Role of the Growth Cone in Neuronal Differentiation

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Abstract

Nerve growth cones are motile, exploring organelles at the tip of a growing neurite. The growth cone is a highly specialized structure, equipped with a complex machinery for reversible membrane expansion and rapid cytoskeletal reorganization, a machinery required for growth cone motility and neurite elongation. It also contains perception systems that enable the growth cone to respond to external signals, thereby steering the trailing neurite to the correct target. Soluble and substrate bound guidance molecules in the environment modulate growth cone behavior either through direct interaction or classical receptor activation coupled to second messengers. A prominent phosphoprotein of the growth cone is B-50. We propose a role for this growth-associated protein kinase C substrate in signal transduction processes in the growth cone.

Index Entries: Nerve growth cone; growth-associated protein; B-50; GAP43; protein kinase C; signal transduction; nerve growth factor; laminin; review.

Introduction

A postmitotic neuron develops a very polar geometry during a relatively short period of its life by sending out axonal and dendritic processes from its cell body in order to establish a complex communication system with other neurons. Since Ramón y Cajal (1890) first described the expanded leading tip of growing axons as "cône de croissance," much attention has been focused on the role of these growth cones as guides, that lead extending neurites to their appropriate synaptic counterparts. On its way to the target, the growth cone meets a spectrum of extacellular cues that contribute to the strength of its adhesion, morphological appearance, the direction and selectivity of its movements and, thereby, to the synaptic architecture of the mature nervous system. The nature of the signals received by growth cones ranges from soluble factors and extracellular matrix molecules to cell surface molecules. The question of how growth cones discriminate between all this information and how they translate it into a cellular response is a leading topic in developmental neurobiology.

The introduction by Harrison (1910) of neuronal cell cultures enabled dissection of the growth cone from its complex surrounding in vivo, and investigation of form and movement in vitro. Using light and electron microscopy, the morphology of the growth cone in vitro has been shown to greatly resemble their structural analog in vivo (Landis, 1983; Johnston and Wessells, 1980; *cf.* descriptions in Bovolenta and Mason, 1987; Argiro et al., 1984). Some aspects of growth cone behavior can now be monitored by means of sophisticated optical methods (e.g., morphology and calcium), whereas many others require a biochemical approach (e.g., changes in protein composition and posttranslational modification). The development of isolation procedures to collect subcellular fractions from vertebrate central nervous systems that are highly enriched in nerve growth cones (Pfenninger et al., 1983; Gordon-Weeks and Lockerbie, 1984) have proven to be valuable tools to gain insight in the nerve growth cone biochemistry.

In this paper, we will discuss several factors that influence growth cone movement and neurite outgrowth in relation to the intracellular biochemical machinery. We will describe the intrinsic features of the growth cone, its morphology, and motile machinery, some extacellular signals that influence growth cone behavior, and, finally, how growth cones may transduce these signals to effector systems.

Growth Cone Structure and Movement

Morphology

The Cytoplasmic Compartment

The nerve growth cone is a very dynamic, irregularly-shaped expansion of a neurite that transiently sends out and retracts one or more extensions (Fig. 1). These extensions are thin, sometimes branched, finger-like filopodia, also called microspikes (diameter 0.1–0.2 μm), or very thin and flat, sometimes curled, veil-like lamellipodia. Although a growth cone's diameter ranges from 0.5 to 10 μm, in invertebrates, filopodia can reach out over a length up to 50 μm to sample its surroundings. The growth cone can be divided into a central part that contains most of the organelles and a microfilament-rich peripheral part that includes the filopodia and lamellipodia. The central part of the growth cone contains microfilaments, microtubules, smooth endoplasmic reticulum, clear vesicles, coated vesicles, large dense core vesicles, mitochondria, and lysosomal structures (Fig.1; Landis, 1983; Johnston and Wessells, 1980; Yamada et al., 1971).

Parallel arrays of neurofilaments (diameter 9–11 nm) run through neuritic shafts and end at the base of a growth cone (Letourneau, 1985). Bundles of microtubules (diameter 24–28 nm) spread from the neurite into the central part of the growth cone as a hand-like fan, but never extend as far as the plasma membrane or into

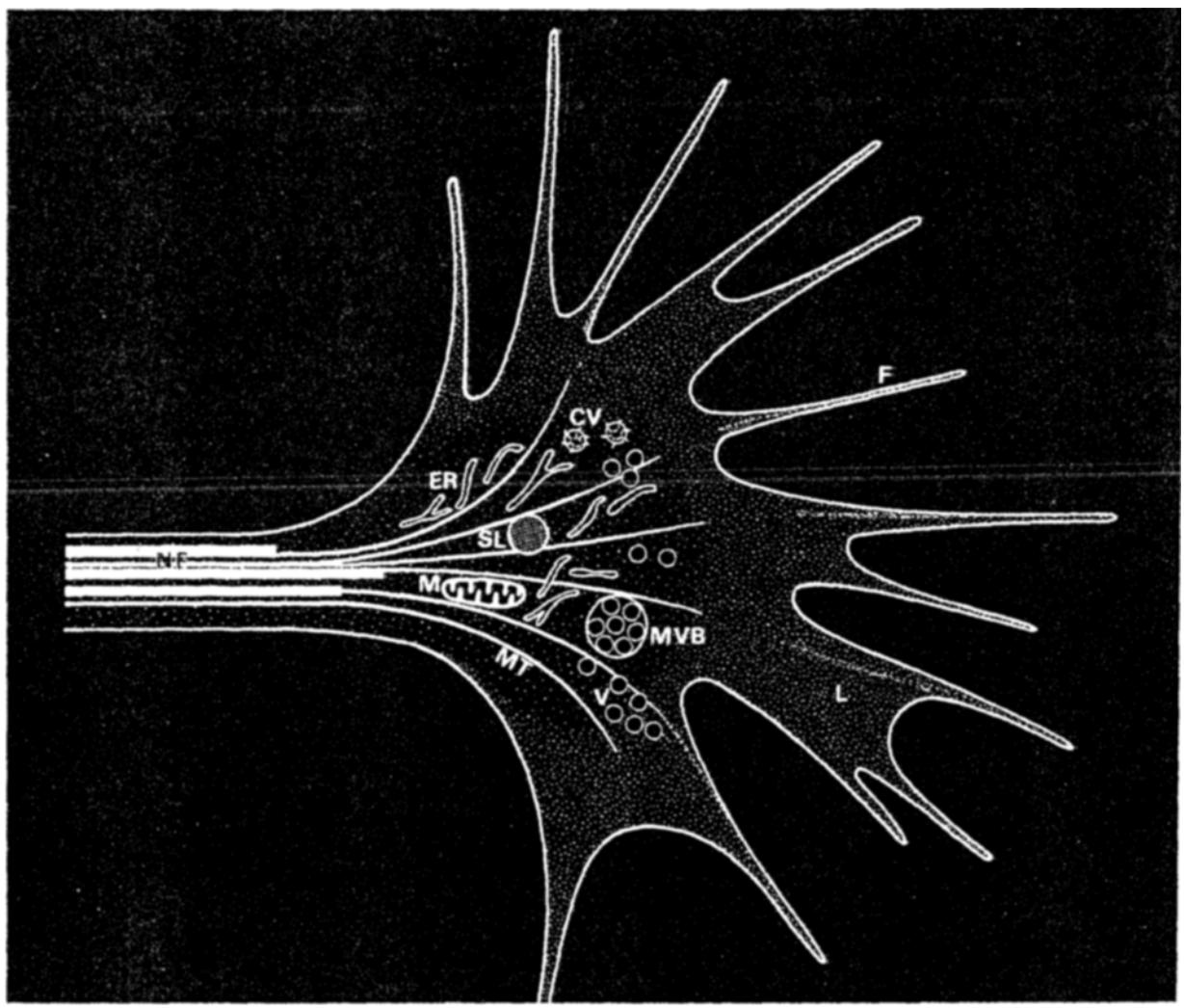

Fig. 1. Cartoon of a nerve growth cone with extended filopodia (F) and lamellipodia (L). The growth cone margin consists of a lattice network of microfilaments (dotted) that reaches into the protrusions and is free of organelles. The growth cone body or center is filled with organelles, such as a branched smooth endoplasmic reticulum (ER), mitochondria (M), vesicles (V), coated vesicles (CV), and secondary lysosomes (SL). Neurofilaments (NF) end at the growth cone base, whereas microtubules (MT) extend as a hand-like fan that ends among microfilaments in the growth cone margin.

filopodia (Yamada et al., 1971; Landis, 1983). Microfilament bundles (diameter 5–7 nm) run from the central part of the growth cone into the filopodia, are embedded in a lattice network of similar microfilaments, and sometimes bear strings of vesicles (Letourneau, 1979). The straight microfilaments are crosslinked to each other, to the surrounding filament network, and to the inner face of the plasma membrane (Letourneau, 1979). The appearance of this network, consisting mainly of actin, can be decorated with heavy meromyosin and is virtually restricted to the growth cone (Letourneau, 1981, 1983). Actin meshworks are the only consti-

