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Molecular mechanisms of neurite extension

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The extension of neurites is a major task of developing neurons, requiring a significant metabolic effort to sustain the increase in molecular synthesis necessary for plasma membrane expansion. In addition, neurite extension involves changes in the subsets of expressed proteins and reorganization of the cytomatrix. These phenomena are driven by environmental cues which activate signal transduction processes as well as by the intrinsic genetic program of the cell. The present review summarizes some of the most recent progress made in the elucidation of the molecular mechanisms underlying these processes.

Keywords: neuronal development; membrane trafficking; neuronal polarity; membrane fusion

1. INTRODUCTION

Neuronal differentiation is a complex neurobiological phenomenon that includes a series of events that are developmentally regulated. The first step in differentiation is a block of proliferation, leading from a round, cycling neuroblast to a flat, quiescent neuron. This is followed by the process of extension of neurites that represents the starting point for the morphological and functional polarization of the cell. Indeed, mature neurons are among the most highly polarized and compartmentalized of all cells.

As neurites start to grow, they are faced with the tremendous task of establishing the appropriate pattern of connectivity. Following axon guidance and the specific fasciculation of processes travelling along the same pathways, the subsequent step is target selection, associated with field invasion, mapping and cell choice, with each neuron ultimately forming the appropriate synaptic architecture with its own specific target(s).

In this review, we will focus our attention on the initial stages of the process, leading from a round neuroblast to a polarized neuron. Neurite extension and the molecular polarization of the cell, accompanied by the establishment of distinct somatodendritic and axonal compartments, are indeed crucial to every aspect of neuronal function, and represent absolute prerequisites for the directionality of the flow of information in the nervous system.

2. SIGNALLING UNDERLYING PROCESS FORMATION: OUTSIDE-IN MECHANISMS

In the context of the developing embryo, extracellular guiding cues, cell–cell interactions and soluble factors create a network of interactions that, in a complex interplay with the genetic programme of the cell, are responsible for neurite outgrowth, polarization, axon guidance to and from choice points, axonal fasciculation and target selection.

The common theme emerging from recent work has been the demonstration of the importance of a balance between positive and negative inputs, producing growth, collapse, attraction or repulsion of the growth cone. It is the integration of all these signals that determines the trajectory of the growing processes and allows a fine-tuning of the directional responses through a continuous sampling of the environment. In addition, the dynamic regulation of the receptors for the extracellular cues contributes to extend the repertoire of possible responses of single growth cones (Stoeckli & Landmesser 1998).

Among the extracellular cues, netrins have been shown to function as midline chemoattractants, members of the semaphorin or collapsin family have been implicated in axon guidance, target recognition and patterning, and the ephrins–Eph receptor system has been shown to play a role in contact repulsion. On the other hand, later-navigating axons may be guided by selective fasciculation on pioneer axons. Fasciculation and defasciculation are controlled by adhesion mechanisms and by tyrosine phosphorylation processes. Indeed, while cell and substrate adhesion molecules of different classes act primarily at stabilizing appropriate cell–cell contacts, receptor tyrosine kinases and phosphatases may dictate the specificity of axonal fasciculation and modulate the process. Since these topics have been the subject of excellent recent reviews (Cook *et al.* 1998; Holland *et al.* 1998; Van Vactor 1998), they will not be addressed here.

Neurite outgrowth is also strongly influenced by signals coming from the extracellular matrix through adhesion receptors, in a complex interplay between adhesion and signalling. In particular, phosphorylation processes have been implicated in adhesion molecule-stimulated neurite outgrowth. The non-receptor tyrosine kinases Src (Beggs *et al.* 1994) and Fyn (Ignelzi *et al.* 1994) have been demonstrated to be necessary for L1-dependent neurite outgrowth *in vitro* and for neural cell adhesion molecule (NCAM) function, respectively, while the $p90^{rsk}$ serine–threonine kinase is involved in neurite outgrowth

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promoted by L1 (Wong *et al.* 1996). In addition, the *in vitro* outgrowth-promoting activities of NCAM, L1 and N-cadherin require the activation of the tyrosine kinase activity of the fibroblast growth factor (FGF) receptor (Saffell *et al.* 1997) and, *in vivo*, dominant-negative FGF receptors inhibit axon extension and target entry of retinal ganglion cells (McFarlane *et al.* 1996). The above-mentioned regulatory interactions between growth factor signalling pathways and adhesion receptors involved in axon extension suggest that growth factors may actually trigger changes in adhesion, locally controlling recognition events at the growth cone (McFarlane & Holt 1997).

