") period of ~ 2–3 s, during which both GDPs and spiking of individual CA3 pyramidal neurons are suppressed (Sipilä et al. 2005; Sipilä et al. 2006). In addition, an inhibitory effect of $GABA_B$ receptors on presynaptic release of glutamate and GABA contributes to GDP termination (McLean et al. 1996), whereas GABA uptake via GAT-1 acts to limit the GDP-associated postsynaptic burst of GABAergic currents (Sipilä et al. 2004).

3.4 Mechanisms of GDP Generation: a Summary

The basic mechanisms underlying GDP generation are summarized in Fig. 3. A crucial property of the immature CA3 pyramidal neurons is their capabil-

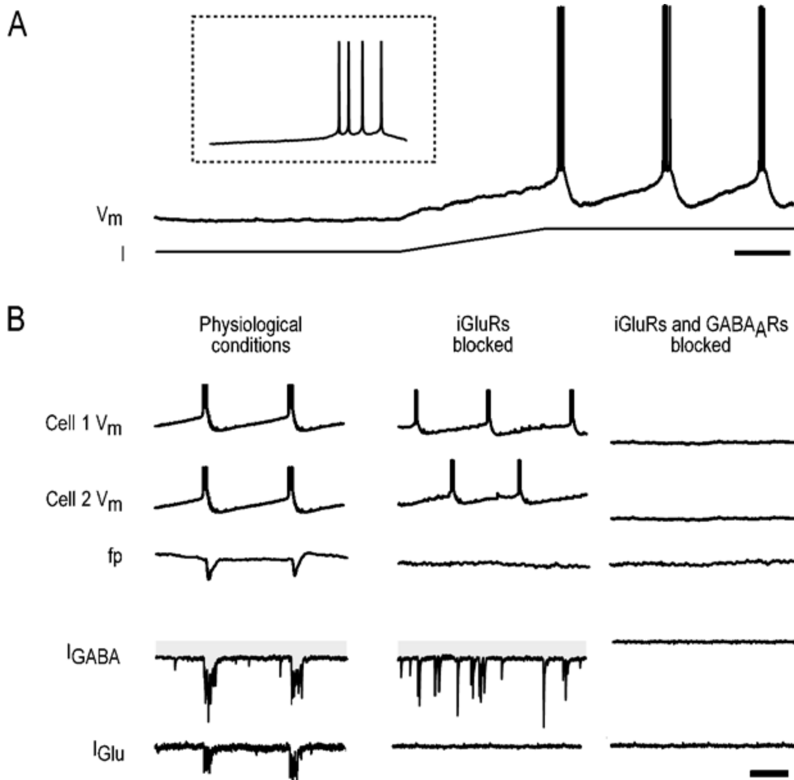

Fig. 3 Cellular and synaptic mechanisms of GDP generation. **A** Immature CA3 pyramidal neurons generate voltage-dependent intrinsic bursts (shown in more detail in the *inset*) in the absence of synaptic transmission. The slow change in V_m is induced by current injection. **B** *Left*: Under physiological conditions, GDPs are seen as synchronous bursts of action potentials in intracellular recordings in immature CA3 pyramidal cells (Cell 1 and Cell 2), paralleled by a slow negative deflection in the field potential (fp). GDPs are associated with ionotropic GABAergic (I_{GABA}) and glutamatergic currents (I_{Glu}). *Middle*: Blockade of ionotropic glutamatergic transmission desynchronizes pyramidal cell bursting and abolishes fp deflections as well as bursts of GABAergic currents that reflect interneuronal activity. *Right*: Blockade of tonic and synaptic $GABA_A$ receptor currents hyperpolarizes immature CA3 pyramidal neurons and, consequently, abolishes intrinsic bursting (the effect of membrane potential on intrinsic bursting is illustrated in *part A*). The traces are semi schematic. *Time calibration bars*: 2 s in **A** and **B**, 200 ms in the *inset* in **A**. For voltage and current amplitudes, see original references cited in the text

ity to produce voltage-dependent intrinsic bursts, which can be observed in the complete absence of ionotropic synaptic transmission (Fig. 3A). Under physiological conditions (Fig. 3B, left traces), GDPs are seen as synchronous bursts in voltage recordings in the immature CA3 pyramidal cells (schematically illustrated by Cell 1 and Cell 2), paralleled by slow negative deflections in the field potential (fp). At the synaptic level, the population discharge underlying GDPs is associated with barrages of ionotropic GABAergic and glutamatergic currents (I_{GABA} and I_{Glu} in Fig. 3B). The role of these two major transmitter systems is readily revealed by the effects of synaptic antagonists. A complete block of ionotropic glutamate receptor-mediated transmission leads to a desynchronization of pyramidal cell bursting, with a consequent abolishment of the fp response. Since the GDP-associated GABAergic bursts, reflecting interneuronal activity, are driven by pyramidal cells under physiological conditions, their synchrony is blurred in the absence of glutamatergic transmission. Thereafter, a blockade of tonic and synaptic $GABA_A$ receptor-mediated currents (indicated by the grey area) hyperpolarizes the immature CA3 pyramidal neurons and, consequently, abolishes intrinsic bursting (Fig. 3B).

Finally, an important conclusion implicit in the scheme in Fig. 3B is that the mechanisms of GDP generation, based on the pacemaking activity of the interconnected network of intrinsically bursting CA3 pyramidal neurons, are largely identical to the mechanisms that generate SPWs and interictal spikes in the adult hippocampus.

4
Are *in vitro* GDPs and early Sharp Waves (SPWs) Homologous?

Mature cortical structures are capable of producing elaborate patterns of neuronal oscillations and network events which have a role in various kinds of cognitive processes (Buzsaki and Draguhn 2004; Traub et al. 2004). A prominent type of these network events in the rat hippocampus are irregularly occurring SPWs (Buzsaki et al. 1983), also known as EEG spikes (Jouvet et al. 1959; O'Keefe and Nadel 1978; Vanderwolf 1969). SPWs have a duration of $\sim 30-120$ ms in field-potential recordings from the CA1 stratum radiatum and are thought to be the main type of self-organized activity pattern produced by the adult rat hippocampus *in vivo* (Buzsaki 1986; Suzuki and Smith 1987). SPWs occur during slow-wave sleep, awake immobility or consummatory behaviors such as drinking, eating and grooming. These observations point to an endogenous origin of SPWs, which is supported by the findings that surgical ablation of afferents to the hippocampus rather increases than decreases the occurrence of SPWs. The hallmark work by Buzsaki (Buzsaki 1986; Buzsaki et al. 1983) as well as by Suzuki and Smith (Suzuki and Smith 1987) points to a mechanism of SPW generation where

endogenously bursting CA3 pyramidal neurons (Hablitz and Johnston 1981; Kandel and Spencer 1961; Wong and Prince 1981), that have mutual excitatory connections (Lebovitz et al. 1971; MacVicar and Dudek 1980), are activated during behavioral-state dependent periods of disinhibition. SPWs in the adult are associated with "ripples", i.e. high-frequency oscillations at 140–200 Hz (Buzsaki et al. 1992; O'Keefe and Nadel 1978).

SPWs are the first organized pattern of activity generated endogenously in the hippocampus during development (Fig. 4) (Karlsson and Blumberg 2003; Leinekugel et al. 2002). They are sometimes followed by a "tail" event

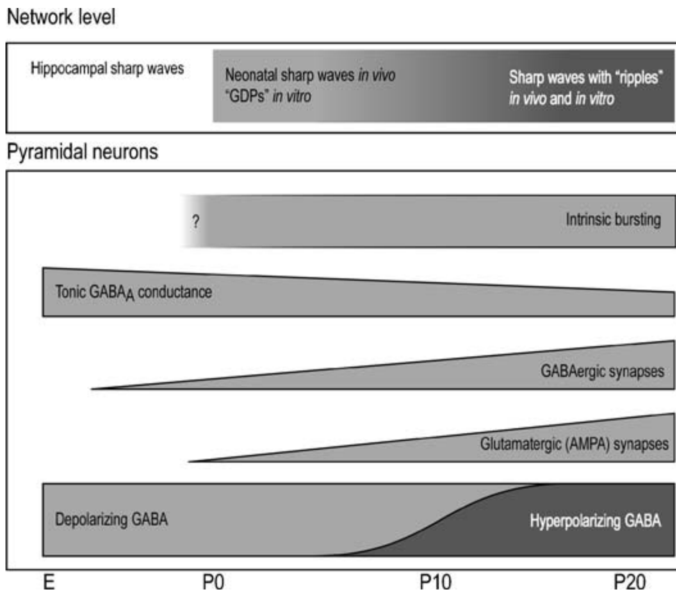

Fig. 4 Developmental profile of CA3-driven hippocampal sharp waves (SPWs) and the *in vitro* network events, GDPs, in the immature rat hippocampus. Already during the embryonic stage, immature pyramidal neurons have a large tonic GABAergic conductance. GABAergic synapses are functional before glutamatergic ones, but GDPs are first seen only after the establishment of functional glutamatergic synapses. CA3 pyramidal neurons generate intrinsic burst activity throughout postnatal development (whether this takes place before birth is not known, as indicated by a question mark in the figure). The probability of GDP occurrence decreases as the $GABA_A$-receptor mediated action shifts from depolarizing to hyperpolarizing. The ability of mature CA3 pyramidal neurons to generate network events *in vitro* is enhanced under conditions where the strength of functional recurrent connections is increased or the efficacy of GABAergic inhibition is decreased. *In vivo*, SPWs are the first endogenous pattern of activity seen during ontogeny. Development of SPWs is further characterized by the emergence of high-frequency "ripple" oscillations that have also been recorded in mature slice preparations. The approximate developmental time scale shows the late embryonic period (E) and the postnatal period from P0 (postnatal day 0; time of birth) to P20 in rats. For details and references, see text

consisting of multi-unit burst activity during the first two postnatal weeks (Leinekugel et al. 2002) while the SPW-associated ripple-activity emerges during the third postnatal week (Buhl and Buzsaki 2005). Both the *in vitro* GDPs and neonatal *in vivo* SPWs are associated with barrages of glutamatergic and GABAergic currents (Ben-Ari 2001), and occur at irregular intervals with a peak at ~ 0.3 Hz (Leinekugel et al. 2002; Sipilä et al. 2005). Moreover, GDPs (Leinekugel et al. 1998; Menendez de la Prida et al. 1998) and SPWs (Buzsaki 1989; Suzuki and Smith 1987) occur simultaneously at various sites of the hippocampi and bilaterally. This type of GDP activity is seen in an *in vitro* preparation of the septohippocampal complex containing both hemispheres (Leinekugel et al. 1998). Both types of events are associated with increased interneuronal and pyramidal cell firing rates (Ben-Ari 2001; Buzsaki 1986). Finally, both the neonatal SPWs and GDPs are blocked by the NKCC1 inhibitor, bumetanide (Sipilä et al. 2006). As a whole, these data are consistent with the view that GDPs are the *in vitro* counterparts of neonatal SPWs (Leinekugel et al. 2002). However, it is also possible that the neonatal SPW and the multi-unit bursts are distinct entities and the latter might be homologous to an *in vitro* GDP that disappears during ontogeny.

When taking a closer look at the similarities between GDPs and SPWs, it is of interest to note that already in the pioneering *in vivo* work cited above, SPWs in the mature hippocampus were concluded to occur as a result of temporary disinhibition from afferent control. In the case of GDPs, there is a steep decline in their rate of occurrence, with a near-abolishment during the first two postnatal weeks, which closely parallels the development of the efficacy of GABAergic inhibition (Fig. 4) (Ben-Ari et al. 1989; Khazipov et al. 2004). Hence, a common feature of both SPWs and GDPs is a state of functional disinhibition.

Our model for GDP generation (Sect. 3.4) predicts that GDPs and GDP-like activity should take place in mature slice preparations under conditions that provide a sufficient amount of functional recurrent coupling between the CA3 pyramidal neurons. Indeed, there is recent evidence that SPW-like (or GDP-like) activity driven by spontaneous population bursts of pyramidal neurons can be observed in adult slices taken from the ventral hippocampus of the rat (Kubota et al. 2003; Papatheodoropoulos and Kostopoulos 2002). In addition, SPWs have been observed in slices from mice hippocampi (Maier et al. 2003; Wu et al. 2005). In both cases, a higher level of connectivity (ventral vs. medial/dorsal hippocampus; and mouse vs. rat slice, respectively) is likely to be a crucial factor in the generation of these spontaneous events *in vitro*.

It has been repeatedly shown that experimental manipulations that block GABAergic inhibition (Miles and Wong 1983; Miles and Wong 1987; Schwartzkroin and Prince 1978) or enhance the functional connectivity among CA3 pyramidal neurons (Bains et al. 1999; Behrens et al. 2005; Miles and Wong 1987; Rutecki et al. 1985) result in CA3-driven spontaneous activity in hippocampal slices. With respect to the underlying cellular and synaptic

mechanisms, the similarity of GDPs and interictal-like events in slice preparations is high. Regarding the *in vivo* situation, Buzsaki (Buzsaki 1986) states that "*the basic assumptions about the mechanisms underlying the generation of the interictal spike and the physiologically occurring SPW appear to be identical*". Of course, this does not imply that interictal events *are* SPWs, and neither do we want to imply that the widely-studied GDPs seen in immature slices *are* interictal events. The main point here is that GDP/SPW-like activity appears to represent an inherent pattern generated by the CA3 pyramidal neurons under diverse conditions (developmental, physiological, or pharmacological) where the efficacy of GABAergic inhibition is reduced and/or overall excitation is enhanced (Fig. 4).

5 Conclusions

Is there a common denominator in the network mechanisms that produce the various kinds of spontaneous, discontinuous activity patterns that are seen in diverse developing structures within the CNS? Looking at the development of the CA3-driven SPW/GDP activity, there appears to be a basic type of pattern that is elaborated during development. In addition to SPWs, the developing hippocampus produces various kinds of oscillations which appear to have their own profiles of maturation (Buhl and Buzsaki 2005; Karlsson and Blumberg 2003; Leblanc and Bland 1979; Leinekugel et al. 2002). On the other hand, on the basis of the available data, it is hard to identify or even postulate a profound similarity in the mechanisms that drive endogenous activity in the spinal cord, retina, and cortical structures (see Introduction). Hence, it might be prudent to conclude that various kinds of developing networks produce distinct kinds of activities. Such a conclusion can be drawn already on the basis of the pioneering work reviewed by Victor Hamburger in his classical 1963 paper (Hamburger 1963). The differences in various kinds of immature motor patterns; the relation of these patterns to overt anatomical and functional development; and the large differences between vertebrate classes: all these aspects point to a substantial heterogeneity of early network mechanisms.

In this review, we have described the basic mechanisms that underlie endogenous events, GDPs, in hippocampal slices. The basic mechanisms involved in the generation of *in vitro* GDPs and neonatal *in vivo* SPWs are retained throughout development, with a key role for the intrinsic bursting characteristics of the interconnected population of CA3 pyramidal neurons (Fig. 4). We conclude that GDPs in neonatal slices represent an immature, "pre-established" activity pattern, which involves mechanisms that are of crucial importance in the state-dependent generation of SPWs in the adult hippocampus.

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Regulation of Excitation by Glycine Receptors

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Abstract Our knowledge of glycine receptor (GlyR) regulation of excitation has advanced significantly in recent years. GlyRs are widespread in the CNS, are heterogeneous, and undergo developmental changes. Activation of GlyRs of immature neurons induces outflow of Cl^-, membrane depolarization, neuronal excitation, calcium influx, and transmitter release, in contrast to the inhibitory effects these receptors have in mature neurons. Thus, GlyRs are important for neuronal excitability in both the developing and the mature CNS. This chapter is an overview of selective studies on the newly discovered roles of GlyRs in regulating neuronal excitation, and inhibition, particularly in the upper brain areas.

1 Introduction

Glycine was discovered as an inhibitory neurotransmitter in the spinal cord about 40 years ago (Aprison and Werman 1965; Aprison 1990). By activating the glycine receptor (GlyR), glycine increases the Cl^- permeability of neuronal membranes, thus yielding inhibitory effects in most of the adult mammalian neurons. Understanding the role of GlyR in regulation of excitation has greatly advanced in recent years. Emerging information indicates that GlyRs are not restricted to the spinal cord and the brain stem, but are distributed more extensively in the CNS, including many forebrain areas. The last few years have seen a surge in interest in the GlyRs in the upper brain areas. Consequently, a wealthy of information has recently emerged concerning the properties and the role of the GlyRs located outside of the spinal cord and brain stem. While the GlyRs in the spinal cord and brain stem are located in the synapses, the GlyRs in many of the forebrain areas are nonsynaptic or extrasynaptic, given that current evidence on glycinergic innervation in many higher brain areas is lacking or rare. There is increasing evidence that these extrasynaptic GlyRs may mediate processes of physiological importance. Accumulating evidence in the past 20 years indicates that during early development, activation of GlyRs causes excitation rather than inhibition of neurons because the intracellular concentration of Cl^- is higher than the equilibrium concentration. Recent studies found that GlyRs also exist on presynaptic terminals. Activation of these receptors has been demonstrated to alter membrane potential, background calcium levels, and transmitter release. Thus, GlyRs in the postsynaptic and presynaptic mem-

brane are important players in the regulation of neuronal excitability in both the developing and the mature CNS.

This chapter is an overview of selective studies on the newly discovered role of GlyRs in regulating neuronal excitation and inhibition, particularly in the upper brain areas. The following topics will be discussed: GlyR and its widespread distribution in the CNS (Sect. 2); developmental changes of GlyRs and intracellular Cl^- (Sect. 3); regulation of excitation by GlyRs (Sect. 4); presynaptic GlyR regulation of transmitter release (Sect. 5); and a newly discovered excitable strychnine-insensitive GlyR (Sect. 5.6).

2
GlyRs and Their Widespread Distribution in the CNS

The strychnine-sensitive GlyR is a ligand-gated anionic channel that is primarily involved in fast inhibitory synaptic transmission (for review see Legendre 2001; Lynch 2004). GlyRs are members of the pentameric Cys-loop receptor superfamily. Two different subunits of GlyR have been characterized so far, an α subunit (48 kDa) and a β subunit (58 kDa). GlyRs are formed either from α subunits alone or from both α and β subunits (Langosch et al. 1988). Synaptically localized heteromeric GlyR consists of three α and two β subunits, which combine to form a pentameric receptor complex. The homomeric form composed of five α subunits is extrasynaptically located (reviewed in Laube et al. 2002; Rajendra et al. 1997). Homo- and hetero-oligomeric GlyRs differ in Cl^- conductance (Bormann et al. 1993) and picrotoxinin sensitivity (Pribilla et al. 1992). The α subunits are thought to contain major determinants of agonist and antagonist binding and exist in four different isoforms ($\alpha1$–$\alpha4$) encoded by regionally and developmentally distinctly expressed genes (Harvey et al. 2004; Malosio et al. 1991a). The unique β subunit gene (Glrb) in contrast is transcribed at all developmental stages in many regions of the mammalian CNS (Fujita et al. 1991; Malosio et al. 1991b). These subunits co-purify with a 93 kDa receptor-associated protein (gephyrin) that is localized to the cytoplasmic face of the neuronal membrane. Gephyrin may serve to anchor the GlyR complex at the synaptic locus (Kirsch et al. 1993). The β subunit interacts with gephyrin (Meyer et al. 1995) and thereby provides for synaptic clustering of heterooligomeric GlyRs (Kneussel and Betz 2000a).

Glycinergic synapses were originally believed to be restricted to the spinal cord and brain stem, where they are essential for the control of motor rhythm generation, coordination of spinal reflex responses, and processing of sensory signals (Legendre 2001). Molecular studies indicate that GlyRs are distributed more extensively than previously thought. In the adult rat, mouse, cat, and guinea pig, many glycine-immunoreactive (IR) cell bodies and fibers exist in widespread brain areas including the cochlear nuclei, superior olivary complex and medial nuclei of trapezoid body, the cerebellar cortex, deep

cerebellar nuclei, and the area postrema. In adult cats, glycine-IR neurons are also described in the vestibular prepositus hypoglossi, in the sensory trigeminal nucleus, in the medullary and pontine reticular formation. An extensive study of glycine-IR in rats reports that glycine-containing cells are also present in the upper brainstem and the forebrain (Danglot et al. 2004; for review see Legendre 2001). More recent electrophysiological, immunocytochemical, and in-situ hybridization evidence indicates that GlyRs exist in the higher brain of mammals, including the prefrontal cortex, hippocampus, amygdala, hypothalamus, cerebellum, nucleus accumbens, ventral tegmental area, and substantia nigra (Flint et al. 1998; Ye et al. 1998, 1999; Chattipakorn and McMahon 2002; McCool and Botting 2000; McCool and Farroni 2001; Mangin et al. 2003; Mori et al. 2002; Gaiarsa et al. 2002; Laube et al. 2002; Zhou 2001; Tapia et al. 2000). Expressions of GlyRs in these areas are regulated distinctly in terms of subunit compositions and amounts.

In a recent study in hippocampal cultures using western blot and immunohistochemical analyses, Brackmann and colleague (2004) found prominent cellular expression of GlyRs in pyramidal neurons and GAD-positive interneurons similar to the calcium-binding protein VILIP-1 with widespread hippocampal distribution. On the subcellular level, they found co-staining of GlyR and the presynaptic marker synapsing I. Furthermore, co-staining with GAD at synaptic terminals indicates partial co-localization of GABA- and GlyRs (Brackmann et al. 2004).

3
Developmental Changes of GlyRs and Intracellular Chloride

3.1
GlyRs are Heterogeneous and Undergo Developmental Changes

Molecular cloning studies reveal heterogeneity of GlyR subunits, which vary with the type of tissue and the age of the animal. In the adult mammalian brain, the major expression sites of GlyRs are the spinal cord and the brain stem. During the early period of development, however, α2 subunits of the GlyR are expressed rather widely in the brain, and most of them disappear or are replaced with other subunits after maturation (with α1 or α3; Eggers et al. 2000; Garcia-Alcocer et al. 2001; Kungel and Friauf 1997; but see Chattipakorn and McMahon 2002, 2003).

During fetal and early postnatal development, α homomeric GlyRs are predominant, whereas adult GlyRs are essentially αβ heteromeric GlyRs (Becker et al. 1988; Takahashi et al. 1992; Kaneda et al. 1995; Kungel and Friauf 1997; Flint et al. 1998; Ye 2000). This maturation change of subunit composition of GlyR may not require the presence of glycinergic innervation (Mangin et al. 2002). Interestingly, mRNA encoding the GlyR α1 subunit was found in the

embryonic encephalon, midbrain, and brainstem (Garcia-Alcocer et al. 2001). There are four α subunit variants (α1, α2, α3, and α4), and they carry binding sites for agonists and for strychnine (Kuhse et al. 1990; Ruiz-Gomez et al. 1989; 1990; Schmieden et al. 1989; Vandenberg et al. 1992). Via in-situ hybridization using sequence-specific oligonucleotide probes, (Malosio et al. 1991a) found that GlyR α1 subunit mRNA is abundant in the spinal cord and is also present in a few brain areas, including the superior and inferior colliculi. The variant of the rat α1 subunit, αl^{ins}, contains eight additional amino acids in the presumed intracellular loop domain close to M4, which represents a potential phosphorylation site (Malosio et al. 1991a). Expression of αl^{ins} is restricted to the brainstem and spinal cord of the adult rat. αl^{ins} mRNA represents 30% of the total αl mRNA, and the ratio of αl^{ins} to αl mRNA remains constant during postnatal brain development (Legendre 2001). α2 subunits are expressed in embryonic neurons. It is likely that homomeric α2-GlyRs mediate nonsynaptic cell-to-cell communication that could be important for neuronal differentiation and synaptogenesis (Kneussel and Betz 2000b).

Glycinergic synapses in immature neurons are probably comprised of α2β-heteromeric GlyRs (Legengre 2001). This is supported by the single-channel conductance of synaptic GlyRs from embryonic neurons (Ali et al. 2000; Singer et al. 1998). α2 transcripts were found in several brain regions, including layer VI of the cerebral cortex and hippocampus. Furthermore, the α2 subunit is expressed predominantly in fetuses and neonatal rats and is progressively replaced by the α1 subunit during the second postnatal week in most structures (Akagi and Miledi 1988; Malosio et al. 1991a). Alternative splicing of the rat α2 subunit generates two splice variants, α2A and α2B. Another version of the α2 subunit, α2*, incorporates a single amino acid substitution (G167E) that confers strychnine insensitivity (Kuhse et al. 1990). The α3 subunit is primarily expressed in the adult cerebellum (Malosio et al. 1991a, b; Matzenbach et al. 1994), and is distinctly expressed in the superficial laminae of mouse dorsal horn. It is an essential target for spinal PGE2-mediated inflammation pain sensation (Harvey et al. 2004). Two transcripts of the human α3-gene, termed α3L and α3K, have been characterized (Nikolic et al. 1994). The α3 subunit is also found in the adult rat amygdala (McCool and Botting 2000).

By contrast, the β subunit seems to be expressed as a single form throughout the brain at all developmental stages and is not required for ligand binding (Grenningloh et al. 1990; Malosio et al. 1991b). However, it might determine the ligand binding properties of synaptic GlyRs (Grudzinska et al. 2005), and might confer resistance to GlyR antagonists (Pribilla et al. 1992). Northern analysis (Grenningloh et al. 1990) and in situ hybridization (Malosio et al. 1991a) indicate high levels of β transcripts in cerebellum and cortex, brain regions where none of the presently known GlyR α polypeptides are expressed at comparable levels. It is relevant to mention that GlyRs mRNAs appear in the embryonic brain well before the mRNAs coding for GABA re-

ceptors, which subsequently become the main inhibitory neurotransmitter receptors in the adult mammalian brain (Carpenter et al. 1988). Furthermore, it is known that GlyRs are activated by taurine during early brain cortical development, suggesting that the presence of functional GlyRs in the neuronal plasma membrane may play a role in the proper development of the brain (Flint et al. 1998; Wang et al. 2005).

3.2 Developmental Switches in Glycine-Induced Responses

The fact that the intracellular Cl^- concentration of some neurons changes dramatically with development was discovered in the last 20 years (Mueller et al. 1984). In early development, intracellular Cl^- is higher than the equilibrium concentration, as estimated from the resting membrane potential (Ehrlich et al. 1999; Kilb et al. 2002; Laube et al. 2002; Rivera et al. 1999), due to an active inward transport of Cl^-: the Na^+–K^+–$2Cl^-$ co-transporter ($NKCC_1$), which is driven by a Na^+ and K^+ gradient, raised $[Cl^-]_i$. The change in intracellular Cl^- with development is mediated by the expression of a K^+–Cl^- co-transporter, KCC_2, which couples Cl^- transport to the K^+ gradient and normally lowers the intracellular Cl^- concentration to more negative than the Cl^- equilibrium concentration, thus converting the action of GlyRs from excitatory to inhibitory (Stein and Nicoll 2003; Rivera et al. 1999). It has been shown that KCC_2 is up-regulated while $NKCC_1$ is down-regulated in the CNS during development (Delpire 2000). As a result, in the early stage of development, glycine and GABA induce outward flux of Cl^-, membrane depolarization, and neuronal excitation, in contrast to the inhibitory effect these neurotransmitters have in mature neurons. This has been shown in many areas, including hippocampus (Ben-Ari et al. 1994), hypothalamus (Chen et al. 1996), auditory brain stem neurons (Backus et al. 1998), spinal neurons (Tapia and Aguayo 1998; Xu et al. 1996), and midbrain neurons (Ye 2000; Wang et al. 2005). The excitation induced by Cl^- out-flux is presumably essential for synaptic maturation or neural development, since depolarization causes activation of voltage-dependent Ca^{2+} channels, thus increasing the intracellular concentration of Ca^{2+} and evoking specific functions of the cell (Eilers et al. 2001; Kanaka et al. 2001; Legendre 2001). However, subthreshold depolarization can be accompanied by an increase in membrane conductance, which will shunt the membrane conductance and inhibit neuronal firing (Lynch 2004). While KCC_2 overexpression in young neurons leads to hyperpolarized E_{Cl} (Fiumelli et al. 2005), KCC_2 expression in adult rat neurons is down-regulated following epileptiform activity and/or neuronal damage by BDNF/TrkB signaling. The down-regulation of KCC_2 under pathophysiological conditions (epilepsy, damage) in mature neurons seems to reflect a “recapitulation” of early developmental mechanisms, which may be a prerequisite for the re-establishment of connectivity in damaged brain tissue (Rivera et al. 2005). Prolonged post-

synaptic spiking of hippocampal neurons was found recently to lead to a shift in the reversal potential of GABA-induced Cl^- current (E_{Cl}) toward positive levels in a duration- and frequency-dependent manner (Fiumelli et al. 2005). This effect depends on cytosolic Ca^{2+} because while blocking cytosolic Ca^{2+} elevation was abolished, and the release of Ca^{2+} from internal stores can mimic the effect of prolonged firing and induce a shift of E_{Cl}. Importantly, the activity and Ca^{2+}-induced shifts of E_{Cl} were larger in mature neurons, which have a higher level of KCC_2.