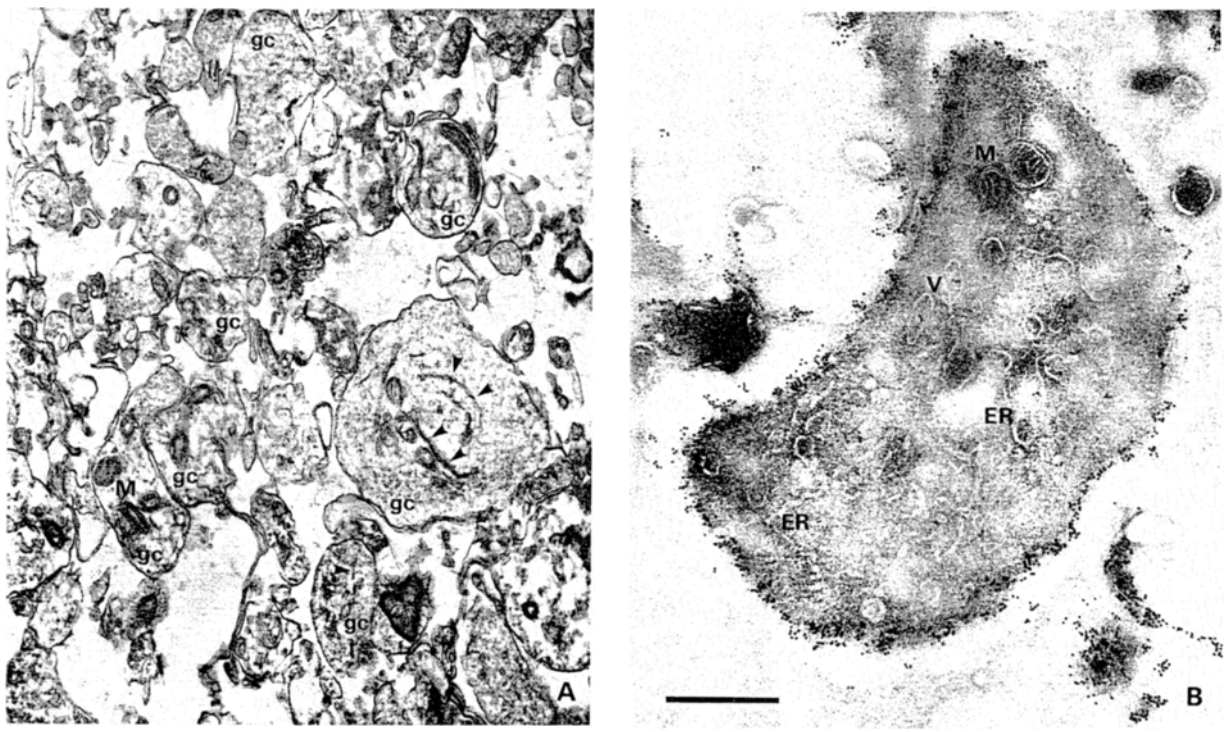

Fig. 2. Ultrastructure and B-50 immunolocalization in nerve growth cones isolated from 5-d-old rat brain. (A) Survey micrograph of and OsO_4-fixed epon section of the growth cone fraction. In intact growth cones (GC), mitochondria (M) and occasional bundles of microfilaments (arrow heads) are visible. (B) Cryosectioned growth cone, immunoglold labeled for B-50, employing affinity-purified polyclonal antibodies. Note the extensive, branched endoplasmic reticulum (ER) and some clear vesicles (V). The plasma membrane is heavily decorated with immunogold, whereas only sparse intracellular B-50 immunoreactivity is found. Bar 2.0 μm (A), 0.5 μm (B). (Micrographs are kindly provided by M. Van Lookeren Campagne; for methods, *see* Van Lookeren Campagne et al., 1988.)

tuents of filopodia (Yamada, 1971) and lamellipodia (DeGeorge et al., 1985), among which sometimes vesicle-like structures are observed (Tsui et al., 1985; DeGeorge et al., 1985). Ultrastructural analysis of isolated growth cones reveal pleiomorphic bulbous sacs, in which both organelles and cytoskeletal elements are conserved (Fig. 2A). Filopodia and lamellipodia retract during isolation from rat brain (Fig. 2A), but will respread when the isolated growth cones are seeded on a coverslip (Gordon-Weeks and Lockerbie, 1984).

In conclusion, the central growth cone body is supported by a microtubule scaffold, among which the organelles for membrane expansion and deletion migrate (Fig. 1). In contrast, the periphery or cortex of the growth cone is supported by a net work of actin without organelles. This characteristic arrangement of organelles and cytoskeletal elements is in continuous motion and may enable the growth cone to expand and move.

The Membrane

Comparison of the growth cone membrane with proximal membranes of neurite and cell body, thus far has focused on some remarkable differences. Freeze-fracture studies have

shown an overall poor intramembrane particle content in the growth cone compared to the somatic membrane (Small and Pfenninger, 1984). A proximodistal gradient with decreasing particle density exists and is characterized by: the larger the particle, the more proximal its localization (Pfenninger, 1987). This distibution suggests somal insertion of integral membrane proteins that diffuse into the neuritic shaft membrane toward the growth cone. The particle distribution at the growth cone plasma membrane is divided into relatively richer and poorer areas. This discrete topography disappears during synaptogenesis, and, therefore may be generated by the process of neuritic growth (Small et al., 1984). Vesicle fusion for membrane expansion may introduce the observed local particle concentrations. Another cause of the irregular particle distribution may be related to receptor clustering, prior to receptor–ligand endocytosis.

Several membrane proteins are organized in a typical proximodistal way. The distribution of lectin-binding sites varies markedly (with neuronal type and lectin species), between cell body and neurites (Schlosshauer, 1985; Pfenninger, 1987). Some lectins bind preferentially to growth cones and others to cell bodies and neurites. The growth cone membrane is enriched in a number of glycoproteins, e.g., a sialic acid-rich 27 kD protein (Pfenninger, 1987) and NGF-induced large external glycoprotein (NILE) (Stallcup et al., 1985). Although growth cones are not enriched in neural cell adhesion molecules (NCAM) (Van den Poll et al., 1986), a more sialic-rich NCAM species is found on growth cones compared with cell bodies (Schlosshauer et al., 1984). High affinity NGF receptors are five times more concentrated at the distal neurite and growth cone than at cell bodies (Carbonetto and Stach, 1982). Voltage-dependent sodium channels (saxitoxin-binding sites) are sparse in distal neurite regions and also found at lower density in isolated growth cones when compared to adult brain synaptosomes (Pfenninger, 1987). In contrast, voltage-sensitive calcium channels appear to be more abundant in growth cones than trailing neurites (Anglister et al., 1982; Bolsover and Spector, 1986).

Taken together, the low particle density at the growth cone and a different protein composition indicate that newly inserted membranes differ from the rest of the cell body wall. High membrane fluidity of neuroblastoma cells has been shown to be a prerequisite for neuritogenesis (De Laat et al., 1978). It is possible that a regional differentiation in membrane fluidity exists. Newly-formed phospholipids are rapidly transported toward the nerve growth cone to become incorporated in its plasma membrane (Pfenninger 1987). This could represent a means to keep the phospholipid/cholesterol ratio and, thus, membrane fluidity high during neurite outgrowth at the growth site. Thereafter, fluidity appears to decrease with aging of the presynaptic membrane (Oestreicher et al., 1986).

Motility and Adhesion

Wessells et al. (1987) have demonstrated that the locomotory capacity of a neuron generally restricted to the growth cone can be extended to cell body and neurites. Both structures regain sprouting activity after mechanical damage or microtubule depolymerization. This suggests that under normal circumstances, proximal membrane motility is suppressed by fixation of the membrane by the intact cytoskeleton. Highly motile growth cone regions, like unattached filopodia and actively extending lamellipodia, comprise an open crisscross array of actin filaments, whereas at substrate adhesion sites, the filaments are aligned in bundles (Tosney and Wessells, 1983; Letourneau, 1981, 1983). Exposure to cytochalasin depolymerizes the linear actin fibers into condensed aggregates (Letourneau et al., 1981) and causes rounding-up of growth cones that retract their filopodia (Yamada et al., 1971). Thus, polymerization of actin into fibers plays an important role in the motile machinery of the growth cone, which can be influenced by external factors, like substrate adhesion. Remarkably, neuronal cells that are plated on very adhesive substrates and grown

in the presence of cytochalasin, grow highly branched neurites without recognizable growth cones. But these "naked" neurites do elongate, albeit slowly, following strange looping tracks (March and Letourneau, 1984; Luckenbill-Edds and Kleinman, 1988).

The pleiomorphic appearance of growth cones, therefore, may reflect their role in neurite guidance and the neurite elongation process. This hypothesis receives further support from experiments in which the outgrowth from pioneering axons in whole grasshopper embryo explants was followed. In the presence of cytochalasin, axons extended without filopodia, but were strongly deviated from their normal paths (Bentley and Toroian-Raymond, 1986). Furthermore, following growth cones of the developing visual pathway in vivo revealed that their morphology is relatively simple when they traverse straight tracts, like the optic nerve. They become more complex at points of decision (the chiasm) and are simple knob-shaped on branched terminals in the target region (Bovolenta and Mason, 1987). Such shape differences may result from differences in adhesive interactions along the optic tract.

In culture dishes, growth cones adhere with their central part to the substratum, whereas the growth cone margin, including filopodia and lamellipodia, is partly, weak, or nonadherent (Letourneau, 1979; Aletta and Greene, 1988). The adhesive property of the growth cone imposes tension upon the trailing neurite, which is attached to the substratum only focally at branching points (Wessells et al., 1978; Shaw and Bray, 1977), and is conserved in sheared-off growth cones after isolation from rat brain (Gordon-Weeks and Lockerbie, 1984). By pulling the growth cone with a mechanical force in a specific direction, elongation will focus toward the source of this force (Bray, 1984), whereas disruption of filopodial adhesion at one site of the growth cone results in growth cone movements toward the opposite site (Bray, 1979). The natural analogous force would be actin-mediated adhesion, consolidation, and, perhaps, contraction of filopodia. The colocalization of actin and myosin in growth cones (Letourneau, 1981) strongly suggests a contribution of actomyosin contractile forces to the motile capacity of the growth cone (Letourneau, 1985; Bray, 1987).

