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REVIEW

ELECTRICAL ACTIVITY, GROWTH CONE MOTILITY AND THE CYTOSKELETON

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Summary

The development of the nervous system takes place in two main steps: first an extensive preliminary network is formed and then it is pruned and trimmed to establish the final form. This refinement is achieved by mechanisms that include cell death, selective growth and loss of neurites and the stabilization and elimination of synapses. The focus of this review is on selective neurite retraction during development, with particular emphasis on the role of electrical activity.

In many developing vertebrate and invertebrate neurones, the frequency and duration of ongoing impulse activity determine the final arborizations and the pattern of connections. When impulse traffic is silenced, axons fail to retract branches that had grown to inappropriate destinations in the mammalian visual system, cerebellum and neuromuscular junctions. Similarly, in crustaceans, *Drosophila melanogaster* and leeches, refinements in axonal morphology during development are influenced by impulse activity. From experiments made in culture, it has been possible to mimic these events and to show a clear link between the density of voltage-activated calcium channels in a neurite and its retraction following stimulation. The distribution of these calcium channels in turn is determined by the substratum with which the neurites are in contact or by the formation of synapses. Several lines of evidence suggest that calcium entry into the growth cone leads to collapse by disruption of actin filaments. One candidate for coupling membrane events to neurite retraction is the microfilament-associated protein gelsolin which, in its calcium-activated state, severs actin filaments. Open questions that remain concern the differential effects of activity on dendrites and axons as well as the mechanisms by which the growth cone integrates information derived from stimuli in the cell and in the extracellular environment.

Key words: calcium, cell motility, neurite retraction, axonal retraction.

Electrical activity and the development of neuronal morphology *in vivo*

The first phase of neural development, the laying down of a preliminary network, is characterized by an initial overproduction of processes and synaptic connections. This phase is followed by a second stage of refinement and pruning, during which synapses are eliminated and neurites retract. Many signals are known that promote neurite outgrowth. In addition, evidence is accumulating that signals leading to cessation of growth and retraction of processes play an important role in defining morphology. Growth-inhibitory activity has been associated with components of the extracellular matrix, membrane-associated proteins and diffusible factors (Schwab *et al.* 1993). Electrical activity of the neurones themselves was one of the earliest factors shown to cause rearrangement of axonal arborizations (Wiesel and Hubel, 1963; LeVay *et al.* 1978; Shatz and Stryker, 1978; Fields and Nelson, 1992).

Mammalian visual system

Retinal ganglion cells

The role that electrical activity plays in the proper development of axonal arborizations and the specific spatial patterns of synaptic connections has been examined in the mammalian visual system (Shatz, 1990; Katz, 1993). Retinal ganglion cells provide signals to the lateral geniculate nucleus (LGN). The terminal arborizations of the retinal ganglion cells in the adult LGN are segregated into eye-specific layers (Minkowski, 1920; Hayhow, 1958; Hickey and Guillery, 1974; Bowling and Michael, 1980) and are arranged within each layer in a retinotopic order, reflecting the neighbour relationships of the ganglion cells in the retina (Hubel and Wiesel, 1961, 1972). Initial outgrowth of foetal retinal ganglion neurones does not reflect this orderly arrangement: the retinogeniculate terminals from the two eyes overlap and form active synapses on the same postsynaptic cell (Rakic, 1976, 1977; Shatz and Kirkwood, 1984). The eye-specific layers in the LGN emerge as axons from the two eyes gradually remodel by withdrawing branches from inappropriate territory and growing extensive terminal arborizations within appropriate areas (Rakic and Riley, 1983; Sretavan and Shatz, 1986). Remodeling of axonal arborizations requires spontaneous firing in the retina and the LGN. Thus, tetrodotoxin (TTX, which blocks Na^+-dependent action potentials) prevents the segregation of retinal terminals into eye-specific layers in the LGN and results in a major increase

in the total length of retinogeniculate axons (Shatz and Stryker, 1988; Sretavan *et al.* 1988). By contrast, the dendrites of the retinal ganglion cells acquire higher-order branches and many spines prenatally which are subsequently lost (Dann *et al.* 1988; Ramoa *et al.* 1988) even in the absence of neural activity (Wong *et al.* 1991).