In addition to KCC_2, the carbonic anhydrase (CA) isoform CAVII also contributes to the switch of intracellular [Cl^-] (Rivera et al. 2005). During early development, at around postnatal day 12 (P12), there is an abrupt, steep increase in intrapyramidal CAVII expression in rat hippocampal pyramidal neurons. This is mainly due to a GABAergic potassium transient and will cause spatially widespread neuronal depolarization and synchronous spike discharges. Thus, CAVII can be a putative target of the CA inhibitors that are used as antiepileptic drugs. In addition, factors that influence the activation/deactivation of CAVII could also alter the ionic modulation of GABAergic responses (Rivera et al. 2005).

3.3 Low Chloride Extrusion Capacity of $GABA_A$Rs Contributes to GABA-Induced Excitation in Developing Neurons

A recent study in rat spinal cord neurons found that the activation of $GABA_A$Rs of rats induced depolarization and an entry of extracellular Ca^{2+} throughout the first two to three postnatal weeks, despite the developmental shift in transmembrane anion gradient (potentially affecting the outcome of glycine and $GABA_A$ transmission), which was shown to be completed within the first week after birth. These investigators proposed that this is due to the low Cl^- extrusion capacity of the $GABA_A$Rs, which causes a rebound depolarization and an ensuring rise in $[Ca^{2+}]_i$ (Cordero-Erausquin et al. 2005). It is unknown whether a similar situation occurs in GlyRs. Future studies should examine this possibility.

4 Regulation of Excitation by Postsynaptic GlyRs

4.1 Activation of Nonsynaptic GlyRs Excites Immature but Inhibits Mature Neurons of the Ventral Tegmental Area

In addition to the phasic postsynaptic currents, which are the consequence of the vesicular release of transmitters, recent studies have identified a tonic cur-

rent mediated by "extrasynaptic" receptors responding to low concentrations of extracellular transmitters (LoTurco et al. 1995; Brickley et al. 1996; Yeung et al. 2003).

There is increasing evidence that these extrasynaptic receptors play important roles in both physiological and pathological situations (Wei et al. 2004; Belelli et al. 2005; Cope et al. 2005; Hemmings et al. 2005; Jia et al. 2005). Interestingly, the "extrasynaptic" $GABA_A$Rs, but not their "synaptic" counterparts, are primary targets for ethanol (Wallner et al. 2003; Hanchar et al. 2004; Hemmings et al. 2005), neurosteroids (Belelli and Lambert 2005) as well as for volatile anesthetics (Caraiscos et al. 2004; Bieda and MacIver 2004). Tonic activation of extrasynaptic GlyRs has also been shown in neocortical neurons (Flint et al. 1998), hippocampal CA3 neurons (Mori et al. 2002), midbrain neurons (Wang et al. 2005), and in neurons of the supraoptic nucleus (Deleuze et al. 2005). This challenges the traditional concept: a functional role for GlyRs in neurons has required the demonstration of strychnine-sensitive synaptic currents (Lynch 2004). Glycine could be released nonsynaptically by glial cells and by some neuronal elements when they are depolarized by, for example, the activation of glutamate receptor (Roux and Supplisson 2000). The physiological implications of such a nonsynaptic release remain unclear. It is possible that this nonsynaptic release glycine may activate the nonsynaptic GlyRs and induce the tonic glycine currents. Taurine, a full agonist of GlyRs (Flint et al. 1998; Hussy et al. 2001; Mori et al. 2002; Wang et al. 2005) is abundant throughout the brain (Jacobsen and Smith 1968; Madsen et al. 1987; Huxtable 1992; Dahchour et al. 1996; Olive 2002), including the ventral tegmental area (Timmerman et al. 1999).

In the spinal cord and brain stem of mammals, GlyRs are primarily involved in fast inhibitory synaptic transmission. Functional ligand-gated ion channels, including GlyRs are present in the immature CNS well before the establishment of synaptic contact (Ben-Ari 2001). As opposite to the adult brain, the activation of GlyRs has an excitatory action in immature neurons resulting from depolarized Cl^- equilibrium potential. GlyR-mediated depolarization of immature neurons induces a calcium entry through voltage-gated calcium channels (Flint et al. 1998; Reichling et al. 1994), which may modulate cell proliferation, migration and differentiation in neural precursors (Nguyen et al. 2001; Ben-Ari 2001) and, later, maturation of the inhibitory synapse (Kneussel and Betz 2000a).

With the gramicidin-perforated technique, which does not disturb the intracellular chloride, exogenous application of taurine (0.01–30 mM), which activates the GlyRs, induced membrane depolarization of neurons from young rats and hyperpolarization of neurons from mature rats (Wang et al. 2005). In the dopaminergic neurons from young rats (1–13 postnatal days), taurine facilitated ongoing discharges at low dosage. At high dosage, taurine induced depolarization and cessation of ongoing firing. The depolarization might be ascribed to a relatively high intracellular $[Cl^-]$ and correspondingly

less negative reversal potential in immature neurons (Cherubini et al. 1991). It is in good agreement with the effect of glycine and GABA on developing neurons of many brain regions (Cherubini et al. 1991; Flint et al. 1998). In contrast, in older rats, the major effect of taurine was hyperpolarization and blockage of ongoing firing. This is consistent with its conventional inhibitory role (Taber et al. 1986; Saransaari and Oja 1997). Furthermore, most of the GlyRs in the neurons of the ventral tegmental area are nonsynaptic, because bicuculline (20 μM), a specific antagonist of $GABA_AR$, blocked the inhibitory postsynaptic currents in the majority of the neurons tested. Taken together, these data indicate that activation of the nonsynaptic GlyRs excites developing dopaminergic neurons but inhibits mature dopaminergic neurons of the ventral tegmental area (Wang et al. 2005).

4.2 Strychnine-Sensitive GlyRs Depress Hyperexcitability in Rat Dentate Gyrus

It was long thought that GABA is the main inhibitory neurotransmitter in the CNS (Curtis et al. 1970), whereas glycine served as an inhibitory neurotransmitter only in spinal cord and brain stem. As discussed in the Sect. 2 of this chapter, recent studies found that GlyRs have a widespread distribution including many forebrain regions, such as amygdala, nucleus accumbens, ventral tegmental area, substantia nigra, hippocampus, and cerebellum (McCool and Botting 2000; Martin and Siggins 2002; Mangin et al. 2002; Ye 2000; Ye et al. 2004; Kawa 2003). It has been reported that functional GlyRs in hippocampus are not expressed beyond the second postnatal week (Ito and Cherubini 1991). However, later work showed that functional GlyRs exist in the hippocampal neurons of both immature and mature rats (Ye et al. 1999). This finding is consistent with Chattipakorn and McMahon (2002), who characterized the pharmacological properties of glycine-gated chloride currents of CA1 pyramidal cells and interneurons in acute hippocampal slices from 3–4 week old rats. This work further suggests that not only $GABA_ARs$ but also GlyRs are important providers of neuronal inhibition in the CNS. Chattipakorn and McMahon (2003) further showed that glycine induced membrane hyperpolarization and a cessation of the action potential firing pattern in hyperexcitable slices induced by elevated extracellular K^+ or by blocking $GABA_ARs$ with bicuculline in neurons of dental gyrus of adolescent rats. Taken together, these findings indicate that GlyRs, like $GABA_ARs$, are widespread in the CNS, including many forebrain regions. GlyRs in many of the forebrain areas are not transient and limited to early development but persist through mature developmental stages (Chattipakorn and McMahon 2003; Ye 2000; Ye et al. 2004; Mangin et al. 2002; McCool and Botting 2000; Martin and Siggins 2002; Kirchner et al. 2003).

These GlyRs have significant roles in physiological and pathological situations. Upon activation, they excite immature neurons. This may be very

important for the early stage of development of the CNS, when excitatory glutamatergic synapses are rather quiescent (Cherubini et al. 1991). On the other hand, activation of GlyRs of neurons from mature animals, similar to the activation of $GABA_A$Rs, induces neuronal inhibition. Thus, GlyRs could be an additional or alternative inhibitory mechanism of $GABA_A$Rs and play important role in maintaining the normal excitatory balance for proper function of the CNS in the physiological and/or pathological conditions. This notion is further supported by a recent report showing that in an epileptiform model induced by reducing extracellular Mg^{2+} concentration in combined rat entorhinal cortex–hippocampal slices, GlyR agonists (in particular taurine) suppressed the seizure-like events, and thus could serve as potential anticonvulsants (Kirchner et al. 2003). This finding further supports an important role of GlyR in cortical function and dysfunction.

5 Presynaptic GlyRs Regulate Neurotransmitter Release

The presynaptic nerve terminal is a major regulatory site for activity-dependent changes in synaptic function. Presynaptic ionotropic receptors for a large range of transmitters have been found recently to be widespread throughout the central and peripheral nervous systems. Ionotropic neurotransmitter receptors on presynaptic nerve endings (McGehee and Role 1996; Miller 1998; MacDermott et al. 1999) often inhibit synaptic transmission (Eccles et al. 1963; Lerma 1997; Casado et al. 2000; Frerking and Nicoll 2000; Satake et al. 2000). As discussed in Sect. 3.2 of this chapter, in adults, glycine is an inhibitory neurotransmitter because it induces Cl^- influx (Werman et al. 1968; Krnjevic 1974; Nicoll et al. 1990; Kuhse et al. 1995). However, during early development, like GABA (Mueller et al. 1984; Cherubini et al. 1991), glycine can be excitatory because the intracellular Cl^- concentration is relatively high (Ben-Ari et al. 1997; Ehrlich et al. 1999; Rivera et al. 1999; Zhou 2001; Kilb et al. 2002; Laube et al. 2002).

Ionotropic presynaptic receptors modulate transmitter release by altering the membrane potential of the synaptic terminal. Classic studies of the neuromuscular junction (Dudel and Kuffler 1961; Takeuchi and Takeuchi 1966) and spinal motor neurons (Eccles et al. 1963) indicated that, at axo-axonic GABAergic synapses, activation of presynaptic $GABA_A$Rs could inhibit transmitter release. Opening the $GABA_A$-gated Cl^- channels will alter the membrane potential, reduce the input resistance of the nerve terminal, and act as an electrical shunt to reduce the amplitude of the action potential. Alternatively, membrane depolarization could reduce the presynaptic action potential by inactivating voltage-gated Na^+ channels in the terminal. This will result in a reduction of action-potential-evoked transmitter release.

That activation of presynaptic ionotropic GlyRs enhances transmitter release was first shown in the giant calyceal synapse in the medial nucleus of the trapezoid body. Several later studies of more conventional (tinier) synapse receptors in the spinal cord and in the brain of young rats have also revealed the facilitating actions of activation of presynaptic GlyRs (Jeong e al. 2003; Kawa 2003; Ye et al. 2004). Currently it is unknown whether the presynaptic GlyRs share the same subunit composition with the postsynaptic GlyRs. However, the presynaptic GlyRs seem to have different physiological and pharmacological properties. They have higher sensitivity to glycine and to picrotoxin, suggesting a different subtype composition (Jeong et al. 2003; Ye et al. 2004). According to expression studies, α-homomeric GlyRs may be more sensitive than αβ-heteromeric GlyRs (Bormann et al. 1993; Handford et al. 1996). Differential pre- and postsynaptic actions of picrotoxin also indicate different properties of pre- and postsynaptic GlyRs (Jeong et al. 2003).

The temporal patterns of actions of presynaptic GlyRs differ with brain areas. Activation of presynaptic GlyRs enhances transmitter release and seems persistent to the maturation in neurons of the medial nucleus of the trapezoid body (Turecek and Trussell 2001; Sect. 5.1), but disappears within two weeks of birth in cerebellum neurons (Kawa 2003; Sect. 5.2). Interestingly, activation of presynaptic GlyRs enhances transmitter release in young neurons but inhibits release in older neurons of the ventral tegmental area (Ye et al. 2004; Sect. 5.4). Further studies are needed to determine the mechanisms underlying the differences. The persistence of the receptors and the changes of intracellular Cl^- levels may be the major contributors to the differences described.

5.1 Presynaptic GlyR Regulation of Glutamate Release in the Giant Calyx Synapse

Using pre- and postsynaptic recordings, Turecek and Trussell (2001) were the first to show that at the calyx synapse exogenously applied glycine enhanced the amplitude of AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) induced by electrical stimulation. This is the first observation in the CNS of enhancement of synaptic transmission by a presynaptic anion channel. These investigators provided the following two major pieces of evidence that glycine acts on the presynaptic site: (i) Glycine greatly reduced paired-pulse facilitation and enhanced the frequency of miniature EPSCs. (ii) More importantly, blocking the voltage-gated Ca^{2+} channels prevented the increase in frequency of miniature EPSCs by glycine. This suggested that glycine might enhance nerve-evoked transmission by elevating intraterminal [Ca^{2+}]. This notion is supported by the following experiments: a membrane permeable EGTA, which increased the Ca^{2+} buffering capacity of the synapse, abolished the glycine-induced facilitation of action-potential-triggered release. These investigators then recorded from the presynaptic terminal itself to fur-

ther investigate the mechanism underlying glycine-induced enhancement of glutamate release. They showed that glycine produced a depolarization of about 10 mV at the nerve terminal resting membrane potential, indicating that the intraterminal [Cl^-] is normally high in the calyx nerve ending. In a later study, the same laboratory showed that this small (10 mV) change in membrane potential activates voltage-gated calcium channels and enhances release (Awatramani et al. 2005; Sect. 5.5).

Turecek and Trussell (2001) also investigated whether synaptically released glycine can activate presynaptic receptors. They showed that the amplitude of the EPSC was enhanced when glycinergic fibers were independently stimulated with a train of action potentials. This result indicates that glycine "spillover" (Isaacson 2000) from synapses on postsynaptic cells in the medial nucleus of the trapezoid body can reach concentrations sufficient to activate glycine channels on the calyx terminal (Vitten and Isaacson 2001).

5.2
Presynaptic GlyRs Regulate Glutamate and Glycine Release on Purkinje Cells of the Cerebellum

Using the whole-cell patch-clamp technique, Kawa (2003) showed that exogenous application of glycine to Purkinje cells in cerebellar slices from P3–P10 rats immediately increased the frequencies of both EPSCs and glycinergic IPSCs. Glycine-induced facilitation showed maximum at P5–P6 for EPSCs and at P9–P10 for IPSCs, and decreased thereafter. Strychnine (1 μM) suppressed glycine-induced facilitation, indicating that the effects of glycine were mediated by the strychnine-sensitive GlyRs. Furthermore, Kawa showed that other glycinergic agonists, including α-L-alanine (1 mM), L-serine (1 mM), and taurine (500 μM) produced facilitation similar to that of glycine. It was postulated that the GlyRs are expressed transiently but profoundly in the developing cerebellum, and that the distributions of these receptors causing excitation are different at excitatory and inhibitory presynaptic neurons. The GlyRs may play distinct roles in the maturation and organization of cerebellar neural circuits (Kawa 2003).

5.3
Activation of Presynaptic GlyRs Facilitates Glycine Release in Spinal Cord

In mechanically dissociated rat spinal sacral dorsal commissural nucleus (SDCN) neurons, in which functional presynaptic nerve terminals remain adherent to the isolated neurons, Jeong et al. (2003) showed that exogenous application of glycine (3 μM) increased the frequency of glycinergic spontaneous glycinergic IPSCs without affecting their amplitudes or decay times. This suggests that glycine acts presynaptically to increase glycine release probability. These investigators further showed that picrotoxin, an antagonist

of the homomeric GlyRs, at a concentration that had little direct effect on spontaneous IPSC frequency and amplitude (30 μM), significantly attenuated glycine-induced presynaptic spontaneous IPSC facilitation. Furthermore, the glycine-induced facilitation of spontaneous IPSC frequency was completely abolished either in a Ca^{2+}-free external solution or in the presence of 100 μM Cd^{2+}, a nonspecific blocker of the voltage-gated calcium channels. These results suggest the involvement of extracellular Ca^{2+} influx into the nerve terminals in glycine-induced facilitation. The glycine-induced facilitation of glycine release was also completely occluded in the presence of tetrodotoxin, a blocker of voltage-gated Na^{+} channels. These investigators also performed experiments in SDCN neurons in spinal cord slices to assess the effects of glycine in more physiological conditions. They found that glycine (10 μM) increased the amplitude of evoked IPSCs and decreased paired-pulse facilitation. Additionally, they showed that in response to brief high frequency stimulus trains the evoked IPSCs displayed a profound frequency-dependent facilitation that was greatly reduced by picrotoxin (30 μM). Based on these results, it was proposed that glycine acts at presynaptic autoreceptors, causing depolarization of the glycinergic nerve terminals, the subsequent activation of voltage-dependent Na^{+} and Ca^{2+} channels, and facilitation of glycine release (Jeong et al. 2003).

5.4 Presynaptic GlyRs Regulate GABA Release in Midbrain

That activation of presynaptic GlyRs regulates the release of GABA was recently shown in the dopaminergic (DA) neurons of the ventral tegmental area (VTA) of rats (Ye et al. 2004). These investigators recorded $GABA_A$ receptor-mediated evoked IPSCs from midbrain slices and spontaneous IPSCs from both the midbrain slices and mechanically dissociated neurons in which many functional terminals were preserved. They showed that in young rats (P3–P10), where GABA is excitatory, glycine (1–3 μM) and taurine (10–30 μM) increased the amplitude of evoked IPSCs and the frequency of spontaneous IPSCs but had minimal postsynaptic effects. They further showed that paired-pulse facilitation, a short-term form of synaptic plasticity that is highly sensitive to manipulations that alter transmitter release, was greatly reduced in the presence of glycine. This indicates that glycine acts presynaptically in enhancing GABA release. Strychnine (1 μM) blocked the action of glycine, indicating that the conventional strychnine-sensitive GlyRs mediate glycine-induced enhancement of GABAergic IPSCs. When applied alone, strychnine reduced the amplitude of evoked IPSCs and the frequency of spontaneous IPSCs. This indicates that GABAergic IPSCs were tonically activated by some endogenous glycine agonist(s), such as glycine and taurine. They further showed that glycine-induced facilitation of GABA release depended on voltage-gated Ca^{2+} channels. In external solution containing no

Ca^{2+}, or when Cd^{2+} or tetrodotoxin was added to the medium, the amplitude and especially the frequency of spontaneous IPSCs greatly diminished, and glycine-induced facilitation was abolished. Furthermore, using fura-2 fluorescent imaging, these investigators were the first to show a glycine-induced increase of $[Ca^{2+}]$ in nerve terminals in the CNS. Furthermore, this increase in $[Ca^{2+}]$ was suppressed by strychnine or 3 μM ω-conotoxin MVIIA. Therefore, the presynaptic GlyR-mediated facilitation of GABAergic transmission seems to be mediated by N- and/or P/Q-type Ca^{2+} channels. Importantly, these investigators further showed that in more mature rats (P22–P30), where GABA causes inhibition, the effect of strychnine on GABAergic IPSCs was reversed to facilitation, indicating a tonic glycinergic inhibition of GABA release in this age group of animals. Furthermore, glycine (1–3 μM) reduced the amplitude of evoked IPSCs and the frequency of spontaneous IPSCs. Based on these findings, it was proposed that the overall effect of the presynaptic action of glycine is to enhance the firing of DA cells, both in very young and older rats (Ye et al. 2004).

5.5 Modulation of Transmitter Release by Presynaptic Resting Potential and Background Calcium Levels

The release of transmitters is triggered and modulated by the rise of Ca^{2+} in the presynaptic nerve terminals. Many factors, including the activation of presynaptic receptors and channels can increase the level of Ca^{2+} in the presynaptic terminals (MacDermott et al. 1999). However, the mechanisms underlying the effects of receptor activation are not fully understood. The activation of GlyRs and $GABA_A$Rs weakly depolarizes the presynaptic terminals, increases intraterminal Ca^{2+}, and facilitates release at some synapses (Jang et al. 2002; Turecek and Trussell 2001, 2002; Ye et al. 2004). It was not known how a small depolarization can increase intraterminal Ca^{2+}, given that, Ca^{2+} channels in the calyx of Held are thought to require depolarization positive to – 45 mV for their activation. This is much higher than achieved by receptor activation (<– 55 mV). In addition, it has been shown that Ca^{2+} accumulation-mediated synaptic facilitation is achieved by conditioning depolarizations to – 30 mV or more (Felmy et al. 2003).

To study the relationship between resting potential, Ca^{2+}, and exocytosis, Awatramani et al. (2005) employed simultaneous pre- and postsynaptic electrophysiological recordings and presynaptic Ca^{2+} measurements at the calyx of Held. They showed that elevation of resting membrane potential between – 80 and – 60 mV led to activation of P/Q-type Ca^{2+} currents of < 1 pA and increased intraterminal Ca^{2+} by < 100 nM, a gradual rise in the background level of Ca^{2+}. They further showed that the small Ca^{2+} elevations were sufficient to enhance the probability of transmitter release up to twofold, with no effect on the readily releasable pool of vesicles. More-

over, these investigators showed that the effects of mild depolarization on release had slow kinetics and were abolished by 1 mM intraterminal EGTA, suggesting that Ca^{2+} acted through a high-affinity binding site. Based on these findings, these investigators proposed that apresynaptic Ca^{2+} channels serve to control release in two ways: (i) the rapid activation and deactivation of the channels mediates phasic exocytosis, while (ii) their sensitivity to resting potential alters ambient Ca^{2+} levels and thus controls release probability. Together, these studies suggest that control of resting potential is a powerful means for regulating synaptic function at mammalian synapses (Awatramani et al. 2005).

5.6
Excitable GlyRs Containing the NR3 Family of NMDA (N-methyl-D-aspartate) Receptor Subunits

Glycine is best known as an independent agonist of a strychnine-sensitive GlyR, which is selective for Cl^{-}. In addition, glycine is also well known for its role as a co-agonist of the NMDA receptor, which is permeable to Ca^{2+} and cations. However, in a recent report, Chatterton et al. (2002) provided evidence that glycine also activates a GlyR that is insensitive to strychnine, is not permeable to Cl^{-}, but is selective to Na^{+}. Upon activation, this GlyR induces neuronal excitation. The glycine response was evoked by glycine alone and inhibited by D-serine but not by 2-amino-5-phosphonopentanoic acid (APV), an agonist of the NMDA receptor, and picrotoxin, or strychnine in Xenopus oocytes co-expressing NR1 and NR3B (Chatterton et al. 2002). It was suggested that glycine-evoked spiking activities originating from out-of-voltage-clamp regions reflected the excitatory GlyR in cortical neurons (Chatterton et al. 2002). However, in a later study, an excitatory effect of these GlyRs was not seen (Matsuda et al. 2003). Apparently, more research is necessary to resolve the difference.

6
Conclusion

Our knowledge regarding GlyR regulation of excitation has advanced significantly in recent years. GlyRs are widespread and not restricted to the spinal cord and brain stem, particularly during early development. GlyRs have also been found in many forebrain areas of mature animals. Further investigations will uncover how these GlyRs, in conjunction with $GABA_A$Rs, control and maintain the balance of the CNS. GlyRs are heterogenous and undergo developmental changes. Activation of GlyRs on immature neurons induces outflow of Cl^{-}, membrane depolarization, neuronal excitation, and Ca^{2+} influx. These effects of GlyRs are particularly important in the context that

GlyRs are present before the synapse genesis and when glutamatergic receptors are quiescent. GlyRs are also found in the presynaptic terminals of both excitatory and inhibitory synapses in several brain areas and in the spinal cord. Activation of these presynaptic GlyRs induces an increase in the release of transmitters in the neurons of young animals but a decrease in neurons of older animals. The regulation of transmitter release is due to the change in presynaptic resting potential and background calcium levels. The physiological role of the GlyRs in the many forebrain areas is not yet fully understood. There has been important progress in analysis of the relationship between development and the functional properties of GlyRs, but correlations between the structure of different GlyR subunits and their functional properties is far from being well understood. Studies on the properties of the presynaptic GlyRs and their function are just starting. The exploration of whether GlyRs exist on all synaptic terminals and how they regulate the release of transmitters, as well as the dynamic changes of their distribution and action will be crucial in understanding how presynaptic GlyRs contribute to signal integration in the CNS.

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Regulation of Excitability by Potassium Channels

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Abstract Neurons express a large number of different voltage-gated potassium (Kv) channels with distinct biophysical and biochemical properties. Possibly, this diversity reflects the need to regulate and fine-tune neuronal excitability at various levels of complexity in space and time. In this context, Kv channels operating in the subthreshold range of action- potential firing are of particular interest. It is likely that these Kv channels play a prominent role in both propagating and integrating dendritic signaling, as well as axonal action-potential firing and propagation.

1
Introduction

K^+ channel α-subunits represent a large family of structurally related proteins. Distinct assembly of the α-subunits to tetramers forms functional K^+ channels which may differ in their biophysical properties, such as voltage range of activation, kinetics and behavior of gating, single channel conductance, sensitivity to protein kinases/phosphatases, and modulation by extra- and/or intracellular signaling pathways (Hille 2001). The resulting diversity of K^+ channels is, in fact, significantly larger than those of Na^+, Ca^{2+} or Cl^- channels. In this context, one of the most intriguing questions is to understand why there are so many different K^+ channels and what makes K^+ channels different from one another. I will discuss the hypothesis that K^+ channel diversity evolved to meet various needs of neurons to regulate and/or modulate their excitability.

K^+ channels are enzymes which catalyze the selective passage of potassium ions across the plasma membrane and thereby produce a K^+ current (for a recent review, see Gouaux and MacKinnon 2005). The concentration of potassium is usually $\sim$25-fold higher on the cytoplasmic than on the extracellular side of the plasma membrane. The respective electrochemical gradient provides the basis for K^+ channels to generate an outward current. This enzymatic K^+ channel activity determines how K^+ channels can contribute to neuronal excitability. K^+ channel activation resulting in an outward K^+ current will dampen neuronal excitability and may shift the membrane potential toward more negative voltages. Accordingly, K^+ channel closure or inactivation may produce an increase in neuronal excitability. I will restrict the

definition of neuronal excitability to the capacity of a neuron to fire action potentials (APs). Thus, we may only consider contributions of K^+ channel activity to neuronal AP firing. Then with respect to K^+ channels one may encounter three distinct scenarios: (1) K^+ channel activity regulates initiation of APs; (2) K^+ channel activity determines AP duration; and (3) K^+ channel activity occurs after AP firing has ended.

Numerous studies have revealed various aspects of the roles of K^+ channel activity in neuronal AP firing. The results showed that K^+ channel activities are involved in all three scenarios, e.g., K^+ channel activity may take a share in the regulation of AP initiation, AP propagation, AP duration, AP frequency, AP back propagation, and AP afterhyperpolarization. This framework sets the stage to consider the role of a particular K^+ channel before, during, and/or after AP firing. In particular, properties like operating voltage range, activation/inactivation, and compartmentalization to dendritic and/or axonal localizations can provide valuable information regarding the role of K^+ channels in regulation and/or modulation of neuronal excitability.

Voltage-gated K^+ (Kv) channels seem to play a prominent role among the many K^+ channels that have been reported to be involved in the three scenarios of AP firing. Kv channels are assembled from Kv α-subunits as homo- or heterotetramers. Approximately 70 genes may encode Kv α-subunits. In many cases the genes give rise to splice variants. Thus, a picture emerges in which each neuron in the mammalian nervous system could possibly express its own individual set of Kv channels. There are good reasons to think that this may not be so, but it is important to keep in mind that this possibility exists. Also, it illustrates the difficulty in describing comprehensively even one K^+ channel subfamily. Therefore, this review is restricted to only a few Kv channels with particular emphasis on their involvement in AP firing and AP propagation.

2 Kv Channel Structure

Several landmark publications, in particular by MacKinnon and collaborators (MacKinnon 2004), have described crystal structures of potassium channels, including one of Kv1.2 (Long et al. 2005). The reader is referred to these publications, which provide, in combination with extensive mutational analyses, considerable insights into the makings of the selectivity filter, the mechanisms of K^+ channel pore opening, and the coupling between K^+ channel gate and the voltage sensor and how these K^+ channel domains may be associated with conformational changes correlated to transitions between the closed and opened K^+ channel. Selectivity filter, pore, gate, and voltage sensor are membrane-integral domains of the Kv channel. A comparison of the available crystal structures indicates important features of K^+ channels:

1. The K^+ selectivity filter has a highly conserved structure and is located in that part of the pore which faces the extracellular side.
2. The structure of the pore on the intracellular side may form the gate of the pore. It appears more variable, which is possibly correlated with conformational changes occurring during opening and closing of the pore.
3. The pore is built from four subunits. Each subunit contributes to the pore structure, from amino- to carboxy-terminus, an outer helix (M1 or S5), a pore helix, and an inner helix (M2 or S6).
4. The pore structure is connected to the voltage-sensor domains via short linkers located at the amino-terminal side of S5. This places the voltage sensor amino-terminal to the pore.
5. Voltage sensors are allosteric sites that influence the enzymatic K^+ channel activity.
6. K^+ channels may contain additional allosteric sites for regulation by ligands or posttranslational modifications.