Together with cell and substrate adhesion molecules, secreted factors play a central role in neurite outgrowth, axon guidance and target selection. Particularly relevant is the function of the neurotrophin family of growth factors, which are involved in the regulation of many aspects of neuronal physiology (Holt & Harris 1998). These proteins play a role in a variety of processes: proliferation and survival of cell precursors, differentiation along specific lineages, programmed cell death, axonogenesis and branching, neurotransmitter metabolism, activity and efficacy of developing synapses, dendritic arborization and sprouting (Snider 1994). A remarkable finding that has emerged in recent years is that the early brain neuroepithelium is highly patterned before axonogenesis begins, and that the growth factors are among the molecules whose regionalized expression divides the brain into distinct domains (McFarlane & Holt 1997).

Nerve growth factor (NGF) has long been known as a potent promoter of neurite extension form several neuronal populations, both *in vitro* and *in vivo* (Levi-Montalcini 1987). The idea that this and other neurotrophins can also act as guidance cues has gained support by the finding that sympathetic fibres fail to innervate the pineal gland and the external ears in mice that lack neurotrophin-3 (NT-3), and fail to innervate the pancreas in transgenic mice ectopically expressing NGF. Both phenotypes can be rescued if a concentration difference between the target and the incoming axons is restored (ElShamy *et al.* 1996; Hoyle *et al.* 1993).

The responses induced by the neurotrophins can be altered by other simultaneous and convergent signals. In *Xenopus laevis* spinal neurons in culture, attractive turning of a growth cone triggered by a gradient of brain-derived neurotrophic factor (BDNF) can be switched to repulsive by altering the intracellular levels of cAMP, suggesting that the behaviour of a growth cone is strictly dependent on the context and involves the integration of multiple signals. While both BDNF and NT-3 act as chemoattractants for *Xenopus* spinal neurons, a reduction in extracellular Ca^{2+} abolishes the effect of the first but not of the second neurotrophin, suggesting that they use different second-messenger pathways (Song *et al.* 1997).

The existence of multiple extracellular signals, interconnected with each other and modulated at multiple levels, accounts for the extreme complexity and precision in the pattern of connectivity observed in the nervous system. Together with the three-dimensional regulation of the process, a fourth dimension—time—plays a crucial role in the building of the exact architecture of the nervous system, since the appropriate signals have to be present at critical stages of development. The same signalling molecules may in fact have opposing effects at different stages, depending on the subsets of receptors present on the neuron and on the concomitant presence of other signalling molecules. Thus, in most neurons of the central nervous system, axonal elongation is a phenomenon possible only during a restricted time-window in the course of development (Skene 1989). The existence of this time-dependence explains the extreme difficulty of correctly repairing damage in the adult brain.

On the other hand, the existence of multiple signals and levels of integration also gives rise to redundance of information, an important security factor to cope with possible mistakes of various origin (mutations, toxicity, etc.) that may occur in the course of neurogenesis. Redundance of information may account for the unexpectedly minor defects observed in the central nervous system of genetically altered mice lacking the expression of the neurotrophins or of their receptors (Snider 1994).

3. CYTOSKELETAL REMODELLING UNDERLYING PROCESS FORMATION

The major cytoskeletal components present in the peripheral cytoplasmic domain of the growth cone are filamentous actin (F-actin) and its associated molecules. Motility in the peripheral region is based on actomyosin dynamics: assembly of filaments at the leading edge; constant retrograde flow of the F-actin network; and proximal recycling of F-actin in the region where it overlaps with microtubules (Welch *et al.* 1997).