Lateral geniculate neurones

Geniculocortical afferents terminate in layer IV of the visual cortical area 17 in alternating eye-specific bands, the ocular dominance columns (Hubel and Wiesel, 1972; LeVay and Gilbert, 1976; Shatz *et al.* 1977; Anderson *et al.* 1988). The terminal arborizations of mature geniculocortical afferents are arranged in discrete clusters of collaterals 400–600 μm in width, alternating with zones relatively free of innervation from that eye (Rakic, 1976, 1977; Ferster and LeVay, 1978; Humphrey *et al.* 1985*a*,*b*). During the initial phase of development, however, segregation of fibres derived from the two eyes is not yet evident and the terminal arborization is formed by long branches that sparsely innervate a wide region of area 17 (Rakic, 1976, 1977; Antonini and Stryker, 1993). These arborizations are then remodelled: the arborization density increases and the terminal arborizations develop short and confined collaterals and form distinct patches, coinciding with the segregation of left and right eye afferents. This remodelling is in part due to elimination of fibres projecting to inappropriate areas and in part to growth extending to the appropriate region (Antonini and Stryker, 1993). Neuronal activity of the afferent visual pathway plays an important role in this reshaping. Blockade by binocular injections of TTX did not change the complexity and extent of terminal arborizations, but perturbed the refinement of ocular dominance columns (Reiter *et al.* 1986; Stryker and Harris, 1986; Antonini and Stryker, 1993). As in retinal ganglion cells, growth of dendrites of LGN neurones is independent of neural activity, except for spine density which increases threefold in the presence of TTX (Dalva *et al.* 1994).

Cortical neurones

Pyramidal cells in layer 2/3 of adult cat striate cortex have long, horizontal axon collaterals in layers 2/3 and 5. These collaterals form periodic 'clusters' of finer axon branches that link columns of similar orientation selectivity (Gilbert and Wiesel, 1989). During the first three postnatal weeks, there is a steady increase in the number of long unbranched horizontal axon collaterals extending from the efferent axons of the pyramidal cells and arranged in crude clusters (Callaway and Katz, 1990). During the fourth postnatal week, the number of these collaterals decreases and axonal arborizations take on a more clustered appearance. This refinement is due mainly to the specific elimination of inappropriately projecting collaterals, but selective axon outgrowth may also occur. Cluster refinement depends on visual activity. Thus, binocular deprivation does not inhibit the maturation of horizontal axonal arborizations and development into crude clusters, but the specificity of axon rearrangements by elimination of incorrectly projecting collaterals and perhaps addition of axons to correct columns is prevented (Callaway and Katz, 1991). In strabismic kittens, in which images on the two retinae cannot be brought into register, tangential collaterals preferentially connect 'orientation clusters' driven by the same eye column, while in normal animals no such preference is obvious. These results show that the proper construction of horizontal connections requires not only visual activity *per se*, but correlations in the patterns of cellular activity (Löwel and Singer, 1992).

Cerebellum and corpus callosum

Another instance of axon elimination occurs in the cerebellum. The Purkinje cells of the newborn rat are innervated by several different climbing fibres, all but one of which are lost over the first few weeks of life (Delhaye-Bouchaud *et al.* 1975; Crepel *et al.* 1976; Mariani and Changeux, 1981). Mutations or treatments that affect afferent input to Purkinje cells result in persistence of multiple innervation by climbing fibres (Mariani, 1982, and references therein) and dramatic alterations in the morphology of Purkinje cell dendritic branching (Berry and Bradley, 1976; Privat and Drian, 1976; Mariani *et al.* 1977; Ito, 1984). Recently it has been shown that activation of cerebellar *N*-methyl-D-aspartate (NMDA) receptors plays a critical role in the elimination of climbing fibres (Rabacchi *et al.* 1992).