In addition to the membrane-inserted core structure comprising pore and voltage sensor, Kv channels contain amino- and carboxy-terminal domains. They have important regulatory and modulatory functions, e.g., in Kv α-subunit tetramerization, in interactions with other proteins (β-subunits, scaffolding proteins, Ca^{2+}-binding proteins), and in ligand binding (Ca^{2+}, cAMP, PIP_2). Tetramerization domains may be found in either the cytoplasmic amino-terminus (Li et al. 1992; Kreusch et al. 1998) or the cytoplasmic carboxy-terminus (Ludwig et al. 1997; Schmidt et al. 2000). This topological difference can have important consequences for the regulation of Kv channel activity. An amino-terminal tetramerization domain connects to the voltage sensor which is linked to the outer helix of the pore. For carboxy-terminal tetramerization domains a comparable topology may be observed. Here, a visual inspection suggests that the tetramerization domain may connect to a ligand-binding domain linked to the inner helix of the pore. Thus, it may be linked directly to the gate of the Kv channel (Jiang et al. 2001). It can be expected that the topological distinction between amino- and carboxy-terminal tetramerization domains has an impact on the control of Kv channel activity, yet details remain to be determined.

A present picture emerges that Kv channels with an amino-terminal tetramerization domain are voltage gated, whereas Kv channels with a carboxy-terminal tetramerization domain are typically sensitive to both changes in voltage and ligands. A well-studied example of the latter is represented by the BK channel. This channel contains a voltage sensor and a carboxy-terminal Ca^{2+} sensor (Jiang et al. 2001). Both sensors may be regarded as allosteric sites like the ones described for the regulation of allosteric enzyme activity (Cui and Aldrich 2000). Accordingly, allosteric mechanisms well describe regulation of BK channel activity by voltage and Ca^{2+}. Possibly, carboxy-terminal ligand-binding sites in other Kv channels contain compar-

able allosteric sites to regulate Kv channel activity and respond to ligands such as the second messengers Ca^{2+}, cAMP, or PIP_2. The presence of allosteric sites in a Kv channel has the important consequence that it enables the respective Kv channel to respond to two different signaling pathways at a time. This property conveys a coincidence detector-like behavior and may be exploited for the integration of neural signaling pathways. Note that ligands can elicit opening (activation) or closing (inhibition) of the respective Kv channel. It follows that a stimulus which activates a Kv channel, a Kv channel opener, will be correlated with a reduction or attenuation of neuronal excitability. Vice versa, a stimulus which inhibits a Kv channel will be correlated with an increase in neuronal excitability.

3 Kv Channels

Many Kv channels are direct relatives of the *Shaker* family (Hille 2001). They contain an amino-terminal tetramerization domain and a voltage sensor. *Shaker*-related channels may activate over a broad range of membrane potentials. Thresholds for the activation of cloned Kv channels in in vitro expression systems vary, e.g., between – 70 and + 40 mV. In broad terms this range corresponds to the one that may be encountered in neurons at rest and during AP firing. The different voltage sensitivities of Kv channels imply that they have different impacts on AP initiation and firing. Kv channels with an activation threshold in the – 70 to – 40 mV range apparently operate in the subthreshold range of AP firing, whereas those which activate at more positive potentials may participate in AP repolarization and afterhyperpolarization. For example, Kv channels of the Kv4 subfamily activate at relatively negative membrane potentials in the subthreshold range of AP firing (Hoffmann et al. 1997). Others, such as the ones of the Kv2 family, behave like delayed rectifiers in AP repolarization, and yet others, the so-called high-threshold Kv channels like the ones of the Kv3 family (Rudy and MacBain 2001; Lien and Peter 2003), may activate at relatively positive membrane potentials.

Most Kv channels appear to rapidly activate and deactivate in the time range of tens of milliseconds, but note important exceptions like the M channel. Some Kv channels, the so-called A-type channels, do not stably activate, but rapidly inactivate. The inactivation kinetics of A-type Kv channels may vary over a relatively large time range, lasting from tens of milliseconds to seconds. Inactivation mechanisms have been studied for cloned A-type Kv channels in great detail (for review, see Kurata and Fedida 2005). The results showed that quite different mechanisms can cause Kv channel inactivation. Discussion of the various mechanisms is not within the scope of this review. In the context of neuronal excitability it is important to note that inacti-

vated A-type Kv channels are refractory to activation for certain time periods until they have recovered from inactivation. Recovery time constants can vary greatly. For some A-type Kv channels, e.g., Kv4 channels, recovery time constants are in the millisecond time range, and for others, e.g., Kv1.4 channels, recovery time constants are in a time range of seconds. The recovery time constant for an inactivated A-type Kv channel may characterize a time window during which a neuron is less or more excitable (Pongs 1999). For example, it may define the time window during which a neuron can propagate or fire APs. Exploiting the refractoriness of A-type Kv channels to activation, a neuron may be able to filter incoming signaling inputs with certain frequencies and thus may obtain bandpass filtering properties. Bandpass filtering properties are an important means for a neuron to control frequencies of signal propagation along dendritic or axonal compartments (Hutcheon and Yarom 2000).

3.1
A-Type Kv Channels

A well-studied example of an A-type Kv channel and its role in neuronal excitability represents the A-type Kv channel family expressed by Kv4 family members. Kv4 A-type channels may be assembled as homo- or heterotetramers with Kv4.1, Kv4.2, and/or Kv4.3 α-subunits. The channels are conspicuously expressed in dendritic compartments, where they may function as a major determinant of dendritic excitability, e.g., in dendrites of CA1 pyramidal neurons (Hoffmann et al. 1997). Kv4 channel density increases in the main apical dendritic trunk with distance from the soma. Various ancillary subunits, such as KChIPs (K-channel interacting proteins) (An et al. 2000) and DPPX (Nadal et al. 2003) proteins may associate with Kv4 channels to multiprotein complexes. The ancillary subunits appear to influence the kinetics of Kv4 channel activation, inactivation, and/or recovery from inactivation in in vitro expression systems. It is not clear how these data reflect Kv4 channel complexes in distal or proximal dendritic compartments in vivo. The composition of native Kv4 channel complexes in distinct dendritic localizations remains to be determined.

Kv4 channels have several unique properties which make them stand out from other A-type Kv channels. Kv4 channels activate at negative membrane potentials, well before voltage-gated Na^+ channels are activated. Thus, Kv4 channels are active in the subthreshold range of AP firing. Furthermore, Kv4 channels inactivate very rapidly and a fast recovery from inactivation takes place with time constants τ_{rec} in the range of $\sim$100 ms. Potentially, these properties can play important roles in several scenarios (see Pongs 1999). In one scenario, Kv4 channels may function as activity-dependent so-called dendritic shock absorbers. Kv4 channel activity prevents back propagation of APs in the dendrites. APs may only back propagate when Kv4 channels are inacti-

vated and the right of passage for a back-propagating AP is determined by the time constants τ_{rec}. In this case, APs could back propagate into the dendrite only with certain frequencies.

According to the high concentration of Kv4 channels in distal dendrites, another scenario proposes for Kv4 channels a regulatory role in dendritic excitability associated with incoming excitatory postsynaptic potentials (EPSPs). An incoming EPSP may depolarize the dendritic membrane and lead to inactivation of Kv4 channels permitting AP back propagation. Thereby, a temporal coincidence of EPSP and a dendritic spike can occur. The coincidence may enhance the dendritic spikes resulting in a greater influx of Ca^{2+} into the dendritic spines (Hoffmann et al. 1997; Schoppa and Westbrook 1999; Watanabe et al. 2002). Thus, the inactivation properties of dendritic Kv4 channels may influence integrations of dendritic excitability signaling and facilitate temporal summation of EPSPs (Wheal et al. 1998; Bernard et al. 2004). Furthermore, Kv4 channel activation, particularly at or near dendritic branch points, may limit the ability of a plateau potential in the stimulated dendrite to propagate into the adjacent dendrite and to trigger activation of voltage-dependent Ca^{2+} channels in the neighboring dendrite. Hence, a further important role of Kv4 channel activity might be to function as a gatekeeper which hinders the spread of a plateau potential from a stimulated dendrite to an unstimulated one (Cai et al. 2004).

Axonal compartments also express A-type Kv channels. They may have properties which are quite distinct from the ones of Kv4 channels. Thus axonal A-type Kv channels are encoded by different members of the Kv family. Interestingly, axonal A-type Kv channels may also have a gatekeeper role and may direct the propagation of APs at axonal branch points (Geiger and Jonas 2000). Two different molecular possibilities are known for how members of the Kv1 family assemble axonal A-type Kv channels. Kv1.4 channels are a typical example where the Kv1 α-subunit harbors an amino-terminal inactivating domain (Kurata and Fedida 2005). This domain confers rapid inactivation to Kv1 channels containing Kv1.4 subunits. An alternative consists in the assembly of Kv1 α-subunits with ancillary Kvβ subunits which may contain an amino-terminal inactivating domain, very similar in structure and function to that of the Kv1.4 α-subunit (Rettig et al. 1994). Both kinds of A-type Kv1 channels are activated in a voltage range which is close to the threshold of Na^{+} channel activation. Thus, A-type Kv1 channel activity in axonal localizations can potentially attenuate propagation of axonal APs, but the difficulties in recording ion channel activities and AP propagation from axons severely limit our knowledge about axonal A-type Kv channels.

In the hippocampus the gyrus dentatus sends out prominent axons to CA3 pyramidal neurons. Because of their typical morphology these axons are called mossy fibers. Mossy fiber boutons represent a primary site of presynaptic transmission of signals from the dentate gyrus to the CA3 region, which is critical for information processing in the hippocampus. Kv1.4 immunoreac-

tivity is associated with mossy fiber axons, particularly with "neck" portions of axons adjacent to synapse-bearing expansions (Cooper et al. 1998). The mossy fiber expansions themselves, i.e., the mossy fiber synapses, did not exhibit Kv1.4 immunoreactivity, but may contain immunoreactivity consistent with the expression of Kv1/Kvβ-type channels in the presynaptic mossy fiber. Thus, Kv1.4 may regulate the efficiency with which the AP may invade a presynaptic expansion (terminal) and Kv1/Kvβ-type channels may contribute in an activity-dependent manner to shaping propagated mossy fiber APs (Cooper et al. 1998). Possibly, two different A-type Kv channels are expressed in mossy fiber axons with different molecular identities and different localizations.

Patch-clamp recordings from hippocampal mossy fiber boutons have provided direct evidence for a dynamic control of presynaptic Ca^{2+} inflow by fast-inactivating Kv1 channels. The presynaptic A-type Kv channels were sensitive to the Kv1 channel antagonists tetraethylammonium (TEA) and dendrotoxin (Geiger and Jonas 2000). By contrast, Kv1.4 channels are relatively insensitive to block by TEA and dendrotoxin. This is consistent with the idea that mossy fiber terminals express Kv1/Kvβ-A-type channel activity. The timing and strength of synaptic transmission is critically dependent on shape and duration of presynaptic APs. They may undergo dynamic changes associated with presynaptic forms of plasticity, such as paired-pulse facilitation, frequency facilitation, and augmentation. Thus, mossy fiber boutons show marked activity-dependent AP broadening during repetitive stimulation (> 10 Hz). The combination of fast inactivation outset and slow inactivation recovery of Kv1 A-type channels generates a cumulative inactivation during repetitive stimulation that is also seen for K^+ currents in mossy fiber boutons. This gating behavior may underlie spike broadening and may implement a dynamic time range for increased synaptic strength on the timescale of seconds. Interestingly, the cumulative inactivation behavior of Kv1.4 channels is very sensitive to CaM-kinase II-dependent phosphorylation, suggesting a modulatory response to changes in presynaptic Ca^{2+} handling (Roeper et al. 1997). Potentially, this property adds to the dynamic changes seen in synaptic neurotransmitter release associated with AP broadening and enhancement of presynaptic Ca^{2+} inflow.

Another interesting A-type Kv channel is assembled from members of the Kv3 family including Kv3.4. A hallmark of Kv3 channels is that they are very sensitive to TEA and 4-aminopyridine (4-AP) (Rudy and McBain 2001). Kv3.4 channels expressed heterologously in vitro activate at potentials positive to – 40 mV very rapidly and also inactivate rapidly. Recovery from inactivation is faster than that of Kv1.4 channels, but markedly slower than that of Kv4 channels. The sea anemone toxin BDS I blocks Kv3.4 channels with high selectivity (Diochot et al. 1998). Thus, Kv3.4 channels and Kv3.4 containing heteromultimers are discernable from other Kv channels by their distinct pharmacology. Kv3.4 channels have been detected in pituitary nerve terminals both by immuno-electron microscopy and in patch-clamp recordings

(Fisher et al. 1993). Furthermore, the channels seem to occur in terminals which secrete vasopressin or enkephalin, but not in those secreting oxytocin. Oxytocin neurons may fire bursts of APs at high frequencies (40–80 Hz); vasopressin neurons, on the other hand, may produce a phasic firing consisting of periods in which APs are fired at 10–20 Hz alternating with silent periods (Wakerley et al. 1978). One may speculate that the silent periods are determined by the inactivation/recovery properties of Kv3.4 channels that are expressed in the vasopressin secreting nerve terminals.

3.2 Delayed Rectifiers

Numerous neurons have the capacity to discharge at high rates. These "fast spiking" (FS) neurons have important physiological functions throughout the mammalian central nervous system (CNS) and are critical participants in central motor and sensory circuits. The ability to fire brief APs at a high rate relies on APs that are nondecremental, of short duration, repolarize rapidly, and possess brief afterhyperpolarizations and interspike intervals. Kv3 channels characterized by positively shifted voltage dependencies and very fast deactivation rates have been proposed to represent major determinants of the FS phenotype and to enable repetitive firing at high frequencies (Rudy and McBain 2001). Apparently, coassembly with a distinct Kv3.4 splice variant enhances the repolarizing efficiency of Kv3.1 channels in FS neurons. It has been shown that Kv3.1/Kv3.4a heteromultimeric channels produce rapidly activating and deactivating currents with properties resembling closely those seen in neurons that spike at high rates in globus pallidus, inferior collicus, substantia nigra, and hippocampus (Lien and Jonas 2003; Baranauskas et al. 2003). The rapid activation of the current during the upstroke of the spike in FS neurons results in rapid repolarization of the membrane keeping the AP short and minimizing Na^+ channel inactivation. Apparently, the narrow spikes reduce the risk of compromising AP generation and membrane responsiveness. Upon repolarization, the Kv3 heteromultimeric channels deactivate quickly, removing any impediment to subsequent depolarizing influences. Thus, the rapid gating properties of Kv3 channels may enable neurons to sustain spiking at high frequencies (Baranaukas 2003). This proposition has been confirmed by studies in Kv3 knockout mice.

The Kv2 subfamily generates Kv channels which mediate currents resembling those of delayed rectifiers. Thus, Kv2 channels may be involved in AP repolarization. Kv2 channels are expressed in a large variety of excitable cells. Kv2 currents activate markedly slower than those mediated by Kv1-, Kv3-, or Kv4-type channels, and inactivate quite slowly in the time range of seconds (Frech et al. 1989). The Kv2 subfamily appears to consist of only two members (Kv2.1 and Kv2.2) which express Kv channels with similar gating characteristics. However, the Kv2 subfamily forms heteromultimers with a large number

of modulatory Kv α-subunits, consisting of Kv5.1, Kv6.1–6.4, Kv8.1–8.2, and Kv9.1–9.3 subunits (Drewe et al. 1992; Post et al. 1996; Salinas et al. 1997; Zhou et al. 1998; Kerschensteiner and Stocker 1999). When expressed in vitro, these modulatory Kv α-subunits are unable to form functional homomultimers, but selectively interact with Kv2 α-subunits. Interestingly, it has been shown in one example that the stoichiometry of Kv2.1 to Kv9.3 subunits is 3 : 1 (Kerschensteiner et al. 2005). Possibly, the presence of one nonfunctional Kv α-subunit can be tolerated to form functional heteromultimers. In comparison to homomultimeric Kv2 channels, currents arising from coexpression with modulatory Kv α-subunits may have diverging gating properties like slowed rise times and slowed inactivation kinetics. Furthermore, the voltages for half-maximal activation at steady state may be shifted either to more negative or to more positive voltages. This suggests distinct roles for the various heteromultimers in AP repolarization.

Some of the Kv2 heteromultimeric channels, e.g., Kv2.1/Kv9.3 heteromultimers, activate at potentials more negative than the threshold potential for AP firing (Kerschensteiner et al. 2005). Kv channels with this property are of particular interest and deserve a special note. The shift of steady-state activation to hyperpolarized potentials suggests that Kv2.1/Kv9.3 channel activity may contribute to the stabilization of the resting membrane potential. Furthermore, Kv2.1/Kv9.3 currents deactivate slowly (τ_{deact} at – 50 mV$\sim$80 ms at 25 °C). Potentially, this channel property may stabilize the resting membrane potential and provide some resilience of the neuron toward incoming depolarizations. Combined with voltage-dependent inactivation properties, which render the Kv2.1/Kv9.3 channel particularly sensitive to inactivation in the ascending phase to firing threshold, this makes an interesting case for a possible role of Kv2.1/Kv9.3 channels in pacemaker cells (Kerschensteiner and Stocker 1999). Thus, it has been proposed that the kinetics fits a possible function of the Kv2.1/Kv9.3 channel in regulating the steepness of pacemaker depolarizations. Probably, other Kv2.1 heteromultimeric channels, e.g., Kv2.1/Kv6.1 and Kv2.1/Kv6.2 channels, may similarly contribute to the resting membrane potential of neurons and other excitable cells. In comparison to the other Kv subfamilies, our knowledge of a modulatory role of a particular Kv2 channel in a distinct neuron is even more sketchy and rudimentary, and knowledge about in vivo functions of particular Kv channel-related delayed rectifiers is scarce. This lack of knowledge is largely due to the fact that delayed rectifiers, especially those expressed in axonal compartments, are still difficult to measure in situ.

3.3
M Channels

Since their discovery Kv7 (KCNQ) channels, denoted as M channels, have been of particular interest. Their name is derived from the original observa-

tion that a noninactivating potassium outward current in frog sympathetic ganglia is attenuated after activation of muscarinic acetylcholine receptors. Subsequently, M currents were recorded from numerous neurons in mammalian CNSs (for review see Delmas and Brown 2005). M channels are activated at membrane voltages negative to the firing threshold of APs. As these noninactivating Kv channels mediate a sustained outward current at negative potentials, they may exert a pivotal control over neuronal excitability and response patterns. M-channel activity potentially attenuates repetitive AP discharges. This proposition is supported by human genetic studies which have correlated a heritable form of epilepsy (BFNC—benign familial neonatal convulsions) with mutations in the genes encoding M-channel subunits (for a review, see Steinlein 2004). The mutations cause the assembly of dysfunctional M channels which may no longer function as efficient "brakes" in regulating neuronal excitability.

M-channel activity may be modulated through the activation of several G-protein coupled receptors (GPCRs). Apparently, the GPCRs have in common that their activation leads to activation of the G-protein Gq and, hence, of Gq-signaling pathways (Haley et al. 2000). How this finally results in inhibition of M-channel activity is still a matter of conjecture. Apparently, several pathways modulate M-channel activity including hydrolysis of PIP_2, changes in local Ca^{2+} concentration, and phosphorylaton by protein kinases (Delmas and Brown 2005). Expression of members of the KCNQ (Kv7) potassium channel family showed that heteromeric KCNQ2/KCNQ3 (Kv7.2/Kv7.3) channels expressed in vitro exhibit electrophysiological and pharmacological properties characteristic of native M channels (Jentsch 2000). The hallmarks are a negative threshold of activation and a slow deactivation at negative test potentials. KCNQ2 and KCNQ3 subunits can be coimmunoprecipitated from lysates of mammalian brain and they were colocalized immunohistochemically in slice preparations of rodent brains (Cooper et al. 2000). This includes colocalization on both the somata and the dendrites of pyramidal and polymorphic neurons in the hippocampus and cerebral cortex, and colocalization on the somata of parvalbumin-positive hippocampal interneurons. The data indicated that M channels may occur in many neurons at different localizations. M channels may be found at high concentrations in axon initial segments. They are also localized to the nodes of Ranvier in peripheral neurons, and in pre- as well as postsynaptic terminals. Thus, M-current inhibition may have important consequences for neuronal excitability.

Attenuation of M-channel activity increases dramatically neuronal firing and excitability in in vitro experiments. It may convert a neuron, such as a pyramidal hippocampal CA1 neuron, from a phasically firing neuron into one that fires tonically. Furthermore, inhibition of synaptic M channels may result in a slow depolarization leading to an enhanced discharge, and also may facilitate the response to glutamate and/or GABA (Delmas and Brown 2000). Also, M-channel activity contributes to afterhyperpolarizations

of medium duration (mAHPs) (Peters et al. 2005). Furthermore, M channels are expressed in inhibitory and excitatory neurons. In conclusion, an increase or decrease in M-channel activity may modulate neuronal excitability: activation of M-channel activity may dampen neuronal AP firing; alternatively, inhibition of M-channel activity may enhance neuronal AP firing frequencies. The various M-channel activities, and the notion that M channels are most likely not the only K^+ channels which modulate neuronal resting membrane potentials and membrane depolarization, make it difficult to delineate M-channel contributions to neuronal excitability in vivo.

A recent transgenic mouse model, in which M-channel activity in the brain was reduced through conditional expression of a human KCNQ2 pore mutant, supports the observations that epileptic seizures, abnormal hyperactivity, and some cognitive deficits may be associated with M-channel activity (Peters et al. 2005). Transgenic mice showed marked changes in electrophysiological properties of hippocampal CA1 pyramidal neurons: a substantially increased excitability, tonic firing properties, reduced spike-frequency adaptation, and attenuated mAHPs. Possibly, the most interesting observation was that membrane properties of transgenic pyramidal CA1 neurons were altered. Normally, CA1 pyramidal neurons exhibit an increased resonance in the theta frequency range in response to sinusoidal current inputs of increasing frequency in the subthreshold voltage range. The subthreshold theta resonance is not seen in pyramidal neurons of transgenic animals. Whether M channels are critical determinants of neuronal membrane properties associated with low- and high-pass filtering properties of single neurons needs further investigation. It remains to be seen whether M-channel activity may influence oscillatory neuronal network behavior in the theta frequency range. Genetically altered mice with attenuated M-channel activity may provide a valuable model to study the proposition that M channels are a primary target in cholinergic afferent pathways involved in mechanisms which selectively upregulate neuronal activity in cognitive processes. In this respect, M channels have the promise to become a prime example of regulation of neuronal excitability by a voltage-gated potassium channel.

4
Kv Channels in Nervous System Diseases

Considering the large Kv channel superfamily, mutants in only a few Kv channel genes have been associated so far with a human disease. Mutations in the KCNA1 gene, which encodes Kv1.1 subunits, have been associated with episodic ataxia type 1 (EA1) (Browne et al. 1994). EA1 is an autosomal dominant neurological disorder affecting central and peripheral nerve function, with symptomatic attacks of uncontrolled movements (Ashizara et al. 1983). EA1 mutations may cause the disorder through dominant negative effects or

haplotype insufficiency. Dominant negative effects are correlated with mutants that alter biophysical properties of Kv1.1 channels when expressed in vitro (Maylie et al. 2002). Haplotype insufficiency seems to be correlated with mutants that predominantly effect protein stability and intracellular transport to the membrane. Curiously, megencephaly is associated with a mutation in the KCNA1 gene. Megencephaly is a fatal condition comprising symptoms such as an enlarged brain volume and seizure-like behavior, and may be a frequent cause of sudden infant death syndrome. A megencephaly mouse model (*mceph/mceph*) showed a mutation in the KCNA1 gene that truncates Kv1.1 protein by $\sim 50\%$ (Klement et al. 2003). Whether similar mutations are associated with megencephaly in humans is, however, not known.

A knockout mouse line has been generated carrying the EA1 mutation V4O8A (Herson et al. 2003). V408A is a missense mutation altering the sequence of Kv1.1 subunits that contributes to the central pore structure of potassium channels. Heterologous expression of homomeric Kv1.1 V408A channels showed that the mutation induces an unstable open state compared to wild type (WT), resulting in a tenfold increase in the deactivation rate, a slowing of Kvβ1-induced N-type inactivation, and a tenfold increase in recovery from N-type inactivation (Maylie et al. 2002). The effects on Kv1.1 channel gating have a dominant negative character when coexpressed with WT Kv1.1 or Kv1.2 subunits. In the cerebellum, Kv1.1 is primarily localized within axon terminals of the plexus region of GABAergic basket cells and interneurons of the granule cell layer. This suggests that Kv1.1-type channels may have a functional role in the control of GABA release and thus may affect control of motoric behaviors. The Kv1.1 V408/+ mice showed stress-induced loss of motor coordination.

At the cellular level, it was shown that Kv1.1 V408A/+ cerebellar Purkinje cells produced spontaneous GABAergic inhibitory postsynaptic currents (IPSCs) at increased frequency and amplitude. These results provided a rational link between Kv1.1 mutations and the EA1 phenotype, suggesting that they are linked to altered GABA release. Kv1.1 subunits are localized to juxtaparanodal regions in cerebellar basket cells and are highly concentrated at axonal branch points, suggesting that Kv1.1-containing channels may influence the success rate of AP propagation down the axon (Wang et al. 1994; Tan and Llano 1999). It has been proposed that the rapid deactivation of V408-containing channels reduces AP propagation failure at axonal branch points, either by decreasing K^+ accumulation and/or by shortening the refractory period, and thus more APs successfully invade the presynaptic GABAergic terminal, raising the IPSC frequency (Zhou and Chiu 2001). The pattern of subcellular localization at or near presynaptic terminals suggests that Kv1.1-containing channels may be important for regulating neurotransmitter release by influencing the local membrane potential and by modulating the amount of calcium that enters the axon terminal, rather than contributing to AP repolarization.

The phenotype of the *mceph/mceph* mouse mutant deviates from the above described mutant mouse carrying the V408A mutation. The KCNA1 V408A mutant mice and also KCNA1 knockout mice have a normal-sized brain (Herson et al. 2003). By contrast, the *mceph/mceph* mice have an enlarged brain and show hypertrophic cells in hippocampal slices (Klement et al. 2003). In addition, the density of other Kv channels like Kv1.2 and Kv1.3 seems to be reduced in *mceph/mceph* mice, possibly explaining both the increased neuronal excitability observed in patch-clamp experiments on mossy cells in hippocampal slices and the epileptic phenotype.

Mutations in the M-channel genes KCNQ2 (Kv7.2) and KCNQ3 (Kv7.3) are associated with the syndrome of BFNC, an autosomal, dominantly inherited epilepsy of the newborn (for reviews, see Jentsch 2000; Steinlein 2004). The mutations may be either missense mutations in the transmembrane region of the subunits or may lead to truncations of the cytoplasmic carboxy-terminus. Most BFNC mutations appear to cause only modest reductions in M-channel current densities in heterologous expression systems. As epileptic discharges correspond to highly synchronized and high-frequency activity of neurons, a modest M-channel deficiency may already lead to an augmented input–output relationship. Intuitively, such changes in neuronal excitability may be the cause of epileptic discharges. However, it is still unclear whether or not modest reductions in M-channel activity are sufficient to cause epilepsy. Recently, a mouse model has been described (Peters et al. 2005) in which a conditional expression of a dominant-negative KCNQ2 (Kv7.2) subunit in the brain caused a reduction in M-channel current density in hippocampal pyramidal neurons, which was associated with increased neuronal hyperexcitability. Since the transgene was expressed in many brain regions, it probably reduced M-channel activity and increased excitability in other neurons as well. Notably, the mice suffered under benign spontaneous seizure attacks. Thus, the mice may provide a good model in which to study a BFNC-related disorder in more detail. Also, patients with BFNC have been described in which the seizures did not respond well to antiepileptic drugs (Dedek et al. 2003). Potentially, the transgenic mice provide a promising model to investigate both drug resistance and new pharmacotherapies.