Axon Elongation

Membrane Expansion at the Growth Cone

Elaboration of new neurites requires the formation of new cytoplasmic structures and membranes to flank them. What makes the nerve growth cone so important in this process? Early observations already suggested that a new membrane was added at the distal end of the neurite: branching points of neurites (Harrison, 1910) or little particles laying on the neurites surface (Bray, 1970) remain at a fixed distance from the cell body, whereas the growth cone moves on. When an actively growing neurite is transected between cell body and growth cone, both distal and proximal ends first retract and coil up their neurites into helical forms. Within 3–20 min after sectioning, growth cone formation and renewed outgrowth is observed on both cut ends and at the original growth site (Shaw and Bray, 1977; Wessells et al., 1978). Furthermore, new synthesized lectin binding sites are inserted at the growth cone (Pfenninger and Pfenninger-Malié, 1981; Feldman et al., 1981). These phenomena corroborate the presumption that neurite growth is mediated by new membrane insertion at the growth cone and not randomly along the neurite nor at the neurite base. These studies also illustrate that the growth cone is endowed with a local growth machinery that is, at least to a certain extent, independent of somal support.

Components for new membranes are synthesized in the endoplasmic reticulum at the cell body and conveyed to the nerve terminal by axonal transport, where they are inserted into the plasma membrane, probably by vesicle fusion (Carbonetto and Muller, 1982). It is uncertain whether the subplasmalemmal mounds of vesicles, described by Pfenninger and Bunge

(1974) represent a local membrane source since their appearance may eventually result from fixation artifacts (Landis, 1983; Carbonetto and Muller, 1982) and are not seen in rapidly fixed preparations (Tosney and Wessells, 1983) or freeze-substituted growth cones (Rees and Reese, 1981). If vesicles for membrane expansion were transported with fast axonal transport (300 μm/min), their number needed to extend one lamellipodium (approx. 60) can be well delivered from further down the neurite and not necessarily accumulate in the growth cone cortex (Tosney and Wessells, 1983). Good candidates for such a membrane pool are varicosities, swollen regions in the neurite shaft often seen within 20 μm proximal to the growth cone in vitro (Koenig et al., 1985; Aletta and Greene, 1988). Such varicosities contain clusters of intracellular organelles, especially during neurite elongation, and can translocate along the neuritic shaft in either direction. Their incidence is highest during active neurite growth, and they seem to supply the growth cone with building material exporting their organelles, thereby decreasing their own size.

Besides the somal supply of membrane constituents, additional local supplies may facilitate outgrowth. For instance, the large amounts of apolipoprotein E, delivered to regenerating nerves by invading macrophages (Snipes et al., 1987) and circulating ketone bodies (Clouet and Bourre, 1988), can be used as precursors for lipid synthesis by regenerating nerve sprouts. In case of a dendritic growth cone, dense packages of polyribosomes at the base of a spine are highest during synapse development (Steward and Falk, 1985). In cultured neurons, newly transcribed RNA is transported into dendrites as far as the growth cone (Davis et al., 1987) and may provide a local source for membrane components.

Endocytotic activity in the growth cone is very high during active growth. Retraction of lamellipodia is accompanied by the appearance of vesicle clusters beneath the irregularly-folded, retracted surface (Tosney and Wessells, 1983). These vesicles may be used as a membrane pool for further protrusive activities. However, much of the endocytosed material is retrieved in lysosomal structures, like mutivesicular bodies (Johnston and Wessells, 1980). Since membrane addition and endocytotic membrane deletion concur in the growth cone, net growth would result from an imbalance between them. Rapid turnover of growth cone membranes could enable a neuron to regulate the expression of (e.g., growth factor) receptors and thus the sensitivity of growth cones to external stimuli with a very high plasticity.

Role of the Cytoskeleton

To support the newly-formed membranes, microtubule and microfilament polymerization is required. Recently, it has been shown by Bamburg et al. (1986) that the growth cone is the major microtubule assembly site. This polymerization is fed from a reservoir of free tubulin in the growth cone (Gordon-Weeks, 1987). However, taxol-induced, uncontrolled tubulin polymerization stops neurite elongation immediately (Letourneau and Ressler, 1984), whereas microtubule depolymerization also stops growth (Yamada et al., 1970). Thus, effective axon elongation requires a balanced, local regulation of tubulin polymerization. Calcium and microtubule-associated proteins (MAP) are important regulators of tubulin polymerization. Actin, tubulin, and MAPs become reversibly phosphorylated by their respective kinases, which are activated through receptor-generated second messengers (Schulman 1984; Akiyama et al., 1986; Hargreaves et al., 1986; Demaille and Pechere, 1983). In vitro studies suggest that this phosphorylation modulates several elements of the cytoskeletal architecture: the self-assembly of tubulin and actin, the interaction of tubulin with actin and membranes, and the interaction of MAPs with tubulin and actin (Demaille and Pechere, 1983; Selden and Pollard, 1983; Hargreaves et al., 1986; Yamamoto et al., 1988). Noteworthy is the fact that growth factors, like NGF, regulate the phosphorylation state of several of these components (Aletta et al., 1988). Thus, phosphoryl-

ation of cytoskeletal elements may serve an important function in the cellular implementation of growth cone guidance (*see* Signal Transduction section).

The Growth Mechanism

High resolution microscopy suggests that the growth cone does not move forward as a body, but that the membrane and cytoplasmic compartment advance separately (Aletta and Greene, 1988; Goldberg and Burmeister, 1988). First, lamellipodia span between an extended filopodial framework (actin-based movements and membrane addition). The lamellipodium then thickens as it fills with cytoplasmic organelles (microtubule polymerization and vesicle movement) to become the proximal growth cone body that adheres to the substratum and from which new motile protrusions are extended. If elongation proceeds, the central growth cone will finally transform into part of the nonadherent neuritic shaft (microtubule and neurofilament polymerization). Bray (1987) summarized ample evidence that contractile forces, provided by actin–myosin interaction, equip the growth cone with a steering mechanism to adequately direct the growing axon to guidance cues. Moreover, there seems to be a balance of opposite forces exerted by microtubules and actin, respectively (Joshi et al., 1985). Taken together, microtubules appear to form the compressive elements that mediate neurite elongation, whereas actin-based activity modulates this force and mediates directional guidance.

Elongation Rate

Neurite elongation rates in vitro, as well as in vivo, vary from 5 to 200 μm/h and are correlated with the differentiation state of the neuron (Argiro et al., 1984). The degree of growth cone spreading, the complexity of its protrusions, and the axon elongation rates increase during embryonic stages (Bray et al., 1987), is highest during perinatal development, and decreases thereafter with aging (Mason, 1985; Argiro et al., 1984). The sensitivity of a neuron to environmental cues also changes during differentiation (Millaruelo et al., 1988; Cohen et al., 1986; Koh and Loy, 1988). Apparently, this is owing to changes in the neuron's genetic program during development. The acquisition of neuronal polarity is suggested to be one of such intrinsic features of the neuron that it is relatively independent from external signals (Dotti et al., 1988; Solomon, 1981) since a neuron conserves its characteristic in vivo cell morphology in culture in vitro (Mattson, 1988). Thus, the interplay between intrinsic features of the neuron with extrinsic signals will concertedly determine the path that a given growth cone chooses.

External Factors Regulate Growth Cone Movement

The influence of external guidance information depends on the composition of signal molecules in the environment surrounding the growth cone. The cell surface of adjacent cells (Bonhoeffer and Huf, 1980), extracellular matrix components in the intercellular space (Carbonetto, 1984), soluble factors that diffuse within this space (Varon, 1985), and electrical fields (Patel and Poo, 1982) have all been shown to influence neurite outgrowth.

Neurotropic signal molecules can roughly be divided into neurotrophic factors (NTF) that enhance neuronal survival and neurite-promoting factors (NPF), that induce neurite outgrowth and guidance. NTF is a group of proteins that, in vivo, may rescue neurons from natural death and, in additon, have the competence to promote neurite outgrowth. NPF stimulates neurite formation, but is only effective in the presence of appropriate NTF activity. NPF activity seems to be associated with substratum-bound molecules, whereas NTF activity is mostly presented in soluble form (Uchida and Tomonaga, 1985; Collins, 1978). For laminin, it has been shown that its NPF activity is only exhibited when laminin is attached to the culture substratum, but not when present in soluble form (Lander, 1987). NGF exhibits NTF as

well as NFP activity and, in additon, NGF can serve as a neurite guiding molecule when attached to a substrate (Gunderson, 1985). Davis et al. (1985) illustrated that a combination of both NTF and NPF is needed for optimal neuritic growth in vitro (Millaruelo et al., 1988). Neurons receiving the NTF alone survived, but failed to grow neurites, whereas neurons receiving only NPF rapidly extended neurites, but then died (Davis et al., 1985). The biological activity of NPF and NTF will be exemplified by laminin and NGF, respectively, in light of their possible mechanism of action (*see* also Signal Transduction section).

Neurite-Promoting Substrates

Laminin

Early experiments show that, given the choice, a neuron prefers to extend its neurites on the most adhesive substrate (Letourneau, 1975). Tissue culture plates, precoated with polycationic material (polylysine or polyornithine) or extracellular matrix-derived proteins possess a marked neurite-promoting activity. A consistent hierarchy in NPF activity is observed when these substrates are tested in tissue culture systems using neurons from different origins. Substrates with increasing NPF potency in vitro are collagen types I and IV, fibronectin, and laminin (Davis et al., 1985). NPF-like action will be further illustrated by laminin.