Restructuring of axonal arborizations is not restricted to the fine-tuning of neuronal circuits. In some situations, process elimination is responsible for the removal of entire neural pathways. The elimination of long collateral projections has been observed during the development of the corticospinal projections (Stanfield *et al.* 1982; Stanfield, 1992) and the corpus callosum (Innocenti, 1981; O'Leary *et al.* 1981; Ivy and Killackey, 1982). Changes in the level or patterning of visual activity to the developing cortex affect the pruning of callosal axons. Thus, neural activity seems to be necessary for the remodelling of callosal projections from the visual cortex (Lund *et al.* 1978; Innocenti and Frost, 1979, 1980; Rhoades and Dellacroce, 1980; Cusick and Lund, 1982; Berman and Payne, 1983; Cowan *et al.* 1984; Innocenti *et al.* 1985; Frost and Moy, 1989; Frost *et al.* 1990; O'Leary, 1992; Grigonis and Murphy, 1994).

Neuromuscular junction

The first evidence for local elimination of neuronal processes came from studies of the developing neuromuscular junction (Redfern, 1970; Brown *et al.* 1976; Van Essen, 1982). In the neonatal rat (and other mammals), skeletal muscle fibres are innervated by as many as five or six separate axons. During early postnatal life, all axons except one are eliminated, so that eventually every muscle fibre is contacted by only one motor axon (Redfern, 1970; Brown *et al.* 1976; Purves and Lichtman, 1980; Thompson, 1986; Goodman and Shatz, 1993). Axonal retraction (rather than degeneration) is the predominant mechanism by which excess neuromuscular synapses are