Changes in the input–output relationship of neurons may be caused by plastic changes on the synaptic level. It has been reported that a decrease in Kv4.2 channel activity may be acquired in experimental temporal lobe epilepsy (TLE) (Bernard et al. 2004). Apparently, transcriptional and posttranslational mechanisms are involved in producing a decrease in Kv4.2 channel activity. At the transcriptional level, Kv4.2 mRNA transcription is reduced leading to a decrease in Kv4.2 channel protein. At the posttranslational level an increased phosphorylation was observed by an extracellular signal-related kinase which leads to a decrease in Kv4.2 channel activity. Given the role of Kv4.2 channels in regulating dendritic AP back propagation, it is likely that a downregulation of Kv4.2 channel activity is likely to increase neuronal

excitability in multiple ways and, thereby, to alter neuronal input–output ratios. Studies showed that dendritic excitability in CA1 pyramidal neurons is increased in an animal model of TLE during the chronic phase of epilepsy (Bernard et al. 2004). This excitability potentially facilitates seizure initiation in the hippocampus, a frequent site of focal seizure onset in human TLE.

5 Concluding Remarks

Neurons need to regulate and fine-tune their excitability properties and input–output relationships in response to many different signals. The responses occur at specific sites in neuronal space, in distinct time windows, and over a wide range of frequencies. It is likely that the complexity in neuronal excitability requires the large number of K^+ channels with distinct biophysical and biochemical properties which the brain expresses. For Kv channels we discern channels that operate in the subthreshold range of AP generation or are activated during and/or after the initiation of an AP. In the three categories, Kv channels may show fast or slow activation/deactivation kinetics. Also, the channels may inactivate and recover from inactivation rapidly, in a medium time range or slowly. Furthermore, Kv channels are targeted to distinct neuronal compartments and localizations. In addition, Kv channels may distinctly respond to different protein kinases or protein phosphatases, and to extracellular and intracellular signaling pathways. All of this adds up to the observed diversity of Kv channels in the nervous system. In recent years, many studies have characterized distinct Kv channels at the single neuron level. But what we know about the physiological function of these channels in the nervous system is largely derived by extrapolation from in vitro studies. Our knowledge is too limited to describe the role of a Kv channel at an integrated in vivo level. Too little is known about densities and localizations of distinct Kv channels at particular dendritic, somatic, and/or axonal sites in excitatory or inhibitory neurons. Hopefully, the future will bring us an advancement in available technologies to study nervous system functions, thus yielding a better understanding of physiological roles of neuronal Kv channels.

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Modulation of Excitation by Metabotropic Glutamate Receptors

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Abstract Metabotropic glutamate receptors, in contrast to ionotropic glutamate receptors, do not form ion channels but instead affect intracellular chemical messenger systems. They couple via GTP-binding proteins ("G-proteins") to a variety of effectors such as ion channels and thus give glutamate, the major excitatory transmitter in the CNS, the ability to modulate processes involved in excitatory synaptic transmission. Therefore, excitatory synaptic transmission is regulated not only by the conventional GABAergic but also by the glutamatergic mechanisms themselves. Many metabotropic glutamate receptors are localized outside the immediate vicinity of transmitter release sites, thereby setting specific requirements for their activation, such as cooperation between synapses, burst activity, and glial involvement in the regulation of ambient glutamate levels.

1 Metabotropic Glutamate Receptors

Glutamate was proposed as a fast neurotransmitter around 50 years ago and glutamate-gated ion channels (ionotropic glutamate receptors) have been recognized as the workhorse for fast excitatory synaptic transmission in the mammalian brain for the last 20 years (Watkins 2000). Subsequently, metabotropic glutamate receptors (mGluRs) were discovered (Masu et al. 1991; Tanabe et al. 1992). They differ from ionotropic glutamate receptors in that they do not form ion channels but instead affect intracellular chemical messenger systems that involve a multitude of signal transduction pathways. These intracellular cascades allow glutamate to function both as an excitatory and inhibitory transmitter (Coutinho and Knöpfel 2002).

mGluRs are a family of proteins that have seven transmembrane segments and that couple to G-proteins. Eight genes coding for different subtypes of mGluRs have been identified to date, numbered in the order in which the cDNAs were cloned. Based on sequence similarities, principal signal-transduction capabilities in recombinant expression systems, and pharmacological properties, the family of mGluR subtypes is divided into three groups. In recombinant expression systems, such as human embryonic kidney (HEK 293) and Chinese hamster ovary (CHO) cells, group I mGluRs (consisting of mGluR1 and 5) couple to phospholipase C and thereby activate

the inositol 1,4,5-trisphosphate (IP_3)/Ca^{2+} signaling pathway. The group II (mGluR2 and 3) and group III (mGluR4, 6, 7, and 8) subtypes inhibit adenylate cyclase and thereby inhibit production of cyclic AMP (Tanabe et al. 1992). While this early classification scheme and the principal signal transduction mechanisms associated with each of the mGluR subtypes have been very useful for pharmacological studies and for development of subtype-specific agonists and antagonists, it needs to be stressed that the mGluR-subtype-related signaling cascades differ significantly between cell types. For instance, mGluR1 affects G-protein-independent transduction pathways and couples to adenylate cyclase in some cellular environments (Heuss and Gerber 2000; Hermans and Challiss 2001). On the effector side, group II/III mGluRs can

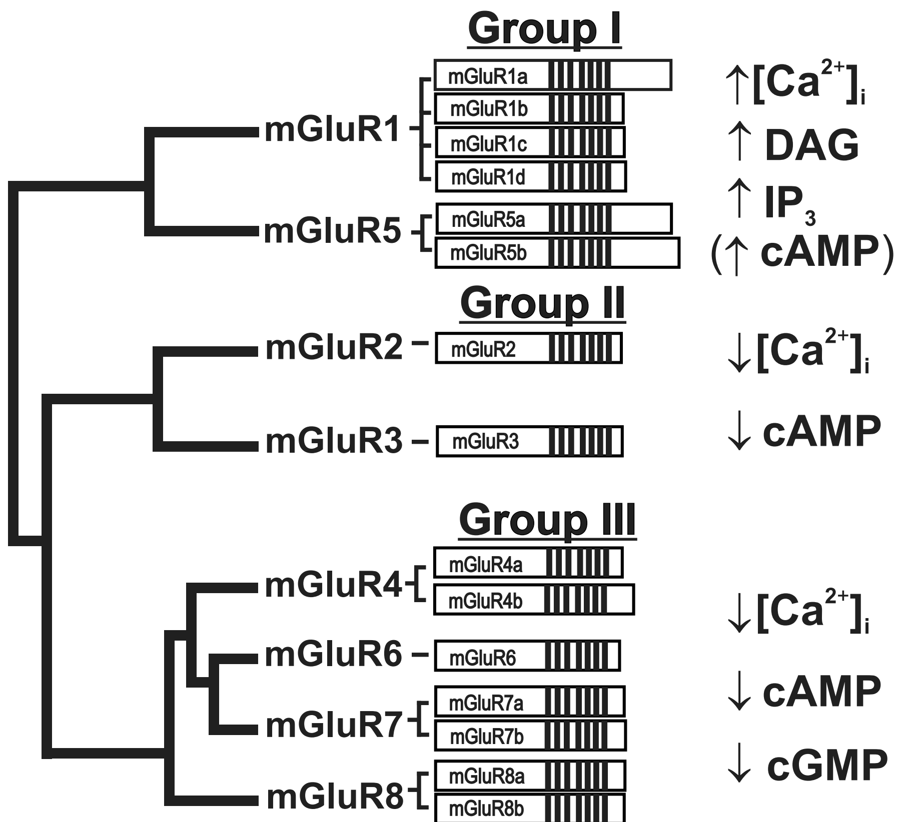

Fig. 1 Classification and chemical signaling pathways of mGluRs. The dendrogram of sequence homology clusters the known mGluR subtypes into three groups. The grouping by homology corresponds well to the signal transductions pathways shown in the *right panel*. Different splice variants are indicated by *boxes*. *Vertical lines* represent transmembrane domains that separate the homologous N-termini and the splice variant specific C-termini

downregulate Ca^{2+} channels (see below). The classification scheme for the family of mGluRs along with the repertoire of second messenger systems affected by mGluRs is shown in Fig. 1. This guideline can be used to systematize the effects on excitatory synaptic transmission and excitability, which will be focused on in the following paragraphs. A second guideline (for which there are also plenty of exceptions) is that group I mGluRs are expressed mainly on the dendrites and cell bodies of neurons; hence, the effect of group I mGluR activation is mainly postsynaptic. Synaptic terminals, in contrast, are typically equipped with group II and III mGluRs.

In the following sections we will review mGluR-mediated regulation of glutamate release and of the conversion of the transmitter signal to synaptic currents. In addition to pre- and postsynaptic regulation of excitatory synaptic transmission, metabotropic regulation of network properties is described under the subheading "Regulation of neuronal excitability". We describe evidence for the notion that physiological activation of mGluRs is, in contrast to ionotropic glutamate receptors, not caused by a fast glutamate transient in the synaptic cleft receptors but by glutamate that spills over from synaptic clefts or that is released from glial cells. Finally, we describe how accumulation of extracellular glutamate can induce slow mGluR-mediated synaptic potentials. Long-term synaptic plasticity, where mGluRs play important roles as well, is reviewed in the accompanying article. This review does not cover potential pharmaceutical applications based on the modulation of synaptic transmission by mGluRs (Knöpfel et al. 1995; Nicoletti et al. 1996).

2
Presynaptic Regulation of Excitatory Synaptic Transmission

The existence of presynaptic glutamate receptors was originally proposed following the observation that glutamate or some of its derivatives depressed excitatory transmission (Koerner and Cotman 1981; Baskys and Malenka 1991; Forsythe and Clements 1990). These glutamate autoreceptors have now been identified as specific members of the mGluR family localized at glutamatergic synaptic terminals where they sense the released transmitter and, in turn, modulate its release.

The most extensively studied example is the inhibition of glutamate release by group II and/or III mGluRs of excitatory synapses in the hippocampus (Cartmell and Schoepp 2000; Capogna 2004; Price et al. 2005; Anwyl 1999). In this structure, group III mGluRs were found at presynaptic active zones of synapses (Shigemoto et al. 1996, 1997), whereas group II mGluRs have been observed at extrasynaptic sites remote from the active zone in the preterminal part of axons (Shigemoto et al. 1996, 1997; Corti et al. 2002). In correlation with this differential localization, group III mGluR activation reduces the release of glutamate through a G-protein-mediated inhibition of presy-

naptic Ca^{2+} channels (Capogna 2004). In contrast, the inhibition mediated by group II mGluRs in the hippocampal stratum lacunosum moleculare acts via activation of K^+ channels (Capogna 2004).

In addition to this function as conventional autoreceptors, mGluRs can also be activated by glutamate that is spilled over from neighboring glutamatergic synapses (see below) and also by ambient extracellular glutamate that is controlled by glial cell activity (Fiacco and McCarthy 2004; Huang

Table 1 Presynaptic regulation of excitatory synapses by activation of mGluRs

System	Group I	Group II	Group III	Refs.
Parallel fiber → Purkinje cell	Inhibition, retro[a], mGluR1/CB1[b] VGCC[c] ↓[d] K^+ channel ↑[e]	Absent	Inhibition, pre[f], mGluR4 VGCC ↓ K^+ channel ↑	Brown et al. 2003, 2004; Daniel and Crepel 2001
Calyx synapse (MNTB)[g]	Inhibition, retro, mGluR1/CB1 VGCC ↓	No function established	Inhibition, pre, mGluR4?	Billups et al. 2005; Kushmerick et al. 2004
Primary afferent synapses in spinal cord	Facilitation, pre, mGluR1/5 (?)	Inhibition, pre	Inhibition, pre, mGluR4/7 (?)	Park et al. 2004; Gerber et al. 2000
Retina → superior colliculus	Inhibition, mGluR1 K^+ channel ↑	No function established	Inhibition, pre, mGluR4/7/8?	White et al. 2003; Lacey et al. 2005
Prelimbic cortex → nucleus accumbens	No function	Inhibition, pre, VGCC (N?)[h] ↓	Inhibition, pre,	Manzoni et al. 1997
Hippocampus	Inhibition, mGluR1 direct? and retro/CB1 VGCC ↓	Inhibition, pre, K^+ channel ↑	Inhibition, pre, mGluR4 VGCC (N) ↓	Capogna 2004; Price et al. 2005; Faas et al. 2002
Layer V of the rat entorhinal cortex	No function established	No function established	Facilitation, pre, mGluR4	Evans et al. 2001; Jones and Woodhall 2005

[a] Retrograde signaling from postsynaptic neurons to presynaptic terminal, postsynaptic mechanism
[b] Cannabinoid receptor 1
[c] Voltage-gated Ca^{2+} channel
[d] Decreased activity
[e] Increased activity
[f] Presynaptic mechanisms
[g] Medial nucleus of the trapezoid body
[h] N-type VGCC

et al. 2004). The physiological role of this glutamatergic negative autofeedback has only been partially elucidated. In addition to a dynamic feedback control function, it appears that the persistently active group II/III receptors (mGluR2/3/8) control the excitability of the hippocampal network (Losonczy et al. 2003).

Despite the general notion that group I mGluRs are typically expressed on the somatodendritic (postsynaptic) membranes, these receptors also modulate presynaptic glutamate release via either retrograde mechanisms or receptors localized directly at the axonal terminal. A presynaptic expression of group I mGluRs is well established at primary afferent nerve fibers to the brain stem (trigeminal caudal nucleus: Liang et al. 2005; superior colliculus: White et al. 2003) and olfactory bulb (Mutoh et al. 2005). Group I mGluRs expressed on these synaptic terminals depress glutamate release by either activating K^+ channels (White et al. 2003) or inhibiting Ca^{2+} channel activity (Mutoh et al. 2005). An unusual mGluR1/5-mediated "self-facilitating" mechanism has been described at glutamatergic primary afferent nerve fibers to the spinal cord (Ikeda and Murase 2004; Park et al. 2004). As for the retrograde mechanisms, the best-characterized pathway where postsynaptic group I mGluRs regulate transmitter release involves presynaptic cannabinoid receptors. This mechanism has been extensively studied at the parallel fiber–Purkinje cell synapse (Brown et al. 2003; Kreitzer and Regehr 2001) as well as at hippocampal synapses (Varma et al. 2001).

The above examples highlight the variety of presynaptic mechanisms by which mGluRs can modulate the release of glutamate. Moreover, the role of glutamate autoreceptors can differ between synapses formed by the same axon: terminals contacting distinct target cells show different expression and functions of presynaptic mGluRs (Shigemoto et al. 1996; Scanziani et al. 1998). Table 1 summarizes and exemplifies the diversity of mechanisms by which presynaptic mGluRs regulate excitatory synaptic transmission at different synapses.

3
Postsynaptic Regulation of Excitatory Synaptic Transmission

mGluRs expressed on the dendritic or somatic membrane can couple directly to ionotropic glutamate receptors and thereby modulate synaptic currents. The *N*-methyl D-aspartate receptor (NMDAR) channel activity can be regulated by protein phosphorylation/dephosphorylation via a variety of protein kinases/phosphatases that are controlled by distinct second-messenger cascades (Grishin et al. 2004 and references below). Activation of group I mGluRs can both up- and downregulate NMDAR activity depending on the particular mGluR subtype (mGluR1 or mGluR5) and cell type (Benquet et al. 2002; Grishin et al. 2004; Heidinger et al. 2002).

There are fewer reports on group II/III mGluR-mediated modulation of NMDAR currents. One example is the group II mGluR-mediated enhancement of NMDAR currents in pyramidal neurons of rat prefrontal cortex via a mechanism dependent on PKC activation (Tyszkiewicz et al. 2004).

A more important factor modulating the strength of glutamatergic synaptic activity than the phosphorylation/dephosphorylation state of receptors is their number in the postsynaptic membrane, controlled by trafficking to and removal (internalization) from the membrane. mGluRs are also involved in the regulation of these processes (Mangiavacchi and Wolf 2004; Snyder et al. 2001). This bidirectional control of ionotropic glutamate receptors by mGluRs is important in the context of synaptic plasticity, metaplasticity, and excitotoxic processes (Grishin et al. 2004; Heidinger et al. 2002).

4 Regulation of Neuronal Excitability

Neuronal excitability is a measure of the ability of neurons to fire action potentials in response to a given stimulus. If neuronal excitability is upregulated, a given excitatory synaptic input will produce more action potentials than in control conditions. Regulation of the excitability of hippocampal pyramidal cells by mGluRs was in fact among the first metabotropic effects of glutamate that clearly differed from the established role of glutamate as the agonist for fast synaptic transmission (Charpak et al. 1990). Thus, at hippocampal CA3 pyramidal cells, group I mGluRs downregulate the activity of voltage-gated potassium channels so that a depolarizing current induces a larger number of action potentials when the mGluRs are tonically activated (Charpak et al. 1990).

Ca^{2+}-activated potassium channels, inwardly rectifying potassium channels, and calcium channels can also be activated via group I mGluRs and this transduction pathway can decrease neuronal excitability (Sodickson and Bean 1998; Fagni et al. 2000). At the neuronal circuit level it has been shown that ambient levels of glutamate tonically activate mGluRs, thereby regulating excitability of cortical cells (Bandrowski et al. 2003), and that the dynamic balance of metabotropic inputs causes dorsal horn neurons to switch functional states (Derjean et al. 2003).

5 Physiological Activation of mGluRs

The affinity of glutamate for mGluRs is in the low micromolar range and for several mGluR subtypes does not differ from that of ionotropic glutamate receptors. So why are mGluRs not unconditionally activated during glutamatergic synaptic transmission? The answer to this question is related to the

fact that the localization of mGluRs is mostly peri- and extrasynaptic, in contrast to ionotropic glutamate receptors that are localized to the synaptic cleft. The physiological activation of mGluRs occurs by glutamate that spills over from synaptic clefts or is released from glial cells.

6
Spillover from Synaptic Clefts and Extracellular Diffusion of Glutamate

The concentration of glutamate in the synaptic cleft reaches millimolar levels during physiological glutamatergic transmission and rapidly decays (within a millisecond, or less) to concentrations that are well below the binding constant of ionotropic glutamate receptors (Conti and Weinberg 1999; Bergles et al. 1999; Clements 1996). This fast decay of glutamate transients mainly results from diffusion of glutamate out of the synaptic cleft into the surrounding extracellular space. Several studies modeling the dynamics of glutamate diffusion, tissue tortuosity, and uptake mechanisms in the vicinity of synapses (e.g., Barbour 2001; Rusakov and Kullmann 1998; Clements 1996) have attempted to resolve whether glutamate released from a single synaptic vesicle can activate extrasynaptic receptors. Even though glutamate released from a single synapse probably can diffuse several microns from its origin and thus potentially reach many hundreds or even thousands of synapses (Arnth-Jensen et al. 2002; Diamond 2002), to our knowledge there is no direct evidence for a mGluR-mediated synaptic potential resulting from binding of glutamate that is spilled over from one single synapse after one single pulse, without blockade of glutamate uptake. It is thus important to consider the factors affecting the accumulation of extrasynaptic glutamate.

7
Clearance of Synaptically Released Glutamate and Regulation of Ambient Glutamate Concentration

The neuronal and glial membranes that define the extracellular space express mGluRs and also carry glutamate transporters that ultimately remove glutamate from the extracellular space. Therefore, physiological activation of mGluRs is tightly related to the efficiency of clearance of synaptically released glutamate and the regulation of ambient extrasynaptic glutamate concentration.

Glial processes ensheathe the synaptic structures and express a very high density of glutamate transporters; in fact, they often act as the most significant sink of glutamate in addition to physically preventing transmitter diffusion from their origin. For this reason, the degree and nature of glial ensheathment, both modifiable by physiological and developmental processes,

crucially influence transmitter spillover and activation of mGluRs (Huang et al. 2004; Sykova 2005).

The actual cycling rate of the transporters is rather slow (order of tens of milliseconds; Clements 1996; Danbolt 2001); therefore, fast glutamate clearance requires expression of transporters at very high densities (Bergles et al. 1999). Glutamate transporters typically colocalize on the perisynaptic dendritic membranes with group I mGluRs (Otis et al. 2004). Since the affinities of glutamate transporters are comparable to those of mGluRs, they directly compete with the receptors for glutamate occupancy. Furthermore, the transporter-to-receptor ratio strongly favors the transporters near the synaptic cleft, often enabling the transporters to dominate the race for binding. Most of the competition occurs on the postsynaptic membrane, just at the edge of the synaptic cleft where a small number of transporters may be sufficient to significantly limit transmitter diffusion during low-frequency activity (Scanziani 2002), but may be overwhelmed during glutamate release at high frequency (Reichelt and Knöpfel 2002).

In addition to activating extrasynaptic mGluRs, glutamate that has escaped immediate clearance by glial cells also feeds the pool of ambient (extracellular) glutamate. Another source of extrasynaptic glutamate are glial cells themselves. Astroglial cells are known to release glutamate in both Ca^{2+}-dependent vesicular and Ca^{2+}-independent nonvesicular manners, either spontaneously or in response to neuronal activity (Hertz and Zielke 2004; Baker et al. 2002; Fiacco and McCarthy 2004). Notably, ongoing nonvesicular, nonneuronal release is one of the causes of a functionally relevant level of extrasynaptic glutamate, since the ambient glutamate level is usually insensitive to tetrodotoxin or to the removal of Ca^{2+} from the perfusion (Cartmell and Schoepp 2000). Thus, even though the high affinity of glutamate transporters could result in low (nanomolar) ambient levels of glutamate, glutamate can be continuously present in high enough concentrations to activate mGluRs. Neurons sense via mGluRs ambient glutamate levels that reflect average local activity and adjust their activity accordingly (Bergles et al. 1999).

Finally, surface expression and turnover rate of glutamate transporters can be regulated by neuronal activity. For example, the neuronal transporter currents in Purkinje cells undergo long-term potentiation (LTP) in response to brief tetanic activation of climbing fibers. This LTP appears to be postsynaptically expressed and it requires activation of a postsynaptic mGLuR/PKC cascade (Shen and Linden 2005).

8
Activation of mGluRs by Extrasynaptic Glutamate

The preceding paragraph provided a framework for understanding the conditions and modes of physiological activation of mGluRs. Group I and III

mGluRs are found at high densities close to synaptic clefts (Martin et al. 1992; Shigemoto et al. 1996, 1997; Mateos et al. 2000). A fraction of group I mGluRs are located at somatodendritic membranes (Martin et al. 1992) and group II mGluRs are mainly residing at preterminal portions of axon terminals (Shigemoto et al. 1996, 1997; Corti et al. 2002). The mGluRs that are localized far from synaptic clefts are most likely activated by ambient glutamate, rather than in response to single synaptic activity. Control of synaptic glutamate spillover ensures that isolated evoked synaptic transmission rarely activates these mGluRs, and it is only when release occurs at high frequency and synchronously at many nearby terminals that extracellular glutamate reaches the threshold for their activation (Reichelt and Knöpfel 2002; Matsukawa et al. 2003; Billups et al. 2005).

9
Slow Synaptic Potentials Mediated by Metabotropic Glutamate Receptors

All mGluR-mediated transduction pathways that affect ion channel activity can be involved in the generation of synaptic potentials if the mGluR is activated by synaptically released glutamate. Because of the requirement of glutamate to reach and accumulate at the extrasynaptic locations of mGluRs, these synaptic potentials are slow as compared to the synaptic potentials mediated by ionotropic glutamate receptors. Group I mGluRs mediate slow excitatory (Batchelor et al. 1997) and inhibitory potentials (Dutar et al. 2000; Fiorillo and Williams 1998). These slow potentials are produced in response to synaptic activity that results in extrasynaptic accumulation of glutamate as described above. Thus, the mGluR-mediated synaptic potentials require activity of nearby synapses at high frequency and are facilitated by weakening of glutamate transporter activity and increasing synaptic release probability (Reichelt and Knöpfel 2002; Matsukawa et al. 2003; Otis et al. 2004). Consequently, the slow mGluR potentials report a high level of excitatory synaptic activity within a time window of tens of milliseconds and in a tissue volume element with a spatial extent of a few micrometers to postsynaptic cells that have dendritic processes within that volume element.

10
Concluding Remarks on the Role of mGluRs in Modulating Synaptic Transmission

Signaling by mGluRs expands the traditional view of glutamate acting as the transmitter in fast, point-to-point synaptic transmission. Glutamate, via mGluRs, can act like a conventional neuromodulator, influencing neuronal

excitation and excitability on the level of local circuits that is on a more integrative scale, beyond simple cell-to-cell communication. Future studies are needed to clarify the exact conditions and consequences of mGluR signaling during behaviorally relevant physiological conditions.

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Presynaptic Inhibition of Glutamate Release by Neuropeptides: Use-Dependent Synaptic Modification

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Abstract Neuropeptides are signaling molecules that interact with G-protein coupled receptors located both pre- and postsynaptically. Presynaptically, these receptors are localized in axons and terminals away from presynaptic specializations. Neuropeptides are stored in dense core vesicles that are distinct from the clear synaptic vesicles containing classic neurotransmitters such as glutamate and GABA. Because they require a stronger Ca^{2+} signal than synaptic vesicles, dense core vesicles do not release neuropeptides with single action potentials but rather require high-frequency trains. Thus, neuropeptides only modulate strongly stimulated synapses, providing negative or positive feedback. Many neuropeptides have been found to inhibit glutamate release from presynaptic terminals, and the major mechanism is likely direct interaction of $\beta\gamma$ G-protein subunits with presynaptic proteins such as SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor). The use of mouse genetic models and specific receptor antagonists are beginning to unravel the function of inhibitory neuropeptides. The opioid receptors kappa and mu, which are activated by endogenous opioid peptides such as dynorphin, enkephalin, and possibly the endomorphins, are important in modulating pain transmission. Dynorphin, nociceptin/orphanin FQ, and somatostatin and its related peptide cortistatin appear to play a role in modulation of learning and memory. Neuropeptide Y has important functions in ingestive behavior and also in entraining circadian rhythms. The existence of neuropeptides greatly expands the computational ability of the brain by providing additional levels of modulation.

1 Introduction

The focus of this chapter is on neuropeptide-mediated presynaptic inhibition of excitatory neurotransmission in mammalian brain. Instead of cataloging the effects of exogenous application of various neuropeptides on synaptic transmission, the focus here will be on mechanisms of peptide release and inhibition, functional consequences, and plasticity of receptor expression and function. Further, I will attempt to point out some characteristics of neuropeptides that distinguish them from other neuromodulators.

Peptides are generated through protein synthesis at the endoplasmic reticulum and processed through the Golgi apparatus. They are packaged in dense core vesicles and transported to presynaptic terminals through axonal trans-

port. Peptides are not released at active zones, and there does not appear to be a specific reuptake system for peptides; after they are released, they are soon degraded by nonspecific peptidases. Thus, peptides are a much more energetically costly signaling system than primary neurotransmitters such as GABA or glutamate, which are synthesized enzymatically at axon terminals and can be recycled back into the terminal after release. It follows that peptides would have very different functions than primary neurotransmitters, and that their release would not be under "hair-trigger" control but would require a quite robust signal. This does in fact appear to be the case.

In the past decade or so, much has been learned about the mechanisms through which neurotransmitters are released, and, more slowly, we are also beginning to understand the processes underlying neuropeptide release (Table 1). Although one might assume that peptide-containing dense core vesicles (DCVs) would require a higher Ca^{2+} threshold for fusion with the membrane, in fact the opposite is true. The levels required for DCV release has been generally estimated to be less than 1 μM compared to 10–100 μM within microdomains for small synaptic vesicles (see Ghijsen and Leenders 2005 for a recent review). It is the precise localization of the small synaptic

Table 1 Distinctions between "classic" neurotransmitter and neuropeptide release and signaling

	Neurotransmitter	Neuropeptide
Location in terminal	Tethered near synaptic structure in synaptic vesicles	Diffusely localized in terminal in DCVs
Synthesis	Enzymatically within terminal	Protein synthesis in soma
Ca^{2+} sensitivity	1–100 μM	< 1 μM
Release kinetics	Fast (submilliseconds)	Slow (10 ms to seconds)
Activation required for release	Single action potential	High-frequency train of action potentials
Diffusion after release	20 nm across the synaptic cleft	Hundreds of μm or more?
Type of signaling	Point-to-point within synaptic structure	Volume transmission
Reuptake	Specific transporters	None
Recycling of vesicles	Yes	Partial?[a]
Duration of signal	Brief	Long

[a] Dense core vesicles (DCVs) may not release all of their content with a single fusion event but may be capable of multiple fusion/release cycles (Artalejo et al. 1998)

vesicles containing neurotransmitters that gives them the capability of rapid release. The readily-releasable pool of vesicles is localized in a subcellular compartment at active release sites that includes voltage-sensitive Ca^{2+} channels. This allows for rapid sensing of a rise in internal Ca^{2+} by synaptotagmin located in the membrane of the small synaptic vesicles, leading to rapid fusion with the presynaptic membrane and release of neurotransmitter into the synaptic cleft. On the other hand, readily-releasable DCVs are not tethered as tightly to the plasma membrane, are not adjacent to Ca^{2+} channels, and are usually released away from the active zone (see. Martin 2003 for a recent review on the differences in release mechanisms). In order for release to be triggered, Ca^{2+} must rise to sufficient levels such that cytoplasmic diffusion to more distal sites occurs. Thus, neuropeptides would not be released from DCVs by a single action potential. Instead, sustained trains are required for internal Ca^{2+} to reach levels sufficient to trigger release.

