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A SURVEY OF CELL BIOLOGY

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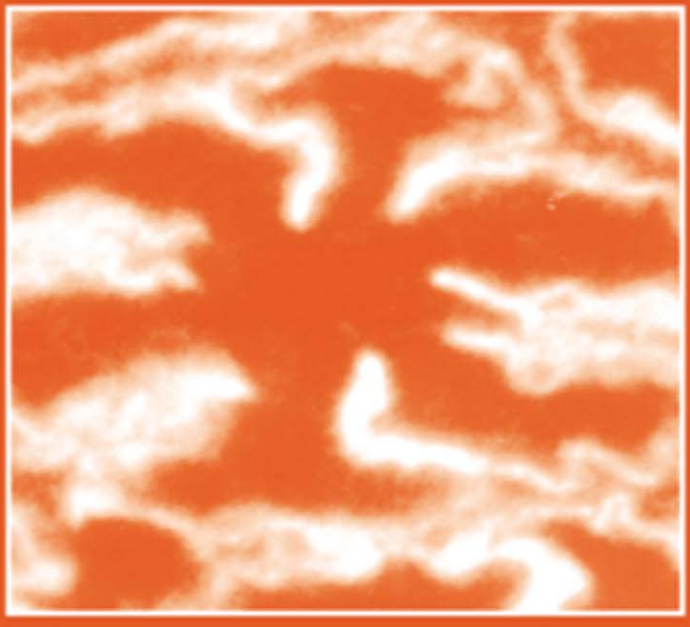
Kwang W. Jeon



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Front cover photograph: Nonreceptive plasma membrane of photoreceptor cell. (For more details see Chapter 4, Figure 7.)

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Biology of the Anococcygeus Muscle

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The anococcygeus is a smooth muscle tissue of the urogenital tract which, in the male, runs on to form the retractor penis. The motor innervation is classically sympathetic with noradrenaline as transmitter, but the relaxant parasympathetic transmitter has only recently been identified as nitric oxide. Indeed, the anococcygeus has provided an extremely useful model with which to probe the mechanisms underlying this novel nitrergic system, including the importance of physiological antioxidants in maintaining the potency of nitric oxide as a neurotransmitter. The cellular mechanisms of contraction and relaxation are slowly being clarified, with particular interest in the contribution of capacitative calcium entry and the guanylyl cyclase/cyclic GMP system. Many questions remain unanswered, however, including the precise physiological role of the muscle, the identity of substances released from subcellular vesicles of nitrergic nerves, the unusual sensitivity of the tissue to certain peptides (oxytocin and urotensin II), and the nature of store-operated channels through which calcium enters the cell to maintain contraction.

KEY WORDS: Anococcygeus, Excitation-contraction coupling, Innervation, Nitrergic nerves, Nitric oxide, Neuropeptides, Smooth muscle.

I. Introduction

A. Early Contributory Studies

Toward the end of the nineteenth century, Langley and Anderson (1895, 1896) published seminal papers in which they described autonomic innervation to organs

of the pelvic viscera, including caudoanal and caudocavernous muscles of the rabbit urogenital tract. During these investigations, they observed that electrical stimulation of the pelvic parasympathetic nerves resulted in penile erection and that this effect was not inhibited following administration of the plant alkaloid atropine, which had blocked parasympathetic responses in most other tissues. In retrospect, this is widely regarded as one of the first descriptions of the phenomenon of nonadrenergic, noncholinergic (NANC) neurotransmission, a general term used to describe autonomic responses not mediated by either of the classical transmitters, noradrenaline or acetylcholine. However, the precise identity of the NANC neurotransmitter responsible for penile erection was to remain a mystery for a further 100 years; resolution of the mystery owed much to unequivocal evidence gained from experiments with the rodent anococcygeus muscle.

B. First Description of the Anococcygeus

In 1972, John Gillespie from the University of Glasgow, Scotland, reported that histological examination of the terminal colon region of the rat, using catecholamine fluorescence techniques, had revealed a paired smooth muscle tissue which, on dissection, he called the anococcygeus (Gillespie, 1972); the muscle received a dense sympathetic innervation, activation of which caused contraction, and a separate NANC innervation, which caused relaxation. As it turned out, the rat anococcygeus was found to be equivalent to the rabbit caudoanal muscle of Langley and Anderson (Gillespie, 1997); the retractor penis, which links the anococcygeus with the penis, was in turn equivalent to the previously identified caudocavernous muscle. Subsequent studies into the NANC innervation of the anococcygeus muscle played a major part in the identification of nitric oxide (NO) as the neurotransmitter responsible not only for relaxation of the anococcygeus and retractor penis but also for penile erection (Rand, 1992; Rand and Li, 1995a, 1995b) and relaxation of a variety of smooth muscles in the respiratory and gastrointestinal tracts. The therapeutic importance of this discovery has of course been amply demonstrated by the high-profile introduction of sildenafil citrate (Viagra^{TM}) for the treatment of male impotence. In addition to its importance in the discovery of the neurotransmitter functions of NO, the anococcygeus has provided useful information on the biology of the sympathetic nervous system, the function of various drug/hormone receptor systems, and the cellular mechanisms associated with smooth muscle contraction and relaxation. To date, however, it would be true to state that the main recognition of this rather arcane tissue has remained predominantly within the realms of physiology and pharmacology. It is the purpose of the present review to introduce and describe the biology of the anococcygeus to a wider audience.

II. Anatomy and Ultrastructure

A. Gross Anatomy

Following on from the original reports of the rat anococcygeus muscle (Gillespie and Maxwell, 1971; Gillespie, 1972), corresponding tissues have been described in the cat (Gillespie and McGrath, 1974), rabbit (Creed *et al.,* 1977), dog (Dehpour *et al.,* 1980), ox (Gillespie, 1980), mouse (Gibson and Wedmore, 1981), and pig (Li and Rand, 1999). The gross anatomy of the tissue is generally similar in all species (Fig. 1). The anococcygeus is a bilaterally paired smooth muscle that arises from true tendonous insertions into the bone of the sacral and coccygeal vertebrae. The muscles then sweep caudally around either side of the rectum to unite on its ventral aspect, where it usually forms a thickened ventral bar. In most species, the ventral bar then continues to form the retractor penis. However, some of the muscle fibers do not extend to the ventral bar, but in fact insert into the rectum, this arrangement being most marked in the rabbit (Creed *et al.,* 1977). Anococcygeus muscles are also found in female animals (Gibson and Gillespie, 1973; Fukazawa *et al.,* 1997; O'Kane and Gibson, 1999) although they are usually smaller than those in the male, with the majority of muscle fibers terminating in the colon.

The precise physiological function of the tissue has yet to be fully determined, but its anatomical location and neurotransmitter profile (see later) strongly suggest a role in penile protrusion and erection. The interaction of some of the anococcygeus fibers with the colon also indicates some coordinating role between the gastrointestinal and urogenital tracts; however, again the exact nature of this role remains unclear.

B. Ultrastructure

The smooth muscle cells of the rat anococcygeus are around 100 μm in length with a maximum diameter of 5 μm (Gillespie and Lullmann-Rauch, 1974). A notable subcellular feature is the numerous caveolae, with associated mitochondria,

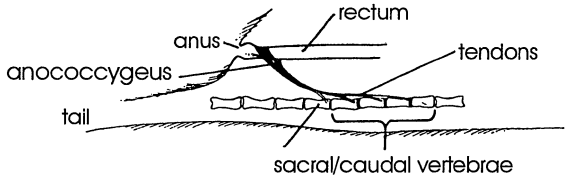

FIG. 1 The anatomical location of the anococcygeus muscles in the mouse.

distributed widely along the internal surface of the plasma membrane; these caveolae appear to have close contact with the sarcoplasmic reticulum (SR) and this association may be important in the processes controlling excitation–contraction coupling in the tissue (see Section V).

The muscle cells are grouped into small bundles of 8–10 cells per bundle, the average diameter of the bundles being 100 μm. Running between the bundles are numerous Schwann cell–axon complexes, containing 1–5 axons per complex, which are believed to represent the terminal regions of the autonomic nerves within the tissue. The axons are always located outside the small muscle bundles and never penetrate between individual smooth muscle cells. This arrangement is typical of smooth muscles that respond with strong and maintained contractions to nerve stimulation, rather than those that respond with rapid, transient twitches where there is usually much closer contact between the nerve and muscle cells.

Gibbins and Haller (1979) examined in detail the profiles of the axons within the rat anococcygeus. They identified three distinct types of neurons, characterized by the nature of the storage vesicle distribution within the terminal region. Type I nerves contained mainly small granular vesicles (40–45 nm), a few small clear vesicles (30–60 nm), and/or large granular vesicles (80–150 nm; all vesicles chromaffin positive), and were destroyed by the sympathetic neurotoxin 6-hydroxydopamine; clearly, these type I nerves represented the sympathetic supply to the muscle, with about 60% of the nerves within the tissue being of this type. Type II nerves contained mainly small clear vesicles and a few large granular vesicles (ratio 20 : 1; both types of vesicle being chromaffin negative); type II nerves (about 5% of the total nerve population) were resistant to 6-hydroxydopamine and appeared to have characteristics similar to those of the cholinergic nerves observed in other tissues. Finally, type III nerves (about 35% of the nerve population) again contained both small clear vesicles and large granular vesicles (both chromaffin negative), but in this case the ratio of small clear vesicles to large granular vesicles was 2 : 1. These nerves were also resistant to 6-hydroxydopamine and appeared similar to peptide-containing neurons in the gut; it was therefore proposed that these represented the NANC parasympathetic nerves to the anococcygeus. Subsequently, Gibbins (1982) reexamined these nerve profiles and concluded that types II and III were likely to represent the same nerve type, but represented sections taken from different points along the terminal region of the neuron. Thus, taking this into account, in the rat anococcygeus the type I sympathetic nerves comprised 60% of the total, while the NANC parasympathetic comprised 40%. However, this ratio is not consistent across species; in the rabbit, the majority of nerve cells within the anococcygeus are of the NANC type (Gibbins, 1982).

Thus, these anatomical and ultrastructural studies revealed some interesting and unusual properties of the anococcygeus—a tissue in which the vast majority of cells are smooth muscle, arranged in parallel bundles (not longitudinal and circular as found in many other smooth muscles), and with a true tendonous insertion into bone. Further, in addition to a dense sympathetic innervation the muscle receives a

second NANC parasympathetic nerve supply. As described later (see Section III), the main transmitter released from these NANC nerves is NO, which is synthesized in the cytoplasm and diffuses out of the neurons; this raises the as yet unresolved question of the nature and role of the substances stored in, and released by, the abundant small clear vesicles and large granular vesicles revealed in these nerves under the electron microscope.

III. Innervation

A. Sympathetic Motor Nerves

As mentioned earlier, the anococcygeus was discovered by the strong catecholamine fluorescence it displayed during a study of the sympathetic innervation of rat terminal colon using the paraformaldehyde technique of Falck and Hillarp (Gillespie and Maxwell, 1971). Although noticeably less dense in the rabbit, a sympathetic innervation has been found in the anococcygeus muscles from all species described to date, and in all cases activation of these nerves results in muscle contraction via the activation of postsynaptic α-adrenoceptors (Gillespie, 1972, 1980; Gillespie and McGrath, 1974; Creed *et al.,* 1977; Dehpour *et al.,* 1980; Gibson and Wedmore, 1981; Li and Rand, 1999); there is clear evidence that it is the α_1 subtype that is located postjunctionally in the anococcygeus (Docherty and Starke, 1981; Coates *et al.,* 1982; Gibson and Yu, 1983; McGrath, 1984; Kenakin, 1984; Adenekan and Tayo, 1985). Using a mobile pithing electrode to stimulate discrete regions of the spinal canal, Gillespie and McGrath (1973) found that the sympathetic nerves to the rat anococcygeus could be activated from two distinct regions of the spinal cord, T11–L3 and L6–S2. Contractile responses of the muscle to stimulation of spinal segment T11–L3 were inhibited by the ganglion blocking agent hexamethonium, and retrograde staining techniques revealed that the ganglia of the sympathetic nerves to the anococcygeus are located mainly in the lower lumbar and upper sacral ganglia (Dail *et al.,* 1990). Apart from the catecholamine fluorescence histology, several lines of evidence strongly support the view that these motor nerves are indeed sympathetic: (1) both nerve stimulation and noradrenaline produce strong contractions of the muscle and both effects are selectively blocked by α-adrenoceptor antagonists; (2) the nerves, and the responses associated with their activation, disappear after treatment with 6-hydroxydopamine (Gibson and Gillespie, 1973; Doggrell and Waldron, 1982); (3) noradrenaline is taken up by nerves within the anococcygeus (Nash *et al.,* 1974; Doggrell and Woodruff, 1977) and released following stimulation of the sympathetic nerves (McGrath and Olverman, 1978); and (4) responses to nerve stimulation and to noradrenaline are enhanced by inhibitors of neuronal uptake of noradrenaline, including a wide variety of antidepressant compounds (Gibson and

Pollock, 1973; Doggrell and Woodruff, 1977; Doggrell, 1980). The release of noradrenaline appears to be initiated by calcium entering the neuron mainly through N-type voltage-operated calcium channels sensitive to ω-conotoxin GVIA (De Luca *et al.,* 1990; Lundy and Frew, 1994; Mudumbi and Leighton, 1994), although some ω-conotoxin GVIA-resistant release has been detected using electrophysiological recordings from innervated smooth muscle cells; the residual release was partially reduced by ω-agatoxin IVA, the remainder being inhibited by ω-conotoxin MVIIC (Smith and Cunnane, 1997). It seems therefore that, under certain stimulation conditions, other types of voltage-operated calcium channels, including P type, may contribute to noradrenaline release.

There is little evidence for any substantial cotransmission within the sympathetic supply to the anococcygeus (Cunnane *et al.,* 1987). However, neurons containing neuropeptide Y-like immunoreactivity have been identified within the rat anococcygeus and it has been suggested that these may be part of the sympathetic innervation (Iravani and Zar, 1997). Functionally, neuropeptide Y by itself causes contractions, enhances contractile responses to noradrenaline, and inhibits the release of noradrenaline from the sympathetic nerves (Vila *et al.,* 1992; Iravani and Zar, 1997). However, more work is required to substantiate this putative neuromodulatory role for neuropeptide Y in the anococcygeus.

Although it is clear that the motor innervation of the anococcygeus is classically sympathetic, there are a couple of unusual aspects. First, the muscle is peculiarly sensitive to contraction by drugs that displace noradrenaline from the sympathetic nerve terminal (indirect sympathomimetics) such as tyramine, tetraethylammonium, guanethidine, and lysergic acid diethylamide (Gillespie, 1972, 1980; Gillespie and Tilmisany, 1976; McGrath and Olverman, 1978; Foster *et al.,* 1978). Secondly, although there is no doubt that the postsynaptic receptors on which the sympathetic neurotransmitter acts are α_1-adrenoceptors, the precise nature of the subtype of this class of receptor which is involved is not clear as yet. Postjunctional β-adrenoceptors mediating relaxations to exogenous catecholamines have been found in anococcygeus muscles of most species other than the rat and mouse (Gillespie, 1980), and prejunctional β-adrenoceptors facilitate release of the sympathetic transmitter (Carr *et al.,* 1983; Li *et al.,* 1988). Inhibitory prejunctional receptors for adenosine (Coates *et al.,* 1994), histamine (Oriowo, 1982; Gwee *et al.,* 1995), and γ-amino-butyric acid (Muhyaddin *et al.,* 1982, 1983; Hills *et al.,* 1989, 1991) have also been reported.

B. Parasympathetic Nitrergic Nerves

1. Background

Ten years after first describing the properties of the rat anococcygeus, Gillespie (1980) observed that its experimental use during that time had largely involved

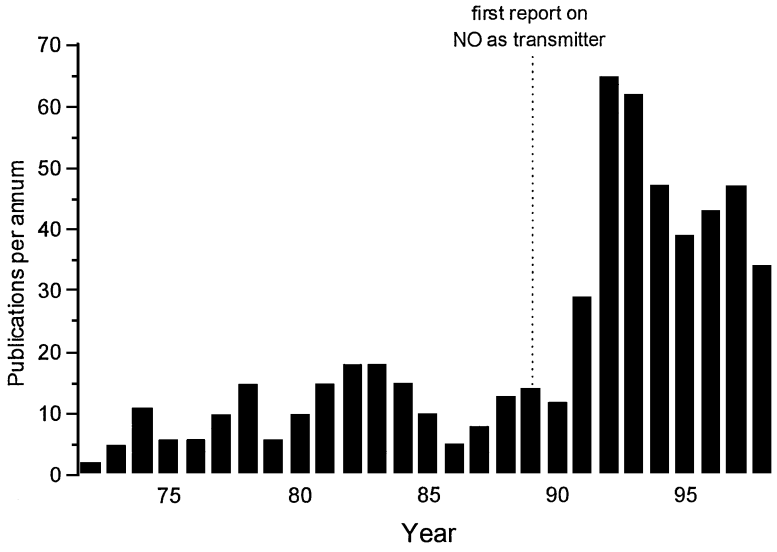

FIG. 2 Number of publications related to the anococcygeus annually from 1973.

investigations involving the sympathetic nervous system, while investigations into the potentially more exciting, and certainly more intriguing, NANC innervation were comparatively sparse. He surmised that this situation would reverse once the nature of the NANC transmitter became apparent and better drugs were available for probing the mechanisms underlying the NANC neurotransmission process. As Fig. 2 clearly shows, he was quite correct; the number of publications relating to the anococcygeus dramatically increased at the beginning of the 1990s and this was entirely due to the identification of the parasympathetic NANC transmitter as NO.

On the assumption that the NANC transmitter of the two tissues would be the same, Gillespie and Martin (1980) identified an extract from both the bovine retractor penis and rat anococcygeus which possessed potent smooth muscle relaxant activity. The extract could exist in two forms, either as a stable, inactive form or as a very unstable, thermolabile, active form, which was generated by exposure of the inactive form to strong acid (Gillespie *et al.,* 1981). The potency and characteristics of the biological actions of the active extract, called inhibitory factor (IF), were consistent with it being the relaxant NANC transmitter of the two tissues; against this possibility, however, was the observation that IF could be extracted from tissues (uterus, umbilical cord, liver) with either sparse, or no significant, NANC innervation. Subsequent experiments revealed that, in common with electrically evoked NANC responses, relaxations to IF were inhibited by drugs that interfered with the guanylyl cyclase/cyclic GMP pathway (including hemoglobin), were

associated with increased cyclic GMP levels within the tissue, and were potentiated by the cyclic GMP phosphodiesterase inhibitor zaprinast (Bowman and Gillespie, 1982; Bowman *et al.,* 1982; Bowman and Drummond, 1984; Martin *et al.,* 1985; Griffith *et al.,* 1985; Gibson and Mirzazadeh, 1989). Indeed the properties of IF showed close similarity to those of endothelium derived relaxing factor (EDRF), the extremely labile vasodilator substance released from vascular endothelial cells that had been discovered by Furchgott and Zawadski (1980). Following identification of EDRF as NO, it was proposed that the stable inactive form of IF extracted from the retractor penis and anococcygeus was, in fact, inorganic nitrite that had been converted to active NO by acid exposure (Furchgott, 1988; Martin *et al.,* 1988).

Confirmation of the proposal that NO was involved in NANC transmission in the anococcygeus was provided by experiments using drugs that inhibit the enzyme nitric oxide synthase (NOS). This enzyme converts L-arginine to L-citrulline with the concomitant production of free radical NO; certain arginine analogs, most potently L-N^G-nitroarginine (L-NOARG) and L-N^G-monoethylarginine (L-NMMA; Moore and Handy, 1997), act as competitive inhibitors of NOS and both of these drugs were shown to selectively inhibit relaxations of the anococcygeus muscle induced by NANC nerve stimulation (Gillespie *et al.,* 1989; Li and Rand, 1989a; Ramagopal and Leighton, 1989; Gibson *et al.,* 1990; Hobbs and Gibson, 1990). The inhibition was stereoselective since D-NOARG was without effect and the reduction of NANC responses observed with L-NOARG and L-NMMA was reversed by application of excess L-, but not D-, arginine. The above initial observations were made on anococcygeus muscles from rats and mice, but similar results have since been obtained in anococcygeus muscles from other species (Graham and Sneddon, 1993; Kasakov *et al.,* 1995; Li and Rand, 1999) and in a wide range of other smooth muscles from the gastrointestinal tract, the respiratory tract, the urogenital tract, and the cardiovascular system; this new and widespread neurotransmission process was termed *nitrergic* (Rand, 1992; Stark and Szurszewski, 1992; Sanders and Ward, 1992; Bredt and Snyder, 1992; Rand and Li, 1995a, 1995b). Following on from these early observations, some of the properties of the nitrergic neurotransmission process have become clear (Fig. 3), with the anococcygeus providing one of the major experimental models used for the investigations.

2. Synthesis of NO

NOS has been detected by both immunofluorescence studies and by NADPH diaphorase histochemistry within nerves coursing through the anococcygeus (Brave *et al.,* 1993c; Dail *et al.,* 1993; Song *et al.,* 1993; Kasakov *et al.,* 1994). NOS-positive cell bodies are found on the surface of the anococcygeus muscle and located in the major pelvic ganglion; about two-thirds of the nerves traced from the anococcygeus to the pelvic ganglion are NOS positive (Dail *et al.,* 1993), and as yet the role of the remaining NOS-negative fibers remains unclear. The

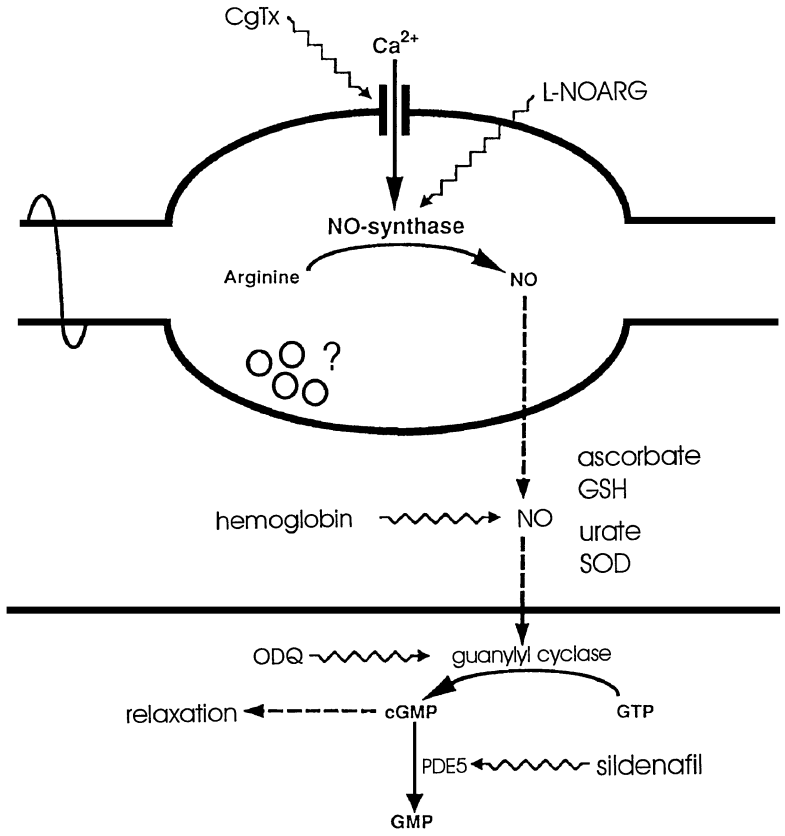

FIG. 3 The nitrergic neurotransmission system in the anococcygeus. Arrival of an action potential at the nerve ending activates voltage-operated calcium channels, which can be blocked by ω-conotoxin (CgTx). Calcium entering the cell via these channels then activates nitric oxide synthase (NOS; a heme containing enzyme requiring calmodulin, FMN, FAD, NADPH, and tetrahydrobiopterin) which converts L-arginine to L-citrulline with the concomitant generation of free radical NO; NOS can be inhibited by arginine analogs such as L-N^G-nitroarginine (L-NOARG). NO readily diffuses through the nerve membrane into the junctional gap; ultrastructural studies have revealed numerous subcellular vesicles in the nitrergic neurone, but the nature of the substances stored in, and released by, these vesicles (?) has yet to be determined (whether cotransmitter, neuromodulator, or antioxidant). While in the gap the NO is vulnerable to attack by hemoglobin, although it is protected from other scavengers by the presence of antioxidants such as ascorbate, glutathione (GSH), urate, and superoxide dismutase (SOD). The receptor for NO is soluble guanylyl cyclase in the cytosol of the smooth muscle cell, which can be selectively inhibited by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). Activation of guanylyl cyclase produces the second messenger substance cyclic GMP (cGMP), which results in relaxation via mechanisms described in Section V. Cyclic GMP is metabolized within the smooth muscle cell by phosphodiesterase 5 (PDE5); this enzyme is inhibited by sildenafil, which therefore increases and prolongs nitrergic relaxations.

NOS-positive fibers are distinct from the sympathetic supply since they are resistant to 6-hydroxydopamine (Brave *et al.,* 1993c). Enzyme activity has also been detected in homogenates of the rat anococcygeus, by measuring the conversion of ^{3}H-arginine to ^{3}H-citrulline (Mitchell *et al.,* 1991). The enzyme was found in both soluble and particulate fractions and was dependent for its activity on calcium, tetrahydrobiopterin, and NADPH. Calcium activation of the enzyme occurs over the range of calcium concentrations which might be achieved in the cytoplasm during nerve depolarization and indeed it is believed that it is the depolarization-induced calcium influx that stimulates NO synthesis.

3. Storage and Release of NO

Although, as described previously, the NANC (nitrergic) nerves in the anococcygeus muscle contain both small clear vesicles and large granular vesicles, it seems clear that NO is synthesized on demand by cytoplasmic NOS and that there is no store of preformed transmitter. Supporting this are observations that NOS immunofluorescence is diffuse within the nerve terminal, and indeed is found along the length of the axon, and that NOS inhibitors reduce nerve-induced relaxations within a few minutes rather than the few hours normally found with synthesis inhibitors in other neurotransmission systems, where the vesicular stores of transmitter must be exhausted before transmission begins to fail. The calcium entry channels allowing access of calcium into the axon to activate NOS are mainly N-type voltage-operated calcium channels since relaxations are largely inhibited by ω-conotoxin GVIA (De Luca *et al.,* 1990; Mudumbi and Leighton, 1994). The mechanisms of synthesis and release of NO are capable of supporting active transmission over long periods of continuous stimulation (Kasakov *et al.,* 1995). The synthesized NO is believed simply to diffuse out of the nerve and into the postjunctional cell. While diffusing across the junctional gap, NO is liable to be scavenged by hemoglobin, thus explaining the early observations that NANC relaxations of the anococcygeus could be blocked by this protein.

The modulation of NO release by receptor mechanisms located on the nitrergic nerve terminal has received some attention. To date, there is evidence for only inhibitory muscarinic receptors (possibly M_1) and excitatory nicotinic receptors (Li and Rand, 1989b; Rand and Li, 1992). Cross-talk between the motor noradrenergic and inhibitory nitrergic innervations of the anococccygeus has not yet been demonstrated at the presynaptic level; postsynaptically, however, nitrergic stimulation can inhibit the contractile response to activation of the sympathetic nerves (Brave *et al.,* 1993a; Kasakov *et al.,* 1994; Mudumbi *et al.,* 1996).

4. The NO Receptor

Unlike most neurotransmitter systems, the postjunctional receptor for NO is not a membrane-spanning receptor on the plasmalemma of the smooth muscle cell

but rather soluble guanylyl cyclase, a heme-containing protein located in the smooth muscle cell cytoplasm (Hobbs, 1997), activation of which results in the generation of the second messenger substance cyclic GMP. That the guanylyl cyclase/cyclic GMP mechanism is the transduction pathway for nitrergic relaxations of the anococcygeus is supported by several pieces of experimental evidence: (1) nitrergic relaxations are mimicked by application of NO-donor drugs and the effects of both stimuli are accompanied by increased cyclic GMP levels in the smooth muscle (Mirzazadeh *et al.,* 1991; Cellek *et al.,* 1996); (2) nitrergic relaxations are selectively reduced by inhibitors of guanylyl cyclase (Gibson and Mirzazadeh, 1989; Cellek *et al.,* 1996; Fonseca *et al.,* 1998); (3) cell permeable analogs of cyclic GMP mimic nitrergic relaxations (Mirzazadeh *et al.,* 1991; Cellek *et al.,* 1996); (4) relaxations to NO-donor drugs and to nitrergic nerve stimulation are selectively potentiated by inhibitors of cyclic GMP phosphodiesterase (Gibson and Mirzazadeh, 1989; Cellek *et al.,* 1996; O'Kane and Gibson, 1999). The mechanism by which cyclic GMP then goes on to elicit relaxation of the smooth muscle is discussed later (see Section V).

5. Nature of the Substance Released from the Nitrergic Nerves

Since the realization of the central role of the L-arginine/NO/cyclic GMP pathway in nerve-induced relaxations of the anococcygeus there has been a continuing debate on the nature of the transmitter molecule actually released from the nitrergic nerves (Gibson *et al.,* 1995; Rand and Li, 1995a). This debate arose as a result of observations from different laboratories that certain NO-scavenger agents could abolish relaxations of the anococcygeus, and other smooth muscles, to exogenous NO but they had no effect on responses to stimulation of the nitrergic nerves. These agents included superoxide anions (Lilley and Gibson, 1995), carboxy-PTIO (Rand and Li, 1995c); hydroquinone (Hobbs *et al.,* 1991), and hydroxocobalamin (Rajayanagam *et al.,* 1993). Subsequently, it was shown that the effects of hydroxocobalamin were dependent on the light conditions under which the experiments were conducted (La *et al.,* 1997). Several hypotheses were put forward to explain the differential effects of the other substances (Gibson *et al.,* 1995).

First, it was possible that the substance actually released from nerves was not free radical NO but rather an NO adduct or perhaps another redox form of nitrogen monoxide; the chemical nature of these adducts or nitrogen monoxide forms would render them resistant to attack by NO scavengers. Candidates for NO adducts were the nitrosothiols, such as nitrosoglutathione or nitrosocysteine; in fact, it has been proposed that the original IF extracted from bovine retractor penis and rat anococcygeus was likely to be a nitrosothiol generated by acidification of nitrite and thiols in the tissue extract (Kerr *et al.,* 1993). Although these nitrosothiols do relax the anococcygeus and show some properties consistent with a neurotransmitter role, the parallelism is far from complete (Gibson *et al.,* 1992; Rand and Li, 1993);

the same is true for nitroxyl, the more likely transmitter candidate of the two other redox forms of nitrogen monoxide (Li *et al.,* 1999).

Another explanation for the differential effects of NO scavengers on exogenous and endogenous NO has been put forward by Wood and Garthwaite (1994). This proposes that the rapid rate of diffusion of free-radical NO over short distances made it relatively resistant to all but the fastest reacting scavenger molecules. However, it has been argued that the rate of reaction of NO with carboxy-PTIO and superoxide anions is theoretically fast enough to have a significant effect on the actions of NO, even over the short distances involved in junctional transmission (Rand and Li, 1995c; Ellis *et al.,* 1998), yet no such effect is apparent experimentally.

A third explanation is that free-radical NO is indeed the substance released as a neurotransmitter but that it is protected in the junctional gap by the presence of "chaperone" molecules (Brave *et al.,* 1993b). One such molecule could be tissue Cu/Zn superoxide dismutase (Cu/Zn SOD). It has now been shown in several tissues, including the anococcygeus, that nitrergic relaxations normally resistant to superoxide anion attack become sensitive following depletion of Cu/Zn SOD activity (Martin *et al.,* 1994; Lilley and Gibson, 1995; Lefebvre, 1996; De Man *et al.,* 1996; Liu *et al.,* 1997; La and Rand, 1999). Further, Cu/Zn SOD was shown to be colocalized with NOS in the nitrergic nerves of the rat anococcygeus (Liu *et al.,* 1997). Other endogenous antioxidants were found to protect exogenous NO against NO scavengers, including ascorbate (protects NO against superoxide anions, hydroquinone, and carboxy-PTIO), α-tocopherol (protects against carboxy-PTIO), uric acid (protects against carboxy-PTIO), and glutathione (protects against hydroquinone; Lilley and Gibson, 1996, 1997). Both ascorbate and uric acid are released by the anococcygeus, the release of ascorbate being increased by depolarization (Lilley and Gibson, 1997). Thus, it seems that the reason for the lack of effect of NO scavengers on free-radical NO released from nitrergic nerves is a combination of fast diffusion kinetics and the presence in the junctional region of physiological antioxidants that protect the NO on its journey from neuronal source to smooth muscle receptor. Exogenous NO on the other hand is clearly vulnerable to attack in the organ bath before reaching the protection of the tissue antioxidants.

C. Other Putative Innervations

1. Purinergic

The anococcygeus muscles of several species display quinacrine-positive nerve fibers indicating the possibility of purinergic transmission; these fibers disappear after section of the nerves running to the anococcygeus but are resistant to 6-hydroxydopamine (Burnstock *et al.,* 1978; Olson and Alund, 1979; Iijima,

1983). Some of these nerves also stain for NOS, suggesting coexistence of the two transmitter systems (Belai and Burnstock, 1994). The putative transmitter adenosine 5′-triphosphate (ATP) relaxes the rabbit anococcygeus, but is generally a contractile agent in muscles from other species; this contraction can be converted to relaxations following administration of indomethacin to inhibit prostaglandin synthesis (Burnstock *et al.,* 1978; Gillespie, 1980; Gibson and Wedmore, 1981). Further supporting a neurotransmitter role for ATP is the observation that the nucleotide is released during electrical stimulation, even in muscles previously treated with 6-hydroxydopamine (Burnstock *et al.,* 1978). Against such a role is the species variability of the mechanical response described above and the lack of specific agents to block the proposed purinergic relaxations. The ATP antagonist arylazido aminopropionyl ATP produced slight inhibition of ATP-induced relaxations of the rabbit anococcygeus, but had no effect on responses to nerve stimulation (Sneddon *et al.,* 1982); similarly, the P_1 purinoceptor antagonist 8-(*p*-sulfophenyl) theophylline inhibited response to both adenosine and ATP but failed to affect relaxations to NANC nerve stimulation.

Because nerve-induced relaxations of the anococcygeus are usually abolished by NOS inhibitors, it seems unlikely that any substance other than NO acts as a relaxant transmitter in this tissue. Recently, however, Selemidis and Cocks (1997) have suggested that the nature of the dominant transmitter may vary, depending on the level of background tone. Thus, when the rat anococcygeus was strongly precontracted with an α-adrenoceptor agonist relaxations to field stimulation were sensitive to NOS inhibitors indicating nitrergic transmission; when much smaller precontractions were induced, the relaxations to nerve stimulation were resistant to NOS inhibitors but were abolished by voltage-operated calcium channel (VOCC) blocking drugs. It was proposed that a low levels of preexisting tone, NANC relaxations were not due to NO but to an as yet unidentified nerve-derived hyperpolarizing factor (NDHF), which produces relaxation via activation of apamin-sensitive potassium channels (Selemidis and Cocks, 1997; Selemidis *et al.,* 1997). However, no evidence for such a factor was obtained in the mouse anococcygeus (Fonseca *et al.,* 1998). A parallel hyperpolarizing factor released from endothelial cells has recently been tentatively characterized as inorganic potassium ions (Edwards *et al.,* 1998); potassium ions do produce relaxations of the anococcygeus but these are due to a combination of direct depolarization of the nerves releasing the inhibitory transmitter and activation of the sodium pump of the smooth muscle cells (Gibson and James, 1977; Ishii and Shimo, 1980).

2. VIPergic

Vasoactive intestinal peptide (VIP)-positive immunofluorescence has been detected in nerve fibers running within the anococcygeus (Gibson and Tucker, 1982; Larsen *et al.,* 1985; Dail *et al.,* 1990). In addition, the anococcygeus muscle of most

species relaxes in response to VIP. However, while relaxations to exogenous VIP can be greatly reduced by VIP antiserum, by α-chymotrypsin, and by long-term exposure to VIP, these procedures have no effect on nerve-induced relaxations (Carvajal *et al.,* 1986). Evidence from histological studies suggests that the VIPergic nerves in the anococcygeus may actually innervate the blood vessels within the tissue rather than the nonvascular smooth muscle cells (Dail *et al.,* 1990).

3. Cholinergic

Early morphological studies revealed the presence of cholinergic-like nerve terminal profiles in the anococcygeus but no evidence for any substantial cholinesterase staining, a commonly used marker for cholinergic neurons (Gillespie and Lullmann-Rauch, 1974; Burnstock *et al.,* 1978; Smith and Spriggs, 1983). In addition, the responses to stimulation of either the motor or inhibitory nerves to the tissue were unaffected by cholinoceptor antagonists and therefore it seemed that there was no functional cholinergic innervation of the anococcygeus. However, biochemical studies indicated the presence of high concentrations of acetylcholinesterase within the rat anococcygeus (Gibson and Pollock, 1975a), levels which in fact were similar to those found in whole brain. Later histological studies confirmed this (Iijima, 1983; Dail *et al.,* 1990), demonstrating a dense plexus of cholinesterase-postive fibers arising from ganglion cells within the muscle itself and from the pelvic plexus. It was also found that motor responses of the rat anococcygeus were potentiated in the presence of a cholinesterase inhibitor (Doggrell, 1981; Smith and Spriggs, 1983) and this raises the possibility that under certain conditions a cholinergic contribution to nerve-induced responses may be present. Certainly, the anococcygeus muscles of most species possess cholinoceptors (Gillespie, 1980). Muscarinic receptors are found postsynaptically, with those causing contraction likely to be of the M_3 subtype (Sideso *et al.,* 1994; Weiser *et al.,* 1997); presynaptic muscarinic receptors modulate the release of NO from the nitrergic nerves, being either inhibitory (M_1 in rat; Li and Rand, 1989b) or excitatory (M_4 in rabbit; Gross *et al.,* 1997). Excitatory nicotinic receptors are found on both sympathetic and nitrergic terminals (Rand and Li, 1992; Gross *et al.,* 1997). Thus, the evidence for or against a functional role for cholinergic nerves in the anococcygeus is equivocal; it is unlikely that they contribute to a direct action on the smooth muscle cells unless the activity of acetylcholinesterase has been reduced; it is more likely that the role of any cholinergic contribution is to modulate the release of the main transmitters from the sympathetic and nitrergic nerves.

4. Sensory Nerves

Although the efferent autonomic nerve supply to the anococcygeus has been extensively investigated, much less attention has been paid to the sensory innervation.

However, one recent, and potentially exciting, observation is that capsaicin, which selectively activates sensory nerves, produces concentration-dependent relaxations of the rat anococcygeus (Davies *et al.,* 1998). These relaxations were blocked by inhibition of NOS or by the NO-scavenger hemoglobin, suggesting that some of the nitrergic nerves within the anococcygeus may have a sensory role. However, because many of the NOS-containing neurons within the anococcygeus have been traced back to cell bodies within the major pelvic ganglia (see Section III), any sensory component to the nitrergic supply can only be partial.

IV. Nonneuronal Influences on Muscle Function

A. Peptides

Numerous peptides, including venoms and toxins, have been shown to modulate the activity of the anococcygeus muscle, as summarized in Table I. The possible role of VIP as a NANC transmitter has already been covered (see Section III) as has the possible cotransmitter role of neuropeptide Y in the sympathetic nerves (see Section III); the effects of two other peptides (oxytocin and urotensin II) are worth further mention.

The sensitivity of the mouse anococcygeus muscle to contraction by oxytocin and to relaxation by urotensin II is unusual in each case. Oxytocin is normally highly selective for the uterus in mammals and has relatively minor effects on smooth muscle from males. However, the male mouse anococcygeus displays powerful and strong contractions to oxytocin, the potency of the peptide being similar to that on the uterus (Gibson *et al.,* 1984; Botting and Gibson, 1985); the anococcygeus receptor system on which the oxytocin acts appears to be similar to that in the uterus, in terms of ion dependence and agonist/antagonist potencies (Gibson, 1985, 1986). This high sensitivity to oxytocin is not shown by the anococcygeus muscles from other species and its biological/physiological significance is not known.

Urotensin II is a osmoregulatory peptide produced in, and released by, the caudal neurosecretory system of teleost fish; the peptide from the goby *Gillichthys mirabilis* is a dodecapetide showing some similarity to somatostatin (Pearson *et al.,* 1980). *Gillichthys* urotensin II relaxes the anococcygeus muscle and inhibits contractions elicited by sympathetic nerve stimulation, an action shared with somatostatin (Gibson *et al.,* 1984; Larsen *et al.,* 1985; Priestley and Woodruff, 1988); urotensin II has also been shown to have potent cardiovascular effects in rats, causing contraction of the isolated thoracic aorta at very low concentrations while producing a long-lasting reduction in blood pressure in the whole animal (Gibson *et al.,* 1986; Gibson 1987). Recently, urotensin II has been identified in the spinal cord and other tissues of frogs and humans (Coulouarn *et al.,* 1998)

TABLE I
Effects of Neuropeptides and Toxins on the Anococcygeus Muscle

	Effects	References
Neuropeptides		
Angiotensin II	Increase sympathetic response (rat)	Li *et al.* (1988)
Bradykinin	Relaxation, release of NO (rat, rabbit, cat)	Gillespie and McKnight (1978); Mudumbi and Leighton (1994)
Eledoisin	Contraction, release of NA; decrease sympathetic response (rat)	Gillespie and McKnight (1978)
Neurotensin	Contraction (mouse)	Gibson *et al.* (1994)
Neuropeptide Y	Contraction; enhance NA; decrease sympathetic response (rat)	Iravani and Zar (1997); Vila *et al.* (1992)
Oxytocin	Contraction (mouse)	Gibson *et al.* (1984)
Peptide histidine isoleucine	Relaxation (rabbit)	Blank *et al.* (1986)
Somatostatin	Relaxation (mouse); decrease sympathetic response (rat, mouse)	Priestley and Woodruff (1988); Gibson *et al.* (1984)
Substance P	Relaxation (cat); contract (mouse)	Gillespie (1980); Gibson *et al.* (1984)
Thyrotrophin	Contraction (mouse)	Gibson *et al.* (1984)
Urotensin II	Relaxation; inhibit sympathetic response (mouse)	Gibson *et al.* (1984); Larsen *et al.* (1985)
Vasopressin	Contraction (rat, mouse)	Gillespie (1980); Gibson *et al.* (1984)
Vasoactive intestinal peptide	Relaxation (rat, mouse, rabbit)	Gibson and Wedmore (1981); Blank *et al.* (1986)
Vasotocin	Contraction (mouse)	Gibson *et al.* (1984)
Toxins		
Makatoxin I (scorpion)	Contraction (release NA, rat); relaxation (release NO, rat)	Gong *et al.* (1997)
Palytoxin	Contraction (release NA, rat)	Amir *et al.* (1997)

raising the exciting possibility that this peptide, formerly believed only to be of importance in fish, may have a role in the biology of mammals. Indeed, human urotensin II has now been reported to be the most potent vasoconstrictor peptide yet discovered, producing dramatic and devastating changes in cardiovascular function in nonhuman primates (Ames *et al.,* 1999).

B. Hormones

The properties of the anococcygeus muscle can be modified by several endocrine hormones (Gibson, 1981). As might be expected for a smooth muscle of the urogenital tract, sex hormones influence both the size of the muscle and its responsiveness to contractile agents. In rats and mice, the anococcygeus muscle of male animals is larger than that in females (Gibson and Gillespie, 1973; Fukazawa *et al.,* 1997). Testosterone injection at birth increased the cross-sectional area

of the anococcygeus of both male and female mice, while injection of estradiol reduced their size. However, neonatal exposure to diethylstilbestrol stimulated growth of the female mouse anococcygeus (Fukazawa *et al.,* 1997). Testosterone also increased the contractility of the rat anococcygeus (Gibson, 1977), as did corticosterone (Gibson and Pollock, 1975b) and thyroxine (Gardiner *et al.,* 1994). Corticosterone also decreased the cholinesterase content of the rat anococcygeus, thus enhancing responses to acetylcholine (Gibson and Pollock, 1975a).

C. Ethanol

Interest in the effects of ethanol on the anoccygeus stems from the well-known effects of alcohol consumption on male potency. Indeed, it was found that ethanol acutely inhibited NANC relaxations of the rat and rabbit anococcygeus, as well as those of the retractor penis. Ethanol also inhibited responses to the putative neurotransmitter, IF (Gillespie *et al.,* 1982). Subsequently, it was confirmed that ethanol could inhibit nitrergic relaxations of the rat anococcygeus and that it might act by sequestering the released NO to form nitroso-ethanol (Knych, 1994; Rand and Li, 1994). However, if ethanol was administered to rats over 2 days the nitrergic relaxations of the anococcygeus muscle were enhanced and the inhibitory effects of ethanol were attenuated (Knych, 1994). Thus, acutely ethanol inhibits nitrergic transmission, but over a period of time the neurotransmission system adapts prejunctionally to produce tolerance to the effects of alcohol.

V. Cellular Mechanisms of Contraction and Relaxation

As with all smooth muscles, the primary stimulus for the initiation of contraction in cells of the anococcygeus is a rise in the concentration of free intracellular calcium. This leads to activation of calcium-calmodulin (CaM)-dependent myosin light chain kinase (MLCK) which phosphorylates the 20-kDa myosin light chains (LC_{20}) with a consequential increase in myosin ATPase activity and enhanced cross-bridge recycling. As mentioned elsewhere in this review (see Section III), contraction of the anococcygeus can be brought about following activation of a number of receptors for excitatory neurotranmitters, including α_1 adrenoceptors (Shimizu *et al.,* 1995) and M_3 muscarinic cholinoceptors (Sideso *et al.,* 1994). In this section, we review the literature relating to the sequence of events coupling receptor activation to contraction. We also discuss the less-than-complete picture concerning mechanisms by which relaxant drugs might interfere with this process. For reference, a schematic representation of the current model of excitation–contraction coupling in the mouse anococcygeus is shown in Fig. 4.

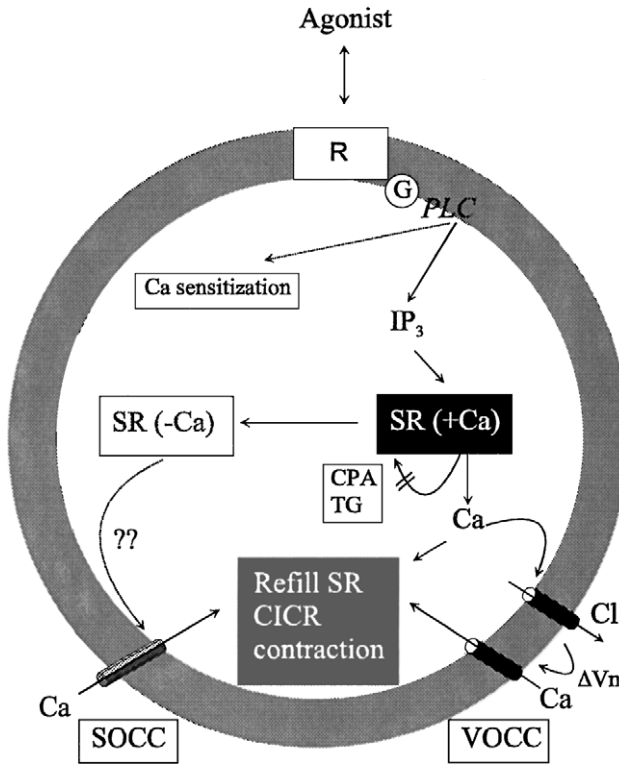

FIG. 4 Model of excitation–contraction coupling in the mouse anococcygeus. Binding of an agonist to its G-protein (G)-coupled receptor (R) activates phospholipase C (PLC), which leads to generation of the second messenger substance inositol trisphosphate (IP_3). IP_3 releases calcium from the sarcoplasmic reticulum (SR), and this calcium then opens calcium-activated chloride channels on the plasma membrane. The resulting depolarization opens voltage-operated calcium channels (VOCCs) through which calcium enters the cell to initiate contraction. Depletion of the SR, in some way which has yet to be established (??), signals the opening of store-operated calcium channels (SOCCs) through which calcium enters to sustain contraction, refill the SR, and possibly activate calcium-induced calcium release (CICR). The SR can also be depleted directly by inhibitors of the sarcoendoplasmic reticulum ATPase such as cyclopiazonic acid (CPA) or thapsigargin (TG). The role of calcium sensitization mechanisms has yet to be determined.

A. Contraction

The vast majority of experiments in this area have been performed on the anococcygeus muscles of mice and rats. In both species, contractile responses to exogenously applied agonists are effectively abolished in the absence of extracellular calcium, suggesting that calcium entry is essential for sustained contractions. One of the main calcium entry pathways found in smooth muscle is the "L-type"

voltage-dependent calcium current, activated by membrane depolarization and selectively inhibited by three classes of organic calcium channel blockers typified by nifedipine, verapamil, and diltiazem. Early electrophysiological recordings showed that both noradrenaline and intramural nerve stimulation (Creed, 1975; Creed and Gillespie, 1975; Creed *et al.,* 1975; Large, 1982, 1983) produced membrane depolarizations from a resting membrane potential of about −60 mV. Such depolarizations were associated with a fall in membrane resistance, indicating that they resulted from the opening of ion channels. Large and coworkers have since shown that that the primary conductance activated by noradrenaline is a calcium-dependent chloride conductance (I_{ClCa}). In rat anococcygeus cells, depolarizations produced by noradrenaline were abolished in low extracellular chloride concentrations (Large, 1984; Byrne and Large, 1985). In whole-cell voltage-clamp experiments carried out using isolated cells, the reversal potential for the current activated by noradrenaline corresponded closely to the chloride equilibrium potential (Byrne and Large, 1987a). The muscarinic receptor agonist carbachol activated a similar conductance (Byrne and Large, 1987b) as did caffeine and the calcium ionophore A23187 (Byrne and Large, 1987a). In mouse anococcygeus cells, the current is activated by carbachol, caffeine, and cyclopiazonic acid (CPA; see later), blocked by the chloride channel blockers 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) and anthracine-9-carboxylic acid (A-9-C), and its activation is prevented in cells dialyzed with an intracellular calcium buffer such as ethyleneglycol-bis(β-aminoethyl)*N,N,N′,N′*-tetraacetic acid (EGTA) or 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′N′*-tetraacetic acid; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (BAPTA) (Wayman *et al.,* 1996).

Neurotransmitter receptors coupled to contraction in the anococcygeus have been shown in other systems to activate the phospholipase C/inositol trisphosphate (IP_3)/diacyglygerol transduction pathway. Using carbachol as an agonist, M_3 receptor activation has been shown to produce a rapid increase in inositol phosphate turnover in rat anococcygeus cells (Gibson *et al.,* 1994b). This response peaks within 20 sec, which corresponds well to the time course of activation of I_{ClCa} in mouse cells using the same agonist (Wayman *et al.,* 1997). Taken together, these observations pointed toward a model in which receptor activation led to the IP_3-mediated release of calcium from CPA- and caffeine-sensitive intracellular stores with a consequential activation of I_{ClCa} and membrane depolarization. This would then be expected to activate voltage-dependent calcium channels, allowing calcium entry. Unfortunately this rather attractive proposition was challenged by a number of pieces of evidence which suggested that voltage-dependent calcium channels are not the primary pathway for calcium entry activated in response to neurotransmitter receptor activation.

Voltage-dependent calcium currents have been recorded in smooth muscle cells isolated from the rat anococcygeus (England and McFadzean, 1995). The current appears to be carried solely by L-type channels as it is abolished by the

dihydropyridine calcium channel blocking agent, nifedipine. Doubts over the role of such currents in mediating contractions however arose as a result of experiments in whole-muscle preparations. As early as 1976, Gillespie and Tilmisany reported that the potassium channel blocker tetraethylammonium (TEA), at concentrations capable of producing membrane depolarizations (Creed et al., 1975) and since shown to be effective at inhibiting the large voltage-dependent potassium current in rat anococcygeus cells (McFadzean and England, 1992), had little effect on the contractions produced by exogenously applied noradrenaline. Blockade of potassium channels would have been expected to potentiate membrane depolarization and enhance contractions mediated by calcium entry via voltage-dependent calcium channels. Removal of extracellular chloride, while abolishing the depolarization produced by noradrenaline, had no effect on the maximum tension produced by exogenous noradrenaline in whole muscle (Large, 1984). Similarly, the chloride channel blockers DIDS and A-9-C have little effect on the contractions produced by carbachol in the rat anococcygeus (McFadzean, unpublished, 19xx). Finally, and perhaps most convincingly, inhibitors of L-type calcium channels including nifedipine (Oriowo, 1984; Gibson *et al.,* 1994), diltiazem (Oriowo, 1984), and D600 (Vila *et al.,* 1984) have relatively little effect on contractions produced by either noradrenaline or carbachol in anococcygeus muscles from mice or rats, producing only partial inhibition of the maximal response to these agonists. This is despite the fact that in each of the studies cited above, the calcium entry blockers abolished contractile responses to raised extracellular potassium. Thus, while there is no doubt that a number of neurotransmitters act to depolarize anococcygeus muscle cells, the physiological role of this depolarization in mediating contraction remains equivocal. One possibility is that entry of calcium via voltage-operated calcium channels is an important determinant of the speed of contraction rather than its absolute amplitude. Large (1984) reported that removal of extracellular chloride slowed the initial, rapid component of the contractions produced by both noradrenaline and phenylephrine in the rat anococcygeus, and similar effects of nifedipine are observed on the contractions to carbachol in the mouse muscle (unpublished).

Thus, we are left with a situation in which contractions of rat and mouse anococcygeus muscles, although dependent on calcium entry, do not rely on entry via voltage-dependent channels. In other smooth muscles, so-called receptor-operated calcium channels (ROCCs) have been suggested to mediate non-voltage-dependent calcium entry (for a review, see Kuriyama *et al.,* 1998). Examples of ROCCS include the P_{2X} receptor activated by extracellular ATP in rabbit ear artery (Benham and Tsien, 1988) and the G-protein-coupled cation channel activated following muscarinic receptor activation in gastrointestinal muscle cells (Pacaud and Bolton, 1991). Extracellular ATP produces contractions of the rat anococcygeus (Byrne and Large, and 1984), and iontophoretically applied ATP has been shown to produce depolarizations of the muscle with a latency significantly shorter than that of the response to noradrenaline, consistent with the involvement of a P_{2X} receptor.

However apart from this there is very little evidence for the presence of ROCCs in anococcygeus muscles.

As mentioned above, contractions of the anococcygeus muscle are initiated by agonists acting on receptors linked to IP_3 production and the associated release of calcium from intracellular stores, in particular the SR. In nonexcitable cells, calcium store depletion has been shown to activate so-called capacitative calcium entry (Putney, 1990), a process responsible for the maintenance of the rise in intracellular calcium produced during prolonged applications of agonists and for the refilling of the stores on termination of the agonist response. We have recently suggested that a similar mechanism exists in some smooth muscle cells, including the anococcygeus, where it plays a pivotal role in excitation–contraction coupling (Gibson *et al.,* 1998). Central to developing the model of capacitative calcium entry in nonexcitable cells have been inhibitors of the sarcoendoplasmic reticulum calcium ATPase (SERCA) responsible for the active transport of calcium into this intracellular organelle from where it can be released via the ion channels associated with IP_3 and/or ryanodine receptors. SERCA inhibitors, including CPA and thapsigargin, cause a receptor-independent, passive depletion of the intracellular calcium stores and as a result activate the capacitative calcium entry process. An early indication that capacitative calcium entry might be important in the anococcygeus came from the observation that CPA produced strong, well-maintained contractions of the mouse muscle (Gibson *et al.,* 1994a). Such contractions were dependent on extracellular calcium, only slightly reduced by nifedipine, abolished by the general calcium entry blockers SKF96365 and cadmium, but insensitive to lanthanum (Wayman *et al.,* 1996a). In these respects the pharmacology of the CPA-induced contractions mirrored those to carbachol. Broadly similar results were obtained using thapsigargin (Wallace *et al.,* 1999). In the rat muscle, CPA produces more variable contractions, significantly smaller than those produced by receptor agonists (Raymond and Wendt, 1996).

Following on from these initial observations, whole-cell patch-clamp experiments were performed on single smooth muscle cells isolated from the mouse anococcygeus and these revealed that CPA, in addition to activating I_{ClCa}, consistent with it being able to release calcium from the SR, also activated a small, but well-maintained inward current (Wayman *et al.,* 1996a). This current had properties consistent with it underlying capacitative calcium entry, as predicted from the whole-muscle experiments using CPA, being insensitive to nifedipine or lanthanum, but blocked by SKF96365 and cadmium. Furthermore, it was still present in cells in which intracellular calcium was buffered to very low concentrations using BAPTA, indicating that it was activated by store depletion, but not as a result of the rise in intracellular calcium so produced. A significant part of the current was carried by calcium as indicated by the positive shift in reversal potential on switching to a calcium-free extracellular solution. Furthermore, experiments in which the current and intracellular calcium concentration were measured simultaneously showed that agents that inhibited the current also inhibited the prolonged

rise in intracellular calcium produced by carbachol (Wayman *et al.,* 1999). This was consistent with an earlier report of CPA producing a sustained rise in intracellular calcium in the rat anococcygeus (Raymond and Wendt, 1996).

As befits a current underlying capacitative calcium entry the so-called depletion operated current (I_{DOC}) could be activated by a range of agents which share the ability to deplete intracellular calcium stores. Thus in addition to CPA, I_{DOC} was activated by carbachol—a response blocked by intracellular heparin, which acts as an IP_3 receptor antagonist—and caffeine. The results obtained with caffeine suggested that, in addition to the IP_3-sensitive store, mouse anococcygeus muscle cells might also contain a ryanodine-sensitive store. Ryanodine receptors are structurally related to the IP_3 receptor but rather than being activated by IP_3 to allow calcium to leave the SR via their integral calcium channel, ryanodine receptors are activated by cytosolic calcium itself and are involved in the process of calcium-induced calcium release. Caffeine acts as an agonist at the ryanodine receptor as does the plant alkaloid ryanodine at low (approx. 3 μM) concentrations; at higher concentrations ryanodine acts as an antagonist. In the mouse anococcygeus low concentrations of ryanodine activated I_{DOC} and produced well-maintained contractions of the whole muscle while high concentrations of the alkaloid failed to activate I_{DOC} but blocked the ability of caffeine to do so (Wayman *et al.,* 1998). Interestingly carbachol was still able to activate I_{DOC} in cells treated with high concentrations of ryanodine and, conversely, low concentrations of ryanodine were still effective in the presence of intracellular heparin, suggesting that depletion of either the ryanodine-sensitive or the IP_3-sensitive component of the store in isolation can activate capacitative calcium influx. Furthermore, activation of I_{ClCa} was dependent on a functional ryanodine-sensitive store, suggesting that it is the release of calcium from this store, presumably triggered by IP_3-mediated calcium release, that is ultimately responsible for neurotransmitter-mediated depolarizations.

Several aspects of the capacitative calcium entry pathway remain unclear, most importantly the nature of the signal between the SR and the store-operated calcium channel (SOCC), and the structure of the channel itself. It has recently been proposed that the signaling mechanism may involve a process analogous to excitation–secretion coupling in neurons (see Putney, 1999), and if this is the case the close association between the SR and the caveolae in the muscle may be of relevance. In terms of the makeup of the channels themselves, it is becoming clear that SOCCs may comprise a family of channels (Trp proteins) related to the transient receptor potential channels of *Drosophila* (Birnbaumer *et al.,* 1996). Several such channels have now been cloned in mammals, some of which are true store-operated channels while others can be activated both by store depletion and/or directly by agonist activation of G-protein-linked receptors; whether a channel of this type contributes to capacitative calcium entry in the anococcygeus (and other smooth muscles) remains to be determined.

A rise in intracellular calcium activates the calcium-calmodulin dependent enzyme myosin light chain kinase (MLCK), which acts to phosphorylate myosin II

light chain (LC_{20}; see reviews by Horowitz *et al.,* 1996, and Somlyo and Somlyo, 2000). The phosphorylation state of LC_{20} is an important determinant of the contractile state of smooth muscle with increased phosphorylation producing an increase in cross-bridge recycling and force production. In addition to MLCK activity, the phosphorylation state of LC_{20} is also governed directly by the activity of a second enzyme, myosin phosphatase (MLCP). In many smooth muscles it is now clear that calcium-*independent* regulation of MLCP, specifically inhibition of this enzyme, plays an important role in so-called calcium sensitization whereby smooth muscle tone is maintained despite a fall in the free intracellular calcium concentration. Calcium sensitization is thought to be of particular importance in tonic smooth muscles, such as the anococcygeus, and while the precise molecular mechanisms underlying the sensitization process remain to be elucidated, accumulating evidence suggests an important role for the small GTPase, RhoA (Somlyo and Somlyo, 2000), and its effector, Rho-kinase. Activation of Rho-kinase phosphorylates, and in doing so inhibits, MLCP. It is not clear what, if any, role calcium sensitization plays in regulating the tone of the anococcygeus muscle. Direct evidence for a calcium-sensitizing effect in response to agonists has been reported in the rat anococcygeus (Boland *et al.,* 1992), though a later study by Shimizu *et al.* (1995) could find evidence for phenylephrine-induced sensitization only in "skinned" preparations of the same muscle. Clearly, further work is required to fully evaluate the importance of calcium sensitization in contractions of the anococcygeus muscle.

B. Relaxation

The major, if not sole, inhibitory neurotransmitter in the anococcygeus is NO (see Section III), released from parasympathetic nitrergic nerves. Here we deal primarily with the proposed cellular mechanism(s) by which NO produces relaxations in the anococcygeus.