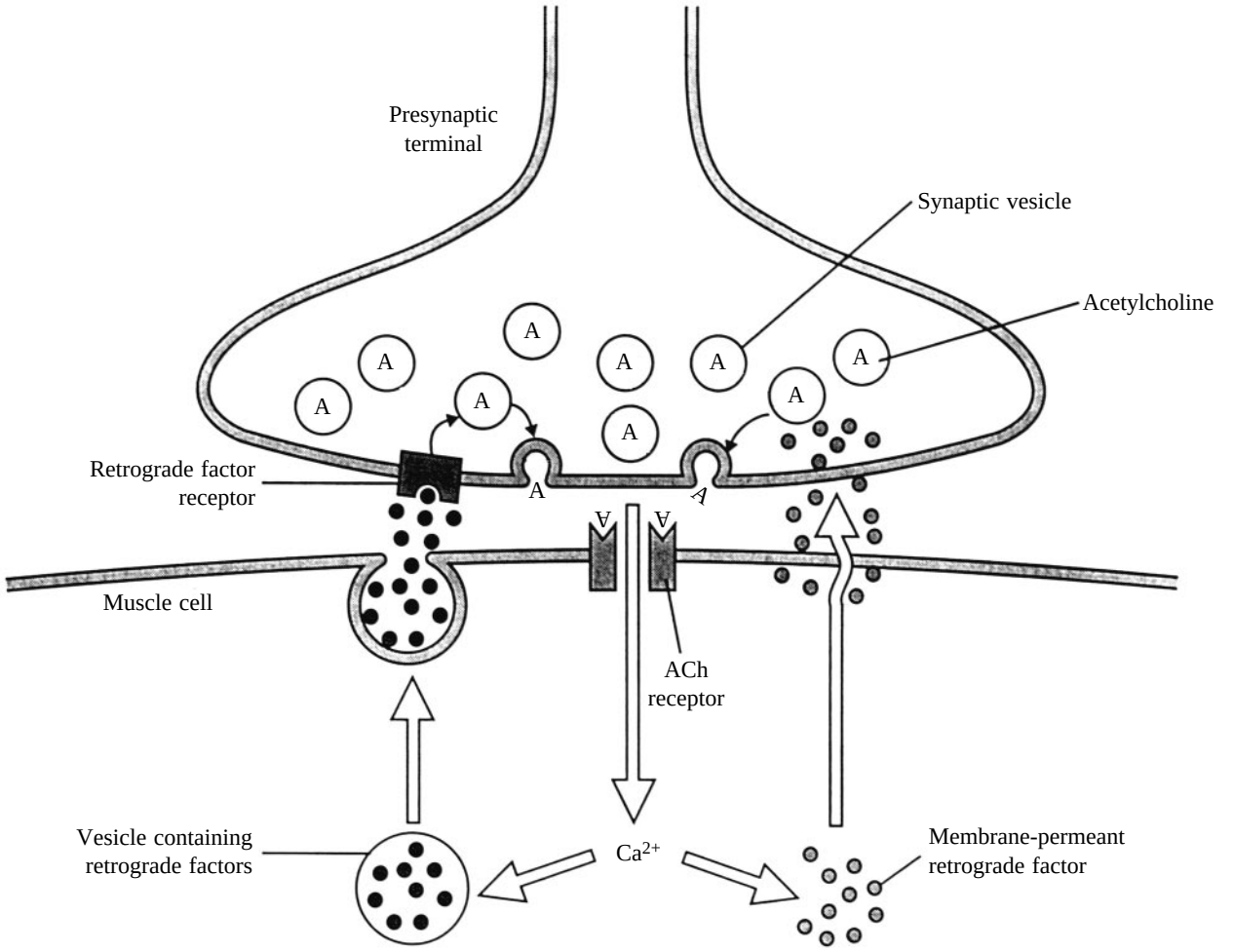


Fig. 1. Scheme for retrograde signalling at the developing neuromuscular junction. In the pathway on the right, calcium influx into the postsynaptic cell leads to the production of membrane-permeant retrograde factors. These factors diffuse from the postsynaptic to the presynaptic cell, where they modulate synaptic activity. Alternatively, according to the scheme on the left, a raised postsynaptic calcium concentration could stimulate the exocytosis of vesicles containing retrograde factors which then bind to retrograde factor receptors localized on the presynaptic membrane. The activation of these receptors eventually results in a change of synaptic activity (reproduced with permission, after Dan and Poo, 1994*a*). ACh, acetylcholine.

removed (Riley, 1977, 1981). This elimination of axonal branches is dependent on neural activity (O'Brien *et al.* 1978; Thompson, 1983). The process of synapse elimination is slowed by decreased neural activity (Benoit and Changeux, 1975; Riley, 1978; Thompson *et al.* 1979) and hastened by enhanced activity (O'Brien *et al.* 1978; Thompson, 1983).

Poo and his collaborators have shown in an elegant system that the elimination of synapses at the neuromuscular junction is a result of competitive interactions between different motoneurones innervating the same muscle fibre. A single embryonic muscle cell in culture was innervated by two spinal neurones. Stimulation of one neurone resulted in functional suppression of the synapse made by the other unstimulated neurone. When both neurones were stimulated concurrently, such synaptic suppression was not observed (Lo and Poo, 1991). The synaptic suppression was dependent on postsynaptic activity and a rise in postsynaptic cytosolic calcium level. It could be elicited by postsynaptic application of acetylcholine in the absence of synchronous presynaptic stimulation. Postsynaptic depolarization in the absence of acetylcholine elicited a reduced response. These results suggest that the activation of the postsynaptic acetylcholine receptors plays a dominant role in the suppression of a synapse at the neuromuscular junction (Dan and Poo, 1992; Lo and Poo, 1994; Lo *et al.* 1994). A transient retrograde messenger has been suggested that acts on the presynaptic terminal to weaken the synapse unless the presynaptic terminal itself has elevated calcium levels due to synchronous activity (Dan and Poo, 1992, 1994*a*,*b*) (Fig. 1). How synaptic suppression leads to axon retraction remains unclear. Loss of adhesion due to the redistribution of receptors away from the inactive synapse and towards the active synapse has been suggested as a first step in the process of axonal retraction (Poo and Young, 1990; Dan and Poo, 1994*a*).