While much neurotransmitter signaling is synaptic, with the synaptic structure limiting diffusion to keep concentrations of ligand relatively high, neuropeptide receptors are generally localized away from the synaptic differentiation. Peptides are also not released at active zones that appose postsynaptic densities, rather DCVs fuse with the terminal membrane at more distal sites. It should be noted that DCVs containing neuropeptides are sometimes observed fusing at active zones (e.g., Drake et al. 2002). However, this is likely random and does not indicate a distinct pathway for regulated synaptic release. Peptide receptors can have more than a 1000-fold higher affinity for their ligand than neurotransmitter receptors, thus allowing them to activate receptors at sites relatively far from the site of release. This likely accounts for the peptide/receptor "mismatch" that is often observed, where localizations of a peptide and its specific receptor do not overlap. This concept of "volume transmission" can be considered as an intermediate between "point-to-point" synaptic transmission and paracrine signaling, in terms of distance from release site to target. Although many, if not most, DCV-containing terminals express more than one neuropeptide, likely existing together within the DCV, no mechanisms of selective neuropeptide release have been described. Multiple neuropeptides would therefore be released together, and may have synergistic actions (see Merighi 2002 for recent review).

Before the development of peptide receptor antagonists and receptor knockout mice, studies on the consequences of endogenous release had been mostly lacking in central neurons. However, with the availability of better and more specific neuropeptide receptor antagonists, and the generation of knockouts for many of the neuropeptide receptors, such studies are becoming more feasible. Correlation of presynaptic actions in vitro with behavioral changes in vivo has suggested that presynaptic inhibition of glutamate release is a powerful mechanism through which neuropeptides modulate brain function.

2
Neuropeptide Receptors on Axon Terminals are Located Extrasynaptically

The cloning of neuropeptide receptors has supported the development of specific, epitope-generated antibodies that allow for cellular and subcellular localization studies using immunohistochemistry with light and electron microscopy. Studies have confirmed that neuropeptide receptors are localized in axons and at terminals; however, co-localization with synaptic specializations is not generally seen. The opioid receptor family has been the most extensively studied at the EM level. For example, the delta opioid receptor in locus coeruleus is associated with plasma membrane in axons and terminals and with DCV membranes, usually distal to the synaptic junction (van Bockstaele et al. 1997). Mu opioid receptors have a similar pattern of expression in axons and terminals in the ventral tegmental area (VTA) (Garzon and Pickel 2001), caudate-putamen (Wang et al. 1996), and nucleus accumbens (Svingos et al. 1996, 1997). Interestingly, in VTA, mu opioid receptors are sometimes found in synaptic vesicle membranes. Kappa opioid receptors in the hippocampus are also located on axons and axon terminals, with labeling of non-synaptic plasma membranes and DCVs (Drake et al. 1996). In rat nucleus accumbens, kappa receptors are localized in axon terminals, where they are often found in synaptic vesicle membranes. Unfortunately, no detailed description of the localization of the final member of this receptor family, the ORL-1 receptor, has been reported, and a previous mapping study (Anton et al. 1996) was confounded by a nonspecific antibody (see discussion in Neal et al. 1999b).

Although not as well-characterized, other inhibitory neuropeptide receptors show similar distribution. Y1 receptors in nucleus accumbens and nucleus tractus solitarius (NTS) are located in axons and axonal terminals, at the extrasynaptic plasma membrane and in dense core and synaptic vesicles (Pickel et al. 1998; Glass et al. 2002). Somatostatin (SST) receptors have also been identified presynaptically. SST_1 is almost exclusively expressed axonally (Schulz et al. 2000), whereas SST_2 has both pre- and postsynaptic distribution (Dournaud et al. 1996b). Immunohistochemical localization of many of the other neuropeptide receptors thought to exist at presynaptic locations (e.g., galanin, Y2, etc.) has not yet been reported with subcellular resolution.

Co-labeling studies of neuropeptide receptors with their peptide ligands confirm that “point-to-point” synaptic communication across the synaptic cleft is not a major function of neuropeptides, as most presynaptic terminals containing neuropeptide receptors are not directly apposed by axons containing their cognate peptide. However, it is not uncommon to find close association between peptide and receptor, suggesting that in many cases relatively short diffusion distances are required (e.g., $< 4\,\mu m$, Drake et al. 2002). In many instances, a receptor is expressed both pre- and postsynapti-

cally in the same region (Svingos et al. 1996; Wang et al. 1996). This is also a common motif of neuropeptide physiology, where presynaptic inhibition is often coupled to postsynaptic hyperpolarization, thus reducing both excitatory input and the responsivity of the neuron (e.g., SST and Noc/OFQ in hippocampus (Madamba et al. 1999; Tallent and Siggins 1999; Tallent et al. 2001), NPY in SCN (van den Pol et al. 1996), dynorphin in NRM (Bie and Pan 2003), etc.). This would obviously result in tighter control over neuronal output as well as protection against hyperexcitability.

The association of opioid receptors with DCV and SV membranes suggests a means by which their functional expression may be regulated. Indeed, there is growing evidence for activity-dependent insertion of neuropeptide receptors "riding" on DCVs into terminal membranes. In hypothalamic magnocellular neurons, terminals in the neurohypophysis contain kappa opioid receptors. Activation of these receptors has been found to inhibit VP release. In the unstimulated condition, most of the kappa receptor is located in membranes of DCVs containing VP within the axon terminal. An in vivo "salt-loading" paradigm that stimulated vasopressin release caused translocation of the kappa receptor into the plasma membrane of the axon terminal (Shuster et al. 1999). This translocation was transient; 1 h after salt-loading the kappa receptor expression returned to the DCVs. Co-expression of dynorphin, the endogenous neuropeptide for the kappa opioid receptor, with VP in DCVs was subsequently demonstrated (Shuster et al. 2000). Thus, this translocation of kappa opioid receptor into the terminal membrane could provide a mechanism for negative feedback. The initial VP/dynorphin surge results in translocation of kappa receptors to the terminal membrane, where they would be poised to inhibit subsequent release.

Similar findings were reported for the delta opioid receptor (Commons 2003). In this study, delta opioid receptor (DOR) localization in the PAG was examined following a cold swim test stressor. In the control condition, the majority of DORs were located on DCV membranes. After swimming, DORs associated with DCVs decreased whereas DORs at the plasma membrane of axon terminals increased. DCVs containing DORs were observed in the process of fusing with the plasma membrane after the swim test. These studies substantiate that DCVs are utilized to control membrane expression of neuropeptide receptors in a highly regulated manner, and suggest a mechanism for stress-induced sensitization to opioids (Shaham et al. 1995; Sutton et al. 1997). Translocation of neuropeptide receptors into the plasma membrane could also be a mechanism of selectively "tagging" previously activated synaptic terminals for subsequent modifications (Frey and Morris 1997). It is interesting to speculate that opioid receptors in membranes of synaptic vesicles could be inserted into the plasma membrane by low frequency firing of action potentials, "priming" the synapse for subsequent neuropeptide modulation at a later time. However, this has not yet been demonstrated, either anatomically or physiologically.

3 Mechanism of Presynaptic Inhibition by Neuropeptides

3.1 Most Inhibitory Neuropeptide Receptors Couple to G_i/G_o G-Proteins

The majority of studies examining presynaptic actions of neuropeptides have not addressed specific mechanisms. Most neuropeptide receptors that inhibit glutamate release are coupled to G_i/G_o G-proteins (e.g., neuropeptide Y, SST, and opioid receptors), but some examples of inhibition by G_q-coupled receptors (oxytocin, orexin/hypocretin, substance P, cholecystokinin) have been demonstrated. For both groups of receptors, both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms have been implicated. Inhibition of Ca^{2+}-dependent release is demonstrated when a neuropeptide inhibits evoked or spontaneous (action potential-dependent) but not miniature EPSCs (mEPSCs) recorded in neurons treated with tetrodotoxin (TTX). However, care must be given in interpreting actions on spontaneous EPSCs, since it is difficult to rule out polysynaptic effects or actions at the presynaptic cell soma. Neuropeptide Y (NPY) inhibited Ca^{2+}-dependent but not Ca^{2+}-independent glutamate release in CA1 (Colmers et al. 1988; Qian et al. 1997) and CA3 hippocampus (McQuiston and Colmers, 1996; Guo et al. 2002). In a striatal synaptosome preparation, kappa opioid receptors were shown to inhibit only the Ca^{2+}-dependent component of evoked glutamate release (Rawls et al. 1999).

Since G_i/G_o-coupled receptors couple negatively to voltage-dependent Ca^{2+} channels, inhibition of Ca^{2+} entry through these channels in axons or terminals would be a logical mechanism of inhibition. Surprisingly, however, this has so far not been demonstrated to be a major mechanism. One example, however, is found in hippocampus. Use of selective Ca^{2+} channel blockers demonstrated N- and P/Q-type channels appear critical for NPY-mediated inhibition in CA1 (Qian et al. 1997). Another common signaling pathway for G_i/G_o-coupled receptors is facilitation of K^+ channels. Increases in K^+ conductances by activation of these receptors would hyperpolarize the membrane and decrease Ca^{2+} influx into terminals to reduce neurotransmitter release. Mu opioid receptors act via this mechanism in several brain regions, including corticostriatal synapses (Barral et al. 2003), central nucleus of the accumbens (Zhu and Pan 2005), and the subthalamic nucleus (Shen and Johnson 2002). Inhibition of GABA release by mu opioid receptors also involves activation of a K^+ current (Vaughan et al. 1997b), suggesting that this may be a general mechanism for this receptor. Inhibition of glutamate release from mossy fiber terminals by kappa opioid receptors is blocked by dendrotoxin, suggesting involvement of A-type K^+ channels (Simmons and Chavkin 1996).

Because mEPSCs recorded in TTX are Ca^{2+}-independent, neuropeptides that inhibit their frequency must act downstream of axonal excitability and Ca^{2+} entry. To date this is by far the most common mechanism found for

G_i/G_o-coupled neuropeptide receptors. However, it should be noted that in most cases effects on Ca^{2+}-dependent release were not ruled out. Therefore, considering that many of these receptors have been shown to inhibit Ca^{2+} channels and/or augment K^+ channels, synergistic Ca^{2+}-dependent and Ca^{2+}-independent mechanisms could be in play. In a study examining presynaptic inhibition of autaptic EPSCs (using a hippocampal microculture system so that recorded glutamate events arose only from terminals of the recorded neuron), SST inhibited Ca^{2+}-independent mEPSCs but also inhibited voltage-dependent Ca^{2+} channels recorded in the cell soma (Boehm and Betz 1997). Thus, SST may inhibit glutamate release by both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms, although it is unclear if SST would have the same actions in the axon terminals as at the cell soma. In lateral amygdala neurons, Noc/OFQ inhibited mEPSCs to the same degree as evoked EPSCs, while inhibition of evoked IPSCs was significantly greater than mIPSCs. Therefore, Meis and Pape proposed that Noc/OFQ inhibition of glutamate release is likely downstream of Ca^{2+} entry, whereas this peptide may inhibit both Ca^{2+}-dependent and Ca^{2+}-independent GABA release (Meis and Pape 2001). In central amygdala, mu opioid receptor-mediated inhibition is sensitive to 4-AP, suggesting actions on terminal excitability, but also decreased mEPSC frequency, suggesting dual mechanisms (Zhu and Pan 2005). In many other instances, inhibition of glutamate release by neuropeptides has been demonstrated to be downstream of Ca^{2+} entry. These include: Noc/OFQ in hippocampus (Tallent et al. 2001) and lateral amygdala (Meis and Pape 2001), mu opioids and Noc/OFQ in periaqueductal grey (Vaughan and Christie 1997; Vaughan et al. 1997a), SST and Noc/OFQ in thalamus (Meis et al. 2002; Sun et al. 2002), galanin, NPY, and melanin concentrating hormone (MCH) in hypothalamus, (Kinney et al. 1998; Gao and van den Pol 2001; Fu et al. 2004), and mu opioids in SON (Honda et al. 2004) and VMH (Emmerson and Miller 1999).

In most cases, further insight into the cellular mechanism by which G_i/G_o-coupled neuropeptide receptors mediate presynaptic inhibition downstream of Ca^{2+} entry has not been established. Since these receptors all should inhibit adenylyl cyclase, which facilitates synaptic release, this could be a common mechanism. SST inhibition of glutamate release in cortical synaptosomes was proposed to be via inhibition of adenylyl cylcase, however, the experimental design used in this study was only correlative (Grilli et al. 2004). Another possible mechanism is direct interaction of $\beta\gamma$ G-protein subunits with synaptic machinery. Direct injection of G-protein $\beta\gamma$ subunits into the presynaptic terminal mimics presynaptic inhibition by G-protein coupled receptors; and serotonin inhibition is occluded by a $\beta\gamma$ scavenger (Blackmer et al. 2001). This effect was independent of typical G-protein mediated pathways and was Ca^{2+}-independent, suggesting a direct interaction with exocytotic pathways. More recently, this group (Blackmer et al. 2005; Gerachshenko et al. 2005) showed that $G\beta\gamma$ interacted directly with SNAP-25 to inhibit vesicular release. SNAP-

25 is a constituent of the presynaptic SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex that mediates vesicle fusion.

Specificity of $G\alpha$ coupling of SST and other receptors to inhibition of presynaptic glutamate release was studied in hippocampal cultured neurons using pertussis toxin-insensitive $G\alpha$ constructs delivered with viral vectors (Straiker et al. 2002). This group found that $G\alpha_i2$, $G\alpha_i3$, and $G\alpha_o1$ coupled the SST receptor (likely SST_2 (Boehm and Betz 1997)) to inhibition of EPSCs, but that $G\alpha_i1$ did not. This somewhat promiscuous $G\alpha$-coupling was similar to $GABA_B$ and the CB1 cannabinoid receptor, which also coupled to all $G\alpha$ subunits tested except $G\alpha_i1$. Interestingly, $G\alpha_i1$ has been shown in several independent studies to couple SST receptors to inhibition of adenlyl cyclase (Tallent and Reisine 1992; Law et al. 1994; Murthy et al. 1996), including SST_2 specifically, in which $G\alpha_i1$ is required for coupling to occur (Kagimoto et al. 1994). These studies provide further evidence that the $G\alpha$ requirement for presynaptic coupling by SST receptors is independent of inhibition of adenylyl cyclase. Because distinct $G\alpha$ subunits show preference for $\beta\gamma$ subunits (Robishaw and Berlot 2004), limitations in $G\alpha$ coupling may confer specificity in free $\beta\gamma$ subunits that are available to interact with SNAP-25.

3.2 G_q-Mediated Presynaptic Inhibition is Likely Indirect

The mechanisms by which G_q-coupled receptors inhibit glutamate release are less clear. Since G_q stimulates PLC, which leads to an increase in internal Ca^{2+}, it is surprising and paradoxical that such receptors would reduce neurotransmitter release. Interestingly, in several cases, mechanistic studies have shown the actions of agonists of G_q-coupled receptors to inhibit glutamate release via an indirect action. It seems likely that similar mechanisms are in play for other G_q-coupled receptors that reportedly have direct presynaptic inhibitory action, including substance P inhibition in PAG (Sekizawa et al. 2003) and CCK and VP in parabrachial nucleus (Saleh et al. 1997; Chen and Pittman 1999).

In the parabrachial nucleus (PBN), a relay nucleus for visceral information, exogenously applied CGRP reduces EPSCs; analysis of mEPSCs suggested a direct presynaptic effect. However, upon further investigation of a synergistic interaction between substance P (SP) and CGRP, Pittman and colleagues (Saleh et al. 1996) found that an SP antagonist but not a CGRP antagonist blocked presynaptic inhibition by CGRP. Further, the actions of a metabolically stable SP analog were not augmented by CGRP. The synergism between SP and CGRP was occluded by phosphoramidon, a peptidase inhibitor, leading the authors to conclude that CGRP competed with SP for endogenous peptidases, which would increase the half-life of SP. Whether this interaction occurs with endogenously released peptides is unknown, especially consider-

ing that although SP is co-localized with glutamate within the PBN, CGRP cell bodies that project to this region originate in the central nucleus of the amygdala (CeA). However, the authors of this elegant study suggest the intriguing hypothesis that intense visceral activation, known to activate neurons in the CeA as well as the PBN, could lead to release of both peptides as a protective measure against hyperexcitability or excitotoxicity.

Another example of an indirect action of a G_q-coupled neuropeptide receptor on glutamate release comes from a study by Haj-Dahmane and Shen (2005). This group showed that orexin B (hypocretin 1) inhibited EPSCs in dorsal raphe nucleus (DRN) slices. Again, use of traditional methods supported a presynaptic site of action. Surprisingly, this group found that inhibiting postsynaptic G-protein signaling abolished the actions of Orx-B, supporting a role of postsynaptic receptors. Further investigation revealed that activation of postsynaptic orexin receptors induced the release of cannabinoids from the postsynaptic cell. The cannabinoids interacted with presynaptic CB1 receptors to inhibit glutamate release. These findings highlight the necessity for cautious interpretation of traditional means (e.g., analysis of mEPSCs, paired-pulse ratios, etc.) for assessing pre- vs. postsynaptic sites of action. A role for endocannibiniods in the presynaptic inhibition of glutamate release by oxytocin had been previously reported in SON (Hirasawa et al. 2004) (see Sect. 4.5), suggesting that this could be a common mechanism for G_q-coupled receptors.

Two studies in the nucleus accumbens also demonstrate that G_q-coupled neuropeptide receptors inhibit glutamate release through indirect mechanisms. Substance P, generally an “excitatory” peptide, inhibits evoked EPSCs through what appeared to be conventional presynaptic mechanisms, since it demonstrated no overt postsynaptic effects, increased paired-pulse facilitation, and was blocked by an NK1 antagonist (Kombian et al. 2003). However, further investigation revealed that the SP depression was also blocked by both a dopamine (D1) and an adenosine receptor (A1) antagonist. The authors conclude that SP induces its effects by enhancing dopamine, which in turn increases adenosine release, although this is not directly shown. However, since it has been shown that SP releases dopamine from nucleus accumbens synaptosomes (Kalivas and Miller 1984), and that dopamine depression of EPSCs in accumbens is blocked by A1 antagonists (Harvey and Lacey 1997) this seems a likely mechanism. This same group also found that cholecystokinin (CCK), another “excitatory” peptide, also depressed EPSCs in the accumbens, while depolarizing the postsynaptic neuron. While DA receptor antagonist partially blocked the inhibition, suggesting that CCK may be increasing DA release similarly to SP, a $GABA_B$ receptor antagonist completely blocked the actions of CCK. This result suggests that CCK receptors on GABAergic terminals increase GABA release, and that GABA acting on presynaptic $GABA_B$ receptors in turn inhibits glutamate release (Kombian et al. 2004).

4 Functional Effects of Neuropeptide-Mediated Presynaptic Inhibition

4.1 Presynaptic Effects of Noc/OFQ and Somatostatin in Hippocampus – Modulation of Learning and Memory

Upon cloning of the three major opioid receptors, mu, kappa, and delta, an orphan receptor was discovered that appeared to belong to the same family but did not bind opioid ligands (Mollereau et al. 1994). This receptor, originally called ORL-1 with the currently accepted nomenclature NOP (Noc/OFQ receptor), also showed high sequence homology to the SST receptor family, although it likewise does not bind SST ligands. The endogenous ligand to this receptor was isolated by two different groups and was named nociceptin (Meunier et al. 1995) or orphanin FQ (Reinscheid et al. 1995); the current commonly accepted terminology is Noc/OFQ. High expression levels of both peptide and receptor were found in hippocampus, and an early study showed that microinjection of the peptide into hippocampus impaired spatial learning in rat (Sandin et al. 1997). An extracellular study in hippocampal slices suggested that Noc/OFQ inhibited glutamate release presynaptically (Yu et al. 1997), and this was later confirmed in voltage-clamp studies in CA3, where we showed that Noc/OFQ inhibited Ca^{2+}-independent release (Tallent et al. 2001). Exogenously applied Noc/OFQ also inhibits CA1 LTP (Yu et al. 1997). Consistent modifications of learning and memory have been described as a consequence of genetic deletion of NOP or its peptide. A mouse with the NOP receptor knocked out displayed a facilitation of hippocampal-dependent learning, and enhanced CA1 LTP (Manabe et al. 1998). Knockout of Noc/OFQ peptide likewise produced an enhancement of spatial learning, although no changes in CA1 LTP were reported (Higgins et al. 2002). Thus, it seems likely that Noc/OFQ negatively regulates learning and may play a role in extinction.

In the hippocampus, somatostatin is localized in GABAergic interneurons of the stratum oriens of CA1 and CA3, and hilus of the dentate. Interestingly, both sets of interneurons project to distal dendrites of the principal neurons (pyramidal neurons and dentate granule cells). Perforant path input from the entorhinal cortex form synapses on distal dendrites of these same neurons, therefore, somatostatinergic terminals are localized to regulate this input. Postsynaptic actions of somatostatin in CA1 are well-characterized and reduce excitability of pyramidal neurons by activating K^{+} channels (Moore et al. 1988; Schweitzer et al. 1998). More recently, presynaptic actions have been described. Inhibition of EPSCs by SST was reported in hippocampal cultures (Boehm and Betz 1997; Straiker et al. 2002) and in CA1 and CA3 of acute hippocampal slices (Tallent and Siggins 1997, 1999). This inhibition is specific to glutamatergic terminals since no effect on GABAergic IPSCs

was reported in cultured neurons (Boehm and Betz 1997) or slices (Tallent and Siggins 1997). In culture, inhibition was likely through the SST_2 receptor subtype (Boehm and Betz 1997), and we reported similar findings in slices (Tallent et al. 1997). The mechanisms of this presynaptic inhibition of glutamate release by SST is still unclear, although in cultured neurons SST actions are downstream of Ca^{2+} entry (Boehm and Betz 1997). In the dentate gyrus, we found no evidence of SST-mediated presynaptic inhibition of glutamate release (Baratta et al. 2002).

Whether SST receptors are localized on terminals of perforant path fibers is currently unknown. Although SST_2 has been localized to axonal sites in hippocampus, it is most robustly expressed somatodendritically (Dournaud et al. 1996b). Other SST receptor subtypes also appear to be largely expressed postsynaptically, including SST_4, which is likely the major CA1 hippocampal receptor (Videau et al. 2003; Qiu et al. 2005). One exception is SST_1, which is almost exclusively expressed in axons (Schulz et al. 2000). Thus, strongly stimulated GABA/SST containing interneurons would control excitability through multiple synergistic mechanisms. Released GABA could act both pre- and postsynaptically to reduce excitability. However, mechanisms such as receptor desensitization, synaptic depletion, and depolarization-induced depression of inhibition may limit the effectiveness of GABA outside a very brief time window. Release of SST, however, would be more prolonged, and its actions would be more sustained and over a wider area. SST acting on presynaptic SST_2 (and perhaps SST_1) receptors would limit glutamate release, while postsynaptic SST receptors would cause membrane hyperpolarization, limiting the responsivity of the neuron to excitatory synaptic input. Interestingly, many of the SST-containing interneurons in hippocampus also contain neuropeptide Y, which also inhibits glutamate release presynaptically (Qian et al. 1997). Thus, multiple modulators of synaptic transmission may be released at the same terminals to limit hyperexcitability. This is an especially important control in hippocampus due to its vulnerability to hypersynchronized firing and the generation of seizures.

The functional role of presynaptic inhibition by SST in hippocampus is still unknown. As would be expected, SST knockout mice show an increased vulnerability to chemically-induced seizures, with more severe seizures and increased mortality (Buckmaster et al. 2002). The role of hippocampal SST in the nonpathological state, however, is still unclear. Both facilitating (Matsuoka et al. 1994; Dournaud et al. 1996a; Van Uden et al. 1999) and inhibitory (Sanchez-Alavez et al. 2000; Dutar et al. 2002) effects by SST on learning and memory have been reported. SST_2 knockout mice show slightly enhanced spatial learning that is correlated with lower thresholds for induction of LTP (Dutar et al. 2002). However, these mice also showed an enhancement of glutamatergic fEPSPs that is difficult to reconcile with endogenous SST function, since one would not expect release of a neuropeptide by a single stimulation. Therefore it is unclear whether this enhancement of spatial learning is a dir-

ect consequence of SST_2 deletion, or whether compensatory mechanisms are involved.

Our group recently showed that overexpression of CST, a neuropeptide that shares 11 of 14 amino acids with SST and interacts with SST receptors, disrupts spatial learning (Tallent et al. 2005b). CST transgenics fail to learn the location of an escape tunnel using the Barnes circular maze. Pharmacological application of CST intrahippocampally also interferes with passive avoidance learning (Sanchez-Alavez et al. 2000). The most simplistic interpretation of these findings is that CST interferes with learning, perhaps raising the threshold of signaling input required for learning to occur. Another interpretation is that the CST (and/or SST) system is critical for learning to occur, and disruption of the endogenous system by genetic or pharmacological manipulation interferes with learning and memory. Thus, it remains unclear whether endogenous SST improves or disrupts learning/memory. Unlike Noc/OFQ actions that are mediated by a single receptor, at least four of the five SST receptor subtypes are expressed in hippocampus, greatly increasing the complexity of the system. Indeed, our data using receptor knockout mice suggests that knockout of SST_2 improves spatial memory, whereas knockout of SST_3 greatly diminishes it (Tallent et al. 2005a). Thus, the discrepancies in the literature regarding the role of SST in learning and memory may be due to distinct actions of the different receptor subtypes.

At the cellular level, the hippocampal actions of SST and Noc/OFQ are quite similar (Moore et al. 1988; Tallent and Siggins 1997, 1999; Madamba et al. 1999; Tallent et al. 2001), therefore it is interesting that SST can have facilitating action on learning while Noc/OFQ is apparently always inhibitory. Noc/OFQ (Neal et al. 1999a) and SST are expressed in distinct populations of interneurons in hippocampus (Morrison et al. 1982; Kosaka et al. 1988), so they would likely be released by different patterns of neuronal activation. A detailed characterization of the presynaptic localization of their distinctive receptors is incomplete; however, they may be localized on different presynaptic terminals. Also, although they have similar actions, the entire repertoire of physiological effects of these two peptides in the hippocampus is not identical. For example, although both robustly inhibit LTP in the dentate gyrus, Noc/OFQ appears to do so through direct postsynaptic actions on NMDA receptors (Yu and Xie 1998), whereas SST likely reduces dendritic Ca^{2+} influx through voltage-gated Ca^{2+} channels (Baratta et al. 2002). In CA3, Noc/OFQ acts on both mossy fiber and associational/commissural terminals to inhibit glutamate release, whereas only associational/commissural-generated EPSCs are sensitive to SST (Tallent and Siggins 1999). Interestingly, NMDA receptor-mediated potentiation of associational/commissural synapses appears to be necessary for certain types of learning, including more complex use of spatial information (Nakazawa et al. 2002). Thus, SST could play a role in use of acquired spatial information. This has been suggested by an in vivo study in which SST injected

into the hippocampus facilitated acquisition of spatial information, but impaired the ability to flexibly use previously acquired spatial information (Lamirault et al. 2001).