On the other hand, microtubules are the prominent components in the neurite shaft and the central domain of the growth cone. Microtubules provide structural support and act as substrates for the fast axonal transport of organelles. Pharmacological studies have revealed that axonal advance depends on the presence of dynamic microtubule ends that are regulated, at least in part, by microtubule-associated proteins (MAPs) (Rochlin *et al.* 1996). MAPs appear to be differentially distributed in the various neuronal compartments. Thus, MAP2 and tau are enriched in dendrites and axons, respectively, and strategies aimed at interfering with either protein induce specific effects on the *in vitro* outgrowth of the corresponding compartment. These results also suggest that the initial establishment of neurites depends on MAP2, whereas neurite elongation relies on tau and on microtubule stabilization (Caceres *et al.* 1992).

A number of *in vivo* and *in vitro* studies indicate that rapid rearrangements of the actin and microtubule cytoskeleton occur when growth cones respond to extracellular guidance cues. Microtubules reorientate and extend towards the interaction sites, and F-actin accumulates distal to the microtubule ends (Tanaka & Sabry 1995). Different models have been proposed to account for this microtubule extension, but in all cases development of tension in the actomyosin network, as a result of adhesive interactions with the underlying substrate, appears to be an essential element. According to the so-called 'clutch hypothesis', cell-surface receptors binding to target substrates establish functional linkages with the actin cytoskeleton, thereby stabilizing it and attenuating the retrograde flow of actin. The anchoring of the distal part

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of the actin network generates tension, promoting microtubule extension and protrusive growth of the leading edge (Suter & Forscher 1998).

The best-characterized system that contributes to the coupling between the extracellular space and the cytoskeleton is represented by the family of integrins, that have been mainly analysed in focal adhesion complexes of non-neuronal cells in culture. These complexes, linking extracellular matrix components to actin stress fibres, contain a large number of structural and regulatory proteins (Burridge & Chrzanowska-Wodnicka 1996). While 'classical' focal adhesions have not been clearly identified in neurons, it is reasonable to assume that analogous complexes exist in growth cones (Gomez *et al.* 1996). Indeed, focal adhesion kinase has been localized to growth cones in hippocampal cells developing in culture (Burgaya *et al.* 1995) and local inactivation of talin and vinculin in the neuronal growth cone results in cessation of filopodial extension and retraction, with bending and buckling of filopodia, respectively (Sydor *et al.* 1996). Furthermore, long-term reduction in the rate of neurite extension was observed in PC12 cells made vinculin deficient by means of antisense oligonucleotides (Varnum-Finney & Reichardt 1994).

Another family of adhesion receptors is that of the cadherins, which are linked to the actin cytoskeleton through the catenins. It has recently been shown that N-cadherin function is required for axonal and dendritic outgrowth from retinal ganglion cells (Riehl *et al.* 1996).

Besides mechanically linking the cell to the extracellular matrix, adhesion molecules can be considered to act as true receptors, able to activate intracellular signal transduction cascades involving tyrosine phosphorylation reactions and the activity of GTPases (Parsons 1996; Suter & Forscher 1998). The activation of these cascades may in turn affect the cytoskeletal organization.

An interesting possibility is that cell-surface receptor signalling may affect cytoskeletal remodelling through polyphosphoinositides. Indeed, it has been shown that FGF receptor activation induced by several cell adhesion molecules stimulates PLCγ and leads to PIP_2 hydrolysis (Doherty & Walsh 1996). Since PIP_2 metabolites are well-known regulators of actin-binding proteins such as gelsolin and profilin (Janmey 1994), the latter process could well account for a modulation of actin dynamics. In addition, in the course of a genetic screen for defects in photoreceptor axon guidance in *Drosophila melanogaster*, an Src homology 2 and 3 (SH2/SH3)-containing adaptor protein has been identified which could provide an *in vivo* link between tyrosine phosphorylation and cytoskeletal regulatory proteins (Garrity *et al.* 1996). Finally, increasing evidence suggests that small GTP-binding proteins of the Rho family link extracellular guidance cues to the actin cytoskeleton in the growth cone (Luo *et al.* 1997).