Invertebrate nervous systems

Crayfish sensory neurones, lobster motoneurones and Drosophila *motoneurones*

The importance of electrical activity for the proper development of synaptic connections has been analyzed for regenerating mechanoreceptor neurones in the crayfish. After the activity of a group of mechanoreceptors known to innervate an identified interneurone is silenced by mechanically preventing the movement of the transducer hair, the target interneurone accepts innervation from inappropriate mechanoreceptors (Krasne, 1987). Nothing is as yet known about the effects of electrical activity on changes in the axonal morphology that might accompany or result from this synaptic competition.

In embryonic, larval and early juvenile lobsters, an identified motoneurone innervates the muscles of its own segment and the two adjacent segments. In late juvenile and adult animals, the same motoneurone innervates only the muscle of its own segment (Stephens and Govind, 1981). These motoneurones establish their target fields by selective elimination of functional synapses initially distributed in a wide peripheral field.

An elegant genetic approach suggests that electrical activity affects the growth and branching pattern of *Drosophila* motoneurones (Budnik *et al.* 1990). *Hyperexcitable Drosophila* mutants show a phenotype of anaesthesia-induced leg shaking and the different alleles have been linked to potassium channels (Ganetzky and Wu, 1986; Baumann *et al.* 1987; Kamb *et al.* 1987; Papazian *et al.* 1987; Tempel *et al.* 1987). In these mutants, higher-order axonal branches of motoneurones are more complex and the density of varicosities on the neurites is higher than in wild-type flies (Budnik *et al.* 1990). Temperature-sensitive mutations that affect sodium channels cause flies to become paralyzed within seconds after exposure to elevated temperatures (Ganetzky and Wu, 1986). In these *hypoexcitable* mutants, a slight decrease in axonal branching of motoneurones has been observed (Budnik *et al.* 1990). Interestingly, the morphology of sensory neurones in both types of mutants reveals no obvious abnormality (Burg and Wu, 1986, 1989).

Changes in the branching pattern of motoneurones were also observed in *Drosophila* mutants that have elevated cyclic AMP concentrations (Zhong *et al.* 1992). In these mutants, as in the *hyperexcitable* mutants, the numbers of terminal varicosities and higher-order motoneurone branches are increased. In double mutants that are *hyperexcitable* and have elevated cyclic AMP concentrations, these changes in axonal morphology are more pronounced than in flies with a single mutation (Zhong *et al.* 1992). These observations led to the hypothesis that enhanced neural activity in the *hyperexcitable* mutants increases calcium influx, stimulating the synthesis of cyclic AMP *via* calcium/calmodulin activation of adenylate cyclase. Cyclic AMP has been shown to affect the growth of neurites in culture (Lohof *et al.* 1992, and references therein).

Leech neurones

During the development of the leech nervous system, several identified neurones exhibit an initial overproduction, followed by a selective loss, of neurites (Kuwada and Kramer, 1983; Wallace, 1984; Gao and Macagno, 1987*a*,*b*; Wolszon and Macagno, 1992). The retraction of the neurites in developing (and regenerating) leech neurones follows one of two patterns. The first is a global retraction, whereby the growth cone that reaches its target seems to send a retrograde message causing the neurone to retract other projections (Jellies *et al.* 1987; Loer *et al.* 1987; Baptista and Macagno, 1988). The second type of retraction is localized so that the growth cone that reaches its target collapses and retracts, while the other neurites of the same cell are not affected (Gao and Macagno, 1987*a*,*b*). Recent observations on the local growth cone response of a neurone known as the anterior pagoda (AP) suggest that activity might play a role in neurite retraction. As AP neurones develop (there are two in each ganglion), they project two axons to the periphery and one axon each into the anterior and posterior connectives. The axons projecting into the connectives grow until they contact the growth cone of the AP cell from the adjacent ganglion; they then stop growing and retract. If one of the AP cells is ablated, the longitudinal projection of its neighbour is retained (Gao and Macagno, 1987*b*). Morphological and physiological evidence shows that the two axons are coupled by gap junctions through which 5-hydroxytryptamine (5-HT) might be transported between AP neurones of neighbouring ganglia (Wolszon *et al.* 1994). Gap junctions have also been implicated in the development of the vertebrate nervous system (Katz, 1993; Peinado *et al.* 1993).