It is generally accepted that NO mediates relaxation of smooth muscle following activation of soluble guanylyl cyclase with a result increase in cyclic GMP. In the rat anococcygeus relaxations to both nitrergic nerve stimulation and the nitrovasodilator, sodium nitroprusside (SNP), are associated with an increase in the cyclic GMP content of the tissue (Mirzazadeh *et al.,* 1991). Furthermore, the inhibitor of the cyclic GMP-dependent phosphodiesterase, M&B22948 (Zaprinast), potentiates the relaxations and the increases in cyclic GMP. More recently, relaxations to electrical filed stimulation in both the rabbit (Cellek *et al.,* 1996) and mouse anococcygeus (Fonseca *et al.,* 1998) have been shown to be blocked by the inhibitor of soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). Taken together, these data provide compelling evidence in support of a role for the guanylyl cyclase/cyclic GMP system in mediating nitrergic relaxations of the anococcygeus. What is less clear is how this raised level of cyclic

GMP produces relaxation. Several possibilities have arisen following experiments in a variety of smooth muscle tissues, including the anococcygeus itself, but a consensus view has yet to emerge.

NANC-mediated relaxations of the rat anococcygeus are associated with a fall in the concentration of free intracellular calcium (Ramogopal and Leighton, 1989; Raymond *et al.,* 1995). As mentioned above, most contractile agonists in the anococcygeus interact with receptors coupled to the phospholipase C/IP_3/diacylglycerol transduction pathway. Experiments in a number of vascular tissues including rat aorta (Rapoport, 1986; Hirata *et al.,* 1990) and superior mesenteric artery (Ghisdal *et al.,* 2000) have shown that the NO/cyclic GMP system inhibits production of IP_3 formation. However there is no evidence that this occurs in the rat anococcygeus (Gibson *et al.,* 1994b). Similarly, the nitrovasodilator, SNP, although reported to inhibit calcium entry through voltage-dependent calcium channels in pulmonary arterial cells (Clapp and Gurney, 1991), had no such effect on smooth muscle cells isolated from the rat anococcygeus (England and McFadzean, 1993). This lack of a direct effect on voltage-operated calcium currents is perhaps not surprising in light of the evidence discussed above that suggests that calcium entry via these channels plays a relatively minor role in mediating contraction. This would also tend to rule out changes in membrane potential, in particular membrane hyperpolarization, as being important. Recently Selemidis *et al.* (1998) have reported that activation of NANC nerves produces biphasic inhibitory junction potentials in the rat anococcygeus, the first phase being mediated by an as yet unidentified, non-NO, "hyperpolarizing factor," which activates apamin-sensitive potassium channels, and the second, slower phase by cyclic GMP-mediated activation of a different potassium conductance. Note, however, that SNP is able to relax contractions of the mouse anococcygeus produced in response to raised extracellular potassium (Gibson *et al.,* 1994a), which is difficult to reconcile with a mechanism involving membrane hyperpolarization. Indeed, no such nerve-derived hyperpolarizing factor was evident in the mouse anococcygeus (Fonseca *et al.,* 1998).

Recently we have shown that SNP inhibits store-operated (capacitative) calcium entry in mouse anococcygeus cells (Wayman *et al.,* 1996b). Given the important role this entry pathway plays in mediating sustained contractions to agonists this offered an attractive mechanism through which nitrates might relax the tissue. Certainly contractions to carbachol, though relatively insensitive to nifedipine, can be inhibited by SKF96365 (Gibson *et al.,* 1994a) as can the store-operated current (Wayman *et al.,* 1996a). The effect of SNP was mediated by the guanylyl cyclase/cyclic GMP transduction pathway, being mimicked by 8-bromo cyclic GMP and blocked by the guanylyl cyclase inhibitor ODQ. Evidence suggests that this effect might be due to the nitrate enhancing uptake into the SR and thus turning off the primary stimulus for capacitative calcium entry, rather than inhibiting the current directly. For example, an indirect measure of the state of filling of the calcium store can be obtained by measuring the size of the small, transient contraction produced by carbachol in the absence of extracellular calcium.

Inclusion of SNP during the refilling period, when calcium is reintroduced to the tissue between agonist responses, increases the size of the contraction subsequently recorded in calcium-free medium (Gibson *et al.,* 1994a). This is in direct contrast to the effect of SKF96365, which inhibits the contraction, presumably by preventing reloading of the stores by directly inhibiting the capacitative calcium entry pathway.

The suggestion that SNP, and NO released in response to NANC nerve stimulation, might enhance calcium sequestration in the anococcygeus was initially put forward by Raymond and colleagues (Raymond *et al.,* 1995; Raymond and Wendt, 1996). These workers measured intracellular calcium in rat anococcygeus muscle directly and showed that the reduction in intracellular calcium produced by both SNP and NO was inhibited by the SERCA pump inhibitor CPA. We have recently obtained broadly similar results in the mouse anococcygeus using another SERCA pump inhibitor, thapsigargin (unpublished). However, yet again, difficulties arise in extrapolating results obtained in single cells to the muscle. In particular, both SNP and NANC nerve stimulation are effective at relaxing contractions produced by both CPA (Gibson *et al.,* 1994a) and thapsigargin (unpublished) in mouse anococcygeus. These findings suggest that despite being unable to lower intracellular calcium in the presence of a SERCA pump inhibitor, SNP can still produce relaxations. It would appear therefore that yet another cellular mechanism must exist through which nitrates interfere with excitation–contraction coupling in the anococcygeus muscle.

VI. Concluding Remarks

It is only some 30 years since Gillespie (1972) introduced (or reintroduced) the anococcygeus muscle into biological research. However, in that relatively short time the tissue has generated considerable interest and has been the subject of much experimental investigation. Its most important contribution to scientific knowledge has been in the discovery of the neurotransmitter role of NO and in the elucidation of the mechanisms underlying nitrergic transmission; from an applied point of view the ripest fruit of this work to date has been the introduction of cyclic GMP phosphodiesterase inhibitors as an effective treatment for impotence; the anococcygeus is also providing some potentially important information on the influence of diseases such as diabetes on nitrergic function (Luheshi and Zar, 1992; Way and Reid, 1994, 1995; Way *et al.,* 1999) and on the cellular mechanisms of excitation–contraction coupling in smooth muscle (see Section V).

We hope this review has stimulated the interest of scientists from a wide range of disciplines in the slowly unravelling mysteries of this little muscle. There are still many questions to be answered: for the comparative biologist, what is the precise function of the muscle and what is its species distribution; for the neuroscientist, what is the significance of the subcellular storage vesicles in the nitrergic

nerves—what do they contain and what do they release; for the molecular biologist, what is the structure of the store-operated calcium channels expressed in the muscle and how do they relate to previously cloned channels from *Drosophila;* and to the pharmacologist—can we identify further selective drugs to modulate the function of the nitrergic nerves, and others which interact specifically with the store-operated calcium channels?

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Growth and Neurotrophic Factors Regulating Development and Maintenance of Sympathetic Preganglionic Neurons[1]

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The functional anatomy of sympathetic preganglionic neurons is described at molecular, cellular, and system levels. Preganglionic sympathetic neurons located in the intermediolateral column of the spinal cord connect the central nervous system with peripheral sympathetic ganglia and chromaffin cells inside and outside the adrenal gland. Current knowledge is reviewed of the development of these neurons, which share their origin with progenitor cells, giving rise to somatic motoneurons in the ventral horn. Their connectivities, transmitters involved, and growth factor receptors are described. Finally, we review the distribution and functions of trophic molecules that may have relevance for development and maintenance of preganglionic sympathetic neurons.
KEY WORDS: Autonomic nervous system, Autonomic motoneurons, Spinal cord, Sympathetic ganglia, Adrenal medulla, Growth factors, Growth factor receptors, Knockout mice.

I. Introduction

The autonomic nervous system comprises three major divisions, the sympathetic, parasympathetic, and enteric nervous systems. The sympathetic and parasympathetic divisions innervate heart, blood vessels, exocrine and endocrine glands, and other organ systems. They consist of an efferent and an afferent portion, i.e.,

[1]This work is dedicated to the memory of Professor Dietrich L. Meyer (1947–1999).

comprise motor and sensory entities. The efferent motor entity is formed by a chain of two neurons: preganglionic neurons have their cell bodies located in the central nervous system (CNS) (hindbrain, spinal cord); receive inputs from supraspinal, spinal, and sensory neurons; and project to peripheral ganglia, where they are synaptically coupled to postganglionic neurons, whose axons innervate the final target cells.

Preganglionic neurons of the sympathetic nervous system project to postganglionic neurons in para- and prevertebral sympathetic ganglia, and chromaffin cells within and outside the adrenal medulla. Somatic and autonomic motoneurons in the spinal cord originate from the same pool of precursor cells (Markham and Vaughn, 1991; Phelps *et al.,* 1991), and both are cholinergic (Barber *et al.,* 1984; Phelps *et al.,* 1991), but differ with respect to their targets and types of synaptic contacts established. Somatic motoneurons establish endplates on striated muscle cells, sympathetic preganglionic neurons make axosomatic and axodendritic synapses on postganglionic neurons.

This review outlines established facts of development, morphological characteristics, and afferent and efferent connections of sympathetic preganglionic neurons, their transmitters, interactions with ganglionic and chromaffin targets, and their dependence on neurotrophic factors. While somatic motoneurons have been extensively studied with regard to their requirements of neurotrophic factors (Oppenheim, 1996; Terenghi, 1999; Thoenen *et al.,* 1993; Sendtner *et al.,* 1996), relatively little is known on trophic factors for preganglionic sympathetic neurons. There is increasing evidence to suggest that somatic motoneurons that project to different groups of muscles along the rostrocaudal and mediolateral axes respond to distinct sets of trophic molecules (Henderson *et al.,* 1998; Kahane *et al.,* 1996; Pettmann and Henderson, 1998; Terenghi, 1999). Sympathetic pre- and postganglionic neurons may be even more heterogeneous: They may not only be different with respect to locations along the neuraxis, but possibly more heterogeneous than somatic motoneurons with respect to their spectrum of targets. It seems safe, therefore, to postulate that preganglionic sympathetic neurons may be at least as heterogeneous as somatic motoneurons with respect to their trophic factor requirements.

II. Anatomy and Physiology of the Sympathetic Preganglionic Outflow from the Spinal Cord

The sympathetic part of the autonomic nervous system appears to be organized in a hierarchical manner and controls many aspects of the body's homeostasis (e.g., cardiac and respiratory rate, vasomotor tone, baroreceptor reflex). Supraspinal and spinal neurons project to preganglionic sympathetic neurons, preganglionic sympathetic neurons innervate postganglionic neurons residing in distinct sympathetic ganglia or in the adrenal medulla (chromaffin cells), and postganglionic

sympathetic neurons send their axons to a variety of peripheral targets (e.g., smooth muscles of thoracic, abdominal, and pelvic viscera; blood vessels; and glands). Early studies of this system led to the interpretation (Cannon and Rosenblueth, 1937) that the entire sympathetic outflow always acts as a functional unit. This view soon had to be corrected, however, because in follow-up studies evidence was provided that different portions of the sympathetic outflow are regulated independently of each other (Appenzeller, 1982; Delius *et al.,* 1972a, 1972b; Okada and Ninomiya, 1983; Stoddard-Apter *et al.,* 1983).

An independent regulation of distinct peripheral targets by the sympathetic nervous system has to fulfill at least two criteria. First, discrete populations of sympathetic preganglionic neurons must innervate discrete populations of postganglionic neurons (targets) and, second, discrete supraspinal neuron populations must innervate distinct populations of sympathetic preganglionic neurons. In studies from the past 30 years a wealth of data has been collected to support both criteria: (1) sympathetic preganglionic neurons are arranged in target-specific columns of the thoracic spinal cord and (2) sympathetic preganglionic neurons receive discrete afferent inputs from different brain regions (Anderson *et al.,* 1989; Appel and Elde, 1988; Bacon and Smith, 1988; Hosoya *et al.,* 1995; Jensen *et al.,* 1995; Markham and Vaughn, 1990; Mohamed *et al.,* 1988; Parker *et al.,* 1988, 1990, 1993; Parker, 1999; Petras and Cummings, 1972; Petras and Faden, 1978; Pyner and Coote, 1994a, 1994b; Schramm *et al.,* 1975; Strack *et al.,* 1988). Taken together, these data clearly support the existence of a functional topography of specific groups of sympathetic preganglionic neurons in the mammalian spinal cord (Cabot, 1996; Taylor *et al.,* 1999).

A. Development of Sympathetic Preganglionic Neurons

1. Migration of Neuronal Progenitors

Sympathetic preganglionic neurons (= autonomic motoneuron/SPN) and spinal somatic motoneurons are generated synchronously (Phelps *et al.,* 1993). In the rat embryo, spinal progenitor cells arise in the ventral ventricular zone and start at E11/E12 (E = day of embryonal development) to migrate radially into the ventral horn of the developing spinal cord, where both somatic motoneurons and SPNs form a single, primitive motor column (Fig.1; Barber *et al.,* 1991; Phelps *et al.,* 1991). In a second phase (E14), the SPNs separate from the somatic motoneurons and are displaced dorsally toward the intermediate spinal cord. When the preganglionic neurons reach the intermediolateral (IML) region (E15), they become multipolar, and many of them undergo a change in alignment, from a dorsoventral to a mediolateral orientation. In the third phase of autonomic motoneuron development (E16), some of these cells are displaced medially and occupy sites between the IML and central canal. The primary and tertiary movements of the SPNs are correlated with radial glial processes in the embryonic spinal cord, an arrangement

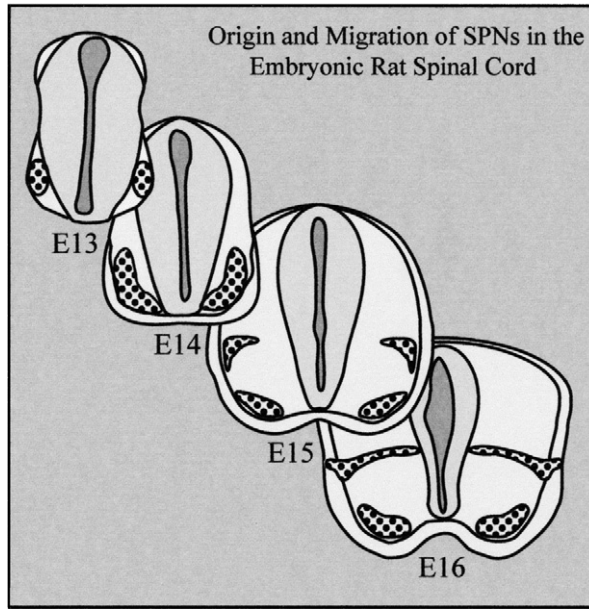

FIG. 1 Origin and migration of SPNs in the embryonic rat spinal cord. Both somatic and SPNs are generated at E11 to E12 and migrate from the ventricular zone into the developing ventral horn of the intermediate zone, where they form a single motor column. This column harbors both the future somatic motoneurons and SPNs. By E14 the future SPNs begin to separate and form a distinct population in the dorsolateral region of the primitive column. As future SPNs approach the intermediolateral area (E15), they become oriented into the mediolateral plane. By E16, most SPNs are oriented in the mediolateral plane. On E16, a majority of SPNs is located in an intermediolateral position. However, a few SPNs are also present in more medial locations (according to Markham and Vaughn, 1991).

that is consistent with the hypothesis that glial elements might guide autonomic motoneurons during these periods of development (Markham and Vaughn, 1991).

2. Ontogenetic Neuron Death

Cell death that occurs naturally is an important aspect of normal pre- and postnatal development. In the nervous system, neuronal death may serve to modulate the interconnections of discrete populations of developing neurons (Oppenheim, 1991). This phenomenon occurs during a specific development time window of a distinct neuron population, and it is regulated by its postsynaptic target and local factors. According to the neurotrophic hypothesis (Korsching, 1993; Oppenheim, 1991; Purves *et al.,* 1986), developing neurons require the contact and a certain amount of trophical support from their target tissue to survive into adulthood. Cell death is initiated if neurons do not receive the appropriate amount of neurotrophic factor produced by their postsynaptic target (Burek and Oppenheim, 1996). Programmed

cell death (apoptosis) has been demonstrated for motoneurons (Oppenheim, 1991), but also for numerous other neuronal cell populations, like peripheral sympathetic, parasympathetic, enteric, and sensory ganglion cells, retinal ganglion cells, and Purkinje cells (Clarke and Clarke, 1996; Oppenheim, 1991; Sanders and Wride, 1995).

It has also been shown, however, that few neuron populations are apparently not affected by ontogenetic cell death. For example, in chicks, cells in the pontine and the red nucleus and spinal interneurons do not show any evidence for programmed cell death (Armstrong and Clarke, 1979; Cowan *et al.,* 1984; McKay and Oppenheim, 1991; Oppenheim, 1981, 1991).

Contrary to the extensively studied cell death of somatic motoneurons in mammals, studies of SPN neurons in this respect are scarce and controversial (Lawson *et al.,* 1997; Lichtman and Purves, 1980; Parker *et al.,* 1988; Wetts and Vaughn, 1998). The elimination of a redundant preganglionic innervation to superior cervical ganglion (SCG) cells of hamsters was observed by Lichtman and Purves (1980). They reported that SCG neurons in the adult hamster are innervated by 6–7 axons, whereas these cells, in the first two weeks of their postnatal life, receive synaptic contacts by 11–12 preganglionic axons. In an ontogenetic study of the innervation of the rat adrenal medulla Parker and coworkers (1988) reported a postnatal, highly significant decrease of retrogradely (Fast-Blue) labeled SPNs that specifically innervate chromaffin cells of the adrenal medulla. A reduction from about 900 SPNs during the postnatal weeks to about 700 SPNs in adult animals was determined in this study (Parker *et al.,* 1988). Early studies on naturally occurring cell death were often based primarily on counts of Nissl stained or retrogradely labeled cells and on the microscopical identification of specific degeneration characteristics (condensation/fragmentation). During the last 10 years, new approaches for studying apoptosis have been developed. New techniques that have been introduced include TUNEL stainings, analysis of ultrastructural characteristics of apoptotic cells, and the correlated immunohistochemical identification of the specific type of dying cell (double labelings).

It has been suggested that cells in the dorsal and intermediate parts of the rat spinal gray matter display significant postnatal programmed cell death (Lawson *et al.,* 1997). Using pan-neuronal markers (NSE; PGP 9.5) the dying cells were identified as neurons. The authors concluded that most apoptotic cells were interneurons rather than autonomic motoneurons. Although spinal interneurons can be identified by their morphology and their characteristic transmitters (e.g., glycine/GABA), these criteria were not applied in the above study (Lawson *et al.,* 1997). We suggest that at least a fraction of the described apoptotic "interneurons" located in the intermediate spinal gray are SPNs.

The most recent study of developmental cell death in rat spinal cord analyzed in great detail different stages of development (pre- and postnatally) for somatic motoneurons and autonomic motoneurons/SPNs (Wetts and Vaughn, 1998). Surprisingly, the authors found the expected amount of apoptotic somatic motoneurons,

whereas SPNs did not display significant signs of ontogenetic neuron death during the time windows investigated (E12–P22). Furthermore, it could be shown that SPNs were able to survive in organotypic slice cultures (*in vitro*), without any target-derived trophic support, during embryonic and postnatal development (Wetts and Vaughn, 1998). These data are contrary to all other studies on this subject at present.

B. Morphology, Neurochemical Markers, and Labeling of Sympathetic Preganglionic Neurons

Two types of SPNs, one with a round cell body and one with a fusiform soma, are commonly described. In addition, a third, rather scarce, and larger SPN cell type has also been detected (Bacon and Smith, 1988; Gilbey *et al.,* 1982a; Pyner and Coote, 1994a). All of these cell types have been shown to be involved in the innervation of three major targets, the superior cervical ganglion, the stellate ganglion, and the adrenal medulla (Bacon and Smith, 1988). Interestingly, it has been reported for rats that SPNs with a round soma are the predominant type in the nucleus intercalatus (see below) and fusiform cell bodies are mainly related to the central autonomic area, whereas both cell types coexist intermingled in the IML region (Bacon and Smith, 1988).

Another interesting finding has been made by studying the dendritic arbors of SPNs. The pattern of dendritic arborization and orientation has been related to a functional organization of SPNs in columns that are target specified (Pyner and Coote, 1994a; Rubin and Purves, 1980). Adult mammalian SPNs possess 6–8 primary dendrites, which branch after passing medially, laterally, and rostrocaudally (Bacon and Smith, 1988). The longitudinally oriented dendrites from an extensive fiber network, running for considerable distances between clusters of SPNs. These extensive bundles extend in both mediolateral and rostrocaudal directions, interconnecting the various autonomic nuclei of the spinal gray and forming a distinct "ladder-like" structure when viewed in longitudinal sections (Barber *et al.,* 1984; Markham and Vaughn, 1990). It has been speculated that these dendritic orientation patterns are specifically designed for receiving similar input categories (Taylor *et al.,* 1999).

Ultrastructural characteristics of rat SPNs were investigated in detail by Markham and Vaughn (1990). According to earlier studies in cat and monkey (Chung *et al.,* 1980; Wong and Tan, 1980), some round cells were present in the IML, but the majority of SPNs in this region were elongated, bipolar neurons that reveal often irregular nuclear profiles and contained an abundance of Nissl substance in their cytoplasm (Markham and Vaughn, 1990). The highest concentration of SPN cell bodies was found at the border between IML area and lateral funiculus (LF). The cell bodies were covered over a large proportion of their surface by a thin astrocytic sheet. The vast majority of synapses of SPNs were from the

axodendritic type, whereas few axosomatic contacts were formed. Axon terminals contained high numbers of round clear vesicles and also several dense core vesicles (Markham and Vaughn, 1990). In the rat, in opposition to other mammals, such as human and cats, the great majority of preganglionic axons are unmyelinated. For example, fewer than 1% of the axons in the rat cervical sympathetic trunk are myelinated (Brooks-Fournier and Cogeshall, 1981; Dyck and Hopkins, 1972; Hedger and Webber, 1976).

Preganglionic neurons of the spinal cord use the classical neurotransmitter acetylcholine (ACh). The only unambiguous identification of cholinergic neurons is based on the immunohistochemical detection of the ACh-synthesizing enzyme, choline acetyltransferase (ChAT; Barber *et al.,* 1984; Phelps *et al.,* 1991). In addition to ChAT immunoreactivity, SPNs also display an intense acetylcholinesterase (AChE) activity (see Figs. 2A–H), the ACh metabolizing enzyme, which can also be detected in sections using a simple enzymehistochemical staining procedure (Karnovsky and Roots, 1964; Navaratnam and Lewis, 1970; Andrä and Lojda, 1986; Paxinos and Watson, 1986; Schober *et al.,* 1997). AChE staining, in contrast to ChAT, is not an unequivocal marker of cholinergic neurons.

Nitric oxide (NO) has been found to be colocalized with ACh in the majority of SPNs located in all nuclei and spinal segments (Anderson, 1992; Blottner and Baumgarten, 1992; Valtschanoff *et al.,* 1992; Saito *et al.,* 1994; Wetts and Vaughn, 1994). NO, a messenger molecule, which acts as a regulator and modulator in a variety of neuronal processes (Blottner *et al.,* 1995; Blottner, 1997, 1999), can be detected in sections (1) by NADPH-diaphorase histochemical staining (Scherer-Singler *et al.,* 1983) and (2) by nitric oxide synthase (NOS) immunohistochemistry.

Subpopulations of spinal SPNs were described, which express moderate amounts of neuropeptides, e.g., substance P (SP), calretinin, neurotensin, enkephalin, somatostatin, and calcitonin gene-related protein (CGRP; Colombo-Benkmann *et al.,* 1995; Gibbins, 1992; Grkovic and Anderson, 1995, 1996; Krukoff *et al.,* 1985a, 1985b).

Cell numbers, distribution pattern, and morphology of SPNs in the spinal cord can be studied by application of retrogradely transported tracer substances [horseradish peroxidase (HRP), DiI, Fast-Blue (FB), Fluorogold (FG), cholera toxin subunit b (CTb), pseudorabies virus (PRV)]. A small amount of an aqueous tracer solution is applied to a distinct target (e.g., sympathetic ganglion or adrenal medulla) of SPNs. In addition, a specific *in vivo* gene transfer using the nonreplicating thymidine kinase-deficient herpes simplex virus type I has been applied for studying SPNs (LeVatte *et al.,* 1997). Another elegant procedure for labeling all autonomic preganglionic neurons by one single tracer application has been described (Anderson and Edwards, 1994; Leong and Ling, 1990; Merchenthaler, 1991). In this approach, the retrograde tracer solution (FG) is injected intraperitoneally (ip). Subsequently, FG distributes throughout the whole body where it can be taken up by all axon terminals outside the blood–brain barrier. Thus, FG labels the entire population of SPNs (see Figs. 3A–C) but also

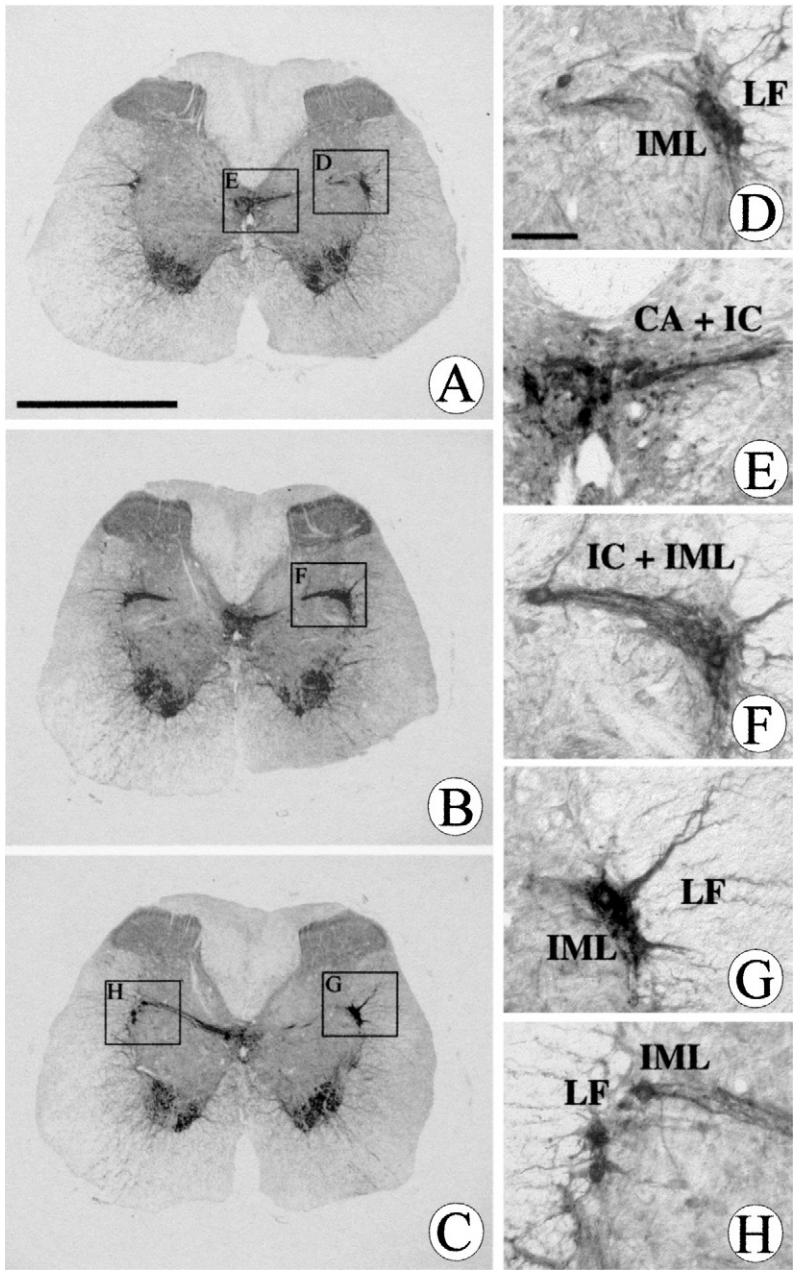

FIG. 2 Acetylcholinesterase (AChE) staining of transverse adjacent sections (A–C) through the mouse spinal cord at the level of Th8 revels the locations of the four spinal autonomic nuclei. Framed areas within (A–C) are enlarged in (D–H). IML, intermediolateral nucleus; LF, lateral funiculus; IC, nucleus intercalatus; CA, central autonomic area. Bar (A–C) = 1 mm. Bar (D–H) = 50 μm.

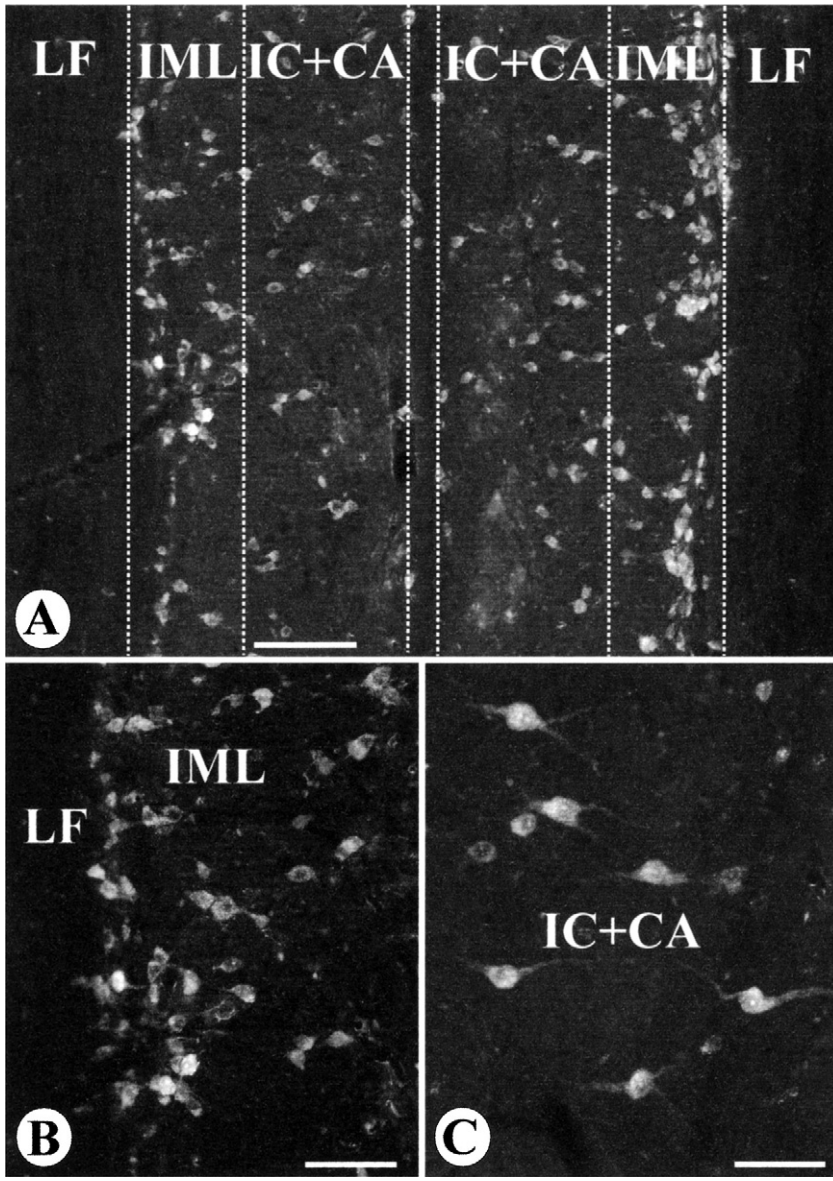

FIG. 3 Longitudinal section of adult mouse spinal cord at the level Th8–Th9. SPNs were identified by intraperitoneal Fluorogold injection (A–C). Enlarged images of SPNs are shown for the LF and IML regions in (B) and and for the IC and CA regions in (C). IML, intermediolateral nucleus; LF, lateral funiculus; IC, nucleus intercalatus; CA, central autonomic area. Bar (A) = 100 μm. Bar (B,C) = 50 μm.

parasympathetic preganglionic neuron and somatic motoneurons (Anderson and Edwards, 1994).

C. Localization and Distribution of Sympathetic Preganglionic Neurons

SPNs are located in the mammalian spinal cord between cervical segment C8 and lumbar segment L2. The detailed pattern of their distribution and morphological characteristics has been intensely studied by retrograde labeling using HRP and other retrogradely transported tracers, e.g., CTb, PRV, FB, or FG (Anderson and Edwards, 1994; Appel and Elde, 1988; Gilbey *et al.,* 1982b; Hosoya *et al.,* 1992, 1994; Jansen *et al.,* 1993; Parker *et al.,* 1993; Pyner and Coote, 1994a, 1994b; Schober *et al.,* 1998a, 1999a; Schramm *et al.,* 1975; Strack *et al.,* 1988). The majority of the preganglionic neurons are located in a column termed the IML column of the spinal cord (Figs. 2A–H and 3A–C). SPNs are organized in clusters of four topographically defined nuclei within the intermediate gray (lamina 7; Molander and Grant, 1995) on either side of the spinal cord. These four nuclei are termed (1) the nucleus intermediolateralis pars principalis (Ilp or IML or IML column); (2) the nucleus intermediolateralis pars funicularis (Ilf or LF); (3) the nucleus intercalatus (IC); and (4) the nucleus intercalatus pars paraependymalis (Icpe), which is also called the central autonomic area (CA) (compare Figs. 2A–H and 3A–C). This organization pattern seems to be the same in all mammals investigated (Coote, 1988).

D. Axonal Connections and Targets of Sympathetic Preganglionic Neurons

In general, SPN axons emerge from the spinal cord within the ventral roots together with somatic motor fibers. From the ventral nerves the fibers pass to the sympathetic chain via the rami communicantes. Depending on the level of origin, preganglionic fibers travel some distances up and down within the sympathetic chain and form synaptic contacts with ganglion cells, frequently within more than one ganglion. After traveling in the paravertebral chain SPN fibers may pass into the splanchnic nerve and run to prevertebral ganglia in the abdominal cavity, to the adrenal gland or extradrenal chromaffin tissues (Gabella, 1995). Major targets of the SPN axons are para- and prevertebral sympathetic ganglia (superior cervical ganglion, middle cervical ganglion, stellate ganglion, aorticorenal ganglion, sympathetic chain, celiac ganglion, superior and inferior mesenteric ganglion) and chromaffin cells inside and outside of the adrenal medulla (see Table I). The majority of SPNs that innervate the SCG (75%) are localized

TABLE I
Segmental Distribution of SPNs Determined by Retrograde Tracing from the Target

Sympathetic ganglion	Segmental distibution	Major cell density/peak
Superior cervical ganglion (SCG)	C8–Th5	Th1–Th3
Middle cervical ganglion (MCG)	C8–Th7	Th2–Th4
Stellate ganglion (SG)	C8–Th8	Th2–Th5
Adrenal medulla (AM)	Th4–Th12	Th7–Th9
Celiac ganglion (CG)	Th4–Th13	Th10–Th12
Aorticorenal ganglion (ARG)	Th7–Th13	Th12
Superior mesenteric ganglion (SMG)	Th7–Th13	Th12
Inferior mesenteric ganglion (IMS)	Th12–L2	L1–L2

Source: Data drawn from Strack *et al.,* 1988.

in the IML, 23% are in the LF, and the remaining 2% join the central and intercalate area (Rando *et al.,* 1981). Other sympathetic ganglia reveal a similar pattern with respect to the localization of innervating neurons within the four autonomic nuclei (IML, LF, IC, CA) as described for the SCG (Strack *et al.,* 1988). Sympathetic neurons that are preganglionic to the adrenal medulla are distributed as follows: IML, 94%; LF, 4%; IC, 1%; CA, 1% (Kesse *et al.,* 1988; Strack *et al.,* 1988).

Evidence has been provided that SPNs are arranged and organized in a target-specific- and spinal segment-correlated manner (Pyner and Coote, 1994a, 1994b; summarized in Table I). In rats, preganglionic neurons innervating the SCG are located in spinal segments C8 to Th5. The majority of these cells, about 90% are found in the segments Th1–Th3. Neurons innervating the adrenal medulla could be localized in the spinal segments Th4–Th12, but the major source of the sympatho-adrenal cell population is clearly restricted to spinal segments Th7–Th9 (Parker *et al.,* 1993; Strack *et al.,* 1988). Preganglionic neurons located in more caudal segments of the thoracic spinal cord (Th4–Th13) also send their axons to the celiac ganglion (CG); the predominant source of the preganglionic input to the celiac ganglion is spinal segments Th10–Th12 (Strack *et al.,* 1988).

An additional column of preganglionic neurons has been identified in lumbar segments L1–L2 (Hancock and Peveto, 1979). This subpopulation projects to the hypogastric nerve and was called the dorsal commissural nucleus (Baron *et al.,* 1985; Hancock and Peveto, 1979a, 1979b; Hosoya *et al.,* 1994).

The numeric ratio between preganglionic and postganglionic neurons differs with respect to different ganglia and different investigators (Gabella, 1995; Wang *et al.,* 1995). For the SCG of the adult rat, Purves and associates (1986) counted about 1000 SPNs, which send their axons to nearly 26,000 SCG neurons. Accordingly, each SPN has to innervate, on average, 240 ganglion neurons, and each ganglionic neuron receives an input from 9 SPNs (Purves *et al.,* 1986).

According to Tomlinson and associates (1987), the adrenal medulla of adult rats (250 g) contains about 650,000 chromaffin cells, and 700 SPN were found to innervate the medulla (Parker *et al.,* 1988). Tomlinson and Coupland (1990) calculated, on the basis of an average of five synaptic contacts per chromaffin cell, that in the rat one single SPN innervates more than 1000 chromaffin cells (Parker, 1999).

E. Afferent Connections to Sympathetic Preganglionic Neurons

The ventrolateral region of the medulla oblongata, the caudal portion of the pons, and hypothalamic nuclei of the diencephalon contain several groups of neurons which project directly to SPN nuclei (Fig. 4). Spinally projecting neurons of the caudal raphe region (raphe obscorus, raphe pallidus, raphe magnus) also send their axons to SPN nuclei. A whole set of supraspinal cell groups that innervate SPNs has been identified by retrograde tracing studies using HRP, CTb, herpes simplex virus type 1 (HSV-1), or pseudorabies virus (Bacon and Smith, 1988; Fuxe *et al.,* 1990a, 1990b; Hosoya *et al.,* 1992; Laskey and Polosa, 1988; Strack *et al.,* 1989a, 1989b). A powerful tool for studying afferent inputs to SPNs and simultaneous targets of preganglionic axons is transneuronal cell body labeling with the pseudorabies virus (PRV, Jansen *et al.,* 1993). The PRV injection into a certain SPN target causes an immunohistochemically detectable retrograde viral infection of a distinct group of SPNs. Furthermore, the virus infection produces a specific labeling of second-order neurons located in the spinal cord and brain that innervate infected SPNs (Strack *et al.,* 1989a, 1989b). Thus, five major cell groups in the CNS were identified to regulate the entire sympathetic outflow (see Table II).

TABLE II
Descending Inputs to SPNs

CNS region	Putative neurotransmitters/marker enzymes
Paraventricular hypothalamic nucleus	SP, TH, met-ENK, OXY, NT, SOM, VP
A_5 cell group	TH, SOM
Rostral ventrolateral medulla oblongata	NPY, PNMT, SP, met-ENK
Ventromedial medulla oblongata	SP, 5-HT, met-ENK, NPY, PNMT
Caudal raphe region	5-HT, SP, met-ENK, SOM
Interneurons of the spinal gray	GABA, GLY

Key: GABA, γ-aminobutyric acid; GLY, glycine; 5-HT, 5-hydroxytryptamine/serotonin; met-ENK, met-enkephalin; NPY, neuropeptide Y; NT, neurotensin; OXY, oxytocin; PNMT, phenylethanolamine *N*-methyltransferase; SOM, somatostatin; SP, substance P; TH, tyrosine hydroxylase; VP, vasopressin.

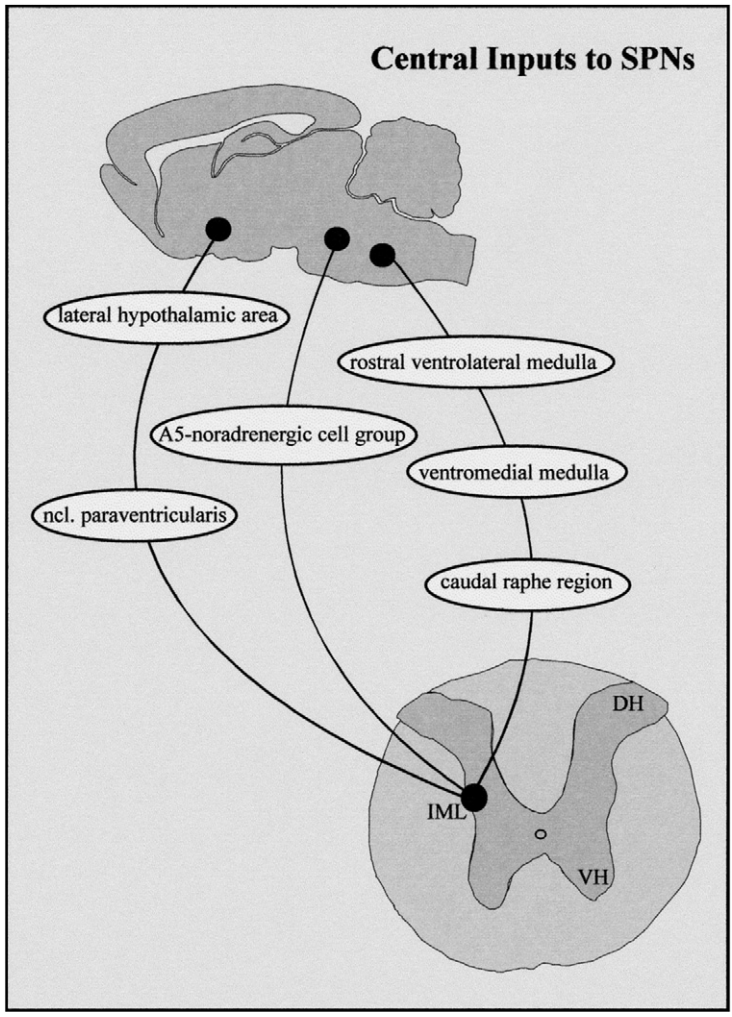

FIG. 4 Schematic drawing illustrating the major supraspinal areas of the rat brain that project to spinal sympathetic preganglionic outflow.

1. Paraventricular Hypothalamic Nucleus (PVH)

Direct projections have been first demonstrated by HRP and True Blue tracing studies (Nicholas and Hancock, 1989; Hosoya *et al.,* 1991; Saper *et al.,* 1976; Sawchenko and Swanson, 1982). Labeled cells are found in the paraventricular nucleus, the dorsal part of the lateral thalamic area, the lateral part of the dorsomedial hypothalamic nucleus, and the posterior hypothalamic area. Double-labeling

studies have provided evidence that catecholamine-, vasopressin-, oxytocin-, somatostatin-, and met-enkephalin-immunoreactive neurons project to the SPN nuclei (Björklund and Skagerberg, 1979; Hosoya *et al.,* 1995; Krukoff *et al.,* 1985a, 1985b; Romagnano and Hamil, 1984; Rousselot *et al.,* 1990; Sawchenko and Swanson, 1982). Dopaminergic diencephalic neurons are described as the A_{11} catecholamine cell group according to Dahlström and Fuxe (1964) and have been shown to provide synaptic input to the dorsal horn and SPN nuclei of the spinal cord (Skagerberg *et al.,* 1982).

2. A_5 Noradrenergic Cell Group

The ventrolateral pontine reticular formation (= A_5 noradrenergic cell group) consists of a loosely clustered column of neurons adjacent to the superior olivary nucleus and the facial nucleus. The majority of these noradrenergic cells send their axons to SPN nuclei, and it has been shown that A_5 neurons are the source of most of the noradrenergic fibers in the SPN nuclei (Loewy *et al.,* 1979). Noradrenergic neurons within this group also project to medullary centers such as the rostral ventrolateral medulla (Sun and Guyenet, 1986), another major region from which SPNs receive axon terminals.

3. Caudal Raphe Region

Anatomical studies show that a large proportion of neurons located in the subnuclei raphe obscorus, raphe pallidus, and raphe magnus can be retrogradely labeled by injection of HRP into the spinal cord (Loewy, 1981). It has been demonstrated that raphe spinal neurons project to the dorsal and ventral horn of the thoracic spinal cord as well as to the SPN nuclei (Loewy and McKellar, 1981; Millhorn *et al.,* 1989; Li *et al.,* 1992; Ding *et al.,* 1993). The major source of serotonergic input to SPNs originates from cells located in the caudal raphe region (Bacon and Smith, 1988; Chiba and Masuko, 1986; Jensen *et al.,* 1995; Pilowsky *et al.,* 1995). Serotonin (5-HT) is known to be the most important neurotransmitter in the caudal raphe region (Laskey and Polosa, 1988). Furthermore, this region also gives rise to a few substance P (SP)/serotonin-immunoreactive fibers, which project to the SPNs located in the IML area (Appel *et al.,* 1986; Chiba and Masuko, 1989; Hökfelt *et al.,* 1978). Ultrastructural, physiological, and pharmacological studies largely support a sympathoexcitatory role for bulbospinal serotonergic pathways (Bacon and Smith, 1988; Gilbey and Stein, 1991; Howe *et al.,* 1983a, 1983b; Minson *et al.,* 1984; Takano *et al.,* 1985; Yusof and Coote, 1988).

4. Rostral Ventrolateral Medulla and Ventromedial Medulla

Neurons of both the rostral ventrolateral medulla (= rostroventrolateral reticular nucleus) and from the more ventromedial region (= lateral paragigantocellular

nucleus and interfascicular hypoglossal nucleus) have been shown to project to SPN nuclei (Caverson *et al.,* 1983a, 1983b; Loewy and McKellar, 1981; Jensen *et al.,* 1995; Zagon and Smith, 1993). The bulk of axon terminals containing SP, which project to SPN nuclei, originates from the ventromedial area of the medulla oblongata (Bacon and Smith, 1988; Grkovic and Anderson, 1996; Helke *et al.,* 1982; Matthews and Cuello, 1982; Oldfield *et al.,* 1985; Pilowsky *et al.,* 1995). In addition to the SP containing fibers, axons immunoreactive for the thyrotropin-releasing hormone (TRH) also originate from this area and form synaptic contacts on SPNs (Appel *et al.,* 1987; Arvidsson *et al.,* 1992; Helke *et al.,* 1986). Both regions of the rostral medulla oblongata play an important role in control of arterial blood pressure and other visceral functions. There is clear evidence that SP-containing fibers participate in these complex regulation processes via an excitatory synaptic input to discrete SPN populations (Loewy, 1981; Loewy and McKallar, 1981; Takano *et al.,* 1985; Yashpal *et al.,* 1985).

Finally, local interneurons, mainly located in lamina V, VII, and X of the spinal cord, innervate identified SPNs in all subnuclei; consequently these interneurons are also involved in the regulation of the sympathetic outflow (Cabot *et al.,* 1994; Clarke *et al.,* 1998; Strack *et al.,* 1989a, 1989b).

III. Growth and Neurotrophic Factors for Sympathetic Preganglionic Neurons

A. Brief Outline of the Neurotrophic Factor Concept

As briefly mentioned above, the classic neurotrophic factor concept (Korsching, 1993) implies that neurons require molecules to ensure their survival and prevent apoptosis during development. At later ages, neurotrophic molecules may stimulate neuronal differentiation, e.g., transmitter synthesis, and may prevent death following axotomy or chemical lesions (Bothwell, 1995; Conover and Yancopoulos, 1997; Klein, 1994; Snider, 1994). The neurotrophic factor concept was largely built on the first discovered neurotrophic molecule, nerve growth factor (NGF). Synthesis in target regions, availability in limited amounts, uptake at nerve terminals via specific membrane receptors, and retrograde axonal transport were key features in the actions of NGF and instrumental in shaping an initial concept to understand how a neurotrophic factor operates. Immunoneutralization experiments in neonatal rodents (Thoenen, 1972; Thoenen and Barde, 1980) and the analysis of mice carrying targeted mutations of NGF and its receptor trkA (Snider, 1994) have fully supported the concept with regard to NGF. However, with the advent of novel neurotrophic factors, both related to NGF or belonging to other gene families, it became clear that the NGF paradigm could not satisfactorily explain the broad range of diverse mechanisms by which these molecules act.

Neurotrophic molecules are found within the families of neurotrophin (NT; Snider, 1994), fibroblast growth factor (FGF; Bieger and Unsicker, 1996), insulin-like growth factor (IGF; Baskin *et al.,* 1988; Ishii *et al.,* 1994), neurokines (CNTF and related factors; Sendtner *et al.,* 1994), transforming growth factor-ß (TGF-ß; Böttner *et al.,* 2000; Krieglstein *et al.,* 1995), and other families, such as interleukins, EGF receptor ligands, or platelet-derived growth factor. These factors employ a large variety of different signaling pathways, e.g., the receptor tyrosine kinase pathways seen in neurotrophin and FGF receptor signaling (Ullrich and Schlessinger, 1990), the serine/threonine kinase-associated SMAD pathways (Böttner *et al.,* 2000); or the JAK/STAT signaling pathways employed by the CNTF family (Watanabe and Arai, 1996). Several neurotrophic factors were initially discovered based on their capacities to act as mitogens or antimitogens, e.g., FGFs 1 and 2 (Bieger and Unsicker, 1996) or the TGF-ßs (Böttner *et al.,* 2000). Neurotrophic factors can be stored in target areas, as shown for several members of the neurotrophin family (Snider, 1994), but they may also be synthesized by glial cells, as shown for CNTF (Rudge *et al.,* 1994). Others are apparently produced by multiple cell types, and in the case of members of the neurotrophin and FGF families, even by the neuron population that requires this particular factor (Acheson *et al.,* 1995; Acheson and Lindsay, 1996). Some neurotrophic factors act during ontogeny and/or in adulthood. Finally, neurotrophic factors may act on their own, or be partially or fully depedent on a synergistic input of another molecule (Unsicker and Krieglstein, 2000). Paradoxically, some neurotrophic factors, such as NGF, may signal neuron survival and death, depending on the development context (Raoul *et al.,* 2000).

In summary, the current general definition of a neurotrophic factor is much broader than the definition based on the original NGF concept and is largely operational—a protein that prevents ontogenetic and/or lesion-induced neuron death, irrespective of its location and mechanisms of action.

B. Growth Factor Receptors on Sympathetic Preganglionic Neurons

The presence and localization of growth factor receptors on SPNs have not been explored yet in sufficient detail to allow extrapolations as to the full range of growth factors addressing this neuron population. In particular, only a few reports have combined receptor mapping using *in situ* hybridization or immunocytochemistry with retrograde tracing to correlate target specificity and growth factor receptor expression in SPNs.

1. Neurotrophin Receptors

Neurotrophin receptors have been mapped in several studies (Schober *et al.,* 1998a, 1999b, 2000b), and there is conclusive evidence for the expression of TrkB, the

cognate receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4), in SPNs of the adult rat spinal cord (Schober *et al.,* 1998a, 1999b). Retrograde tracing of SPNs by injecting Fluorogold (FG) into the adrenal medulla combined with *in situ* hybridization for TrkB revealed TrkB expression by most of the FG-labeled neurons. As expected, there were also a large number of TrkB-positive, FG-negative SPNs that presumably project to targets other than the adrenal medulla. This is consistent with data from another study (Schober *et al.,* 1999b) that showed TrkB expressing neurons in the IML over a wide range of spinal cord segments of the adult rat.

The same study also revealed expression of TrkC in neurons located in the IML column. However, expression of TrkC was weaker and numbers of positive neurons were smaller than for the TrkB-positive population. Whether TrkB and TrkC are expressed by distinct or partly overlapping SPN populations has not been clarified. Both TrkB and TrkC are more prominently expressed during prenatal development of the rat IML (Schober *et al.,* 1999b) starting at the time when IML precursors separate from the ventral motoneuron pool. Expression of TrkA has not been found in the IML; even so, both numbers and the chemistry of SPNs are indirectly and severely affected in TrkA-deficient mice (Section IV.B).

2. Fibroblast Growth Factor (FGF) Receptors

FGFs are coded by four different genes (Bieger and Unsicker, 1996) and exist in a variety of alternatively spliced forms. FGFRI is expressed by many neuron populations in the CNS including SPNs (Blottner *et al.,* 1997; Stapf *et al.,* 1997) consistent with responsiveness of SPNs to FGF-2 (Blottner *et al.,* 1989a; Blottner and Unsicker, 1990). FGFRI immunoreactivity appears in virtually all neurons of the lateral column, suggesting that SPNs may express FGFRI independent of their target projections.

3. Ciliary Neurotrophic Factor (CNTF)

CNTF and related molecules including leukemia inhibitory factor (LIF), cardiotrophin I, Oncostatin M, and interleukin-6 (IL-6) signal through a heterodimeric or heterotrimeric receptor complex (Watanabe and Arai, 1996). CNTF employs a lipid-anchored α receptor in conjunction with two transmembrane receptors, LIFRß and gp 130. Both the α-receptor component and LIFRß have been localized to SPNs (Forger *et al.,* 1998; Schober, unpublished, 1999), again consistent with a role for CNTF in maintaining target-deprived SPNs (see below).

4. Receptors for Transforming Growth Factor-ßs (TßR)

TGF-ßs constitute a superfamily of multifunctional cytokines with wide distribution. Members include those of the TGF-ßs proper, the bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), the glial cell line-derived

neurotrophic factor (GDNF) family, and several other proteins with common structural motifs (Böttner *et al.,* 2000; Krieglstein *et al.,* 1995; Unsicker *et al.,* 1998). TGF-ß2 and -ß3 are widely expressed in neurons and glial cells of the normal nervous system (Unsicker *et al.,* 1991). They signal through a heteromeric receptor complex of TßRI and TßRII serine/threonine kinases (ten Dijke *et al.,* 1996). TßRII has been localized to SPNs (Schober *et al.,* 1999a). Members of the GDNF family employ a different receptor complex, which consists of the tyrosine kinase c-Ret and an α-receptor component, GFRα1–GFRα4 (Airaksinen *et al.,* 1999). *In situ* hybridization has provided evidence for both c-Ret and GFRα1 expression by SPNs (Schober *et al.,* 1999a). Receptors for other members of the TGF-ß superfamily have not been localized to the IML column so far.

C. Growth Factors in Target Regions of Sympathetic Preganglionic Neurons

1. Neurotrophins

Members of the NGF family, the neurotrophins, have long been considered to be prototypic target-derived molecules, but are now widely recognized to be also synthesized by nontarget cells, including glia and afferent neurons. RT–PCR, *in situ* hybridization, and immunocytochemistry are the most widely used tools to document local synthesis and expression of a protein of interest. However, it should be noted that evidence for mRNA and immunoreactivity does not prove the presence of a biologically active and functionally relevant molecule. In particular, data provided by using highly sensitive methods, e.g., RT–PCR, should only be interpreted in conjunction with protein and functional data.

Messenger RNA and protein expression of most neurotrophins is scarce in target regions of preganglionic sympathetic neurons, i.e., in sympathetic ganglia and adrenal medulla (Schober *et al.,* 2000b). NGF, the prototype of a neuronal survival and differentiation factor (Levi-Montalcini, 1987), or its mRNA, respectively, can be detected by RT–PCR in rat adrenal medulla (Lachmund *et al.,* 1994), but its presence has not been revealed in sympathetic ganglia or adrenal medulla by *in situ* hybridization or immunocytochemistry. Using Northern blots, Shelton and Reichardt (1984) have reported the presence of NGF mRNA in rat and rabbit adrenal glands. There is also an early report by Harper and associates (1976) on a NGF-like biological activity in explants of mouse adrenals that could be blocked by neutralizing antibodies to NGF. Adrenal chromaffin cells, like sympathetic neurons, express TrkA, the receptor for NGF (Michael and Priestley, 1996; Schober *et al.,* 1997, 1998b). However,while sympathetic neurons can recruit NGF from their targets, NGF sources for chromaffin cells that do not project outside the adrenal gland are enigmatic. Roles for TrkA on chromaffin cells have been revealed by analyzing TrkA-deficient mice (Schober *et al.,* 1997). TrkA mutants

lose the activity of the acetylcholine-hydrolyzing enzyme AChE in chromaffin cells and preganglionic neurons after birth. The effect on AChE seen with preganglionic neurons projecting to the adrenal medulla must be indirect, since these neurons do not express TrkA. Retrograde factors regulating preganglionic AChE activity through a TrkA-mediated mechanism have not been identified. Likewise, the reduction of SPN numbers in TrkA mutants caused by the dramatic loss of paravertebral sympathetic neurons (Smeyne *et al.,* 1994) must also be indirect.

BDNF mRNA and protein occur in low amounts in sympathetic ganglia (Causing *et al.,* 1997; Wetmore and Olson, 1994), and RT–PCR has revealed BDNF mRNA in whole postnatal rat adrenal gland (Lachmund *et al.,* 1994). With regard to the synthesizing cell type(s), *in situ* hybridization has shown BDNF mRNA in embryonic rat (E16) adrenal glands in what has been tentatively identified as cortical cells (Schober *et al.,* 1998a). Moreover, low levels of BDNF mRNA can be detected in the reticularis zone of the adult rat adrenal cortex, but not in adrenal chromaffin cells (Schober *et al.,* 1998a). Lack of BDNF synthesis in postnatal chromaffin cells suggests that BDNF is probably not the physiological ligand for TrkB-positive sympathetic preganglionic neurons projecting to the adrenal medulla.

Neurotrophin-3 (NT-3) is synthesized in sympathetic ganglia during development (Verdi *et al.,* 1996), but hardly detectable by RT–PCR in adult sympathetic ganglia. In adult rat adrenal gland, cells in the innermost cortical layer are weakly labeled by *in situ* hybridization, while adrenal chromaffin cells appear negative. Synthesis of NT-3 by nonneural cells, as in the adrenal cortex, are not surprising given its presence in many nonneural tissues (Ernfors *et al.,* 1992, 1994; Pirvola *et al.,* 1992; Scarisbrick *et al.,* 1993). Although few SPNs express TrkC, the cognate receptor for NT-3, the protein is apparently not retrogradely transported from the adrenal gland to the spinal cord (Rush, personal communication, 1998). Together, these data suggest that a retrograde trophic role of NT-3, at least for adult SPNs, is unlikely.

NT-4 is still one of the more enigmatic neurotrophins (Ibanez, 1996). The significance of its wide distribution in neural and nonneural tissues and redundancy with BDNF in many *in vitro* assays is still poorly understood. On the other hand, both the NT-4 knockout (Conover *et al.,* 1995; Liu *et al.,* 1995; Lewin and Barde, 1996) and targeted mutations of tyrosine residues on the TrkB tyrosine kinase domain affecting NT-4, but not BDNF signaling (Minichiello *et al.,* 1998), have revealed specific deficits distinct from those seen in BDNF and TrkB knockouts. NT-4 mRNA is expressed in pre- and postnatal rat adrenal glands (Timmusk *et al.,* 1993) as well as in sympathetic ganglia (Roosen *et al.,* 2001). The protein has been localized to chromaffin cells and a subpopulation of adrenal medullary macrophages (Schober *et al.,* 1998b). Although retrograde axonal transport from the adrenal gland to the IML does not seem to occur (Schober *et al.,* 1998a), its presence in chromaffin cells, biological effects on SPNs (see below), and a clear phenotype seen with IML neurons in NT-4 knockouts (Roosen *et al.,* 2001) underscore the significance of NT-4 as a ligand for TrkB expressing SPNs (for a summary, see Fig. 5).

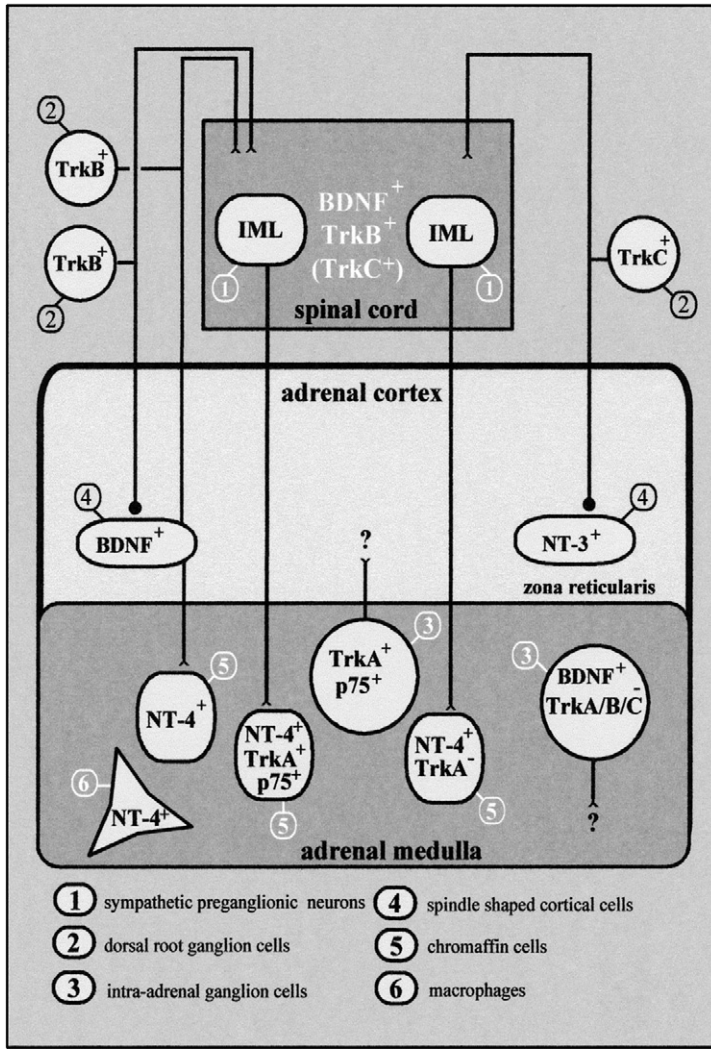

FIG. 5 Summarizing sketch showing cells in the adult rat adrenal cortex and medulla, as well as in the IML region of the spinal cord, which synthesize neurotrophins and their cognate receptors. Within the adrenal cortex BDNF and NT-3 synthesis occurs in cells of the zona reticularis, an area supplied by sensory nerves. We assume that these cells may supply BDNF and NT-3 to TrkB- and TrkC-positive subpopulations of DRG neurons. Within the adrenal medulla BDNF is synthesized by a subpopulation of ganglion cells. Medullary chromaffin cells store NT-4 and can be subdivided into a larger supopulation of TrkA/p75 expressing and a smaller TrkA-negative subpopulation. NT-4 is also expressed by a subpopulation of adrenal medullary macrophages. SPNs in the spinal cord, which innervate adrenal medullary chromaffin cells, express TrkB and low levels of BDNF and TrkC mRNAs. (Reproduced from Schober *et al.* (1999b), Fig. 6, copyright notice of Springer-Verlag.)