4.2
Heterosynaptic Inhibition by Endogenously Released Dynorphin from Mossy Fiber Terminals

Analysis of the functional consequences of endogenously released neuropeptides has been difficult. Specific antagonists for many neuropeptide families have still not been fully developed. One exception to this is the opioid receptor, for which specific antagonists to kappa, delta, and mu predated the cloning of the receptors. These have been used in brain slice studies in an attempt to observe a physiological effect of endogenously released glutamate, e.g., synaptic depression. One such study from Roger Nicoll's group examined the effect of dynorphin, the endogenous ligand of kappa opioid receptors, on mossy fiber transmission in guinea pig hippocampus. Dynorphin is expressed in glutamatergic mossy fibers of dentate granule cells that synapse onto CA3 proximal dendrites (Weisskopf et al. 1993). They found that exogenously applied dynorphin depressed mossy fiber but not associational fiber CA3 fEPSPS. This depression was associated with a decrease in paired-pulse facilitation, suggesting a presynaptic site of action. The opioid antagonist naloxone or the kappa-selective antagonist nor-BNI did not affect mossy fiber fEPSPs generated with low frequency trains. However, high-frequency trains induced a long-lasting synaptic depression of unstimulated mossy fiber inputs that was blocked by naloxone. These results suggest that release of dynorpin from stimulated mossy fibers can depress nearby unstimulated mossy fiber synapses. This phenomenon of heterosynaptic depression is distinct from associative forms of synaptic plasticity in that it does not require activity in the affected synapse. The authors demonstrated that dynorphin released from these synapses could cause homosynaptic depression as well. When mossy fiber inputs were stimulated with four brief high frequency trains, no LTP was generated in control conditions. However, in the presence of nor-BNI these same trains resulted in LTP. Thus, dynorphin released from mossy fiber synapses modulates synaptic plasticity induced by high-frequency trains. Another interesting finding from this study is that exogenously applied dynorphin inhibits potentiated synapses to a greater degree than naive synapses. In the light of subsequent findings that kappa receptors are located on DCV membranes in axon terminals (Drake et al. 1996), and that their expression can be regulated in an activity-dependent manner (Shuster et al. 1999), it is tempting to speculate that this increased responsivity is due to incorporation of additional kappa receptors into the terminal membrane by fusion of dynorphin-containing DCVs.

4.3
Modulation of Pain by Presynaptic Kappa Receptors in the Dorsal Raphe Magnus

Mu opioid receptors influence pain transmission at both the spinal and supraspinal level. Two major components of the descending modulatory nociceptive circuitry are the periaqueductal gray and its major target, the nucleus raphe magnus (NRM). Activation of mu opioid receptors on inhibitory interneurons of the NRM causes "disinhibition" (leading to activation) of projection neurons and inhibition of ascending nociceptive pathways in the dorsal horn of the spinal cord (Aimone and Gebhart 1986). Plasticity of mu opioid receptor responses occurs at the level of the NRM, including tolerance and dependence to mu agonists and withdrawal-induced hyperalgesia. Activation of kappa opioid receptors in NRM reduces the analgesic actions of mu opioid agonists such as morphine. Cellular studies suggest that the mechanism involves presynaptic inhibition of glutamate release onto the projecting neurons of the NRM, which would opposing MOR-mediated disinhibition (Bie and Pan 2003). Interestingly, kappa agonists also hyperpolarize the projecting neurons, thus synergistic pre- and postsynaptic inhibitory actions of kappa receptors would act to turn off the descending analgesic pathway. In contrast, during the hyperalgesic (increased pain threshold) state that occurs during opioid withdrawal, activation of kappa opioid receptors in NRM results in an increase in pain threshold to near baseline levels (Bie and Pan 2003). Under this condition, therefore, kappa opioid receptors are analgesic. Hyperactivity of secondary neurons in the NRM is thought to underlie hyperalgesia during morphine withdrawal. Slice studies show kappa agonists inhibit glutamatergic input onto these neurons (Bie and Pan 2003), suggesting that the ability of kappa agonists to reduce this hyperalgesia is likely due to inhibition of presynaptic glutamate release by activation of kappa opioid receptors. Thus, under conditions where the primary neurons are active, such as during morphine treatment, the hyperalgesic actions of kappa agonists would predominate, whereas when the secondary neurons are active (during morphine withdrawal), kappa agonists would be analgesic.

4.4
Presynaptic Modulation of SCN Activity by NPY is Important for Circadian Entrainment

The hypothalamic suprachiasmatic nucleus (SCN) generates circadian rhythms. Photic input from the retina reaches the SCN via a direct glutamatergic projection and an indirect projection from the intergeniculate leaflet (IGL). Neurons of the IGL that project to SCN co-express NPY (Chronwall et al. 1985), and injection of NPY into the SCN causes long-term phase shifts in diurnal rhythms, similar to photic input (Medanic and Gillette 1993). Van

den Pol and colleagues (van den Pol et al. 1996) examined the cellular mechanism of this effect using SCN slices and cultured neurons. They found that brief applications of NPY caused robust hyperpolarizations of SCN neurons and large decreases in glutamatergic EPSPs. Although the postsynaptic actions were transient, inhibition of EPSPs lasted for an hour or more. They also examined inhibition of intracellular Ca^{2+} by NPY, and found that although NPY transiently inhibited glutamate-induced Ca^{2+} transients in the presence of TTX, spontaneous, action-potential mediated transients were inhibited more robustly and for much longer. Inhibition of mEPSC frequency by NPY was also long-lasting, suggesting that NPY inhibited glutamate release downstream of Ca^{2+} entry. Interestingly, this group also reported that NPY must be applied in the presence of intact glutamatergic transmission for it to generate its long-lasting effects; however, NMDA receptors were not required. They suggest that only active synapses are sensitive to the long-lasting changes induced by NPY. The mechanism by which NPY mediates long-lasting inhibition of glutamate release is unknown, but would appear to involve additional signaling besides $\beta\gamma$ interaction with presynaptic vesicular release. However, NPY-induced phase shifts in circadian rhythms are insensitive to inhibition of protein synthesis (Hall et al. 1999). Thus, NPY may act as a "coincidence detector" to integrate photic information entering the SCN.

4.5
Dendritically Released Neuropeptides Can Inhibit Neurotransmission

In the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus, dendritic release of the neuropeptides oxytocin (OXT) and vasopressin (VP) occurs in magnocellular neurons. These two nuclei project to the neurohypophysis, providing the major source of circulating OXT and VP. Release is distinctly regulated in dendrites vs. projection terminals, and can occur independently (Ludwig 1998; Wotjak et al. 1998). As in terminals, dendritic release of neuropeptides likely requires elevations in intracellular Ca^{2+} (Ludwig et al. 2002). Also, both L-type Ca^{2+} channels and NMDA receptors, as well as release from intracellular stores, can support peptide release (Shibuya et al. 1998; Bains and Ferguson 1999).

In the SON, inhibition of glutamate neurotransmission by dendritically released OXT allows for reciprocal communication between glutamate terminals and dendrites. Glutamatergic input regulates firing patterns of MCGs, thus controlling release of neuropeptides in the neurohypophysis as well as locally from dendrites. Exogenously applied OXT causes presynaptic inhibition of glutamatergic EPSCs (Pittman et al. 1998). To assess the actions of endogenously released OXT, Pittman and colleagues used an aminopeptidase inhibitor and showed it had similar effects on synaptic depression as OXT and was blocked by a nonspecific antagonist to OXT/VP. These results

suggested that an OXT/VP tone exists in the densely packed SON that is normally masked by degradative enzymes. This group showed that high frequency stimulation (100 Hz) of the glutamatergic inputs also led to synaptic depression that was blocked by the OXT/VP antagonist (Pittman et al. 1998). Thus, high frequency activation of the inputs would lead to sufficient peptide release to overcome the endogenous peptidases and induce presynaptic inhibition of glutamate release. The mechanism of presynaptic inhibition in the SON was assessed. Using specific Ca^{2+} channel blockers, presynaptic inhibition by OXY was found to be dependent on intact N-type and to a lesser degree P/Q-type Ca^{2+} channels. Ca^{2+}-independent spontaneous release was not affected by OXT (Hirasawa et al. 2001). Interestingly, a more recent study from this group showed that the inhibition of glutamate release by OXT was not direct but instead was a result of stimulation of cannabinoid release from the postsynaptic neuron (Hirasawa et al. 2004). This is similar to the action of ORX-B in the DRN (Haj-Dahmane and Shen 2005), described above.

In guinea pig hippocampus, dynorphin is expressed in the axons of granule cells that synapse onto CA3 pyramidal neurons. The receptor for this peptide, the kappa opioid receptor, is located largely in the molecular layer of the dentate gyrus, where granule cell dendrites are localized. Dense core vesicles containing dynorphin were observed using electron microscopy in dendrites of hippocampal dentate granule cells (Drake et al. 1994). A series of studies by Chavkin and colleagues provided insight into physiological consequences of release. Bath-applied kappa ligands reduced dentate granule cell population spikes and field EPSPs (fEPSPs) evoked by perforant path stimulation; IPSPs were not affected (Wagner et al. 1992). Analysis of paired-pulse facilitation of fEPSPs suggested a presynaptic site of action (Simmons et al. 1994). High frequency stimulation caused depression of granule cell fEPSPs that was blocked by a selective kappa receptor blocker, suggesting inhibition was due to endogenous release of dynorphin. Focal application of dynorphin B suggested that the response was mediated by receptors in the molecular layer (Drake et al. 1994). L-type Ca^{2+} channels were required for dendritic release of dynorphin, whereas N-type channels were necessary for axonal release (Simmons et al. 1995). A physiological consequence of the dendritic release of dynorphin is the inhibition of long-term potentiation, a cellular model of learning and memory, at the perforant path synapses onto dentate granule cells (Wagner et al. 1993). Blockade of kappa receptors during the induction phase leads to an enhancement of LTP (Terman et al. 1994). These results suggest that dendritically-released dynorphin can modulate hippocampal-dependent learning and memory, although this hypothesis has not been confirmed in studies in kappa receptor knockout mice, which do not show alterations in spatial learning (Jamot et al. 2003). However, it should be noted that distribution of kappa receptors in hippocampus of mice, in contrast to guinea pigs, is quite low (Williams and Johnston 1996).

5
Conclusions

Presynaptic inhibition of glutamate release has been demonstrated in multiple brain regions (and spinal cord) to be a major mechanism by which neuropeptides mediate their actions. We are only just beginning to understand the functional impact of this synaptic modulation. However, because endogenous neuropeptides would only modulate robustly active synapses, this allows for use-dependent modulation and coincidence detection. Because neuropeptide receptors are "stored" in DCV membranes, they would be inserted into the plasma membrane at release sites. This would allow for increased inhibitory modulation during times when the GABA system may be depressed due to synaptic depletion, depolarization-induced suppression of inhibition, GABA receptor desensitization, etc. The increased expression of neuropeptide receptor at presynaptic sites could also serve as a synaptic "tag" for strongly stimulated synapses, leading to subsequent long-term alterations. Use of selective receptor antagonists and peptide/receptor knockout mice are beginning to shed light on specific functions for neuropeptides. Because of the wide distribution of neuropeptides and their receptors in the brain, it is not surprising that a diverse array of functions have been identified, with many more likely to follow.

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Regulation of Excitation by GABA Neurotransmission: Focus on Metabolism and Transport

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Abstract The vast majority of excitatory synapses in the central nervous system (CNS) utilize glutamate as the neurotransmitter. The level of excitation appears to be under regulatory control by the major inhibitory neurotransmitter GABA, which is synthesized from glutamate by its decarboxylation catalysed by glutamate decarboxylase (GAD). The inactivation of GABA is brought about by high affinity GABA transporters located in the presynaptic GABAergic neurons as well as surrounding astrocytes and subsequently GABA may be metabolized by GABA-transaminase (GABA-T) ultimately allowing the carbon skeleton to enter the tricarboxylic acid (TCA) cycle for oxidative metabolism. In the presynaptic GABAergic neuron, GABA taken up seems, however, preferentially to enter the vesicular GABA pool and hence it is recycled as a transmitter. It has become clear that compounds acting as inhibitors at either the transporters or GABA-T are capable of regulating the inhibitory tonus thus controlling excitation. This has led to development of clinically efficatious antiepileptic drugs. This paper shall review recent progress in targeting these pharmacological entities.

1 Introduction

1.1 Excitatory Activity Mediated by Glutamate

Classical studies of electrophysiological actions of glutamate in spinal cord and CNS preparations carried out around 1960 clearly indicated that this ubiquitous and abundant amino acid could play a major role as an important neurotransmitter with excitatory actions (Curtis et al. 1959). While at that time this notion was highly controversial, by now it is firmly established that glutamate is the major excitatory neurotransmitter in the CNS. This is based on the abundant presence in essentially all brain areas of vesicular glutamate transporters, the most likely candidate for a selective molecular marker of glutamatergic synapses (Reimer et al. 2001). Inactivation of glutamate is analogous to that of GABA (see below) since it is mediated by high affinity transporters, the majority of which are located on astrocytes surrounding the glutamatergic synapses (Danbolt 2001; Schousboe et al. 2004).

The presence and functional importance of glutamate transporters in the presynaptic nerve endings have been debated (Danbolt 2001) but experimental evidence indicates that such transporters exist and that they may play a role in the maintenance of the synaptic glutamate pool (Waagepetersen et al. 2005). It is, however, clear that astrocytic glutamate transport is of critical importance for the maintenance of low extracellular glutamate levels (Schousboe et al. 2004) and that conditions such as energy failure, which lead to reversal of carrier mediated glutamate transport and glutamate overflow (Benveniste et al. 1984; Nicholls and Attwell 1990) result in extracellular glutamate levels exceeding the threshold for glutamate mediated excitotoxic neuronal death (Benveniste et al. 1984; Choi and Rothman 1990). In keeping with this, inhibition of the glutamate transporters by pharmacological agents also results in excitotoxic neuronal degenerations (O'Shea et al. 2002; Bonde et al. 2003). However, during energy failure, these agents may prevent glutamate overflow caused by reversal of the carriers, thus preventing or reducing neuronal degeneration (Bonde et al. 2003).

Hyperactivity of glutamatergic synapses may lead to not only excitotoxic insults but may also cause seizures and epileptic episodes (Meldrum 1975). Hence, it is important that glutamatergic synaptic activity can be regulated, a process which is mediated by GABA, the major inhibitory neurotransmitter in the CNS (Roberts 1991). Since it is currently pharmacologically difficult to regulate glutamatergic activity an alternative approach may be to enhance GABAergic activity which may balance the excitatory-inhibitory tonus (Meldrum 1975). The following sections will focus on pharmacological strategies involving GABA transport and metabolism which have been developed to achieve this goal.

1.2 Inhibitory Activity Mediated by GABA

In analogy to glutamate, detailed electrophysiological studies of the actions of GABA in different neuronal preparations resulted in the notion that GABA acts as an inhibitory neurotransmitter (Curtis et al. 1959). Studies of the distribution of the synthesizing enzyme GAD which is almost exclusively associated with GABAergic synapses (Saito et al. 1974; Roberts 1991) have underlined the abundance of such synapses in the CNS. Again, in analogy to glutamate, GABA is inactivated by high affinity transporters and subsequent metabolism via GABA transaminase (GABA-T) (Schousboe et al. 2004). However, in contrast to glutamate, these transporters are mainly located on presynaptic GABAergic neurons which allows for recycling of GABA as a neurotransmitter (Schousboe et al. 2004).

1.2.1
GABA Metabolism

The major biosynthetic route for GABA is that involving decarboxylation of glutamate leading to formation of GABA and CO_2 catalyzed by GAD (Martin and Rimvall 1993). GAD exists in two isoforms having different molecular weights (65 and 67 kD) and they are therefore refered to as GAD_{65} and GAD_{67}, respectively (Martin and Rimvall 1993). They exhibit different properties with regard to regulation via coenzyme binding and they also have different subcellular distribution. Thus, GAD_{65} is associated with synapses while GAD_{67} is distributed ubiquitously in the cytoplasm of GABAergic neurons (Martin and Rimvall 1993). Recent detailed studies of GABA biosynthesis have revealed that the TCA cycle may be involved which may further complicate the regulatory machinery governing GABA synthesis (Waagepetersen et al. 1999, 2001).

Metabolic degradation of GABA involves two enzymes, GABA-T and succinic semialdehyde dehydrogenase, the concerted action of which leads to conversion of GABA to succinate, a TCA cycle constituent (Waagepetersen et al. 2003). This metabolic route is called the "GABA shunt" since the overall result is the conversion of α-ketoglutarate which is funnelled out of the TCA cycle, converted to GABA, and subsequently degraded to succinate that re-enters the TCA cycle. It is estimated that 8–10% of the glucose metabolism in GABAergic neurons are funnelled through the "GABA shunt" (Balazs et al. 1970).

1.2.2
GABA Transaminase

The initial work by Bessman and co-workers in the field of GABA research identified the GABA-T (4-aminobutyrate-2-ketoglutarate aminotransferase) in 1953. However, it was not until 1973 that Schousboe and colleagues purified mouse GABA-T to homogeneity and characterized it kinetically (Schousboe et al. 1973) and in a subsequent study determined its cellular and subcellular localization (Schousboe et al. 1980).

GABA-T was shown to have a molecular weight of 110 kDa and to consist of two almost identical subunits weighing 53 and 58 kDa. The K_m values for GABA and α-ketoglutarate were estimated to be 1.1 mM and 0.25 mM, respectively. GABA-T is located in both neurons and astrocytes with the highest activity residing in the former cell type. The subcellular localization of GABA-T has primarily been coined to the inner mitochondrial membrane, but evidence suggests that it may also be located to the plasma membrane. GABA-T has also been found in other tissue among other, kidney and liver, and no existence of isoenzymes was indicated (Schousboe et al. 1973, 1980). Several other laboratories have reported the existence of isoenzymes in dif-

ferent species, however, studies conducted by Silverman and colleagues on pig brain GABA-T, also reported the existence of several different active GABA-T forms, via MALDI-TOF mass spectral analysis and N-terminal sequence they identified the different active species to be N-terminal truncated forms rather than isoenzymes (Koo et al. 2000). The activity of GABA-T relies on the co-factor pyridoxal 5′-phosphate (PLP) which is bound to LYS-329 via a Schiff base linkage (Toney et al. 1995). As inhibition of GABA-T leads to elevated synaptic GABA levels, this enzyme constitutes an interesting pharmacological target by which GABAergic neurotransmission may be enhanced (see below).

1.2.3 GABA Transport

Early studies by Iversen and coworkers (1968) proved the existence of high affinity uptake sites for GABA in both neurons and astrocytes, which differ in chemical specificity for diaminobutyric acid (DABA) and β-alanine, respectively (Iversen and Neal 1968; Iversen and Kelly 1975), revealing different cellular components which facilitate the termination of the GABAergic neurotransmission. Neuronal uptake has been estimated to be three to six-fold more efficient than glial uptake, which could indicate that GABA taken up into neurons serves as a mechanism to replenish the GABA pool (Hertz and Schousboe 1987).

The event leading to the cloning of GABA transporters began when Radian and coworkers isolated an 80 kDa glycoprotein transporter from the synaptic plasma in the rat brain, with an apparent K_m for GABA of 3 μM and a Na^+ and Cl^- dependence for transport. The protein showed no immunoprecipitation with GABA receptor complexes, which suggest little homology between these proteins (Radian et al. 1986). Guastella and coworkers isolated a 67 kDa transporter protein designated GAT-1 consisting of 599 amino acids, using a cDNA made from purified GABA transport protein. GAT-1 showed a Na^+ and Cl^- requirement for transport, and when tested with previously established inhibitors of either neuronal or glial GABA transport, it had similar pharmacology as the neuronal subtype with a K_m for GABA of 7 μM (Guastella et al. 1990). Subsequently, Yamauchi and colleagues reported the isolation of a renal betaine/GABA transporter (BGT-1) encoding a 614 amino acid protein weighing 69 kDa with a dependence on Na^+ and Cl^- for transport. The protein transports the osmolyte betaine with a K_m of 398 μmol and GABA with a K_m of 93 μM, hence the affinity of GABA is four-fold greater than that of betaine (Yamauchi et al. 1992). Through low stringency screening of rat brain cDNA library with probes encoding GAT-1, Borden et al. (1992) isolated two GABA transporters designated GAT-2 and GAT-3 having K_m values for GABA of 8 and 12 μM, respectively. GAT-2 and GAT-3 encode proteins of 602 and 627 amino acids, which are sensitive to β-alanine suggesting a greater similarity to the glial transporter than GAT-1. Though sensitive to

β-alanine, GAT-2 and GAT-3 showed much lower activity for nipecotic acid, guvacine, and hydroxynipecotic acid than did glia cells. Liu and colleagues cloned and characterized four GABA transporters from a mouse neonatal cDNA library and designated them GAT1, GAT2, GAT3, and GAT4 (without hyphen). They encode a protein of; 598, 614, 602, and 627 amino acids, respectively with Na^+ and Cl^- dependence for transport. Interestingly, nipecotic acid, DABA, and guvacine were more potent inhibitors of GAT1 and GAT4 than of GAT2 and GAT3 (Liu et al. 1992, 1993). GAT3 showed 69% and 51% amino acid identity with GAT2 and GAT1, respectively, and GAT4 showed 70%, 65%, and 53% amino acid identity with GAT3, GAT2, and GAT1, respectively (Liu et al. 1993). A summary of the rather confusing nomenclature of the GABA transporters is shown in Table 1. Throughout this chapter the nomenclature associated with the cloned mouse GABA transporters will be used in general terms.

The GABA transporters belong to a superfamily of transporters that can be divided into four groups; 1) Transporters of biogenic amines (noradrenalin, dopamine, and serotonin). 2) Various GABA transporters as well as transporters of taurine and creatine. 3) Transporters of proline and glycine and 4) "Orphan" transporters. Their amino acid sequence reveals an identity of 30–65% between different members of the family.

The GABA transporters are all highly dependent on Na^+ and to a lesser extent Cl^- for transport. The three GABA transporters cloned from rat brain all show an absolute dependence for Na^+ but when Cl^- is substituted with acetate GAT1, GAT3, and GAT4 retained 0%, 43% and 20% of their total transport activity, respectively, arguing for differences in uptake mechanisms (Borden et al. 1992). The stoichiometry of the transport system is 2–3 Na^+ ions and 1 Cl^- ion for each molecule of GABA translocated across the membrane. The transporter is capable of generating a gradient in the order of

Table 1 GABA transporter nomenclature across species

Species	Nomenclature			
Rat	GAT-1 [a]	BGT-1 [b]	GAT-2 [c]	GAT-3 [c]
Human	GAT-1 [d]	BGT-1 [e]	NC	GAT-3 [f]
Mouse	GAT1 [g]	GAT2 [g]	GAT3 [g]	GAT4 [g]

[a] Guastella et al. 1990
[b] Yamauchi et al. 1992
[c] Borden et al. 1992
[d] Nelson et al. 1990
[e] Borden et al. 1995
[f] Borden et al. 1994
[g] Liu et al. 1992, 1993 NC not cloned

10^5 between intra- and extra-cellular GABA (Schousboe et al. 1981b; Beleboni et al. 2004).

The transporters share similar secondary structure composed of 12 hydrophobic putative transmembrane (TM) domains organized in dimers (Beleboni et al. 2004). A highly conserved region within the superfamily (> 50% homology) including TM1, extracellular loop (EL) 2 and 3, and intracellular loop (IL) 2 are thought to be essential for stabilizing the tertiary structure and thereby function. The N- and C-terminals face the cytoplasm and contain putative phosphorylation sites probably involved in regulation of the transport process, and it is the least homologous part of the transporters (Jursky et al. 1994; Kanner 1994). Shown in Fig. 1 is the secondary structure of the bacterial *Aquifex aeolicus* LeuT_{Aa} transporter, which is the first crystal structure of a Na^+/Cl^- dependent neurotransmitter transporter presented. The analysis of this transporter has revealed a repeat in the internal structure in the first ten TM helices, where TM1-5 and TM6-10 folds in a pseudo-twofold structure which is believed to represent the essential core of Na^+/Cl^- dependent neurotransmitter transporters. The breaks in helical structure observed in TM1 and TM6 together with TM3 and TM8 are believed to comprise the substrate and sodium ion binding site (Yamashita et al. 2005).

Through site directed mutation studies it has been found, that W68, R69, E101, Y140, and W222 are essential for transport activity and binding of GABA in GAT1. A minimal length of the extracellular loop EL4 and intracellular loop IL 4 has also been shown to be necessary for transport activity (Schousboe and Kanner 2002). The large extracellular loop EL2 contains potential glycosylation sites, which have been implicated in the final stage of insertion of the transporter into the plasma membrane (Beleboni et al. 2004). Perhaps of more interest is the crucial role of the extracellular loops in conferring chemical selectivity among the GABA transporters. Mutations of EL4 in GAT1 led to GAT4-type structure in the loop and a K_m similar to GAT4. Exchanging the amino acid sequence of EL6 in GAT1 with that of GAT2 led

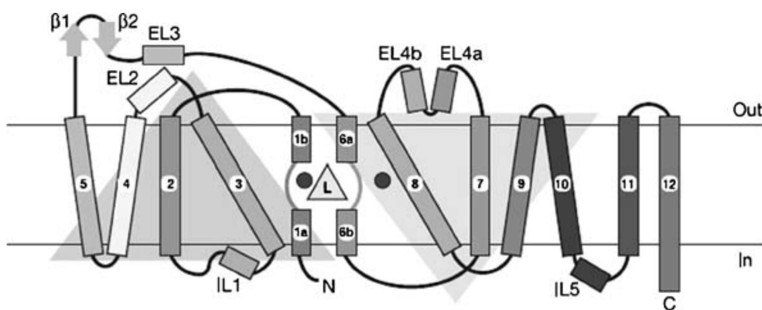

Fig. 1 Topological representation of the crystal structure of LeuT_{Aa}, with Leucine and the two sodium ions depicted as a *triangle* and *circles*, respectively (Yamashita et al. 2005)

to a mutant with low affinity (K_m of 35 μM) for GABA and at least a two-fold increase in V_{max}. As described earlier GAT1 is insensitive to β-alanine, when substituting the EL5 region in GAT1 with that of GAT3, the mutant was sensitive to β-alanine. The reverse mutation in GAT3 with the EL5 region of GAT1 revealed a mutant with less sensitivity to β-alanine. Hence it was suggested that EL5 participates in binding of β-alanine. The mutation in EL6 of GAT1 conferred to the mutant a slight sensitivity to betaine. It was proposed that the extracellular loops 4–6 are involved in substrate binding (Tamura et al. 1995).

GAT1 is highly abundant and widely distributed throughout the brain, particularly in the retina, olfactory bulb, cortex, ventral pallidum, hippocampus, interpeduncular nucleus, and cerebellum closely paralleling that of GABAergic neurons (Durkin et al. 1995; Borden 1996). GAT1 is found to be restricted to the apical surface in polarized MDCK cells (Pietrini et al. 1994). Furthermore, it is found exclusively in axonal segments of GABAergic neurons, where it mediates a presynaptic uptake of GABA (Radian et al. 1990; Pietrini et al. 1994; Borden 1996; Conti et al. 1998). The perikarya are negative for GAT1 labelling (Radian et al. 1990). GAT1 labeling of glia cells in cerebellum, hippocampus and substantia nigra is observed and cortical astrocytes express GAT1 in their distal processes close to axon terminals forming symmetric synapses (Radian et al. 1990; Conti et al. 1998, 2004). However, the glial expression of GAT1 might be underestimated, due to the intense neuronal labeling which might obscure the glial signals (Borden 1996).

Low to moderate labeling of GAT2 is detected in the cortex and hippocampus (Zhu and Ong 2004) and throughout the brain (Borden 1996). Borden et al. (1995) found that GAT2 was not located closely to GABAergic synapses, and suggested that its function was to sequester GABA that had diffused away from the synaptic region. GAT2 transfected in polarized MDCK reveals basolateral targeting under normal and hyperosmolar conditions (Pietrini et al. 1994; Ahn et al. 1996). Consistent with this statement is the finding that CA neurons in the hippocampus and pyramidal neurons of the cortex, have many GAT2 labeled dendrites along with moderately labeled cell bodies (Zhu and Ong 2004). The dendrites make asymmetric synaptic contact with glutamatergic axons. The GAT2 label is observed in an extra-perisynaptic region, not located within the postsynaptic region. The authors state that GAT2 does not have a direct influence in fast termination of GABAergic neurotransmission, rather the ability of GAT2 to transport betaine, thereby participating in osmoregulation, might be its primary role in the CNS (Zhu and Ong 2004).

Liu et al. (1993) only found GAT3 in the neonatal brain. Northern blot analysis reveals mRNA of GAT3 in cerebellum and olfactory bulb. However, the only in-situ localized GAT3 is found in the leptomeninges (Durkin et al. 1995) suggesting a regulatory role of GABA levels in CSF (Borden 1996; Conti et al. 2004). The low levels of protein may obscure the detection of

transporter protein in the mature brain. Conti and coworkers obtained high resolution using electron and light microscopy and found that GAT3 is expressed throughout the mature cortex in both neurons and astrocytes in low levels. Neuronal GAT3 is primarily located to the basolateral domain of polarized MDCK cells (Ahn et al. 1996), hence a dendritic localization, strikingly where neither GAT1 nor GAT4 has been found. Distal astrocytic processes are also labeled with GAT3. It is concluded that GAT3 is primarily located to the extra-synaptic region, and is involved in regulation of extra-synaptic GABA levels (Conti et al. 1999, 2004).