Biological Activity of Laminin. In vivo, the extracellular matrix component laminin (mol wt 900 kD) is a major glycoprotein of basal laminae of different cell types, including Schwann cells (Bunge and Bunge, 1983). When presented as part of the tissue culture substratum, it provides a track for selective growth cone extension (Lander, 1987) and induces neurite outgrowth in a variety of embroyonic neurons (Baron van Evercooren et al., 1982; Rogers et al., 1983; Manthorpe et al., 1983; Davis et al., 1985). Anti-laminin antibodies have been shown to inhibit the biological NPF activity in conditioned media derived from different sources (Lander et al., 1985), suggesting that laminin is a general and potent NPF. Studying primary outgrowth in tissue culture from neural tube cells of 40 h chick embryos, a stage at which neurite outgrowth in vivo has not yet started, neuronal adhesion, survival, and neurite extension is strongly increased by laminin coating (Heaton and Swanson, 1988). But these studies do not answer the question of what is laminin's role during normal embryonic development.

In vivo laminin is expressed on neuroepithelial cells along the developing visual pathway during the earliest stages of development (E3–E7), becoming restricted to the basement membrane thereafter (Cohen et al., 1986). Interestingly, chick retinal ganglion cells lose their responsiveness to laminin between E6 and E11 (Cohen et al., 1986). These findings illustrate that, during embryonic development, NPF expression and neuronal NPF responsiveness are closely tuned in time and space. During regeneration, laminin is present on Schwann cell membranes and Schwann cell-derived basement membranes (Longo et al., 1984), both of which are thought to act as a surface for regenerating axonal sprouts to grow along (Ide et al., 1983). Apparently, the same mechanism that enables embryonic development also facilitates reestablishment of neuronal connections after injury. Besides its NPF action, laminin improves trophic supported survival of neurons (Skaper and Varon, 1986; Edgar et al., 1984; Pixley and Cotman, 1986; Millaruello et al., 1988).

Mechanism of Action of Laminin. Employing rotary shadowing electron microscopy, laminin is shown to be a cross-shaped molecule with one long branch and three shorter ones (Engel et al., 1981). The long arm is involved in heparin binding, as well as the promotion of neurite outgrowth (Edgar et al., 1984). However, these properties are located on different sites of the molecule (Engvall et al., 1986; Hopkins and Agranoff, 1987). Calculations by Davis et al. (1985) reveal that, under experimental conditions a ciliary ganglion growth cone can cover or contact 600–1500 substratum-bound laminin molecules and that a filopodial tip (0.01–0.02

μm^2) may respond to only one single molecule at a given time.

We are confronted with the question of what the underlying mechanism is for the observed effects. It has long been thought that NPF activity is mediated primarily through enhancement of substratum adhesion (Letourneau, 1975). But this hypothesis has been questioned recently by measurements of adhesive forces between growth cones and different substrates. Growth cone adhesion to collagen is stronger than to laminin, despite the obvious choice to navigate their neurites on laminin in favor of collagen (Gunderson, 1987). Lander (1987) postulated that neurites prefer an adequate adhesive substratum to a poorly adhesive one but, once within the range of adequate adhesion, they will grow faster and remain restricted to an area of substratum containing a specific NPF molecule. Noteworthy is the observation that the more adhesive the substratum, the longer the growth cone protrusions remain extended (Bray and Chapman, 1985; Gunderson, 1987). Indeed, the action of laminin is thought to imply stabilization of microfilaments, since laminin can counteract cytochalasin-induced growth arrest (Luckenbill-Edds and Kleinman, 1988). Thus, preferred substrates, like extracellular matrix molecules, may achieve their effect through stabilization of microfilaments.

Recently, structurally-related receptors for extracellular matrix molecules like laminin and fibronectin, but also for molecules involved in platelet and immune function, have been collected in a receptor family named "Integrins" (Hynes, 1987). Considering that laminin effects are mediated by membrane receptors and that local microfilament polymerization is involved in the subsequent cellular response, some transmembrane signaling should occur. Indication for a direct coupling has been presented by Horwitz et al. (1986), who carefully isolated an intrinsic membrane protein that has two distinct binding domains, one for extracellular matrix molecules and another for cytoskeletal proteins like talin, an actin binding protein. Thus, laminin may, by direct transduction of receptor occupation, influence cytoskeletal organization. Furthermore, neurites elaborating on a laminin-like substrate display a higher calcium channel density when compared with those growing on a lectin substrate (Ross et al. 1988). This may indicate that laminin induces changes in calcium fluxes that are presumed to be of crucial importance for effective neurite extension (*see* Signal Transduction section).

Neurotrophic Factors

NGF

Diffusion of chemical messengers from the target region to an innervating neuron is important as trophic support to ensure neuronal survival. In additon, these messengers can comprise guiding information and promote neurite branching and outgrowth. Several of such factors have been described and isolated from different sources. NTF-like action will be further illustrated by the best studied NTF, nerve growth factor (NGF). NGF is a 130 kD polypeptide complex, containing three subunits and stabilizing zinc ions. The biological activity, residing in the dimeric β-subunit (mol wt 26.5; IEP 9.3; Greene and Shooter, 1980), can be divided into three major actions: trophic, tropic, and differentiative.

Biological Activity of NGF. Anti-NGF antibody interference experiments in vivo have revealed that developing sensory and sympathetic neurons depend for their survival on NGF. Sympathetic neurons continue this NGF dependency during maturity, whereas sensory neurons do not although they seem to require NGF for normal biochemical and morphological homeostasis (Johnson et al., 1986). It is now recognized that central neurons also are responsive to NGF; the strongest evidence for a role of NGF in CNS function is seen in the basal forebrain cholinergic systems of rodent, as well as human, brain (Whittemore and Seiger, 1987; Hefti et al., 1986). In additon to a trophic action, NGF also induces neuronal sprouting of NGF-responsive neurons toward a NGF source. For example, NGF injections in the fourth ventricle

of neonatal rats causes fibers, originating from sympathetic ganglia, to grow all the way to the intracerebral NGF pool (Levi-Montalcini, 1976). Contribution of the target produced NGF to attract their correct neurons to become innervated in vivo (neurotropic action); however, it may not be as dramatic as these experiments indicate. Davies et al. (1987) have shown that target NGF synthesis and NGF receptor expression on ingrowing neurons does not begin until axons are in the vicinity of this target. Moreover, the density of sympathetic innervation correlates well with the level of NGF in corresponding peripheral target tissues (Shelton and Reichardt, 1984; Thoenen and Edgar, 1985). This may be attributed to its potency to enhance survival and induce local branching of neurites and growth cones (Campenot, 1982) especially since NGF is shown to induce collateral sprouting rather than stimulating regeneration in vivo (Diamond et al., 1987). In conclusion, NGF may have a major task in matching neuronal innervation with the size of the target area by rescuing innervating neurons from death and regulating their terminal arborization.

Biological actions of NGF can be divided in short- and long-latency effects (Greene, 1984). Long-term effects (after at least 1 d) are dependent on protein synthesis in the cell body and include induction of neurotransmitter synthesizing enzymes, neurite growth, development of electrical excitability, and other features of neurotransmisson-competent neurons. Early NGF effects (minutes to hours) are characterized by their independence on protein synthesis and their local influence independent of the cell body. These effects comprise regulation of growth cone shape and motility, initial neurite sprouting, neuronal survival, changes in the uptake of nutrients and precursors, and activation of neurotransmitter-synthesizing enzymes. The pheochromocytoma PC12 cell line is often used as a model system to study NGF-induced neuritogenesis since NGF induces these cells to differentiate into sympathetic-like cells bearing neurites with growth cones, whereas they are not dependent on NGF for survival. We will now discuss the major proposed cellular mechanisms that mediate NGF action.

The Intracellular Signal of NGF. NGF AND NGF RECEPTORS. By separating the cell body and its extending neurite into different compartments, Campenot (1977) has demonstrated that NGF is needed at the distal neurite to maintain this neurite and promote its growth, irrespective of the presence of NGF at the cell body compartment. NGF in the cell body compartment alone cannot prevent neurite degeneration. It is not surprising that, indeed, the highest NGF receptor density is found in dorsal root ganglion neurons at the distal neurites (Carbonetto and Stach, 1982; Campenot, 1982). However, this NGF-receptor distribution over the cell appears to be dependent on the NGF-concentration (Stach and Perez-Polo, 1987) since NGF appears to modulate the density of its own receptor on responsive cells (Bernd and Greene, 1984). Two different NGF receptors have been described, one with a high affintiy and slow NGF releasing (158 kD) and the other with low affinity, fast NGF releasing properties (100 kD) (Stach and Perez-Polo, 1987; Hosang and Shooter, 1987). An interesting observation is that Schwann cells transiently exhibit NGF receptors after axotomy of the axons they support (Taniuchi et al., 1986a). The authors postulate an attractive role for these receptors during regeneration: low affinity Schwann cell receptors, localized in bands of Büngner, would concentrate NGF and offer this to high affinity receptors of ingrowing nerve sprouts. This idea is supported by the finding that cells with low affinity receptors bind, but do not respond to nor internalize NGF (Green et al., 1986; Stach and Perez-Polo, 1987). Upon binding of NGF to high-affinity receptors at the distal part of the central and peripheral neurons, the whole NGF-receptor complex is internalized and retrogradely transported at a rate of approximately 6–7 mm/h to the cell body, where the complex evokes cell body responses (Johnson et al., 1986, 1987; Leonard et al., 1987).