2. Fibroblast Growth Factors

The family of FGFs has significantly expanded in the past few years and comprises approximately 20 members as of 2000 (Bieger and Unsicker, 1996). However, only the role of FGF-2/basic FGF has been explored in some detail with regard to SPNs. Adrenal medullae and chromaffin cells of bovine and rat express FGF-2 mRNA and show FGF-2-like immunoreactivity. In the rat adrenal medulla, FGF-2 seems to be localized in the noradrenergic subpopulation of chromaffin cells (Bieger *et al.,* 1995; Blottner *et al.,* 1989a; Blottner and Unsicker, 1990; Grothe and Unsicker, 1989).

Immunoelectron microscopy suggests that, similar to other peripheral organs, a substantial portion of FGF-2 associated with chromaffin cells is located extracellularly (Bieger *et al.,* 1995). Perfusion of bovine adrenal glands with collagenase-containing buffers, which leaves cells intact, liberates large amounts of FGF-2 into the perfusate, consistent with its extracellular location. Intracellularly, FGF-2 is localized in the cytosol, in endosome-like structures, and nuclei of chromaffin cells (Stachowiak *et al.,* 1994; Bieger *et al.,* 1995), which is consistent with the occurrence of the 18-kDa cytosolic and higher molecular weight nuclear forms of FGF-2 in chromaffin cells (Grothe and Meisinger, 1997; Meisinger *et al.,* 1996), as in many other types of cells.

How FGF-2 is secreted from chromaffin cells and neurons is not known (Bieger and Unsicker, 1996). Although earlier reports had suggested that FGF-2 may be localized in secretory granules of chromaffin cells (Presta and Rifkin, 1991; Westermann *et al.,* 1990), follow-up studies were not able to corroborate these findings (Bieger *et al.,* 1995). FGF-2 does not seem to become expressed in rat adrenal chromaffin cells until the first postnatal week (Grothe and Unsicker, 1990). The end of the first and beginning of the second postnatal week also mark the beginning of a functional innervation of chromaffin cells that links preganglionic impulse flow to exocytosis of chromaffin cell products. It may therefore be conceivable that the preganglionic input triggers expression of FGF-2, which may have a role in synapse stabilization or regulation of differentiated properties of chromaffin cells. Denervation of adult rat and bovine chromaffin cells by isolation in culture causes a rapid decline in FGF-2 expression (Bieger *et al.,* 1995) consistent with its positive regulation by presynaptic input.

Despite their close ontogenetic relationship to chromaffin cells (Unsicker, 1993; Unsicker and Krieglstein, 1996) sympathetic neurons express only very low levels of FGF-2 mRNA and seem to lack FGF-2 immunoreactivity (Weise *et al.,* 1992). It has been speculated (Unsicker and Krieglstein, 1996) that the difference in FGF-2 expression by chromaffin cells and sympathetic neurons may be related to the persistent capacity of chromaffin cells to divide (Tischler *et al.,* 1989; Wolf *et al.,* 1999). Apparently, FGF-2 may have a role as a mitogen for chromaffin cells (Frödin and Gammeltoft, 1994; Wolf *et al.,* 1999). Other putative intra-adrenal functions of FGF-2 may be related to the maintenance of differentiation, as shown by its ability

to induce tyrosine hydroxylase activity, the rate-limiting enzyme in catecholamine synthesis (Unsicker and Westermann, 1992). FGF-2 does not seem to induce the adrenaline synthesizing enzyme PNMT, which is not entirely unexpected because of its restriction to noradrenergic cells.

3. CNTF and CNTF-like Neurokines

CNTF and LIF are expressed by astroglial cells in the CNS and by (myelinating) Schwann cells in the peripheral nervous system (Sendtner *et al.,* 1994; Ip, 1998). CNTF, LIF, and cardiotrophin-1 promote survival of somatic motoneurons and may therefore be of interest concerning the trophic regulation of SPNs. CNTF and LIF are expressed at low levels in adrenal medulla (Krohn, personal communication, 1995) and sympathetic ganglia (Banner and Patterson, 1994). LIF is upregulated in ganglia following axotomy (Sun and Zigmond, 1996; Zigmond and Sun, 1997). Rat and bovine adrenal chromaffin cells express CNTF, and isolated rat chromaffin cells in culture show CNTF-like immunoreactivity (Krohn, Meyer, data unpublished, 1994, 1995). Schwann cells of preganglionic sympathetic axons are an unlikely source for CNTF in rat and mouse, since preganglionic nerves in these species are largely unmyelinated (see above).

4. Transforming Growth Factor-ßs

TGF-ßs are among the most ubiquitous cytokines, and members of the different subfamilies are found in both developing and adult neurons and glial cells. Sympathetic ganglia have not been studied in great detail with regard to expressions of TGF-ßs. However, parasympathetic autonomic ganglia, such as the chick ciliary ganglion, and sensory ganglia, such as dorsal root ganglia (DRG), show stereotypical patterns of TGF-ß2 and -ß3 immunoreactivities, both with regard to the temporal sequence during embryogenesis and immunoreactive cell types (Flanders *et al.,* 1991; Krieglstein and Unsicker, 1995; Unsicker *et al.,* 1991, 1996). In E5 chick and E13.5 mouse embryos TGF-ß immunoreactivity surrounds neuroblasts, i.e., is located at the outer or inner surface of the cell membranes. This also holds true for lumbar sympathetic ganglia at E7 (Combs *et al.,* 2000). At E9–E11 in chick and E15.5. in mouse embryos, neuronal perikarya begin to show TGF-ß2 and -ß3 immunoreactivity. In adult rat both neurons and satellite cells are TGF-ß2 and -ß3 immunopositive, although at different intensities. The TGF-ß receptor TßRII can be detected on sensory neurons (Krieglstein *et al.,* 1998). Together, these data make it likely that sympathetic neurons and Schwann cells may employ TGF-ß for signaling in auto- and paracrine loops.

The adrenal medulla and its chromaffin cells have been extensively analyzed with respect to TGF-ß synthesis, storage, and release (Blottner *et al.,* 1996; Flanders *et al.,* 1991; Krieglstein and Unsicker, 1995; Wolf *et al.,* 1999; Unsicker and Krieglstein, 1996). Chromaffin cells of chick, mouse, rat, and bovine synthesize at

least two isoforms of TGF-ßs, either -ß2 and -ß3 (chick), -ß1 and -ß3 (rat), or -ß1 and -ß2 (bovine), or all three mammalian isoforms (mouse). TGF-ß biological activity determined by the mink lung epithelial cell assay can be released by exocytosis from cultured bovine chromaffin cells (Krieglstein and Unsicker, 1995). TGF-ß can inhibit chromaffin cell proliferation *in vitro* (Wolf *et al.,* 1998). Functions with regard to SPNs are described below.

Another member of the TGF-ß superfamily that is synthesized and stored by adrenal chromaffin cells is GDNF (Deimling *et al.,* 1997; Krieglstein *et al.,* 1998). Like TGF-ß, GDNF is stored in secretory granules and can be released upon stimulation by cholinergic agonists (Krieglstein *et al.,* 1998). As outlined below, GDNF is a potent trophic factor for SPNs, but may also affect chromaffin cells through mechanisms employing the GFRα2 and GFRα4 receptors without c-Ret (Airaksinen *et al.,* 1999; Lindahl *et al.,* 2000; Schober *et al.,* 2000a).

5. Interleukins

Interleukins are multifunctional cytokines with important roles in immune and inflammatory reactions. Interleukin-1 (IL-1) has been localized in rat and mouse noradrenergic chromaffin cells (Schultzberg *et al.,* 1989). Synthesis can be induced by cholinergic stimulation (Andersson *et al.,* 1992) and be released through reserpine (Schultzberg *et al.,* 1989). IL-6 transcripts and the IL-6 receptor mRNA occur in rat adrenal medulla (Gadient *et al.,* 1995). Sympathetic ganglia express both IL-1 and IL-6 (Freidin *et al.,* 1992; Gadient and Otten, 1996; Marz *et al.,* 1996; Carlson *et al.,* 1996). IL-1 has been implicated in the induction of LIF mRNA following axotomy and modulation of neuropeptide synthesis.

6. Insulin-like Growth Factors

Insulin-like growth factors (IGF-I, IGF-II) have been detected in embryonic sympathetic ganglia (Zackenfels *et al.,* 1995). It also occurs in adrenal medulla and pheochromocytoma cells (El Badry *et al.,* 1989; Suzuki *et al.,* 1989) and is involved in the regulation of chromaffin cell proliferation (Frodin and Gammeltoft, 1994).

D. Local Growth Factors in the Spinal Cord

Very little is known about factors that may regulate SPN performances in auto-/paracrine fashions. As shown by *in situ* hybridization, embryonic rat SPNs synthesize BDNF and NT-3 mRNAs (Schober *et al.,* 1999b) suggesting that TrkB and TrkC expression by SPNs might also serve for responding to local neurotrophins. Similarly, FGF-2 immunoreactivity in SPNs has been reported along with expression of the FGFR-1 (Blottner *et al.,* 1997; Stapf *et al.,* 1997).

IV. Experiments Revealing the Physiological Significance of Growth Factors

A. Target Organ Ablation and Growth Factor Substitution

Target organ destruction, e.g., the adrenal medulla, has been known for more than a decade to result in degeneration and numerical losses of the corresponding SPN population (Blottner *et al.,* 1989a, 1989b; Blottner and Baumgarten, 1994). In this respect, adult SPNs are apparently different from somatic motoneurons that fail to degenerate on axotomy, unless root convulsion is performed (Koliatsos *et al.,* 1994). The adrenomedullectomy lesion model uses electrocauterization for the selective destruction of the adrenal medulla in adult rat (Blottner *et al.,* 1989a, 1989b; Schober *et al.,* 1998a, 1999a) and subsequent implantation of gelfoam soaked with growth factors of interest or nontrophic control proteins, respectively (see Fig. 6). Complete destruction of the medulla can be monitored using histology and quantitative determinations of catecholamines. Results with regard to SPNs are evaluated after 4 weeks, when SPNs identified by FG-labeling have degenerated under control conditions. The precise time course of SPN degeneration has not been revealed. Another important issue that also remains to be clarified concerns delayed administration of factors.

Factors that have been tested in the adrenomedullectomy lesion paradigm and found to exert protective effects on SPNs include FGF-2 (Blottner *et al.,* 1989a; Blottner and Baumgarten, 1992; Blottner and Unsicker, 1990), CNTF (Blottner *et al.,* 1989b), IGF-I (Blottner and Baumgarten, 1992), TGF-ß2 (Blottner *et al.,* 1996), NT-3 (Schober *et al.,* 1998a), NT-4 (Schober *et al.,* 1998a), and GDNF (Schober *et al.,* 1999a; for a summary, see Table III). All factors are produced within the adrenal medulla, and IML neurons express their cognate receptors. For FGF-2 a dose response study has shown that a single dose of 60 ng of FGF-2 is sufficient for mediating protection. Moreover, FGF-2 is the only factor for which retrograde axonal transport to IML neurons has been documented (Blottner *et al.,* 1997). Since the proportion of specifically transported iodinated FGF-2 in relation to the total injected amount was very small, it has been speculated that FGF-2 may locally induce second messengers, e.g., GTP-binding proteins, for which retrograde transport has been documented in other systems (Hendry and Belford, 1991; Crouch *et al.,* 1994). FGF-2 also seems to act as an endogenous factor for target-deprived SPNs, since implantation of gelfoam with neutralizing antibody to FGF-2 resulted in increased rates of IML neuron degeneration (Blottner and Baumgarten, 1992).

Of particular interest may be the neuroprotective actions of NT-4 and GDNF, respectively. NT-4 administered in the gelfoam implant induces massive sprouting of axons in the innermost layer of the adrenal cortex (Schober *et al.,* 1998a). Axons even enter into the gelfoam, which has been shown to be colonized by fibroblasts and macrophages (Blottner and Unsicker, 1990). Because iodinated NT-4 is not retrogradely transported to the spinal cord, it is conceivable that NT-4

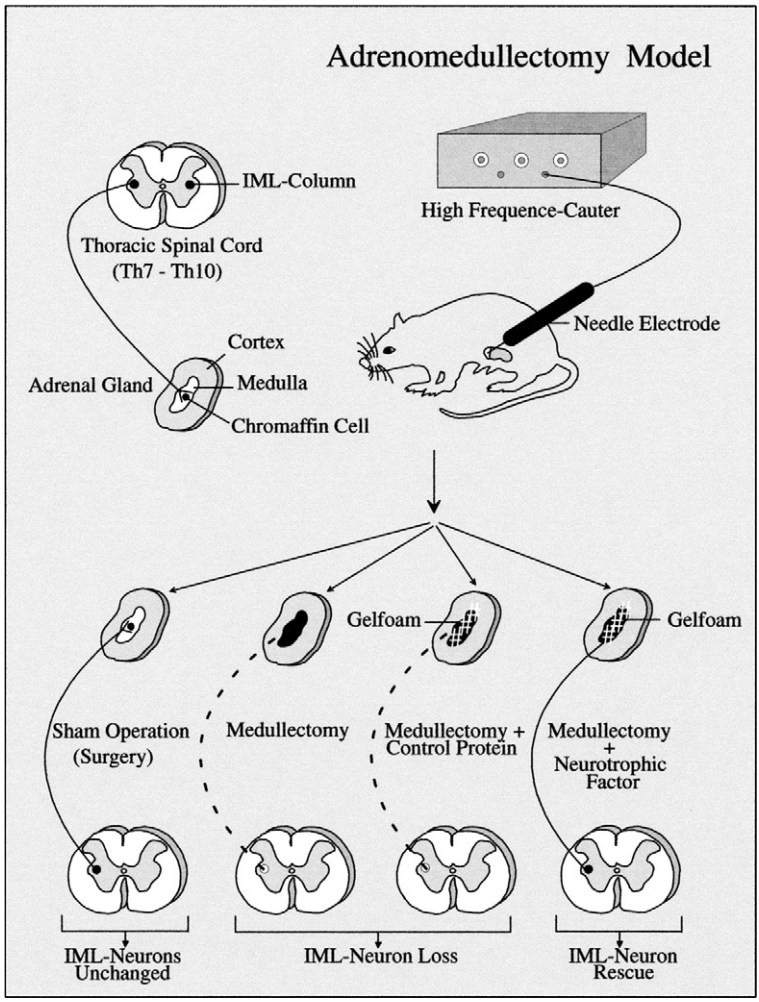

FIG. 6 Illustration of the adrenomedullectomy model employed for studying the neuroprotective effects of FGF-2, CNTF, IGF, TGF-ß2, GDNF, NT-3, and NT-4 for target-deprived SPNs *in vivo.* (Reproduced from *The Journal of Neuroscience,* 1999, vol. 19(6), pp. 2008–2015 by copyright permission of the Society for Neuroscience.)

may exert its protective effect indirectly by inducing branching and improving access of preganglionic axon terminals to other trophic factors. GDNF is of special interest, because its trophic functions fully depend on the simultaneous presence of TGF-ß (Schober *et al.,* 1999a). Combining GDNF and neutralizing antibodies to the TGF-ß isoforms -ß1, -ß2, and -ß3 abolishes the rescue effect of GDNF. Together, these data suggest that several growth factors expressed in the adrenal

TABLE III
Summary of Adrenomedullectomy Data

Factor	Amount/gelfoam	SPN labeling	Loss of SPNs	Rescue of SPNs	References
CNTF	7.2 μg	Nissl	25%	95%	Blottner *et al.* (1989b)
FGF-2	2.0 μg	Nissl	25%	100%	Blottner *et al.* (1989a)
FGF-2	60 ng	Nissl	25%	92%	Blottner and Unsicker (1990)
FGF-2	100 ng	Fast Blue (retrograde)	85%	74%	Blottner and Baumgarten (1992)
IGF-1 + FGF-2	1 μg/0.5 μg	NADPH-diaphorase	20%	no effect	Blottner and Baumgarten (1992)
TGF-ß2	0.5 μg	NADPH-diaphorase	26%	96%	Blottner *et al.* (1996)
NT-3	2.0 μg	Fluorogold (ip)	24%	no effect	Schober *et al.* (1998a)
NT-4	6.0 μg	Fluorogold (ip)	24%	98%	Schober *et al.* (1998a)
GDNF	1 μg	Fluorogold (ip)	26%	97%	Schober *et al.* (1999a)

medulla share a capacity to rescue the respective SPNs projecting to the adrenal medulla in a pharmacological of target organ deprivation.

Despite local expression of these factors in the target organ and expression of their receptors by SPNs, it is important to realize that these experiments may emphasize the pharmacological potential rather than the physiological significance of these factors for SPNs.

B. Knockout Animals and Immunological Sympathectomy

Mice deficient for the neurotrophin receptors TrkA and TrkB, NT-4, and FGF-2 have been analyzed so far with respect to their significance for SPN development and maintenance. TrkA-mutant mice (Schober *et al.,* 1997) show increasing loss of AChE activity in SPNs and their intra-adrenal axons during the first and second postnatal week. These effects are specific for TrkA because they are not observed in mice deficient for TrkB. Disappearance of AChE occurs during the period of onset of TrkA expression in chromaffin cells. Because SPNs do not express TrkA, the effect must be indirect and probably due to lack of a chromaffin cell-derived factor, whose expression is controlled by TrkA. In contrast to paravertebral sympathetic neurons, chromaffin cells do not die in TrkA mutant mice. This accounts for the survival of SPNs projecting to the adrenal medulla, in contrast to those SPNs that project to paravertebral sympathetic ganglia and which are severely impaired.

The precise pattern of SPN death in TrkA mutants still needs to be evaluated. Survival or death of sympathetic neurons in locations other than the paravertebral ganglia has not been studied as yet. It is therefore conceivable that SPN death in TrkA deficiency is restricted to the subpopulation that projects to paravertebral ganglia. Results of TrkA knockout analyses corroborate previous data obtained by systemic application of NGF antibodies ("immunosympathectomy"), which have revealed substantial death in paravertebral ganglia, but only relatively subtle changes in the adrenal medulla (Bode *et al.,* 1986; Thoenen, 1972). Using antibodies to AChE (Brimijoin *et al.,* 1993) it has been shown massive death of SPNs and increased levels of peptides and their mRNAs in rat adrenal chromaffin cells (Dagerlind *et al.,* 1994a). This manipulation also eliminated all calcitonin gene-related peptide and enkephalin-immunoreactive preganglionic fibers to the SCG and upregulated galanin in the ganglion (Dagerlind *et al.,* 1994b).

Mice lacking TrkB do not show loss of SPNs during the first and second postnatal week, suggesting that a TrkB ligand is not required during this period for the maintenance of IML neurons. This is consistent with the observation that early postnatal mice (postnatal day 7) lacking NT-4 have normal numbers of SPNs during this period (Roosen *et al.,* 2001). However, electron microscopy has provided evidence for signs of degeneration in axon terminals making synaptic contacts with chromaffin cells (Schober *et al.,* 1998a).

Analysis of NT-4-deficient mice that survive into adulthood has provided evidence for significant losses of SPNs projecting to the adrenal medulla (Schober *et al.,* 1998a) and paravertebral and prevertebral ganglia (Roosen *et al.,* 2001). Thus, NT-4 is the first identified molecule that is apparently required for postnatal maintenance of SPNs.

Lack of FGF-2 has been shown to cause neuron losses in the cerebral cortex (Dono *et al.,* 1998) and a significant reduction in arterial blood pressure. As with NT-4, FGF-2 knockout mice become adult. However, counts of SPNs labeled by FG have failed to provide evidence for SPN losses (Dictus, unpublished, 2000) suggesting that FGF-2 may not be absolutely required for the maintenance of this neuron population. Given the established redundancy in the actions of about 20 members of the FGF family, it is also conceivable, however, that other FGFs compensate FGF-2 effects in FGF-2-deficient mice.

V. Concluding Remarks

As summarized in Fig. 7, SPNs and their targets are catching up with somatic motor and sensory neurons with regard to neurotrophic factors/neurotrophic factor receptors that are relevant during and after development and in lesion models.

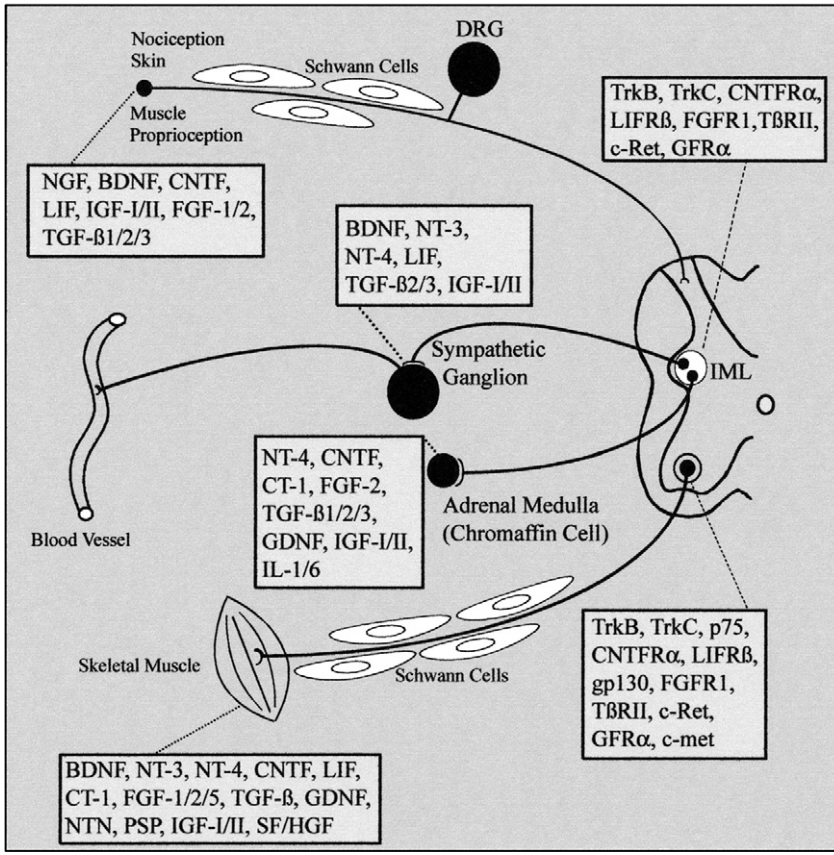

FIG. 7 Summary showing neurotrophic factor ligands and receptors for sensory neurons (DRG), preganglionic sympathetic (IML) neurons, and somatic motoneuons. *Ligands:* NGF, nerve growth factor, BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; NT-4 neurotrophin-4; CNTF, ciliary neurotrophic factor; LIF, leukemia inhibitory factor; CT-1, cardiotrophin-1; FgF, fibroblast growth factor; TGF-ß, transforming growth factor-ß; IGF insulin-like growth factor; GDNF, glial cell line-derived neurotrophic factor, NTN, neurturin; PSP, persephin, SF/HGF, scatter factor/hepatocyte growth factor; IL, interleukins. *Receptors:* TrkB, TrkC, p75, neurotrophin receptors; CNTFRα, LIFRß, gp130, CNTF family receptors; FGFR fibroblast growth factor receptor(s), TßR, TGF-ß receptor(s), *c*-Ret, GFRα, GDNF family receptors; *c*-met, SF/HGF receptor.

As outlined above, the issue of ontogenetic neuron death in the SPN population is controversial and needs to be resolved. If programmed neuron death does not occur in the sympathetic preganglionic neurons, this will have to be reconciled with the multitude of factors and receptors expressed in the sympathetic preganglioninc outflow. Their presence, then, could not be related to putative roles in the regulation of ontogenetic survival and would probably be restricted

to functions in the regulation of neuron differentiation and in emergency situations. In return, this might also shed new light on the significance of the known plethora of neurotrophic factors acting on motoneurons as well as on sensory neurons.

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Factors Controlling Axonal and Dendritic Arbors

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The sculpting and maintenance of axonal and dendritic arbors is largely under the control of molecules external to the cell. These factors include both substratum-associated and soluble factors that can enhance or inhibit the outgrowth of axons and dendrites. A large number of factors that modulate axonal outgrowth have been identified, and the first stages of the intracellular signaling pathways by which they modify process outgrowth have been characterized. Relatively fewer factors and pathways that affect dendritic outgrowth have been described. The factors that affect axonal arbors form an incompletely overlapping set with those that affect dendritic arbors, allowing selective control of the development and maintenance of these critical aspects of neuronal morphology.
KEY WORDS: Neuronal development, Neuronal polarization, Axonal outgrowth, Dendritic outgrowth, Neurotrophins.

I. Introduction

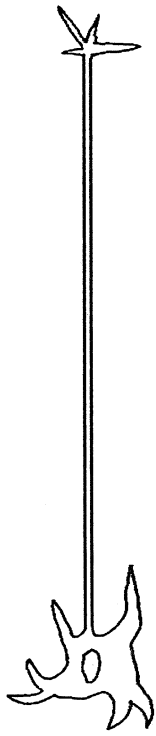
The manner in which the connectivity of the nervous system is established—especially of the neurons of the central nervous system—is an area that has challenged cell and developmental biologists for many years, and in which considerable progress has been made in recent years.

It has long been recognized that the amount of information necessary to fully encode the connectivity of the nervous system is orders of magnitude beyond that which could be encoded in the genome alone. Therefore, it stands to reason that the pathfinding and branching of neuronal processes—the axonal and dendritic arborization—must be largely determined by extracellular factors. An

understanding of the manner in which these extracellular factors modulate the development and maintenance of axonal and dendritic arborization may therefore be seen as one of the basic elemental processes that leads to nervous system patterning.

The control of axonal arbors by extracellular factors may be simplified to a consideration of factors that encourage axonal outgrowth, factors that encourage axonal branching, and factors that inhibit axonal outgrowth and can cause axon or branch retraction. In general, extracellular factors affecting axonal outgrowth act at the growth cone, so the difference between factors that modulate axonal outgrowth and those that modulate axonal branching is usually one of presentation. Insofar as dendrites are concerned, there is less evidence for a distinction between factors that act at the dendritic growth cone and the neuronal cell body. Nevertheless, there is little evidence for factors that encourage dendritic branching without also enhancing dendritic outgrowth. For example, in some cases effects early in development on dendritic outgrowth later appear as effects on branching of more mature dendrites. With dendrites, moreover, we must also consider factors that influence the development and retraction of dendritic spines.

Over the last few years an impressive menagerie of factors that act to enhance or inhibit the growth of axons and/or dendrites has been described. Both outgrowth-enhancing and outgrowth-inhibiting factors can act either as substrate-bound or soluble factors, and may have an effect on a neuronal process that is specific to its tissue of origin, physiological state, or age. Factors that can encourage or inhibit local process response include neurotransmitters, which lead to electrical activity in the target cells, substrate-bound and soluble neurotrophins and collapsins, cytokines, and various other species. In the amalgam, these factors give rise to the observed initial developmental pathfinding and sculpting of axonal and dendritic arbors, as well as later and continuing activity-dependent strengthening or pruning of synapses, and hence to the structure and function of the vertebrate nervous system. While the sculpting of axonal and dendritic arbors has been studied for many years, it is only recently that we have begun to understand the chemistry of the extracellular mediators involved. It has been even more recently that we have begun to understand the chains of events that led to the action of these extracellular mediators on the motile machinery of the neuronal processes. Our understanding of these processes is complicated by the fact that some of the data are contradictory and not always distinguished by the type of neuronal process (axon or dendrite) under study. In addition, our knowledge about factors that guide axonal elongation is considerably more advanced than our knowledge about factors that control dendritic arbors. Over the last few years, a number of comprehensive reviews have dealt with signals that control axonal outgrowth (Colamarino and Tessier-Lavigne, 1995; Kennedy and Tessier-Lavigne, 1995; Goodman, 1996; Tessier-Lavigne and Goodman, 1996; Goodman *et al.,* 1997; Stoeckli, 1997, 1998; Varela-Echavarria and Guthrie, 1997; Chen *et al.,* 1998; Cook *et al.,* 1998; Key, 1998; Stoeckli and Landmesser, 1998; Tear, 1998, 1999; de Castro *et al.,* 1999;



Axons:

+ :	Cell Adhesion Molecules Other Extracellular Matrix Molecules Neurotrophins
- :	Chondroitin Sulfate Ephrins
+/- :	Netrins Sempahorins Electrical Activity

Dendrites:

+ :	Neurotrophins Osteogenic Protein - 1 Candidate Plasticity Gene - 15 Dermatan Sulfate Amyloid Precursor Protein
- :	Ciliary Neurotrophic Factor Heparan Sulfate
+/- :	Neurotransmitters/depolarization β-amyloid

FIG. 1 Examples of factors that can enhance (+), inhibit (−), or either enhance or inhibit (+/−) the growth of axons and dendrites, depending on conditions.

Gallo and Letourneau, 1999; Joosten and Bar, 1999). It is our intention in this article to review the factors that control axonal outgrowth and arborization and to compare them with those that control dendritic arborization. We then discuss transduction mechanisms involved, again contrasting axonal to dendritic mechanisms (Fig. 1).

Both activity-independent and activity-dependent mechanisms operate on the axonal (Antonini and Stryker, 1993; Goodman and Shatz, 1993; Jessell and Kandel, 1993; Davis and Murphey, 1994; Zou and Cline, 1999) and on the dendritic (Kossel *et al.,* 1995, 1997; Maletic-Savatic *et al.,* 1999; McAllister *et al.,* 1999) side of the developing synapse. Activity-independent mechanisms controlling axonal and dendritic arbors appear to involve a variety of different intracellular second messenger pathways, as described below. Relatively fewer activity-dependent mechanisms have been described, although interactions between activity-dependent and activity-independent mechanisms complicate matters (McAllister *et al.,* 1996, 1999; Zou and Cline, 1996; Morrison and Mason, 1998). Some of the major

activity-dependent mechanisms appear to involve elevation of postsynaptic $[Ca^{2+}]_i$, which can affect dendritic growth directly (Maletic-Savatic *et al.,* 1999; Wu and Cline, 1998; Rajan and Cline, 1998; Rajan *et al.,* 1998; Meberg *et al.,* 1999) and axonal growth either directly (Lankford and Letourneau, 1989; Bentley *et al.,* 1991; Song *et al.,* 1994; Williams *et al.,* 1995; Ramakers *et al.,* 1998) or indirectly through actions involving retrograde signaling mechanisms (Zou and Cline, 1996; Van Wagenen and Rehder, 1999).

The molecular signals operating during development may operate in the mature animal to give rise to structual rearrangements that result in changes in synaptic efficiency (Bailey and Kandel, 1993; Bailey *et al.,* 1996; Martin *et al.,* 1997). For example, in studies that deal with postembryonic process outgrowth, it has been observed that prolonged stimulation augments the presynaptic field of Aplysia synapses (Abel and Kandel, 1998), that serotonin increases process outgrowth from thalamic neurons (Lieske *et al.,* 1999), and that hippocampal dendrites show increased filopodial sprouting in response to synaptic activity (Maletic-Savatic *et al.,* 1999). It is attractive to speculate that such responses could contribute to the long-term activity-dependent enhancement of synapse efficiency, and thus to the elemental processes of learning and memory.

In addition to playing a critical role in the normal developmental sculpting of the vertebrate nervous system, extracellular signals may be critically involved in pathological changes in axonal and dendritic arbors that may occur in events such as stroke, epileptic seizure, and other central nervous system diseases (Purpura, 1982; Catala *et al.,* 1988; Ferrer *et al.,* 1991). Both the density of dendritic spines and the number of dendritic branches are reported as being reduced in anatomical studies of epileptic patients and in animal models of epilepsy (Isokawa, 1998; Jiang *et al.,* 1998; Eid *et al.,* 1999). Exposure of cultured central neurons to excitatory signals can induce rapid remodeling of dendritic spines in the short term (Brandon and Coss, 1982; Halpain *et al.,* 1998), and can induce wholesale remodeling of dendritic branches on longer exposure (Mattson *et al.,* 1988a, 1988; Wilson *et al.,* 2000).

Like the normal developmental sculpting of axonal and dendritic arbors, a number of the pathological changes, such as spine (Halpain *et al.,* 1998; Morrison and Mason, 1998) and dendrite retraction (Mattson *et al.,* 1988a; Nuijtinck *et al.,* 1997; Wilson and Keith, 1998), and, ultimately, axonal regression (Lankford and Letourneau, 1989; Cohan, 1992; Neely, 1993; Song *et al.,* 1994; Turnell *et al.,* 1995; Job and Lagnado, 1998) may be driven by changes in $[Ca^{2+}]_i$ resulting from excessive or prolonged exposure to neurotransmitter or other depolarizing stimuli. Additionally, there is evidence that normal age-dependent changes in calcium metabolism may modulate synaptic efficiency (Norris *et al.,* 1998a, 1998b) in rats, although there is little evidence as to whether there are accompanying structural changes. Thus, many of the extracellular and intracellular factors that guide neurite differentiation during development may also be involved in the induction of pathological changes in neuron morphology during disease.

II. Factors Regulating Axonal Arborization

The development of axons starts with the initial sprouting of neurites and the specification of one of those minor processes as an axon. The subsequent development of axons encompasses their guidance by extracellular factors, their bundling or fasciculation, and the formation of terminal branches in the region of their postsynaptic target. Subsequently, it often involves retraction of supernumerary branches. Both substrate-bound and non-substrate-bound factors can regulate these aspects of axonal development. Substrate-bound factors include transmembrane, membrane-associated, and extracellular matrix-associated molecules. These factors include growth promoting molecules, such as elements of the extracellular matrix and cell adhesion molecules, and growth inhibiting molecules, such as ephrins, semaphorins, and elements of the extracellular matrix. Non-substrate-bound factors include molecules such as neurotransmitters, neurotrophins, netrins, and secreted semaphorins, which can act either as growth promoters or growth inhibitors. These factors work in concert to induce neuritogenesis, guide axonal trajectories, promote axonal bundling, and direct axonal targeting to synaptic sites. Thus, the combinatorial effect of these factors on the several aspects of axonal development determines long- and intermediate-range neural connectivity. This section reviews the structure and function of these families of molecules with an emphasis on their impact during axonal growth, guidance, and fasciculation.

A. Substrate-Associated Factors That Affect Axonal Arbors

The substrate along which axonal and dendritic elogation, branching, and retraction occurs *in vivo* includes the somatodendritic and axonal membranes of other neurons, the cellular membranes of nonneuronal cells, basement membranes, and the extracellular matrix (proteinaceous and nonproteinaceous) associated with it. *In vitro,* it can include all of the above, plus nonspecific adhesive or antiadhesive moieties associated with the culture substrate.

1. Substrate-Bound Factors That Enhance Axonal Outgrowth

a. Cell Adhesion Molecules of the Immunoglobulin Superfamily The cell adhesion molecules (CAMs) were among the first factors identified that mediated neuronal adhesion either to other neurons (homophilic CAMs) or to other cell types (heterophilic CAMs; Brackenbury *et al.,* 1977; Thiery *et al.,* 1977). They all have multiple domains structurally related to the immunoglobulins (Ig domains) and are therefore referred to as Ig-superfamily molecules. In addition, they have repeats related to the extracellular matrix protein fibronectin (FNIII repeats). The

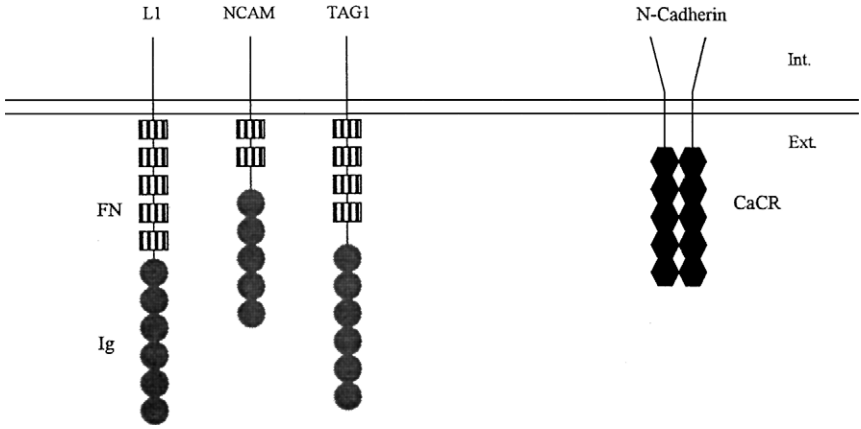


FIG. 2 The structure of cell adhesion molecules in the immunoglobulin (Ig) superfamily and cadherin family. CAMs, such as L1 NCAM, and TAG1, contain multiple fibronectin type III repeats (FN) and Ig domains, and a short cytoplasmic domain. N-cadherin is found as dimer with five calcium-binding cadherin repeats (CaCR) in the extracellular region, and short cytoplasmic domains that interact with catenins and tyrosine kinases (see text). Int., intracellular; Ext., extracellular face of the membrane.

CAMs, including NCAM, L1, and TAG1, appear to be substantially involved in both neuronal precursor migration and in axonal guidance; their roles in the latter process have been described in a number of recent reviews (Kamiguchi *et al.,* 1998; Key, 1998; Sonderegger *et al.,* 1998; Tear, 1998; Joosten and Bar, 1999). In addition to homo- or hetereophilic interactions with CAMs, the CAMs can interact with other receptors, such as the fibroblast growth factor receptor (a receptor tyrosine kinase) or with receptor tyrosine phosphatases (Fig. 2) (Walsh and Doherty, 1997). The neural cell adhesion molecule, NCAM, was the first CAM identified based on functional criteria (Brackenbury *et al.,* 1977; Thiery *et al.,* 1977). Three isoforms of NCAM include 180-, 140-, and 120-kDa forms. Both the 180 and 140 forms have transmembrane regions, while the 120 is glycosylphosphatidylinositol-anchored (GPI-anchored). The 180-kDa isoform is expressed only in neurons, while the smaller forms are expressed by neurons, astrocytes, oligodendrocytes, and Schwann cells. *In vitro,* expression of the different isoforms of NCAM in fibroblasts promotes neurite outgrowth from chick retinal ganglion cells (Doherty *et al.,* 1990a, 1990b; Doherty and Walsh, 1992). In these studies, NCAM-specific antibodies blocked the growth promoting effects, and NCAM 140 was shown to be a more potent growth promoter than NCAM 180. *In vivo,* injection of function blocking NCAM antibodies causes alterations in axonal growth and fasciculation during frog retinotectal development (Fraser *et al.,* 1988) and chick hindlimb innervation (Landmesser *et al.,* 1988). Genetic studies show that the NCAM homolog in *Drosophila* (Fas), is required for axonal fasciculation and sprouting (Lin *et al.,* 1994; Lin and Goodman, 1994), while mice lacking all forms of NCAM have

defects in the fasciculation of mossy fiber projections in the hippocampus (Cremer *et al.,* 1997).

Many of the effects of NCAM can be modulated by polysialylation. During late embryonic and early postnatal stages of development, NCAM is highly polysialylated. This polysialylic acid (PSA) is thought to promote axonal outgrowth and reduce axonal fasiculation. *In vitro,* removal of PSA from NCAM by endoneuraminidase suppresses the enhancement of retinal ganglion cell neurite outgrowth on fibroblasts that express NCAM (Doherty *et al.,* 1990a). *In vivo,* PSA removal reduces the defasiculation of motor neurons entering the sacral plexus (Tang *et al.,* 1994). On the other hand, removal of PSA from NCAM in the developing hippocampus (Seki and Rutishauser, 1998) or retinotectal tracts (Yin *et al.,* 1995) leads to defasciculation and aberrant axonal targeting. Thus, the role of PSA in NCAM-mediated regulation of axonal outgrowth may be dependent both on the cell type and extracellular environment.

L1 (or NgCAM and NrCAM in chick) is another CAM that functions in a fashion similar to NCAM. *In vitro,* L1-coated substrata or L1 expressing cells promote axonal outgrowth in variety of neuron types, including retinal ganglion cells (Lemmon *et al.,* 1992), cerebellar neurons (Williams *et al.,* 1992), dorsal root ganglion neurons (Dou and Levine, 1995), and hippocampal nerons (Lochter *et al.,* 1995). In addition, antibody perturbation studies *in vitro* demonstrate that L1 is required for dorsal root ganglion axonal fasiculation (Honig *et al.,* 1998). Neurons from L1 knockout mice grown *in vitro* show impaired neuritogenesis and fasciculation on L1-containing substrata (Dahme *et al.,* 1997). A role for L1 in axonal development *in vivo* was suggested when mutations in the L1 gene were associated with multiple neurological deficits. Additionally, *in vivo* L1 antibody perturbation studies showed inhibition of axonal outgrowth in the projection of rat retinal axons to the tectum (Brittis *et al.,* 1995), as well as inhibition of midline crossing and defasciculation of chick commissural axons (Stoeckli and Landmesser, 1995; Stoeckli *et al.,* 1997). In L1 knockout mice, a substantial proportion of corticospinal axons do not cross the midline to the opposite dorsal column (Cohen *et al.,* 1998), and few callosal axons cross the midline in the corpus callosum (Demyanenko *et al.,* 1999).

TAG1 (TAX1 in human, axonin-1 and F3/F11 in chick) is a third CAM that may be important for axonal outgrowth and fasciculation (Furley *et al.,* 1990; Felsenfeld *et al.,* 1994). Antibody perturbation studies of chick commissural axons *in vivo* show that its interactions with NrCAM are required for accurate axonal pathfinding at the midline of the spinal cord (Stoeckli and Landmesser 1995). In addition, Chinese hamster ovary (CHO) cells that express F3 inhibit neurite outgrowth and induce axonal fasciculation in cerebellar granule cells, while TAG-1 coexpression with F3 in the same CHO cells blocks the inhibitory influences of F3 alone (Buttiglione *et al.,* 1998). Thus, the role of CAMs during axonal development is probably determined by combinatorial homophilic and heterophilic interactions between specific CAMs, as well as by the post-translational modifications present on distinct CAMs.

b. Cadherins Like the CAMs, the cadherins are cell surface proteins that mediate cell-to-cell adhesion. They are transmembrane proteins with five Ca^{2+}-binding cadherin repeats in the extracellular region, and a cytoplasmic tail that interacts with cytoskeletal-associated catenins (Fig. 2) (Vleminckx and Kemler, 1999). The defining characteristic of cadherins is that their interactions are dependent on the presence of calcium in the extracellular milieu. The predominant neuronal cadherin, N-cadherin, is broadly expressed in nervous tissue at developmental stages in which extensive axonal outgrowth and fasciculation occur (Redies, 1997; Vleminckx and Kemler, 1999). *In vitro,* purified or cell-surface expressed N-cadherin promotes axonal outgrowth from retinal ganglion (Matsunaga *et al.,* 1988; Kljavin *et al.,* 1994), dorsal root ganglion (Bixby and Zhang, 1990), hippocampal (Doherty and Walsh, 1992) and forebrain (Bixby *et al.,* 1994) neurons. In addition, retinal ganglion and superior cervical ganglion axonal fasciculation on a laminin substratum can be inhibited by antibodies against N-cadherin (Drazba and Lemmon, 1990). *In vivo,* antibody perturbation studies show that N-cadherin influences the lamina-specific branching and arborization of axons in the chick retina (Inoue and Sanes, 1997), while N-cadherin and integrins are required for accurate pathfinding during the development of frog retinotectal projections (Stone and Sakaguchi, 1996). N-cadherin is also required for fasciculation and routing of neurites in the developing plexus of the chick hindlimb (Honig *et al.,* 1998). Loss of function mutations in the homolog of N-cadherin in *Drosophila* (DN-cadherin), causes abnormal axonal trajectories and bundling (Iwai *et al.* 1997), while expression of dominant negative N-cadherin in single frog retinal ganglion cells leads to impaired axonal outgrowth (Riehl *et al.,* 1996). Thus, N-cadherin is another important cell adhesion molecule that, like the CAMs, regulates both axonal outgrowth and fasciculation.

c. Molecules of the Extracellular Matrix The extracellular matrix contains a variety of components that promote both axonal growth cone adhesion and axonal outgrowth. Laminin, collagen, fibronectin, thrombospondin, and vitronectin are extracellular matrix components that have been implicated in neurite outgrowth *in vitro* and *in vivo* (Letourneau *et al.,* 1992, 1994). Laminins are secreted into the extracellular matrix and are heterotrimeric glycoproteins with varying types of α, β, and γ chains (Reichardt and Tomaselli, 1991; Letourneau *et al.,* 1994). Two domains in the γ chain, V and VI, are related to similar domains in netrins (Tessier-Lavigne, 1994). Laminins have been shown to promote neurite outgrowth from a variety of neuron types *in vitro* (Powell and Kleinman, 1997). Laminin, like many other ECM molecules, mediates its axonal growth, thus promoting actions predominately through activation of specific receptors termed integrins (Hynes, 1992; McKerracher *et al.,* 1996). Integrins are formed as heterodimeric complexes of α and β subunits, in which different combinations have different binding specifities. Intergrin α subunits have metal binding sites in the N-terminal region that are required for heterodimer formation, while the β subunits have cysteine-rich repeats near the transmembrane region and disulfide loops in the N-terminal region

that contribute to the ligand binding domain. Both α and β subunits typically have small cytoplasmic domains without catalytic domains. Thus, integrin signaling events that modulate axonal outgrowth must involve intracellular adaptor proteins (see below).

The tenascins are another family of extracellular matrix molecules that have been implicated in regulating axonal outgrowth. They have a cysteine-rich region, multiple epidermal growth factor-like repeats, multiple FNIII repeats, and a fibrinogen-like domain. The number of EGF domains is characteristic for each tenascin, but the number of FNIII domains differs. Studies of tenascin C demonstrate that it may have both growth promoting and growth inhibiting roles *in vivo* (Gotz *et al.,* 1997) and *in vitro* (Faissner and Kruse, 1990; Lochter *et al.,* 1991; Fischer *et al.,* 1997; Meiners and Geller, 1997). *In vitro,* tenascin R alone can promote neurite outgrowth (Lochter and Schachner, 1993; Lochter *et al.,* 1994), but when copresented with growth promoting molecules tenascin R has an inhibitory influence on axonal outgrowth (Pesheva *et al.,* 1993; Taylor *et al.,* 1993). In addition, tenascin R can regulate cerebellar axonal fasciculation *in vitro* (Xiao *et al.,* 1998). Mice deficient in tenascin C (Settles *et al.,* 1997) or tenascin R (Weber *et al.,* 1999) do not demonstrate major abnormalities during axonal development. Thus, the role of tenascins during axonal development *in vivo* may be redundant with other axonal guidance molecules.

d. Adhesion In a series of classic experiments, Letourneau demonstrated that outgrowing neurites would selectively extend along nonspecifically adhesive substrates (Letourneau, 1975a, 1975b). This led to the hypothesis that substrate adhesion was a major cue for axonal guidance *in vivo,* which is consistent with many observations that indicate that tension is an important regulator of process elongation (Bray, 1984; Letourneau *et al.,* 1987; Dennerll *et al.,* 1989; Zheng *et al.,* 1991; Baorto *et al.,* 1992; Heidemann *et al.,* 1995). Most of the axon-promoting molecules described in this section have been characterized as adhesion molecules, and that is demonstrably one of their functions (Heidemann, 1996). Recently, though, it has become apparent that there is poor correlation between the adhesivity of most biologically relevant substrates and their promotion of axonal outgrowth (Lemmon *et al.,* 1992; Zheng *et al.,* 1994a; Heidemann, 1996; Lamoureux *et al.,* 1998; Isbister and O'Connor, 1999). These and other observations have led to the current view that, for most biological substrata, adhesion is more likely to play a "permissive" rather than an "instructive" role for process extension (Lemmon *et al.,* 1992), and that the major role of these substrates in promoting axonal outgrowth is accomplished through specific signaling mechanisms.

2. Substrate-Bound Factors That Inhibit Axonal Outgrowth

a. Ephrins The Eph family of receptor tyrosine kinases and their ligands, ephrins, have been implicated in the regulation of axonal guidance in a variety of CNS

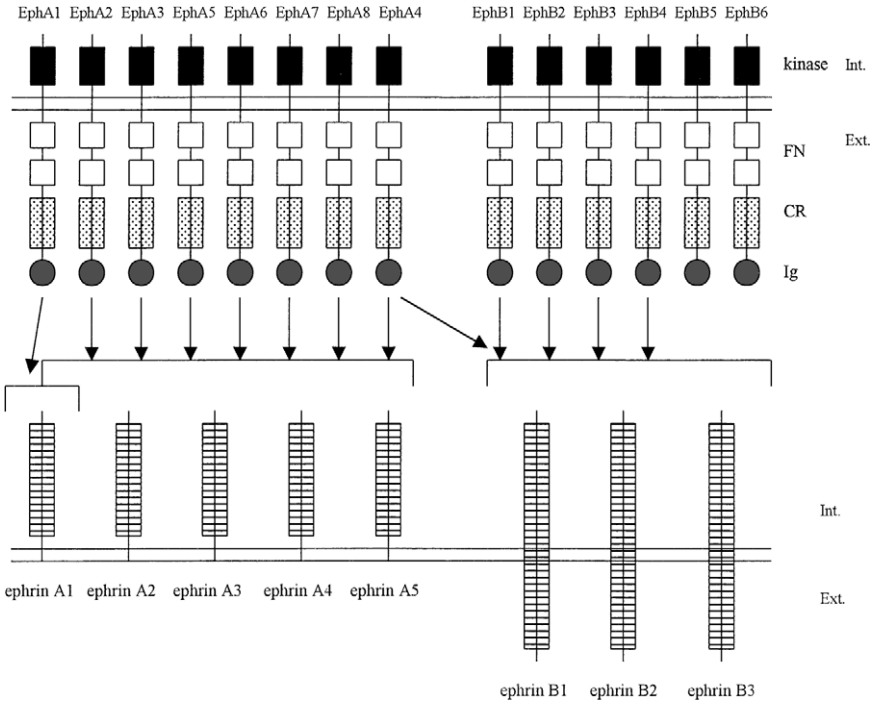


FIG. 3 Structure of the Eph receptors and their ephrin ligands. The full-length structures of the Eph receptors include extracellular FNIII repeats, a cysteine-rich region (CR), an Ig domain, and an intracellular kinase domain. There are many variant forms of these Eph receptors that are not shown, including truncated variants that are transmembrane, membrane-attached, or secreted forms. The ephrins are divided into type A GPI-anchored ephrins and type B transmembrane ephrins. Both type B ephrins and Eph receptors contain intracellular PDZ motifs that may be involved in receptor clustering and protein–protein interactions. In addition, Eph receptors contain a SAM domain that may be important for signal transduction from the active receptor to downstream signaling molecules.

regions. Fourteen Eph receptors and eight ephrin ligands have been identified to date, and they constitute one of the most important negative regulatory systems controlling axonal elongation (Fig. 3). For example, ephrins A2 and A5 are felt to underlie a classical example of CNS axonal guidance, the ordered mapping of retinal axons onto the surface of the optic tectum (Drescher *et al.,* 1995; Frisen *et al.,* 1998, 1999). The ephrins, their receptors, and their functions in nervous system development have been well described in a number of recent reviews (Gale and Yancopoulos, 1997; Bruckner and Klein, 1998; Cook *et al.,* 1998; Krull, 1998; Holder and Klein, 1999; O'Leary and Wilkinson, 1999). In the extracellular region, Eph receptors contain a ligand binding globular domain in the N terminal, a central cysteine-rich region, and two fibronectin repeats involved in receptor dimerization, while the intracellular region contains a tyrosine kinase domain,

a sterile alpha motif (SAM) domain, and a postsynaptic density/disc-large/ZO1 (PDZ) binding motif (Frisen *et al.,* 1999). Ephrins are divided into type A and type B ephrins based on their interaction with EphA and EphB receptors. Type A ephrins are attached to the membrane by a GPI anchor, while type B ephrins are transmembrane proteins. Both types of ephrins contain four conserved cysteines in their extracellular region, while the intracellular portion of type B ephrins has a C-terminal PDZ binding motif.

Eph receptor–ephrin interactions have inhibitory influences on axonal outgrowth *in vitro* and *in vivo* (Flanagan and Vanderhaeghen, 1998; Frisen *et al.,* 1999). For example, medial hippocampal neurons and entorhinal neurons will not extend neurites along NIH 3T3 cells that express ephrin A2 and ephrin A3, respectively (Gao *et al.,* 1996; Stein *et al.,* 1999). In addition, retinal axons and ventral spinal cord axons are repelled by substrate-bound ephrin A5 *in vitro* (Drescher *et al.,* 1995; Yue *et al.,* 1999), while substantia nigra neuron neurite outgrowth is inhibited by ephrin B2 *in vitro* (Yue *et al.,* 1999). *In vivo,* genetic studies have shown an array of axonal guidance errors in various regions of the CNS in ephrin mutants (Frisen *et al.,* 1999). For instance, superior collicular neurons in EphA8-deficient mice display misrouting of axons to the ipsilateral cervical spinal cord, rather than the contralateral inferior colliculus (Park *et al.,* 1997), while in EphB3-null mice axons fail to cross the midline leading to defects in the formation of the corpus callosum (Orioli *et al.,* 1996). Similarly, mice deficient in Eph4 and EphB2 have axonal guidance errors that cause disruption of the corticospinal tract and malformation of the anterior commissure, respectively (Henkemeyer *et al.,* 1996; Dottori *et al.,* 1998). In ephrin A5 null mice, there are errors in the projections of retinal axons to the superior colliculus and lateral geniculate nucleus (Feldheim *et al.,* 1998; Frisen *et al.,* 1998). Overexpression of ephrin A2 or ephrin A5 on retinal axons leads to errors in targeting in the tectum, while ectopic expression of ephrin A2 and ephrin A5 disrupts midline crossing of axons in the optic chiasm (Dutting *et al.,* 1999; Hornberger *et al.,* 1999). Thus, Eph receptor–ligand interactions appear to play a major inhibitory influence in axonal guidance and targeting during development of many regions of the CNS.

b. Semaphorins The semaphorins—named after semaphore flags—are a family of axonal guidance molecules characterized by a conserved 500-amino-acid N-terminal domain. They include both membrane-associated and soluble forms; at present the absolute number of characterized membrane-associated forms exceeds that of secreted forms. The semaphorins are widely distributed throughout the animal kingdom, and constitute a second major negative regulatory system for axonal pathfinding. The semaphorins, their receptors, and their functions have been well described in recent reviews (Fujisawa and Kitsukawa, 1998; Giger *et al.,* 1998; Kolodkin, 1998; Roskies, 1998; Sanes and Yamagata, 1999; Van Vactor and Lorenz, 1999; Yu and Kolodkin, 1999). Invertebrate semaphorins include class 1 and 2, while vertebrate semaphorins include classes 3 to 7 (Fig. 4). Class 2, 3, 4, and

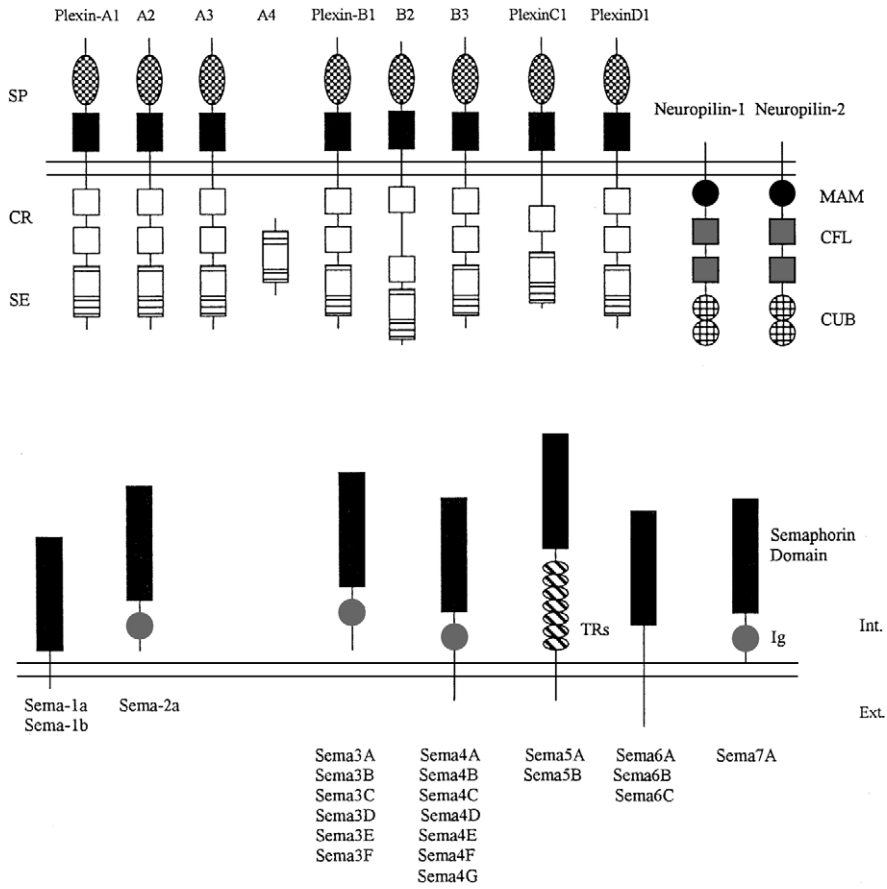


FIG. 4 The semaphorin family of axon guidance molecules and their neuropilin and plexin receptors. Secreted and GPI-anchored semaphorins contain a semaphorin domain and an Ig domain, while transmembrane semaphorins similar extracellular domains, short cytoplasmic domains, and in some cases thrombospondin repeats (TRs). Plexin contain multiple cysteine- rich regions and a semaphorin domain in their extracellular region, and a sex and plexins domain (SP) in the intracellular region. The SP domain is conserved in the sex/plexins gene family, and contains multiple tyrosine phosphorylation sites. Neuropilins have short cytoplasmic domains, and multiple extracellular domains, including a MAM domain, a two coagulation factor-like domain (CFL), and two complement binding domains (CUB). Plexins and neuropilins may form receptor complexes that mediate semphorin-induced effects on axon guidance.

7 semaphorins contain immunglobulin domains, with class 2 and 3 being the only secreted semaphorins. Class 5 semaphorins contain extracellular thrombospondin repeats, while class 7 semaphorins are GPI anchored. All of the transmembrane semaphorins (class 1, 4, 5, and 6) have small cytoplasmic domains. The first representative of the semaphorin family was identified in chick brain membranes

as a factor (collapsin-1, now called Sema3a) that induced growth cone collapse in chick sensory axons (Kapfhammer *et al.,* 1986; Luo *et al.,* 1993). This semaphorin was found to be homologous to a glycoprotein (G-sema I, now called Sema1a) that had repulsive roles during axonal development in the grasshopper CNS (Kolodkin, 1998). *In vitro,* Sema3a has been shown to inhibit the growth of sensory and motor axons from many species (Messersmith *et al.,* 1995; Puschel *et al.,* 1995; Kobayashi *et al.,* 1997; Varela-Echavarria and Guthrie, 1997; Chedotal *et al.,* 1998; Shepherd and Raper, 1999). Sema3b and Sema3c repel sympathetic ganglia axons (Adams *et al.,* 1997), while Sema3e and Sema3f can collapse growth cones from dorsal root ganglion axons and hippocampal axons, respectively (Chedotal *et al.,* 1998; Miyazaki *et al.,* 1999). *In vivo,* mice deficient in Sema3a display abnormal targeting of nociceptive axons in the spinal cord (Behar *et al.,* 1996), and reduced fasciculation of the trigeminal projection (Ulupinar *et al.,* 1999). However, these are mild anomalies in axonal development considering the large number of *in vitro* studies that demonstrate roles for Sema3a in many regions of the CNS (Catalano *et al.,* 1998; Ulupinar *et al.,* 1999). It is therefore likely that other molecular mechanisms act in concert with Sema3a in these regions of the CNS for accurate axonal guidance (Catalano *et al.,* 1998; Ulupinar *et al.,* 1999).