It has recently been proposed that Cdc42 and Rac1 are involved in growth-cone remodelling by inducing filopodia and lamellipodia formation, respectively (Kozma *et al.* 1997), similar to what has previously been demonstrated to occur in fibroblasts (Nobes & Hall 1995), whereas RhoA may be involved in myosin contractility (Jalink *et al.* 1994; Gebbink *et al.* 1997). In addition, Rac1 has been implicated in collapsin-1-mediated growth cone collapse (Jin & Strittmatter 1997). Expression of a constitutively active form of Rac1 in Purkinje cells of transgenic mice induces a reduction in the number of presynaptic terminals and an increase in the number of dendritic spines (Luo *et al.* 1996), suggesting that the various neuronal compartments are differentially affected by the protein. A novel form of Rac1, specifically expressed in the nervous system, has recently been described (Malosio *et al.* 1997). Overexpression of this protein (called cRac1B) induces enhanced neuritogenesis and branching in primary neurons (Albertinazzi *et al.* 1998).

Recently, it has been demonstrated that all three GTPases play a role in the dendritic development of cortical neurons *in vitro*, since expression of dominant negative mutants leads to a reduction, while constitutively active proteins cause an increase in the number of dendrites (Threadgill *et al.* 1997). The future challenge in formulating hypotheses on the molecular mechanisms underlying growth-cone guidance and neurite extension will be to understand how these signalling pathways interact with each other to regulate the proteins involved in cytoskeletal remodelling.

4. MEMBRANE ADDITION TO GROWING NEURITES: MECHANISMS AND MOLECULES

The supply of newly synthesized membrane components necessary to sustain neurite growth is enormous. The average surface area of a neuronal cell body (10 μm in diameter) is approximately 300 μm^2. Since a typical rate of growth for an axon (1 μm in diameter) is approximately 20–50 $\mu m\ h^{-1}$, it follows that the neuronal surface area grows at 60–150 $\mu m^2\ h^{-1}$, i.e. one-quarter to one-half of the cell body area per hour is added to the growing neurites (Futerman & Banker 1996). The site of insertion of the new membrane is still controversial. Various models have been put forward, suggesting preferential insertion at the growth cone, at the region of the axon closest to the cell body or proposing that new membrane is intercalated all along the growing process. In any case, it appears clear that the vesicles to be inserted into the plasma membrane do not move freely in the cytoplasm, but rather travel along microtubules. Consistently, suppression of the expression (with antisense oligonucleotides) of microtubule-based motors appears to inhibit axonal elongation (Ferreira *et al.* 1992; Morfini *et al.* 1997), and depolymerization of microtubules by treatment with nocodazole impairs the delivery of proteins to both the axonal and the dendritic compartments (Cid-Arregui *et al.* 1995).

New membrane is delivered at the sites of insertion into the plasma membrane via vesicle carriers, whose nature and composition is still debated. In developing neurons, organelles bearing synaptic vesicle antigens undergo active exo-endocytosis, and this recycling is present in all developing processes, rather than being restricted to a specific compartment, as it occurs in mature neurons (Matteoli *et al.* 1992). Whether these organelles represent real synaptic vesicles or precursors thereof is, however, still unclear. Since synaptic vesicle antigens are normally not found on the plasma membrane, one must assume that either selective retrieval of vesicle antigens occurs, or that this exocytotic process does not significantly contribute to the permanent addition of new membrane to the

growing neurites. It has also been proposed that membrane addition is accomplished by fusion of large 'plasmalemma precursor vesicles' different from synaptic vesicles and belonging to the constitutive secretory pathway (Pfenninger & Friedman 1993). Studies with antisense oligonucleotides as well as with clostridial toxins suggest that insertion of the new membrane into the growing axon is accomplished by means of a fusion machine similar to that employed for the fusion of synaptic vesicles (Higgins *et al*. 1997).