When NGF is internalized and coupled to its receptor, either of these molecules or fragments

thereof could function as a cellular messenger. It has been shown that NGF is not fully degraded during transport through the axon and is still biologically active when arriving at the cell body (Thoenen and Edgar, 1985). As far as NGF itself is considered for such a second message role, cytoplasmic and nuclear injections of NGF fail to mimic any biological effect (Thoenen and Edgar, 1985). In NGF-responsive neurons of the PNS and CNS, the NGF receptor is transported both anterogradely and retrogradely through the axon (Johnson et al., 1987). Several indications have led to the idea that a certain portion of internalized NGF receptors on retrograde transport are unoccupied and not dependent on NGF binding. Furthermore, NGF receptor subunits (80 and 210 kD) are phosphorylated in intact primary neurons and PC12 cells by the cAMP independent protein kinase. The degree of phosphorlyation is not influenced by NGF binding to the receptor (Taniuchi et al., 1986b). Phosphorylation of the receptor may be a step in the internalization or transport process, but much more experimental evidence is needed to ascribe a second messenger role to the NGF receptor. Whatever the signal resulting from the retrograde transport of NGF-receptor-complex, it could only account for delayed effects that influence transcription in the cell body.

K^+ Ions. Like NGF, high potassium exposure also increases neuronal survival (Wakade et al., 1983), mimics (Schubert et al., 1978), and potentiates NGF-induced neurite outgrowth (Koike, 1986) in PC12 cells. The latter may be explained by the rapid appearance and internalization of NGF receptors during depolarization in PC12 cells (Koike, 1987). The survival enhancing action of NGF has been ascribed to activation of the sodium/potassium pump by NGF (Varon, 1985; Boonstra et al., 1983). Upon removal of NGF, the sodium gradient is dramatically disturbed, but quickly restored when NGF is replaced. Using a compartmentalized culture system, Campenot (1986) showed that proximodistal increases in potassium concentration along neurites cause abrupt neurite retraction and degeneration. It should be considered that, in this case, only the neurites are exposed to high potassium. The destructive effects of high K^+ may be owing to interference of the imposed gradient with local ionic currents and, therefore, be unrelated to NGF effects. Cellular events evoked by NGF and high potassium only partly overlap. A PPI response (*see* below; Traynor, 1984) and dephosphorylation of a 70 kD chick sympathetic neuronal protein is induced by both treatments (Acheson et al., 1986). Phosphorylation and stimulation of tyrosine hydroxylase (60 kD) in PC12 cells is different for NGF and high potassium (Lee et al., 1985). Although high potassium induction of the expression of the oncogen c-*fos* in PC12 cells is mediated by calcium entry through voltage-dependent channels in a calmodulin-dependent manner, c-*fos* induction by NGF is independent of both extracellular calcium and calmodulin (Morgan and Curran, 1986). The treatments further differ in susceptibility to inhibition by protein methylation inhibitors: NGF, but not high potassium effects are inhibited (Acheson et al., 1986). Protein methylation may be a very early event in the NGF effector path (Seeley et al., 1984), perhaps directly coupled to NGF receptor activation or internalization. It appears that the biological short-term actions by NGF and high K^+ are initially divergent in nature, though converge further down the cellular chain of reactions. However, it should be emphasized that none of the long-term NGF effects can be mimicked by high K^+ (Leonard et al., 1987).

cAMP. Contradictory results have been presented concerning changes in cellular cAMP levels by NGF. Cellular cAMP increases, as well as decreases, have been described. Exposure of PC12 cells to the membrane-permeable cAMP analog dibutyryl-cAMP mimics NGF induction of neuritogenesis (Heidemann et al., 1985; Boonstra et al., 1987; Rydel and Greene, 1988). However, NGF- and cAMP-induced neurite formation appears to be mediated by different pathways (Rydel and Greene, 1988). The reports are conflicting: direct effects of NGF and

cAMP on growth cone motility are similar in dorsal root ganglion (Gunderson and Barrett, 1980), but not in PC12 cell growth cones (Seeley and Greene, 1983). cAMP and NGF synergistically promote microtubule stability (Heidemann et al., 1985). Drugs that increase cAMP levels, such as forskolin and cholera toxin, enhance initial NGF-induced sprouting, but delay long-term NGF neuritogenesis (Greene et al., 1986). In any case, a rise in cellular cAMP level appears not to be an essential step in NGF differentiation (Richter-Landsberg and Jastorff, 1986; Katoh-Semba at al., 1987).

PPI Response, Protein Kinase C and Calcium. A good candidate for local short-term effects is the so-called polyphosphoinositide (PPI) response (*see* Signal Transduction section). Membrane phosphoinositides are rapidly and transiently (within 1 min) broken down upon NGF-receptor activation in PC12 cells (Contreras and Guroff, 1987; Traynor, 1984). Calcium appears to play a central role in this PPI signaling since the calcium ionophore A23187 not only mimics NGF-induced neurite outgrowth, but also causes a dramatic PPI response in PC12 cells that is totally dependent on the influx of extracellular calcium ions (Contreras and Guroff, 1987; Traynor, 1984). Concomitantly, protein kinase C (PKC) activation by PPI breakdown products is also presumed to be a key step in NGF-induced sprouting (Hama et al., 1986; Hall et al., 1988).

Cell–Cell Contact, Electrical Fields, and Neurotransmitters

Cell–Cell Contact

When a growth cone meets and touches an inappropriate neurite, it will retract its extensions and collapse to resume translocation in another direction (Kapfhammer and Raper, 1987; Letourneau, 1987). This indicates that contact inhibition plays a role in growth cone guidance. In vitro filopodial tips have been shown to display high affinity for each other and become linked by extracellular filaments (Tsui et al., 1985). In this regard, another phenomenon, release of the protease plasminogen activator by the growth cone (Krystosek and Seeds, 1981, 1984; Lander et al., 1987), seems of particular interest. If release of proteases will prove to be a common phenomenon, it would enable the growth cone to modulate the features of the extracellular matrix and become involved in the regulation of these peculiar, interneuronal filamentous links. Another form of communication is seen in filopodia of developing grasshopper growth cones, which have been shown to insert deeply into neighboring growth cones, inducing the formation of coated pits and vesicles (Bastiani and Goodman, 1984). Moreover, direct signal transmission between filopodia and contacting cells may also be mediated by a transient formation of tight junctions, across which intracellular information molecules can be directly exchanged (Taghert et al., 1982). Formation of these junctional connections appear to be facilitated or even induced by NCAM-mediated cell adhesion (Keane et al., 1988).

A number of studies have shown that growth cones selectively translocate along preferred pathways marked by spatially organized surface molecules on neighboring cells (Bonhoeffer and Huf, 1980; Goodman et al., 1984; Edelman, 1985; Kuwada, 1986; Bastiani et al., 1987; Matsunaga et al., 1988; Rathjen et al., 1987, Rathjen, 1988). Such recognition molecules interact in a specific homophilic or heterophilic way that is often calcium dependent. Antibody localization and interference experiments have elegantly shown that this form of information can direct growth cone movement, determine axonal growth rates, or give rise to selective fasciculation (Henke-Fahle and Bonhoeffer, 1983; Rutishauser, 1985; Stallcup et al., 1985; Chang et al., 1987). Interestingly, such guiding glycoproteins, like NCAM, NILE, Thy-1, Cadherin, and L1, belong to a big surface immunoglobulin family that exhibit structural homologies between surface glycoproteins involved in cell–cell contact (Williams and Gagnon, 1982, Williams, 1987). Thus, the surface composition of neighboring cells appear to be capable of modulating the interaction of passing growth

cones with these cells, thereby providing guidance information.

Electrical Fields

Lectins are often used to localize and crosslink surface glycoconjugates (glycoproteins and glycolipids). During active sprouting, concanavalin A (conA) is retrogradely transported along the protruding surface toward the growth cone body (DeGeorge et al., 1985). A shift of surface glycoconjugates is also induced by an extracellularly-applied electrical field. Such an electrical field causes growth cones to adjust their growth direction toward and to accelerate this growth specifically at the cathodal site (Patel and Poo, 1982; Patel et al., 1985). The observed shift in membrane components may be mediated by lateral electrophoresis along the membrane toward the cathodal site of the growth cone (Jaffe, 1977). Interestingly, these field effects are abolished in the presence of conA, which may diminish the mobility of glycocomponents in the membrane by crosslinking. These studies point to a possible contribution of differential distribution of membrane components along the cell membrane to growth cone translocation. Analogously, endogenous electrical currents also occur, generated in the growth cone along the membrane, entering at the filopodial tip and leaving at the filopodial base (Freeman et al., 1985). These much weaker endogenous currents, however, have not proven so far to induce significantly lateral electrophoresis or alter motility of the growth cone.

Neurotransmitters

Before being incorporated in a functional synapse, growth cones are known to release neurotransmitters spontaneously or upon depolarization (Young and Poo, 1983; Hume et al., 1983; Gordon-Weeks et al., 1984; Lockerbie and Gordon-Weeks, 1985). This premature neurotransmission may imply a contribution to the neuronal network architecture since, beside their signal function in the synapse, neurotransmitters also influence growth cone motility, neurite outgrowth, and neuronal degeneration. Such modulatory actions have been described for invertebrate and vertebrate neurons in vitro, as well as in vivo (Mattson, 1988). Some neurotransmitters stimulate outgrowth or provide a trophic support for neurons, whereas others lead to growth cone arrest or even degeneration. This influence is dependent on the concentration of the neurotransmitter. The inhibitory effects of serotonin on a *Helisoma* neuron in vitro (McCobb and Kater, 1986) and glutamate on hippocampal neurons (Mattson et al., 1987) can be counteracted by acetylcholine and GABA, in combination with diazepam respectively. Therefore, the contribution of neurotransmitters to outgrowth control in vivo will be the result of the various neurotransmitters present. The major site of this neurotransmitter action appears to be the growth cone. A growth cone maintains its sensitivity to the neurotransmitter when cut apart from its trailing neurite (Mattson, 1988), again emphasizing the autonomy in the intergration of guiding signals. Not only is the effect of glutamate the opposite for cerebellar granule cells and hippocampal pyramidal cells, glutamate also selectively inhibits dendrites, while leaving axons of the same neuron unaffected (Mattson et al., 1988b; Pearce et al., 1987). Some of these neurotransmitter influences have been shown to be mediated via receptor-generated second messengers (Lockerbie et al., 1988; Lankford et al., 1988; Mattson, 1988; Mattson et al., 1988b). Furthermore, the neurotransmitter action may be mediated analogously to the adult synapse, because the inhibitory effects of a depolarizing neurotransmitter can be prevented by a hyperpolarizing neurotransmitter (McCobb and Kater, 1986; Mattson et al., 1987). Thus, selectivity of neurotransmitter effects are dependent on a range of variables: the temporal and spatial expression of different neurotransmitters, the expression of neurotransmitter receptors on the growth cone, and the differential coupling of the receptor with the intracellular outgrowth machinery.