There is also evidence that semaphorins may have attractive roles during axonal guidance. *In vitro,* increasing concentrations of Sema3c act as a chemoattractant for cortical axons (Bagnard *et al.,* 1998), and Sem3b can act as a chemoattractant for olfactory bulb axons (de Castro *et al.,* 1999). *In vivo,* ectopic expression of Sema1a in grasshopper epithelium steers Ti1 axons toward these regions (Wong *et al.,* 1999). Thus, the concentration of semaphorin relative to other guidance cues, as well as the neuron type involved, may be important determinants of axonal behavior. Additional factors may also interact with the semaphorins to modulate their actionss.

c. Molecules of the Extracellular Matrix Some extracellular matrix proteoglycans have also been implicated as important molecules inhibiting the growth of axons. Proteoglycans are structurally and functionally diverse depending on the type and number of glycosaminoglycans, composition of their protein core, and sulfated position of each carbohydrate moiety (Margolis and Margolis, 1993, 1994). Some proteoglycans are decorated with varying patterns of sulfation (i.e., chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate) and some are not (i.e., heparan and hyaluronate). *In vitro* and *in vivo* studies have shown that secreted chondroitin sulfate proteoglycans (CSPGs) may have important inhibitory influences on axonal outgrowth. *In vitro,* studies of the actions of CSPG in dorsal root ganglion and retinal ganglion cell cultures demonstrate inhibitory influences on cell adhesion, axonal outgrowth, and growth cone motility (Snow *et al.,* 1991, 1994, 1996; Snow and Letourneau, 1992). *In vivo,* CSPG expression patterns correlate with boundary regions that are not crossed by axons in spinal cord, optic tectum, retina, olfactory bulb, somatosensory cortical barrels, and thalamus (Snow *et al.,*

1990; Steindler *et al.,* 1990; Tosney and Oakley, 1990; Brittis *et al.,* 1992; Pindzola *et al.,* 1993; Gonzalez-Romero *et al.,* 1994). Additionally, removal of chondroitin sulfate residues from CSPGs expressed in specific regions of retina demonstrates that CSPGs can function as barriers to axonal growth (Brittis *et al.,* 1992). These inhibitory actions of CSPGs may involve interactions with CAMs, such as L1 and NCAM. The CSPGs neurocan and phosphacan (secreted receptor-type protein tyrosine phosphatase-β) bind to CAMs primarily via their chondroitin sulfate chains, and this binding inhibits cell adhesion and neurite outgrowth (Friedlander *et al.,* 1994; Milev *et al.,* 1994; Retzler *et al.,* 1996). Alternatively, CSPG interactions with CAMs or other growth promoters may suppress these inhibitory effects, since studies have shown that CSPGs promote axonal outgrowth when expressed in combination with strong growth promoters, such as L1 or laminin (Snow and Letourneau, 1992; Bicknese *et al.,* 1994; Dou and Levine, 1994).

B. Soluble Factors That Affect Axonal Arbors

1. Non-Substrate-Bound Factors That Enhance Axonal Outgrowth

In addition to substrate-bound factors that enhance axonal outgrowth and steer axons along them, a number of soluble factors enhance axonal outgrowth and, when presented in a gradient, can act as chemoattractants, causing axons to turn toward them. This chemotropism may act at short range, steering axons toward appropriate targets (Tessier-Lavigne and Placzek, 1991).

a. Netrins The netrins are a family of soluble axonal guidance molecules found in both inverterbrates (UNC-6 in *Caenorhabditis elegans* and netrin A and B in *Drosophila*) and vertebrates (netrin 1, netrin 2, and netrin 3) (Cook *et al.,* 1998; Culotti and Merz, 1998; Tear, 1998; Cooper *et al.,* 1999). Netrins are secreted proteins that have a highly basic carbonic anhydrase domain (C domain), and two domains that are homologous to domain V and VI from the B2 chain of laminin (Fig. 5). The receptors for the vertebrate netrins include the protein encoded by the gene deleted in colorectal cancer (DCC), neogenein, and the product of the nematode gene Unc5h1-3 (Fig. 5). DCC and neogenin contain four immuoglobulin-like domains, six fibronectin type III domains, a transmembrane domain, and an intracellular domain (Fearon *et al.,* 1990; Vielmetter *et al.,* 1994). Unc5h proteins contain two immunoglobulin domains and two thrombospondin type 1 domains extracellularly, a long cytoplasmic domain that has a ZU-5 domain, and a "Death Domain" (Leonardo *et al.,* 1997; Leonardo *et al.,* 1997).

Data obtained both *in vitro* and *in vivo* suggest that netrins have a positive chemotropic influence on axons. *In vitro,* soluble netrin 1 or cells expressing netrin 1 produce chemoattractant effects on axons from spinal cord (Kennedy *et al.,* 1994; Serafini *et al.,* 1994; Shirasaki *et al.,* 1996; Ming *et al.,* 1997; Saueressig *et al.,* 1999), retinal ganglion cells (de la Torre *et al.,* 1997; Hopker *et al.,* 1999),

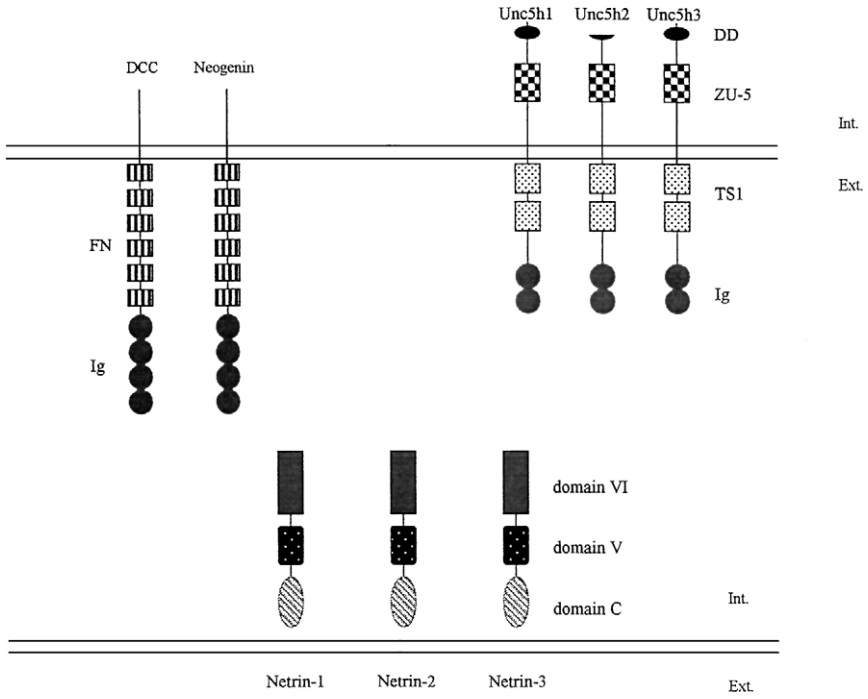


FIG. 5 The structure of netrins and their receptors. The netrin receptors, DCC and neogenin, contain FNIII repeats and Ig domains in the extracellular region similar to axon guidance molecules in the Ig superfamily, while the netrin receptors Unc5h1, Unc5h2, and Unc5h3 contain two thrombospondin type1 repeats and two Ig domains in their extracellular region, and a zonula occludens-like domain (ZU-5) and death domain in the cytoplasmic region. Netrin 1, netrin 2, and netrin 3 are secreted molecules with regions similar to domains V and VI in the B2 chain of laminin, and a highly basic C domain.

and cortical neurons (Richards *et al.,* 1997). In addition, antibody perturbation studies show that the DCC receptor is required for spinal commissural axonal growth toward a netrin 1 source *in vitro,* while netrin 1 is required for corticofugal axonal growth toward the netrin 1 secreting ganglionic eminence *in vitro*. Netrin 3 may also have a role during axonal development because it can bind all of the netrin receptors-DCC, neogenin, and Unc5h1-3, and it can regulate axonal outgrowth of spinal commissural and trochlear motor neurons (Wang *et al.,* 1999). *In vivo,* netrin 1, and DCC deficient mice show defects in axonal trajectories and targeting in the spinal commissure, forebrain commissure, hippocampal commissure, corpus callosum, optic disc, and hypothalamus (Serafini *et al.,* 1996; Deiner *et al.,* 1997; Fazeli *et al.,* 1997; Deiner and Sretavan, 1999; Wang *et al.,* 1999). Thus, netrins and their receptors appear to have important chemoattractant roles during the development of axonal projections in a variety of CNS regions.

In addition to their chemoattractant roles, netrins may also have important chemorepulsive actions on axons. *In vitro,* axons from the cranial motor nerves, such as trochlear, trigeminal, facial, and glossopharyngeal, avoid cells and explants that secrete netrin 1 or netrin 3 (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria *et al.,* 1997; Wang *et al.,* 1999). However, mice deficient in netrin 1 do not show abnormalities in the development of trochlear projections (Serafini *et al.,* 1996). Thus, netrins may not be the only repulsive factors that affect trochlear axonal development *in vivo*. The repulsive effects of netrins may require Unc5 family receptors, since the expression of Unc5 in *Xenopus* spinal neurons converts netrin 1/DCC-dependent chemoattraction into chemorepulsion (Hong *et al.,* 1999), and Unc5h3 mutant mice lack chemorepulsive events required for formation of the rostral cerbellar boundary (Przyborski *et al.,* 1998). Thus, netrins may have bifunctional roles during axonal development dependent on the expression of specific netrin receptors.

b. Neurotrophins The neurotrophins (NGF, BDNF, NT-3 and NT-4/5) are another family of soluble factors that may be important for regulating axonal and dendritic (see below) development. *In vitro,* NGF acts as a chemoattractant for developing neurites in dorsal root ganglion neurons (Gallo *et al.,* 1997), sympathetic neurons (Campenot 1982) cochleovestibular ganglion neurons (Staecker *et al.,* 1996), and hippocampal pyramidal neurons (Brann *et al.,* 1999). Similarly, NT-3 promotes neurite outgrowth in dorsal root ganglion neurons (Dijkhuizen *et al.,* 1997), hippocampal pyramidal neurons (Morfini *et al.,* 1994), and cochleovestibular ganglion neurons (Avila *et al.,* 1993). *In vivo,* NGF overexpression or injection leads to hyperinnervation by sympathetic axons (Avila *et al.,* 1993), while cutaneous overexpression of NT-3 enhances innervation of skin by sensory axons. Innvervation and branching of sympathetic axons into the pineal gland is inhibited in NT-3 -/- and NT-3 +/- mice, respectively (ElShamy *et al.,* 1996). BDNF also has growth promoting, as well as branch-inducing effects on axonal development in many CNS neurons *in vitro* (Avila *et al.,* 1993; Rabacchi *et al.,* 1999) and *in vivo* (Marty *et al.,* 1997; Shieh and Ghosh, 1999). BDNF may also have inhibitory effects on axonal outgrowth in dorsal root ganglion neurons and spinal neurons *in vitro* (Paves and Saarma, 1997; Wang *et al.,* 1998) and sympathetic neurons *in vivo* (Kohn *et al.,* 1999). Thus, neurotrophins may also be important diffusible factors that regulate axonal outgrowth and branching during nervous system development.

c. Neurotransmitters Many different neurotransmitters are present in the developing nervous system, and have been implicated in regulating axonal development. Glutamate is a neurotransmitter that is found throughout the CNS, and activates both ionotropic [α-amino-3-hydroyl-5-methyl-4-isoxazole-propionate (AMPA) and *N*-methyl-D-aspartate (NMDA)] and metabotropic glutamate receptors. In cerebellar granule cell neurons, retinal ganglion neurons, and spinal cord neurons,

NMDA receptor activation promotes axonal outgrowth and branching *in vitro* (Pearce *et al.,* 1987; Cuppini *et al.,* 1999; Heng *et al.,* 1999). In other neuron types activation of NMDA receptors may inhibit neurite outgrowth. For instance, NMDA decreases neurite length in basilar pontine neurons (Baird *et al.,* 1996), and stabilizes retinotectal axons by reducing branching behavior *in vivo* (Rajan *et al.,* 1999). Similarly, activation of AMPA receptors inhibits axonal outgrowth and growth cone activity in spinal cord neurons *in vitro* (Owen and Bird, 1997). Thus, in some tissues glutamate may act as a "stop signal" for axons, so that they can begin synaptogenesis (Baird *et al.,* 1996).

Other neurotransmitters have also been implicated in the regulation of axonal development. Axonal growth cones of spinal motor neurons turn toward higher concentrations in a gradient of acetylcholine *in vitro* (Zheng *et al.,* 1994b). Serotonin causes the filopodia of snail axons to retract (Haydon *et al.,* 1987; Mattson and Kater, 1987) and inhibits neurite outgrowth from retinal explants (Matus *et al.,* 1997), but enhances neurite outgrowth in thalamic neurons (Lieske *et al.,* 1999; Lotto *et al.,* 1999). The effect of neurotransmitters is not specific to excitatory neurotransmitters and is not uniformly stimulatory. γ-Amino butyric acid ($GABA_a$) receptor activation promotes neurite outgrowth and branching in hippocampal neurons (Barbin *et al.,* 1993), while $GABA_b$ receptor activation inhibits neurite outgrowth and growth cone motility in spinal cord neurons (Bird and Owen, 1998). In addition, dopamine acting on D_1 and D_2 dopamine receptors can enhance or inhibit neurite outgrowth in cortical neurons dependent on the concentration used (Reinoso *et al.,* 1996). Thus, the role of neurotransmitters during axonal development may be determined by the neurotransmitter receptor activated, as well as by the concentration of neurotransmitter.

2. Non-Substrate-Bound Factors That Inhibit Axonal Outgrowth

As mentioned in Section II.A.2.b, the semaphorins include both cell-bound and soluble forms. Because the number of membrane-associated semaphorins exceeds the number of soluble forms, they were covered in the context of substrate-associated axonal growth inhibitors.

a. Slit and Robo Slit is a large secreted extracellular matrix protein that has been implicated in the repulsion of axonal growth cones in both invertebrates and vertebrates. Three slit proteins have been identified in vertebrates, Slit1, Slit2, and Slit3, which have four tandem leucine-rich repeats, multiple EGF repeats, and a cysteine knot at the C terminus (Fig. 6) (Brose *et al.,* 1999). The receptor for slit proteins is thought to be the protein encoded by the roundabout gene, Robo. Two robo species have been identified in vertebrates, Robo1 and Robo2, which have five Ig domains followed by three fibronectin type III repeats, and a cytoplasmic domain with proline-rich reigons (Fig. 6) (Kidd *et al.,* 1998). In invertebrates, slit–robo interactions have been implicated as a midline repellent for axons in

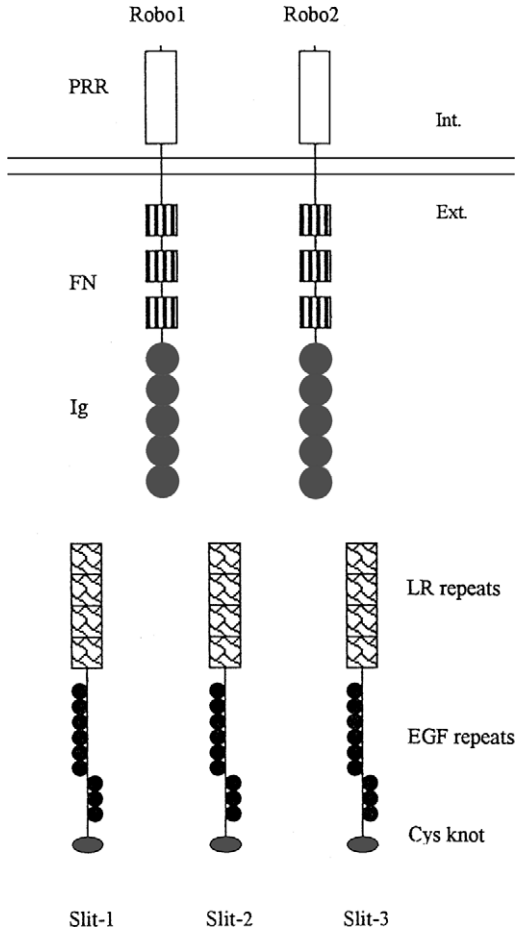


FIG. 6 The structure of Slit proteins and their Robo receptors. Robo1 and Robo2 contain proline-rich regions (PRRs) in their cytoplasmic region, and FNIII and Ig domains in their extracellular region similar to netrin receptors (DCC and neogenin), CAMs (L1, NCAM, TAG 1), and RPTPs (CRYPα1 and CRYPα2). Slit1, Slit2, and Slit3 are secreted proteins with leucine-rich repeats (LRRs), multiple EGF repeats, and a cysteine knot at the C terminus, which may be involved in dimerization.

Drosophila and *C. elegans* CNS (Kidd *et al.,* 1998; Zallen *et al.,* Battye *et al.,* 1999; Kidd *et al.,* 1999). In vertebrates, Slit2 expressing cells can repel olfactory bulb, hippocampal, and spinal motor axons (Liang *et al.,* 1998; Brose *et al.,* 1999; Nguyen Ba-Charvet *et al.,* 1999), while slit induces growth cone collapse of axonal growth cones from olfactory bulb explants (Nguyen Ba-Charvet *et al.,* 1999). However, slit proteins may also promote axonal outgrowth and branching, since recombinant human Slit2 increases the elongation and branching of dorsal root

ganglion axons *in vitro* (Wang *et al.,* 1999). Thus, slit–robo interactions may have important chemorepellent and chemoattractant roles during axonal outgrowth in the vertebrate CNS.

III. Factors Regulating Dendritic Arborization

Several technical factors have limited our knowledge about the factors that regulate dendritic outgrowth relative to the factors that regulate axonal outgrowth: dendrites grow much more slowly than do axons, they develop later than do axons, and they are much closer to the cell body (and to each other), so it is more difficult to study strictly local effects. However, factors that impinge on dendritic outgrowth and branching are equally as important as those that affect axons, since it is critical that the size and configuration of the dendritic arbor match that of the axonal arbor. It is clear that some of the features of the dendritic arbor are programmed into the cell; for example, hippocampal pyramidal neurons will elaborate dendrites in isolated cell culture that are reminiscent of those seen *in vivo* (Dotti *et al.,* 1988; Goslin and Banker, 1989), even when they are dissected from an embryo before extensive process outgrowth has occurred. However, it is equally clear that extracellular factors can play an extensive role in controlling dendritic arbors; for example, Purkinje neurons, which have an elaborate two-dimensional dendritic arbor in the cerebellum, elaborate a much simpler arbor in tissue culture, which, while constrained to be two dimensional by the culture dish, has limited resemblance to that seen *in vivo* (Fig. 7).

The factors that modulate the growth of dendrites can be broadly classified into factors that affect overall arborization and factors that affect the density and configuration of dendritic spines. Many of the same factors that modulate dendritic outgrowth and branching may also affect dendritic spines. It is difficult to draw a single uniform model, since studies of dendritic motility have been performed in systems that range over a variety of different cell types and preparations (*in vivo,* in long-term organotypic cultures, and in dissociated cell cultures at either high or low cell density). However, it appears in general that the growth of dendrites (or presumptive dendrites) can be divided into three different phases as neurons mature, either *in vivo* or *in vitro* (Craig and Banker, 1994; Dunaevsky *et al.,* 1999; Wu *et al.,* 1999). In the first phase (polarization), neurons bear an array of different short, highly dynamic processes, one of which is specified as the presumptive axon and begins to elongate rapidly. The remaining processes—presumptive dendrites—are largely unbranched and highly plastic (Craig and Banker, 1994), although they extend multiple lateral filopodia (Dailey and Smith, 1996). In the second phase (dendritic maturation), the dendritic arbor grows rapidly (Craig and Banker, 1994; Wu *et al.,* 1999), both

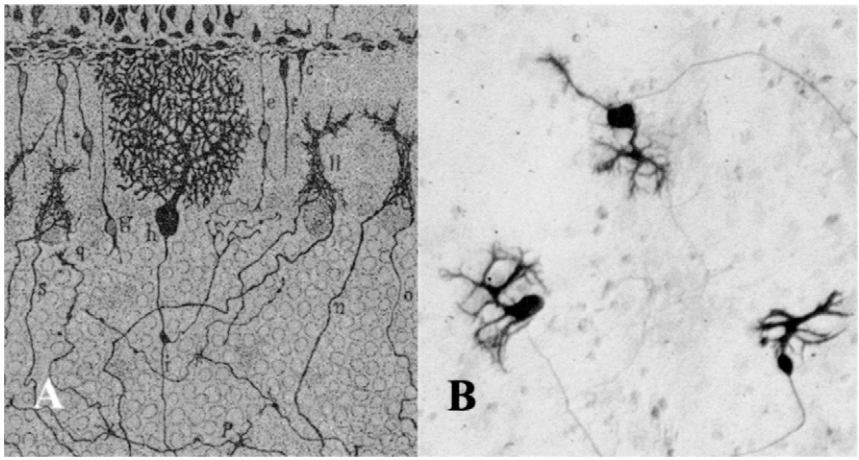


FIG. 7 Comparison of the dendritic arbor of a Purkinje neuron of a 16-day-old puppy (A— cell h), as drawn by S. Ramon y Cajal (Ramon y Cajal, 1895), to those of three Purkinje neurons cultured from embryonic rats and maintained in culture for 19 days (B—a gift of John Connor, Department of Neurosciences, University of New Mexico Medical Center). Note that the dendritic arbor of Purkinje neurons in culture, while generally similar to that *in vivo,* is subtantially simpler and less constrained.

through increases in length of individual dendrites and because of the maturation of lateral filopodia into new dendritic branches (Dailey and Smith, 1996). In the third phase, the dendritic arbor appears to stabilize (Wu and Cline, 1998), with total dendritic branch length increasing slowly (Wu *et al.,* 1999). However, at this third stage, the dendritic spines (and presumably other postsynaptic specializations), although in general persistent, are still quite dynamic, and new spines and dendritic branches continue to be initiated, giving the overall dendritic arbor a good deal of structural plasticity (Dunaevsky *et al.,* 1999; Wu *et al.,* 1999).

In general, there seems to be a gradient whereby factors that affect both dendritic branching and dendritic spine development tend to modulate branching at earlier and spine density at later developmental times. For example, postsynaptic electrical activity appears to promote the normal initial branching of *Xenopus* tectal dendrites (Rajan and Cline, 1998; Maletic-Savatic *et al.,* 1999). Activity also can influence both spine density and overall dendritic branch patterns in the mammalian cortex *in vivo* and in slice preparations (Annis *et al.,* 1994; Dalva *et al.,* 1994; Baker *et al.,* 1997). By contrast, at later times, when long-term potentiation is extensively induced, the primary structural effect of stimulation is on the density and structure of dendritic spines (Andersen and Trommald, 1995; Papa and Segal, 1996; Collin *et al.,* 1997; Schuster *et al.,* 1998). There are, however, many exceptions to this general rule, as in the relatively aspinous magnocellular neurosecretory cell (MNC) of the rat supraoptic nucleus, which show

substantial suckling-induced expansion (vasopressin-producing MNC) or contraction (oxytocin MNC) of their dendritic arbors in adult females (Stern and Armstrong, 1998).

As with axons, in addition to factors that encourage or modulate the form of dendritic outgrowth, there are factors that cause active retraction of dendritic spines or dendritic branches. These factors may come into play both in normal development and in pathological conditions, and may profoundly affect the form of the dendritic arbor, and hence neuronal activity. For example, during development of the rat neocortex, the pyramidal cells of layer V of the visual cortex initially form apical dendrites that extend to layer I. In subsequent development, those neurons that project through the corpus callosum retract their apical dendrites from the more superficial layers of the visual cortex, whereas those that project to the tectum do not, giving rise to two functional classes of cortical projection neurons (Koester and O'Leary, 1992). Additionally, retraction of dendritic spines or remodeling of dendritic arbors has been reported in models of neuropathological conditions such as epilepsy (von Campe *et al.,* 1997; Jiang *et al.,* 1998), Alzheimer's disease (Flood and Coleman, 1990; Anderton *et al.,* 1998), Parkinson's disease (Arendt *et al.,* 1995), and dementia (Catala *et al.,* 1988) as well as in normal aging (Flood and Coleman, 1993; Norris *et al.,* 1998b).

As with axons, one of the major factors that can lead to dendritic spine and branch retraction is one of the factors that can also lead to increased dendritic outgrowth: exposure to neurotransmitters and concomitant depolarization (Morrison and Mason, 1998; Shimada *et al.,* 1998; Wilson and Keith, 1998; Okada *et al.,* 1999). This suggests that neurotransmitters may be involved in multiple phases of dendritic growth modulation—from initial stimulation of outgrowth (Wilson and Keith, 1998), to titration of dendritic growth against synaptic activity, all the way to dendritic spine and branch collapse resulting from excess exposure to excitatory neurotransmitters (Halpain *et al.,* 1998; Bravin *et al.,* 1999). In other words, as described by McEachern and Shaw (1996, 1999), there is a continuum between dendritic plasticity and pathology, and a given stimulus may fit onto that continuum at various points, depending on stimulus paradigm, neuronal age, and various other factors. An understanding of the downstream details of these pathways—including second messenger cascades and cytoskeletal alterations that lead to observed effects on dendritic structure—will vastly enhance our understanding of and our control over the elemental mechanisms that lead to nervous system function and pathology.

Like the factors the modulate axonal outgrowth and arborization, the factors that affect dendritic arborization include both substrate-bound and soluble factors, which can act either to encourage or inhibit dendritic arborization. Of particular interest is that many factors show different effects on dendrites than they do on axons, and thus they may either enhance neuronal polarization by encouraging the growth of axons and inhibiting dendritic outgrowth, or they may suppress polarization by selectively inhibiting axonal outgrowth.

A. Substrate-Associated Factors That Affect Dendritic Arbors

1. Substrate-Bound Factors That Enhance Dendritic Outgrowth

a. Dendritic Shafts

i. Substrate Adhesion As with axons, substrate adhesion appears to play at least a permissive role in dendritic outgrowth. Most neurons do not thrive on untreated glass, but rather require that it be treated with nonspecific adhesive molecules, such as polylysine, polyornithine, or polyethyleneimine, or with specific extracellular matrix (ECM) molecules, or with both (Higgins and Banker, 1998). Dendritic outgrowth, in particular, appears to be quite sensitive to the adhesivity of the substrate (Prochiantz, 1995). Neurons mature more rapidly on the highly negative substrate polyethyleneimine than on polylysine (Lelong *et al.,* 1992), and this is reflected in the earlier outgrowth and maturation of the dendritic arbor on polyethyleneimine (Wilson and Keith, 1998) than on polylysine (Craig and Banker, 1994). Additionally, the growth of hippocampal neurons has been examined on mixed substrates of a nonspecific adhesive molecule and the ECM glycoprotein laminin. In these experiments, the concentration of laminin was held constant and the adhesivity of the substrate was varied by varying the concentration of polyornithine, or by masking polyornithine with serum albumin. It was found that the rate of growth of the minor processes—presumptive dendrites—was enhanced on the more adhesive substrata, but that the growth of major processes—axons—was unaffected (Lochter *et al.,* 1995). However, it has not yet been determined whether dendrites will orient up a gradient of substrate adhesivity. Accordingly, it is not known whether the role of substrate adhesivity is permissive or instructive for dendritic outgrowth (Lemmon *et al.* 1992).

ii. Molecules of the Extracellular Matrix Aside from molecules that promote adhesion, most of the ECM molecules that have been found to modulate dendritic outgrowth do so in a negative manner, and will be covered below. However, a number of molecules commonly associated with the ECM can enhance dendritic outgrowth. An interesting example is the proteoglycan dermatan sulfate, which is often associated with extracellular matrix and which measurably increases both the number and average length of the dendrites of some cortical neurons in culture (Lafont *et al.,* 1992). However, this enhancement does not require that that sugar be bound to the substrate, as enhancement is observed when the cultures are exposed to soluble fragments of dermatan sulfate either before or after being plated on the substrate (Lafont *et al.* 1994).

Another ECM-associated molecule that appears to specifically enhance dendritic outgrowth is the matrix associated growth factor osteogenic protein-1 (OP-1), also known as bone morphogenetic protein-7 (BMP-7) (Lein *et al.,* 1995), as well as certain other members of the bone morphogenetic protein family (Guo *et al.,* 1998). OP-1 is a soluble proteinaceous growth factor that has a high affinity for

type IV collagen, and is therefore found in association with basement membranes (Vukicevic *et al.,* 1994; Lein *et al.,* 1996). Its dendrite-promoting activity was originally discovered when it was recognized that a urea extract of the artificial basement membrane produced by the EHS tumor line (trade name Matrigel) had potent dendrite-promoting activity toward sympathetic neurons (Lein and Higgins, 1989). Subsequent investigations revealed that OP-1 was the component of this extract that increased both the fraction of cultured sympathetic neurons bearing dendrites and the number of dendrites per neuron (Lein *et al.,* 1995; Lein *et al.,* 1996), and other studies have indicated that it can increase the average length of dendrites grown from cultured hippocampal neurons (Higgins and Banker, 1998).

Two final examples of ECM-associated proteins that enhance dendritic outgrowth are the soluble fragment of the β-amyloid precursor protein, sAPPα, and the intact (cell surface) β-amyloid precursor protein (βAPP) (Mattson, 1997). The soluble fragment is released from cells in response to electrical activity and binds to proteins of the extracellular matrix, where it both enhances neurite outgrowth and serves a neuroprotective function (Mattson, 1994; Small *et al.,* 1994). Cell-surface βAPP expressed in nonneuronal cells enhances minor process outgrowth from cocultured embryonic hippocampal neurons (Qiu *et al.,* 1995). The function of βAPP has been somewhat difficult to assess in detail because it is both produced by and affects neurons. However, recent studies with hippocampal neurons derived from APP-deficient mice have revealed that intact APP enhances axonal outgrowth, whereas sAPPα both enhances dendritic outgrowth and increases the number of dendrites produced by hippocampal neurons in culture (Perez *et al.,* 1997).

It is likely that other proteinaceous ECM molecules that enhance dendritic outgrowth remain to be identified. It is well established that astrocytes—particularly homotypic astrocytes—enhance dendritic outgrowth by cortical neurons (Tropea *et al.,* 1988; Le Roux and Reh, 1995, 1996). This property is shown even by reactive astrocytes, which actively inhibit axonal outgrowth (Le Roux and Reh, 1996). While the enhancement of dendritic growth is in part due to diffusable factors, it also appears to be in part due to substrate effects (Le Roux and Reh, 1994). It is notable that integrins alpha8beta1 (Einheber *et al.,* 1996) and alphaVbeta8 (Nishimura *et al.,* 1998) are restricted to the dendritic arbors in many parts of the brain, and that antibodies to beta1 integrins inhibit dendritic outgrowth from cultured sympathetic neurons (Lein and Higgins, 1996). Because integrins are often associated with productive cell–substrate interactions, it is possible that these integrins interact with as yet unidentified ECM molecules to enhance dendritic outgrowth. Alternatively, they may mediate the effects of extracellular matrix on dendritic spines (see below).

iii. CPG15 In addition to ECM molecules and molecules that associate with the extracellular matrix, dendritic outgrowth may be modulated by cell surface molecules, particularly those presented on ingrowing afferent axons. Particularly notable in this respect is the protein CPG15, which is the gene product of cpg15

(candidate plasticity gene 15) (Nedivi *et al.,* 1996). CPG15 appears to be an activity-induced GPI-anchored protein expressed on axons and neuronal cell bodies throughout the CNS in vertebrates (Nedivi *et al.,* 1998; Corriveau *et al.,* 1999). Expression of CPG15 in *Xenopus* tectum leads to promotion of dendritic outgrowth in neighboring tectal projection neurons, without affecting axons (Nedivi *et al.,* 1998). Presumably, under normal circumstances CPG15, expressed in an activity-dependent manner on the surface of ingrowing retinal ganglion cells, may be responsible for inducing activity-dependent changes in the dendritic arbor of target tectal neurons. It is also observed that entorhinal axons enhance the formation of dendritic branches of hippocampal neurons growing over them, and do so in an activity-independent fashion. That enhancement of branching appears to be due to cell surface molecules expressed in the axons, since it is specific to the dendrites that form contact with the bed (Kossel *et al.,* 1997).

b. Dendritic Spines In addition to the evidence for enhancement of dendritic outgrowth specifically by extracellular matrix-associated proteins, there is solid evidence for an effect of extracellular matrix on dendritic spines. Immunolocalization indicates that the astrocyte-secreted extracellular matrix molecule laminin alpha-2 is extensively localized to the dendritic spines of the cortex, and its levels are upregulated during periods of synaptogenesis (Tian *et al.,* 1996, 1997). Furthermore, in cerebellar cultures that have been depleted of granule cells and glia, the addition of laminin causes the proliferation of dendritic spines (Seil, 1998). The latter observation is particularly interesting in light of the observation that laminin enhances axonal outgrowth and does not affect the initial growth of the dendritic arbor in both sympathetic neurons (Lein and Higgins, 1989) and hippocampal neurons (Lein *et al.,* 1992) in culture. This dichotomy suggests that the growth of the dendritic trunk and the growth of dendritic spines are separately regulated events (Seil, 1998). A potentially related observation is that expression of the spine-localized cell-surface heparan sulfate proteoglycan syndecan-2 induces the maturation of dendritic filopodia in cultured hippocampal neurons. It has been suggested that this induction of spine maturation is due to an autoendocrine effect of the extracellular domain of syndecan-2 on the spine (Ethell and Yamaguchi, 1999).

2. Substrate-Bound Factors That Inhibit Dendritic Outgrowth

a. Dendritic Shafts

i. Molecules of the Extracellular Matrix As mentioned in the introduction to this section, most molecules of the extracellular matrix that enhance axonal outgrowth tend to be inhibitory or to be without effect on dendritic outgrowth. Accordingly, in cultured neuronal systems, most ECM molecules tend to enhance the development of neuronal polarity, leading either to unipolar neurons, in the cultured sympathetic neuron model system (Lein *et al.,* 1992), or to neurons that

have a much more extensive axonal than dendritic arbor, in the cultured hippocampal neuron model system (Lein *et al.,* 1992). Thus, when sympathetic neurons are cultured in serum-free medium on simple adhesive substrata, such as polylysine, they tend to develop one axon-like process and no dendrites (Bruckenstein and Higgins, 1988a; Lein *et al.,* 1992); in the presence of serum (Bruckenstein and Higgins, 1988b) or OP-1 (Lein *et al.,* 1995), they grow dendrites. Laminin both increases the number of axon-like processes extended by these neurons and the rate of their growth, thus increasing neuronal polarity. In cultured hippocampal neurons, laminin causes a significant increase in the rate of growth of the axon-like major process (Lein *et al.,* 1992; Lochter and Schachner, 1993), either without consequence on the rate of dendritic outgrowth (Lein *et al.,* 1992; Craig and Banker, 1994), or inhibiting their growth (after 12 hr in culture) (Lochter and Schachner, 1993). Recent results indicate that the enhancement of major process outgrowth, and any concomitant (and, in these experiments, temporary) consequences in terms of dendritic outgrowth, occurs at the level of the growth cone of that major process, rather than at the level of the whole cell. Other ECM glycoproteins, such as tenascin, fibronectin, and janusin, seem to exhibit similar influences on hippocampal neuron polarity, enhancing major process outgrowth while eithr inhibiting or not affecting minor processes (presumptive dendrites) (Lochter and Schachner, 1993; Lochter *et al.,* 1994).

In addition to the ECM molecules mentioned above, other molecules associated with the extracellular matrix may have profound effects on the establishment and regulation of neuronal polarity. Proteoglycans, including dermatan sulfate (see Section II.A.1.a.ii), are found throughout the developing nervous system (Wight *et al.,* 1992; Margolis and Margolis, 1994). Many of these proteoglycans appear to inhibit process outgrowth altogether or to function in a fashion opposite to that of dermatan sulfate: to enhance axonal outgrowth (Wang and Denburg, 1992) and inhibit dendritic development and outgrowth (Lafont *et al.,* 1992). In particular, heparan sulfate, and defined syntheitic heparan-sulfate-like oligosaccharides, profoundly inhibit the initiation and outgrowth of dendrites from cortical neurons, while enhancing the growth of their axons (Lafont *et al.,* 1994). This effect of heparan sulfate on neuronal polarity appears to be specific to cortical neurons, as the growth of axon-like and dendrite-like processes from spinal motorneurons is unaffected by these oligosaccharides (Lafont *et al.,* 1994). Unlike the effect of extracellular matrix glycoproteins above, the effect of proteoglycans on cortical neuronal polarity appears to involve signaling through the somatodendritic compartment of the neurons (Calvet *et al.,* 1998), and to require internalization of the sugar molecules (Lafont *et al.,* 1994). (Transductional pathways for all molecules that modulate dendritic outgrowth and development are dealt with below.)

ii. Other Insoluble Species There is a significant amount of evidence that extracellular deposits of insoluble β-amyloid, giving rise to amyloid plaques, can substantially affect dendritic arborizations in Alzheimer's disease or animal models thereof (for example, Flood and Coleman, 1990; Arendt *et al.,* 1995; Beeri

et al., 1997). Unfortunately, there is less agreement as to whether the dendritic arbor is expanded or collapsed, and whether those morphological changes occur selectively in basal or apical dendrites. Part of the variation in observed effects may arise because insoluble β-amyloid and, presumably, other plaque-forming species, appear to be toxic toward neurons (Mattson, 1997) in addition to any actions they may have as proteins of the extracellular matrix.

b. Dendritic Spines There is also considerable agreement that Alzheimer's, as well as other neurodegenerative diseases such as scrapie, can substantially reduce dendritic spine density (Catala *et al.,* 1988; Flood and Coleman, 1990; Jeffrey *et al.,* 1997; Garey *et al.,* 1998; Wozniak *et al.,* 1998; Seabrook *et al.,* 1999), and that such changes may play a significant role in the cognitive deficits seen in these diseases. However, it is clear that the cell death seen in cultured hippocampal neurons exposed to insoluble β-amyloid is largely a toxic response that is oxidative in nature (see, for example, Mattson, 1997, 1999; Guo *et al.,* 1999a, 1999b). It is therefore quite possible that the loss of dendritic spines is due to this toxic response rather than to effects of the extracellular matrix surrounding the dendrites. Besides these data related to pathological conditions, little is known about the role(s) of substrate-bound inhibitory factors that are involved in dendritic spine formation.

B. Soluble Factors That Influence Dendritic Arbors

1. Non-Substrate-Bound Factors That Enhance Dendritic Outgrowth

a. Dendritic Shafts

i. Neurotrophins There is a long history that indicates that, in addition to their effects on axons, the neurotrophins, acting either alone or in combination with other factors, can promote dendritic outgrowth in a wide variety of neurons *in vivo* and *in vitro*. Thus, surgical manipulations of sympathetic inputs have indicated that the dendrites of sympathetic neurons can develop even in the absence of afferent input (Voyvodic, 1987), and that target-derived nerve growth factor (NGF) is at least in part responsible for this afferent-independent dendritogenesis (Snider, 1988; Ruit *et al.,* 1990; Ruit and Snider, 1991). *In vitro,* however, NGF alone supports only axonal outgrowth from sympathetic neurons; additional factors, such as the aforementioned substrate-adherent growth factor OP-1 (Lein *et al.,* 1995), are required to encourage dendritic outgrowth (Bruckenstein and Higgins, 1988; Tropea *et al.,* 1988; Lein *et al.,* 1995, 1996).

In slices of developing cerebral cortex, perfusion with any of the four neurotrophins (NGF, BDNF, NT-3, and NT4/5) leads to the expansion of dendritic arbors in specific layers and areas (McAllister *et al.,* 1995; Baker *et al.,* 1998). The reverse manipulation—treatment of slices with agents (Trk-IgG fusion proteins) that deplete specific neurotrophins—leads to the selective contraction of dendritic

arbors. The neurotrophins appear to act in concert with other factors—either electrical activity of the target neurons or activity-dependent factors—because inhibition of electrical activity in these slice preparations in various ways abolishes the effect of neurotrophins (McAllister *et al.,* 1996).

Cortical neuronal tissue culture systems also present evidence that soluble neurotrophins can enhance dendritic outgrowth from central neurons. One example that has already been discussed is the soluble, but frequently substrate-adherent OP-1, which specifically enhances dendritic outgrowth from hippocampal neurons in culture (Higgins and Banker, 1998). Additionally, there is solid evidence that both BDNF and NT-3 can increase the complexity of the dendritic arbor established by embryonic day 16 rat hippocampal neurons in culture. Curiously, of the two neurotrophins, only BDNF also enhances axonal branching, implying that NT-3, like OP-1, can act as a specific trophic factor for dendrites in this particular model system (Vicario-Abejon *et al.,* 1998). Recent evidence indicates that overexpression of BDNF by layer 4 cortical pyramidal neurons induces rapid sprouting of their basal dendrites through an autocrine mechanism (Horch *et al.,* 1999). Finally, there is evidence that all neurotrophins can enhance the initial outgrowth of prepolarization minor processes from hippocampal neurons by signaling though the pan-neurotrophin low-affinity (p75) receptor (Brann *et al.,* 1999).

ii. Neurotransmitters Dendritic outgrowth from many neurons can be enhanced by activity, as elicited by exposure to appropriate excitatory neurotransmitters, and the depolarization resulting from it. Depolarizing stimuli may either act alone or in combination with neurotrophins to modulate filopodial extension, dendritic outgrowth, and dendritic branching from neurons. Thus, in primary dissociated cells of mouse Purkinje neurons, dendrites elongate during the first week in culture, but thereafter, as electrical activity develops, the growth rate of individual processes slows, but they then begin to branch, so that total dendritic branch length increases. If, however, these cultures are exposed to tetrodotoxin, outgrowth continues without branching (Schilling *et al.,* 1991). Similarly, when cultured hippocampal neurons are bathed in glutamate, the rate of outgrowth of individual presumptive dendrites is initally enhanced (Wilson and Keith, 1998), and when dendrites are exposed focally to electric fields, both filopodial sprouting and the conversion of filopodia into dendritic branches are locally enhanced (Meberg *et al.,* 1999). In *Xenopus* optic tectum, blocking the NMDA class of glutamate receptors with aminophosphovalerate (APV) significantly inhibits increases in the total dendritic branch length of simple neurons, apparently by inhibiting the addition of new branches (Rajan and Cline, 1998; Rajan *et al.,* 1999). In hippocampal slices, it is found that synaptic activity causes rapid filopodial sprouting, which also appears to be due to the activation of NMDA receptors (Maletic-Savatic *et al.,* 1999). In adult female rats, lactation, which increases excitation of the supraoptic nucleus of the hypothalamus, causes expansion of the dendritic arbor of vasopressin-secreting magnocellular neurosecretory cells (Stern and Armstrong, 1998). Finally, in cats deprived of binocular stimulation in early development, dendritic asymmetries of

spiny stellate cells near the boundaries of ocular dominance columns are markedly reduced (Kossel *et al.,* 1995).

Additionally, in many of the studies of the effect of neurotrophins on dendritic outgrowth, it is found that neurotrophins and neurotransmitters interact to cause an effect on dendritic architecture. It is in fact entirely possible that some of the growth enhancements listed in the discussion of neurotrophins are due to the combination of activity and neurotrophins, since the Purkinkje neurons were grown in serum (Schilling *et al.,* 1991), the hippocampals in the presence of astrocytes (Wilson and Keith, 1998), and the intact animal and hippocampal slices would have had various growth factors present. The enhancement of dendritic and dendritic spine outgrowth by a combination of electrical activity and neurotrophins—provided that neurotrophin release is activity triggered—is particularly noteworthy because it represents a molecular mechanism that may explain how coincident electrical activity in the presynaptic and postsynaptic neurons of a synapse may lead to structural synapse enhancement (McAllister *et al.,* 1999). Such activity-dependent neurotrophin release has been demonstrated in a number of systems. Depolarization of hippocampal neurons with or, in an immature state, with GABA enhances their expression of BDNF and NGF (Thoenen *et al.,* 1991; Berninger *et al.,* 1995). Additionally, as mentioned in section *i,* above, the enhancement of dendritic outgrowth in ferret cortical slices by neurotrophins requires electrical activity (McAllister *et al.,* 1996, 1999), and the NT-3-mediated enhancement of pyramidal neuron dendritic outgrowth in rat cortical slice requires spontaneous electrical activity (Baker *et al.,* 1997, 1998).

iii. Semaphorins A recent study (Polleux *et al.,* 2000) has demonstrated that semaphorin 3a (Sema3a), mentioned earlier as an inhibitor of cortical axon outgrowth, can act as a chemoattractant for the apical dendrites of cortical pyramidal cells. In an electronic supplement to that study (Polleux *et al.,* 2000) and in earlier studies (Polleux *et al.,* 1998, Skaliora *et al.,* 1998), it was also demonstrated that Sema3a maximally expressed in the cortical plate in late embryonic and early postnatal rat cortex. Sema3a is therefore well suited to serve as a major morphogenetic factor for pyramidal cortical neurons (Polleux *et al.,* 2000).

b. Dendritic Spines Both neurotrophins and electrical activity can affect the density of dendritic spines in spiny neurons and, presumably, of direct axodendritic (and axosomatic) synapses in aspiny neurons. As with dendritic outgrowth, they tend to intract with each other, which makes the analysis of their individual actions complex.

Although there are abundant indications that electrical activity can modulate the density of dendritic spines on spiny neurons, the evidence in intact animals is mixed as to the direction of such modulation. In visual cortex and in other areas of the brain, the onset of sensory stimulation is correlated with increased spine density (Walsh, 1981; Juraska, 1982), and inhibition of activity by sensory deprivation or deafferentation is correlated with reduced spine density (Deitch and

Rubel, 1984; McMullen and Glaser, 1988; Bryan and Riesen, 1989; Zafirov *et al.,* 1994). On the other hand, blockade of input activity by infusion of tetrodotoxin (Dalva *et al.,* 1994; Bravin *et al.,* 1999) or by climbing fiber depletion in the cerebellum (Sotelo *et al.,* 1975; Baetens *et al.,* 1983) increases spine density on Purkinje neuron dendrites.

With *in vitro* preparations, the evidence largely tends to indicate that activity tends to lead to increases in spine density. In slice preparations, electrical activity, whether generated electrically or through disinhibition, is correlated with an increase in spine density (Annis *et al.,* 1994; Collin *et al.,* 1997). In cultured cortical neurons, spine density is decreased by treatments that inhibit electrical activity (van Huizen *et al.,* 1985; Kossel *et al.,* 1997) and is increased by multiple direct and indirect treatments that increase electrical activity (van Huizen *et al.,* 1987; Schilling *et al.,* 1991; Morrison and Mason, 1998; Murphy *et al.,* 1998a).

Because neurotrophins can interact with electrical activity to cause changes in circuits and in direct effects on dendrites, the analysis of their mode of action is extremely complex in intact animals, in slice preparations, and even in dissociated cell cultures that contain multiple cell types. For example, estradiol causes a dramatic increase in the density of dendritic spines on CA1 hippocampal neurons *in vivo* (Woolley and McEwen, 1992; McEwen and Woolley, 1994; McEwen *et al.,* 1995), in hippocampal slice preparations (Woolley *et al.,* 1997); and in dissociated hippocampal cultures (Murphy and Segal, 1996). Additionally, estradiol can increase the density of dendritic spines on the granule cells that form the principal excitatory input to the CA1 pyramidal neurons (Miranda *et al.,* 1999). It appears that the increase in spine density on the CA1 neurons comes about because estradiol down-regulates the neurotrophin BDNF, and that other treatments that lower BDNF can produce similar responses in cultured hippocampal neurons (Murphy *et al.,* 1998b). However, it now appears likely that the up-regulation of spines following depletion of BDNF occurs because BDNF increases the production of glutamic acid decarboxylase (GAD) and hence GABA in inhibitory interneurons in the cultures. These inhibitory interneurons then synapse on hippocampal pyramidal neurons, lowering their activity. Lowering BDNF down-regulates these interneurons, thereby increasing activity in the culture, and it is this increase in activity that is proposed to modulate spine density (Murphy *et al.,* 1998a, 1998b). Since the granule cells have no estrogen receptors, the same or a similar indirect pathway is likely to hold true for them (Miranda *et al.,* 1999).

With cautions about the complexity of analysis in mind, a number of data indicate that the same types of factors that enhance dendritic outgrowth can, under other circumstances (later in development), lead to an increase in the density of dendritic spines. Dendritic outgrowth and increases in spine density seem to create a complementary relationship, so that the same stimulus at a given point in development may either cause dendritic sprouting or an increase of spine density, but is unlikely to do both. (In fact, an increase in dendritic sprouting is generally correlated with a decrease in spine density; Horch *et al.,* 1999.)

Under the proper conditions neurotrophins—particularly BDNF, but in some cases NT-3 as well—may directly modify the density of dendritic spines and other postsynaptic specializations with or without electrical activity. For example, in cocultures of purified cerebellar granule and Purkinje cells, BDNF causes an increase in the density of surviving Purkinje cell dendritic spines, without altering the complexity of their dendritic arbor (Morrison and Mason, 1998; Shimada *et al.,* 1998). Additionally, in cultures of embryonic day 16(E16) rat hippocampal pyramidal neurons, BDNF induced the formation of both excitatory and inhibitory synapses, whereas NT-3 induced only excitatory synapses (Vicario-Abejon *et al.,* 1998). Finally, in electron microscope immunolocalization studies, TrkB, the receptor for BDNF, is found on dendritic spines on hippocampal pyramidal cells and interneurons (Drake *et al.,* 1999). Thus, neurotransmitters, neurotrophins, and other factors involved with changes in electrical activity may regulate either dendritic outgrowth or spine formation depending on the circumstances.

2. Non-Substrate-Bound Factors That Inhibit Dendritic Outgrowth

In addition to dendritic outgrowth, dendritic retraction is also a part of the normal developmental program of the nervous system. For example, the initial extension of rat phrenic dendrites throughout the spinal white matter is subsequently remodeled into the typical rostrocaudal arborization seen at birth (Allan and Greer, 1997). In zebrafish, the initial extension of dendrites to the ventral aspect of the tectum is subsequently pruned back and remodeled as a much limited arborization (Kaethner and Stuermer, 1997). Finally, rat Purkinje cells initially extend multiple primary dendrites; most of these initial dendrites subsequently retract, leaving the cells with their characteristic single apical primary dendrite (see Fig. 7) (Armengol and Sotelo, 1991). Additionally, dendritic retraction—and the retraction of dendritic spines—can also occur in response to non-substrate-bound factors in neuropathological conditions. Some of the factors that cause dendritic retraction do so by their absence; the lack of a factor such as a neurotrophin that is reponsible for enhancing normal dendritic outgrowth and maintenance can cause dendritic retraction. Other mediators, however, seem specifically to cause dendritic retraction when present, and to do so in a manner specific to neuronal cell types.

a. Neurotrophins A particularly interesting and potentially developmentally relevant case of dendritic outgrowth comes from studies of the effect of neurotrophins on dendritic growth in cerebral cortical slices. While, as previously mentioned, the growth of most dendrites is enhanced by BDNF, the growth of basal dendrites in layer 6 is inhibited by that neurotrophin and enhanced by NT-3 (McAllister *et al.,* 1995). Subsequent studies in which neurotrophins were selectively depleted with

IgG/Trk fusion proteins have expanded on that theme, and indicated that in layer 4 of the visual cortex the opposite pattern holds: NT-3 inhibits the growth of basal dendrites, so that its depletion enhanced their outgrowth.

b. Cytokines Another group of factors that can inhibit dendritic outgrowth in a fashion that may be relevant to their function in the developing animals are the neuropoetic cytokines, ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF). These cytokines cause the retraction of OP-1-induced sympathetic neuron dendrites without reducing cell viability (Nobes and Tolkovsky, 1995; Guo *et al.,* 1997, 1999). Induction of dendritic retraction *may* be specific to sympathetic neurons, since the related cytokine interleukin-6 (IL-6), and IL-6/receptor complex, promotes general neurite outgrowth from enteric neurons (Schafer *et al.* 1999), and it only minimally affects dendritogenesis by hippocampal neurons (Gadient *et al.,* 1998). The effect of LIF/CNTF on sympathetic neurons is likely to be relevant to their normal development, since a CNTF-like cytokine produced by sweat glands induces the transdifferentiation of sympathetic neurons into cholinergic neurons (Fann and Patterson, 1994; Landis, 1996; Cheng and Patterson, 1997; Habecker *et al.,* 1997).

c. Neurotransmitters Paradoxically, one further factor that can induce dendritic retraction is electrical activity resulting from the exposure of that neuron to its neurotransmitter. Neurotransmitter-induced dendritic retraction may be particularly important in some forms of neurodegenerative damage. Thus, when hippocampal neurons are exposed to modest levels of bath-applied glutamate for extended periods, the dendrites begin to retract, while the axons continue to grow (Mattson *et al.,* 1988a, 1988b, 1988c; Reese and Drapeau, 1998). On particularly adhesive substrata, this retractive phase may be seen following a period of enhanced dendritic outgrowth in the same cells (Wilson and Keith, 1998; Wilson *et al.,* 2000). Similarly, in rat motoneurons, glutamate can inhibit dendritic outgrowth in a dose-dependent and reversible manner without reducing cell viability (Metzger *et al.,* 1998). In primary cultures from mouse neocortex, hypoxia or exposure to NMDA causes segmental dendritic beading and retraction of dendritic spines, even under protocols that result in little cell death (Park *et al.,* 1996), and exposure of cultured hippocampal neurons to brief pulses of glutamate causes spine collapse (Halpain *et al.,* 1998). Both dendritic spines and branches appear to be reduced, both in postmortem sections of epileptic humans (Isokawa and Levesque, 1991; Isokawa and Mello, 1991; Multani *et al.,* 1994; Belichenko and Dahlstrom, 1995; Isokawa *et al.,* 1997) and in animal models of focal epilepsy (Paul and Scheibel, 1986; Jiang *et al.,* 1998). Therefore, neurotransmitters, such as glutamate, and growth factors, such as neurotrophins, CNTF, and LIF, can negatively regulate dendritic outgrowth or stability during development and disease.

IV. Transduction Mechanisms in the Control of Axonal Arborization

The pattern of axonal outgrowth is determined by signaling events in the growth cone, which is the motile enlargement at the distal end of the axon. (Dendritic outgrowth may involve both growth cone-specific events and somatodendritic compartment-wide events—see below.) Growth cone motility is generated by the protrusion and retraction of filopodial and lamellar membranous structures where the net effect of these membrane movements determines the direction of growth (extension, turning, retraction, or stalling; Mueller, 1999). The structural components that facilitate growth cone motility are proteins that make up the cytoskeleton. In the growth cone, actin filaments (microfilaments, MFs) extend into the filopodia and form a meshwork near the plasma membrane within the lamellipodia, while microtubules (MTs) fill the central region of the growth cone and interact with the peripherial located MFs (Burgoyne, 1991). A variety of MF (actin depolymerization factor, cofilin, profiling, and gelsolin) and MT (MAPs; tau, MAP2, and MAP4) associated proteins regulate the assembly of MFs and MTs, as well as the interaction between MFs and MTs (Burgoyne, 1991). The activation of signaling pathways that regulate the activity of these cytoskeletal proteins is thought to be the mechanism by which guidance molecules manipulate the pattern of axonal and dendritic development.

An array of signaling pathways has been implicated in the regulation of growth cone motility including Ca^{2+}-linked, cyclic nucleotide-linked, receptor tyrosine kinase, and receptor protein tyrosine phosphatase pathways. The role of Ca^{2+} signaling pathways in the regulation of growth cone motility and neurite outgrowth has been intensely studied during the last two decades (Kater *et al.,* 1988; Kater and Mills, 1991; Neely and Nicholls, 1995; Goldberg and Grabham, 1999). These pathways include calcium-dependent regulation of the activity of a variety of signaling and cytoskeletal proteins, such as kinases (Goldberg and Wu, 1994; Tang and Wang, 1996; Daniels and Bokoch, 1999), phosphatases (Maness *et al.,* 1996; Stoker and Dutta, 1998; Gallo and Letourneau, 1999a; Morioka *et al.,* 1999), MF-binding proteins (Sobue, 1993; Neely and Nicholls, 1995; Letourneau, 1996), and MAPs (Tucker, 1990; Gordon-Weeks, 1993; Mattson, 1999). Thus, it is not suprising that many axonal guidance molecules mediate their effects on growth cone motility through Ca^{2+}-dependent mechanisms (see below). 3′-5′ Cyclic adenosine monophosphate (cAMP) is also a widely studied signaling element in growth cone motility, and in many systems, cAMP signaling works in concert with Ca^{2+}-dependent pathways (see below). Less is konwn about the signaling cascades that facilitate G-protein, receptor tyrosine kinase, and receptor protein tyrosine phosphatase regulation of growth cone motility. However, a role for the Rho family of monomeric GTPases in many of these pathways is beginning to emerge.

The Rho family of small GTPases has been implicated in the regulation of actin filament dynamics during cell adhesion and motility in many cell types (Hotchin and Hall, 1996; Tapon and Hall, 1997; Hall, 1998). In neurons, both *in vitro* and *in vivo* studies have implicated the Rho family GTPases in the regulation of axonal outgrowth by a variety of axonal guidance molecules (Luo *et al.,* 1997; Gallo and Letourneau, 1999a). Injection of specific GTPases that are constitutively active or are dominant-negative mutants cuase distinct effects on growth cone motility depending on the neuron type and guidance cue involved. In PC12 pheochromocytoma and N1E-115 neuroblastoma cells, Cdc42 and Rac promote the formation of lamellipodia and filopodia, as well as neurite outgrowth, while Rho induces growth cone collapse and inhibits neurite outgrowth (Kozma *et al.,* 1997; Lamoureux *et al.,* 1997; Kranenburg *et al.,* 1999). In contrast semaphorin-induced collapse of DRG axonal growth cones involves Rac1, while constitutively active Rho increases DRG growth cone spreading, but decreases the rate of neurite outgrowth (Jin and Strittmatter, 1997). In addition, there is evidence the Rac and Rho signaling pathways may have antagonistic interactions during neurite outgrowth (Leeuwen *et al.,* 1997). *In vivo,* the expression of constitutively active and dominant-negative mutant GTPases in *Drosophila* causes an array of abnormal effects on axonal development (Luo *et al.,* 1997). Thus, axonal guidance molecules may activate a variety of signaling pathways that utilize GTPases (Rho, Rac, and Cdc42) to coordinate the activity of effectors, such as signaling and cytokeletal proteins, in a manner that produces specific patterns of growth cone motility (see examples below).

A. Calcium-Dependent Signaling Pathways

Cell adhesion molecules, such as N-cadherin, NCAM, and L1, may mediate many of their effects on axons through interactions with fibroblast growth factor receptors (FGFR) that lead to changes in Ca^{2+} influx (Viollet and Doherty, 1997). Activation of FGFR through interactions with CAMs is thought to cause activation of phospholipase Cγ (PLCγ), as well as production of diacylglycerol (DAG) and arachidonic acid (AA). AA-induced Ca^{2+} influx through N- and L-type calcium channels may activate calmodulin-dependent protein kinase II, as well as other downstream effectors, such as GAP-43 and protein phosphatases, leading to modulation of neurite outgrowth (Doherty and Walsh, 1994; Klinz *et al.,* 1995; Saffell *et al.,* 1997; Lom *et al.,* 1998; Meiri *et al.,* 1998; Archer *et al.,* 1999). Alternatively or in concert, tyrosine and serine-threonine phosphorylation pathways may also be involved in calmodulin-induced changes in neurite outgrowth. Both NCAM and N-cadherin have been shown to cause MAP kinase-dependent changes in neurite outgrowth (Perron and Bixby, 1999; Schmid *et al.,* 1999). In the case of NCAM, recruitment and tyrosine phoshorylation of local adhesion kinase (FAK) may activate the Ras-MAP kinase pathway leading to ELK-1 or CREB activation and modulation of neurite outgrowth promoting genes (Perron and Bixby, 1999;

Schmid *et al.,* 1999). L1 has also been shown to interact with alphaVbeta3 integrin receptors and promote neurite outgrowth in dorsal root ganglion neurons (Yip *et al.,* 1998). Integrin signaling pathways also activate FAK, as well Ras-MAP kinase pathways (Giancotti and Ruoslahti, 1999) that regulate gene transcription. In addition, direct interactions between specific CAMs and the cytoskeleton may work in concert with these intracellular pathways to regulate growth cone behviors (Takei *et al.,* 1999). Thus, CAMs may use multiple signaling mechanisms to altter the pattern of axonal outgrowth.

Netrin regulation of axonal outgrowth involves both Ca^{2+}-dependent and cAMP-dependent signaling pathways (Ming *et al.,* 1997; Hopker *et al.,* 1999). In *Xenopus* spinal neurons, axonal growth cone turning toward a netrin 1 source requires extracellular Ca^{2+}, is converted to repulsion by inhibition of cAMP or PKA, and is blocked by antibodies against DCC (Ming *et al.,* 1997). In addition, laminin may convert netrin 1 chemoattraction to chemorepulsion through reduction in cAMP levels (Hopker *et al.,* 1999). Similarly, many neurotransmitters may regulate axonal outgrowth through Ca^{2+}-dependent and cAMP-dependent pathways (Mattson *et al.,* 1988c; Neely and Nicholls, 1995). Acetylcholine-induced growth cone turning involves both Ca^{2+} influx and cAMP dependence (Song *et al.,* 1994; Zheng *et al.,* 1994b), while glutamate activation of AMPA or NMDA receptors can regulate axonal outgrowth in a Ca^{2+}-dependent fashion (Cuppini *et al.,* 1999; Heng *et al.,* 1999).