In all the examples mentioned, neurite retraction is limited to a selected set of processes, while other neurites of the same neurone continue to grow. Why then are not all processes affected by depolarization and what is the nature of the mediator of electrical activity on growth cone motility?

Electrical activity and neurite outgrowth in culture

Effect of electrical activity on Purkinje cell development in culture

The Purkinje cell is one of the few neurones that has been analyzed both *in vivo* and *in vitro* with respect to the effect of electrical activity on its growth pattern. Electrophysiological, biochemical and morphological changes in cultured Purkinje cells mirror the *in vivo* developmental programme (Schilling *et al.* 1991). The emergence of electrical activity and dendritic branching coincide. Blockage of action potentials with TTX results in a dramatic change in dendritic morphology. Whereas control cells have a dense tree of branched dendrites with numerous protuberances (probably spines), silenced Purkinje cells have dendrites that are 3–4 times longer and exhibit little branching and no spines (Schilling *et al.* 1991). Axon morphology is not affected by changes in electrical activity. As electrical activity and dendritic branching appear, intracellular calcium levels become sensitive to changes in membrane potential. Calcium conductances in Purkinje cells are prominent in the dendritic tree and reduced in the axon (Llinas and Sugimori, 1979, 1980*a*,*b*; Tank *et al.* 1988; Ross *et al.*

1990). These observations suggest that calcium may be involved by acting as a second messenger that couples membrane events to changes in Purkinje cell dendritic development.

The role of calcium

Calcium currents have been localized on growing tips of regenerating neurites *in vivo* (Meiri *et al.* 1981) and growth cones of cultured neurones (Grinvald and Farber, 1981; Anglister *et al.* 1982); moreover, calcium channels are the first of the voltage-sensitive channels to be detected during the development of a variety of neurones (Spitzer and Baccaglini, 1976; Spitzer, 1979). The role calcium influx may play in activity-induced changes in neurite outgrowth has been investigated in several types of neurones grown in culture.

Depolarization-induced neurite retraction is mediated by a rise in intracellular calcium concentration

A phasic pattern of stimulation of dorsal root ganglion (DRG) neurones causes immediate growth cone collapse with retraction of filopodia and lamellipodia. This response is often followed by retraction of the neurite. Growth cone morphology and growth rate do not change, however, when the action potentials are blocked with TTX (Fields *et al.* 1990). Depolarization of DRG neurones with either a high extracellular potassium concentration ($45\,mmol\,l^{-1}$) or bradykinin also inhibits neurite outgrowth (Robson and Burgoyne, 1989). Electrical stimulation or depolarization with a high extracellular potassium concentration results in an elevation of intracellular calcium concentration. This increase in calcium concentration is due to a transmembrane flux of calcium ions through voltage-dependent L-type calcium channels. Blocking the calcium influx abolishes the growth-inhibitory effect of depolarization (Robson and Burgoyne, 1989; Fields *et al.* 1993). These experiments show that the inhibition of neurite outgrowth by depolarization is mediated by a rise in intracellular calcium concentration. DRG growth cones also collapse after exposure to a myelin-associated protein (NI-35). Interestingly, this response is due to release of calcium from intracellular stores (Bandtlow *et al.* 1993).