The last few years have seen tremendous advances concerning the molecular mechanisms of synaptic vesicle fusion and have led to the development of a model believed to hold true for several (if not all) intracellular fusion events (for review, see Südhof 1995). According to the so-called SNARE hypothesis, a central role in the process is played by the compartment-specific coupling between proteins localized on the donor vesicular compartment (v-SNAREs) and the acceptor target compartment (t-SNAREs) (Söllner *et al*. 1993). These molecules are selectively cleaved by the clostridial neurotoxins that are hence responsible for the block of neurotransmitter release caused by the corresponding pathogens (Schiavo *et al*. 1995*a*).

Elimination of the t-SNAREs by treatment with botulinum neurotoxin type C, which cleaves syntaxins 1, 2, 3 (Schiavo *et al*. 1995*b*) and synaptosome-associated protein of 25 kDa (SNAP-25) (Williamson *et al*. 1996), induces rapid growth-cone collapse and impaired neurite outgrowth in chick dorsal root ganglion and retina explants (Igarashi *et al*. 1996), and inhibits neurite growth in cultured rat cortical neurons (Osen-Sand *et al*. 1996). These results suggest that the introduction of new membrane required for neurite formation comes from SNARE-dependent vesicle fusion processes. Indeed, abnormal vesicle accumulation could be evidenced in the neurotoxin-treated neurons. The total surface area of the accumulated vesicles roughly equalled the membrane expansion underlying normal neurite growth (Igarashi *et al*. 1996).

On the other hand, dendritic growth is apparently less affected by toxin cleavage of the t-SNAREs (Osen-Sand *et al*. 1996), suggesting the existence of alternative pathways. Furthermore, the observation that cleavage of the v-SNAREs, despite the efficient blockade of neurotransmitter release, produces appreciable effects on axon growth neither *in situ* (Sweeney *et al*. 1995) nor in culture (Osen-Sand *et al*. 1996; Ahnert-Hilger *et al*. 1996), confirms the existence of differences between the machinery for neuroexocytosis and that mediating membrane insertion during axonal growth.

The rat phaeochromocytoma cell line, PC12, is a well-characterized model for the study of both neurosecretion and neuron-like differentiation (Greene & Tischler 1976). Nerve growth factor (NGF) induces morphological and biochemical changes in PC12 cells, ultimately resulting in their differentiation into a sympathetic neuron-like cell. After the application of NGF, PC12 cells stop dividing, extend neurites and express a battery of neuronal genes, including components of the neuronal cytoskeleton, voltage-gated ion channels and neurotransmitter synthesizing enzymes.

We have performed experiments on a PC12 cell clone (Trk-Fck) previously transfected with the cDNA for the human form of the high-affinity receptor for NGF, TrkA (Hempstead *et al*. 1992). In these cells, TrkA is constitutively active, albeit at moderate levels, and therefore the cells undergo spontaneous differentiation and extend neurites. Neurite extension is strongly accelerated by the presence of NGF in the medium. These morphological changes are accompanied by changes in the expression pattern of cytoskeletal proteins. Thus, during neurite extension the microtubule-binding protein tau, which in neurons appears to be specifically localized to the axonal compartment, is strongly up-regulated and so is the growth-cone marker GAP-G3. In addition, in the cell clone investigated, both undifferentiated and NGF-treated cells are completely defective in the expression of proteins of the secretory apparatus, including proteins of synaptic vesicles, neurotransmitter transporters and neurotransmitter-synthesizing enzymes. The defect does not extend to the constitutive pathway of secretion, which operates in all cells, independently of their origin and function (Leoni *et al*. 1998). It appears therefore that these cells are capable of carrying out a normal developmental programme from the point of view of morphological differentiation and organization of the cytoskeletal apparatus, despite being completely defective from the point of view of neurotransmitter secretion.

The phenotype of Trk-PC12 cells does not depend merely on TrkA overexpression, since other clones stably transfected with TrkA appear normal from the point of view of the expression of the secretory machinery, independently of the levels of expression of the transfected receptor. Thus, such a phenotype most likely represents a clonal variation of the parental cells. Indeed, another variant PC12 cell clone, clone 27, which completely lacks the machinery for neurosecretion, has previously been described (Corradi *et al*. 1996), suggesting that the achievement of the secretory phenotype depends on the coordinate expression of a set of genes, as though a 'master switch' for regulated secretion existed. The prompt and potentiated response of Trk-PC12 cells to the differentiating action of NGF suggests that if a 'master switch' for neurosecretion does exist, it does not control other aspects of PC12 differentiation into neuron-like cells, i.e. neurite extension and cytoskeletal remodelling. In addition, our results raise the possibility that neurite extension can occur in the absence of the SNARE proteins which constitute the fusion machine for the discharge of neurosecretory vesicles (see below).