We have illustrated that several types of molecules can influence the growth cone's behavior and that the interaction of a growth cone with its

surroundings is not a one way communication. The growth cone responds to extracellular signals by changing its appearance, adhesion, motile behavior, neurotransmitter release, protease release, surface glycoprotein distribution, and filamentous or junctional connections.

Signal Transduction in the Growth Cone

In the preceding sections, we have concentrated on extracellular signals that influence several aspects of growth cone behavior. We will now focus on the intracellular machinery that growth cones use to autonomously respond to these signals. Implementation of guiding signals at the growth cone requires rather discrete steps. First, sensation of the signal through interaction at the growth cone surface, e.g., by specific receptors. Second, by transduction of the signal across the membrane. Third, responding to the signal by the activation of effector systems, such as the motile apparatus. This latter step is, in many cases, accomplished through mediation of second messengers. An overview of second messenger systems in the growth cones is depicted below. Despite the conspicuous importance of second messengers in signal transduction at the growth cone, it appears conceivable that some signals influence the effector system of the growth cone by direct interaction. Some examples arise from the previous sections of this article. Extracellular matrix molecules may transduce their signal by mechanical forces through membrane-spanning receptors that are directly coupled to cytoskeletal elements. Aggregation of receptor molecules, ion channels, or intercellular exchange of molecules, through induction of endocytosis or tight junctions, are alternatives that may bypass second messengers.

In search for proteins that play a specific role in neurite growth, anterogradely-transported proteins in intact vs regenerating nerves have been compared (Skene and Willard, 1981a–c). These authors discovered a small family of neurite growth-associated proteins, synthesized at levels up to 100-fold higher during neurite outgrowth, compared to nongrowing states of the neuron. Based on these initial metabolic labeling studies, the GAP hypothesis was postulated: induction of a small subset of growth-associated proteins (GAPs) may be a prerequisite for axonal growth during development and regeneration (Levine et al., 1982). In this review, we will confine ourselves to the best characterized member of this family, the growth-associated protein GAP43 (= B-50) and propose a role for this protein in signal transduction at the growth cone.

The Growth-Associated Protein GAP43/B-50

Different laboratories have independently studied the acidic phosphoprotein B-50 (GAP43) as a presynaptic, neuron-specific PKC substrate, with a putative interaction with the PPI cascade (Zwiers et al., 1980; Gispen et al., 1985b) as a phosphoprotein F1 that is involved in hippocampal long-term potentiation (Routtenberg and Lovinger, 1985) as a neurite growth-associated protein, GAP43 (Skene and Willard, 1981a–c) and GAP48 (Benowitz and Lewis, 1983), and as a component of isolated nerve growth cones, pp46 (Katz et al., 1985). Cross-laboratory studies and sequencing data then corroborated the presumption that all these molecules are equivalent (*see* Benowitz and Routtenberg, 1987). This list has recently been extended with a neuron-specific, atypical calmodulin-binding protein, P–57 (Cimler et al., 1987). We will further refer to the protein as B-50.

Increased Levels of B-50

During development of the central nervous system, B-50 levels are highest in the perinatal period when axon outgrowth and synaptic organization occur in rabbit (Skene and Willard, 1981a,b), rat (Zwiers et al., 1987; Jacobson et al., 1986), hamster (Moya et al., 1987), and human (Neve et al., 1987; Ng et al., 1988). A sharp de-

cline in synthesis (mRNA level) then is seen, followed by a slower decrease of B-50 levels (Jacobson et al., 1986). In human brain, B-50 expression declines with age, but remains relatively high in some associative brain areas (Neve et al., 1987; Ng et al., 1988). Induction of the protein accompanies successful regeneration of peripheral nerves, but does not occur in damaged central nerves that fail to restore their projections (Benowitz and Routtenberg, 1987). Whether the amount of B-50 molecules determines the axonal growth rate is not clear. In tissue culture, growth cones of young neuronal origin grow faster and also display stronger B-50 immunoreactivity than morphologically-matched ones from older animals (Johnson et al., 1986). Interestingly, when neurite outgrowth is accelerated, following a conditioning lesion in the rat sciatic nerve, the B-50 levels rise earlier and higher than after a single crush lesion (Van der Zee et al., 1988). Furthermore, B-50 levels decline to normal several weeks after the lesion (Skene and Willard, 1981; Van der Zee et al., 1988), and this normalization may be independent of whether a successful target connection has taken place (Yoon et al., 1986). All these studies of the last 5 yr confirm that its expression is highly correlated with axon growth by in vitro and in vivo studies. Nonetheless, we have still not established a causal relationship between expression of the protein and axonal outgrowth and the synaptic organization. The first approach to attack this question is determining its exact localization in growing neurites.

B-50 Localization

Immunostaining for B-50 in explanted dorsal root ganglia grown in culture, shows a halo of strong immunoreactivity in the distal growth cone region with low intensity staining in neurites (Meiri et al., 1986; Schmidt-Michels et al., 1988). Monitoring the developing pyramidal tract at the third cervical spinal segment, a transient wave of high B-50 immunoreactivity coincides with passing of the growth cones of outgrowing corticospinal axons (Gorgels et al., 1987). In the regenerating sciatic nerve, B-50 is associated with newly-formed sprouts (Verhaagen et al., 1986), whereas in the developing hippocampus, outgrowing neurites display a strong B-50 immunoreactivity (Oestreicher and Gispen, 1986). B-50 immunoreactivity is absent from intact neuromuscular junctions, but appears during reinnervation in association with the presynaptic membrane and synaptic vesicle-like structures (Verhaagen et al., 1988). In all these neuronal specimens, a conspicuous punctuate staining of growing neurites is observed. In mature neurons B-50 levels are much lower and its localization is restricted to presynaptic membranes (Gispen et al., 1985a).

Ultrastructural immunolocalization in isolated rat brain growth cones reveals B-50 to be predominantly associated with plasma membranes and, to a lesser degree, intracellular vesicles (Fig. 2B; Van Lookeren-Campagne et al., 1988). Since virtually all growth cones are labeled in this ultralocalization study, the presence of B-50 may be a general property that is common to all developing neurons of the central nervous system. For comparison, immunogold labeling revealed synaptosomes containing B-50, as well as unlabeled synaptosomes of adult brain. Perhaps this represents a certain degree of plasticity that is conserved throughout adulthood in some, but not all neurons (Van Lookeren-Campagne et al., 1988). Ultralocalization of B-50 in PC12 cells shows the protein to be mainly associated with organelles of the lysosomal family in proliferating chromaffin-like cells, whereas the plasma membrane is virtually free of B-50. In contrast, during NGF-induced differentiation into sympathetic neuron-like cells, B-50 becomes associated with PC12 cell plasma membranes. B-50 is most pronounced at the thinnest, distal protruding regions of the growth cone's plasma membrane in differentiating PC12 cells. Moreover, several other treatments that induce neuritogenesis in PC12 cells, by mechanisms different from NGF, are all accompanied with a similar redistribution of the protein. Therefore, we conclude that the expression of B-50 at the membrane appears to be

correlated with morphological differentiation (Van Hooff et al., 1988b).

B-50 immunoreactivity is highest in growth cones and much lower in neurites of cultured dorsal root ganglion cells. This typical distribution is abolished when axonal transport is inhibited by colchicine, so that B-50 immunoreactivity becomes evenly distributed between neurites and growth cones (Schmidt-Michels et al., 1988). Thus, the proximodistal gradient of B-50 appears to be built up by fast axonal transport. Preliminary studies of Meiri and Gordon-Weeks (1987) describe that, in growth cones isolated from fetal and neonatal rat brain, B-50 is detectable in cytoskeleton-associated membranes, but not in a pure cytoskeleton preparation of the growth cone. From these correlative and immunolocalization studies, B-50 appears to be transported along the cytoskeleton toward the growth cone, where it somehow becomes associated with the plasma membrane. They further suggest that the function of B-50 is associated with events in the distal nerve end, the growth cone, or its mature counterpart, the presynaptic terminal.

B-50 and Protein Kinase C

In isolated nerve growth cones, B-50 is present as a major substrate for endogenous calcium/phospholipid-dependent PKC (De Graan et al., 1985; Katz et al., 1985; Van Hooff et al., 1988a,c). PKC is concentrated in differentiating, neuropile-rich regions and nerve fibers of developing rat brain (Murphy et al., 1983; Girard et al., 1985), whereas in adult rat brain, the kinase is closely associated with presynaptic terminals (Wood et al., 1986; Girard et al., 1986). A very similar localization has been described for B-50 (Oestreicher et al., 1981; Oestreicher and Gispen, 1986; Gispen et al., 1985a; Benowitz et al., 1988). Like B-50 (Zwiers et al., 1987), the kinase C system develops during prenatal (Burgess et al., 1986), or perinatal (Hashimoto et al., 1988) development of rat brain. The colocalization and copurification of B-50 with its kinase through several steps (Zwiers et al., 1980; Aloyo et al., 1983), suggest that PKC phosphorylation of the protein is very important for its function (*see* below).