Environment and synapse formation affect calcium channel expression

The effect of environmental influences on the response of neurites to electrical activity was analyzed in identified cultured leech neurones. Neurite morphology is strongly influenced by the substrata on which leech neurones are plated. When grown on laminin, neurites are long and thin and rarely branch, whereas on the plant lectin Concanavalin A , they are broad, curly and have a complex branching pattern (Chiquet and Acklin, 1986; Chiquet and Nicholls, 1987; Grumbacher-Reinert, 1989). Leech neurones growing on laminin respond with growth cone collapse and neurite retraction to electrical stimulation or elevated extracellular potassium, a response that is dependent on calcium influx (Fig. 2). The same cells cultured on Concanavalin A continue to grow when they are stimulated or depolarized (Grumbacher-Reinert and Nicholls, 1992; Neely, 1993). Ross *et al.* (1988) have shown that voltage-dependent calcium channels are abundant in leech neurites growing on laminin, but scarce in processes of neurones growing on Concanavalin A. The hypothesis that these differences in calcium channels might be responsible for differences in response to depolarization is suggested by the finding that treatment of leech neurones growing on Concanavalin A with the calcium ionophore A23187 results in cessation of outgrowth (Neely and Gesemann, 1994). The environment with which the growth cone is in contact therefore influences the expression of calcium channels and the response of a neurite to neural activity.

The establishment of contacts between leech neurites prevents their retraction, while the free neurites on the same cell shorten following electrical stimulation (R. von Bernhardi, unpublished observations). Synapse formation has been shown to change calcium channel distribution in cultured leech neurones. After the formation of chemical synapses between two identified leech neurones, calcium currents at the cell stump (region of outgrowth) in the postsynaptic cell are reduced (Fernandez-de Miguel *et al.* 1992). This change is accompanied by reduced outgrowth in the postsynaptic cell when compared with single neurones (Cooper *et al.* 1992). Synapse formation can therefore alter calcium channel activity and neurite outgrowth.

Variety of growth cone responses to calcium

Whereas a rise in intracellular calcium concentration inhibits growth cone motility in leech neurones (Grumbacher-Reinert and Nicholls, 1992; Neely, 1993) and DRG neurones (Robson and Burgoyne, 1989; Fields *et al.* 1993), stimulation of growth occurs in neuroblastoma cells (Anglister *et al.* 1982; Bedlack *et al.* 1992), ciliary ganglion neurones (Nishi and Berg, 1981), neurones from the diencephalon (Connor, 1986), embryonic spinal neurones (Holliday and Spitzer, 1990) and embryonic retinal neurones (Suarez-Isla *et al.* 1984). Neurites of *Helisoma trivolvis* neurones stop growing when the intracellular calcium concentration is raised or reduced, suggesting that there is perhaps an optimal range of calcium concentration, above or below which neurite outgrowth is impaired (Cohan and Kater, 1986; Cohan *et al.* 1987; Mattson and Kater, 1987; Kater *et al.* 1988; Cohan, 1992).

In cultured sympathetic neurones, electrical stimulation increases the cytosolic calcium concentration, but has no effect on the rate of neurite outgrowth (Garyantes and Regehr, 1992). Voltage-sensitive calcium channels also do not seem to play a critical role for neuronal differentiation of PC12 cells. PC12 cells of a line devoid of such channels grow normal neurites after they are induced to differentiate (Usowicz *et al.* 1990).

We conclude that electrical activity and the concomitant calcium influx are not universal regulators of neurite outgrowth. Inherent properties of neurones, environmental influences (such as the substratum) and synapse formation, can all affect calcium currents and modify the response of a neurone to electrical activity.