Neurotrophins (BDNF, NGF, NT-3, and NT-4) regulate axonal outgrowth through activation of tyrosine kinase receptors (TrkA, TrkB, TrkC, and p75), and in some cases both Ca^{2+}-dependent and cAMP-dependent pathways may be involved downstream of tyrosine phosphorylation (Song *et al.,* 1997; Wang and Zheng, 1998; Ming *et al.,* 1999). In *Xenopus* spinal neurons, increases in cAMP or inhibition of Ca^{2+} influx suppress BDNF-induced growth cone collapse, while competitive analogs of cAMP and inhibition of PKA convert BDNF-induced attraction into repulsion. Similarly, NGF-induced growth cone attraction requires extracellular Ca^{2+} and inhibition of PKA converts this attraction into repulsion. In both cases, the Ca^{2+}-mediated and cAMP-mediated regulation of growth cone behaviors may involve the activity of PI3 kinase and PLCγ (Iwasaki *et al.,* 1998; Ming *et al.,* 1999). In addition, BDNF and NGF activation of Trk receptors associated with p75 may regulate Rho-dependent pathways, since neurotrophin binding to p75 inhibits Rho activation and promotes neurite outgrowth in ciliary neurons (Yamashita *et al.,* 1999). Thus, BDNF and NGF may use similar pathways to modulate growth cone motility. On the contrary, NT-3 regulation of neurite outgrowth may involve Ca^{2+}-independent pathways (Song *et al.,* 1998). NT-3 acts as a chemoattractant for *Xenopus* axonal growth cones *in vitro*. This chemoattraction is converted to chemorepulsion by inhibiting cGMP or PKG activity, but is unaffected by manipulation of cAMP or Ca^{2+} pathways. These differences in signaling pathways activated by NT-3 relative to NGF/BDNF are most easily reconciled by binding specific receptors, TrkC receptors relative to TrkA/TrkB receptors.

B. Calcium-Independent Signaling Pathways

The receptors for semaphorins include homo- and heterocomplexes formed by two families of transmembrane proteins: neuropilins (Chen *et al.,* 1997; He and Tessier-Lavigne, 1997; Kolodkin, 1998) and plexins (Winberg *et al.,* 1998; Tamagnone *et al.,* 1999). Neuropilins (neuropilin-1 and neuropilin-2) contain multiple extracellular domains that include complement binding domains, coagulation factor-like domains, and a mephrin- and A5 glycoprotein-like (MAM) domain that may be important for neuropilin–neuropilin homo- and heterointeractions (Fig. 3). Plexins (plexins A1-4, B1-3, C1, and D1) contain a semaphorin domain and multiple cysteine rich met-related sequences in the extracellular region, and an intracellular domain containing multiple tyrosine phosphorylation sites (Fig. 3). Neuropilin-1 binds with high affinity to semaphorin 3a (Sema3a), Sema3c, and Sema3f, and both *in vitro* and *in vivo* studies demonstrate that neuropilin-1 is a receptor for Sema3a (He and Tessier-Lavigne, 1997; Kitsukawa *et al.,* 1997; Chen *et al.,* 1998a; Nakamura *et al.,* 1998). Neuropilin-2 binds Sema3b, Sema3c, and Sema3f, but not Sema3a, and mediates the repulsive effects of Sema3f *in vitro* (Chen *et al.,* 1997; Giger *et al.,* 1998; Takahashi *et al.,* 1998). In grasshopper, plexin A binds Sema1a and Sema1b *in vitro,* and plexin A loss-of-function mutants show similar abnormalities as Sema1a mutants. Additionally, plexin A overexpression disrupts axonal guidance *in vivo* (Winberg *et al.,* 1998). Human plexin C1 binds Sema7a, plexin B1 binds Sema4d, and class 3 semaphorins will not bind plexins (Tamagnone *et al.,* 1999). However, plexin A1, A3, and B1 can associate with either neuropilin-1 or neuropilin-2 (Tamagnone *et al.,* 1999). In addition, plexin A1 and neuropilin-1 form a stable complex that binds with a higher affinity to Sema3a, and overexpression of dominant-negative plexin A1 in sensory neurons blocks Sema-3a-induced growth cone collapse (Takahashi *et al.,* 1998). Thus, the existence of multiple neuropilin and plexin homo- and heterocomplexes may be an additional factor in determining the role of specific semaphorins during axonal development.

The semaphorin receptors may activate diverse pathways to regulate axonal outgrowth. As with NT-3, Sema3a regulation of neurite outgrowth may involve Ca^{2+}-independent pathways, such as cGMP and G-protein pathways (Igarashi *et al.,* 1993; Song *et al.,* 1998). In dorsal root ganglion (DRG) neurons, Sem3a-induced growth cone collapse can be inhibited by the G-protein inhibitor, pertussis toxin, or by activation of cyclic guanosine monophosphate (cGMP) or protein kinase G (PKG). Similarly, cGMP or PKG activation, but not low extracelluar Ca^{2+}, convert Sema3a-induced chemorepulsion to chemoattraction in *Xenopus* spinal neurons. However, blocking Ca^{2+} influx can suppress Sema3a-induced growth cone collapse in mouse DRG neurons (Behar *et al.,* 1999). Thus, calcium may be involved in pathways that cause growth cone collapse, but may have lesser roles in growth cone turning away from Sema3a. Downstream of Ca^{2+} and cGMP pathways, Rac1, Cdc42, and collapsing response mediator protein (CRMP-62) have

been implicated in Sema3a-induced growth cone collapse in DRG and motor neurons (Goshima *et al.,* 1997; Jin and Strittmatter, 1997; Kuhn *et al.,* 1999). These effectors may target specifically the microfilament cytoskeleton, since Sema3a-induced growth cone collapse results from rapid depolymerization of actin filaments (Fritsche *et al.,* 1999; Kuhn *et al.,* 1999). In addition to growth cone collapse, some semaphorins (Sema1a, Sema3b, Sema3c, Sema3e) can promote axonal outgrowth (Bagnard *et al.,* 1998; de Castro *et al.,* 1999; Sakai *et al.,* 1999; Wong *et al.,* 1999). In PC12 cells, Sema3e-induced neurite outgrowth requires Ca^{2+} influx, as well as activation of Ras-MAP kinase pathways (Sakai *et al.,* 1999). Thus, the signaling pathways involved in semaphorin-regulated axonal outgrowth may be quite diverse depending on the neuron type and the neuropilin/plexin receptor activated.

Eph receptors and their ephrin ligands modulate (inhibit) axonal outgrowth through many pathways that are associated with tyrosine kinase receptor signaling. Tyrosine phosphorylation of Eph receptors after ephrin binding facilitates binding of SH2 domain containing proteins, including PI3 kinase and PLCγ. Both PI3 kinase and PLCγ may play roles in mediating neurite outgrowth in PC12 and neuroblastoma cells (Kimura *et al.,* 1994; Inagaki *et al.,* 1995; Sarner *et al.,* 2000). In neuroblastoma cells, EphB2 forms a trimolecular complex that consists of $p62^{dok}$, Ras-GAP, and Nck (Holland *et al.,* 1997). Ras-GAP is bound to a GTPase activating protein for Rho (p190 Rho-GAP), which acts as a negative regulator of Rho (McGlade *et al.,* 1993). In addition, EphB2 also interacts with a novel protein, SHEP1, which binds the GTPases R-Ras and Rap 1 A (Dodelet *et al.,* 1999). This implicates the Ras-MAP kinase pathway in Eph receptor regulation of axonal outgrowth. However, ephrin-induced growth cone collapse in retinal ganglion neurons appears to involve a different Ca^{2+}-independent pathway: a $G_{o/i}$ pathway (Nakayama *et al.,* 1999). This type of collapse requires the microtubule-associated protein kinase (cdk5) and its phosphorylation of the prominent axonal microtubule-associated protein tau. The latter interacts with both microtubuless and microfilaments (Kempf *et al.,* 1996) and has been implicated in neuritogenesis (Caceres and Kosik, 1990). Changes in tau phosphorylation affect its microtubule binding characteristics (Bramblett *et al.,* 1993) and, hence, the efficiency of linkage between the microtubular and microfilamentous cytoskeleton. Similar to semaphorins, then, the ephrin signaling involved in regulation of axonal outgrowth may include an array of signaling pathways which are determined by the neuron type and receptors activated.

Receptor tyrosine phosphatases (RPTPs) are another family of receptors that appear to be important for regulating axonal development. In *Drosophila,* three axonal RPTPs, DPTP99A, DPTP69D, and DALR, are required for targeting of several motor neuron axons to their appropriate muscles *in vivo* (Desai *et al.,* 1996; Krueger *et al.,* 1996). In vertebrates, RPTPs that have been implicated in the regulation of axonal development include RPTPβ, RPTPδ, RPTPμ, RPTPκ, and CRYPα. RPTPs contain intracellular protein tyrosine phosphatase domains,

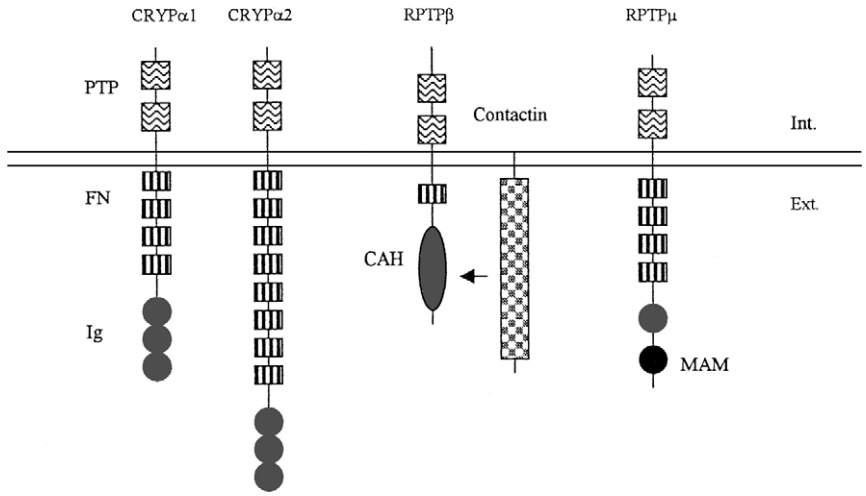


FIG. 8 The structure of four vertebrate receptor protein tyrosine phosphatases (RPTP). All of these RPTPs have protein tyrosine phosphatase (PTP) domains in the cytosplasmic region. CRYPα isoforms have multiple FNIII and Ig repeats in the extracellular region similar to CAMs. RPTPβ has a carbonic anhydrase domain (CAH) in the extracellular region that may allow heterophilic interactions with the GPI-anchored contactin. RPTPμ contains multiple FNIII repeats, an Ig domain, and a MAM domain that may be involved in homophilic interactions.

and extracellular FNIII repeats, Ig domains, and some cases carbonic anhydrase domains and MAM domains (Fig. 8). RPTPβ is a glial RPTP expressed in short and long transmembrane forms, and in an aforementioned secreted form (phosphacan), which contains an extracellular carbonic anhydrase domain, a unique spacer region (short form), and glycosaminoglycan side chains (phosphacan and long form) (Peles *et al.,* 1998). It binds to a variety of molecules implicated in the control of axonal development, including contactin, tenascin, Ng-CAM, Nr-CAM, N-CAM, and TAG1 (Peles *et al.,* 1998). *In vitro,* RPTPβ interactions with contactin and Nr-CAM promote neurite outgrowth (Sakurai *et al.,* 1997; Peles *et al.,* 1998). CRYPα is a RPTP found in two isoforms, CRYPα1 and CRYPα2, and expressed on retinal axons and growth cones at early stages of development (Stoker *et al.,* 1995; Ledig *et al.,* 1999). *In vitro,* CRYPα antibodies perturb retinal axonal outgrowth along retinal basal membranes (Ledig *et al.,* 1999). In addition, RPTPδ (Wang and Bixby, 1999) and RPTPκ (Drosopoulos *et al.,* 1999) promote neurite outgrowth *in vitro* in forebrain and cerebellar neurons, respectively, while RPTPμ promotes retinal ganglion cell neurite outgrowth and also potentiates N-cadherin-dependent retinal ganglion cell neurite (Burden-Gulley and Brady-Kalnay, 1999). Thus, vertebrate RPTPs appear to be important regulators of axonal development *in vitro,* but further studies are required to determine the role of RPTPs during axonal development *in vivo*.

V. Transduction Mechanisms in the Control of Dendritic Arborization

Most of the factors that modulate the growth of dendrites and dendritic spines are factors that have effects on other neuronal and nonneuronal systems and on the initial stages of the transductory pathways used in other cell types. Unfortunately, in dendrites, knowledge of downstream targets and pathways is not nearly as well advanced as in axons.

A. Calcium-Dependent Signaling Pathways

1. Dendritic Outgrowth

As with axons, calcium is probably the intracellular second messenger in the regulation of dendritic growth about which most is known. Many of the extracellular signals listed above can give rise to changes in intracellular free-calcium levels. Thus, the common CNS neurotransmitter can interact with NMDA receptors, which are calcium-permeable ionotropic receptors, and can directly increase calcium conductance (Burnashev, 1996, 1998). Additionally, however, the AMPA/kainate class of glutamate receptors can cause an elevation of intracellular free calcium, either directly, through ionotropic receptors (Metzger *et al.,* 1998; Sakurai *et al.,* 1998; Okada *et al.,* 1999), or indirectly, by activating voltage-gated calcium channels at subthreshold levels (Dailey and Smith, 1994; Mills *et al.,* 1994; Christie *et al.,* 1996; Seamans *et al.,* 1997). Similarly, other excitatory neurotransmitters, such as serotonin (Lieske *et al.,* 1999) and acetyl choline (Egorov and Muller, 1999), can cause or contribute to elevation of calcium levels by similar mechanisms.

There is significant evidence that calcium is involved in the activity-dependent enhancement of dendritic outgrowth and arborization (Schilling *et al.,* 1991; Mattson, 1992, 1996; Hentschel and Fine, 1996; Metzger *et al.,* 1998; Meberg *et al.,* 1999), and of dendritic spine outgrowth (Fifkova, 1985; Maletic-Savatic *et al.,* 1999). Additionally, calcium appears to be involved in the excitoxic retraction of dendrites (Mattson *et al.,* 1988a, 1988b, 1988c, 1989; Mattson and Kater, 1989; Mattson, 1992, 1996; Song *et al.,* 1994; Johnston *et al.,* 1998; Okada *et al.,* 1999) and of dendritic spines (Murphy and Segal, 1996; Papa and Segal, 1996; Halpain *et al.,* 1998) induced by excessive activity. Because the same intracellular second messenger appears to trigger both dendritic and spine outgrowth and retraction, the targets activated by the increase in $[Ca^{2+}]_i$ must determine the consequence of calcium influx.

When stage 2 hippocampal neurons in culture are exposed to modest levels of glutamate, the initial response is an enhancement of the outgrowth of minor processes and the filopodia thereof (Mattson, 1992, 1996; Wilson and Keith, 1998). This initial enhancement is correlated with a transient elevation of $[Ca^{2+}]_i$ and is

inhibited by the anticalmodulin agent calmidazolium (Wilson *et al.,* 2000). Furthermore, tetanic stimulation of presynaptic inputs leads to increased accumulation of calmodulin-dependent protein kinase II (CaMKII) (total and autophosphorylated) in dendrites (Ouyang *et al.,* 1997, 1999; Steward and Halpain, 1999), indicating that the total activity of calmodulin-dependent kinases is substantially up-regulated by activity. There is substantial evidence that activity can lead to increased dendritic filopodial sprouting (Maletic-Savatic *et al.,* 1999) and increases in dendritic spine density (van Huizen *et al.,* 1987; Schilling *et al.,* 1991; Morrison and Mason, 1998; Murphy *et al.,* 1998a). It can therefore be hypothesized that glutamate, acting through either NMDA or non-NMDA receptors, causes a rise in intracellular free calcium that activates, among other mediators, CaMKII, and that this may lead to dendritic or dendritic spine outgrowth (Halpain and Greengard, 1990; Mattson, 1992, 1996; Muller and Connor, 1992; Quinlan and Halpain, 1996b; Okada *et al.,* 1999; Pozzo-Miller *et al.,* 1999).

A similar enhancement of dendritic outgrowth can be seen when hippocampal neurons, plated on a bed of entorhinal axons, are exposed to focal electrical field stimulation by an extracellular electrode: at the part of the dendrite closest to the electrode, local filopodial sprouting is induced. When the field is applied for a longer period, consolidation of the filopodia into dendritic branches is also focally enhanced. Furthermore, focal increases in intracellular calcium are temporally and spatially Correlated with the regions of enhanced dendritogenesis, and blocking calcium influx blocks the enhancement of sprouting (Meberg *et al.,* 1999). In those studies the downstream effectors were not assayed but by analogy to the above cases, it is likely to involve calmodulin and calmodulin-dependent kinases (Maletic-Savatic *et al.,* 1999).

It has further been shown that in neonatal mammals (where dendritic sprouting tends to predominate), or in cultures established from young animals, activity gives rise to a net increase in the phosphorylation of the prominent dendritic cytoskeletal protein MAP2 (Fukunaga *et al.,* 1995, 1996; Quinlan and Halpain, 1996a, 1996b). Similarly, in mammalian olfactory bulb neurons, activity seems to induce increased phosphorylation of MAP2 (Philpot *et al.,* 1997). By contrast, in the intact hippocampus of adult rats, activity induces an initial burst of MAP2 phosphorylation, which is followed by MAP2 dephosphorylation (Halpain and Greengard, 1990; Quinlan and Halpain, 1996b; Angenstein *et al.,* 1999). Both phosphorylation and dephosphorylation in adults are linked to calmodulin activation. As in neonates, the phosphorylation appears to come about because of CaMKII activation; the subsequent dephosphorylation is then hypothesized to occur because of the activation of the calmodulin-dependent phosphatase calcineurin (Quinlan and Halpain, 1996b). It has been suggested that this change from net increases to net decreases in MAP2 phosphorylation on activity correlates with reductions in dendritic plasticity (Aoki and Siekevitz, 1985), because dephosphorylated MAP2 binds more efficiently to microtubules, increasing their stability (Quinlan and Halpain, 1996b). In neonates, and in olfactory bulb, where neurons

constantly turn over, activity increases MAP2 phosphorylation, which increases dendritic plasticity; in older animals, however, activity decreases MAP2 phosphorylation, which stabilizes dendritic arbors, and may lead to increases in spine density (Aoki and Siekevitz, 1985; Quinlan and Halpain, 1996a; Philpot *et al.,* 1997). This correlation of plasticity with decreases in phosphorylation, however, may be specific to mammalian tissue. In *Xenopus* optic tectum, older neurons, which have more stable dendritic arbors, have higher levels of CaMKII than do younger neurons, suggesting that in this system increases in kinase activity may stabilize dendritic arbors. Furthermore, in that system, the CaMKII antagonist KN93 causes an overall increase in total dendritic branch length, and overexpression of CaMKII results in premature stabilization of dendritic arbors (Wu and Cline, 1998; Wu *et al.,* 1999).

There is also evidence that other elements of the microtubular cytoskeleton may also be involved in driving dendritic outgrowth, either by effects on microtubule polymerization or through interactions with the microfilamentous cytoskeleton. For example, inhibition of the microtubule motor protein CHO1/MKLP1 is essential for dendritic development (Sharp *et al.,* 1997; Baas, 1998). Furthermore, the microtubule-associated protein MAP2, whose phosphorylation is affected by activity (Quinlan and Halpain, 1996a), is colocalized with actin in dendrites and dendritic spines (Morales and Fifkova, 1989). Additionally, excitatory amino acid stimulation of cortical neurons increases the extent of cytoskeletal association of MAP2 (Bigot and Hunt, 1990). It is quite possible that changes in MAP2 localization and phosphorylation may affect the linkage between microtubules and microfilaments, resulting in alterations in the pattern of neurite outgrowth (Joshi *et al.,* 1985; Letourneau *et al.,* 1987; Dennerll *et al.,* 1988, 1989; Lankford and Letourneau, 1989; Heidemann and Buxbaum, 1991; Heidemann *et al.,* 1995).

Further details of the mechanisms by which the signaling cascades affect the activity of cytoskeletal proteins during dendritic motility have not been fully worked out. In terms of general process outgrowth, it is known that actin is involved in motility (Letourneau, 1996) and that its polymerization is affected by calcium (Lankford and Letourneau, 1989). In dendritic growth cones (as in axonal growth cones), both actin and actin-binding proteins are highly enriched (Markham and Fifkova, 1986; Bassell *et al.,* 1998; Micheva *et al.,* 1998; Horch *et al.,* 1999; Mattson, 1999; Norenberg *et al.,* 1999). There is evidence that actin and actin-binding proteins such as myosin are involved in dendritic outgrowth (Mattson, 1999; Wylie, 1998), and extensive evidence that they are involved in dendritic spine motility (Hayashi *et al.,* 1996; Dunaevsky *et al.,* 1999; Hayshi and Shirao, 1999; Kaech *et al.,* 1999). Additionally, there is evidence that stimulation that causes growth cones to retract is correlated with a loss of growth cone microfilaments (Lankford and Letourneau, 1989; Bernstein and Bamburg, 1992; Neely, 1993; Neely and Geseman, 1994). These signaling pathways may involve small Rho-family GTPases (Threadgill *et al.,* 1997; Kuhn *et al.,* 1998),

but the details of their activity have not yet been worked out. It is known that the expression of factors that down-regulate Rho, Rac, and/or Cdc42 inhibit dendritic outgrowth from cortical neurons, and the expression of constitutively active forms of these GTPases results in dendritic outgrowth (Threadgill *et al.,* 1997).

2. Dendritic Retraction

At higher levels or over a more protracted time course neurotransmitter exposure can cause retraction of dendrites and dendritic spines, as well as cell death, in models of excitoxicity (Choi, 1992, 1995). Thus, in human epileptic patients and in animal models of epilepsy, there is a significant reorganization in the dendritic arbors of a variety of neuronal cell types (Pettigrew *et al.,* 1996; von Campe *et al.,* 1997; Zhu *et al.,* 1997; Jiang *et al.,* 1998). Furthermore, in cultured hippocampal neurons, exposure to excitoxic stimuli at modest levels causes dendritic retraction while sparing axons (Mattson *et al.,* 1988a, 1988b, 1988c; Mattson and Kater, 1989; Mattson, 1992, 1996; Wilson and Keith, 1998). The effect of excitoxins on dendritic spines is also dramatic, and occurs in a more rapid time frame than effects on the overall dendritic arbor. Accordingly, hypoxia or treatment of experimental animals with kainate induces dendritic varicosities and loss of spines within hours of exposure (Olney *et al.,* 1979; Choi, 1995; Rothman and Olney, 1995). Exposure of cultured hippocampal neurons to glutamate or NMDA causes a rapid (within 5 min) and extensive loss of dendritic spines, correlated with a depolymerization or redistribution of their filamentous actin (Halpain *et al.,* 1998; Norenberg *et al.,* 1999; van Rossum and Hanisch, 1999); these effects are correlated with influx of calcium into the spines (Fifkova, 1985; Papa and Segal, 1996; Halpain *et al.,* 1998).

In addition to the extreme effects seen in excitotoxicity, normal levels of electrical activity may lead to reductions of dendritic spine density. For example, the proximal region of the Purkinje cell dendritic arbor has an extremely low density of dendritic spines, which can routinely be detected only by electron microscopy (Larramendi and Victor, 1967). However, when the climbing fiber input to these Purkinje cells is eliminated, the proximal regions of the Purkinje cells develop a large number of spines (Sotelo *et al.,* 1975). This same heterospinous transformation is mimicked—in a reversible manner—by eliminating electrical activity in the adult rat cerebellum by infusing tetrodotoxin (Bravin *et al.,* 1999). This suggests that spine formation is an inherent property of Purkinje cell dendrites and that it is drastically reduced by electrical activity in the climbing fibers (Baptista *et al.,* 1994; Bravin *et al.,* 1999).

Beyond calcium influx, multiple transductory pathways have been implicated in the retraction of dendrites and dendritic spines induced by excessive or prolonged calcium influx. There is an extensive literature indicating that the calcium-activated protease, calpain, is activated following experimentally or naturally

induced seizure or focal ischemia (Bi *et al.,* 1996; Minger *et al.,* 1998; Liebetrau *et al.,* 1999; Lipton, 1999), and that glutamate receptor agonists can reduce that activation (Minger *et al.,* 1998). It has further been noted that calpain inhibitors can protect against the necrotic sequelae of seizure (Wang and Yuen, 1997; Tsuchiya *et al.,* 1999) and of ischemia (Chen, Z., *et al.,* 1997; Wang and Yuen, 1997; James *et al.,* 1998; Markgraf *et al.,* 1998; Yokota *et al.,* 1999). Furthermore, calpain inhibitors block the calcium-induced retraction of the dendrites of neurons in culture (Song *et al.,* 1994; Wilson *et al.,* 2000). This retraction of dendrites appear to come about, at least in part, because calpain affects microtubules, either directly or indirectly (Pettigrew *et al.,* 1996; Minger *et al.,* 1998; Wilson *et al.,* 2000), although other cytoskeletal elements may certainly be involved (James *et al.,* 1998; Domanska-Janik *et al.,* 1999).

In addition to activating calpains, excess influx of calcium can activate other intracellular processes that may lead to dendritic retraction. The activation of caspases in excitotoxicity begins in the dendrites and spreads to the cell body (Mattson *et al.,* 1998; Duan *et al.,* 1999), and this activation can have effects below the threshold inducing cell death (Chan *et al.,* 1999). Since caspases, like calpains, are cysteine proteases that can act on abundant cytoskeletal proteins such as actin (Kayalar *et al.,* 1996; Chan *et al.,* 1999), it may be supposed that subthreshold activation of caspases might cause retraction of dendrites and dendritic spines (Mattson and Duan, 1999).

The mechanisms by which increased calcium influx causes a reduction in the number of dendritic spines or the collapse of individual spines have some overlap with the mechanisms by which dendritic branch retraction is caused. However, calcium may also lead to reductions in spine density and/or individual spine collapse by mechanisms other than the activation of calpains or caspases. For example, rat hippocampal neurons maintained in low density culture for 18–23 days show extenive spine-like f-actin-rich punctae, which collapse rapidly on exposure to 50 μM NMDA. This collapse is fully blocked by the preincubation of cultures in the NMDA receptor antagonists MK-801 or D-amino-5-phosphonopentanoic acid (AP-5), and could be mimicked by treatment of the cultures with the calcium ionophore ionomycin. In addition, spine collapse is attenuated by preincubation of cells with the calcineurin inhibitor ascomycin, suggesting that this calmodulin-dependent phosphatase may be involved in the pathway. Spine collapse appears to involve the microfilamentous cytoskeleton, since preincubation of the cells with the membrane-permeant f-actin-stabilizer jasplakinolide prevents spine loss (Halpain *et al.,* 1998). The f-actin linkage to spine motility may involve myosin as a motor and the actin-binding protein drebin, which is localized to the dendritic spines of rat cortical neurons, where it complexes with actin and myosin (Hayashi *et al.,* 1996). Deletion of the actin-binding portion of drebin prevents its localization to spines, and overexpression of native drebin modulates the shape of spines (Hayashi and Shirao, 1999).

B. Calcium-Independent Signaling Pathways

As mentioned, neurotrophins can act alone or in combination with electrical activity to modulate dendritic outgrowth and dendritic spine outgrowth (Snider, 1988; McAllister *et al.,* 1995, 1996; 1999; Snider and Lichtman, 1996; Shimada *et al.,* 1998; Vicario-Abejon *et al.,* 1998; Lu and Chow, 1999). Studies of the effect of neurotrophins on dendritic and dendritic spine outgrowth have largely indicated that signaling pathways through the corresponding tyrosine kinase receptors (Trk A for NGF, Trk B for BDNF, and NT4/5, and trk C for NT3) are largely responsible for such effects (McAllister, Katz *et al.,* 1996; McAllister, Katz *et al.,* 1997; Morrison and Mason, 1998; Shimada *et al.,* 1998; Lu and Chow, 1999). Downstream signaling from the receptors presumably involves the Ras-MAP Kinase cascade, and may involve CREB activation (Lewin and Barde, 1996; Segal and Greenberg, 1996; Segal and Murphy 1998), but the downstream details of the signalling cascades are not known.

In addition to signaling through the tyrosine kinase receptors, neurotrophins can also signal through the low-affinity p75 neurotrophin receptor. Recent evidence indicates that such signalling may enhance the initial growth of minor processes from hippocampal neurons (Brann, Scott *et al.,* 1999), as it enhances neurite outgrowth in general (Kontny, Ciruela *et al.,* 1997; Kimpinski, Jelinski *et al.,* 1999; Pollack, Young *et al.,* 1999), and that this signaling may involve modulation of the production of sphingelin by the neutral sphingomyelinase (Brann, Scott *et al.,* 1999). However, as for the high-affinity neurotrophin receptors, downstream mechanistic details of this pathway are not known.

As mentioned above, sema3A has been implicated as a chemoattractant for the apical dendrites of cortical pyramidal neurons, while acting as a chemorepellent for their axons. This assymetry of action is correlated with an assymetry in the distribution of soluble guanylate cyclase (SGC) in these neurons, with SGC concentrations being significantly higher in the apical dendrites than in the axons [Polleux, 2000]. This correlation suggests that sema3A chemoattraction of dendrites may be mediated by cyclic GMP. That hypothesis is further supported by the observations that the oriented growth of dendrites toward the pial surface, but not the differentiation of the apical dendrite, is disrupted when slice overlay cultures are exposed to inhibitors of SGC [Polleux, 2000].

Beyond the above, little is known about the signaling pathways responsible for the enhancement of dendritic outgrowth by exogenous factors. Cell bound β-amyloid protein and soluble amyloid precursor protein lower intracellular free calcium levels in hippocampal dendrites and cell bodies, but it is not known whether that effect is necessary for structural plasticity, or merely excitoprotective (Mattson, Cheng *et al.,* 1993). Additionally, dermatan sulfate, which enhances dendritic outgrowth, must be internalized in order to achieve its effect (Lafont *et al.,* 1994), but little is known about its mechanism of action.

VI. Concluding Remarks

It is clear that there is an overlapping set of factors that control axonal and dendritic arborization (Fig. 9). For example, neurotransmitters, neurotrophins, and some of the general class of integrin-binding molecules of the extracellular matrix can affect both types of process. By contrast, there is no evidence that dendrites respond to netrins, ephrins, semaphorins, or laminin, and there is no evidence that axons respond to OP-1 or CPG15. Some factors, such as CNTF, have opposing effects on axons and dendrites (Jordan, 1996; Guo *et al.,* 1997, 1999; Cho *et al.,* 1999; Cui *et al.,* 1999). As a general rule, it appears that those factors that affect both axonal and dendritic outgrowth are those that impinge on the overall health of the cell. Thus, neurotrophins are necessary for neuronal survival, and appear to enhance process outgrowth from both axons and dendrites (Vicario-Abejon *et al.,* 1998). Exposure to neurotransmitters and the resultant calcium influx can enhance both axonal and dendritic outgrowth at low levels (Lipton and Kater, 1989; Kossel *et al.,* 1997; Metzger *et al.,* 1998; Wilson and Keith, 1998; Wilson *et al.,* 2000), and can cause retraction of both at higher levels (Mattson and Kater, 1987; Mattson *et al.,* 1988a, 1988c; Mills and Kater, 1990; Wilson and Keith, 1998; Wilson *et al.,* 2000). Additionally, both amyloid precursor protein and beta amyloid tend to affect both axons and dendrites in a direction consistent with their neurotoxicity (Mattson, 1997; Mattson *et al.,* 1998; Mattson and Duan, 1999; Ohyu *et al.,* 1999; Phinney *et al.,* 1999a, 1999b). Conversely, the factors that have specific effects on axons tend not to affect overall cellular health, but tend to act specifically on the axonal growth cone. Thus, ephrins, netrins, and semaphorins do not enhance

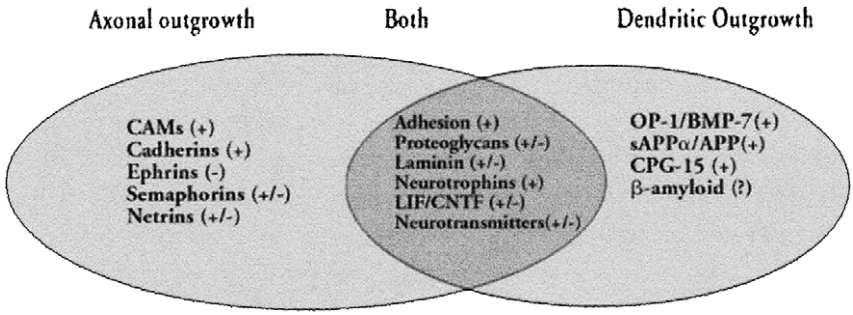


FIG. 9 Examples of factors that selectively affect axonal outgrowth, affect both axonal and dendritic outgrowth, and that selectively affect dendritic outgrowth. (+) indicates enhancement of outgrowth, (−) inhibitions, and (+/−) indicates that either enhancement or inhibition of outgrowth may be seen depending on conditions of exposure or on the type of process.

or reduce neuronal survival. Insofar as specific dendritic outgrowth modulators are concerned, evidence is mixed as to whether effects can be generated locally or must uniformly involve the entire somatodendritic compartment. For those effects driven by calcium influx, there is substantial evidence for local activity. For example, with dendritic spines, there is substantial evidence that ionic changes occuring in the spine do not spread efficiently to the dendritic shaft (Harris and Kater, 1994). The localized effects produced on dendritic arbors by focal electrical stimulation (Meberg *et al.,* 1999), suggest that the spread of Ca^{2+} in dendritic shafts is similarly limited. However, other mediators, like dermatan and heparan sulfates, may mediate their actions through internalization into the soma, and so are unlikely to operate locally at the dendritic growth cone.

As the external factors responsible for controlling axonal and dendritic arborization form an incompletely overlapping set, so too do the signaling pathways known to be used. Both axonal and dendritic outgrowth can be controlled by calcium, cAMP, tyrosine kinases, arachidonic acid, and the monomeric G proteins. However, to date only the control of axonal outgrowth is known to be mediated by receptor phosphatases or guanidylate cyclases. While the list of signaling pathways controlling dendritic arborization may expand, it is likely that, like the list of extracellular factors, it will continue to overlap incompletely with the list of factors that control axonal outgrowth. The localization of specific receptor families exclusively to the axon, such as Eph receptors and plexin-neuropillin receptors, indicates that there are signaling pathways distinct to the axon. Equally, the presence of distinct targets of signaling pathways in the axonal and somatodendritic compartments indicates that, even for shared signals, axonal and dendritic responses may differ.

The combination of these mechanisms, including local responsivity to global signals, local presentation of signals, and limited spread of intracellular second messengers, makes possible the polarization of neurons, leading to the formation of axonal and dendritic compartments that have distinct morphology and function. Additionally, the different responses of the two compartments to stimuli present after maturation may allow differential plasticity in the axonal and dendritic compartments of the mature neuron, thus optimizing responses to environmental cues. Future studies that better identify the molecules distinctly involved in the developmental and postdevelopmental plasticity of the axonal and dendritic compartments of the neuron will be important to further our understanding of the functioning of the nervous system.

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Endoplasmic Reticulum of Animal Cells and Its Organization into Structural and Functional Domains

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The endoplasmic reticulum (ER) in animal cells is an extensive, morphologically continuous network of membrane tubules and flattened cisternae. The ER is a multifunctional organelle; the synthesis of membrane lipids, membrane and secretory proteins, and the regulation of intracellular calcium are prominent among its array of functions. Many of these functions are not homogeneously distributed throughout the ER but rather are confined to distinct ER subregions or domains. This review describes the structural and functional organization of the ER and highlights the dynamic properties of the ER network and the mechanisms that support the positioning of ER membranes within the cell. Furthermore, we outline processes involved in the establishment and maintenance of an anisotropic distribution of ER-resident proteins and, thus, in the organization of the ER into functionally and morphologically different subregions.
KEY WORDS: Endoplasmic reticulum, Ca^{2+} regulation, Protein synthesis, Lipid synthesis, Organelle motility, Cytoskeleton, f-Actin, Microtubules.

I. Introduction

The endoplasmic reticulum (ER) is a three-dimensional network of interlinked membranous tubules and cisternae that extend throughout the cell. This organelle represents by far the largest membrane system in animal cells (Griffiths *et al.,* 1984). Classically, the ER is subdivided into three domains, the nuclear envelope (NE), the smooth ER (sER), and the rough ER (rER), with the latter being involved in the synthesis of secretory and membrane proteins. In addition to protein

synthesis, the ER participates in a variety of other cellular functions, such as the biosynthesis of phospholipids, cholesterol, and steroids, the degradation of glycogen, detoxification reactions, and the regulation of intracellular Ca^{2+}. The fine structure of the ER and the extent of its development in any given cell type depends on which of these functions predominates. This is particularly obvious in the case of the sarcoplasmic reticulum (SR) of skeletal muscle cells, an ER specialized in Ca^{2+} regulation and with a highly characteristic morphology and topography.

Many of the diverse functions of ER have been individually discussed in numerous reviews (see sections below). However, the limited scope of most of these reviews has prevented them from pointing out the full extent of the biochemical and structural versatility of this organelle and the anisotropic distribution of these various functions across the ER network. In this review, we highlight the organization of the ER into morphological and functional subregions in a manner that goes significantly beyond the classical ER domains. In this context, we also summarize current knowledge about the mechanisms that establish and stabilize the location and fine structure of this complex organelle within the cell, and the way in which the polarized distribution of proteins in the ER membrane is maintained. In view of the central role of ER in the multitude of biochemical pathways and intracellular processes, an understanding of the biogenesis and organization of this organelle will have implications for many areas in cell biology and cell physiology.

II. Structural Organization of the ER

A. The ER—A Single Continuum?

The question of whether the ER encloses a single continuous lumen has puzzled cell biologists for a long time. Because not all ER regions function equally (see below), this issue is important for understanding how compartmentalization of the ER is achieved, and whether some molecules, e.g., calcium ions, can diffuse and equilibrate within the entire ER network. Although several ultrastructural studies have provided results in favor of ER continuity (Droz *et al.,* 1975; Walz, 1982; Qvortrup and Rostgaard, 1990; Martone *et al.,* 1993), the interpretation of these data is open to criticism, because most of the studies involve the use of chemical fixation techniques, which are prone to artifacts. Moreover, the determination of ER continuity requires not only the examination of part of the cell, as in the studies mentioned above, but also a three-dimensional reconstruction of the entire membrane system. Because of the enormous size and complexity of this organelle, this is a practically impossible task.

The issue of ER continuity has also been addressed by fluorescence light microscopy of live cells injected with the lipophilic fluorescent dye $DiIC_{16}(3)$. This dye diffuses only within a continuous lipid bilayer and labels a membrane network

that costains with ER markers (Terasaki *et al.,* 1991). Upon introduction of $DiIC_{16}(3)$ into cerebellar Purkinje cells, a neuron with a very elaborate ER network, fluorescence spreads throuthout the cell at a rate that is consistent with diffusion in a membrane, but that is too high to be accounted for by vesicular traffic (Terasaki *et al.,* 1994). The results of this and similar studies on other cell types (Terasaki and Jaffe, 1991; Feng *et al.,* 1994; Kline *et al.,* 1999) have demonstrated that the $DiIC_{16}(3)$-stained ER forms a continuous membrane network. However, because $DiIC_{16}(3)$ does not label any ER membranes discontinuous with this system, these studies do not provide definitive evidence that the entire ER represents an uninterrupted membrane system.

Direct evidence in support of a single continuous ER network has been provided by recent studies using GFP (green fluorescent protein) and FLIP (fluorescence loss in photobleaching) techniques (Cole *et al.,* 1996; Subramanian and Meyer, 1997; Dayel *et al.,* 1999; Terasaki, 2000). Chimeric GFP proteins targeted to the lumen or the membrane of the ER were expressed in cultured cells and imaged by fluorescence microscopy. By using a laser beam, a small spot within the cell was repeatedly illuminated to bleach GFP within this area. At the same time, fluorescence intensity in the unbleached region of the cell was also uniformly reduced, because unbleached GFP molecules diffused into the bleaching spot. This ultimately resulted in a complete loss of ER fluorescence within the entire cell, demonstrating that all GFP-tagged molecules could diffuse within a continuous membrane system to the bleaching zone. However, although these studies strongly support the concept that the ER forms a single continuum, they also provide some evidence that this may not be the case all the time. The ER reversibly fragments under some conditions, e.g., during fertilization of starfish oocytes (Terasaki *et al.,* 1996) or persistent increases in cytosolic Ca^{2+} (Subramanian and Meyer, 1997).

B. Morphology and Topography of ER Membranes

The ER network is not homogeneous in its structure but adopts different morphologies in different cell areas or in conjunction with different functions (Fig. 1). A selection of diverse ER structures in various cell types has been presented by Fawcett (1981). For instance, ER membranes may form either a tubular network or flattened cisternae. Morever, tubules and cisternae may be organized in ordered arrays, such as cisternal stacks of rER (Fig. 1b) or crystalloid/paracrystalline ER (Fig. 1c,d), the morphologically most spectacular subregion of the ER. The crystalloid ER is either a hexagonally packed array of sER tubules or an aggregate of undulating sER tubules and has been observed in various cell types *in vivo* and under special experimental conditions (Table I). These variations in ER shape directly affect the surface-to-volume ratio and may thus be of importance for optimizing different functions in different ER regions.

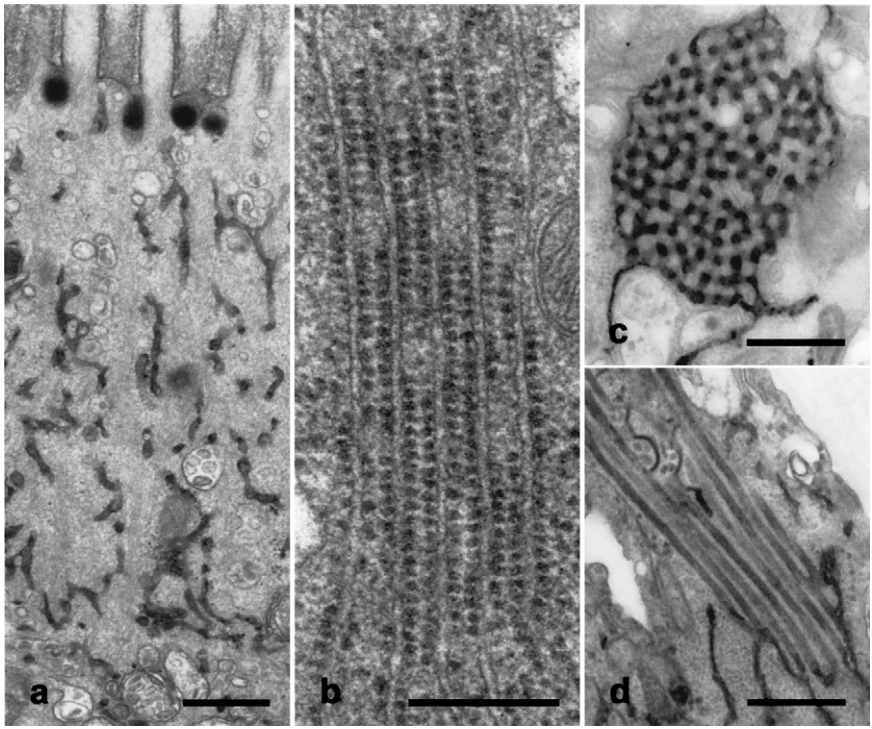

FIG. 1 Morphology of ER domains. (a) Network of ER tubules in the terminal web region of a cockroach midgut epithelial cell. The specimen was fixed by the OsFeCN method (see Walz and Baumann, 1989) to stain ER elements selectively. (b) Stacked rER cisternae densely studded with polysomes in a locust photoreceptor. (c,d) Crystalloid ER in OsFeCN-fixed photoreceptor cells of the medical leech. Note that the geometrical arrays of sER tubules are continuous with other ER elements. Scale bars, 0.5 μm. (Reproduced from *The Journal of Cell Biology,* 1982, vol. 93, pp. 839–848 by copyright permission of The Rockefeller University Press.)

Figure 2 presents an example from our own work (Baumann and Walz, 1989a) showing that distinct ER regions can have a different morphology within a single cell. Another reason for selecting this image is because it illustrates impressively that ER cisternae may adopt a characteristic position within a cell. Some ER cisternae are juxtaposed to the plasma membrane, whereas others lie in intimate contact with mitochondria or are located close to Golgi membranes. Because some functions of the ER require interplay with other cellular membrane systems, the topography of the ER may enhance the efficiency of the exchange of molecules between the compartments. Moreover, the layout of the ER within a cell may also influence the shaping of an intracellular Ca^{2+} signal, as discussed below.

TABLE I
Occurrence of Crystalloid (Paracrystalline) ER in Animal Cells

Cell type	Condition	Reference
Annelid photocytes	*In vivo*	Bassot and Nicolas, 1987 (and references therein)
Quail bird uropygial gland	*In vivo*	Fringes and Gorgas, 1993
Insect germ cells	*In vivo*	Wolf and Motzko, 1995
Leech photoreceptor cells	*In vivo*	Walz, 1982
UT-1 cells	Inhibition of HMG CoA reductase	Pathak *et al.,* 1986 (and references therein)
Hepatocytes	Inhibition of HMG CoA reductase	Singer *et al.,* 1988
CHO cells	Overexpression of HMG CoA reductase	Jingami *et al.,* 1987; Roitelman *et al.,* 1992
COS cells	Overexpression of microsomal aldehyde dehydrogenase	Yamamoto *et al.,* 1996
Human kidney cell line	Overexpression of cytochrome *P*-450	Sandig *et al.,* 1999

III. Organization of the ER into Functional Subregions

The ER has a variety of different functions that are not homogeneously distributed throughout the entire organelle but are often concentrated within distinct subregions. In the following subsections, we summarize data on the localization of various ER functions and demonstrate that there is a significant degree of complexity in the organization of the ER beyond the classical ER domains, namely, the rER, the sER, and the NE. We focus on those functions shown to have an anisotropic distribution.

A. Nuclear Envelope and Annulate Lamellae

The NE separates the nuclear and the cytoplasmic compartments of interphase cells and comprises about 10% of the total ER surface (Bannykh *et al.,* 1996). Its lumen is connected to the bulk ER as demonstrated by the diffusion of ER luminal proteins (Terasaki *et al.,* 1996). The NE can be further subdivided into three morphologically and biochemically different areas, the outer nuclear membrane (ONM), the inner nuclear membrane (INM), and the "pore membrane" that links the ONM and INM and is associated with the nuclear pore complex (NPC). The organization of these membrane domains is presented in schematic form in

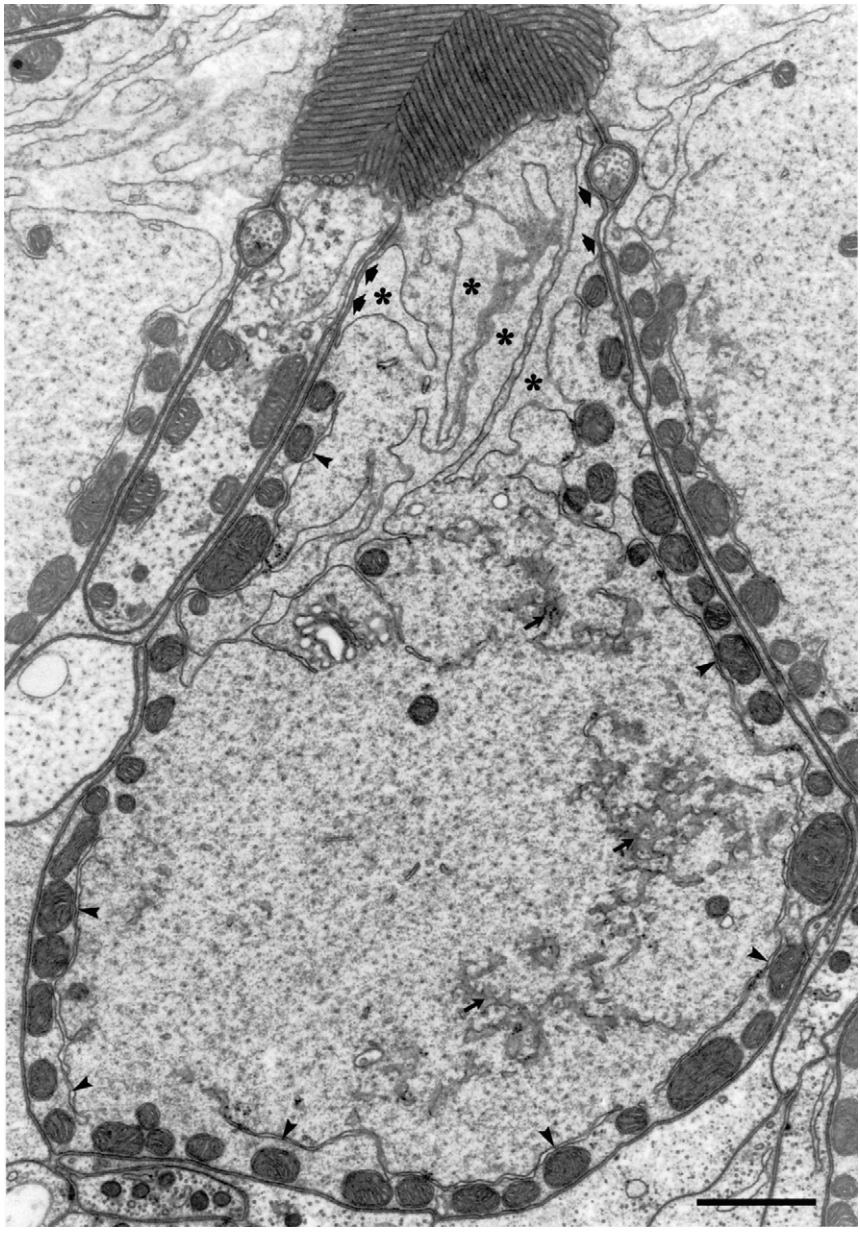

FIG. 2 Structural layout of the ER. The electron micrograph shows a cross section through a honeybee photoreceptor, a highly polarized epithelial cell. The ER is characterized by morphologically different subdomains, e.g., sac-like cisternae (asterisks) at the base of the photoreceptive microvilli, or flattened cisternae that are traversed by microtubules and seen in face view (thin arrows). Moreover, each ER domain has a very characteristic position within the cell, e.g., next to the subsurface layer of mitochondria (arrowheads) or in close apposition to the plasma membrane (broad arrows). Scale bar, 1 μm. (Reproduced from Baumann and Walz (1989a), Fig. 3, copyright notice of Springer-Verlag.)

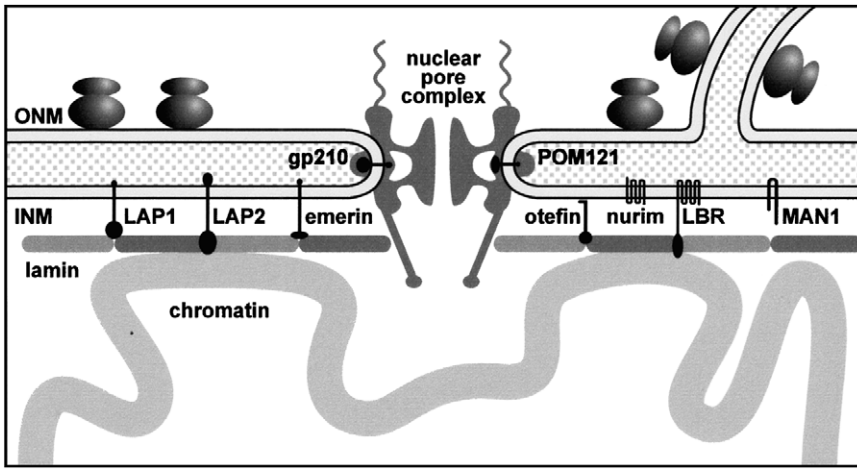

FIG. 3 Organization of the nuclear envelope. The outer nuclear membrane (ONM) is continuous with the bulk ER and has ribosomes bound to it. The inner nuclear membrane (INM) is undercoated by the nuclear lamina, a network of lamin filaments. Several INM-specific proteins attach the membrane to lamin and to chromatin. The nuclear pore complex mediates molecular traffic between the nucleoplasma and the cytoplasma. It is associated with the pore membrane via the integral membrane proteins gp210 and POM121.

Fig. 3 and has been the subject of several recent reviews (Gerace and Foisner, 1994; Goldberg and Allen, 1995; Gant and Wilson, 1997).

The ONM is morphologically continuous with the remaining ER network. Its equipment with ribosomes and with integral membrane proteins, e.g., the Ca^{2+} ATPase (Humbert *et al.,* 1996) and cytochrome P-450 (Matsuura *et al.,* 1983), suggests that the ONM also performs functions similar to bulk ER membranes. Moreover, the ONM can even serve as a budding site for vesicles that transport proteins to the Golgi apparatus (Bannykh *et al.,* 1996; Hager *et al.,* 1999). The ONM may thus not be regarded as a functionally distinctive ER subregion, but only as an ER domain with a characteristic morphology and topography.

The INM, in contrast, is distinguished by a unique protein equipment that serves the attachment of the nuclear lamina and of chromatin to the NE during interphase and that is important for the structural organization of the nucleus. The nuclear lamina is an orthogonal network of intermediate-filament-type proteins, lamins, that undercoat the INM (Stuurman *et al.,* 1998). Lamins have been classified into A and B subtypes and can bind to core histone proteins (Taniura *et al.,* 1995; Stuurman *et al.,* 1998). Four different integral membrane proteins on the INM have been demonstrated to be involved with the anchorage of the nuclear lamina and of chromatin to the NE in vertebrate cells (Gerace and Foisner, 1994; Gant and Wilson, 1997): the lamina-associated polypeptides LAP1 and LAP2, the lamin-B receptor (LBR, also called p58), and emerin. LAP1, LAP2, and emerin are type

II membrane proteins with a large N-terminal nucleoplasmic domain that binds to lamin and, in the case of LAP2, also to chromatin. LBR consists of eight putative transmembrane segments and an N-terminal nucleoplasmic domain binding to B-type lamins and chromatin. Immunogold localization has demonstrated that these proteins are resticted to or at least highly concentrated on the INM and can thus be regarded as molecular markers for this ER domain (Senior and Gerace, 1988; Furukawa *et al.,* 1995; Yorifuji *et al.,* 1997). Moreover, two other presumptive INM membrane proteins, called nurim and MAN1, have been characterized recently, but their functions are still elusive (Rolls *et al.,* 1999; Lin *et al.,* 2000). The only INM-specific protein known in invertebrates is otefin. This *Drosophila* protein binds to lamin but is a peripheral rather than a transmembrane protein (Ashery-Padan *et al.,* 1997; Goldberg *et al.,* 1998).

The NPC and the associated pore membrane connect the ONM and INM and mediate bidirectional molecular traffic of proteins and RNA between the nucleoplasm and the cytoplasm (Nigg, 1997; Talcott and Moore, 1999). In addition, the NPC sustains the passive diffusion of ions and small molecules through aqueous pores with a physical diameter of ~10 nm (Paine *et al.,* 1975; Peters, 1984) and possibly the diffusion of integral membrane proteins between the ONM and the INM (Powell and Burke, 1990). The NE of a typical animal cell contains several thousand individual NPCs that are quite homogeneously distributed over the nuclear surface (Maul and Deaven, 1977). Each NPC is a macromolecular assembly of ~125 MDa made up of probably more than 100 different polypeptides called nucleoporins (Rout and Wente, 1994; Bastos *et al.,* 1995; Panté and Aebi, 1995; Stoffler *et al.,* 1999). Structurally, the NPC consists of three rings stacked on top of each other, with the middle ring being attached by eight radial spokes to a central gated channel (transporter). Eight short filments extend from the cytoplasmic ring into the cytoplasm, whereas the nucleoplasmic ring has attached filaments that form a basket-like assembly (Panté and Aebi, 1995; Stoffler *et al.,* 1999). Two integral membrane proteins, gp210 and POM121, have been identified as components of the NPC and have been localized exclusively to the pore membrane by means of immunoelectron microscopy (Greber *et al.,* 1990; Hallberg *et al.,* 1993). These may serve as an anchor for the NPC to the pore membrane.

Membrane-associated pore complexes are not restricted to the NE. Pore complexes with a structural organization similar to NPCs occur in cytoplasmic cisternae of various cell types, e.g., oocytes, embryonic cells, tumor cells, and cultured cells (Kessel, 1992). These cytoplasmic cisternae often assemble into stacks to form a structure termed annulate lamellae, with the pore complexes being packed in geometric arrays (Fawcett, 1981). At their ends, annulate lamellae are morphologically continuous with other ER cisternae, and they colocalize with rER elements at the light-microscopic level (Cordes *et al.,* 1996). Besides their structural similarity to NPCs, pore complexes in annulate lamellae also share several nucleoporins with the latter, including some molecular components of the cytoplasmic and intranuclear filaments (Meier *et al.,* 1995; Cordes *et al.,* 1995, 1996; Ewald *et al.,* 1996). It has been suggested that these pore-associated filaments are involved in packing

the cisternae into stacks (Ewald *et al.,* 1996). However, pore complexes of annulate lamellae are devoid of some NPC proteins, raising the question of whether they are functional in terms of transport processes. Annulate lamellae also lack a lamina (Chen and Merisko, 1988; Dabauvalle *et al.,* 1991; Cordes *et al.,* 1996) and can be assembled *in vitro* in the absence of lamin and chromatin (Dabauvalle *et al.,* 1991; Meier *et al.,* 1995). The physiological function of annulate lamellae is still elusive (Kessel, 1992). As one possibility, annulate lamellae have been suggested to represent a cytoplasmic pool of pore complexes for later use. Alternatively, annulate lamellae have been considered to represent a by-product of nucleoporin synthesis and a storage site for surplus pore complexes. In support of this hypothesis is the finding that the number of annulate lamellae increases in lamin mutant *Drosophila* cells with a defective NE (Lenz-Böhme *et al.,* 1997).

B. ER Domains and Their Role in Protein Synthesis

The biogenesis of the luminal proteins of the ER, Golgi apparatus, endosomes, and lysosomes, of secretory proteins, and of membrane proteins represents one of the main functions of the ER. Protein biogenesis at the ER is a highly complex process that involves numerous distinct steps: the targeting of ribosomes with the nascent polypeptide to the ER membrane, cotranslational translocation, and the folding and maturation of the polypeptide chain.

1. Cotranslational Translocation on the Rough ER

Translation of secretory and membrane proteins and their translocation across the ER membrane occurs at the rER, which is characterized and identified by membrane-bound ribosomes. The rER displays quite different morphologies, depending on the cell type and the physiological state. In cells with high secretory activity, the rER is very prominent and often consists of flattened cisternae arranged in stacks that are densely occupied by polysomes. In other cells, the rER forms a loose network of tubular cisternae that are only sparsely studded with polysomes.

When a signal sequence emerges from the ribosome, it is recognized by the signal recognition particle (SRP), and the ribosome–SRP complex with the nascent polypeptide chain is targeted to the ER membrane by interaction with the heterotrimeric SRP receptor (Rapoport *et al.,* 1996; Johnson, 1997; Matlack *et al.,* 1998; Johnson and van Waes, 1999). As translation proceeds, the nascent polypeptide chain is translocated across the ER membrane via an evolutionally highly conserved macromolecular machinery, termed the translocon (Fig. 4). The core component of the translocon is the Sec61p complex, which is composed of an α-, a β-, and a γ-subunit (Görlich *et al.,* 1992a). Oligomers of the Sec61p complex form an aqueous channel with a 2- to 6-nm pore that has been imaged by electron microscopic techniques (Hanein *et al.,* 1996; Beckmann *et al.,* 1997; Matlack *et al.,* 1998). It is thought that the Sec61p complex makes a tight seal with the

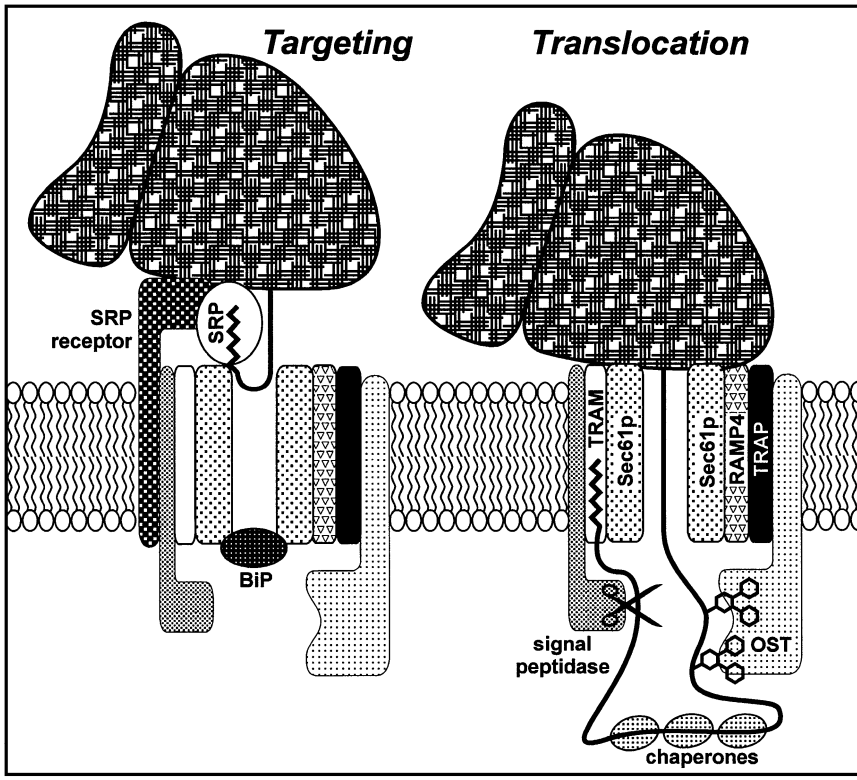

FIG. 4 Cotranslational protein translocation at the rER. When a signal sequence (zigzag end of the nascent polypeptide chain) emerges from the ribosome, it is recognized and bound by the signal recognition particle (SRP). The ribosome–SRP complex is then targeted to the ER membrane by interaction with the SRP receptor. The ribosome forms a tight seal with the translocon, a protein complex composed of Sec61p, the translocating chain-associated membrane (TRAM) protein, the signal peptidase complex, the oligosaccharyl transferase (OST) complex, and presumably the small ribosome-associated membrane protein 4 (RAMP4) and the translocon-associated protein (TRAP) complex. Upon binding of the ribosome, an aqueous pore formed by the Sec61p complex opens by dissociation of BiP (Hamman *et al.,* 1998), and the emerging polypeptide chain passes through the pore. During translocation, the OST complex containing ribophorins I and II transfers the oligosaccharide $Glc_3Man_9GlcNAc_2$ from the lipid carrier dolichol pyrophosphate onto certain aspargine residues within the polypeptide chain. The signal peptidase cleaves the signal sequence in the case of luminal or secretory proteins. The functions of the other translocon components are not fully resolved.

ribosome and thereby provides a physically protected path for the nascent polypeptide chain as it passes from the ribosome through the protein-conducting channel into the ER. Several other proteins have been shown to be involved with cotranslational translocation and to be possibly associated with the translocon (see Fig. 4): the translocating chain-associated membrane (TRAM) protein (Görlich *et al.,*

1992b), the translocon-associated protein (TRAP) complex (Wiedmann *et al.,* 1987; Hartmann *et al.,* 1993), the small ribosome-associated membrane protein 4 (RAMP4; Görlich and Rapoport, 1993; Yamaguchi *et al.,* 1999), the signal peptidase complex (Evans *et al.,* 1986), and the oligosaccharyl transferase (OST) complex containing ribophorins I and II (Kelleher *et al.,* 1992). Furthermore, p180 (Savitz and Meyer, 1990) and p34 (Tazawa *et al.,* 1991) have been implicated in the binding of the ribosome on the ER membrane of mammalian cells, although the contribution of these to ribosome targeting and protein translocation is still controversial.