The distribution of GAT4 is more restricted compared to GAT1. It shows strong intensity in retina, olfactory bulb, certain brainstem nuclei, and structures in the diencephalons (e.g., thalamus, hypothalamus). Low levels are found in the cortex, hippocampus, caudate putamen, and cerebellar cortex (Durkin et al. 1995; Minelli et al. 1996). Its distribution seems to comprise both neurons and astrocytes. However, GAT4 is predominantly found in glial cells, and most neuronal GAT4 is found within the retina and olfactory bulb (Durkin et al. 1995). Neuronal GAT4 expression in the cortex cannot be re-

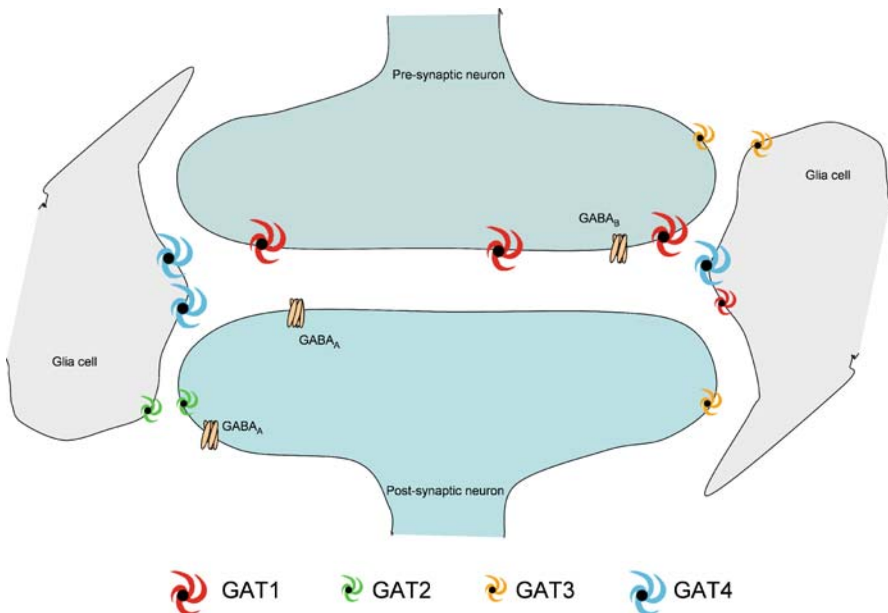

Fig. 2 Schematic representation of the subcellular localization of the four GABA transporters in cortical synapses. GAT1 is primarily located presynaptic and in astrocytes near the synapsis. GAT2 is located to the soma and dendritic, with the dendrites making asymmetric synaptic contact with glutamatergic neurons in the extra-synaptic region. GAT3 is located to both astrocytes and neurons, but primarily dendritic in neurons. GAT4 is located primarily to the distal astrocytic processes in the synapses

jected, but the levels are too low to be detected (Minelli et al. 1996). GAT4 transfected in polarized MDCK cells reveals localization to the apical domain (Ahn et al. 1996; Borden 1996). Minelli et al. (1996) investigated the expression of GAT4 utilizing immunolabeling, and gaining a higher level of resolution than achieved before, enabled them to find higher levels in the cortex than previous reported. GAT4 was found throughout the cortex located exclusively in distal astrocytic processes. The stained GAT4 astrocytes are in direct contact with GABAergic neurons, thus contributing to termination of fast GABAergic neurotransmission, and establishing the importance of glial selective GABA uptake inhibitors.

Figure 2 summarizes the different localizations of the GABA transporters. Within the brain the mRNA for GAT1 and GAT4 are in general, differentially distributed between neuron and astrocytes, and GAT1 appears to be more abundant than GAT4 (Durkin et al. 1995). Although GAT2 and GAT3 is present in the cerebral cortex, GAT1 and GAT4 mediates the largest uptake of GABA (Conti et al. 1998; Zhu and Ong 2004). The differences in pharmacological, physiological, and subcellular localization that exist between the GABA transporters, presents a challenging task in elucidating the fine tuning of extracellular GABA levels.

2
Inhibitors of GABA-transaminase and GABA Transporters as Anticonvulsants

To date, at least 18 AEDs are available on the European market but only Vigabatrin and Tiagabine display a mechanism of action that is solely dependent on an interaction with the GABA system namely inhibition of GABA-T and GAT1, respectively. However, numerous initiatives worldwide have been undertaken in the search for new potential antiepileptic drugs for this system. Several new drugs for the treatment of epilepsy have been introduced to the market in the past decade; however, they do not exclusively potentiate the GABAergic tonus.

2.1
GABA-T Inhibitors

The first drug available on the market for the treatment of epilepsy with a mechanism of action targeting GABA-T, at least to some extent, was Valproate (VPA). VPAs mechanism of action although not understood completely, is most likely a combination of three different cellular interactions as discussed below. VPA enhances the GABAergic tonus due to an inhibition of GABA-T (Larsson et al. 1986a) and displays an anticonvulsant activity in maximal electroshock treated rats as demonstrated previously (McLean and MacDonald 1986). However, the anticonvulsant effect mediated by VPA has

not been directly associated with an enhancement in GABA levels in the cerebrospinal fluid (CSF) and cortex in all studies undertaken, as seen in studies on the prevention of audiogenic seizures in mice (Anlezark et al. 1976) and electroshock induced seizures in rats (Kerwin and Taberner 1981). It has become more evident that VPA also displays a direct membrane effect. VPA exerts an use- and voltage-dependent limitation of high-frequency repetitive firing of Na^+-dependent action potentials (McLean and MacDonald, 1986), and reduces the Ca^{2+} T-current to a modest extent in a concentration dependent manner within the therapeutic range of VPA free plasma levels in humans (Kelly et al. 1990).

4-Amino-hex-5-enoic acid (γ-vinyl GABA) also known as Vigabatrin (VGB) is accepted as a false substrate by GABA-T and transformed into an active metabolite which binds irreversibly to the active site on GABA-T and subsequently causes an irreversibly inhibition of the enzyme, therefore VGB is classified as a suicide inhibitor (Lippert et al. 1977). VGB also possesses a minimal inhibitory effect on the GAD isoenzyme when tested in a very high dose (Jung et al. 1977). When administered i.p. VGB produces a long lasting protection against audiogenically induced seizures in DBA/2 mice, offering a 56% level of protection 72 hours after administration of a 1500 mg/kg dose. The same dose was administered i.p. to CD_1 male mice and the elevation of brain GABA levels was measured. The results showed increased GABA levels up to 5 days post administration, when compared to a control group. To achieve a 50% reduction in seizure incidence, an increase of about 324% in brain GABA concentration was needed in DBA/2 mice (Schechter et al. 1977).

Utilizing the information about the mechanism of action of VGB, the synthesis of other suicide inhibitors has resulted in several series of compounds acting as either competitive inhibitors or time- and concentration-dependent inactivators of GABA-T. Rigid conformational analogues of VGB such as 4-amino-2-cyclopentane-1-carboxyclic acid were synthesized and displayed only a competitive mode of action (Qiu et al. 1999). To determine whether this mechanism of action applies to all cyclic analogues of VGB, a series of cyclic mono- and di-halogen substituted analogues of 4-amino-5-halopentanoic acids were produced. They were all competitive inhibitors of GABA-T, however, several were also mechanism based inactivators (Qiu and Silverman, 2000). The less confined cyclohexene analogues of VGB allow for more flexibility in the ring system and the cis-forms of (3-aminocyclohex-4-ene-1-carboxylic acid and 2-aminocyclohex-3-ene-1-carboxylic acid) are mechanism based inactivators of GABA-T following a time- and concentration dependent interaction, however, the trans-forms were competitive inhibitors (Choi and Silverman 2002). For a more detailed discussion regarding the molecular intricacies resulting in the two inhibition mechanisms, i.e., whether the compounds follow a Michael addition mechanism or an enamine mechanism, please review the above-mentioned papers by Silverman and colleagues.

2.2
GABA-transport Inhibitors

Muscimol derived from of the mushroom *Amanita muscaria* is a GABA analogue, and a potent $GABA_A$ agonist, a weak inhibitor of glial and neuronal GABA uptake, and substrate for GABA-T (Krogsgaard-Larsen et al. 2000). THIP and THPO are two restricted analogues of muscimol and the $GABA_A$ agonist and transport inhibitor properties of muscimol were “isolated”, respectively, in these two structures (Krogsgaard-Larsen et al. 2000).

Guvacine and nipecotic acid are monocyclic bioisosteric analogues of THPO which is a moderately potent inhibitor of neuronal and glial transport being 2-fold more potent on the latter cell type. Nipecotic acid and guvacine are powerful inhibitors of both neuronal and glial transport and targets GAT1, GAT3, and GAT4 but not GAT2, however, they do not display a selectivity towards astrocytes as do their parent compound THPO (Schousboe et al. 1979, 1981a). The structures of these compounds are shown in Fig. 3. Although being unable to penetrate the blood–brain barrier, mostly due to their zwitterionic character and their low lipophilicity, they have been used as parent compounds in the subsequent search for new compounds, which have turned out to represent interesting pharmalogical tools in the characterization of GATs (Krogsgaard-Larsen et al. 2000).

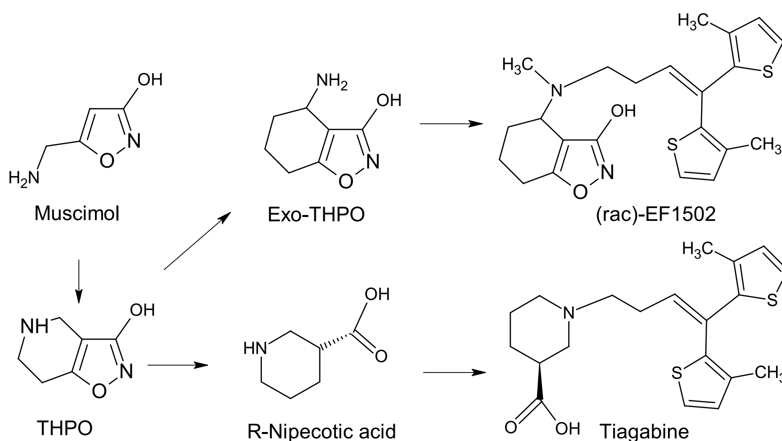

Fig. 3 Depicts the progress in synthesis from muscimol to nipecotic acid and then tiagabine. Also exo-THPO which has been used as parent structure for a several new inhibitors of GABA transport is displayed

THPO being two-fold more selective for astrocytic versus neuronal transport, resulted in the synthesis of a series of drugs with exo-THPO as parent compound. Some of the most important compounds from this series are

listed in Table 2 which displays their inhibitory activity in cultured cortical neurons, astrocytes, and cloned GAT1-4 expressed in HEK-293 cells.

Some of the derivatives of exo-THPO are more potent on astrocytic versus neuronal transport. N-Methyl-exo-THPO and N-acetyloxyethyl-exo-THPO both displaying a ten-fold selectivity towards glial GABA uptake are, however, moderately potent inhibitors of GAT1 mediated GABA transport. The anti-

Table 2 Inhibitory patterns of GABA analogues on neurons, astrocytes, and cloned mouse GABA transporters GAT1-4

Compound	GABA uptake inhibition IC_{50} or $^*K_{m/i}$ (μM)					
	Neurons	Astrocytes	GAT1	GAT2	GAT3	GAT4
GABA	8[a]	32[a]	17[a]	51[a]	15[a]	17[a]
Nipecotic acid	12	16	24	> 1000	113	159
Tiagabine	0.45	0.18	0.11	> 100	> 100	800
Guvacine	32	29	39	> 1000	228	378
DABA	1000	> 5000	128	528[c]	300	710
ACHC	200	700	132	1070[c]	> 1000	> 10 000
β-alanine	1666[b]	843[b]	2920	1100[c]	66	110
THPO	501[b]	262[b]	1300	3000	800	5000
Exo-THPO	780	250	1000	3000	> 3000	> 3000
N-methyl-exo-THPO	405	48	450	> 3000	> 3000	> 3000
N-ethyl-exo-THPO	390	301	320	> 1000	> 1000	> 1000
N-2-hydroxyethyl-exo-THPO	300	200	> 500	> 500	> 500	> 500
N-4-phenylbutyl-exo-THPO	100	15	7	> 500	> 1000	> 1000
N-acetyloxyethyl-exo-THPO	200	18	550	> 1000	> 1000	> 1000
(R/S)-EF1502	2	2	7	26	> 300	> 300
(R)-EF1502	1.5	0.65	4	22	> 150	> 150
S)-EF1502	> 100	> 100	120	34	> 150	> 150
DPB-THPO	38[b]	26	30	200	> 300	> 1000
DPB-Nipecotic acid	1.3[b]	2.0[b]	0.64	7210[c]	550	4390
N-DPB-exo-THPO	1.4	0.6	6	100	> 100	> 100
N-DPB-N-methyl-exo-THPO	5	2	2	200	> 100	> 100

Data summarized from (Bolvig et al. 1999; Borden 1996; Clausen et al. 2005; Falch et al. 1999; Larsson et al. 1981, 1983, 1986b, 1988; Sarup et al. 2003a; Schousboe 1979; White et al. 2002)

[a] K_m

[b] K_i

[c] Human BGT-1

convulsant activity of exo-THPO, N-Methyl-exo-THPO, N-Ethyl-exo-THPO, and tiagabine has been established in audiogenic seizure (AGS)-susceptible Frings mice following intracerebro-ventricular injection. Figure 4 shows ED_{50} values as a function of their IC_{50} value for inhibition of GABA uptake in both neurons and astrocytes. There is a high correlation between the inhibitory potency in astrocytes and anticonvulsant properties ($r^2 = 0.988$) compared to their inhibitory potency in neurons and anticonvulsant properties ($r^2 = 0.5336$) (White et al. 2002). Albeit GAT1 is located primarily to the presynaptic neuronal terminals and to a minor extent to distal processes of astrocytes near the synapses, the subcellular localization of GAT offers no

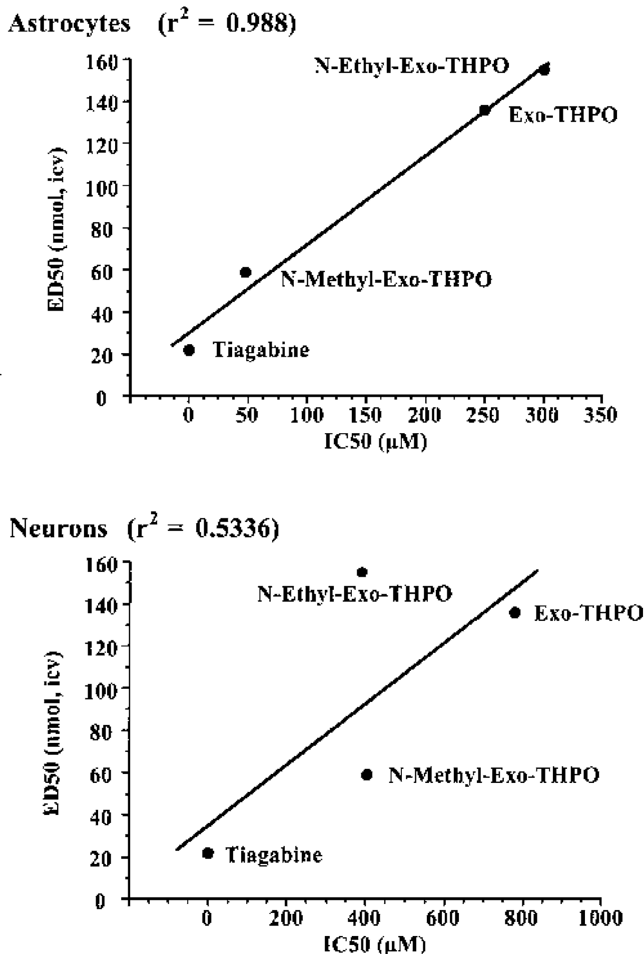

Fig. 4 Correlation between the ED_{50} for protection against sound induced seizures in AGS-susceptible Frings mice and the IC_{50} for GABA uptake into astrocytes (*top*) and neurons (*bottom*) (White et al. 2002)

immediate explanation for this glial selectivity. It has been suggested that co-expression of GAT1 with the other GATs could lead to a modification of the transporter protein complex in astrocytes, thus leading to the observed pharmacology of these drugs (White et al. 2002; Sarup et al. 2003a), however, this question has not yet been answered.

Tiagabine shows the highest degree of minimal motor impairment in the rotarod test of the four compounds tested, suggesting better tolerability of the exo-THPO derivates than tiagabine itself (White et al. 2002). Interestingly, the large difference observed in inhibitory effect between N-methyl-exo-THPO and tiagabine on cultured cells is far greater than the difference in anticonvulsant effect, where N-methyl-exo-THPO is only three-fold less potent than tiagabine (White et al. 2002). However, the lack of pharmacokinetic parameters makes an explanation on this difference purely speculative.

Due to the glial selectivity of the N-substituted exo-THPO analogues another effort was undertaken to produce a second generation of compounds more potent with enough lipophilic character to penetrate the BBB. Most of these compounds do not display any cell type selectivity, but EF1502 is an equipotent inhibitor of GABA mediated uptake at GAT1 and GAT2. EF1502 is an analogue of N-Methyl-exo-THPO, and it bears the same lipophillic substituents as tiagabine (*n*-(4,4-di(3-methyl-thien-2-yl)-but-3-enyl). EF1500 bears the exact same bulky N-substituent as EF1502 but its parent compound is exo-THPO and its potency at GAT2 is five times weaker compared to EF1502 (Clausen et al. 2005; White et al. 2005).

EF1502 like tiagabine possesses a broad anticonvulsant profile and it offers protection against sound induced seizures in AGS-susceptible Frings mice, pilocarpine induced seizures in mice, and in two models for human partial seizures; 6 Hz psychomotor seizure test and the amygdala kindled rat, suggesting that it would be effective against human partial seizures and partial seizures with secondary generalization. EF1502 also seems to possess a superior tolerability profile to that of tiagabine, all the evidence taken together suggests that EF1502 possesses more beneficial properties compared to tiagabine. To evaluate whether GAT2 mediated inhibition by EF1502 contributes to its anticonvulsant activity, a combination study utilizing a 2D-isobologram was conducted. EF1502 was tested in combination with tiagabine and Lu-32-176B, both being GAT1 selective inhibitors of GABA uptake. Tiagabine and Lu-32-176B were also evaluated in combination (White et al. 2005). The 2D-isobolograms at the ED_{50} effect level are shown in Fig. 5. In both studies with EF1502, a synergistic effect was observed (Fig. 5B,C). However, only a pure additive effect was observed between tiagabine and Lu-32-176B (Fig. 5A), suggesting that the GAT2 mediated GABA uptake of EF1502 was indeed contributing to the anticonvulsant effect. Interestingly, the tolerability of the mixture of EF1502 and tiagabine was not synergistic which could suggest an advantage in blocking GAT2 mediated GABA uptake (White et al. 2005). Interestingly the S enantiomer of EF1502 has been found to

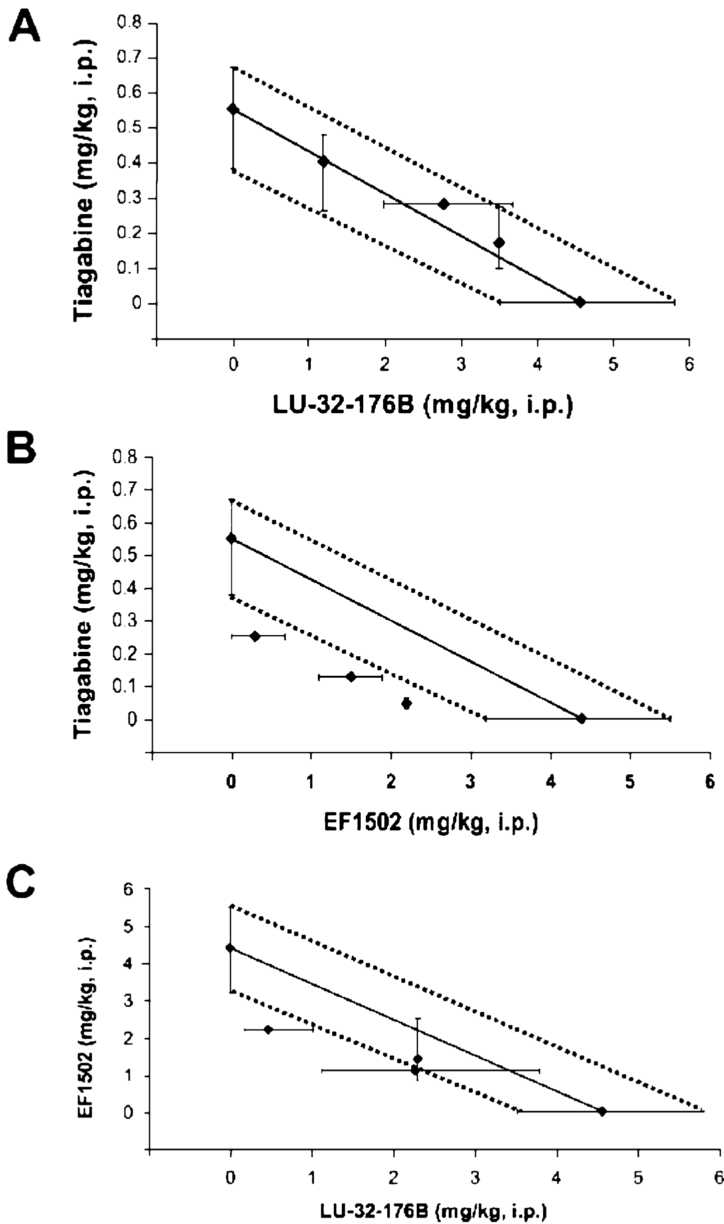

Fig. 5 The isobologram results are plotted as a function of their ED_{50} and 95% confidence intervals. The predicted line of additivity and 95% confidence interval connect the individual ED_{50} values for each drug tested independently. Additive anticonvulsant effect is observed between Lu-32-176B in combination with tiagabine (**A**). Synergistic anticonvulsant effects of EF1502 are observed when combined with tiagabine (**B**) or Lu-32-176B (**C**). The study is preformed in the audiogenic seizure-susceptible Frings mouse (White et al. 2005)

possess anticonvulsant activity when tested in the Frings audiogenic seizure-susceptible mouse following intraperitoneal administration. The ED_{50} values for R and S EF1502 are 3.1 and 41.0 mg/kg, respectively. Taking the IC_{50} values of R and S EF1502 into consideration (Table 2) (S)-EF1502 is a selective inhibitor of GAT2 that also displays anticonvulsant activity, which further establishes GAT2 as a relevant target in antiepileptic drug research (Clausen et al. 2005b).

Introducing the N-4,4-diphenyl-3-butenyl (DPB) group as a substituent on the primary amino group of exo-THPO markedly increases the potency with a factor of 100 for inhibition at neuronal and GAT1 transport (see Table 2). As seen with the N-DPB substitution of exo-THPO and nipecotic acid this group seems to convey selectivity towards GAT1, similarly results are observed with N-DPB substitution of guvacine (Ali et al. 1985; Sarup et al. 2003a, 2003b; Schousboe et al. 2004). Greater lipophilicity and size of the N substituents lead to higher potency of these analogues regardless of their parent compounds. Previous results indicate that the lipophilic side chain does not interact with the GABA binding site. Competitive inhibitors are probably most likely to bind to the GABA binding site, whereas non-competitive inhibitors probably have another binding site different from the GABA binding site, making their interaction more illusive and complex (Sarup et al. 2004).

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Human Disorders Caused by the Disruption of the Regulation of Excitatory Neurotransmission

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Abstract The nicotinic acetylcholine receptors (nAChRs) are members of the large family of ligand-gated ion channels, and are constituted by the assembly of five subunits arranged pseudosymmetrically around the central axis that forms a cation-selective ion pore. They are widely distributed in both the nervous system and non-neuronal tissues, and can be activated by endogenous agonists such as acetylcholine or exogenous ligands such as nicotine. Mutations in neuronal nAChRs are found in a rare form of familial nocturnal frontal lobe epilepsy (ADNFLE), while mutations in the neuromuscular subtype of the nAChR are responsible for either congenital myasthenia syndromes (adult subtype of neuromuscular nAChR) or a form of arthrogryposis multiplex congenita type Escobar (fetal subtype of neuromuscular nAChR).

1 Introduction

Dysregulation of the precarious balance between excitatory and inhibitory neurotransmission due to inherited or acquired gene mutations has been implicated in a wide range of human disorders, including different epilepsy syndromes, familial migraine and neurodevelopmental disorders. The possible pathophysiological mechanisms include loss-of-function mutations in neurotransmitter genes, neurotransmitter receptor subunit genes or genes necessary for the synthesis of neurotransmitters, as well as mutations in genes involved in epigenetic regulation of the neurotransmitter system. Several of the disorders that are caused by disruption of either excitatory or inhibitory neurotransmission and their pathomechanisms will be discussed in this chapter.

2 Epilepsy and GABAergic Neurotransmission

The GABAergic system has long been implicated in epileptogenesis, however, most of the defects in GABA neurotransmission observed in human epileptic syndromes are likely to be the indirect rather than direct result of

uncontrolled neuronal activity. Nevertheless, animal studies support a possible etiological role of disturbed GABAergic transmission in epilepsy. In the normal brain, phasic and tonic $GABA_A$ inhibition is necessary to counterbalance excitatory stimuli. Thus, loss-of-function or function-altering mutations within the GABAergic system can be expected to result in hyperexcitability of the affected neurons (Fig. 1). To date several genes associated with the GABAergic system have been disrupted in animal models. These include genes for glutamic acid decarboxylase (*GAD*), the enzyme that converts the excitatory neurotransmitter glutamate into the inhibitory neurotransmitter GABA, as well as genes for different subunits of the $GABA_A$ receptor. An example is GAD65, an enzyme contributing to the increase of GABA levels observed during normal postnatal brain development. Mice with homozygous disruptions of the *gad65* gene did not show this postnatal increase in GABA levels. Phenotypically they exhibited a largely abnormal neural activity with frequent paroxysmal discharges and spontaneous seizures (Storck et al. 2000). Additional arguments for the close association

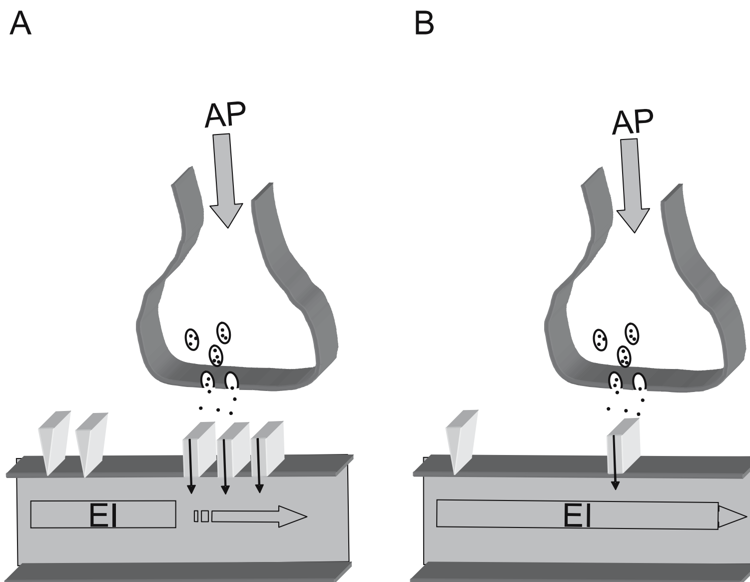

Fig. 1 Mutations in $GABA_A$ receptor subunits reduce GABA-mediated inhibition. **A** The fusion of GABA vesicles with the presynaptic membrane of the GABAergic interneuron is induced by an incoming action potential (*AP*). The released GABA triggers the opening of postsynaptic $GABA_A$ receptor ion channels, increasing their permeability for bicarbonate and chloride. **B** This leads to increased membrane conductance via a net inward flow of anions and, subsequently, to a reduction of the postsynaptic excitatory input (*EI*). Loss-of-function mutations in $GABA_A$ receptors have been shown to cause impaired receptor trafficking and reduce the numbers of cell surface receptors. The subsequent decrease of inhibitory $GABA_A$ receptor current is likely to result in increased neuronal excitability

between GABAergic transmission and neuronal hyperexcitability are presented by *Gabrg3* knockout mice, which also exhibit EEG abnormalities and develop spontaneous seizures (DeLorey et al. 1998). In humans, GABAergic receptor mutations have been found in families with rare monogenic epilepsies.