5. POLARIZED SORTING AND DEVELOPMENT OF AXONS AND DENDRITES

Neurons exhibit a high variety of different shapes and sizes. However, one feature all neurons share is that they are highly polarized cells in which several compartments can be recognized. In particular, neuritic processes can be classified in two kinds: dendrites and axons. In this respect, nearly all neurons are characterized by having one single axon and several dendrites. Axons and dendrites differ in their shape, molecular composition of plasma membrane and cytoskeletal apparatus, as well as in their function. Thus, axons are typically dedicated to the transport and transmission of signals, whereas dendrites are generally devoted to the reception and processing of information. In addition, axons and dendrites cannot be

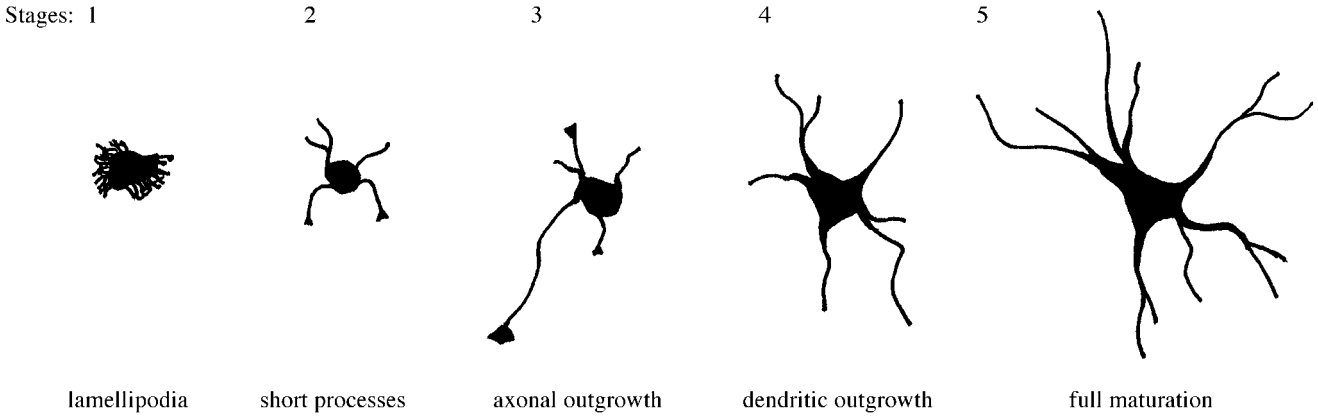

Figure 1. Stages of development of E18 hippocampal neurons in culture. Soon after adhering to the culture dish, freshly plated neurons first extend lamellipodia (stage 1), and soon thereafter establish several short, unpolarized processes (stage 2). After several hours, one of the processes begins to grow at a much higher rate, and becomes the axon (stage 3). A few days later, the other processes also start to elongate, becoming the dendrites (stage 4). By the end of the first week in culture, the neurons have reached full maturation and have established a synaptic network with surrounding cells (stage 5). (Modified from Dotti *et al*. (1988).)

considered as homogeneous compartments. The emergence of the axon from the cell body is characterized by the presence of an ill-defined compartment, called the axon hillock, which is thought to represent a barrier for the diffusion of proteins and lipids, with ensuing preservation of the differences in the molecular make-up of the plasma membrane of the soma and axon. In addition, a highly specialized compartment is found at the distal part of the axon—the nerve terminal—which is devoted to the transfer of information from one neuron to another by transforming the stereotyped electrical signal that travels along the axon into a graded chemical signal that is released into the extracellular space and reaches specific receptors in the postsynaptic neurons. In the case of axo-dendritic synapses, the specific receptors are often localized in specialized, distal compartments of the dendrites known as dendritic spines (Craig & Banker 1994).