Immunogold localization of the SRP receptor (Hortsch *et al.,* 1985) and of some translocon components, namely, the TRAP α- and β-subunits (previously called signal sequence receptor; Vogel *et al.,* 1990), clearly demonstrates that these proteins are essentially restricted to the rER. Biochemical fractionation experiments suggest that this also applies to OST complex proteins, namely, the ribophorins (Kreibich *et al.,* 1978; Marcantonio *et al.,* 1984). These molecules are thus generally regarded as markers for the rER (Krijnse-Locker *et al.,* 1995). The subcellular distribution of other translocon components, in contrast, has not been studied in detail. Although their role in protein translocation and their association with the translocon is suggestive of a localization to only the rER, this assumption may be misleading. Results of a freeze-fracture study (Hanein *et al.,* 1996) and of biochemical cross-linking experiments (Kalies *et al.,* 1998) indicate that the translocon complex is not a permanent structure but is assembled *de novo* during the initiation of cotranslational translocation. Some of the translocon components, such as the ribophorins, may thus be restricted to rER membranes by forming a stable supramolecular network (Ivessa *et al.,* 1992), whereas other translocon components may be relatively mobile within the ER membrane and be recruited to the translocon complex by the onset of the cotranslational translocation process. Indeed, a recent immunofluorescence study on cultured cells has visualized Sec61p throughout the ER network and (together with TRAM) also in a post-ER compartment (Greenfield and High, 1999), indicating that Sec61p is not confined to the rER. However, this interpretation requires reconfirmation by Sec61p localization at the electron microscopic level or by colabeling with established markers for rER and other ER domains. Because Sec61p is not only involved with cotranslational translocation, but also with retrograde transport of misfolded proteins for destruction within the cytosol (Wiertz *et al.,* 1996), its widespread distribution raises the possibility that this protein complex has different functions in different ER regions.

2. Polypeptide Folding and Maturation

Co- and posttranslational protein folding and maturation is assisted by a panoply of molecular chaperones localized in the ER (Helenius *et al.,* 1992, 1997; Ellgaard *et al.,* 1999; Zapun *et al.,* 1999). Chaperones associate transiently to folding

intermediates, promote their proper folding and assembly, and prevent their aggregation. Moreover, chaperones function as a "quality control" system in the secretory pathway; misfolded or incompletely assembled proteins remain bound to the chaperones, preventing their export to the Golgi complex. The chaperones themselves remain localized within the ER, because they contain retention and retrieval signals. Most luminal ER proteins carry a C-terminal KDEL sequence that is recognized by the KDEL-receptor in downstream organelles in the secretory pathway (see below) and that ensures the retrieval of escaped proteins to the ER. Type I membrane proteins have a C-terminal KKXX or KXKXX (X is any amino acid) sequence that functions similarly (Pelham, 1990; Teasdale and Jackson, 1996).

Prominent examples of ER-resident chaperones are BiP/Grp78, endoplasmin/Grp94, calnexin, and calreticulin. BiP, a member of the heat shock protein 70 (HSP70) family, is an ER-luminal ATPase that binds to hydrophobic stretches of amino acids in a large number of folding intermediates (Gething, 1999). Endoplasmin belongs to the HSP90 family and interacts only with a restricted subset of protein substrates (Argon and Simen, 1999). Calnexin is a type I membrane protein that binds with its luminal domain to monoglycosylated glycoproteins, and calreticulin is a soluble ER-luminal homolog of calnexin (Helenius *et al.,* 1997; Michalak *et al.,* 1999). In addition to these chaperones, there are folding catalysts, such as the luminal protein disulfide isomerase (PDI), which promotes the formation and breakage of disulfide bonds and, thereby, corrects errors in disulfide pairing (Noiva, 1999), and peptidylprolyl *cis-trans* isomerases (e.g., cyclophilin), which catalyse the isomerization of X-P peptide bonds (Shadidy *et al.,* 1999; Zapun *et al.,* 1999). Indeed, most ER-resident luminal proteins have functions related to protein folding and maturation (Helenius *et al.,* 1992).

Results obtained by subcellular fractionation, immunofluorescence microscopy, and immunoelectron microscopy suggest that BiP, calreticulin, calnexin, and PDI are present thoughout the ER network (Akagi *et al.,* 1988a; Terasaki and Reese, 1992; Villa *et al.,* 1992; Nori *et al.,* 1993; Ioshii *et al.,* 1995; Krijnse-Locker *et al.,* 1995; Soltys *et al.,* 1996; Kellokumpu *et al.,* 1997). The ability to promote protein folding and maturation may thus be a function shared by all ER domains (however, see below). Moreover, because of their apparently universal distribution within the ER, these proteins are generally regarded as good molecular markers for determining the extent of the ER and for probing whether a membrane system is part of the ER. For example, the identification of BiP, calreticulin, calreticulin, calnexin, and PDI in the SR of muscle cells has provided direct support for the concept that this organelle is a subdomain of the ER, although being highly specialized for Ca^{2+} regulation (Volpe *et al.,* 1992; Villa *et al.,* 1993).

Despite their presence in the entire ER, chaperones may not be uniformly concentrated throughout the organelle (Ioshii *et al.,* 1995; Kellokumpu *et al.,* 1997; Simpson *et al.,* 1997; Chao *et al.,* 1999) and may be enriched in the rER (Opas *et al.,* 1991). Biochemical studies suggest further that chaperones and other proteins

in the ER are weakly associated to form a dynamic network that could account for the inhomogeneous chaperone distribution (Baksh *et al.,* 1995; Kellokumpu *et al.,* 1997; Tatu and Helenius, 1997). This matrix of chaperones may enhance the efficiency of protein folding and maturation and limit the mobility of newly synthesized folding and assembly intermediates, thus preventing their premature exit from the ER. There is also the possibility that chaperone aggregation is regulated and thus adapted to the physiological needs of the cell. The interaction between calreticulin and PDI, at least, depends on the luminal Ca^{2+} concentration (Corbett *et al.,* 1999), and the ability of calnexin to associate with the translocon can be modified by phosphorylation (Chevet *et al.,* 1999).

Although chaperones are generally regarded as markers for the entire ER, exemptions to this rule have been detected. First, chaperones may be absent or at least be highly diluted in ER exit sites, the regions where transport vesicles for delivery to post-ER organelles are made (for details, see Section III.C). Second, chaperone proteins are excluded from spaces occupied by insoluble protein aggregates, such as zymogen granules in exocrine pancreatic cells (Tooze *et al.,* 1989). Finally, several molecular chaperones seem to escape ER retention, to be present along the entire secretory pathway, and to be exported to the cell surface in various cell types (Akagi *et al.,* 1988b; Wiest *et al.,* 1997; Andrin *et al.,* 1998; Bruneau *et al.,* 1998; Mezghrani *et al.,* 2000). In conclusion, the use of molecular chaperones as markers for the entire ER and only the ER should be exercised with some prudence.

3. Organization of the Rough ER into Functional Subcompartments

The rER might not represent a homogeneous domain, but instead may be organized into subregions that are specialized with respect to mRNA translation and/or posttranslational protein processing. This concept is based on the results of *in situ* hybridization studies demonstrating that particular mRNAs encoding for membrane proteins or secretory proteins are restricted to specific areas of the rER (Trembleau *et al.,* 1994; Trembleau and Bloom, 1996; Ralston *et al.,* 1997; Deshler *et al.,* 1997; Racca *et al.,* 1997). For instance, in skeletal myotubes, the mRNA encoding for the transferrin receptor resides on a subset of rER membranes, whereas the mRNA for the secreted immunoglobulin κ light chain has a uniform distribution over the rER (Ralston *et al.,* 1997). Such a segregation of particular mRNAs to distinct areas of the rER seems to be especially prominent in highly polarized cells, namely, neurons, muscle cells, and oocytes. These rER subregions may provide unique environments for the biogenesis of substrates and may thus support different pathways for folding and processing of the translation products (Hegde and Lingappa, 1999). Alternatively, translation of mRNAs on distinct rER subregions might be a mechanism for targeting and confining the encoded proteins to different cell regions or surface domains, and thus of supporting cell polarity

(Racca *et al.,* 1997). This latter scenario requires that the synthesized proteins do not intermix in post-ER compartments, a condition that seems to contradict the findings that the Golgi apparatus of cultured vertebrate cells forms a single unit (Rambourg and Clermont, 1990) and that proteins can rapidly move throughout the Golgi system (Cole *et al.,* 1996). However, keep in mind that, in insect cells (Ripoche *et al.,* 1994; Baumann, 1998a), in sea urchin blastomeres (Terasaki, 2000), and even in some vertebrate cells (de Vries *et al.,* 1993; Rahkila *et al.,* 1997), the Golgi complex consists of numerous discrete membrane stacks dispersed in the cytoplasm; these may represent morphologically separated post-ER pathways.

How are these different mRNA localization patterns over the rER established and maintained? Three possible mechanisms that may, independently or in combination, be involved with determining mRNA positioning come readily to mind. First, the distribution of mRNAs over the ER may be established by differences in mRNA lifetime, with mRNA of a high turnover rate being restricted to the vicinity of the nucleus, and stable mRNA being distributed over a larger area of the ER. This model, however, has been challenged by the finding that the subcellular distribution of transferrin receptor mRNA over the rER in skeletal myotubes is independent of mRNA lifetime (Ralston *et al.,* 1997). Moreover, stability differences cannot explain such complex mRNA localization patterns as those observed in neurons (Trembleau *et al.,* 1994; Racca *et al.,* 1997). Second, mRNA subsets may be actively transported to a defined area of the cell and then may associate with ER membranes in the target region. Transport of mRNAs along either microtubules or actin filaments has been documented in a variety of cell types (Bassell and Singer, 1997; Nasmyth and Jansen, 1997), and there is also evidence from yeast that mRNA molecules can bind to a motor protein either directly or via an adaptor protein (Takizawa *et al.,* 1997; Münchow *et al.,* 1999). Finally, mRNA could be captured on the ER by specific mRNA-anchoring molecules, and the mRNA-carrying ER membranes could then be translocated to their final destination (Deshler *et al.,* 1997).

Some recent studies have identified proteins that are assumed to be involved with mRNA targeting and localization. The best known of these is Staufen, a *Drosophila* protein required for the localization of mRNA in oocytes. Mammalian homologs of Staufen have been characterized and shown to bind double-stranded mRNA, to be transported in a complex with mRNA along microtubules, and to codistribute with rER markers at the light-microscopic level in various cultured mammalian cells (Kiebler *et al.,* 1999; Köhrmann *et al.,* 1999; Marión *et al.,* 1999; Wickham *et al.,* 1999). Moreover, Deshler *et al.* (1997) have identified a protein called Vera that is associated with the rER *in Xenopus* oocytes and that may determine the distribution of mRNA encoding for Vg1, a transforming growth factor. Identification of further ER-associated mRNA-anchoring proteins and their role in mRNA positioning will be interesting fields of future research.

C. ER Exit Sites

After passing the quality control system of folding factors, newly synthesized proteins destined for non-ER membrane systems or secretion are packaged into vesicular carriers that mediate their transport to the Golgi apparatus. This event occurs on specialized areas of the ER that have been termed exit/export sites or transitional elements (Kuehn and Schekman, 1997; Bannykh *et al.,* 1998). A characteristic of exit sites is a cytosolic COPII coat–complex that promotes cargo packaging and vesicle budding. After release from the ER, COPII vesicles may become uncoated and undergo homotypic fusion to form vesicular-tubular clusters (VTCs; Balch *et al.,* 1994; Bannykh *et al.,* 1996, 1998). VTCs, also refered to as the ER–Golgi intermediate compartment (ERGIC), *cis*-Golgi network, or salvage compartment, are then forwarded to the Golgi complex in a microtubule-dependent manner (Presley *et al.,* 1997; Scales *et al.,* 1997). Moreover, VTCs are prominent sites of protein sorting. Escaped ER-resident proteins with C-terminal retrieval motifs and the molecular machinery required for vesicle budding at the ER become segregated from cargo proteins within the VTCs and recycled back to the ER in a COPI-dependent manner (Aridor *et al.,* 1995).

The structural organization and the subcellular distribution of ER exit sites has been examined in detail by stereological analysis of serial thin sections taken from cultured mammalian cells (Bannykh *et al.,* 1996). COPII-coated buds are localized at the tip of smooth tubules emerging from rER cisternae. Budding activity is not randomly distributed over the entire ER surface but is restricted to hot spots. Several ER cisternae with COPII-coated buds enclose a VTC to form a higher order structure termed the export complex (Fig. 5; Balch *et al.,* 1994; Bannykh *et al.,* 1996, 1998). By electron microscopy and by fluorescence labeling, about 50–100 export complexes can be counted within a cultured mammalian cell; they are prominent in the neighborhood of the Golgi apparatus but are also found at the NE and the cell periphery (Bannykh *et al.,* 1996; Presley *et al.,* 1997; Scales *et al.,* 1997; Chao *et al.,* 1999; Nishimura *et al.,* 1999). The number of export complexes, however, may be variable between cell types, there being just one in the intracellular parasite *Toxoplasma gondii* (Hager *et al.,* 1999). Interestingly, this export site in *Toxoplasma* is localized to a discrete area of the ONM, providing further support for the concept that budding activity is confined to specialized areas of the ER.

ER exit sites are small and highly dynamic, making it difficult to determine the way in which their molecular composition differs from other ER subregions. However, in cells expressing the rubella virus E1 glycoprotein but not E2, exit sites appear largely expanded because E1 can leave the ER only as an E1/E2 heterodimer. Immunoisolation of these presumptive exit sites and analysis of their protein content have demonstrated an enrichment in COPII coat components and in ERGIC-53, an integral membrane protein that continuously cycles between

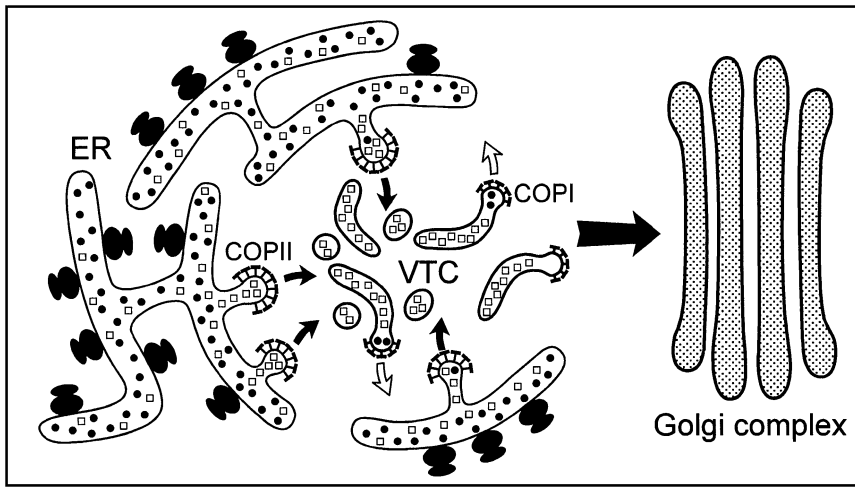

FIG. 5 Organization of export complexes. Protein export from the ER is mediated by COPII-coated vesicles. Vesicle budding occurs at distinct subregions of the ER termed exit sites and involves the exclusion of ER-resident proteins (circles) and the concentration of cargo proteins (squares). ER cisternae carrying exit sites enclose a vesicular-tubular cluster (VTC) to build an export complex. VTC membranes represent the target of ER-derived vesicles, are characterized by a COPI coat, and are carried toward the Golgi complex along microtubules. Moreover, escaped ER-resident proteins and the molecular machinery of vesicle budding are segregated from cargo proteins in the VTCs and recycled back to the ER in a COPI-dependent manner.

the ER and the ERGIC (Hobman *et al.,* 1998). PDI, BiP, and glucosidase II are undetectable in this membrane preparation by Western blot analysis, suggesting that ER-resident proteins are excluded from exit sites. Further evidence in support of this conclusion has been provided by the characterization of COPII-coated prebudding complexes (Aridor *et al.,* 1998) and of ER-derived vesicular carriers (Rowe *et al.,* 1996). These observations, together with the findings that COPII interacts with cargo proteins (Kappeler *et al.,* 1997; Aridor *et al.,* 1998) and that cargo proteins are concentrated before being included into transport vesicles (Balch *et al.,* 1994; Aridor *et al.,* 1998; Nishimura *et al.,* 1999), have led to the concept that cargo packaging is a selective process. Only proteins that have passed the ER quality control system may have access to the exit sites, and these proteins may then be sorted and concentrated by binding to transport receptors that bridge the cargo to the cytosolic COPII coat (Kuehn and Schekman, 1997; Hobman *et al.,* 1998; Ellgaard *et al.,* 1999). Several potential transport receptors have been identified, one of them being ERGIC-53 (Appenzeller *et al.,* 1999). However, although the data in support of this selective export model continue to solidify, this mechanism may not be used by all secretory proteins. In particular, secretory proteins synthesized in abundant amounts may be exported by bulk flow, without

a concentration step at the ER exit sites (Martínez-Menárguez *et al.,* 1999; Warren and Mellman, 1999).

D. ER Domains and Their Role in Regulating Intracellular Ca^{2+}

The ER/SR is the major intracellular Ca^{2+} store in muscle and nonmuscle cells. Ca^{2+} pumps (SERCAs), inositol 1,4,5-trisphosphate receptor ($InsP_3R$) and ryanodine receptor (RyR) Ca^{2+} channels, and intraluminal Ca^{2+}-binding proteins all contribute to the ability of the ER/SR to function as a Ca^{2+} source and Ca^{2+} sink (Pozzan *et al.,* 1994; Meldolesi and Pozzan, 1998a). Tremendous progress has been made in recent years in understanding the way in which the ER contributes to intracellular Ca^{2+} regulation in general, and to the generation of complex spatiotemporal Ca^{2+} signaling patterns, such as intracellular Ca^{2+} oscillations and Ca^{2+} waves, in particular. Because a multitude of physiological activities within a cell are sensitive to changes in cytosolic Ca^{2+}, the question arises regarding how the cell can use this second messenger system to regulate these processes individually. Key aspects in this context are cell geometry, the three-dimensional layout of the ER within the cell, the spatial distribution and functional properties of ER-resident Ca^{2+} channels and pumps, and the kinetics of the interaction of Ca^{2+} with target proteins. The basic mechanisms of intracellular Ca^{2+} regulation, the properties of ER-resident Ca^{2+} channels and SERCAs, and the mechanisms underlying the generation of Ca^{2+} oscillations and waves have been the subject of many recent reviews (McPherson and Campbell, 1993; Berridge, 1993, 1997; Berridge *et al.,* 1998; Clapham, 1995; Meldolesi and Pozzan, 1998a, 1998b; Patel *et al.,* 1999). In the following, we focus on the way in which the topography of the ER and its heterogeneity (inhomogeneous distribution of pumps and channels) affect intracellular Ca^{2+} signaling and permit spatially localized Ca^{2+} signals.

1. Ca^{2+} Uptake and Storage

The ER/SR contains a high intraluminal calcium concentration that has been measured *in situ* in various cell types by several different methods. Electron probe X-ray microanalysis of frozen-dried ultrathin cryosections through cryofixed tissues has revealed intraluminal concentrations for total Ca ranging from 5 mmol/kg dry wt. in hepatocyte ER (Somlyo *et al.,* 1985) through intermediate concentrations of 28 mmol/kg dry wt. in smooth muscle SR (Bond *et al.,* 1984), 47 mmol/kg dry wt. in honeybee photoreceptor ER (Baumann *et al.,* 1991), and up to 120 mmol/kg dry wt. within the terminal cisternae of skeletal muscle SR (Somlyo *et al.,* 1981). Much of this total Ca is bound to low-affinity high-capacity Ca^{2+}-binding proteins within the ER lumen (Niki *et al.,* 1996). Thus, reported concentrations of free intraluminal Ca^{2+}, measured with either Ca^{2+}-sensitive fluorescent dyes or ER-targeted aequorin, range from 100 μM to 5 mM (Meldolesi and Pozzan, 1998a).

The luminal Ca^{2+} concentration (Ca_L) at every point along the morphological continuum of the ER is the combined result of Ca^{2+} uptake and release. If SERCAs and Ca^{2+} channels are distributed inhomogeneously over the ER, it might be expected that Ca_L is not the same throughout the ER. This possibility has been tested and confirmed by monitoring Ca_L with high-resolution imaging techniques and the ER-targeted recombinant aequorin (HeLa cells; Montero *et al.,* 1997) or Ca^{2+}-sensitive fluorescent dyes loaded into the ER of intact cells (cultured astrocytes and mesenteric artery myocytes; Golovina and Blaustein, 1997). These studies show that the ER can indeed establish and maintain spatial subregions with different Ca_L. A striking observation is that Ca_L can fall in some areas of the ER upon application of physiological agonists, of caffeine (an activator for RyR), or of SERCA blockers, whereas it increases in others. Thus, although the ER forms a morphological continuum, it should not be expected that stored Ca^{2+} easily equilibrates throughout its lumen.

The high Ca_L is the result of active Ca^{2+} uptake mediated by SERCAs that, together with the plasma membrane Ca^{2+} ATPase and Na^+/Ca^{2+} exchanger, contribute to the setting of the resting cytoplasmic Ca^{2+} concentration (MacLennan *et al.,* 1997). Three principal isoenzymes, namely, SERCA1, SERCA2, and SERCA3, have been cloned in vertebrates, and all of them exist in alternatively spliced isoforms (Hussain and Inesi, 1999). These SERCA isoforms exhibit subtle differences in their functional properties and show tissue-specific expression patterns. SERCA1 is expressed exclusively in fast-twitch skeletal muscle, SERCA2a is expressed in cardiac and slow-twitch skeletal muscles, SERCA2b is almost ubiquitously expressed, and SERCA3 is expressed in a variety of nonmuscle cells. Some isoforms are coexpressed in some cells, such as SERCA2a, SERCA2b, and SERCA3 in Purkinje neurons (Plessers *et al.,* 1991; Baba-Aissa *et al.,* 1998), SERCA2a and SERCA2b in pancreatic acinar cells, and SERCA2b and SERCA3 in salivary gland acinar and duct cells (Lee *et al.,* 1997a).

Attempts to localize ER subdomains involved in active Ca^{2+} uptake have revealed that, generally, all subdomains including the NE can act as functional Ca^{2+} stores, i.e., that SERCAs have a relatively homogeneous distribution in the ER membrane (Walz, 1982; Payne *et al.,* 1988; Takei *et al.,* 1992; van de Put and Elliot, 1997). Notable exceptions are (1) the junctional regions of the SR terminal cisternae (Fig. 6; Jorgensen *et al.,* 1982), as the high density of RyR Ca^{2+} channels may exclude other proteins at this site; (2) cisternal stacks of sER enriched in $InsP_3R$ in the somata of Purkinje neurons (Satoh *et al.,* 1990; Takei *et al.,* 1992; Villa *et al.,* 1991); and (3) paracrystalline sER in an invertebrate photoreceptor (Walz, 1982). Although, with these few exceptions, the entire ER appears to have SERCAs, there may be differences in the localization of SERCA isoforms in cells that express multiple isoforms. For instance, in pancreatic acinar cells, SERCA2b is localized to the basal cell pole and the NE, whereas SERCA2a is concentrated at the luminal cell pole and in the zymogen granule region. Submandibular salivary gland acini and duct cells, in contrast, have SERCA2b at the luminal pole and

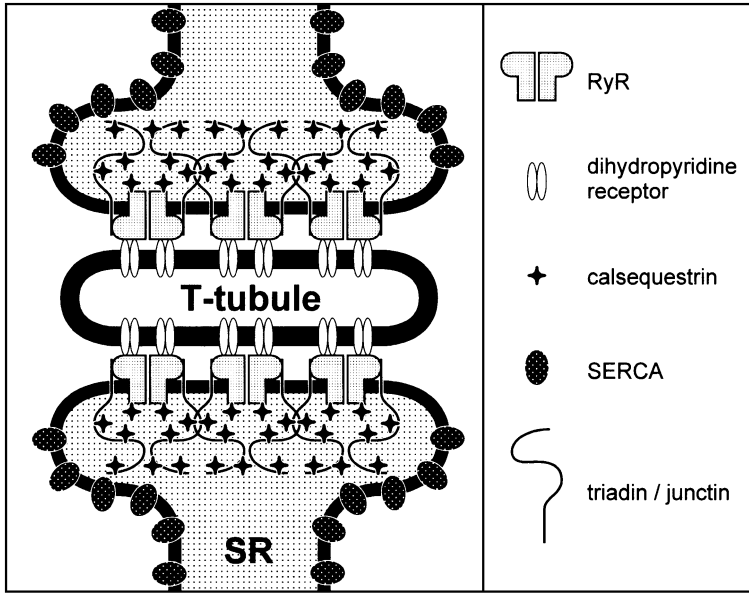

FIG. 6 Structural and molecular organization of the SR in skeletal muscle. The SR is composed of two structurally and functionally distinct domains, the longitudinal SR and the terminal SR, the latter abutting plasma membrane T-tubules. RyR on the terminal SR interact with dihydropyridine receptors located in the T-tubule membrane. Moreover, junctin, triadin, and the Ca^{2+}-binding protein calsequestrin are restricted to the terminal SR and bind to the RyR. The SERCA molecules reside at high density in the longitudinal SR and at the nonjunctional area of the terminal SR.

close to the lateral surface, but SERCA3 in their basal region (Lee *et al.,* 1997a). The physiological significance of this polarized distribution of SERCA isoforms is still unknown, because the small differences in their functional properties do not express themselves in a measurable manner. Nevertheless, these observations raise the possibility that SERCAs contribute, in a cell-specific way, to compartmentalized intracellular Ca^{2+} signaling.

The Ca^{2+}-storing capacity of the ER/SR is greatly influenced by ER/SR-resident low-affinity high-capacity Ca^{2+}-binding proteins. Calsequestrin is the major Ca^{2+}-binding protein in skeletal muscle SR, and calreticulin, originally discovered in skeletal muscle, is a major Ca^{2+}-binding protein in the ER of nonmuscle cells (MacLennan and Wong, 1971; Michalak *et al.,* 1992, 1998, 1999; Franzini-Armstrong and Jorgensen, 1994; Niki *et al.,* 1996). Other ER-luminal proteins, such as BiP, endoplasmin, and PDI, may contribute to the Ca^{2+} storage capacity of the ER (Niki *et al.,* 1996; Michalak *et al.,* 1998; Lucero *et al.,* 1998). The latter proteins and calreticulin are also involved in the post-translational maturation of newly synthesized secretory proteins and are

relatively homogeneously distributed throughout the ER network as discussed in Section III.B.2.

Calsequestrin is special. In skeletal muscle cells at least, it is preferentially localized to the terminal cisternae of the SR (Fig. 6), as demonstrated by light-microsopic and electron microscopic immunocytochemistry and by subcellular fractionation (Meissner, 1975; Jorgensen *et al.,* 1983). Electron microscopy has further revealed that calsequestrin forms an electron-dense network in the center of the terminal cisternae and is connected by fine strands to the SR junctional membrane containing RyR (Saito *et al.,* 1984; Franzini-Armstrong *et al.,* 1987). This proximity between calsequestrin and RyR seems to be important for excitation–contraction coupling not only because it provides high Ca^{2+} storage capacity next to the Ca^{2+} channels, but also because the phosphorylation state of calsequestrin influences RyR Ca^{2+} channel activity (Szegedi *et al.,* 1999).

2. Distribution of Ca^{2+} Channels and Localized Ca^{2+} Signaling

Ca^{2+} release from the ER is mediated by two different types of Ca^{2+} channels, namely, $InsP_3R$ and RyR, that exhibit a high degree of homology in their amino acid sequence, particularly within the membrane-spanning regions (Berridge, 1993; McPherson and Campbell, 1993; Pozzan *et al.,* 1994; Patel *et al.,* 1999). Three isoforms of $InsP_3R$ ($InsP_3R1$, $InsP_3R2$, $InsP_3R3$) have been identified in vertebrates, and even more molecular heterogeneity is produced by alternative splicing. RyR also occurs as three family members (RyR1, RyR2, RyR3) in vertebrates. Both $InsP_3R$ and RyR Ca^{2+} channels are large tetrameric complexes that display a four-leaf-clover-like structure in electron microscopic images, as first recognized for RyR channels (Radermacher *et al.,* 1994; Katayama *et al.,* 1996; Patel *et al.,* 1999). These constitute the "feet" structures spanning the gap between SR terminal cisternae and T tubules in skeletal muscle cells (Fig. 6; Franzini-Armstrong and Jorgensen, 1994).

Of immediate relevance for intracellular Ca^{2+} signaling is the Ca^{2+} sensitivity of $InsP_3Rs$ and RyRs. The opening of both channel types (but not of all isoforms) is regulated by positive and negative feedback of cytosolic Ca^{2+} (Baumann and Walz, 1989b; Iino, 1990; Bezprozvanny *et al.,* 1991; Finch *et al.,* 1991). This property of the Ca^{2+} channels is supposed to provide the mechanistic basis for regenerative Ca^{2+} release and for intracellular Ca^{2+} oscillations and waves that have been observed in many cells (Berridge, 1993; Clapham, 1995; Berridge *et al.,* 1998).

Both $InsP_3R$ and RyR isoforms show tissue-specific expression patterns. Some cells express only RyRs or $InsP_3Rs$ (Pozzan *et al.,* 1994; Bennett *et al.,* 1996). A large number of cell types, however, contains both channel types (Galione *et al.,* 1993; Lee *et al.,* 1993; Walz *et al.,* 1995; Zhang *et al.,* 1999). Possession of two release pathways may have the advantage of allowing the separate modulation of the channels by different agonists and of keeping ready two release pathways with

different Ca^{2+} sensitivities (Bezprozvanny *et al.,* 1991) that may be sequentially activated to amplify and propagate Ca^{2+} signals. To understand the way in which $InsP_3Rs$ and RyRs interact to organize the spatiotemporal pattern of intracellular Ca^{2+} signaling or the manner in which either type of Ca^{2+} channel alone generates spatially localized Ca^{2+} signals, and which downstream functions are activated by such signals, we need to know the precise spatial distribution of $InsP_3Rs$ and RyRs over the ER, supplemented by high-resolution Ca^{2+}-imaging experiments and the identification of the physiological and/or biochemical effects of localized Ca^{2+} signals. Such combined data are available for only a few cell types, but these examples are particularly instructive.

(1) The classical well-established example is the striated muscle cell. Here, the RyR Ca^{2+} release channels are localized at the terminal cisternae of the SR where they are bound to dihydropyridine receptors in the T-tubular membrane by direct protein–protein interactions (Fig. 6). In skeletal muscle cells, the dihydropyridine receptors act as voltage sensors for sarcolemmal depolarization and forward a conformational change to the RyR. In cardiac muscle cells, sarcolemmal L-type Ca^{2+} channels (also dihydropyridine receptors) are activated by depolarization and mediate an influx of Ca^{2+}; this initial Ca^{2+} signal is then amplified by Ca^{2+}-induced Ca^{2+} release from the SR via RyRs. In both muscle cell types, the elementary Ca^{2+} signaling events, called Ca^{2+} sparks and Ca^{2+} quarks, can be monitored by high-resolution Ca^{2+} imaging. These spatially highly localized elementary events represent the openings of Ca^{2+} release channels located in the SR membrane and contribute to myofibrillar Ca^{2+} elevation during muscle contraction (Franzini-Armstrong and Jorgensen, 1994; Niggli, 1999).

(2) The first demonstration that $InsP_3R$ Ca^{2+} channels may not be homogeneously distributed over the ER came from work on ventral nerve photoreceptors in the horseshoe crab *Limulus*. These large cells have two lobes, an arhabdomeric and a rhabdomeric lobe, and only the latter is equipped with photoreceptive microvilli and is light sensitive. Photostimulation activates the phosphoinositide signaling cascade, $InsP_3$ formation, and Ca^{2+} release from the ER (Payne, 1987). Light-induced Ca^{2+} release is initiated at the base of the microvilli, spreads throughout the rhabdomeric lobe, but remains confined to this cell portion (Ukhanov and Payne, 1995). Payne, Fein, and coworkers (Payne and Fein, 1987; Payne *et al.,* 1988) have demonstrated that, although the ER extends through both lobes, only $InsP_3$ injections into the rhabdomeric lobe produce a Ca^{2+} elevation. This observation suggests that $InsP_3Rs$ are enriched on ER within the rhabdomeric lobe and are presumably localized to the submicrovillar ER, an elaborate system of sER cisternae underneath the microvilli (Feng *et al.,* 1994). This interpretation has recently been confirmed by localized photolysis of caged $InsP_3$ and simultaneous confocal imaging of Ca^{2+} changes (Ukhanov *et al.,* 1998). The tight spatial relationship of $InsP_3$ to the phototransduction compartment, the microvilli, may be a precondition for fast excitation and for the efficient modulation of the light sensitivity of the cells, because several steps in the phototransduction cascade,

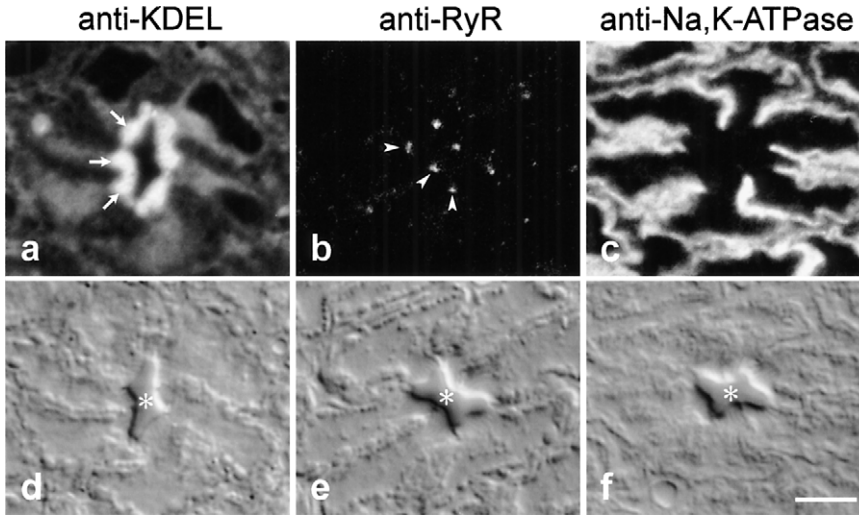

FIG. 7 Restricted distribution of RyR Ca^{2+} channels in honeybee photoreceptors (for details, see Baumann, 2000). (a) Labeling with an antibody against the C-terminal KDEL sequence of ER-resident proteins reveals the distribution of the ER. The intense staining (arrows) corresponds to the voluminous submicrovillar ER cisternae (compare with Fig. 2). (b) Labeling with an antibody against RyR demonstrates that these Ca^{2+} channels are concentrated at discrete sites (arrowheads) within the cells. (c) Labeling for Na^+, K^+-ATPase visualizes the nonreceptive plasma membrane of the photoreceptor cells and, thus, reveals the morphological organization of the tissue. (d–f) The corresponding Nomarski contrast images show the position of the rhabdom, the microvillar light-receptive structure (asterisks) of the photoreceptors. Scale bar, 5 μm.

including the opening of the light-activated cation channels, are sensitive to $[Ca^{2+}]$ changes.

Submicrovillar sER cisternae have been identified in all invertebrate microvillar photoreceptors and constitute the major intracellular Ca^{2+} store in these cells (Walz and Baumann, 1995). Studies on insect photoreceptors have provided evidence for the presence of RyR Ca^{2+} channels in addition to IP_3Rs in these cells (Hasan and Rosbash, 1992; Walz *et al.,* 1995; Arnon *et al.,* 1997). Analysis of RyR distribution in bee photoreceptors has demonstrated that these Ca^{2+} channels are highly enriched at discrete ER elements close to the microvilli (Fig. 7; Baumann, 2000). RyR may thus contribute, together with $InsP_3R$ Ca^{2+} channels, to Ca^{2+} regulation next to the phototransduction area, at least in bee photoreceptors.

(3) Cerebellar Purkinje neurons are among the most intensively studied cell types with respect to Ca^{2+} signaling (Pozzan *et al.,* 1994; Berridge, 1998; Svoboda and Mainen, 1999). Their elaborate ER forms a continuous network extending from the soma into both the axon and the dendritic tree, and from there reaching with small sER tubules into the synaptic spines (Martone *et al.,* 1993; Terasaki *et al.,* 1994).

Purkinje cells express $InsP_3Rs$ and RyRs, and both have a distinctive, yet overlapping, intracellular distribution. They coexist on ER membranes in most regions of the cell, including the dendritic trunk, whereas the ER in synaptic spines has only $InsP_3Rs$ (Walton *et al.,* 1991). The dendritic tree of Purkinje neurons receives synaptic input from afferent fibers that release glutamate as a neurotransmitter. High-resolution confocal Ca^{2+} imaging in Purkinje cell dendrites has shown that electrical stimulation of a few afferent fibers produces a biphasic intradendritic Ca^{2+} elevation, with a fast component resulting from Ca^{2+} influx through voltage-gated Ca^{2+} channels and a slower component based on $InsP_3$-induced Ca^{2+} release. The spatial spread of the Ca^{2+} elevation depends on the frequency of the stimulation and ranges from individual spines to larger spinodendritic compartments (Finch and Augustine, 1998; Takechi *et al.,* 1998). $InsP_3$-dependent Ca^{2+} release induces long-term synaptic depression (LTD) at the Purkinje cell synapse, as mice without the $InsP_3R$ type 1 gene or neurons injected with an antibody against $InsP_3R$ type 1 completely lack LTD (Inoue *et al.,* 1998). Finch and Augustine (1998) have further demonstrated that the Ca^{2+} rise upon focal photolysis of caged $InsP_3$ in Purkinje cell dendrites causes LTD only on synapses with an increased Ca^{2+} concentration. Most notably, mice and rat mutants termed *dilute* have dendritic spines that lack ER cisternae, whereas the ER in the dendritic trunk appears unaffected (Dekker-Ohno *et al.,* 1996; Takagishi *et al.,* 1996; Bridgman, 1999). These animals seem normal at birth except for a light (dilute) coat color, but later develop cerebellar defects, including symtoms of ataxia and motor coordination deficiencies, suggesting that the absence of $InsP_3Rs$ in dendritic spines impairs postsynaptic calcium signaling and leads to severe neuronal malfunctions. These studies on Purkinje cell neurons thus illustrate nicely the importance of $InsP_3R$ Ca^{2+} channel distribution for spatiotemporal Ca^{2+} patterning and for local Ca^{2+}-dependent information processing. The role of RyR in Ca^{2+} regulation within Purkinje neurons, however, is still elusive.

(4) The functional heterogeneity and the coordinated interplay of different Ca^{2+} release channels in the organization of intracellular Ca^{2+} signaling has also been extensively studied in pancreatic acinar cells. The main functions of these cells are transepithelial fluid secretion and apical secretion of enzymes. Stimulus-secretion coupling involves a cytosolic Ca^{2+} elevation caused by $InsP_3$-induced Ca^{2+} release (Yule and Williams, 1994). Ca^{2+} imaging studies have shown that agonist stimulation produces a fixed pattern of Ca^{2+} signaling. Ca^{2+} spikes are initiated at the so-called trigger zone in the apical secretory-granule-containing pole of the cell and then spread as a nondiminishing Ca^{2+} wave toward the basal pole (Kasai and Augustine, 1990). Pancreatic acinar cells express both $InsP_3Rs$ (all three isoforms; Lee *et al.,* 1997b) and RyRs (RyR2; Leite *et al.,* 1999) and position them in a polarized fashion. $InsP_3Rs$ are concentrated in the apical zone, whereas RyRs are localized throughout the basolateral region of the acinar cells (Nathanson *et al.,* 1994; Yule *et al.,* 1997; Leite *et al.,* 1999). Upon stimulation of pancreatic acinar cells with acetylcholine, serial activation of first apical $InsP_3Rs$ and then

basolateral RyRs may generate an apicobasal Ca^{2+} wave (Pfeiffer *et al.,* 1998). This Ca^{2+} wave may sequentially activate physiological processes within the cell, first exocytosis of zymogen granules (Ito *et al.,* 1997) and Ca^{2+}-dependent Cl^- channels on the apical side and then Ca^{2+}-dependent Cl^- channels on the basolateral surface domain (Kasai and Augustine, 1990), and thus coordinate electrolyte and water flow across both surface domains.

(5) Oligodendrocytes provide another instructive example for a cell type that has contributed significantly to our current knowledge of the role of ER organization in determining the spatiotemporal Ca^{2+} pattern. Oligodendrocytes respond to a variety of phosphoinositide-pathway-activating neurotransmitters with intra- and intercellular Ca^{2+} waves that may convey signaling information over long distances within the nervous tissue (Simpson and Russell, 1996; Simpson *et al.,* 1997). Ca^{2+} waves elicited by Ca^{2+} release from the ER do not migrate at constant rates along oligodendrocyte processes but rather propagate by a regenerative saltatory mechanism that involves amplification sites with enhanced Ca^{2+} release kinetics (Simpson and Russell, 1996; Simpson *et al.,* 1997). These amplification sites correspond to ER domains enriched in SERCA, $InsP_3R$, and calreticulin (Simpson and Russell, 1997; Simpson *et al.,* 1997). Notably, mitochondria are found in close apposition to these distinctive ER domains but not elsewhere along the cell processes (Simpson and Russell, 1996). There seems to be a bidirectional functional interplay between both organelles at these specialized cell areas (Simpson and Russell, 1996, 1998a, 1998b). Increases in cytosolic Ca^{2+} concentrations cause mitochondrial Ca^{2+} elevations that may activate mitochondrial metabolism. Because of this Ca^{2+} uptake, on the other hand, mitochondria may act as a local Ca^{2+}-buffering system and thus modulate $InsP_3R$-dependent Ca^{2+} release from the adjacent ER. This model is also supported by results from studies of a variety of other cell types (Rizzuto *et al.,* 1993, 1998, 1999; Jouaville *et al.,* 1995; Csordás *et al.,* Montero *et al.,* 1999; Zimmermann, 2000), suggesting that mitochondria may generally participate in the local regulation of Ca^{2+} channels residing in nearby ER cisternae and that the spatial layout of the ER in relation to mitochondria plays a role in shaping intracellular Ca^{2+} responses.

E. ER Domains and Their Role in Lipid Metabolism

Most steps in the biosynthesis of membrane lipids and lipophilic compounds occur on ER membranes (Bishop and Bell, 1988; Dawidowicz, 1987a, 1987b). In the following, we summarize the results of studies demonstrating that several steps in the synthesis of phospholipids and cholesterol are compartmentalized between the ER and mitochondria (Black *et al.,* 1994), and that some key enzymes of lipid synthesis, cholesterol synthesis, and the synthesis of glycosylphosphatidylinositol seem to be distributed inhomogeneously over the ER. We must state, however,

that all of these studies provide examples of functional heterogeneity in the ER in a descriptive sense, and that we are far from understanding the physiological significance of this heterogeneity for lipid metabolism.

1. Synthesis of Phospholipids

Synthesis of phosphatidylcholine (PtdCho) occurs by a biochemical pathway, the enzymes of which reside on the ER for the most part, whereas an intervening step, the conversion of phosphatidylserine (PtdSer) to phosphatidylethanolamine (PtdEtn) by PtdSer decarboxylase, is associated with the inner mitochondrial membrane (Dennis and Kennedy, 1972; Zborowski *et al.,* 1983). Hence, PtdSer made on ER membranes must be transferred to mitochondria for processing to PtdEtn, which is then shuttled back to the ER for methylation by PtdEtn N-methyltransferase (Fig. 8). This tight functional interplay between ER and mitochondria in phospholipid synthesis raises the question of whether mitochondria-associated ER domains, often observed in electron microscopic images (e.g., Baumann and Walz, 1989a; Takei *et al.,* 1994; Perkins *et al.,* 1997), are involved in or even specialized for lipid transfer between the two organelles. To investigate this possibility, Vance (1990) has isolated, from liver cells, a crude mitochondrial fraction that is able to synthesize PtdSer, PtdEtn, and PtdCho. PtdSer synthase and PtdEtn *N*-methyltransferase activity are associated with a mitochondria-associated membrane (MAM) fraction that is characterized as ER by its phospholipid composition and content of ER-marker enzymes. However, Vance (1990) has noted

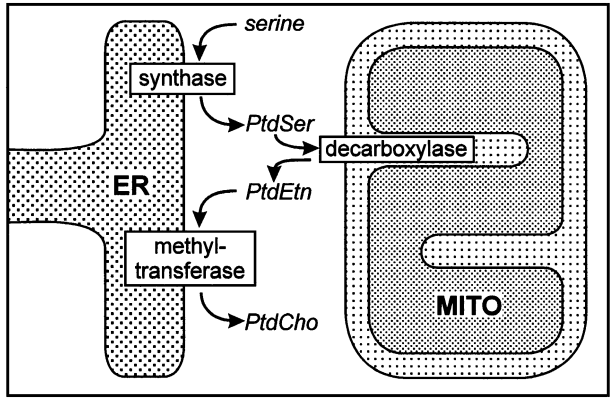

FIG. 8 Phospholipid translocation between ER and mitochondria. Phosphatidylserine (PtdSer) is synthesized on the ER and translocated to mitochondria (MITO) for decarboxylation by mitochondrial PtdSer decarboxylase. The resulting phosphatidylethanolamine (PtdEtn) is then transferred back to the ER and methylated by PtdEtn *N*-methyltransferase to form phosphatidylcholine (PtdCho). Both PtdSer synthase and PtdEtn *N*-methyltransferase are concentrated on a mitochondria-associated ER domain.

quantitative differences between MAM and pure microsomes in the specific activities of these enzymes, e.g., an enrichment in PtdSer synthase activity and a reduced NADPH:cytochrome C reductase activity in the MAM fraction. Similar results have been obtained by other authors (Ardail *et al.,* 1990, 1991, 1993; Gasnier *et al.,* 1993) and strengthen the concept that mitochondria-associated ER domains are involved in the transfer of lipids between these organelles. Moreover, since several lipid-biosynthetic enzymes, particularly PtdSer synthase and PtdEtn *N*-methyltransferase, are enriched on ER zones in contact with mitochondria (Cui *et al.,* 1993; Rusinol *et al.,* 1994), these ER regions may be specialized in the biogenesis of phospholipids.

2. Synthesis of Glycosylphosphatidylinositols

Mitochondria-associated ER domains also seem to be involved in some key steps of the synthesis of glycosylphosphatidylinositols (GPIs) that serve as a membrane anchor for various cell surface glycoproteins (e.g., Ferguson and Williams, 1988). GPIs are synthesized in the ER by the sequential addition of monosaccharides, fatty acid, and phosphoethanolamine (PE) to phosphatidylinositol (PI). A recent analysis (Vidugiriene *et al.,* 1999) of the distribution of various GPI biosynthetic reactions in subcellular fractions prepared from mammalian cells indicates that the initial reactions of GPI synthesis, namely, the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to PI to form GlcNAc-PI, are uniformly distributed over the ER, whereas subsequent steps in the pathway, namely, the de-*N*-acetylation of GlcNAc-PI to GlcN-PI and the conversion of GlcN-PI to H5 (= a single mannosylated GPI structure containing one phosphoethanolamine side chain) are largely confined to mitochondria-associated ER. The physiological significance of these findings is unknown. Vidugiriene *et al.* (1999) speculate, however, that the PE residues in GPI may be preferentially derived from PE synthesized via the mitochondrial phosphoserine decarboxylation pathway, but alternative interpretations are possible. Another study (Watanabe *et al.,* 1996) has addressed the question of where the first reaction step of GPI synthesis (transfer of GlcNAc from UDP-GlcNAc to PI) takes place in the ER of mammalian cells. The authors show that the products of two genes, PIG-A and PIG-H, which participate in this first step, are ER membrane proteins that form a protein complex localized to the rER.

Studies on trypanosomes have contributed considerably to our knowledge of the biosynthetic reactions leading to GPIs, because they are important components for the anchoring of major surface glycoproteins in these protozoan parasites (Ferguson, 1999). Ilgoutz *et al.* (1999) have reported a stable tubular ER subdomain that contains dolichol-phosphate-mannose synthase (DPMS), a key enzyme in GPI biosynthesis, and other enzymes involved in GPI synthesis, in *Leishmania mexicana*. They call this prominent organelle the DPMS tubule. Because it is closely associated with the single Golgi apparatus of these cells and appears to exclude luminal ER-resident proteins, they hypothesize that the DPMS tubule

might be a stable transitional ER. It is noteworthy that the DPMS tubule is also located next to a mitochondrion.

3. Cholesterol Biosynthesis and Metabolism

Reinhart *et al.* (1987) have investigated the distribution of several enzymes of cholesterol synthesis and metabolism in rat liver microsomes. They have found that all enzymes assayed from the pathway between lanosterol and cholesterol (lanosterol 14-demethylase, steroid 14-reductase, steroid 8-isomerase, cytochrome *P*-450, and cytochrome b_5) are distributed in both rER and sER. Identical results have been obtained for 3-hydroxy-3-methylglutaryl-CoA reductase (HMG CoA reductase), the rate-limiting enzyme in this pathway (Brown *et al.,* 1978). Most notably, Reinhart *et al.* (1987) have found that acyl-CoA-cholesterol acyltransferase, an enzyme that catalyzes the esterification and removal of free cholesterol from the membrane, is only present in the rER fraction. The authors suggest that acyl-CoA-cholesterol acyltransferase could be an important component of a protective system to maintain a low cholesterol level in the rER. Moreover, the lamin B receptor (LBR), a membrane protein confined to the INM (see Section III.A.), has been demonstrated to have sterol C_{14} reductase activity (Moebius *et al.,* 1998; Silve *et al.,* 1998), providing further evidence for a compartmentalization of at least some steps in cholesterol metabolism.

A pronounced anisotropic distribution of HMG CoA reductase is observed in cells that are starved of cholesterol by experimental inhibition of this enzyme. This treatment results in a 100-fold to 500-fold overexpression of HMG CoA reductase (Chin *et al.,* 1982) and a marked proliferation of sER that forms either lamellar stacks or crystalloid ER (Table I). Immunolocalization of HMG CoA reductase in such cells has demostrated that this enzyme is highly enriched within the morphologically distinctive sER regions and almost nondetectable on rER (Pathak *et al.,* 1986; Singer *et al.,* 1988).

4. Leukotriene Synthesis

The lipoxygenase-mediated formation of active leukotrienes from arachidonic acid is another example of the compartmentalization of an ER-resident reaction of lipid metabolism. Leukotrienes are products of arachidonic acid metabolism and are made by leukocytes. Two related lipoxygenase (LO) enzymes (5-LO and 15-LO) use arachidonic acid as a substrate. Both lipoxygenases are cytoplasmic enzymes in resting cells, but 5-LO translocates to the NE upon cell stimulation (Woods *et al.,* 1993; Christmas *et al.,* 1999). Critical for 5-LO activity is an 18-kDa membrane-bound 5-LO-activating protein (FLAP) that possibly presents arachidonic acid to 5-LO. By immunogold labeling, FLAP has been shown to be highly concentrated on the NE in both resting and stimulated cells (Woods *et al.,* 1993). Moreover, a cytosolic phospholipase A2 releasing arachidonic acid from membrane

phospholipids exhibits a stimulus-induced translocation to the NE (Glover *et al.,* 1995; Gijón *et al.,* 1999), suggesting that several key enzymes of leukotriene biosynthesis assemble on the NE following cell stimulation.

F. ER Domains and Their Role in Carbohydrate Metabolism

Since the early days of electron microscopy, it has been known that sER cisternae and glycogen particles are closely juxtaposed in liver cells and many other cell types (Fawcett, 1981). These specialized areas have been termed SERGE foci (Cardell *et al.,* 1985) and may represent a microenvironment favorable for glycogen metabolism (al-Habori, 1995). Although key enzymes of glycogen metabolism, such as glycogen synthase phosphatase and phosphorylase phosphatase, have been found in association with sER membranes (Margolis *et al.,* 1979), this classical and more intuitive concept has not yet been substantiated.

IV. Dynamics of the ER Network

The ER is a highly dynamic membrane system and displays mobility on three hierarchy levels: (1) diffusional mobility of the molecular ER components, (2) movement of individual ER cisternae, and (3) reorganization of the entire ER network.

A. Diffusional Mobility of ER Proteins

To examine the diffusional mobility of luminal ER proteins, the movement of GFP with an N-terminal secretory sequence and a C-terminal KDEL retention signal was followed within cultured mammalian epithelial cells (Dayel *et al.,* 1999). Translational diffusion of the GFP construct was analyzed by using FRAP (fluorescence recovery after photobleaching). In this method, a laser bleaches the fluorescence within a small defined area of a live cell; recovery of fluorescence within the photobleached area, because of diffusion of unbleached molecules into this region, can then be used to determine the mobility of the fluorescent protein (White and Stelzer, 1999). These measurements have demonstrated that the GFP-KDEL construct is fully mobile within the entire ER lumen (Table II; Dayel *et al.,* 1999). The diffusion coefficient D of 5–10 μm^2/sec for GFP-KDEL corresponds to an average diffusion distance of 10 μm in 5–10 sec. Diffusion of the ER luminal GFP construct is thus 3–6 times lower than that for GFP within the cytoplasm (Swaminathan *et al.,* 1997), suggesting that translational mobility of ER luminal proteins is slightly reduced by collisional interactions (Dayel *et al.,* 1999). However, not all ER luminal proteins may have such a high mobility, as

TABLE II
Diffusional Mobility of GFP-Tagged Proteins in the Lumen or the Membrane of the ER

Molecule	Location	D (μm^2/sec)	Reference
Galactosyltransferase	ER membrane[a]	0.28 ± 0.04	Zaal *et al.,* 1999
	ER membrane[b]	0.30 ± 0.04	Zaal *et al.,* 1999
	ER membrane[c]	0.21 ± 0.02	Cole *et al.,* 1996
KDEL-receptor	ER membrane[c]	0.43 ± 0.05	Cole *et al.,* 1996
Lamin B receptor	ER membrane[d]	0.41 ± 0.01	Ellenberg *et al.,* 1997
Cytochrome *P*-450	ER membrane	0.06 ± 0.01	Szczesna-Skorupa *et al.,* 1998
Emerin	ER membrane[d]	0.32 ± 0.01	Östlund *et al.,* 1999
MHC class I	ER membrane		
Free		~0.4	Marguet *et al.,* 1999
Bound to TAP		~0.2	Marguet *et al.,* 1999
Lamin B receptor	NE	Immobile	Ellenberg *et al.,* 1997; Rolls *et al.,* 1999
Emerin	NE	0.10 ± 0.01	Östlund *et al.,* 1999
LAP2	NE	Restricted mobility	Rolls *et al.,* 1999
Nurim	NE	Restricted mobility	Rolls *et al.,* 1999
Elastase	ER lumen	0.5	Subramanian and Meyer, 1997
GFP-KDEL	ER lumen	5–10	Dayel *et al.,* 1999

[a] Galactosyltransferase pool in the ER of interphase cells.
[b] Mitotic cells.
[c] Brefeldin A treated cells, resulting in a redistribution of Golgi proteins into the ER.
[d] Overexpression of emerin-GFP and LBR-GFP causes a partial localization to the ER network.

elastase-GFP exhibits a 10-fold lower D than GFP-KDEL (Subramanian and Meyer, 1997). This lower diffusion rate may result from protein aggregation.

By use of a similar approach, several different groups have demonstrated that various integral ER membrane proteins, such as LBR, emerin, KDEL receptor, galactosyltransferase, and MHC (major histocompatibility complex) class I, are also extremely mobile and can diffuse throughout the ER network with a D of 0.2–0.4 μm^2/sec in interphase and mitotic cells (Table II; Cole *et al.,* 1996; Ellenberg *et al.,* 1997; Östlund *et al.,* 1999; Zaal *et al.,* 1999; Marguet *et al.,* 1999). This value is comparable to the D for proteins in other intracellular membrane systems, such as rhodopsin in the disc membrane of rod outer segments (Wey *et al.,* 1981) and mannosidase II and galactosyltransferase in Golgi membranes (Cole *et al.,* 1996), and is near the limit set by membrane viscosity (Marguet *et al.,* 1999).

Although only a few ER membrane proteins have been examined with respect to their lateral diffusion coefficient, these measurements indicate that mobility may be quite different for various ER membrane proteins, in particular for those

that form protein complexes or have a restricted distribution (Table II). A *D* of only about 0.06 μm^2/sec has been determined for cytochrome *P*-450, probably because this protein forms aggregates (Szczesna-Skorupa *et al.,* 1998). Moreover, the lateral mobility of MHC class I is reduced from ~0.4 to ~0.2 μm^2/sec by binding to TAP, a protein complex that supplies peptides generated by cytosolic proteasomes to MHC molecules (Marguet *et al.,* 1999). Emerin has a 3-fold lower mobility in the NE than in the bulk ER (Östlund *et al.,* 1999), and LBR appears to be practically immobile when concentrated in the INM (Ellenberg *et al.,* 1997). Similar results have been observed for other integral membrane proteins of the INM (Rolls *et al.,* 1999; Östlund *et al.,* 1999). This restricted mobility of integral membrane proteins within the INM indicates stable association with the nuclear lamina and chromatin.

In conclusion, the GFP technique in conjunction with FRAP provides a promising approach for determining protein mobility in the ER. Future challenges will include determining the mobility of ER chaperones in order to define further their proposed organization into a macromolecular network. Moreover, the mobility of integral membrane proteins with a restricted distribution, such as the translocon components, the $InsP_3R$ and the RyR, requires more attention.

B. Motility of ER Membranes

In thinly spread areas of cultured epithelial cells, the ER network can be imaged *in vivo* by fluorescence microscopy after staining with fluorescent lipophilic dyes, such as $DiOC_6(3)$ (Terasaki *et al.,* 1984; Terasaki, 1990), rhodamine B hexyl ester (Terasaki and Reese, 1992), and $DiIC_{18}(3)$ (Terasaki and Jaffe, 1991; Terasaki *et al.,* 1991). In these preparations, the ER is practically two dimensional and thus entirely within the focal plane. It appears as a polygonal network of branching and anastomosing tubules (Fig. 9a) that is continuously rearranged (Lee and Chen, 1988; Sanger *et al.,* 1989). Network dynamics are based on three basic motion types: "tubule branching," "tubule sliding," and "ring closure" (Lee and Chen, 1988; see Fig. 9c). During tubule branching, a free-ending tubule extends linearly at a velocity of about 1 μm/sec from the side of another tubule. The tubule tip may then either fuse with another tubule to form a new polygon, or it may retract again until complete elimination (Fig. 9b). Tubule sliding is characterized by the lateral movement of a three-way junction along a tubule. Finally, ring closure results from the contraction of a polygon until it disappears. These three motion types lead to continuous modifications in the fine structure of the ER. However, because these motion types appear to have random directions within the cell, the general outline of the ER pattern remains largely unchanged.