Signal Transduction

The well-known second messenger systems: calcium, cAMP, and PPI are all shown to be operative in nerve growth cones (Kater et al., 1988; Lockerbie et al., 1988; Garofalo and Pfenninger, 1986; Van Hooff et al., 1988a,c). We now try to summarize how they can mediate growth cone behavior. It is worthwhile to consider that this diagram for transmembrane signaling at the growth cone is certainly not complete. The addition of arachidonic acid and its metabolites, other phosphorylated inositol derivatives, phosphatase activity, and more cross-talk arrows between the cAMP, the PPI, the calmodulin, and calcium pathways (*see* e.g., Yoshima et al., 1987) will undoubtedly lead to further completion and complication of the diagram as drawn below.

PPI

The activation of certain receptors, coupled to phospholipase C (PLC), stimulate phosphatidylinositol 4,5-bisphosphate (PIP_2) breakdown, thus raising intracellular levels of the second messengers inositol trisphosphate (IP_3) and diacylglycerol (Berridge and Irvine, 1984). IP_3 mobilizes calcium from intracellular stores that, in concert with diacylglycerol, translocates PKC to the plasma membrane and stimulates PKC activity. We have recently shown that stimulation of muscarinic receptors on isolated growth cones is accompanied by a dose-dependent increase in B-50 phosphorylation (Van Hooff et al., 1988c). Since muscarinic receptors are coupled to an IP_3 response in neonatal rat brain (Heacock et al., 1987) and in the growth cone's adult counterparts, synaptosomes (Audigier et al., 1988), a causal relationship between the PPI response and B-50 phosphorylation is presumed. It is not clear what component of the muscarinic-induced cellular message, DG, IP_3-mediated intracellular calcium mobilization or extracellular calcium influx, stimulates B-50

phosphorylation. DG mediation is likely since diacylglycerol analogs, such as phorbol dibutyrate and dioctanoyl glycerol, increase B-50 phosphorylation in intact (Van Hooff et al., 1988b) and permeabilized (Hyman and Pfenninger, 1987) growth cones. The higher degree of B-50 phosphorylation appears to be mediated through stimulation of the endogenous kinase, PKC, although a (concurring) effect on a B-50 phosphatase cannot be excluded. Furthermore, K^+-depolarization enhances B-50 phosphorylation in intact nerve growth cones (Van Hooff et al., 1988c) similar to intact adult brain synaptosomes (Dekker et al., 1988). In the latter preparation, this effect is shown to be abolished in the absence of extracellular calcium and mimicked by the calcium ionophore, A23187. Thus, a rise of intracellular calcium in the growth cone, upon depolarization or muscarinic receptor activation, may mediate the stimulation of B-50 phosphorylation.

In search for a role of PKC in neurite outgrowth, phorbol diesters and membrane-permeable diaylglycerol analogs have shown to be valuable tools to stimulate PKC activity in vivo and in vitro (Castagna et al., 1982). Local phorbol diester application to growth cones of cultured *Helisoma* and hippocampal pyramidal neurons results in growth arrest and decreased growth cone motility within hours (Mattson, 1988). Long-term phorbol diester exposure of neurons in culture for several d, however, stimulates neurite outgrowth (Hsu, 1985; Montz et al., 1985; Honegger et al., 1986). This stimulation may be secondary to downregulation of the PKC content owing to long-term phorbol diester treatment (Matthies et al., 1987). In apparent contrast is the observation that PKC activation appears to be an essential step in NGF-induced outgrowth in PC12 cells (Hama et al., 1986; Hall et al., 1988). Therefore, the inhibition of NGF-induced outgrowth by simultaneous phorbol diester treatment (Ishii, 1978) may reflect the importance of PKC activation for NGF-induced outgrowth.

Studies on adult rat brain membranes have led to the proposal of a feedback role for B-50 phosphorylation on the PPI cascade (Gispen et al., 1985b). Phosphorylated B-50 (but not dephospho-B-50) may by inhibition of phosphatidylinositol 4-phosphate (PIP)-kinase deplete the PIP_2 pool. Thus, the degree of phosphorylation of B-50 is thought to modulate the availability of PIP_2 for PLC-stimulated breakdown. Extrapolation of these studies to the growth cone seems legitimate since the IP_3-signaling pathway is present in isolated growth cones (Garofalo and Pfenninger, 1986) and a comparable inverse relationship between PKC phosphorylation of B-50 and PIP_2 formation exists in growth cone membranes (Van Hooff et al., 1988a). Interestingly, PKC appears to play a dual role in muscarinic receptor signal transduction. Although phorbol diesters mimic muscarinic-like activity, they also promote desensitization of the receptor (El-Fakahany et al., 1988). This latter function may be served by B-50 phosphorylation that, by inhibiting PIP-kinase, depletes the PIP_2 pool for further PLC-mediated breakdown. The hypothesis that B-50 phosphorylation dampens the IP_3 response lends further support from studies in which a feedback inhibition on the PPI metabolism is found to be mediated through PKC activation (Labarca et al., 1984; Vicentini et al., 1985; Jope et al., 1987).

cAMP

A rise in the intracellular cAMP-level either promotes (Schubert et al., 1978; Nirenberg et al., 1984; Rydel and Greene, 1988) or inhibits (Lankford et al., 1988; Mattson et al., 1988a) neurite outgrowth in different species. Pharmacological interference with cAMP levels markedly changes growth cone morphology and motility (Gunderson and Barrett, 1980; Forscher et al., 1987; Mattson et al., 1988a; Lankford et al., 1988), suggesting a local influence on the growth cone. Indeed, cAMP-binding proteins, cAMP-dependent phosphorylation (Ellis et al.,1985), and receptor coupling to adenylate cyclase activation (Lockerbie et al, 1988) have all been found in isolated nerve growth cones. Thus, a complete cAMP messenger system exists in the

growth cone. The cAMP effect on some growth cones is independent of extracellular calcium, whereas on others it is mediated by cAMP-induced calcium influx (Forscher et al., 1987; Mattson et al., 1988a). This differentiation between subsets of neurons emphasize a differential coupling to cellular elements in different growth cones. In *Aplysia* bag cell neurons, agents that elevate intracellular cAMP levels not only decrease growth cone motility, but also promote organelle transport from the center of a growth cone into its peripheral extensions (Forscher et al., 1987), a region that normally remains free of organelles. In contrast to calmodulin-mediated phosphorylation, cAMP-induced phosphorylation is believed to enhance the polymerization of microtubules (Manalan and Klee, 1984), along which the export of organelles into the extensions may be guided. Moreover, long-term exposure of neuronal cell lines to cAMP-analogs promote neurite extension and the formation of synaptic contacts (Nirenberg et al., 1983). cAMP-stimulated processes may mediate the reorganization of growth cone organelles, and, thereby, the transition of a growth cone into a presynaptic terminal.

Calcium

There is considerable evidence that calcium plays central role in the transduction chain of many signals also in the growth cone. Calcium ionophores mimic and calcium channel blockers abolish the effects of many neurite growth modulating factors (Kater et al., 1988 and refs. therein). Active neurite growth is associated with high intracellular-free calcium levels in growth cones (Connor, 1986; Cohan et al., 1987). However, increases, as well as decreases, in calcium levels have been associated with growth cone arrest. Kater et al. (1988) postulated that each growth cone has its own "calcium set point," with a narrow optimal calcium concentration range. Optimal calcium levels for growth cone motility and neurite elongation appear not to be the same (Cohan et al., 1987; Mattson and Kater, 1987). This illustrates once more that they are different components of outgrowth (*see* Growth Cone Structure and Movement section). It is conceivable that tubulin association, actin-based movement, and vesicle fusion for membrane expansion have different calcium requirements.

A proposal of how calcium is mobilized and regulated in a growth cone, including a suggestion of how increases in calcium levels may lead to growth cone movement, is depicted in Fig. 3. Receptor activation or depolarization may stimulate calcium entry through receptor-activated and voltage-sensitive calcium channels, respectively (Inoue and Kenimer, 1988), hereby increasing the cytosolic free calcium levels in the growth cone (Anglister et al., 1982; Bolsover and Spector, 1986; Cohan et al., 1987). In addition, caffeine-sensitive intracellular calcium stores are present in growth cones. Such stores contribute, for example, to neurotransmitter release (Lockerbie and Gordon-Weeks, 1986) and organelle transport (Koenig et al., 1985). Receptor-induced IP_3 generation, shown to occur in isolated fetal brain growth cones (Garofalo and Pfenninger, 1986), may represent the physiological trigger to mobilize calcium from these stores. Increased calcium levels in the growth cone may directly or indirectly contribute to growth cone motility by promotion of actomyosin interaction, stimulation of membrane expansion (Goldberg, 1987) through vesicle fusion, and dissociation of cytoskeletal elements before reorganization. Following a rise in cytosolic-free calcium, several calcium-buffering systems come into play to modulate this signal. Such a modulation of the calcium signal is important: first, to differentiate the biological response and second, to control the calcium level to prevent calcium-activated protease damage (Nixon, 1986). These functions are performed by, e.g., calcium-dependent phosphorylation, calcium binding proteins, ATP-dependent reuptake into intracellular stores and calcium extrusion (e.g., McBurney and Neering, 1987).