In recent years the molecular mechanisms which lead to the establishment and maintainance of polarity in neurons have been subject of considerable interest. Studies carried out on neurons in culture indicate that the acquisition of polarity is a late process in the development of neurites. The phenomenon has been best characterized in cultures of embryonic hippocampal neurons. In this system, five stages in the development of the neuron have been identified (figure l; Dotti *et al*. 1988).

Initially, the cell is not polar. In fact, when a process is formed from the cell body (between stage 1 and stage 2), it is still multipotential and can evolve into either an axon or a dendrite. Polarity is acquired only later, between stages 2 and 3, through a process of membrane sorting, which becomes more efficient at later stages (Dotti & Simons 1990). Specifically, one of the processes starts to differentiate into an axon, and this appears to inhibit the ability of the other processes to evolve into axons, therefore driving them to become dendrites. However, the potential to become an axon persists for some time at least, since the experimentally induced excision of the developing axon leads to the redirection of one of the dendrites into an axon (Dotti & Banker 1987).

It is unclear how one process among others is chosen to become the axon. However, it has been suggested that bulk cytoplasmic flow plays a critical role in this choice. According to this hypothesis, the process which has a larger growth cone and a greater transport of membrane organelles starts to elongate more rapidly, well before the establishment of polarized sorting of molecular components (Bradke & Dotti 1997). Bulk cytoplasmic flow might provide the force necessary for extending the growing neurite, thus explaining the higher initial rate of elongation of the process bound to become the axon, and resembling the situation observed at the leading edge of migrating cells.

The establishment and maintenance of neuronal polarity implies the existence of mechanisms for the sorting and segregation of specific axonal and dendritic membrane proteins. The problem has been widely studied in epithelial cells, which are polarized in distinct apical and basolateral cell surface domains, segregated by tight junctions (Simons & Fuller 1985). This has led to the hypothesis that common sorting mechanisms might be shared by neurons, in spite of obvious differences in their functional and morphological features of polarization.

In the Madin–Darby canine kidney (MDCK) cell line, apical and basolateral sorting has been studied by infecting the cells with vesicular stomatitis virus (VSV) and influenza virus, and following the fate of the viral proteins (Rodriguez-Boulan & Sabatini 1978). The application of these studies to hippocampal cells has suggested that in neurons the segregation of proteins in the axonal and somato-dendritic compartment presents analogies with apical and basolateral targeting of proteins in epithelial cells, respectively. In fact, the VSV glycoprotein, which is sorted basolaterally in MDCK cells, is found on the somato-dendritic domain of fully mature infected neurons, whereas influenza haemagglutinin, which is sorted apically in epithelial cells, is targeted preferentially, but not exclusively, to the axon (Dotti & Simons 1990). These findings have been extended to the study of several other segregated proteins. The data obtained thus far confirm the suggestion of a parallel between basolateral and somato-dendritic sorting, whereas the analogy between apical and axonal sorting is likely not to be of general significance. In particular, it

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appears that the sorting information present in apical proteins is not sufficient to guarantee targeting of these proteins to the axon (Jareb & Banker 1998).

The apical and basolateral routes of epithelial cells have been suggested to differ not only in terms of the sorting machinery, but also in terms of mechanism of fusion of the exocytic vesicles with the plasma membrane. In MDCK cells, whereas the basolateral pathway requires SNAREs integrity as well as Rab protein function, the apical delivery of proteins seems to be SNARE- and Rab-independent, suggesting that fusion of apical exocytic vesicles occurs through alternative (and as yet poorly defined) mechanisms (Ikonen *et al*. 1995; Yoshimori *et al*. 1996).

Recently, a model has been put forward according to which protein cargos destined to the apical membrane are sorted in the trans-Golgi network in closely packed membrane microdomains (rafts) formed by the clustering of glycosphingolipids within the exoplasmic leaflet of the Golgi membrane (Simons & Ikonen 1997). Rafts are then transported in vesicles to the apical surface and their fusion with the plasma membrane possibly involves proteins of the annexin family (Fiedler *et al*. 1995).