The ER of most cells is not two dimensional as in the systems described above, but a complex three-dimensional network that is difficult to resolve by light-microscopic techniques. Nevertheless, a few studies have succeeded in imaging

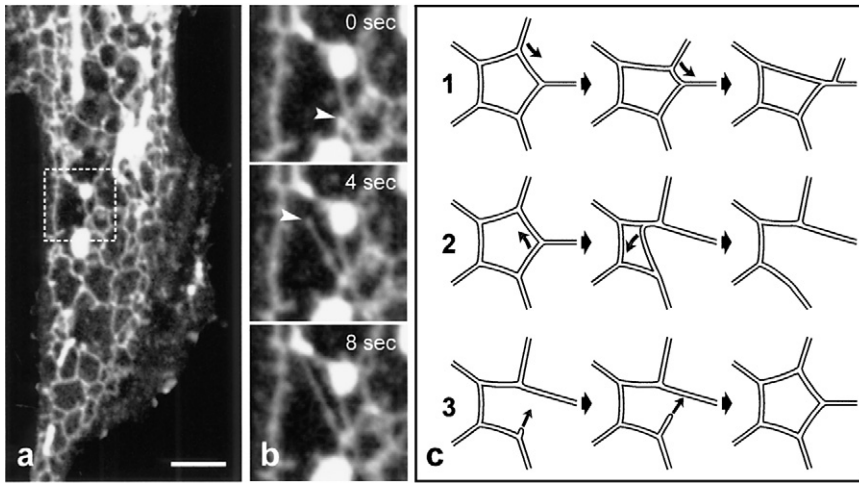

FIG. 9 Motility of ER membranes. (a) Fluorescence micrograph showing the $DiOC_6(3)$-labeled network of ER tubules in the cell periphery of a live BHK cell. The most intensely fluorescent structures represent mitochondria. Scale bar, 5 μm. (b) Time series of the area outlined in part (a). An ER tubule (arrowheads) emerges from the side of another tubule, extends linearly, and finally contacts a prexisting tubule to form a new polygon. (c) Diagrammatic representation of the three basic motion types resulting in network dynamics: (1) tubule sliding, (2) ring closure, and (3) tubule branching.

similar ER dynamics in cells with an elaborate ER network, such as sympathetic neurons (Dailey and Bridgman, 1989), photoreceptors of the horseshoe crab *Limulus* (Feng *et al.,* 1994), and sea urchin eggs (Terasaki and Jaffe, 1991). The dynamics of ER tubules are thus not confined to the thin cell periphery of cultured cells but are a general phenomenon within animal cells. However, the imaging of ER dynamics in sea urchin eggs has demonstrated differences in the motility of different ER regions (Terasaki and Jaffe, 1991). This finding indicates that only some domains within a complex ER system are subjected to constant rearrangement, whereas other subregions, e.g., ER cisternae bound to the plasma membrane or to mitochondria, are relatively stationary or move in conjuction with the associated structure. This issue requires further investigation by means of fluorescent probes specific for discrete ER domains.

C. Reorganization of the ER Network

1. The ER during the Cell Cycle

The organization of the ER changes dramatically during mitosis. In prophase, the peripheral ER collapses around the nucleus as the cells round up (Sanger *et al.,* 1989). Subsequently, at prometaphase, the NE breaks down into elements that

structurally cannot be distinguished from the remaining ER (Zeligs and Wollman, 1979). The physical state of these NE-derived membranes is discussed further below (Section V.A). In metaphase, ER motility is stalled at least in some systems (Allan and Vale, 1991; Niclas *et al.,* 1996), and protein export from the ER is blocked (Featherstone *et al.,* 1985; Farmaki *et al.,* 1999). Moreover, Golgi membranes become absorbed into the ER (Zaal *et al.,* 1999; Terasaki, 2000), suggesting that mitotic ER and interphase ER differ in their molecular composition. In late anaphase and during telophase, the NE reassembles around the condensing chromatin (Zeligs and Wollman, 1979). Finally, the Golgi apparatus reforms (Zaal *et al.,* 1999), and the ER network is extended again toward the cell periphery during cytokinesis (Sanger *et al.,* 1989).

The structural organization of the ER during metaphase and anaphase is still a matter of debate. In electron micrographs of thin sections, the ER of mitotic cells appears to be fragmented into individual vesicular and cisternal elements (Zeligs and Wollman, 1979; Koch *et al.,* 1988). However, the interpretation of these ultrastructural data is open to criticism, because it is impossible to determine, from thin sections, whether round membranous structures represent distinct vesicles or whether they reflect cross sections through a continuous three-dimensional tubular system. Indeed, recent data obtained by using GFP techniques demonstrate that proteins can freely diffuse within the entire ER system in metaphase cells (Ellenberg *et al.,* 1997; Zaal *et al.,* 1999; Terasaki, 2000), providing strong evidence for a continuous ER network thoughout mitosis.

2. Changes in ER Organization during Cell Differentiation or in Response to an Exogenous Stimulus

The ER is a versatile organelle with a variety of functions. However, because different functions predominate in different cell types, ER organization must change during cell differentiation to suit its final prevailing duty. For instance, the rER becomes very prominent in cells with high protein secretion capacity, such as B lymphocytes/plasma cells and pancreatic acinar cells. Cells specialized in steroid synthesis, e.g., adrenal cortical cells or Leydig cells, are characterized by a marked increase in sER (Fawcett, 1981). Yet another cell type that exhibits an impressive reorganization of the ER is the myoblast, in which the ER differentiates into the SR with its peculiar structural and functional subdomains (Flucher, 1992).

Reorganization of the ER not only occurs during cell differentiation, but may also happen on a shorter timescale upon exposure to an exogenous stimulus, e.g., fertilization of eggs (Jaffe and Terasaki, 1993; Terasaki *et al.,* 1996), drug exposure of hepatocytes (Remmer and Merker, 1963), or electrical stimulation of scale worm photocytes (Bassot and Nicolas, 1987). Figure 10 presents a cell in which the stimulus-induced remodeling of the ER is particularly impressive, namely, in the locust photoreceptor. In dark-adapted visual cells of the locust eye, voluminous ER cisternae termed submicrovillar ER back the photoreceptive microvilli. Upon

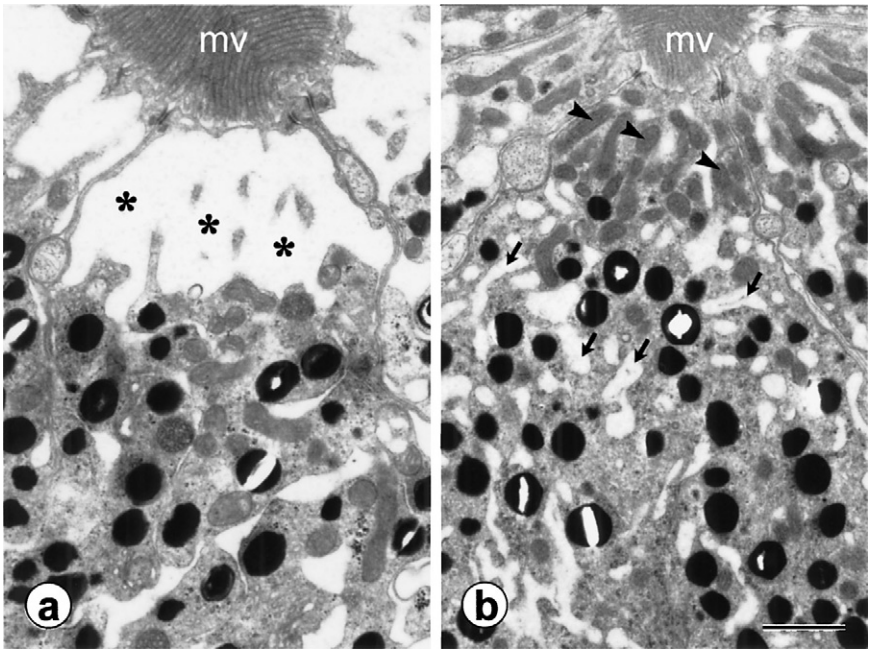

FIG. 10 Light-induced restructuring of the ER in locust photoreceptors. (a) In dark-adapted visual cells, sac-like ER cisternae (asterisks), called submicrovillar ER, back the photoreceptive microvilli (mv). (b) During light exposure, the submicrovillar ER is remodeled into smaller ER cisternae (arrows) that are translocated away from the microvilli, whereas numerous mitochondria (arrowheads) migrate toward the microvilli. Scale bar, 1 μm.

photostimulation, the submicrovillar ER is reorganized into smaller cisternae that are translocated away from the microvilli (Horridge and Barnard, 1965; Stürmer *et al.,* 1995).

Little is known, however, about the molecular mechanisms that lead to ER reorganization and differentiation (Pahl and Baeuerle, 1997). These may be of general interest with respect to understanding the way in which cells actively regulate ER biogenesis.

V. Mechanisms Involved with the Motility of ER Membranes and with the Organization of the ER into Subregions

As is evident from the text so far, the ER of animal cells represents a patchwork of subregions that differ in structure, protein equipment, and motility. The subsequent parts of this review focus on the mechanisms that establish and maintain the

distribution of ER membranes within the cell and the compartmentalization of the ER into subdomains. Because of the complexity of the ER network and the variability of ER organization between cell types, a variety of different mechanisms can be expected to be involved with this task, and various mechanisms may work together in different cell types. Generally, these mechanisms can be assigned to two groups: (1) the binding of ER membranes to other cellular elements, namely, cytoskeletal components and non-ER membranes; and (2) interactions within the ER, e.g., homotypic binding of ER membranes and formation of macromolecular complexes by ER-resident proteins.

A. Interaction with the Lamin Network

Although some ultrastructural studies have reported a close spatial relationship between ER cisternae and intermediate filaments (Franke *et al.,* 1987; Katsumoto *et al.,* 1990; Ohno and Fuji, 1991), there is generally no mechanistic interaction between them (Terasaki *et al.,* 1986; Lee *et al.,* 1989; Houliston and Elinson, 1991). The NE is thus the only ER domain that interacts with components of the intermediate filament system, namely, the nuclear lamina.

The importance of the lamin network for NE organization has been demonstrated by genetic disruption of lamin genes. P-element insertion into the *Drosophila* gene encoding for B-type lamin results in a partially fragmented or a completely absent NE, a clustering of NPCs within the NE remnants, and an abundance of annulate lamellae (Lenz-Böhme *et al.,* 1997). Similarily, mice lacking A-type lamins exhibit defects in nuclear morphology, NE integrity, and NPC clustering (Sullivan *et al.,* 1999). These data lend direct support to the view that the nuclear lamina stabilizes the morphology of the NE and maintains a homogeneous NPC distribution.

In addition to its role in maintaining NE structure, the nuclear lamina is involved with establishing and stabilizing the distinct protein equipment of the INM and the pore membrane. Based on observations of mitotic disassembly and reassembly of the NE, two different models have been generated for the manner in which the nuclear lamina, in conjunction with chromatin, fulfills this function (Collas and Courvalin, 2000). In the first model, the NE fragments at prometaphase, generating vesicles that specifically harbor INM and pore membrane proteins and that do not fuse with bulk ER membranes. Moreover, lamin B may remain associated with the LBR-containing vesicles, whereas lamin A becomes fully solubilized (Meier and Georgatos, 1994). At the end of anaphase, these INM/lamin B vesicles reassemble and fuse to a new NE on the surface of chromatin, which serves as a receptor matrix for vesicle sorting and docking. The nuclear pores and the lamina then reform during telophase and cytokinesis. Support for this model has been provided by the biochemical isolation of distinct vesicle populations enriched in either INM markers (LBR, LAP2) or pore membrane proteins (gp210) from mitotic cells and

by the *in vitro* reconstitution of NE assembly with these vesicles (Vigers and Lohka, 1991; Buendia and Courvalin, 1997; Drummond *et al.,* 1999).

The above model, however, has been severely challenged by recent data. The costaining of INM/pore membrane proteins and ER markers (Yang *et al.,* 1997) and the *in vivo* imaging of the distribution and mobility of GFP-tagged INM proteins (Ellenberg *et al.,* 1997) have demonstrated that the NE proteins are not retained in distinct vesicles but are present on the entire ER system during mitosis. Therefore, Yang *et al.* (1997) and Ellenberg *et al.* (1997) have proposed a new model for NE assembly, termed selective retention mechanism: NE proteins may diffuse throughout the ER network after dissassembly of the NE at the onset of mitosis or after their biosynthesis on the rER, and they become immobilized as they diffuse into ER regions in contact with lamin/chromatin. The binding and release of INM proteins to lamin/chromatin during the cell cycle is regulated by mitotic kinase-induced phosphorylation. Moreover, not only may the chromatin/lamina system serve as a passive receptor for the INM proteins, but reassembly of the lamina and of the NE may also involve cooperativity (Yang *et al.,* 1997). Direct support for this selective retention model has been provided by FRAP experiments, demonstrating that the GFP-labeled LBR is highly mobile in the ER network but immobile on the INM in interphase cells. During mitois, the immobile fraction relocates to the ER network, leaving only highly mobile LBR molecules (Ellenberg *et al.,* 1997). Also in accordance with this model is the finding that, in A-type lamin-defective mice, the INM-specific emerin distributes over the ER network, whereas LAP2, which is known to interact with B-type lamins and chromatin, remains concentrated at the nuclear periphery (Sullivan *et al.,* 1999).

The selective retention model may also elucidate the way in which other ER domains are established and maintained. ER membrane proteins with the ability to bind to distinct cellular structures, e.g., cytoskeletal components (see below), may diffuse from their site of synthesis through the ER network and be retained on ER cisternae within reach of this cellular structure. Studies on the diffusion dynamics of proteins restricted to a distinct ER domain, e.g., the rER, may be helpful in examining this possibility.

B. Interactions with the Microtubule System

1. The Role of Microtubules in Organizing the ER Network

By electron microscopic techniques, a close relationship of ER cisternae and microtubules has been observed in a variety of animal cells, suggestive of an interaction between these structures. For example, analyses of chemically fixed or rapidly frozen whole-mount cells have visualized ER membranes that run closely parallel to microtubules over considerable distances but that do not extend beyond distal microtubule tips (Buckley and Porter, 1975; Dailey and Bridgman, 1991).

Moreover, 20- to 30-nm-long cross-linking structures have been identified between ER membranes and microtubules (Dailey and Bridgman, 1991), possibly representing attachment proteins.

The role of microtubules in the organization of the ER network has been investigated in more detail by fluorescence imaging of $DiOC_6(3)$-labeled ER in the thinly spread cell periphery of cultured vertebrate cells. When fixed cells are costained for ER and microtubules, ER tubules often coalign with microtubules, and the ends of both structures coincide at the resolution of light microscopy (Terasaki *et al.,* 1986; Lee *et al.,* 1989; Dailey and Bridgman, 1989; Terasaki and Reese, 1994). Disruption of the microtubule system by pharmacological agents (colchicine, colcemide, nocodazole) results in a collapse of the ER, which forms an aggregate of membranes around the nucleus. After the microtubule-disrupting drugs are washed out, the cultured cells reconstruct full ER networks within 15–30 min (Terasaki *et al.,* 1984, 1986; Lee *et al.,* 1989; Terasaki and Reese, 1994). Depolymerization of the actin filament system by cytochalasin treatment, in contrast, has no apparent effect on ER organization (Terasaki *et al.,* 1984; Lee *et al.,* 1989). These data have led to a model for ER–microtubule interaction suggesting that the ER uses the microtubule cytoskeleton as a framework for extending and maintaining its reticular organization in animal cells (Terasaki, 1990).

To analyze the role of microtubules in ER construction further, *in vitro* assays have been developed in which polygonal networks of membrane tubules form in a microtubule-dependent manner (Dabora and Sheetz, 1988; Vale and Hotani, 1988; Allan and Vale, 1991, 1994; Waterman-Storer *et al.,* 1995). The identity of the tubule networks as ER membranes has been confirmed by labeling with $DiOC_6(3)$ and antibodies against ER-specific proteins. These *in vitro* assays have allowed the simultaneous imaging of ER and microtubules by video-enhanced differential interference microscopy and have revealed three possible mechanisms for the microtubule-dependent construction and motility of ER networks (Fig. 11): (1) the transport of ER tubules along stationary microtubules by motor proteins (membrane sliding mechanism); (2) the static attachment of ER membranes to the side of microtubules that are translocated over a substrate or along other microtubules (microtubule movement mechanism); and (3) the extension of ER tubules via stable association with the growing plus-ends of microtubules by a structure termed the tip attachment complex (TAC mechanism).

A recent study by Waterman-Storer and Salmon (1998) has examined which of these mechanisms drives ER remodeling in newt lung epithelial cells *in vivo*. After the staining of ER with $DiOC_6(3)$ and of microtubules by microinjection of rhodamine-tubulin, microtubule and ER dynamics has been imaged in parallel by multiple-wavelength time-lapse fluorescence microscopy. Waterman-Storer and Salmon (1998) have elegantly demonstrated that new ER tubules extend in the microtubule plus-end direction toward the cell periphery, about two-thirds via the membrane sliding mechanism and the remaining one-third by the TAC mechanism. The mean transport rate of both mechanisms is $\sim$4 μm/min in this system. When

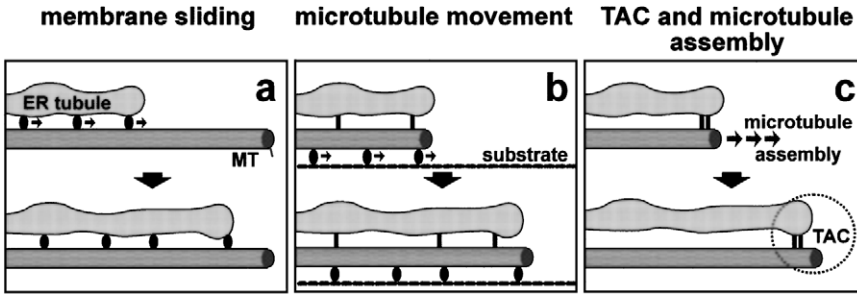

FIG. 11 Models for microtubule-dependent ER motility. Three different mechanisms may account for microtubule-dependent ER translocation (a) An ER tubule slides along a stationary microtubule by the activity of ER-bound motor proteins, such as kinesin or cytoplasmic dynein. (b) The ER tubule is stably attached to a microtubule, and the microtubule is translocated along the substrate (some other cellular structure). (c) The tip of an ER tubule is associated with the microtubule plus-end by a structure called the tip attachment complex (TAC), and ER extension is driven by microtubule polymerization.

a microtubule switches from the growing to the shrinking phase, the ER tubule retracts toward the cell body along the microtubule end. In addition, microtubules with statically attached ER membranes show a steady retrograde movement at $\sim$0.4 μm/min. Inhibition of this movement by the f-actin depolymerizing drug cytochalasin D and by the myosin inhibitor 2,3-butanedione monoxime indicates that it occurs through actomyosin-based retrograde flow, in aggreement with the results of Terasaki and Reese (1994) from a frog kidney cell line.

Taken together, there is thus ample evidence that ER extension into the cell periphery occurs by microtubule-dependent mechanisms, and that the microtubule cytoskeleton has a major share in establishing and maintaining the distribution of ER membranes in animal cells. However, why do cells have redundant mechanisms for the extension of ER tubules? Waterman-Storer and Salmon (1998) postulate that usage of the membrane sliding mechanism or of the TAC mechanism may depend on the organization of the microtubule cytoskeleton. Cells may preferentially employ the TAC mechanism when microtubule plus-ends are abundant, e.g., after cell division. When microtubules are long and unipolar, as in the axon, the sliding mechanism may predominate.

2. Motor Proteins Involved with Microtubule-Dependent ER Motility

Because microtubules within cultured cells and many somatic cell types are oriented with their plus-ends peripherally, the membrane sliding mechanism requires the activity of a plus-end-directed motor, such as conventional kinesin or other members of the kinesin superfamily (Hirokawa, 1998). There is indeed evidence from several different experimental approaches for the presence of kinesin on ER

membranes and for its involvement in ER motility. Kinesin and the kinesin-binding protein kinectin are associated with an ER-like structure and partially colocalize with ER markers at the light-microscopic level in several cell types (Houliston and Elinson, 1991; Henson *et al.,* 1992; Toyoshima *et al.,* 1992). Kinesin has also been identified on ER vesicles in squid axoplasm by immunogold labeling (Tabb *et al.,* 1998). The inhibition of ER motility by anti-kinesin or anti-kinectin antibodies in *in vitro* assays (Kumar *et al.,* 1995; Lane and Allan, 1999) and the retraction of ER from the cell periphery in astrocytes depleted of kinesin heavy chain by antisense oligonucleotides (Feiguin *et al.,* 1994) provide further support for the notion that kinesin mediates ER transport. However, results regarding kinesin distribution and function conflict considerably, and several studies have reported an association of kinesin with cargo organelles other than ER membranes (Marks *et al.,* 1994; Lippincott-Schwartz *et al.,* 1995). Moreover, no change in ER morphology is apparent after microinjection of function-blocking anti-kinesin antibodies into sea urchin embryos (Wright *et al.,* 1993) and after knockout of the kinesin gene in mouse cells (Tanaka *et al.,* 1998). These negative results may reflect the preferential use of the TAC mechanism for ER extension in some cell types. Alternatively, other members of the kinesin superfamily with plus-end directionality may substitute for kinesin to translocate ER membranes. Indeed, the relatively low plus-end-directed sliding rate of ER tubules ($\sim$4 μm/min) compared with the transport rate of kinesin ($\sim$60 μm/min) and with ER tubule extension in other cell types ($\sim$60 μm/min; see Section IV.B.) is in favor of a kinesin-related protein driving ER transport in newt lung epithelial cells (Waterman-Storer and Salmon, 1998).

The microtubule cytoskeleton in polarized cells is not always organized with the plus-ends peripherally. In epithelial cells, microtubules are arranged with their minus-ends in the apical cell region and their plus-ends directed toward the basal region (Troutt and Burnside, 1988; Bacallao *et al.,* 1989) and, in neuronal dendrites, microtubules have mixed polarity (Baas *et al.,* 1988). Microtubule-dependent organization of the ER network in some cells may thus require minus-end-directed motors, namely, cytoplasmic dynein. Compelling evidence for the existence of ER transport toward the microtubule minus-end has been provided by *in vitro* studies of *Xenopus* egg extracts (Allan and Vale, 1991; Allan, 1995; Steffen *et al.,* 1997; Lane and Allan, 1999). The formation of tubular ER networks in the motility assays is blocked after photocleavage or immunodepletion of cytoplasmic dynein, demonstrating that it is powered by this motor (Niclas *et al.,* 1996; Steffen *et al.,* 1997). *In vitro* assays have also provided preliminary evidence for a contribution of cytoplasmic dynein to ER network formation in rat hepatocytes (Lane and Allan, 1999). However, in mammalian cells, the overexpression of dynamitin, which is a component of the dynactin complex that binds cytoplasmic dynein to cargo organelles, has no obvious effect on ER distribution, whereas other organelles (endosomes, lysosomes, Golgi apparatus) are redistributed (Burkhardt *et al.,* 1997). This negative result does not rule out a role for cytoplasmic dynein

in ER organization, as subtle changes in ER motility and distribution may have escaped detection by immunofluorescence imaging, and as the contribution of different transport mechanisms and different motors may vary between cell types. Thus, more detailed studies are needed to determine whether, and to what extent, cytoplasmic-dynein-driven ER motility occurs *in vivo*.

3. Proteins Mediating Static Microtubule–ER Interactions

Stable interactions between microtubules and membranes must be involved with the maintenance of ER position and with ER motility via the microtubule sliding mechanism and the TAC mechanism (Figs. 11b and 11c). Indeed, as only a fraction of cells contains a highly motile ER with the ER appearing relatively stationary in the remainder (Lee and Chen, 1988), static association of ER membranes with microtubules may predominate over dynamic motor-dependent interactions.

A recent study by Klopfenstein *et al.* (1998) has identified p63 as a candidate for a nonmotor protein involved in stable ER–microtubule interactions. p63 is a 63-kDa type II integral membrane protein that directly binds to microtubules with its cytoplasmic domain. By immunofluorescence colocalization with rER markers and by immunogold electron microscopy, p63 has been localized to the rER in cultured COS (African green monkey kidney) cells (Schweizer *et al.,* 1993, 1995). Overexpression of p63 in COS cells rearranges the ER and bundles microtubules along ER membranes, suggesting that p63 can function as a linker between ER membranes and microtubules, and contributes to ER positioning (Klopfenstein *et al.,* 1998). Assuming that other organellar membrane proteins with similar properties and functions will be identified, Klopfenstein *et al.* (1998) have proposed the name CLIMPs (cytoskeleton-linking membrane proteins) for this class of proteins and have accordingly renamed p63 as CLIMP-63.

In addition to CLIMPs, another group of nonmotor proteins termed CLIPs (cytoplasmic linker proteins) is implicated in the attachment of membranous organelles to microtubules (Rickard and Kreis, 1996). CLIPs, such as CLIP-170, which links endosomes to microtubules (Pierre *et al.,* 1992; Rickard and Kreis, 1996), and CLIP-115, which operates in the localization of dendritic lamellar bodies (De Zeeuw *et al.,* 1997), are cytoplasmic proteins that presumably require receptor proteins to bind to their target organelle. The subcellular distribution of CLIP-170 is particularly interesting with respect to ER–microtubule interaction. CLIP-170 localizes specifically to microtubule plus-ends *in vivo* and treadmills on growing microtubule ends in association with dynactin, a putative binding protein for cytoplasmic dynein (Perez *et al.,* 1999; Vaughan *et al.,* 1999). This localization and behavior of CLIP-170 is reminiscent of that of TACs, and it is thus attractive to speculate that CLIP-170, or an unidentified CLIP with similar properties, is somehow involved with ER extension via the TAC mechanism. Studies simultaneously visualizing CLIP-170 and ER dynamics may help to investigate this possibility further.

4. Regulation of ER–Microtubule Interaction

The interaction of ER membranes with the microtubule system can be modulated by several mechanisms. One of them is the regulation of the microtubule-dependent motors, either by direct inhibition/stimulation of motor activity or by regulating recruitment of motors to the cargo membrane. Evidence in favor of these events has been provided by *in vitro* experiments. Treatment of *Xenopus* extracts with phosphatase inhibitors results in a marked increase in cytoplasmic-dynein-dependent movement of ER tubules along microtubules but does not effect membrane association of cytoplasmic dynein (Allan, 1995). Phosphorylation in metaphase *Xenopus* extracts by miotic kinases, in contrast, causes a detachment of cytoplasmic dynein from membranes and an inhibition of ER motility (Allan and Vale, 1991; Niclas *et al.,* 1996), suggesting that cytoplasmic-dynein-driven ER transport in *Xenopus* eggs is regulated in a cell-cycle-dependent manner by various kinases. Similarly, the phosphorylation of kinesin is closely coupled to its cargo binding and modulates its ATPase activity (Sato-Yoshitake *et al.,* 1992; Matthies *et al.,* 1993; Lee and Hollenbeck, 1995). By using these mechanisms, the cell could turn on and off ER motility during the cell cycle or in response to a physiological stimulus.

Other mechanisms for the regulation of ER–microtubule interaction include the coating of microtubules with nonmotor MAPs (microtubule-associated proteins), such as MAP2, tau, and MAP4. Tau and MAP2 are neuron-specific proteins localized to the axon and dendrites, respectively, and MAP4 is present in nonneuronal vertebrate cells. These structural MAPs promote tubulin assembly and stabilize microtubules, often in the form of bundles (Drewes *et al.,* 1998). Modulation of microtubule dynamics by MAPs, on the other hand, should have an impact on TAC-dependent ER motility. Moreover, MAPs may inhibit the activity of ER-associated motor proteins by preventing or reducing motor–microtubule interaction. During *in vitro* motility assays, MAP2 blocks kinesin- and cytoplasmic-dynein-driven transport and reduces the formation of ER networks, because a sidearm of MAP2 interferes with the binding of motors to microtubules (Lopez and Sheetz, 1993). Expression of tau in CHO (Chinese hamster ovary) cells dramatically alters ER distribution, and the ER no longer extends into the cell periphery (Ebneth *et al.,* 1998), providing direct evidence for an influence of MAPs on ER organization *in vivo*. The positioning of different MAPs in different cell areas, as in the case of MAP2 and tau within neurons, the regulation of MAP binding to microtubules (Drewes *et al.,* 1998), and the expression of mapmodulin, an ER-associated protein that may displace MAPs along microtubules during organelle translocation (Ulitzur *et al.,* 1997), provide a multitude of ways in which a cell could modulate ER–microtubule interaction.

5. Microtubules and ER Compartmentalization

A question that has not been examined in detail as yet is whether ER–microtubule interactions contribute to the organization of the ER network into functional

domains, that is, whether ER–microtubule association is confined to a distinct ER domain and is involved with the establishment of this ER domain. Although p63 has been located on the rER in COS cells by Schweizer *et al.* (1995), their data are still too preliminary to conclude that p63 is involved in the binding of only rER to microtubules, because no molecular markers for other ER domains were applied in their study. Moreover, the ER in cultured cells may be less elaborate in terms of functional compartmentalization than the ER of cells *in situ*. Evidence in favor of the selective interaction of ER domains with microtubules has been presented by Lane and Allan (1999). These authors report differences in the behavior of rat liver sER and rER in microtubule-dependent motility assays. To examine this topic further and, importantly, *in vivo,* cells with a highly compartmentalized ER and ER-domain-specific probes are needed with which to visualize the motility of various ER subregions and their interactions with the microtubule cytoskeleton.

C. Interactions with the f-Actin System

1. Actomyosin-Dependent ER Motility

As a result of studies demonstrating that the disruption of actin filaments has no obvious effect on ER construction in cultured cells (see above), it was well accepted by the end of the 1980s that ER motility is mediated in a microtubule-dependent manner in animal cells, but by actomyosin in plant cells (Quader *et al.,* 1987; Kachar and Reese, 1988; Knebel *et al.,* 1990; Lichtscheidl *et al.,* 1990; Staehelin, 1997). Data obtained during the 1990s then paved the way for the notion that ER membranes in animal cells can also be translocated by actomyosin-dependent mechanisms. These studies were performed on three distinct systems and used quite different experimental approaches.

Stürmer *et al.* (1995) have analyzed the light-induced movement of ER membranes in locust photoreceptors (see Fig. 10). By electron microscopic techniques, they have demonstrated that motile ER cisternae coalign with actin filaments and reside in a cell area without microtubules. Cytochalasin-B-induced disruption of the f-actin system results in a blockade of ER mobility, suggesting that movement occurs along actin filaments. The motor involved with f-actin-dependent ER transport in this system, however, is still unknown.

Kuznetsov and collegues (Kuznetsov *et al.,* 1992; Langford *et al.,* 1994) have succeeded in directly visualizing active organelle translocation along actin filaments by video analysis of organelle motility in extruded squid axoplasm. f-Actin-dependent transport of axoplasmic organelles occurs at a velocity of about 1 μm/sec and is directed to the plus-ends of actin filaments. These motile organelles have subsequently been identified as ER membranes by use of an anti-PDI antibody (Tabb *et al.,* 1998). Immunogold labeling of ER membranes and inhibition of ER transport with function-blocking anti-myosin V antibodies have further

characterized the motor mediating f-actin-dependent ER motility in this system as a myosin V (Tabb *et al.,* 1998).

Morphological studies on ER organization in cerebellar Purkinje cells of *dilute* mice and rats (see also Section III.D.2) with a mutation in a myosin V gene support the involvement of myosin V with ER motility in neuronal cells. Electron microscopic analysis of Purkinje cells in *dilute* animals has revealed that small tubular ER cisternae normally found in dendritic spines are missing, whereas the ER system in the dendritic trunk appears to be normal (Dekker-Ohno *et al.,* 1996; Takagishi *et al.,* 1996; Bridgman, 1999). Because actin filaments, but no microtubules, have been detected in dendritic spines (Landis and Reese, 1983; Hirokawa, 1989), these results suggest that ER cisternae are transported into and held within dendritic spines by myosin V and f-actin.

Common to the above studies is that they were performed on neuronal cells with a structurally highly complex ER. Moreover, the studies on locust photoreceptors and on Purkinje cells suggest that only part of the entire ER network is transported via an actomyosin system, whereas the distribution of the remaining ER is maintained by different means, probably by interaction with the microtubule system. The findings that a single organelle can move on both actin filaments and microtubules and switch between both structures (Kuznetsov *et al.,* 1992), that myosin V and kinesin colocalize on ER vesicles of squid axoplasm (Tabb *et al.,* 1998), and that myosin V can directly interact with kinesin (Huang *et al.,* 1999) provide support for the concept that both cytoskeletal systems work together to organize the ER network. Whereas the microtubule system may determine the gross distribution of the ER, the actomyosin activity may only be necessary for local movements or for the positioning of ER in specialized regions of the cell (DePina and Langford, 1999; Bridgman, 1999). This may also explain why f-actin-dependent ER motility has escaped detection in studies employing cultured cells, because their ER is far less elaborate and differentiated and may thus be organized by employing fewer mechanisms.

2. Association of Spectrin and Ankyrin with ER Membranes

In addition to myosin, other actin-binding proteins have been detected on ER membranes in animal cells. These proteins, however, may not be directly involved with ER motility but rather may provide some static linkage to actin filaments. Immunolocalization studies on cerebellum have identified nonerythroid spectrin on intracellular organelles, including membranes with an ER-like structure (Zagon *et al.,* 1986; Malchiodi-Albedi *et al.,* 1993). Spectrin is well known as a major component of the submembrane cytoskeleton bound to the cytoplasmic face of the plasma membrane; there, spectrin filaments are cross-linked by short actin filaments to form a polygonal network connected to the plasma membrane via ankyrin and other proteins.

A recent study on honeybee photoreceptors demonstrates more vigorously that a morphologically distinct ER domain, the submicrovillar ER, is associated with

a cytoskeletal network composed of f-actin and spectrin (Baumann, 1998b). The submicrovillar ER is a palisade of ER saccules that are traversed by narrow cytoplasmic bridges (Fig. 2). The membrane of this ER domain is laid down on an array of parallel actin filaments (Baumann, 1992) cross-linked by spectrin filaments (Baumann, 1998b). Notably, this f-actin/spectrin system is confined to the submicrovillar ER, and other ER regions are devoid of these proteins. Cytochalasin treatment of bee photoreceptors causes the actin filaments to depolymerize but leaves spectrin on the ER membranes, suggesting that spectrin is bound to the ER membrane independent of its interaction with actin filaments (Baumann, 1998b). A likely explanation is that linkage occurs via an ankyrin-like protein. Isoforms of ankyrin have been recently identified on several intracellular organelles (Golgi apparatus, lysosomes), including the SR of skeletal muscle fibers (Zhou *et al.,* 1997; Kordeli *et al.,* 1998).

Lateral ER cisternae in the mammalian outer hair cell provide another example of an ER domain possibly bound to f-actin/spectrin. This sER domain consists of a multilayered stack of flattened cisternae closely apposed to the lateral membrane of the cell. Between the outermost ER cistern and the plasma membrane, and probably connected to both membrane systems, is a network of parallel actin filaments, interlinked by spectrin filaments (Holley and Ashmore, 1990; Holley *et al.,* 1992). Again, the molecular identities of the proteins anchoring the ER membrane to f-actin/spectrin have not yet been determined.

The f-actin/spectrin on ER membranes may assume functions analogous to those of the submembrane cytoskeleton associated with the plasma membrane. The ankyrin/spectrin-based cytoskeletal network undercoating the cell surface has been implicated in stabilizing cell shape. Moreover, it segregates diverse proteins (e.g., Na^+,K^+-ATPase, voltage-gated Na^+ channel) within defined membrane domains by a mechanism analogous to the selective retention model described above for NE construction.

Evidence for a mechanical support function of f-actin/spectrin on ER membranes has been obtained by cytochalasin experiments on honeybee photoreceptors. The submicrovillar ER cisternae in the visual cells of honeybees are regularly arranged like a palisade and, in contrast to the submicrovillar ER in locust photoreceptors, are stationary. Cytochalasin-B-induced disruption of the f-actin system in bee photoreceptors causes a structural reorganization of the submicrovillar ER (Baumann and Lautenschläger, 1994). The f-actin/spectrin system may thus provide a means of immobilizing this ER region and of supporting the shape of distinctive ER cisternae.

The spectrin network may also bind to and laterally segregate selected proteins within the plane of the ER membrane, possibly via ankyrin-like proteins. Evidence in support of a direct interaction of ankyrin with RyRs and $InsP_3Rs$ has been provided by *in vitro* binding assays (Bourguignon *et al.,* 1995; Bourguignon and Jin, 1995). Moreover, ankyrin and $InsP_3Rs$ are co-immunoprecipitated from rat cerebellar membrane extracts by antibodies to either protein (Joseph and Samanta, 1993). A recent study by Tuvia *et al.* (1999) on mice lacking the ankyrin-B

isoform demonstrates directly that ankyrin proteins are involved with the restriction of diverse proteins to specialized ER domains. In cardiomyocytes of ankyrin-B-deficient mice, the RyR, the $InsP_3R$, and the SERCA are mislocalized, although the SR and T tubules are present at the correct location and linked by junctions. The abnormal localization of these proteins can be "rescued" by transfection of ankyrin-B-*Null* cardiomyocytes with cDNA encoding for the 220-kDa ankyrin-B isoform, but not the 150-kDa isoform. $InsP_3R$ also exhibits an altered distribution pattern in ankyrin-B-*Null* lymphocytes, suggesting that the anykrin-B isoform may be involved in determining the positioning of Ca^{2+}-regulating proteins not only in muscle cells.

D. Other Mechanisms Involved with ER Organization

1. Interactions of ER with Other Cellular Membranes

Some ER cisternae maintain a close spatial relationship with other cellular membranes, indicating a molecular linkage of the two membrane systems. For instance, the terminal cisternae of the SR are juxtaposed to the cell surface T tubules to form the triad in skeletal muscle (Franzini-Armstrong and Jorgenson, 1994). The gap between both membranes is of constant width and bridged by feet structures, representing the cytoplasmic domains of the RyR Ca^{2+} channels (Section III.D.2; Fig. 6). Couplings between ER and the plasma membrane can be also observed in nonmuscle cells with a highly differentiated ER network (Henkart *et al.,* 1976; Bassot and Nicolas, 1987; Baumann and Walz, 1989a; Takei *et al.,* 1994; Metuzals *et al.,* 1997), although bridging structures, if observed at all, are scarce, and the molecular identity of these cross-links is still unknown. Moreover, ER cisternae may form intimate contacts with mitochondria in various cell types (Baumann and Walz, 1989a; Takei *et al.,* 1994; Perkins *et al.,* 1997). These ultrastructural data suggest that distinct ER membranes have the ability to bind to other membrane systems by some kind of heterotypic interaction.

So far, only the triad junction in muscle cells has been studied with respect to the way in which the ER/SR is bound to a different membrane system. In light of the proximity of RyRs and dihydropyridine receptors within the triad junction and based on results of biochemical experiments demonstrating a direct physical interaction between RyRs and dihydropyridine receptors (Marty *et al.,* 1994; Murray and Ohlendieck, 1997; Leong and MacLennan, 1998), these two proteins can be assumed to be directly involved with the formation of the junction and with holding the two membranes together. However, we now know that mechanical coupling between these two Ca^{2+} channels does not play a primary role in the initial association of the SR with the plasma membrane (Flucher and Franzini-Armstrong, 1996). Triad junctions can form in muscle cells in the absence of either RyR or dihydropyridine receptor proteins (Franzini-Armstrong *et al.,* 1991;

Flucher *et al.,* 1993; Takekura *et al.,* 1995a; Powell *et al.,* 1996; Barone *et al.,* 1998; Protasi *et al.,* 1998). Moreover, the docking of SR membranes to the surface membrane precedes the localization of RyRs and dihydropyridine receptors at the junctions in developing cardiac muscle cells (Protasi *et al.,* 1996). Finally, when these proteins are coexpressed in nonmuscle cells, no junctions are formed between the ER and the surface membrane (Takekura *et al.,* 1995b). These findings suggest that components other than RyRs/dihydropyridine receptors (but still unidentified) are responsible for the development and stabilization of the SR/T-tubule couplings.

2. Homotypic Binding of ER Membranes

ER membranes are sometimes arranged in stacks of regularly spaced, flattened cisternae, such as the rER in cells with high secretory activity or the sER in Purkinje cells. Moreover, sER tubules may form tightly packed sets with constant intercisternal spacing, the crystalloid ER (see Section II.B.). Typically, ER cisternae organized in such geometrical arrays are highly enriched in a single or a few ER membrane proteins and are thus specialized to perform a distinct function; this could be protein synthesis in the case of stacked rER membranes or cholesterol biosynthesis in the case of some crystalloid ER.

The formation of cisternal stacks or crystalloid ER can be artifically induced by overexpression of various ER proteins, thus providing model systems for studying the way in which ER membranes are bound to each other. Takei *et al.* (1994) have reported that expression of $InsP_3R$ at very high levels causes the formation of ER cisternal stacks in COS cells. Moreover, overexpression of RyR results in the formation of closely apposed flat ER cisternae in CHO cells (Takekura *et al.,* 1995b). The ER membranes in these systems are regularly spaced and the gap between adjacent cisternae is bridged by periodically arranged projections that may represent head-to-head appositions of the cytoplasmic domains of the $InsP_3Rs$ and RyRs, respectively. Similar stacks of ER membranes enriched with $InsP_3Rs$ are observed in Purkinje cells under stress conditions (Takei *et al.,* 1994). Formation of crystalloid ER can be induced in several cell types by inhibitors for HMG CoA reductase (Table I; Chin *et al.,* 1982; Singer *et al.,* 1988). There is a large increase in HMG CoA reductase on inhibition of this enzyme, and the protein is concentrated to account for about one-quarter of the total protein within the crystalloid ER (Kochevar and Anderson, 1987). Crystalloid ER is also formed upon overexpression of microsomal aldehyde dehydrogenase in COS cells (Yamamoto *et al.,* 1996) and of a cytochrome *P*-450 isoform in a human embryonic kidney cell line (Sanding *et al.,* 1999). Moreover, expression of the putative 180-kDa ribosome receptor in yeast and mammalian cells results in a proliferation of ER membranes and the formation of rER cisternal stacks (Becker *et al.,* 1999). It thus appears that expression of a single ER protein at high levels can induce the formation of a regularly ordered ER domain in which this protein becomes extremely enriched.

The proteins that cause the formation of either cisternal stacks or crystalloid sER upon overexpression have a feature in common: All of these membrane proteins have a large cytoplasmic domain. Thus, the association of membranes probably occurs by homotypic head-to-head interactions of the cytoplasmic portion of ER proteins. Deletion mutations demonstrating the requirement of the cytoplasmic domain for membrane association (Takei *et al.,* 1994; Yamamoto *et al.,* 1996; Becker *et al.,* 1999) support the above model. Furthermore, quick-freeze deep-etch electron microscopy of the silk gland of silkworms, a tissue specialized for the synthesis and secretion of silk proteins, has visualized short bridge-like structures between adjoining cisternae within rER cisternal stacks (Senda and Yoshinaga-Hirabayashi, 1998); these structures can be also reconciled with homotypic protein interactions mediating membrane binding and defining the regular spacing. Alternatively, other proteins, e.g., cytoskeletal components, may serve as a linker between the overexpressed proteins on adjacent membranes. However, since the depolymerization of f-actin and of microtubules has no effect on crystalloid ER formation (Yamamoto *et al.,* 1996), this possibility seems unlikely.

3. Protein Aggregation and Formation of Macromolecular Complexes

Some ER-resident proteins have a tendency to aggregate into homopolymers or to form heteromolecular complexes, resulting in an enrichment of these molecules within some ER areas and a corresponding depletion in the remaining regions. For example, when the RyR is expressed in CHO cells, it forms extensive arrays in the ER membrane with a spacing similar to that in the junctional SR (Takekura *et al.,* 1995b). Triadin, another prominent intrinsic membrane protein of the junctional SR with a highly charged (basic) luminal domain, makes homomultimers through disulfide bonds (Knudson *et al.,* 1993; Froemming *et al.,* 1999). Moreover, RyR and triadin have been shown to bind to each other and to additional components of the junctional SR, namely, calsequestrin and junctin (Guo and Campbell, 1995; Murray and Ohlendieck, 1997; Zhang *et al.,* 1997; Groh *et al.,* 1999), the latter being an integral membrane protein with considerable sequence homologies to triadin (Jones *et al.,* 1995). Therefore, RyR, triadin, junctin, and calsequestrin have been suggested to form a quaternary complex that immobilizes these proteins and restricts their distribution to the junctional membrane (Zhang *et al.,* 1997; see also Fig. 6).

Molecular chaperones represent another group of ER-resident proteins that have been proposed to form protein aggregates. Biochemical cross-linking experiments on mammalian cell cultures have demonstrated that the chaperones BiP, calreticulin, Grp94, calnexin, and ERp57, a member of the PDI family of proteins, are weakly associated in a variety of different combinations and in the absence of their substrates (Tatu and Helenius, 1997; Oliver *et al.,* 1999). By means of these interactions, chaperones may assemble into a macromolecular network within the ER lumen (Tatu and Helenius, 1997). Moreover, this may also provide an explanation for the observed inhomogeneities in the distribution of some chaperones within the ER network (see Section III.B.2.).

VI. Concluding Remarks

Our understanding of the diverse ER functions in molecular detail is rapidly progressing. Moreover, at least some of these functions, e.g., Ca^{2+} regulative mechanisms or the synthesis of membrane and secretory proteins, are restricted to discrete areas of the ER, despite the continuity of the ER network; these ER domains may have a very characteristic positioning within the cell. The segregation of functions to ER subcompartments and the topography of these ER domains may be critical for coordinating and optimizing ER functions and for their interplay with other cellular compartments. This concept is based on and mainly supported by studies of the role of the ER in intracellular Ca^{2+} regulation, whereas the importance of ER topography and compositional diversity for other functions has so far been largely neglected. Similarly, the ways in which the cell restricts certain components to a distinct ER domain and maintains the topography of an ER domain remain elusive. Are proteins that are involved in the same biochemical pathway assembled into macromolecular complexes and networks, as suggested for the protein folding machinery? Do microtubules, actin filaments, and spectrin/ankyrin molecules contribute to the sorting of ER-resident proteins? Does direct molecular interaction with other cellular membranes cause a partitioning of proteins within the ER membrane? Although some data have been presented in support of these mechanisms, there is still a long way to go in order to understand completely the biogenesis of this complex organelle.

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Apoptosis and Syncytial Fusion in Human Placental Trophoblast and Skeletal Muscle

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Skeletal muscle fibers and placental villous trophoblast are the main representatives of syncytia in the human. Both syncytia are derived from fusion of mononucleated stem cells, show a high degree of differentiation, and have lost their generative potency. Consequently, for their growth both depend on fusion of additional stem cells. There is evidence that syncytial fusion is directly or indirectly related to apoptotic events: As early as in the differentiated stages of the mononucleated stem cells, initiation stages of the apoptosis cascade have been observed. After syncytial fusion progression of the cascade is retarded or blocked by a variety of mechanisms. In this review we emphasize the links between apoptosis cascade, differentiation pathways and syncytial fusion. It needs to be elucidated whether these processes simply take place in parallel, both temporally and spatially, or whether there are causal connections between apoptosis cascade and syncytial fusion. Based on recent data obtained for placental villous trophoblast, it is tempting to speculate that early molecular mechanisms of the apoptosis cascade are involved in differentiation and syncytial fusion. Data obtained in skeletal muscles support this assumption and reveal a considerable degree of homology in genesis, maintenance and turnover of both tissues.

KEY WORDS: Apoptosis, Syncytial fusion, Differentiation, Placenta, Trophoblast, Skeletal muscle, Myotube

I. Introduction

In biological tissues normal turnover involves a balance between proliferation of cells and removal of excess cells by apoptosis. This turnover is generally accepted as a mechanism to reach numerical equilibrium in tissues composed of mononucleated cells. Respective data and concepts for turnover and equilibrium of syncytial tissues such as skeletal muscle fibers, osteoclasts, and villous trophoblast are rare.

Two different modes have evolved to generate multinucleated systems:

1. For the formation of a plasmodium, a single mononucleated cell undergoes acytokinetic mitoses (multiple nuclear divisions) producing a multinucleated giant cell—a plasmodium. True plasmodia are rare; their presence in normal human tissues is still under discussion. One example is found in myxomycetes (true slime funghi).
2. By contrast, a syncytium is formed by fusion of mononucleated cells, forming a larger polynucleated structure. Additional mononucleated cells may subsequently be incorporated, supplying further growth. Syncytia are surrounded by a single plasma membrane and contain the complete cytoplasmic and nuclear contents of all fused cells.

Syncytial tissues are common throughout phylogeny: In *Scolecidae* (Rotifera) nearly all organs are syncytial in nature. In *Gastrotricha, Nematoda, Trematoda,* and *Cestoda,* the epidermal layer is represented by a syncytium. In vertebrates, known examples for syncytia comprise skeletal muscle fibers, osteoclasts and the placental trophoblast in many species including humans.

The two main representatives of syncytia in the human, villous trophoblast and skeletal muscle fibers, survive for 9 months (placental trophoblast) or for several decades (skeletal muscle) as highly differentiated, multinucleated syncytial structures. Throughout this period, the syncytia may grow or recede, according to the demands of the respective organism. We are just beginning to understand the underlying mechanisms of their formation, longevity, and turnover.

In this review we summarize data available on proliferation, syncytial fusion, differentiation, and apoptosis of both the placental trophoblast and skeletal myotubes. Initially, we briefly summarize those stages and steps of the apoptosis cascade in both syncytia that may be involved in their turnover. In the second part we analyze the correlation between apoptosis cascade and villous trophoblast differentiation. This part is followed by a summary of respective data for the differentiation and turnover of myotubes. The final section identifies gaps in our understanding where further studies are required.

II. Summary of the Apoptosis Cascade

A. Apoptosis and Necrosis Are Types of Cellular Death

Programmed cell death as developmental necessity was described by Glücksmann in 1951. Using electron microscopy, about 20 years later Kerr (1969, 1970) analyzed a process in hepatocytes that was similar to programmed cell death as described by Glücksmann. Since it was structurally different from necrosis, Kerr originally termed it *shrinkage necrosis* (Kerr, 1971). Shortly later, Kerr *et al.* (1972) introduced the term *apoptosis,* thereby following the suggestion of James Cormick, professor of Greek Language at the University of Aberdeen (the Greek term "apoptosis" means falling off of leaves from a tree).

Apoptosis is morphologically and functionally clearly distinct from the accidental cell death, necrosis. Necrosis is accidentally induced by lethal chemical, biological or physical events and usually encounters groups of neighboring cells. By contrast, apoptosis is an energy-dependent biological cascade that leads to the death of a single cell, controlled by a genetic program (Cotter *et al.,* 1990). During necrosis, cells undergo cellular edema resulting in disintegration of plasma membrane, cell body, and nucleus. Cellular contents are released and may cause inflammatory reactions. In contrast, the apoptotic cell undergoes shrinkage, which finally results in the formation of sealed cell fragments, apoptotic bodies. The latter are engulfed by neighboring cells without inflammatory reaction (Fadok, 1999). Necrosis implies uncoordinated breakdown of cellular metabolism with subsequent uncontrolled lysis of the cellular contents. During apoptosis, the coordinated action of activators and inhibitors and effectors and regulators establishes a complex pathway causing coordinated cleavage and disposal of the cell. The process can be regarded as a cascade-like sequence of events, although the precise sequence and follow-up of events are not yet clear (Villa *et al.,* 1997) and not necessarily identical in all tissues.

In this review we focus on the caspase cascade of events (Fig. 1), which finally leads to degradation of cytoskeletal proteins and DNA as well as the formation of apoptotic bodies. Other specific pathways resulting in an altered gene expression, activation of protein kinases, or phosphorylation of distinct proteins are beyond the scope of this review.

B. Initiation Stages of Apoptosis Are Still Reversible

Apoptosis is a complex molecular cascade of events that can be induced by a variety of stimuli. These stimuli include ligands binding to respective receptors (e.g., FasL binding to Fas; TNFα binding to TNF-R1), cytolytics secreted by

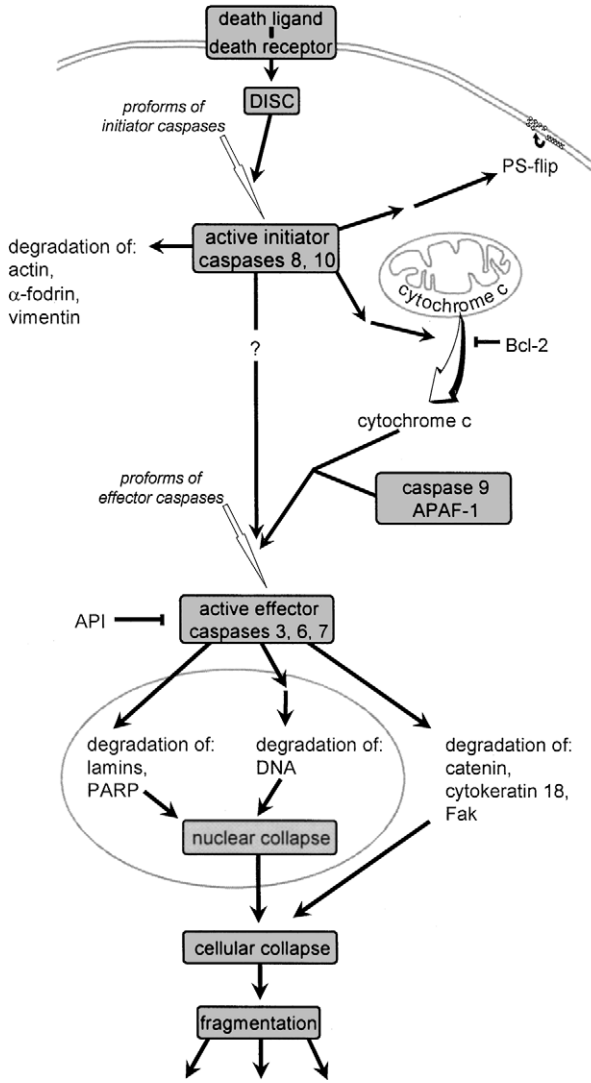

FIG. 1 Schematic overview of those parts of the apoptosis cascade that are important for turnover of placental trophoblast and skeletal muscle fibers. Induction of the cascade via ligand–receptor interactions leads to the activation of initiator caspases and first proteolytic events. The subsequent activation of effector caspases may be achieved by direct cleavage or via the mitochondrial pathway, which is regulated by the Bcl-2 family of proteins. Once activated, the effector caspases cleave cytoplasmic as well as nuclear proteins, finally resulting in the collapse of the cell and its fragmentation into apoptotic bodies. For details see Figs. 2 to 4.

cytotoxic lymphocytes (e.g., granzyme B, perforin), disruption of cell–cell or cell–matrix interactions (e.g., disturbed interactions of cadherins or integrins with their ligands), presence (e.g., TGFβ) or absence (e.g., CSF) of specific growth factors, increasing or decreasing levels of specific hormones (e.g., steroid hormones), or nonphysiological stimuli (such as hypoxia, serum starvation, or irradiation). In some cases programmed cell death is thought to be initiated by activation of an intrinsic program. But even in these cases there seems to be the need for external activators such as bone morphogenetic proteins (BMPs) during limb formation (Chen and Zhao, 1998).

The most prominent apoptosis-inducing ligands, tumor necrosis factor α (TNFα; Pennica *et al.,* 1984) and Fas-ligand (FasL, Apo-1L, or CD95L) (Trauth *et al.,* 1989; Yonehara *et al.,* 1989), belong to the family of type II membrane proteins. They induce apoptosis by autocrine or paracrine loops. Receptor binding can be accomplished either by cell–cell contacts or by ligand diffusion since both FasL and TNFα may be shed from the cell surface by enzymatic cleavage.

The superfamily of TNF receptors is steadily growing. The most prominent members of this family comprise TNF-R1 (tumor necrosis factor-receptor-1, p55 or CD120a) and Fas (also known as Apo-1 or CD95). Others are known as death receptors (DR3 to DR6) (Kidd, 1998; Hunt *et al.,* 1999). All apoptosis-inducing receptors of this family share a specific intracellular domain, the death domain, activation of which triggers the apoptosis cascade (Yuan, 1997; Kidd, 1998). Also the so-called decoy receptors (DcR-1 and DcR-2) belong to the same superfamily and bind the same ligands. They prevent apoptosis by binding the ligand. They cannot activate the subsequent caspases because they lack the intracellular death domain (Hunt *et al.,* 1999).

Finally, it needs to be mentioned that activation of TNF-R1 and Fas may also result in proliferation rather than in apoptosis (Liu *et al.,* 1996; Ponton *et al.,* 1996; Packham *et al.,* 1997). The respective kind of cellular response obviously depends on cell type and cellular environment (Kidd, 1998).

1. Inducers of Apoptosis Ultimately Activate Initiator/ Signaling Caspases

Binding of a ligand to its respective death receptor leads to clustering of the receptors and subsequently to the formation of the death inducing signaling complex (DISC) (Kischkel *et al.,* 1995). The clustered receptors facilitate protein–protein interactions via their death domains. Signaling proteins share sequence homologies to these death domains and bind to the receptors, thus starting the signal transduction pathway for apoptosis (Fig. 2). Receptor-associated proteins such as FADD or TRADD (Fas or TNF receptor-associated death domain) interact directly with the death domain of the activated receptor, upon binding they initiate distinct pathways leading to the activation of caspases (Hsu *et al.,* 1995; Muzio *et al.,* 1996).

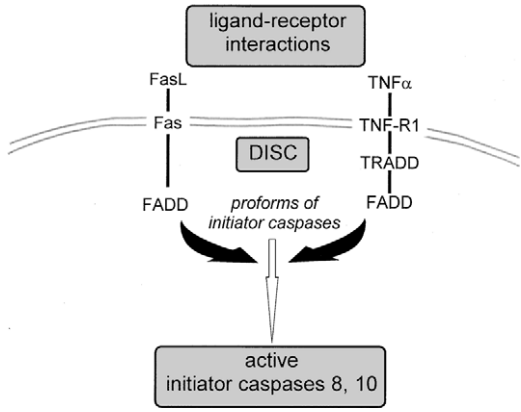

FIG. 2 Activation of initiator caspases via ligand–receptor interactions. Binding of the death ligand to its respective death receptor leads to the formation of the death inducing signaling complex (DISC). Association of proteins involved in the DISC leads to binding and cleavage of the initiator caspases. The cleaved initiator caspases can leave the DISC and show first proteolytic activity in the vicinity of the plasma membrane.

Also apoptosis-inducing stimuli other than receptor–ligand interactions ultimately result in the activation of caspases. Caspases are a family of intracellular proteases that cleaves their targets next to aspartic acid residues. Accordingly they were named cysteine aspartases or caspases. Fourteen members of this protease family have been reported thus far (Slee *et al.,* 1999a). Based on substrate preferences and structural homologies, the family has been divided into various subfamilies (Cryns and Yuan, 1998; Kidd, 1998). In this review, we focus on the caspase 3-like caspases including caspases 3, 6, 7, 8, 9, and 10, which play central roles in the apoptosis cascade (Miller, 1997). This subfamily is further subdivided into initiator/signaling caspases 8, 9 and 10 as well as effector/execution caspases 3, 6, and 7 (Fraser and Evan, 1996; Mignotte and Vayssiere, 1998). Both subgroups differ mainly in one aspect: the initiator caspases are active during early, still reversible stages of the apoptosis cascade. By contrast, activity of the effector caspases is a secondary step that unavoidably leads to apoptotic death.

2. Active Initiator Caspases Are Proteolytic and Cause Early Apoptotic Events

Among initiator caspases, caspases 8 and 10 were found to be directly activated by the signaling complex (DISC) by cleavage of their prodomains. Activation of the initiator caspases leads to first proteolytic events; preferential substrates include cytoskeletal proteins such as actin (Kayalar *et al.,* 1996), α-fodrin (Cryns *et al.,* 1996), and vimentin (Engeland van *et al.,* 1997). Cleavage of these proteins results

in shrinkage of the cell and formation of cell surface blebs, which are typical for early stages of the apoptosis cascade.

The flip of phosphatidylserine from the inner to the outer leaflet of the plasma membrane is another early event (Martin *et al.,* 1995). All mammalian cells actively sustain an asymmetrical distribution of phospholipids in their plasma membrane: in the outer leaflet normally neutral phospholipids including phosphatidylcholine prevail. By contrast, negatively charged aminophospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) are mostly confined to the inner leaflet (Bevers *et al.,* 1996). This asymmetrical distribution is achieved by an ATP- and Mg^{2+}-dependent aminophospholipid translocase, which counteracts spontaneous flipping of PS and PE to the outer leaflet (Williamson and Schlegel, 1994). Inactivation or reversion of translocase activity following initiator caspase activation (within short) results in the appearance of PS in the outer leaflet of the plasma membrane (PS flip). This flipping of PS to the outer leaflet is characteristic of the early stages of apoptosis; moreover, it is thought to be crucial for the induction of syncytial fusion (Lyden *et al.,* 1993; Adler *et al.,* 1995).

PS and PE translocation from the outer to the inner leaflet is driven by translocase activity and is a relatively fast process ($t_{1/2}$ of 5–10 min). PS and PE flip in the opposite direction, from the inner to the outer leaflet, can be due to different mechanisms: (1) spontaneous diffusion, (2) actively driven by slow ATP- and Mg^{2+}-dependent floppases ($t_{1/2}$ of 1–2 hr), or (3) actively driven by fast Ca^{2+}-dependent scramblases ($t_{1/2}$ of less than 1 min). Floppases interact specifically with negatively charged aminophospholipids, PE and PS; scramblases do not show any headgroup specificity.

Current views suggest that PS flip during apoptosis is due not only to spontaneous PS diffusion, but also to an active process (Martin *et al.,* 1995) that is thought to be supported by activation of scramblases. By contrast, PS flip during differentiation prior to syncytial fusion is expected to be due to activation of slower floppases (N. Rote, personal communication; Huppertz and Hunt, 2000). Consequently, accumulation of PS in the outer leaflet of the plasma membrane is the result of (1) scramblase activation or (2) floppase activation, both with or without (3) inhibition of translocases.

The resulting PS flip is known as signal for cell–cell recognition, and for induction of the coagulation cascade (Bevers *et al.,* 1996). During apoptosis, it acts as an "eat-me" signal for phagocytes (Savill, 1998), the latter eliminating apoptotic cells and their fragments. Moreover, PS flip in opposing cells was shown to be a prerequisite for their syncytial fusion (Lyden *et al.,* 1993; Adler *et al.,* 1995): forskolin treatment of choriocarcinoma cells resulted in externalization of PS and in syncytial fusion; application of PS antibodies prevented fusion (Adler *et al.,* 1995).