Calmodulin

Many of calcium's effects are mediated through binding to calmodulin (Cheung, 1986). Calmodulin (CaM) is a ubiquitous 17 kD protein that binds calcium to its four binding sites in the

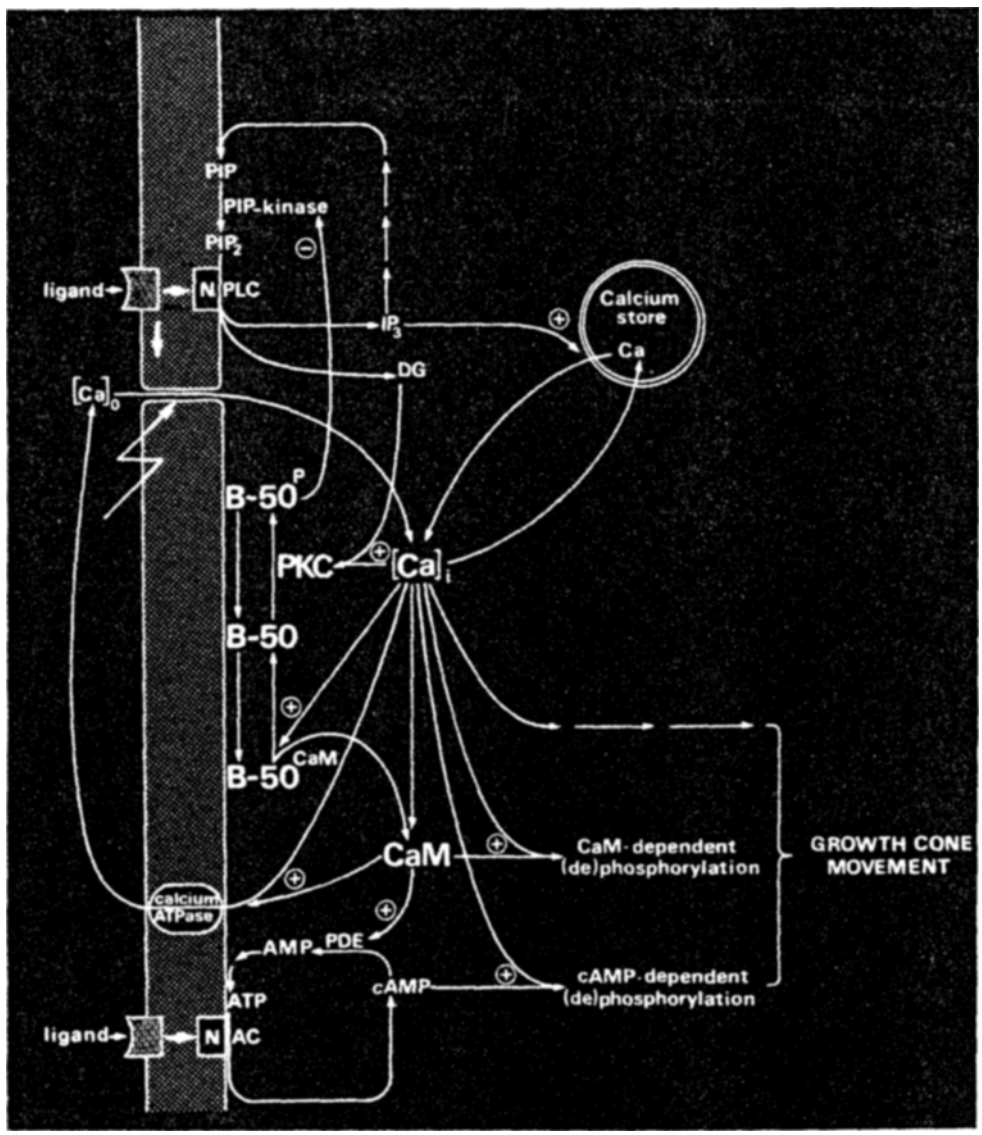

Fig. 3. Diagram of the hypothetical implication of B-50 phosphorylation in the regulation of second messenger responses in the growth cone. Intracellular free calcium ($[Ca]_i$) can be elevated through receptor stimulation or depolarization. Upon this calcium influx, calmodulin (CaM) is released from B-50. Freed CaM then binds calcium and cooperatively stimulates CaM-dependent protein kinase and phosphatase activity. APTase mediated extrusion of calcium and regulates cyclic nucleotide levels, e.g., by inhibiting phosphodiesterase (PDE). Receptor activation is coupled via *N*-proteins (*N*) to the activation of phospholipase C (PLC) to generate inositol trisphosphate (IP_3) and diacylglycerol (DG). IP_3 mobilizes calcium from intracellular stores, whereas DG stimulates PKC phosphorylation of B-50, which in turn, inhibits phosphatidylinositol 4-phosphate (PIP)–kinase. Other receptors are coupled to the activation of adenylate cyclase (AC) to increase cAMP levels that mediate cAMP-dependent phosphorylation or indirectly cause dephosphorylation. For further explanation, *see* text.

presence of physiological Mg^{2+} concentrations. With respect to calcium buffering, calmodulin binds calcium to decrease cytosolic-free calcium concentration and cooperates with calcium itself to increase calcium-ATPase mediated calcium extrusion (Manalan and Klee, 1984). Though cytosolic calcium buffering may be dominated by much stronger calcium-binding proteins, such as calbindin and paravalbumin, rather than by calmodulin (McBurney and Neering, 1987). In isolated growth cones, CaM and a family of CaM-binding proteins are present, most of which appear to have equivalents in adult synaptosomes (Hyman and Pfenninger, 1985). CaM represents an important modulatory factor in many cellular processes, primarily by stimulating CaM-dependent protein phosphorylation and CaM-dependent protein phosphatase activity and regulating the levels of cyclic nucleotides (Manalan and Klee, 1984 and refs. therein). CaM is involved in the regulation of growth cone motility, as illustrated by tissue culture experiments, in which the CaM antagonist trifluoperazine is shown to inhibit lamellipodium formation (DeGeorge et al., 1985; Goldberg, 1987).

In the mediation of biological effects, calmodulin binds to and stimulates CaM-depenent phosphorylation of several cytoskeletal elements, hereby changing their cohesion. For example, phosphorylation of microtubule-associated proteins or tau-factor decreases their microtubule stabilizing properties (Yamamoto et al., 1988). CaM-binding and -phosphorylation of fodrin, a spectrin-like component of the growth cones cytoskeleton, may influence its actin anchoring and crosslinking properties (Baitinger et al., 1983; Manalan and Klee, 1984; Koenig et al., 1985). Furthermore, by its calcium trapping power, CaM may (locally) decrease the free cytosolic calcium level, hereby favoring the assembly of microtubules. Moreover, phosphorylation of synapsin I by CaM-dependent kinase is shown to occur in growth cones (Pfenninger et al., 1986). This phosphorylation is believed to facilitate vesicle fusion for neurotransmission in mature nerve endings (Llinas et al., 1985; Schiebler et al., 1986; Baines, 1987) and may analogously contribute to vesicle fusion for growth cone expansion. In conclusion, membrane addition and a network of cytoskeletal elements, used for motility (actin) and elongation (tubulin), appear to be under control of CaM and calcium. If indeed its action in the growth cone is so versatile, then what regulates CaM? The recent identification of B-50 with the CaM-binding, neuron-specific protein P-57 (Cimler et al., 1987) invites the proposition of B-50 for such a role (Fig. 3). It has been shown that P-57 binds CaM in the absence of calcium, but releases CaM under high calcium conditions in vitro (Andreassen et al., 1983). This discriminates B-50 from all known CaM-binding proteins, for which CaM-binding is stimulated by calcium. Extrapolating these findings to the in vivo situation, B-50 could act as a local CaM-concentrator at the plasma membrane of the resting cell near the site of calcium entry. Upon stimulus-induced calcium influx, CaM will then rapidly be mobilized by this signal to perform its modulating functions. Concentration of CaM on B-50, together with a local increase in calcium, would then contribute to focal changes in cytoskeletal organization. To prolong the CaM-activity beyond a calcium transient, phosphorylation of B-50 by PKC may delay reassociation of CaM with B-50 (Alexander et al., 1987). Interestingly, L. H. Schrama (personal communication) found that CaM-stimulated phosphatase calcineurin, present at high concentrations in synaptosomes (Anthony et al., 1988), can dephosphorylate B-50 in vitro. Moreover, CaM inhibits PKC-mediated phosphorylation of several substrates (Albert et al., 1984), including B-50 (Chan et al., 1986). Taken together, when cytosolic CaM levels follow a calcium increase, CaM may subsequently stimulate the conversion of B-50 into the dephosphoform to perform its putative CaM-storage function. Such CaM-induced dephos-

phorylation of B-50 eventually may explain the transient increase in B-50 phosphorylation (1 min) during persistent K^+-depolarization (5 min). However, during prolonged receptor activation (5 min carbachol) in isolated intact nerve growth cones, the stimulus for B-50 phosphorylation appears to be stronger than a possible inhibition by CaM (Van Hooff et al., 1988c).

This hypothetical calcium/CaM regulatory function of B-50 may provide a clue to its growth-associated expression. First, B-50 is concentrated predominantly at actively sprouting regions of the plasma membrane (Van Hooff et al., 1988b), where a critical and fast calcium/CaM regulation is vitally important for effective growth cone motility and neurite extension. Second, B-50 may serve a feedback function during persistent growth cone activation. The sustained presence of B-50 in its phospho-form may prevent CaM reassociation with B-50 to guarantee maximal calcium buffering and desensitize receptor activation through depletion of the PIP_2-pool by inhibition of the PIP-kinase.

Conclusion

Biochemical manipulation and characterization of intact growth cones, isolated in great quantities, rapidly expand our knowledge about the signal transduction systems that effectuate growth cone motility and extension. It turns out that the growth cone displays many similarities with its adult counterpart, the synaptic terminal. Differences between growth cones and synaptosomes appear to be merely quantitative, rather than qualitative. One such difference is the content of B-50. The simultaneous presentation to the growth cone of several signals of strongly diverging nature and the high degree of plasticity of its behavior, requires strictly controlled transduction-effector mechanisms. We propose that the prominence of B-50 in growth cones may reflect its participation in the very dynamic biochemical activity of this organelle.

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