It is unclear also whether in the case of neurons, apical and basolateral exocytic vesicles exploit differential fusion mechanisms. Recent results suggest that in fully polarized neurons at least a subset of axolemmal proteins are sorted by a mechanism which requires the formation of lipid (sphingolipid-cholesterol) rafts (Ledesma *et al*. 1998). However, experiments carried out with clostridial toxins which cleave v- or t-SNAREs indicate that, whereas v-SNAREs are apparently dispensable for neurite extension, cleavage of t-SNAREs impairs axonal growth (Osen-Sand *et al*. 1996). The integrity of the Rab cycle seems also to be required for neurites to extend, although this requirement differs in the various stages of neuronal development.

Depletion of Rab8, achieved by treatment of hippocampal neurons at stages 1 or 2 (i.e. with no molecularly distinguishable axons and dendrites) with antisense oligonucleotides impairs neurite outgrowth, possibly by interfering with the budding and/or transport of exocytic vesicles (Huber *et al*. 1995). It is noteworthy that in fully polarized neurons Rab8 is confined to the somatodendritic compartment, whereas in immature neurons it is ubiquitously distributed (Huber *et al*. 1993). It is therefore possible that, before polarization, neurites behave as dendrite-like processes and that axon formation requires a further specialization and/or a switch in the mechanisms of growth and sorting.

The application of antisense oligonucleotides to GDIα (a regulator of the Rab cycle) to hippocampal neurons in culture, when carried out at early stages of development, is also able to block neurite outgrowth almost completely. When the same treatment is performed on polarized neurons (after stage 3), retraction of the growing neurites is observed. Morphometric analysis performed after labelling with selective markers of the compartments indicates that withdrawal affects solely axons, inasmuch as dendrites appear to continue growing (D'Adamo *et al*. 1998). These results suggest that early neuritogenesis is totally dependent on Rab cycle integrity, whereas in polarized neurons only axonal outgrowth continues to require Rabs, and dendrites are probably not dependent on these proteins, possibly being more inherently stable. It is interesting to note that GDIα depletion is also effective in blocking neurite outgrowth in Trk-PC12 cells, which are devoid of both v- and t-SNAREs (Leoni *et al*. 1998). This result might suggest a possible dissociation between SNARE and Rab function.

We do not know which, among the more than 30 known Rab proteins (Novick & Zerial 1997), is responsible for the observed effects of GDIα depletion, since this protein (at least *in vitro*) appears to bind several, and possibly all, Rabs. Whereas the effect observed on immature, unpolarized neurons could be ascribed to an effect on Rab8 function, the effect observed on polarized neurons is unlikely to depend on Rab8 since, as already mentioned, Rab8 appears to be excluded from the axonal compartment. The only Rab protein which is known to be selectively enriched in the growing tips of axons is Rab3a. Depletion of Rab3a at early stages of development is not effective in inhibiting process formation (Huber *et al*. 1995). Whether the same occurs in more mature neurons has not yet been investigated.

Extrapolation of results obtained with cultured neurons to the *in vivo* situation requires great caution.

In fact, whereas neurons in culture are soaked in a homogeneous environment, in the brain it is conceivable that developing neurites are exposed to different sets (or at least to gradients) of environmental cues soon after their emergence from the cell body. It is therefore possible that the acquisition of polarization occurs much earlier *in vivo*. However, the fact that neurons can still polarize in a homogeneous *in vitro* environment indicates that the acquisition of polarity is part of an intrinsic developmental programme of the cell.

6. CONCLUSIONS

The past few years have witnessed a remarkable advancement in our knowledge of the mechanisms underlying the process of neurite outgrowth. However, to date such knowledge is still far from complete. In particular, most of the work carried out thus far concerns the description of the cellular events occurring during neurite formation and the identification of molecules which are necessary for the process to occur. What is still lacking is a conceptual framework of the process, correlating membrane trafficking phenomena with both changes in the organization of the cytoskeletal network and signal transduction processes. In particular, it will be important to elucidate the genetic mechanisms which direct the development of neurites and the establishment of their polarity.

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