PS flip seems to be a basic, but not the only prerequisite for cell fusion. This is underlined by two facts: (1) not all early apoptotic cells fuse syncytially, and (2) in most cases only cells of the same type fuse syncytially with each other. From this we conclude that additional cell-specific signals are required. A few

years ago, members of the ADAM family (membrane proteins with a disintegrin and a metalloprotease domain) were detected and their supporting roles for cell–cell fusion of myotubes and osteoclasts as well as for sperm-oocyte fusion were analyzed (Huovila *et al.,* 1996). The disintegrin domain provides specificity of binding to integrins of fusion partners. An additional fusion peptide is involved in the fusion process itself. Interestingly, recent data from Martin *et al.* (1998) suggest that the effects of fusion peptides are strongly enhanced by increasing the concentration of negatively charged lipids (such as phosphatidylserine) in the lipid bilayer. The authors discuss the fact that two factors are required for successful fusion: the presence of members of the ADAM family and changes of electrostatic properties of the membranes.

C. Most Mechanisms Controlling Apoptosis Are Directed to Activation or Inhibition of Effector Caspases

Activation of effector/execution caspases 3, 6, and 7 is another direct or indirect effect of initiator caspases 8 and 10. This step of the cascade marks the transition from reversible to irreversible stages of apoptosis. Moreover, in this stage of the cascade most of its regulation takes place. At this point, the cascade can be retarded or even completely blocked. Alternatively, it can be promoted and accelerated.

Most of these regulatory activities are linked to mitochondrial proteins. Accordingly, there is no possibility of controling caspase activity as long as the initiator caspases are located close to the plasma membrane. Only after their diffusion into the vicinity of mitochondria, can regulation of the downstream caspases take place (Mignotte and Vayssiere, 1998). Consequently, early proteolytic events such as cleavage of cytoskeletal proteins, blebbing, and PS flip take place in the periphery of the cell, but they are not necessarily followed by the cell's execution (McCarthy *et al.,* 1997).

1. Bcl-2 Family of Proteins Regulates the Apoptosis Cascade at the Mitochondrial Level

The main players in apoptosis regulation belong to the Bcl-2 family of mitochondria-associated proteins. This family is divided into two subfamilies with anti-apoptotic (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1/BfI-1, Boo/Diva, NR-13), and pro-apoptotic properties (Bak, Bax, Bok/Mtd, Bcl-x_s, Bid, Bad, Bik/Nbk, Hrk, Bim/Bod, Nip3, Nix/BNip3), respectively (Gross *et al.,* 1999).

All of these proteins share homology domains with the Bcl-2 protein (Bcl-2 homology domains, BH domains). Members of the anti-apoptotic subfamily normally contain four such BH domains (BH1 to BH4). Proapoptotic members contain the domains BH1 to BH3 for the most part, but lack the BH4 domain (Li and Yuan, 1999). In some of the pro-apoptotic members (Bid, Bad, Bik/Nbk,

Hrk, Bim/Bod, Nip3, Nix/BNip3) homology is restricted to the BH3 domain, which is a critical death domain. Most of these proteins bind to the outer mitochondrial membrane by a carboxyterminal hydrophobic domain (Nguyen *et al.,* 1993).

The mode of interaction with the caspases is still under discussion. Homodimerization of pro-apoptotic Bcl-2-like proteins is thought to promote progression of apoptosis (Oltvai *et al.,* 1993; Sedlak *et al.,* 1995). Heterodimerization of anti- and pro-apoptotic proteins is believed to have the opposite effect (Oltvai *et al.,* 1993; Sedlak *et al.,* 1995). Hsu and Youle (1997, 1998) have shown that the ability to form dimers depends on the presence of detergents. According to the same authors, during apoptosis the cytoplasmic members of the Bcl-2 family (Bax, Bcl-x_L) are translocated to mitochondria where they act as agonists or antagonists of apoptosis, respectively (Hsu *et al.,* 1997). Bcl-2 family members control the release of proforms of execution caspases from mitochondria into the cytoplasm; only in the latter location can activation by active initiator caspases take place (Mancini *et al.,* 1998; Samali *et al.,* 1998). Finally, Bcl-2 family members control the release of the apoptosis-promoting molecule Apaf 2 (apoptosis protease activating factor-2, see Section II.C.2) from mitochondria into the cytoplasm (Kluck *et al.,* 1997; Rosse *et al.,* 1998). Additionally, Bcl-2 family members (Bcl-x_L) were found to directly interact with the Apaf molecules in the cytoplasm (Pan *et al.,* 1998).

2. Release of Apaf 2 from the Mitochondria into the Cytoplasm Triggers the Apoptosis Cascade

For several years it was assumed that initiator caspase 8 directly activates effector caspase 3 by cleavage of its proform (Gross *et al.,* 1999; Huppertz *et al.,* 1999a). Most recent studies favor more complex interactions, in which Apaf proteins are involved (Gross *et al.,* 1999). Three members of this group, Apaf 1 to 3, were identified. Apaf 2 is better known as cytochrome *c*, Apaf 3 as caspase 9. In nonapoptotic cells, these proteins are separated by the outer mitochondrial membrane and do not interact with each other: Apaf 2 is present in the intermembraneous mitochondrial space, whereas Apaf 1 and 3 are cytoplasmic proteins. Apaf 2 must be released into the cytoplasm in order to form an active Apaf complex (Zou *et al.,* 1999).

Release of Apaf 2 (cytochrome *c*) is achieved by a complex pathway (Fig. 3): in the cytoplasm, the activated initiator caspase 8 cleaves Bid, a member of the pro-apoptotic Bcl-2 subfamily (Li *et al.,* 1998). The resulting carboxyterminal part of Bid (p15) is inserted into the outer mitochondrial membrane as an integral membrane protein. In this position it induces conformational changes of other pro-apoptotic proteins (Bax, Bak), finally resulting in the release of cytochrome *c* (Apaf 2) into the cytoplasm (Luo *et al.,* 1998; Desagher *et al.,* 1999). In the cytoplasm cytochrome *c* binds to Apaf 1 and activates the latter (Zou *et al.,* 1997). This complex in turn activates initiator caspase 9 (Apaf 3) by binding to it (Pan *et al.,* 1998). This process specifically involves caspase 9 since binding of the Apaf complex requires the presence of the caspase activation and recruitment

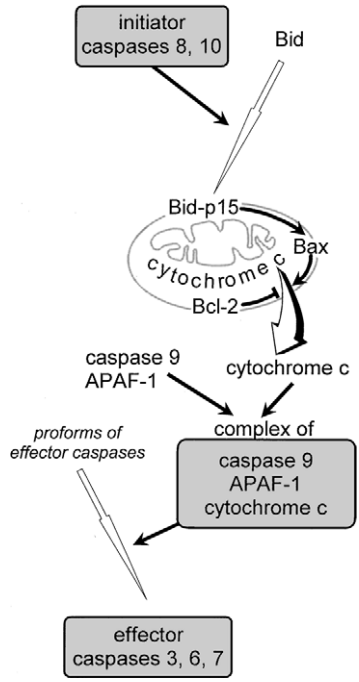

FIG. 3 The mitochondrial pathway of effector caspase activation. Activity of initiator caspase 8 (or 10) leads to cleavage of Bid (a Bcl-2 related pro-apoptotic protein). The resulting p15 cleavage product of Bid integrates into the outer mitochondrial membrane and activates other pro-apoptotic proteins such as Bad or Bax. These proteins induce the release of cytochrome *c* (Apaf-2) into the cytoplasm where it complexes with Apaf-1 and caspase 9 (Apaf-3) in order to activate caspase 9. The release of cytochrome *c* from the mitochondria can be inhibited by the anti-apoptotic members of the Bcl-2 family. In the cytoplasm the Apaf-complex (Apaf-1, -2, -3) cleaves and thereby activates the effector caspases.

domain (CARD) in both interacting Apafs (Chou *et al.,* 1998). The CARD is missing in initiator caspases 8 and 10 as well as in effector caspases 3, 6, and 7.

According to the above data, only the Apaf complex containing active caspase 9 can initiate the downstream caspase cascade by cleaving and activating the proform of effector caspase 3 (Cecconi *et al.,* 1998; Zou *et al.,* 1999). The general validity of this concept is still under discussion. In agreement with this concept, Casp9−/− embryonic stem cells and embryonic fibroblasts do not show activation of effector caspases. By contrast, in Casp9−/− mature thymocytes and splenocytes cellular sensitivity to Fas-mediated apoptosis is not affected (Hakem *et al.,* 1998). The tissue-specific importance of the Apaf complex can be explained by the following hypothesis: in embryonic cells, early stages of the apoptosis cascade are often used for differentiation purposes (such as syncytial fusion) without subsequent cellular execution. This requires intricate control mechanisms at the transition

from initiation to execution stages of apoptosis. These are provided by the highly complex Apaf cascade. In mature cells, induction of the apoptosis cascade is usually used only for the disposal of cells. This objective does not require complex control mechanisms of the cascade, so that the Apaf complex can be bypassed to the benefit of quicker but irreversible shortcuts from initiator to effector caspases.

3. API Family of Proteins Inhibits Active Effector Caspases

API (apoptosis inhibitor) proteins provide a second inhibitory mechanism. This is obviously not available in all cells. The family of inhibitors of apoptosis proteins (IAPs or APIs) act independently and downstream of the Bcl-2 family (Deveraux and Reed, 1999). All members of the API family (API-1 to API-4) were shown to bind directly to activated effector caspases 3 and 7, thereby inhibiting the latter (Ambrosini *et al.,* 1997; Roy *et al.,* 1997; Duckett *et al.,* 1998). It remains unclear whether the inhibitory effect is specific for effector caspases; new studies suggest that initiator caspase 9, but not caspase 8, can be inhibited by some of the API family members (Roy *et al.,* 1997; Deveraux *et al.,* 1999).

The functional role of caspase inhibition by API remains to be elucidated. Does it retard or even completely block a running cascade? Does it only prevent deleterious action of accidentally activated execution caspases? Marks and coworkers have shown that effector caspases require a defined cell-specific time interval to drive a cell to death (Marks and Berg, 1999; Marks *et al.,* 1998). Activation of caspase 3 *in vitro* does not result in apoptotic death when the caspase is inactivated within short, the allotted time ranging between minutes and hours, depending on size and type of the cell type. Accordingly, short-term activation of effector caspases can be definitely blocked by inhibitors such as the API family.

D. On Activation of Effector Caspases, the Cell Enters Irreversible Execution Stages of Apoptosis

Effector caspases 3, 6, and 7 always act downstream of the initiator caspases 8, 9, and 10. The order of activation of these caspases depends on the type of cell. In some tissues, caspase 3 was found to be activated by caspase 6 (Grossmann *et al.,* 1998). In others, caspase 3 is activated by caspase 8, and active caspase 3 then activates caspase 7, which in turn activates caspase 6 (Cohen, 1997). In cell-free extracts obtained from Jurkat T cells and MCF-7 cells, Slee *et al.* (1999b) reported a hierarchical activation of effector caspases with caspase 9 activating caspases 3 and 7; only subsequently was caspase 6 activated by caspase 3. The localization of the proforms of effector caspases is not yet fully clear. Recent experiments suggest that inactive proforms are localized in mitochondria and/or cytoplasm whereas active forms are restricted to cytoplasm and nucleus (Mancini *et al.,* 1998; Samali *et al.,* 1998).

1. Within the Cytoplasm, Active Effector Caspases Cleave Proteins and Activate Enzymes

Also the effector caspases are proteases cleaving a variety of cytoplasmic proteins. Caspases 3 and 6 cleave α- and β-catenin, both of which link cadherins to actins (Brancolini *et al.,* 1997; Herren *et al.,* 1998). Also the focal adhesion kinase (Fak) is cleaved by caspases 3 and 6. This molecule promotes contacts between cell and extracellular matrix (Wen *et al.,* 1997; Gervais *et al.,* 1998). The intermediate filament cytokeratin 18 is cleaved by caspases 3, 6, and 7 (Caulin *et al.,* 1997). Interestingly, cytokeratin 18 and other intermediate filament proteins share a conserved specific cleavage site for caspases. Active effector caspases shift transglutaminase II (tissue transglutaminase) from a diffuse cytoplasmic to a subplasmalemmal localization. Only in the latter position does transglutaminase II become activated and form cross-links between subplasmalemmal proteins, resulting in large protein scaffolds (Cummings, 1996; Fesus *et al.,* 1996). These are thought to prevent the release of cytoplasmic contents from the apoptotic cell via a possibly damaged plasma membrane (Piredda *et al.,* 1997).

2. Also within the Nucleus, Active Effector Caspases Cleave Proteins and Activate Enzymes

The following nuclear cleavage substrates are of importance (Fig. 4). Lamins constitute a network of intermediate-sized filaments at the inner surface of the nuclear membrane, supporting the latter (Georgatos *et al.,* 1994; Bosman, 1999). Lamin degradation during the execution stages is preferentially a function of caspase 6 (Takahashi *et al.,* 1996; Cuvillier *et al.,* 1998). Degradation starts with lamin B, followed by lamins A and C. It results in nuclear collapse and fragmentation (Lazebnik *et al.,* 1995; Zhivotovsky *et al.,* 1995). Poly-(ADP-ribose)polymerase (PARP) is another nuclear substrate of effector caspases (Tewari *et al.,* 1995). It catalyzes ADP-ribosylation of nuclear proteins at sites of spontaneous DNA strand breaks and thus facilitates DNA repair. Tanaka *et al.* (1984) showed that PARP also inhibits Ca^{2+}/Mg^{2+}-dependent endonucleases, which are responsible for DNA cleavage during apoptosis. Degradation of PARP consequently results in increased degradation of DNA.

DNA degradation is further supported by caspase-mediated activation of endonucleases: DFF40/CAD (DFF = DNA fragmentation factor; Liu *et al.,* 1997; CAD = caspase-activated DNase; Enari *et al.,* 1998) and its inhibitor DFF45/ICAD (inhibitor of caspase-activated DNase) form a heterodimeric complex. The nuclease stays soluble and does not cleave DNA, as long as the inhibitor binds to the nuclease (CAD–ICAD complex) via specific functional domains (McCarty *et al.,* 1999). As soon as effector caspases cleave the inhibitor ICAD, the nuclease CAD binds to DNA and starts cleaving it. This process results in characteristic fragments that can be visualized by laddering in an agarose gel or by the TUNEL test.

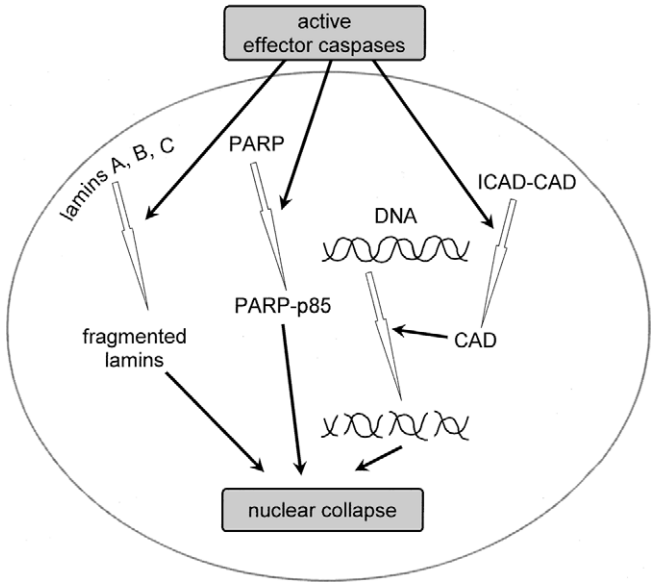

FIG. 4 Intranuclear proteolytic activities of the effector caspases. Besides the cleavage of structural proteins (e.g., lamins) and repair proteins (e.g., PARP), the active effector caspases lead to activation of endonucleases (e.g., caspase-activated DNase, CAD). Activated CAD produces the characteristic DNA fragments typical for late apoptosis. These events result in the destruction of DNA and in collapse of the nucleus.

DNA laddering and positivity of the TUNEL reaction are often understood to be typical and indispensable signs of apoptosis. One has to bear in mind that the significance of both methods is restricted: DNA fragmentation takes place only in the very final stages of the apoptosis cascade and is no indicator of the apoptosis cascade in general (Huppertz *et al.,* 1999a). DNA fragmentation as revealed by the TUNEL reaction also occurs in later stages of necrotic nuclear destruction. Consequently, it is an unsuitable discriminator between apoptosis and necrosis (Didenko and Hornsby, 1996). In the final stages of apoptosis, activation of endonucleases can be bypassed. This results in apoptotic death without DNA laddering and without TUNEL positivity (Pampfer, 2000).

E. Apoptotic Death Is the Final Stage of the Apoptosis Cascade

Structural alterations including cellular shrinkage, nuclear shrinkage, and chromatin condensation (Kerr *et al.,* 1972) are direct consequences of apoptotic degradation of cytoskeleton, nuclear skeleton, and DNA. These structural changes

indicate progression of the apoptosis cascade beyond the point of no return. Chromatin condensation results in spots of maximal chromatin density beneath the nuclear envelope. The final stage is annular chromatin condensation beneath the nuclear membrane. This is accompanied by deformation of cellular and nuclear shape as well as loss of nuclear volume. Given enough time, these events are followed by fragmentation of the cell body and the nucleus, resulting in apoptotic bodies.

F. Apoptotic Bodies Are Engulfed by Macrophages

Phagocytosis of apoptotic cells and their fragments, the apoptotic bodies, is the "last and often forgotten stage of apoptosis" (Fadok, 1999). Engulfment by macrophages or other neighboring cells prevents the release of potentially proinflammatory material from apoptotic bodies. Immediate ingestion of apoptotic fragments, prior to lysis and release of their intracellular contents, is one of the key phenomena to prevent inflammatory responses. Various "eat-me" signals (Savill, 1998) have been described. These include exposure of carbohydrates and phosphatidylserine flip (see Section II.B.2) (Fadok *et al.,* 1998). Receptors recognizing an apoptotic cell, comprise lectins; ABC1 (a mammalian ATP binding cassette transporter; Luciani and Chimini, 1996); integrin $\alpha_v\beta_3$ (associating with CD36 and binding via secreted thrombospondin to an unidentified ligand on apoptotic cells; Savill *et al.,* 1992); and members of the scavenger receptor family (CD36 and CD 68; Savill *et al.,* 1992; Ramprasad *et al.,* 1996). The exact nature of signal/receptor interactions is still largely unknown. This is underlined by the fact that one and the same signal, e.g., PS flip, may result in very different reactions such as phagocytosis, start of the coagulation cascade, or syncytial fusion.

III. Syncytial Fusion and Apoptosis

A. Placental Trophoblast

Throughout human pregnancy, maternal blood is separated from fetal blood by the placental barrier. The latter is actively involved in most transport processes between mother and fetus. Moreover, this barrier represents the main site of metabolic and endocrine activities of the placenta. Its main constituent is a largely uninterrupted multinucleated layer of syncytiotrophoblast. The syncytiotrophoblast evolves by syncytial fusion from trophoblastic stem cells, the villous cytotrophoblast (Fig. 5), and thus forms a true syncytium (Benirschke and Kaufmann, 2000).

During syncytial fusion, the syncytiotrophoblast has lost its generative potency. First respective proof was presented by Richart (1961) using ^{3}H-thymidine

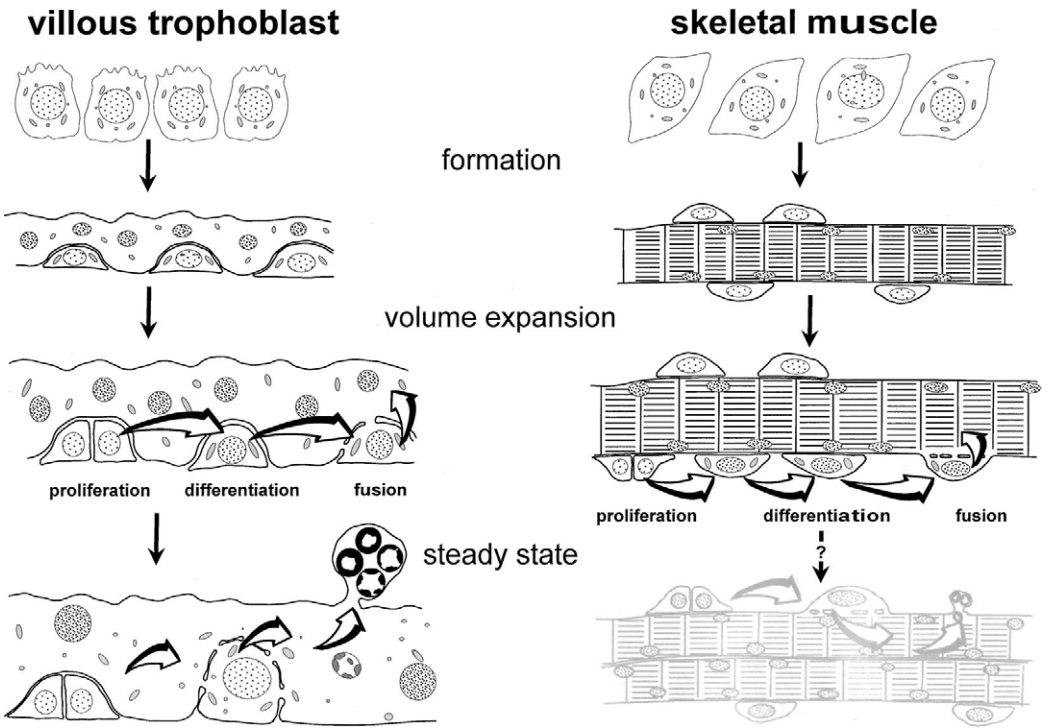

FIG. 5 Early formation, volume expansion, and steady state of villous trophoblast as compared to skeletal muscle. A multinucleated syncytium is formed by syncytial fusion of mononucleated precursor cells. Once formed, the syncytium grows due to continuous proliferation and subsequent fusion of mononucleated cells with the syncytium. In the villous trophoblast a steady state is maintained by continuous syncytial fusion of fresh and extrusion of aged trophoblast from the syncytiotrophoblast. It is still unclear whether the same mechanisms are also valid for skeletal muscle.

incorporation. He demonstrated the absence of DNA synthesis from syncytiotrophoblastic nuclei while incorporation was present in cytotrophoblastic nuclei. Interestingly, the same data were obtained for ^{3}H-uridine incorporation as a measure of transcription; also, this process is highly active in cytotrophoblast but down-regulated to a barely measurable degree in syncytiotrophoblast (Kaufmann *et al.,* 1983; Huppertz *et al.,* 1999b). The deficiency of nucleic acid metabolism in the syncytiotrophoblast explains the necessity of the presence of a layer of villous cytotrophoblast beneath, throughout pregnancy. These are the stem cells that continuously proliferate and subsequently fuse, thus providing both growth of the syncytiotrophoblast and its supply with new mRNA, proteins, and organelles (Benirschke and Kaufmann, 2000).

1. Differentiation of Trophoblast Stem Cells

The layer of cytotrophoblast is nearly complete during early pregnancy and becomes rarefied in later stages. At the end of pregnancy only about 20% of the syncytiotrophoblast layer is in contact with cytotrophoblast beneath. This relative rarefaction of stem cells is due to an enormous surface expansion of the syncytiotrophoblast, increasing from 0.3 m^2 at 12 weeks of pregnancy to about 12.5 m^2 at term. The absolute amount of stem cells in the same period even multiplies from about 2 g to about 20 g (Benirschke and Kaufmann, 2000). This implies continuous proliferation of cytotrophoblast throughout pregnancy; this was proven by ^{3}H-thymidine incorporation and application of various proliferation markers (Kosanke *et al.,* 1998).

According to ultrastructural and enzyme histochemical data, the trophoblast cells, after leaving the cell cycle, undergo differentiation prior to syncytial fusion (Fig. 6). Studies on cyclin D3 expression (DeLoia *et al.,* 1997) suggest that about 50% of the cells are in the cell cycle. They display an ultrastructurally undifferentiated phenotype with a large euchromatic nucleus and few cell organelles (Kaufmann, 1972; Martin and Spicer, 1973). Enzyme histochemistry revealed only low activities of enzymes belonging to anaerobic and aerobic glycolysis (Kaufmann *et al.,* 1974). Nearly 50% of cells display the same undifferentiated phenotype but are immunonegative for cyclin D3. They very likely represent G_o cells.

Several trophoblast mitogens have been described: hepatocyte growth factor (HGF) is expressed by villous mesenchyme; it is a potent stimulator of trophoblast proliferation (Uehara *et al.,* 1995). Its deficiency results in trophoblast maldevelopment (Uehara *et al.,* 1995) and in intrauterine growth restriction (Somerset *et al.,* 1998). Its receptor, the proto-oncogene protein product c-met is expressed by both villous cytotrophoblast (Furugori *et al.,* 1997) and myoblasts (Tatsumi *et al.,* 1998, see Section III.B.1).

In vitro data by McKenzie *et al.* (1998) revealed that interactions between (1) increased expression levels of the Cdk inhibitor Kip1, (2) inactivation of cyclin-dependent kinase-2 (Cdk2), (3) down-regulation of cyclin E, and (4) accumulation

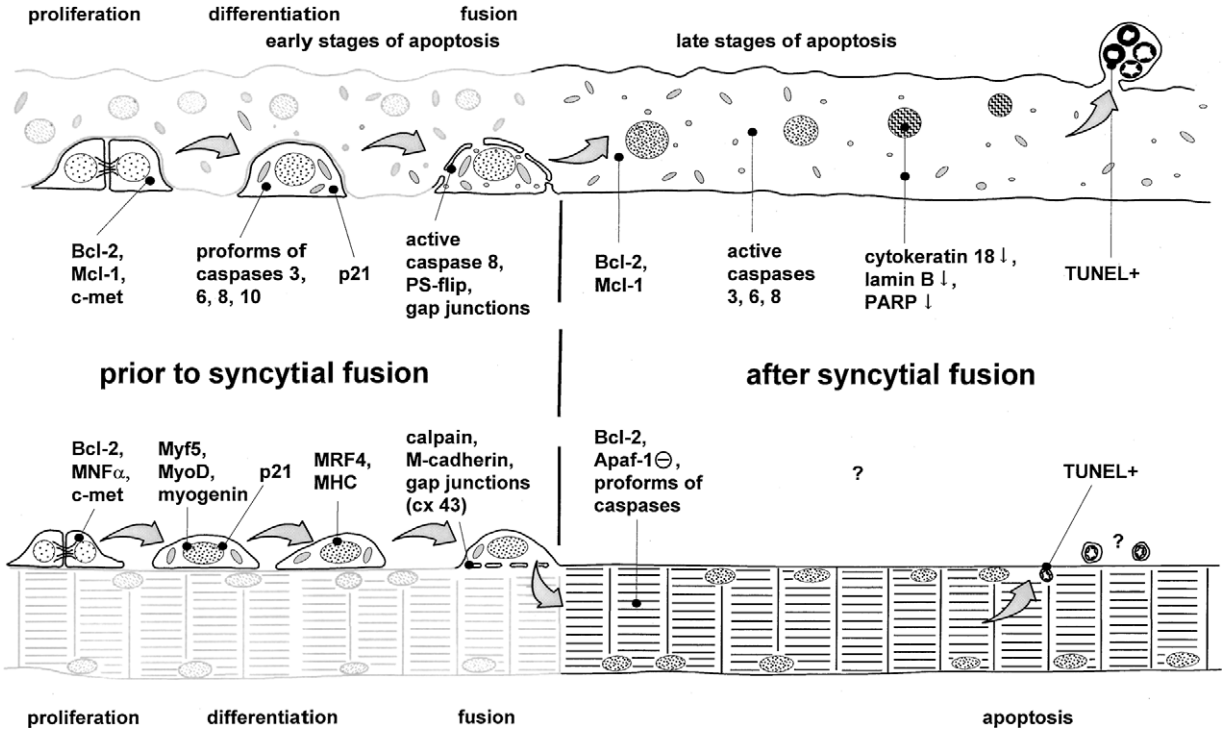

FIG. 6 Markers for differentiation and apoptosis in villous trophoblast as compared to skeletal muscle. For the mononucleated precursor cells of the skeletal muscle, a variety of differentiation markers have been decribed, while in cytotrophoblast mostly apoptosis markers have been analyzed. After syncytial fusion, the syncytiotrophoblast reveals clear signs of slow progression of the apoptosis cascade. For skeletal muscle fibers, few data on progression of apoptosis are available.

of active (hypophosphorylated) retinoblastoma gene product (pRb) are responsible for blockage of the S phase of cytotrophoblast and the cells' entrance into the differentiation pathway. This happens obviously only to a small percentage of cells; a minority of cells shows higher concentrations of organelles including mitochondria, rough endoplasmic reticulum, and polyribosomes. Finally, these cells acquire a concentration of organelles that clearly exceeds that of the covering syncytiotrophoblast (Kaufmann, 1972; Benirschke and Kaufmann, 2000). In this stage of differentiation, activities of enzymes related to energy metabolism, transport processes, and steroid metabolism considerably exceed respective activities in the syncytiotrophoblast (Kaufmann *et al.,* 1974). Only these highly differentiated cells reveal structural signs of syncytial fusion such as partial dissolution of separating plasma membranes (Boyd and Hamilton, 1966; Kaufmann, 1972; Kaufmann *et al.,* 1977). Marker proteins for the various stages of differentiation of trophoblast cells are still unknown.

2. Fusion of Cytotrophoblast with the Syncytiotrophoblast

Only in the implantation period (days 7–12 of human pregnancy) does syncytial fusion of trophoblast cells with other trophoblast cells take place. In all subsequent stages of pregnancy, cytotrophoblast fuses only with syncytiotrophoblast. Several data suggest that this is a tightly controlled event. The absolute increase in the number of villous trophoblast cells throughout pregnancy (see Section III.A.1) implies that the proliferation rate of cytotrophoblast always exceeds the fusion rate. Out of the large pool of cytotrophoblast in the G_o phase, only the highly differentiated cells fuse with syncytiotrophoblast. Fusion was only observed with syncytiotrophoblast, which shows degranulation of its endoplasmic reticulum and loss of polyribosomes (Kaufmann, 1972; Kaufmann and Stark, 1972; Benirschke and Kaufmann, 2000). These data imply that syncytial fusion of trophoblast requires both a high degree of differentiation in one and a certain grade of regressive changes in the other fusion partner.

In pseudopregnant rabbits Winterhager *et al.* (1984) showed that the formation of a uterine epithelial syncytium is introduced by the formation of gap junctions. These junctions establish an intercellular coupling of neighboring epithelial cells and finally induce disintegration of the separating membranes. A comparable role of gap junctions for syncytial fusion of trophoblast has been shown in guinea pigs (Firth *et al.,* 1980). Moreover, Contractor and coworkers (1969, 1977) suggested that lysosomes are actively involved in the disintegration of the plasma membranes during fusion of villous trophoblast.

The molecular mechanisms of syncytial trophoblast fusion and its control are still poorly understood. Recent data make it very likely that the molecular machinery of early stages of the apoptosis cascade is involved (Fig. 6). Cytotrophoblast cells express TNF-R1 (Yui *et al.,* 1996; Huppertz *et al.,* 1999a). The respective ligand, TNFα, is secreted by placental macrophages (Hofbauer cells) (Steinborn *et al.,*

1998). Addition of TNFα to trophoblast cells in culture or co-culture of trophoblast cells with activated macrophages induces trophoblast apoptosis; the latter can be inhibited by addition of TNF-R1 antibodies (Yui *et al.,* 1994; Reister *et al.,* 2001).

In tissue sections, villous cytotrophoblast cells show immunoreactivities of the proform of initiator caspase 8; only in some of these cells is the caspase activated; additional proof for activation of caspase 8 was also obtained biochemically in cytotrophoblast lysates (Huppertz *et al.,* 1999b). Fodrin immunoreactivity is absent from a subset of cytotrophoblast cells (Huppertz *et al.,* 1999b); this plasma membrane-associated cytoskeletal protein is a substrate of initiator caspases and is typically cleaved in early stages of apoptosis. Some villous cytotrophoblast cells show a phosphatidylserine flip from the inner to the outer plasmalemmal leaflet, as is typical also for early stages of apoptosis (Huppertz *et al.,* 1998). The proform of effector caspase 3 is present in villous cytotrophoblast (Huppertz *et al.,* 1998); however, indications for its activation as well as other signs of progression of the apoptosis cascade are missing in cytotrophoblast (Huppertz *et al.,* 1998, 1999b).

Rote and coworkers (Adler *et al.,* 1995; Vogt *et al.,* 1997) showed that exposure of phosphatidylserine to the extracellular space is a prerequisite for syncytial fusion of trophoblast cell lines *in vitro:* The trophoblast-derived choriocarcinoma cell lines BeWo and JAR proliferate *in vitro* and show an undifferentiated phenotype. Stimulation with forskolin induces the cells to leave the cell cycle, to start differentiation, and, finally, to fuse syncytially. Incubation with a phosphatidylserine antibody inhibited syncytial fusion (Adler *et al.,* 1995; Vogt *et al.,* 1997).

The above data suggest that the PS flip is a consequence of activation of initiator caspases (e.g., caspase 8) and that the molecular machinery of early apoptosis is involved in the fusion process. The roles of further fusion relevant molecules such as ADAMs (see Section II.B.2) still need to be elucidated. mRNA of ADAM proteins was also found to be expressed in high levels in human placenta (Huovila *et al.,* 1996). According to Gilpin *et al.* (1998), this mRNA is likely to represent ADAM 12 (meltrin α), which is also involved in the fusion of myoblasts with myotubes. As discussed above (see Section II.B.2), the pro-fusion effects are strongly enhanced by externalized phosphatidylserine.

3. Maintenance and Turnover of the Syncytiotrophoblast

Syncytial fusion of trophoblast has been shown to take place in all stages of pregnancy. The number of fusing cells exceeds the need for growth of syncytiotrophoblast by a factor of ∼6 (Huppertz *et al.,* 1998). The resulting excess amounts of syncytiotrophoblast, after a few weeks of aging, are accumulated as syncytial knots and are shed from the syncytial surface into the maternal blood (Martin and Spicer, 1973; Jones and Fox, 1977; Cantle *et al.,* 1987; Huppertz *et al.,* 1998; Mayhew *et al.,* 1999). Rough calculations have shown that the mean intrasyncytial survival time for a nucleus, between incorporation by fusion and shedding into the maternal blood, is about 3–4 weeks (Huppertz *et al.,* 1998).

These data raise two questions: (1) in most cells, once the apoptosis cascade is started, its execution takes place within less than 24 hr. Which mechanisms within the syncytiotrophoblast prolong the cascade up to 3 weeks? (2) Why does the syncytiotrophoblast require this excess degree of syncytial fusion, which by far exceeds the syncytial needs for growth?

The answer to question 1 may be sought in the special situation of a syncytium. As has been shown by Zhou *et al.* (1997) in hematopoietic cells, overexpression of Mcl-1 delays death for several days. Similar effects are known for Bcl-2 (Hawkins and Vaux, 1994). Also in cytotrophoblast considerable amounts of the apoptosis inhibitors Mcl-1 and Bcl-2 are transcribed and are transferred into the syncytiotrophoblast by syncytial fusion (Huppertz *et al.,* 1998, 1999c). In the latter, both mitochondrial proteins may be involved in inhibition of the cascade. Moreover, API (X-IAP), a potent inhibitor of active effector caspases, was revealed in syncytiotrophoblast (A. Gruslin, 1998, personal communication). Marks *et al.* (1998) have shown that activation of effector caspases in neurons does not necessarily result in apoptotic death, provided that the caspases were inactivated again within a critical time interval. This interval varied between minutes and hours, depending on the size of the cell.

The above data may raise the question of whether later progression of the apoptosis cascade in the syncytiotrophoblast is only a matter of duration of inhibition. Rather, as soon as inhibition exceeds the life span of active effector caspases, the molecular machinery for restart of the cascade is required. Interestingly, pro- and active forms of initiator caspases were found in the syncytiotrophoblast (Huppertz *et al.,* 1998, 1999b) as well as mechanisms for their activation: Fas receptor is expressed along the microvillous surface of the syncytiotrophoblast (Uckan *et al.,* 1997; Huppertz *et al.,* 1998). The data concerning its ligand, FasL, are controversial. It was found to be present (Runic *et al.,* 1996; Zorzi *et al.,* 1998) or absent (Huppertz *et al.,* 1998; Hammer *et al.,* 1999) in the syncytiotrophoblast. This leaves the question open as to whether restart is an endogenous syncytial process or requires external triggering.

As for question 2, the necessity of continuous turnover of syncytiotrophoblast can be explained by down-regulation of transcription (Kaufmann *et al.,* 1983; Huppertz *et al.,* 1999b; Benirschke and Kaufmann, 2000). Overall incorporation of ^{3}H-uridine as compared to cytotrophoblast and other placental cells is more or less unmeasurable (Kaufmann *et al.,* 1983; Huppertz *et al.,* 1999b). Down-regulation of transcription is a widespread phenomenon after induction of apoptosis (Owens *et al.,* 1991; Leist *et al.,* 1994; Kockx *et al.,* 1998). However, this does not necessarily imply that it is valid for all proteins. In the special case of syncytiotrophoblast showing a retarded apoptosis cascade, survival and functional activity depend on transfer of mRNA, proteins, and organelles from the cytotrophoblast by syncytial fusion (Kaufmann *et al.,* 1977; Benirschke and Kaufmann, 2000). Respective mRNA and/or protein transfers from cyto- to syncytiotrophoblast have been demonstrated for apoptosis-related proteins such as Bcl-2, Mcl-1, and procaspase

3. Under normal culture conditions, in villous explants all three proteins can be found immunohistochemically in syncytiotrophoblast and in its stem cells. As soon as syncytial fusion is blocked *in vitro* by low oxygen partial pressure (about 3%), immunoreactivities of these proteins get lost in the syncytiotrophoblast within 2 days, but become accumulated in the cytotrophoblast within the same period (Huppertz *et al.,* 1999c).

The aging of syncytiotrophoblast from its fusion until its extrusion into the maternal circulation (Fig. 6) is reflected by changes of nuclear shapes. Syncytial nuclei from freshly fused trophoblast cells are large, ovoid, and rich in euchromatin. With continuous aging they become smaller, denser, and finally show marginal or even annular chromatin condesation (Martin and Spicer, 1973; Nelson, 1996; Huppertz *et al.,* 1998; Mayhew *et al.,* 1999). Freshly incorporated nuclei are surrounded by impressive amounts of rough endoplasmic reticulum and polysomes; parallel to nuclear aging, the endoplasmic reticulum degranulates into smooth endoplasmic reticulum (Benirschke and Kaufmann, 2000). Enzyme histochemistry revealed that syncytiotrophoblastic aging and turnover are accompanied by inactivation of mitochondrial and transport-related enzymes, but activation of lysosomal enzymes and aminopeptidases (Kaufmann and Stark, 1972).

These events are accompanied by restart and progression of the apoptosis cascade (Huppertz and Kaufmann, 1999; Huppertz *et al.,* 1998, 1999b). All syncytial nuclei, independent of the degree of nuclear aging and condensation, lack autoradiographic signs of ^{3}H-uridine incorporation, a finding that points to distinctive down-regulation of overall transcription directly on syncytial fusion. Freshly fused syncytiotrophoblast, as defined by its nuclear shape, shows strong immunoreactivities for inhibitory mitochondrial proteins such as Bcl-2 and Mcl-1. Neither activities of initiator nor of effector caspases are detectable. Corresponding hints to cleavage action of effector caspases are missing. With increasing nuclear shrinkage and chromatin condensation, active initiator caspases and finally also active effector caspases can be seen. Immunoreactivities for their nuclear substrates lamin B, PARP, and topoisomerase IIα are gradually lost. TIAR is displaced from a nuclear to a cytoplasmic position. Transglutaminase II is shifted to a subplasmalemmal position. Moreover, parts of the syncytial surface bind annexin V, thus suggesting a phosphatidylserine flip (Huppertz *et al.,* 1998, 1999b).

In some areas of the syncytiotrophoblast, immunoreactivities for the caspase cleavage-dependent cytokeratin neoepitope indicate degradation of cytokeratin 18 by caspases. Degradation of cytoskeletal proteins in general is thought to be responsible for impaired anchorage of syncytial nuclei. This results in local accumulation of nuclei, the so-called syncytial knotting. The driving force for nuclear accumulation is probably provided by shear stress caused by maternal blood flow in the neighboring intervillous space. This assumption is supported by the fact that nuclear accumulation does not take place either *in vitro* or at sites with arrested maternal circulation *in vivo.* Under *in vivo* conditions, endonucleases become activated and respective DNA degradation with TUNEL positivity can be found only

following nuclear accumulation in syncytial knots. In most placentas, this is a rare finding. This may be due to the fact that syncytial knots together with their accumulated, condensed nuclei are shed into the maternal circulation even prior to DNA laddering. Pampfer (2000) provided another explanation for paucity of TUNEL-positive nuclei; the author found that activation of endonucleases and DNA degradation in otherwise normal apoptosis cascades can also be bypassed. With and without DNA degradation, the trophoblastic knots following shedding are deported into the maternal lung (Ikle, 1964) where they are engulfed and degraded by alveolar macrophages.

B. Skeletal Muscle

Skeletal muscle has the highest functional efficiency regarding contractile speed, contractile power, and stroke capacity among all types of muscle tissues evolved during evolution. This efficiency goes in line with the accumulation of the highest concentrations of contractile proteins and with the acquisition of multinucleated myotubes. The latter are derived by syncytial fusion of mononucleated precursor cells ("myoblasts" during development of immature myotubes, "satellite cells" around muscle fibers in fully mature muscle). The formation of myotubes takes place in the embryonic/fetal stage of development. After birth, growth of skeletal muscle is due to expansion of preexisting myotubes by incorporation of additional myoblasts (Fig. 5), resulting in larger and higher differentiated muscle fibers (Miller *et al.,* 1999).

1. Differentiation of Myoblasts

Myoblasts are mononucleated muscle precursor cells (Miller *et al.,* 1999). Most myoblasts have limited proliferative capacity (Quinn *et al.,* 1984; Nicolas *et al.,* 1996). Only a subpopulation, the stem cells, are in the cell cycle (Miller *et al.,* 1999, and references therein) until they reach proliferative senescence (Webster and Blau, 1990). Their daughter cells arise from asymmetric cell division, producing one daughter cell that retains stem cell properties and a second, differentiating one (Nicolas *et al.,* 1996; Yoshida *et al.,* 1998). Mampuru et al. (1996) provided *in vitro* evidence for the existence of a special myoblast stem cell population that remains in the cell cycle. After serum withdrawal most Sol8 myoblasts in culture underwent apoptosis; only a small proportion of cells survived and showed proliferation rather than apoptosis.

Gene expression patterns of stem cells hardly differ from that of other myoblasts. So far only Bcl-2 has been found to be differentially expressed by stem cells from that of differentiating myoblasts (Dominov *et al.,* 1998; Miller *et al.,* 1997). The role of Bcl-2 in these cells is unclear. Two hypotheses are under discussion: (1) In fibroblasts, lymphocytes, and a variety of other cells, Bcl-2 induces the cells

to enter a quiescent G_0/G_1 state and thereby reduces proliferation (Vairo *et al.,* 1996; O'Reilly *et al.,* 1997). Fruthermore, Mazel et al. (1996) showed that elevated levels of Bcl-2 lead to an increase in the amount of hypophosphorylated retinoblastoma gene product (pRb). In myogenic cells, similar to villous cytotrophoblast (see Section III.A.1), hypophosphorylated pRb is related to inhibition of the cell cycle (Molkentin and Olson, 1996). (2) The classical role of Bcl-2 is the inhibition of the apoptosis cascade prior to activation of the effector caspases (Reed, 1997, and Section II.C.1). This role may also be linked to pRb since hypophosphorylated pRb, according to Wang et al. (1997), inhibits apoptosis during myoblast differentiation.

Taken together, the above hypotheses let us suggest a dual function for Bcl-2 in skeletal myoblasts: reduction of proliferation and inhibition of apoptosis at the same time. As a consequence, these myoblasts can survive as satellite cells in a quiescent state for a human lifetime. In normal mature skeletal muscle, the muscle fibers together with their satellite cells express only low levels of Bcl-2 (Olive and Ferrer, 1999) as compared to the levels found in most of the bigger perimysial vessels (Tews and Goebel, 1997a). Only under pathological conditions was an up-regulation of the apoptosis inhibitor Bcl-2 found; but this was accompanied by an up-regulation of apoptosis-promoting factors such as bax and caspase 3, both contributing to increased apoptosis and loss of muscle fibers (Tews and Goebel, 1996, 1997b; Tews *et al.,* 1997a; Libera *et al.,* 1999; Olive and Ferrer, 1999).

Two further proteins are expressed in differentiating myoblasts (Fig. 6): MNFα (myocyte nuclear factor-α; Garry *et al.,* 1997) is a member of the winged-helix family of transcription factors and is found to bind to promoter regions of muscle-specific genes. The function of MNFα is not yet clear but it was proposed that MNFα is involved in maintaining myogenic capacity of myoblasts as long as the MRFs (see below) are not expressed. The membrane protein c-met (Cornelison and Wold, 1997) is the receptor for the hepatocyte growth factor. The latter is a mitogen for myoblasts (Tatsumi *et al.,* 1998) and is required for migration of myoblasts (Bladt *et al.,* 1995).

After leaving the cell cycle, myoblasts undergo a series of differentiation steps (Fig. 6) that finally lead to their fusion with each other or with preexisting myotubes. Different from proliferating myoblasts, differentiating myoblasts no longer express Bcl-2 but rather start sequential expression of several members of the family of MRFs (muscle regulatory factors). MRFs belong to the basic helix-loop-helix (bHLH) factor family of transcription factors (Yun and Wold, 1996; Chen and Goldhamer, 1999). The first MRFs expressed in early mouse myoblasts are Myf-5 and MyoD, followed by myogenin and finally by MRF4 (Smith *et al.,* 1993, 1994; Andres and Walsh, 1996; Cornelison and Wold, 1997).

Following up-regulation of myogenin, but still before MRF4 expression, transcription of the cyclin-dependent kinase (cdk) inhibitor p21 is upregulated (Walsh and Perlman, 1997), p21 is a cell cycle regulator that induces an irreversible arrest of the cell cycle (Guo *et al.,* 1995; Halevy *et al.,* 1995). Consequently, incorporation

of bromodeoxyuridine (BrdU) can only be seen before onset of p21 expression. Mice homozygous null for p21 appear to have a normal development of skeletal muscle (Deng *et al.,* 1995), suggesting that p21 is not the only cell cycle regulator involved in myoblast differentiation (Walsh and Perlman, 1997). Other candidate regulators include p57 (expressed in high levels in adult myofibers; Matsuoka *et al.,* 1995), p27 (slightly up-regulated during *in vitro* myogenesis), and p18 (markedly increased during *in vitro* myogenesis; Franklin and Xiong, 1996). Only p21-positive myoblasts that have definitively left the cell cycle express the myosin heavy chain (MHC) (Andres and Walsh, 1996).

2. Fusion of Myoblasts with Syncytial Myotubes

It is important to note that myoblasts undergo syncytial fusion with other myoblasts or with preexisting myotubes only after passing all of the above differentiation steps (Andres and Walsh, 1996). A variety of molecules is directly or indirectly involved in the fusion process (Fig. 6).

M-calpain, a Ca^{2+}-activated cysteine protease, plays a crucial role in myotube formation. In differentiating myoblasts, the concentration of M-calpain remains constant while mRNA and protein levels of its inhibitor, calpastatin, significantly decrease just prior to fusion (Barnoy *et al.,* 1996; Balcerzak *et al.,* 1998). At this stage of differentiation, but not in proliferating myoblasts, limited proteolysis of a selected subset of proteins occurs, including desmin, fodrin, integrin ß1, talin, and ß-tropomyosin (Barnoy *et al.,* 1998; Huang and Forsberg, 1998; Dourdin *et al.,* 1999). In addition, m-calpain is thought to be secreted into the extracellular space shortly before fusion and leads to the degradation of fibronectins surrounding the cells (Dourdin *et al.,* 1997).

These data suggest that M-calpain, prior to fusion, degrades proteins involved in anchorage of the plasma membrane toward both cytoskeleton and extracellular matrix; and the altered balance of calpain and calpastatin results in destabilization of the membrane and thus creates fusion-potent regions (Barnoy *et al.,* 1998).

Cell–cell adherence mediated by the family of Ca^{2+}-dependent cadherins plays key roles in the morphogenesis of a variety of tissues. M-cadherin (but not N-cadherin; Charlton *et al.,* 1997) is important for myoblast fusion (Eng *et al.,* 1997). Syncytial fusion of myoblasts was inhibited in the presence of antagonistic peptides to M-cadherin (Zeschnigk *et al.,* 1995). Fusion was impossible when the contact areas lacked M-cadherin, due to the absence of divalent cations (Eng *et al.,* 1997), or due to expression of M-cadherin antisense RNA (Zeschnigk *et al.,* 1995).

Also for syncytial fusion of myoblasts cell–cell communication via gap junctions seems to be a prerequisite (Proulx *et al.,* 1997). Mege *et al*. (1994) have demonstrated connexin 43 immunoreactivity at contact areas between myoblasts prior to fusion. In the presence of gap junction blockers, rat L6 myoblasts in culture did not fuse; following removal of the blocker, fusion took place (Mege

et al., 1994). According to Constantin *et al.* (1997), gap junctions are involved in preparation of the fusion rather than in the fusion process itself since they disappear prior to fusion.

Presence of the extracellular protein merosin, the predominant laminin variant in skeletal muscle basal laminas, is essential for syncytial fusion (Vachon *et al.,* 1996). Fusion-deficient myoblast cell lines fused syncytially following addition of merosin to the culture medium; clonal variants of these cell lines expressing laminin rather than merosin fuse syncytially but form unstable myotubes.

As described above (Section II.B.2), members of the ADAM family of membrane proteins play a decisive role in syncytial fusion (Huovila *et al.,* 1996). Their disintegrin domain provides specific binding to integrins of the fusion partner. Their fusion domain is involved in the fusion process itself. From this family, ADAM 12 (meltrin α) is involved in formation of myotubes and muscle fibers (Gilpin *et al.,* 1998). Two splicing variants are known: a larger membrane-bound form (ADAM 12-L) and a shorter secreted form (ADAM 12-S). Rhabdomyosarcoma cells, deficient for ADAM 12, do not differentiate forming myotubes; only following transfection with an ADAM 12-S minigene do they fuse syncytially. It is interesting to note that mRNAs for both forms of ADAM 12 are also abundant in human term placenta (Gilpin *et al.,* 1998).

3. Maintenance and Turnover of Immature Myotubes and Adult Muscle Fibers

Expansion and differentiation of myotubes results in formation of muscle fibers. Even following this process, quiescent mononucleated myoblasts persist as satellite cells (Mauro, 1961) between plasmalemmas of muscle fibers and their basal laminas throughout adult life. These cells are regarded as a kind of emergency reserve: as long as muscle fibers stay intact, satellite cells are mostly in the G_0 stage and do not express MRFs such as Myf-5, MyoD, myogenin, or MRF4. Only following injury of muscle fibers are their satellite cells found to proliferate, to differentiate subsequently expressing MRFs (Koishi *et al.,* 1995), and finally to fuse syncytially.

Muscle fiber injury and muscle fiber regeneration are found in "necrotizing myopathies," especially in muscular dystrophies. Interestingly, regenerating muscle fibers with proliferating satellite cells display expression of apoptosis-associated proteins such as Bcl-2, Bax, and caspases as well as numerous TUNEL-positive nuclei (Tews and Goebel, 1997b; Olive and Ferrer, 1999). This is also true for denervated muscle fibers, which reveal up-regulated expression of a number of pro-apoptotic factors such as Bax and caspases as well as anti-apoptotic factors such as Bcl-2 (Tews and Goebel, 1996, 1997b; Tews *et al.,* 1997a, 1997b). At present it is still under discussion whether these observations point to apoptotic processes or are in agreement with necrosis of muscle fibers. At least some of the apoptosis-related events (e.g., increase in mitochondrial membrane permeability)

are discussed to play roles also in necrotic cell death (Kroemer *et al.,* 1998). Moreover, as discussed above (see Section II.D.2), DNA cleavage with subsequent TUNEL-positivity may occur during necrosis.

With increasing age, the low proliferative capacity of satellite cells is decreased even further; accordingly the pool of stem cells diminishes continuously (Schultz and Lipton, 1982; Musaro and Rosenthal, 1999). These data were confirmed by Lescaudron *et al.* (1997) using transgenic mice; moreover, according to these authors the capacity for regeneration of injured muscle fibers was reduced with increasing age.

The percentage of satellite cell nuclei in the soleus muscle of 8-month-old mice is about 4%; it decreases to about 2.4% at 30 months of age (Snow, 1977). This is thought to be a relative decrease due primarily to an increasing number of nuclei inside the muscle fibers rather than to a decreasing number of satellite cells (Snow, 1977). Also in nongrowing muscles, the number of satellite cells was found to decrease with age (Schultz and McCormick, 1994). The authors speculated on the mechanisms involved: "Finally, the reduction in satellite cells could also be related to myonuclear turnover in the adult muscles, but this phenomenon has never been documented." To our best knowledge, this is the only reference in which turnover mechanisms similar to those in syncytiotrophoblast have also been discussed for muscle fibers.

Evident apoptosis in skeletal muscle including DNA fragmentation and TUNEL positivity is a rather rare event. It has been reported as a mechanism for removal of undesired myotubes during development (Sandri and Carraro, 1999). Also apoptotic death of single nuclei in otherwise normal muscle fibers has been shown; an incidence of 0.1% (Sandri *et al.,* 1998) to 0.3% (Migheli *et al.,* 1997) TUNEL-positive nuclei in muscle fibers was reported.

For the stability and survival of myotubes intact myotube–ECM interactions are required. Among others, the roles of integrins, merosin, laminin, and laminin-binding proteins such as α-dystroglycan have been studied (Vachon *et al.,* 1996, 1997; Miyagoe *et al.,* 1997; Montanaro *et al.,* 1999); perturbations of these proteins led to loss of maintenance of myotubes and muscle fibers and to enhanced apoptosis. Dystrophin, the protein product of the human Duchenne muscular dystrophy gene, is thought to provide a linkage between merosin and cytoskeletal components; defects of this sarcolemmal protein result in increased apoptosis (Tews and Goebel, 1997b; Hack *et al.,* 1998; Sandri and Carraro, 1999).

An increased incidence of apoptosis has also been reported for denervated muscles (Tews and Goebel, 1996, 1997b; Tews *et al.,* 1997a, 1997b) and chronic heart failure-related myopathies (dalla Libera *et al.,* 1999). The respective parameters include DNA fragmentation, typical ultrastructural features including formation of membrane-lined fragments of muscle fibers (Fidzianska *et al.,* 1990), and up-regulated expression of bax and caspases, but also up-regulation of bcl-2 as potential anti-apoptotic strategy (Tews and Goebel, 1996, 1997b; Tews *et al.,* 1997a, 1997b). Furthermore it was found that not all nuclei of a single multinucleated muscle fiber display TUNEL-positive DNA-fragmentation (Tews and Goebel,

1996; Tews *et al.,* 1997b). Rather, the presence of TUNEL-negative, normal nuclei may provide a mechanism for survival and continuous function of the respective muscle fibers until a critical number of nuclei is degraded. Only then will the muscle fiber finally die. The same authors reported evidence that this apoptotic loss of nuclei contribute to the atrophy process of muscle fibers seen under pathological conditions including denervation and muscular dystrophies.

Taken together, the above data suggest the following role of the apoptosis cascade in skeletal muscle (Fig. 6): Upon syncytial fusion, in myotubes and muscle fibers the apoptosis cascade is stopped, rather than in an unstable balance of blockage as is the case in syncytiotrophoblast. Apoptosis can be executed in single nuclei in otherwise normal muscle fibers. Apoptosis is considerably enhanced in pathological conditions such as myopathies and denervation, and may contribute to atrophy and loss of muscle fibers. However, it is still open whether muscle fibers undergo a continuous turnover of nuclei, starting with continuous syncytial inclusion of precursor cells and resulting in extrusion of "aged" nuclei.

IV. Concluding Remarks

There are analogies between the mechanisms of syncytial fusion in trophoblast and in skeletal muscle. In many cellular systems it has been shown that the molecular machinery for apoptosis is not only used for execution but also for certain steps of differentiation. Activation of caspases, cleavage of lamin B, and generation of single-strand breaks inside the DNA (TUNEL positivity) are examples described for differentiation of chondrocytes (Feng *et al.,* 1999), erythroid cells (Morioka *et al.,* 1998), keratinocytes (Weil *et al.,* 1999), lens fiber cells (Ishizaki *et al.,* 1998; Dahm, 1999; Wride *et al.,* 1999), and neurons (Urase *et al.,* 1999). In many tissues, leaving the cell cycle is obviously a starting signal for both differentiation and/or apoptosis; and both processes partly share identical molecular events.

Apoptosis inhibitors allow stem cells to employ the molecular machinery of early apoptosis for differentiation purposes. Both a subset of the myoblasts (Miller *et al.,* 1997; Dominov *et al.,* 1998) and a subset of villous cytotrophoblast cells (Sakuragi *et al.,* 1994; Huppertz *et al.,* 1998) show high expression levels of Bcl-2, a potent inhibitor of both apoptosis and proliferation. Also Mcl-1, another anti-apoptotic protein, has been reported in both systems (Krajewski *et al.,* 1995; Huppertz *et al.,* 1998). As has been shown by Marks *et al.* (1998), early blockage of the apoptosis cascade is compatible with survival of the cell. Accordingly, availability of anti-apoptotic mitochondrial proteins enables cells to make use of early steps of the apoptosis cascade without immediate commitment to death.

Fodrin cleavage by initiator caspases or calpain precedes syncytial fusion. Both, initiator caspases and calpains are involved in early proteolysis during apoptosis. Both families of proteases interact with each other (caspase-mediated activation of calpain, Wood and Newcomb, 1999; calpain-mediated activation of caspases,

Ruiz-Vela *et al.,* 1999). Both proteases cleave fodrin to a 150-kDa fragment (Jaenicke *et al.,* 1998; Waterhouse *et al.,* 1998). Fodrin, a member of the spectrin family, is part of the subplasmalemmal cell cortex that mechanically supports the plasma membrane. In trophoblast, both activation of initiator caspases and cleavage of fodrin precede syncytial fusion (Huppertz *et al.,* 1999b). Little information is available on the expression of initiator caspases in normal skeletal muscle (Ng *et al.,* 1999). By contrast, calpain has been shown to be necessary for syncytial fusion of myoblasts (Barnoy *et al.,* 1997). Calpain is present also in the human placenta (Kubota *et al.,* 1984; Shastri and Anandaraj, 1986); however, data on its precise localization and role are not available.

Externalized negatively charged membrane lipids are involved in syncytial fusion. The flip of phosphatidylserine from the inner to the outer plasmalemmal leaflet is involved in syncytial fusion. It is induced by initiator caspase-mediated activation of scramblases and cleavage of translocases, events that have been described as both steps of the early apoptosis cascade or signs of differentiation. In trophoblast this flip precedes fusion (Huppertz *et al.,* 1998) and has been shown to be a prerequisite for it (Lyden *et al.,* 1993; Adler *et al.,* 1995). Its role during formation of myotubes is not yet clear. As discussed above, formation of myotubes by syncytial fusion is induced by members of the ADAM family (meltrin α, Gilpin *et al.,* 1998), which is expressed in villous trophoblast, too. The fusiogenic action of ADAMs, in turn, seems to depend on the presence of negatively charged membrane lipids (Martin *et al.,* 1998).

Following syncytial fusion the apoptosis cascade can be retarded or completely stopped. It seems to be a question of definition whether events leading to syncytial fusion in both tissues are defined as initiation stages of apoptosis or as steps of differentiation. In both cases they involve early parts of the molecular machinery of apoptosis. These include activation of initiator caspases, which are likely to result in progression of the apoptosis cascade if not tightly controlled by inhibitors. Early, successful inhibition is of particular importance since execution to apoptotic death in normal tissues takes place within 24 hr (Saraste, 1999). Both syncytia analyzed here are long-lived structures with life spans from 9 months to many decades however.

In the syncytiotrophoblast, the control of progression of the cascade is provided by cytotrophoblast fusion itself: Both, the inhibitors of apoptosis as well as the effectors (initiator and effector caspases) are only transcribed in cytotrophoblast and are transferred into the syncytium by syncytial fusion (Huppertz *et al.,* 1999b, 1999c). The extent of syncytial fusion regulates activation and blockage of apoptosis and thus the turnover of the syncytiotrophoblast. The normal time lapse between nuclear inclusion by syncytial fusion and nuclear extrusion by apoptotic shedding is 3–4 weeks. Reduced fusion results in enhanced apoptosis; missing fusion in necrosis within a few days (Huppertz *et al.,* 1998, 1999c).

The respective situation in myotubes is still unclear in many aspects. Both low levels of apoptosis inhibitors, such as Bcl-2 in normal myotubes (Sandri and

Carraro, 1999) as well as the long survival period of many decades suggest that the apoptosis cascade is completely stopped rather than only balanced by inhibitors. This is feasible even after initial activation of the cascade, provided that the cascade is sufficiently inhibited for a certain period and reinduction does not take place. This can be achieved by two muscle-specific findings in myotubes/muscle fibers: (1) the presence of a mitochondria-associated inhibitor of caspase 8 (Koseki *et al.,* 1998; Ekhterae *et al.,* 1999) and (2) the absence of Apaf-1 in myotubes (Burgess *et al.,* 1999). Both result in an interruption of the cascade at the transition from initiator to effector caspases. It is still unclear whether the TUNEL-positive nuclei present in normal adult muscle fibers point to a slow nuclear turnover similar to that in syncytiotrophoblast or whether they are accidental in nature. During muscle development and in various pathologies, apoptosis plays an important role in the removal of excess tissue or damaged fibers.

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