2.1
GABRG2 and GABRA1 Genes in Human Epilepsy

Mutations within the γ2-subunit of the $GABA_A$ receptor gene (*GABRG2*) were described as one of several causes of generalized epilepsy with febrile seizures plus ($GEFS^+$) (Baulac et al. 2001; Wallace et al. 2001; Harkin et al. 2002; Kananura et al. 2002; Marini et al. 2003). Another cause are mutations in subunits of the voltage-gated sodium channels (see below). A wide spectrum of epilepsy phenotypes can be present in $GEFS^+$ families. Some of the more common phenotypes are febrile seizures, febrile seizures plus (attacks with fever continue beyond six years of age, FS^+), FS^+ and absence seizures, FS^+ and myoclonic seizures, FS^+ and atonic seizures or myoclonic-astatic epilepsy. Childhood absences seem to be the main afebrile seizure type in $GEFS^+$ individuals with *GABRG2* mutations (Baulac et al. 2001; Wallace et al. 2001; Harkin et al. 2002; Kananura et al. 2002; Marini et al. 2003), while mutations in voltage-gated sodium channels seem to cause a more heterogenic epilepsy phenotype (see below). The second subunit of the $GABA_A$ receptor associated with epilepsy in humans is *GABRA1*, which is mutated in a single family with autosomal dominant juvenile myoclonic epilepsy (Cossette et al. 2002). The mutant $GABA_A$ subunits are likely to disturb or prevent the receptor's normal activity. At least some mutations seem to interfere with receptor trafficking, causing increased retention of the receptor in intracellular compartments (Bianchi et al. 2002; Kang and MacDonald 2004). The reduced GABA-mediated synaptic inhibition is likely to cause increased neuronal excitability and raise the likelihood of seizures.

3
Neurodevelopmental Disorders and GABAergic Transmission

Both Rett syndrome and Angelman syndrome have phenotypic overlap with autism, a neurodevelopmental disorder of complex genetic etiology. Autistic features such as impaired communication and social interactions or repetitive behaviour can be observed in most Rett or Angelman patients, at least to some degree. Dysregulation of GABAergic neurotransmission, which plays a role in both Angelman syndrome and Rett syndrome, is also suspected to be involved in the pathogenesis of idiopathic autism.

3.1 Angelman Syndrome

Angelman syndrome is characterized by severe psychomotoric retardation, absence of speech, ataxic gait, epilepsy and typical EEG abnormalities. The characteristic EEG findings in patients with Angelman syndrome consist of rhythmic 2–3 Hz delta waves of high-amplitude with a maximum over frontal regions, and 3–4 Hz spikes and sharp wave over posterior regions. Such distinctive rhythmic EEG patterns can be observed in almost all patients with Angelman syndrome irrespective of genotype, clinical severity and the presence or severity of a seizure disorder. The EEG patterns are consistent with a model of cortical and thalamo-cortical dysfunction resulting from impaired synaptic GABAergic neurotransmission (Dan and Boyd 2003). The Angelman syndrome is an imprinting disorder that results from a physical and/or functional loss of the maternally inherited chromosomal region 15q11–q13, a region that contains the ubiquitin–protein ligase E3A gene (*UBE3A*) and the $GABA_A$ receptor β3-subunit (*GABAB3*) gene. Most cases of Angelman syndrome are associated with a functional defect of *UBE3A*, however, the *GABAB3* gene also showed significantly reduced expression in multiple Angelman brain samples as well as in mouse models of Angelman syndrome (Sinkkonen et al. 2003). Several Angelman patients with a maternal deletion including the *GABAB3* gene locus have been described. GABAergic receptor expression plays an important role in brain development, thus the loss of specific subunits could be expected to elicit severe neurodevelopmental abnormalities. This would be especially true for *GABRB3*, a subunit that is expressed early in embryogenesis. In fact, *GABRB3* knockout mice exhibit EEG abnormalities, seizures and behavioural changes typically associated with Angelman syndrome (DeLorey et al. 1998; DeLorey and Olsen 1999). The behavioural changes include learning and memory deficits, poor motor skills, hyperactivity and a disturbed rest–activity cycle, features that are also found in Angelman patients. These observations suggest that an impaired expression of *GABRB3* contributes to the overall phenotype of Angelman syndrome. It remains to be established how important this contribution is in comparison to the other genes that have been implicated in Angelman syndrome, especially *UBE3A* (Holopainen et al. 2001).

3.2 Rett syndrome

Rett patients are almost exclusively female, their clinical features include autism, seizures, loss of speech and the typical hand wringing. Typically, the deterioration starts after normal development up to the age of 6–18 months. The syndrome is caused by mutations in the X-linked methyl-CpG

binding protein 2 gene (*MECP2*) (Amir et al. 1999). *MECP2* is thought to selectively bind to methyl CpG dinucleotides in the mammalian genome and to function as a transcriptional repressor. MECP2 protein associates with both histone H3-Lys9-methyltransferase and DNA-methyltransferase DNMT1, linking DNA methylation to histone methylation. Such epigenetic modifications are required for genome reprogramming during development, for tissue-specific gene expression regulation and for global gene silencing. *MECP2* is highly expressed in brain and its expression pattern correlates with the maturation of neurons, an observation that might explain the progressive neurological phenotype of Rett syndrome (Shahbazian et al. 2002). Recent studies have shown that MECP2 protein targets and silences only a small subset of imprinted genes. One of its targets is an imprinted gene cluster on chromosome 6 that contains the *distal-less homeobox* gene (*DLX5)* (Horike et al. 2005). Binding of MECP2 protein in the *DLX5* region promotes the formation of a transcriptionally silent chromatin loop. Loss-of-function mutations of *MECP2* might therefore cause a loss-of-imprinting of *DLX5* in Rett syndrome individuals. Such a scenario would be likely to cause severe disturbances in GABAergic transmission (Fig. 2). Members of the DLX homeobox gene family regulate the development of primordial basal ganglia, large nuclei that consist of GABAergic projection neurons. DLX expression is also found in embryonic neurons that give rise to GABAergic interneurons in the cerebral cortex, hippocampus and olfactory bulb. It has also been shown that *DLX5* expression can induce cortical neurons to express the glutamic acid decarboxylase genes,

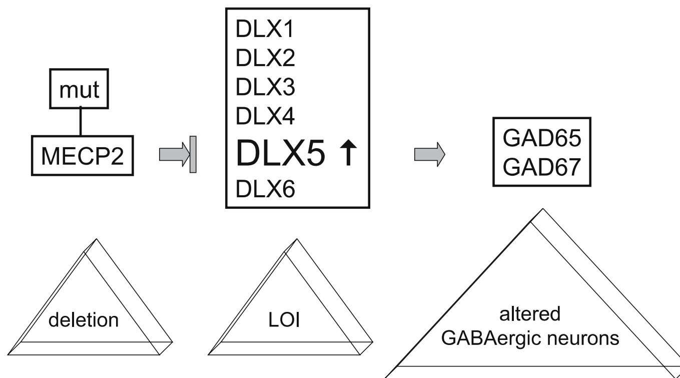

Fig. 2 Possible consequences of *MECP2* deficiency in Rett syndrome. Mutations (*mut*) in *MECP2* interfere with the protein's ability to function as a transcriptional represser of *DLX5*, a member of the DLX-homeobox gene family. DLX genes play a significant role in the specification of GABAergic neurons. Loss-of-imprinting of *DLX5* is suspected to affect the GABAergic neurotransmission system in Rett patients. There is also evidence that *MECP2* affects the level of *UBE3A* expression, pointing to a possible overlap in the pathways leading to either Rett syndrome or Angelman syndrome (Samaco et al. 2005)

enzymes that synthesize GABA. Thus the loss of *DLX5* imprinting and subsequent upregulation of its expression might alter the anatomy and activity of GABAergic neurons in individuals with Rett syndrome. Such a dysregulation of neuronal activity might contribute to at least some of the phenotypic features of this severe neurodevelopmental disorder (Samaco et al. 2005; Bradbury 2005).

3.3 Autism

Autistic spectrum disorders (ASD) are neuropsychiatric and behavioural syndromes affecting social behaviours and communication development. ASD are characterized by phenotypic and genetic complexity. In some patients the autistic behaviour can be attributed to disorders that affect the brain (like tuberous sclerosis or neurofibromatosis type 1). Furthermore it is a common symptom in patients with mental retardation of unknown cause. In most cases the autism occurs without known cause other than a possible familial predisposition. These cases are summarized under the term "idiopathic autism". Support for a genetic basis for idiopathic autism comes from family and twin studies. Siblings of autistic individuals have a 5% risk for the disorder, a number which is 50 times higher than in the general population. The observation of a striking disparity in concordance rates between monozygotic (concordance approximately 90%) and dizygotic (approximately 25%) twins further argues for a genetic basis of idiopathic autism (Ritvo et al. 1985; Bailey et al. 1995). One of the candidate regions for autism genes is the above-mentioned Prader Willi/Angelman syndrome region on chromosome l5q11–q13, which contains a $GABA_A$ gene cluster. Cytogenetic abnormalities of this chromosomal region have been described in several autistic individuals (Tager-Flusberg et al. 2001). Furthermore, a significantly reduced expression of *GABRB3* has been found in brain samples from patients with autism (Samaco et al. 2005). Based on these observations, different research groups investigated polymorphic markers from the Prader Willi/Angelman syndrome region for a possible linkage or association with autism. One of these markers, 155CA-2 in *GABRB3*, was found to be associated with autism in two of these studies (Cook et al. 1998; Buxbaum et al. 2002) but not in others (Martin et al. 2000). Martin et al. (2000) reported suggestive evidence for linkage disequilibrium with a marker located approximately 60 kb beyond the 3′ end of *GABRB3*, supporting a possible role for genetic variants within the 15q11–q13 $GABA_A$ gene cluster in autism. Further evidence for an involvement of *GABRG3* or a gene nearby was provided by the reported association of two additional single nucleotide polymorphisms (539T/C, 687T/C) and autism (Menold et al. 2001).

4 Disorders Due to Mutations in Voltage-Gated Potassium Channels

Voltage-gated potassium channels have been implicated in several paroxysmal disorders. The best-studied examples are the two members of the KCNQ-subfamily of voltage-gated potassium channels that play an essential role in the regulation of action potential frequency. KCNQ-subunit genes code for proteins with six transmembrane regions (TM) and one potassium-selective pore region each. Proteins from the KCNQ family can assemble as homo- or heterotetramers, the latter being the more likely formation in brain (Tinel et al. 1998). Mutations in the subunit genes *KCNQ2* and *KCNQ3* can cause a rare monogenic seizure disorder of the newborn (Biervert et al. 1998).

4.1 Benign Familial Neonatal Convulsions

Benign familial neonatal convulsions (BFNC) is a rare autosomal dominantly inherited epilepsy of the newborn. The syndrome is characterized by unprovoked, generalized or multi-focal seizures typically starting within the first few days of life. The course of the disorder is usually benign and self-limiting, and, with or without pharmacotherapy, the seizures remit spontaneously within a few weeks or months (Ronen et al. 1993; Wakai et al. 1994). Later seizures occur in up to 15% of the patients, but they tend to occur infrequently. Some BFNC patients with a more severe phenotype including therapy-resistent seizures and/or delayed development or psychomotor retardation have been described (Dedek et al. 2003; Schmitt et al. 2005) (Fig. 3). The major gene for BFNC is the voltage-gated potassium channel gene *KCNQ2* (20q13.3) (Biervert et al. 1998; Singh et al. 1998), while mutations in only three families have been found in the homologous gene *KCNQ3* (8q24) (Charlier et al. 1998; Singh et al. 2003). *KCNQ2* and *KCNQ3* contribute to the M-current, a very slowly opening and closing potassium current that regulates excitability of numerous types of neurons (Wang et al. 1998). The M-current, first discovered several years ago (Brown 1988), is a powerful controller of neuronal repetitive firing. It is able to reduce the number of action potentials of individual neurons by opposing sustained membrane depolarization. The BFNC mutations in *KCNQ2* are often truncating and located in the large C-terminal part downstream of the subunit's six TMs, but several missense mutations within the TMs have also been described. Electrophysiological studies demonstrated that most BFNC mutations cause only modest reductions (20–30%) of potassium currents, indicating that even a slight alteration of M-current activity is sufficient to cause epilepsy (Biervert et al. 1998). One of the exceptions to this haploin-sufficiency concept is the *KCNQ2*/R207W mutation, which neutralizes a charged amino acid residue

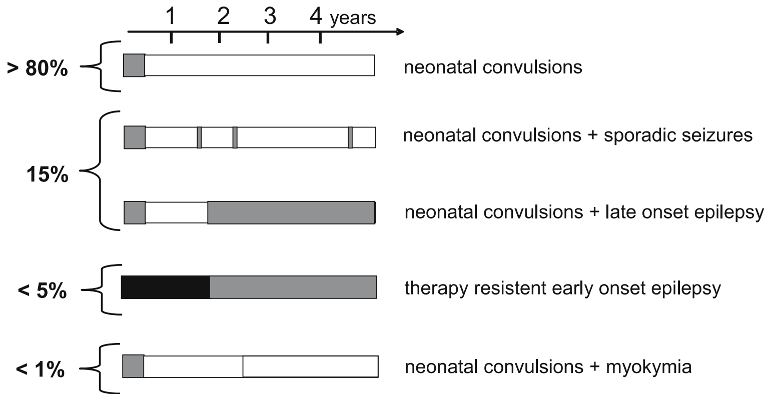

Fig. 3 Different clinical phenotypes associated with KCNQ2/KCNQ3 mutations. Mutations in voltage-gated potassium channel subunits KCNQ2 (and sometimes KCNQ3) are mostly associated with benign clinical courses. However, patients with more severe clinical phenotypes have been described, as well as one family in which the neonatal convulsions were followed by the onset of myokymia during childhood

within the channel's voltage-sensor in TM4. R207W causes a more severe change of potassium channel properties by drastically slowing the voltage-dependent activation. On the clinical level this results not only in BFNC, but also in myokymia, a spontaneous and repetitive involuntary contraction of muscle fibre groups (Dedek et al. 2001). However, the affection of the peripheral in addition to the central nervous system can not be explained by the dominant negative effect alone. Another *KCNQ2* mutation, 867insGGGCC, also reduces the current magnitude by more than 50% but is not associated with any neurological features except epilepsy (Singh et al. 2003). In mice, *KCNQ2* knockout experiments resulted in early postnatal lethality in homozygous animals, while heterozygous mice developed and behaved normal but showed an increased sensitivity to epileptic inducers (Watanabe et al. 2000). The absence of spontaneous seizures might indicate that *KCNQ2* is not as important for the M-current in the murine brain as it is in the human brain.

5
The Phenotypic Spectrum of Calcium Channel Mutations

At least three inherited paroxysmal neurologic syndromes have been linked to mutations in the voltage-gated P/Q-type calcium channel gene *CACNA1A* on chromosome 19p13: episodic ataxia type 2 (EA2), familial hemiplegic migraine (FHM1) and spinocerebellar ataxia type 6 (SCA6) (Ophoff et al. 1996; Zhuchenko et al. 1997). The *CACNA1A* gene codes for a main transmembrane

component of the neuronal P/Q-type calcium channel, and is particulary expressed in the cerebellum and at the neuromuscular junction. P/Q-type calcium channels respond to membrane depolarization, letting calcium ions flow into the cell. They have a major role in the control of neuronal excitability and neurotransmitter release. The *CACNA1A* gene is composed of 47 exons, the last one containing a polymorphic CAG repeat predicted to code for a polyglutamine stretch in a subset of transcripts (Ophoff et al. 1996; Zhuchenko et al. 1997). Knockout mice without a functional *Cacna1a* gene are born with severe ataxia and die within a few days, while mice carrying different *Cacna1a* mutations display various paroxysmal phenotypes called *tottering*, *leaner* or *rocker* (Fletcher et al. 1996; Doyle et al. 1997; Zwingman et al. 2001). The mutants also have neuroanatomical abnormalities, suggesting that P/Q channels may also be involved in neuronal development.

5.1 Episodic Ataxia Type 2

The clinical phenotype of EA2 is characterized by spontaneous episodes of ataxia typically lasting for a few hours or even days. The patients develop cerebellar symptoms like interictal nystagmus or mild clumsiness. Episodes can be triggered by stress and exertion and respond favourably to treatment with acetazolamide (Bain et al. 1996). In some families additional features like episodic weakness, dystonia, epilepsy and cognitive impairment occur. Both missense and truncating mutations of the *CACNA1A* gene have been described in EA2 families (Ophoff et al. 1996). Expression studies showed that EA2 mutation affects several channel properties, including reduced current density, increased rate of inactivation, and shifts in the voltage dependence of activation to more positive values. Although these findings are consistent with an overall loss of P/Q-type channel function, the mutation also caused some biophysical changes consistent with a gain of function effect (Spacey et al. 2004).

5.2 Migraine

Migraine is a very common disorder, affecting about 15% of western populations (Lipton et al. 2001). The pathomechanisms are far from understood, but familial aggregation strongly suggests a genetic component. In most patients migraine is probably of multifactorial origin, but a few subtypes of the disorder have been identified in which the underlying mode of inheritance is autosomal dominant. One of them is familial hemiplegic migraine (FMH), a rare disorder characterized by attacks of unilateral motor weakness associated with at least one other aura symptom such as motor deficit, vis-

ual symptoms or aphasia (Ophoff et al. 1996). The aura often lasts for about 1–2 h and is followed by a migrainous headache. The mean age of onset is 11 years, which is lower than in other types of migraine. About one third of the FMH patients experience at least one attack with confusion or even coma with prolonged hemiplegia, fever, and meningismus during their life. FMH is genetically heterogeneous, *CACNA1A* on chromosome 19 is one of the known genes that can cause this autosomal dominant disorder (FMH1) (Ophoff et al. 1996). Other FMH loci are located on chromosome 1q21–q31 (FMH2, *ATP1A2* gene) (De Fusco et al. 2003) and 1q31 (FMH3, gene not identified yet) (Gardner et al. 1997). Several different *CACNA1A* mutations have been identified in FHM1 families, some of them were found more than once. The commonest mutation, T666M, has been detected in at least 12 unrelated families and one sporadic patient (Ducros et al. 2002). All mutations are missense mutations located in functionally important domains of the gene. Electrophysiological studies showed that the FHM1 mutations both increase the calcium ion influx and decrease the maximum current density in neurons. The authors hypothesized that these two apparently contradictory effects may underlie parallel processes that result in either migraine or aura (Tottene et al. 2002). However, it cannot be ruled out that the functional changes caused by FMH1 mutations might be different among different types of neurons and that the single cell studies might not truly reflect the situation in the patient's brain (Müllner et al. 2004).

5.3 Spinocerebellar Ataxia Type 6

Depending on the ethnic origin, SCA6 accounts for 1–11% of the autosomal dominant cerebellar ataxias. Clinically, SCA6 is characterized by an early phase of episodes with ataxia, dysathria, vertigo and nausea, accompanied by visual disturbances and tinnitus. These episodes might last minutes or days and can be triggered by head movement and physical or emotional stress. This early phase lasts several years before the onset of a permanent and progressive ataxia, usually of the pure cerebellar type. Pathologically, SCA6 is marked by the severe loss of Purkinje cells in the cerebellum. SCA6 is caused by an expansion of the CAG repeat embedded in the cytoplasmatic 3′ region of the *CACNA1A* gene (Zhuchenko et al. 1997). The normal allele sizes of the CAG repeat range from 4–18 units, while expanded alleles range from 20–30 repeats. So far the pathophysiological consequences of the expanded CAG repeat are controversial. Some researchers reported that the extended stretch induces a hyperpolarizing shift in the voltage dependence of channel inactivating. This could dramatically decrease the calcium influx and lead to neuronal cell death, especially in Purkinje cells. Other researchers observed increased cell surface expression of mutated CACNA1A channels resulting in a sharp increase of current density in cells expressing

the mutated channel. One plausible explanation for these experimental discrepancies is that the different groups used different structural *CACNA1A* variants for their expression studies. The *CACNA1A* pre-mRNA undergoes extensive splicing, producing several isoforms. The presence or absence of a 5-nucleotide stretch between exons 46 and 47 is critical for the expression of the CAG repeat, which is only translated when this 5-nucleotide stretch is left in place (Toru et al. 2000; Restituito et al. 2000; Piedras-Renteria et al. 2002).

5.4
CACNA1H and Childhood Absence Epilepsy

Another calcium channel gene, *CACNA1H* on chromosome 16p13.3 is likely to be a susceptibility factor for childhood absence epilepsy (CAE). Absence epilepsies are characterized by short seizures that cause a sudden impairment of consciousness and interruption of ongoing activity (Sander 1996). The ictal EEG shows a rhythmic 3 Hz spike and slow-wave discharge that is likely to be generated by generalized synchronization of neuronal firing mediated by thalamic circuits. CAE is one of the more common idiopathic epilepsies with a mean age of onset between 6–7 years. Several missense mutations affecting highly conserved residues of the T-type calcium channel gene have been identified in CAE patients and their healthy parents but not in controls. Functional characterization indicated that some of these mutations allow for greater calcium influx during physiological activation, a behaviour that could result in enhanced neuronal bursting activity. Other *CACNA1H* mutations shift the voltage dependence of the channel's activation to levels that are closer to resting membrane potentials. In most neurons this would result in increased calcium influx and hyperexcitability of neuronal tissue (Khosravani et al. 2004, 2005; Chen et al. 2003). It has recently been shown that common *CACNA1H* polymorphisms can further alter the effect of the above-mentioned CAE-specific mutations, highlighting the importance of the individual genetic background (Vitko et al. 2005).

6
Acetylcholine Receptors and Human Disorders

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that are found throughout the peripheral and central nervous system. They are associated with numerous transmitter systems and their expression is altered during development and ageing as well as in diseases like Alzheimer disease or Morbus Parkinson. There is evidence that nAChRs can be located both presynaptically or on axon terminals (Wonnacott et al. 2000).

They might therefore be involved in both fast synaptic transmission and the modulation neuronal activity. At least 11 genes are known that code for the different α- and β-subunits of the neuronal nicotinic acetylcholine receptor (nAChR). Most but not all of these subunits are able to co-assemble in various combinations to form a wide variety of functional nAChR channels. One of the most abundant nAChR subtypes in mammalian brain is made up of α4 and β2 gene products (Whiting et al. 1991). Both genes are known to cause a rare monogenic form of epilepsy in humans.

6.1 Autosomal Dominant Nocturnal Frontal Lobe Epilepsy

Mutations in two subunits of the neuronal nAChR have been described in families with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), a rare partial epilepsy characterized by clusters of brief motor seizures. The seizures, which occur mostly during non-REM (rapid eye movement) sleep, consist of thrashing hyperkinetic activity or tonic stiffening with superimposed clonic jerking. Secondary generalization with loss of consciousness can occur, but most patients remain conscious through their seizures (Scheffer et al. 1995). ADNFLE can be caused by mutations in either the α4-subunit (*CHRNA4*) or the β2-subunit (*CHRNB2*) genes of the neuronal nAChRs (Steinlein et al. 1995, 1997; Fusco et al. 2000; Phillips et al. 2001). One of the most common nAChR in mammalian brain assembles from CHRNA4 and CHRNB2 subunits, thus it is not surprising that both subunits can cause the same type of epilepsy. The surprising and so far not very well understood part is that a nAChR subtype ubiquitously present in brain is able to cause a partial type of epilepsy instead of a generalized one. All known ADNFLE mutations are located within or close to the second or third TM (Fig. 4). Both TMs contribute to the walls of the ion channel, thus it seems that only mutations that have a direct effect on the ion pore are able to cause ADNFLE. Reconstitution experiments in *Xenopus* oocytes or HEK cells and patch clamp characterization of the known ADNFLE mutations revealed different electrophysiological and pharmacological profiles. Some mutants (α4-S248F, α4-776ins3) showed a reduction of calcium permeability, while others (α4-S252L) did not change their ionic selectivity. Several of the mutant receptors (α4-S248F, α4776ins3, β2-V287M) are effectively blocked by the antiepileptic drug carbamazepine, while the drug response observed for some other mutants did not differ from that seen for the wild-type receptor (Bertrand et al. 2002). The different functional properties might explain why some of the nAChR mutations are associated with distinct clinical features. For example, carriers of the α4-776ins3 mutation are often affected by psychiatric disorders, while families with the α4-S252L mutation seem to have an increased risk of mental retardation (Magnusson et al. 2003; Cho et al. 2003). The one characteristic that all mutations have in common is that they increase

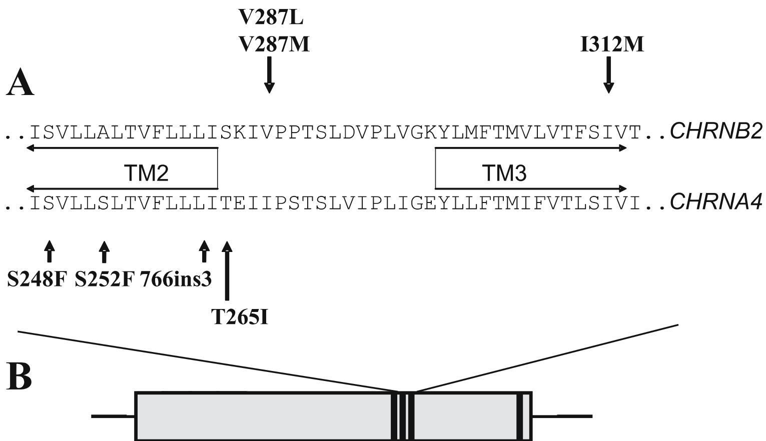

Fig. 4 Nicotinic acetylcholine receptor genes in human epilepsy. *A* Positions of the known ADNFLE mutations within TM2–TM3 of CHRNB2 and CHRNA4 (TM2–3 sequences are only partially shown). *B* Position of TM2–TM3 within the CHRNB2/CHRNA4 cDNA. *Black vertical bars*: TM1–TM4

the nAChRs acetylcholine sensitivity, suggesting that a gain-of-function effect underlies this type of epilepsy.

7
Mutations in Voltage-Gated Sodium Channels

Voltage-gated sodium channels become selectively permeable to sodium ions when the cell membrane depolarizes. The resulting voltage-dependent fast inactivating, but transient, sodium current is the principal current responsible for the depolarizing phase of the action potential and thus is the essential current for neuronal excitation in general. Voltage-gated sodium channels have further shown to produce a persistent, non-inactivating sodium current. This current is particularly instrumental in finely modulating neuronal excitability and thus possibly in determining epileptogenicity, as it lacks the "safety factor" of automatic inactivation. It is still not clear whether the persistent sodium current originates from the same voltage-gated sodium channel that produces the fast inactivating sodium current, or if different channels are responsible for those currents.

7.1
GEFS+/Benign Familial Neonatal–Infantile Convulsions

Mutations in three voltage-gated sodium channel subunit (*SCN1A*, *SCN2A*, *SCN1B*) genes are known to cause the above-mentioned human epilepsy syn-

drome of GEFS$^+$ (Wallace et al. 1998; Escayg et al. 2000; Sugawara et al. 2001a,b). *SCN1A* is the most frequently involved gene, while *SCN2A* and *SCN1B* can be regarded as minor genes for GEFS$^+$. The large α-subunits encoded by *SCN1A* and *SCN2A* are characterized by four domains each containing six TMs and a pore region, while the β-subunits are small accessory proteins with only a single TM. Several studies indicated that the mutations might led to hyperexcitability by increasing the activity of the sodium ion channels. The proposed mechanisms include a slowing of inactivation, an increase of sodium channel availability or a reduction of sodium current rundown during high frequency channel activity (Wallace et al. 1998; Meadows et al. 2002). However, other studies showed a reduced or a complete absence of sodium currents if the mutated channels were expressed (Alekov et al. 2001; Spampanato et al. 2001, 2003). The functional consequences of the mutations are therefore still a matter of debate. *SCN1A* mutations are also found in severe myoclonic epilepsy of infancy (SMEI or Dravet's syndrome), an intractable epilepsy of early childhood that sometimes co-occurs in GEFS$^+$ families (Claes et al. 2000). So far the genetic relationship between GEFS$^+$ and SMEI remains unclear. Mutations in *SCN2A* not only cause GEFS$^+$ but are also found in a dominantly inherited idiopathic epilepsy with an mean age of onset at 3.5 months, named benign familial neonatal/infantile seizures (Heron et al. 2002).

8 Future Goals

Most of the genes described in this chapter contribute to rare Mendelian forms of disorders rather than to the common forms. Those rare Mendelian forms are the exception, the suspected genetic etiology in most cases of migraine, epilepsy or other neurological disorders is oligo- or polygenic rather than monogenic. It has already proven much more difficult to approach the genetics of complex disorders, with their unknown number of contributing genes. We can nevertheless hope that the same classes of genes that can cause monogenic forms of a disorder are also involved in the more common forms. This would mean that genes involved in the balance of excitatory and inhibitory input in the brain are candidates for common neurological disorders. The increasingly complete information on the human genome, as well as large scale collections of well-characterized cases, will provide the essential tools for the search for such susceptibility genes. The identification of these genes will help us to understand the pathophysiology of neurological disorders and, hopefully, will represent the first step towards the discovery of new therapeutic targets.

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