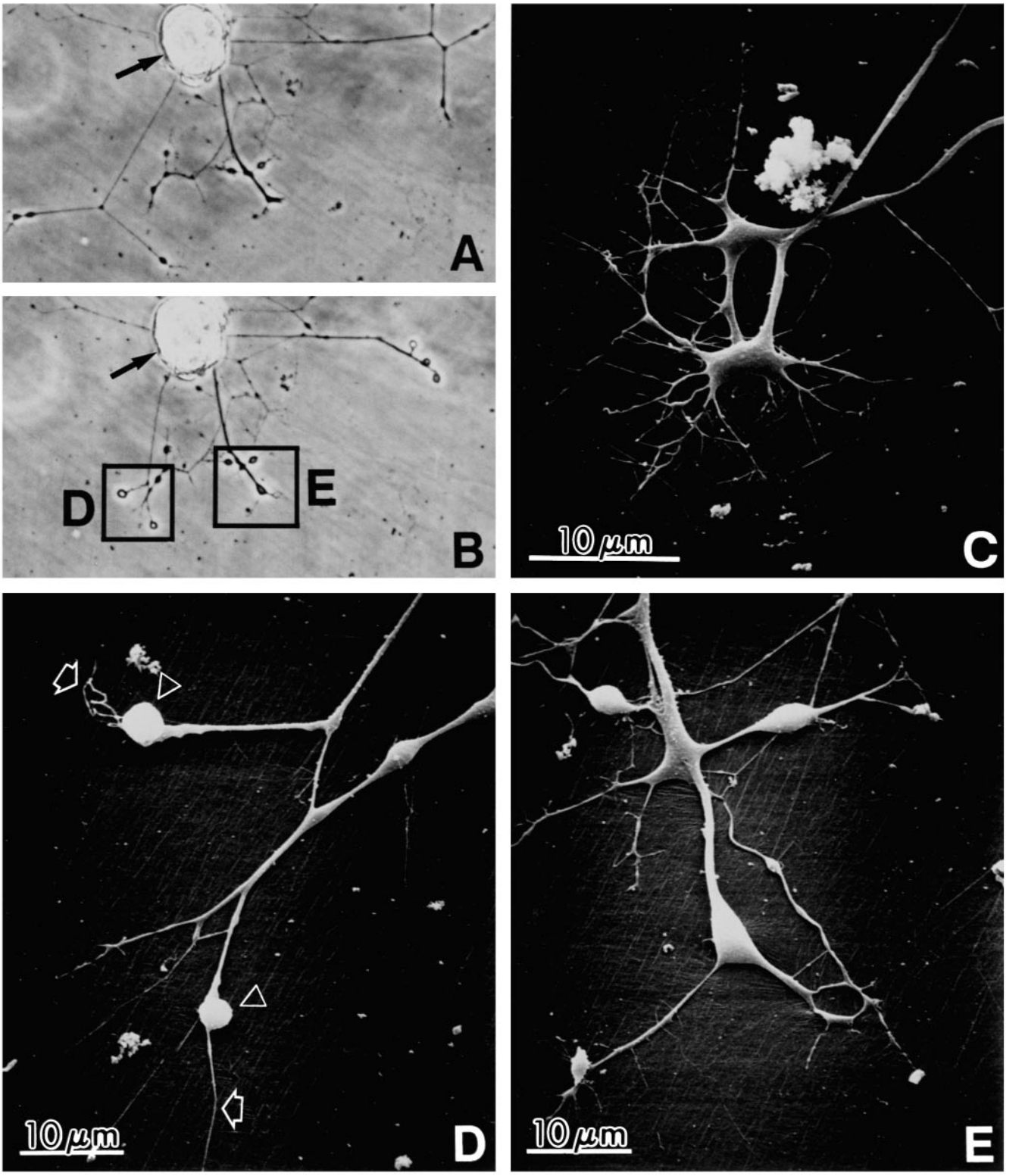


Fig. 2. Growth cones of leech neurones cultured on a laminin-enriched substratum collapse and neurites retract after depolarization. An anterior pagoda (AP) neurone in culture was photographed before (A) and after (B) 30 min of exposure to medium with elevated $[K^+]$ (60 mmol l^{-1}) (arrow points to cell body). This depolarization caused growth cone collapse (box E) and neurite retraction (box D). Scanning electron microscopy revealed retraction bulbs (arrowheads) and retraction fibres (arrows) on neurites that had retracted (D). Loss of filopodia and rounding up of lamellipodia are characteristic features of collapsed growth cones (E) (compare E with C, which shows a control growth cone). D and E are scanning electron micrographs of the boxed regions in B. The cell body in A and B has a diameter of 50 μm. (reproduced with permission, after Neely, 1993).

Calcium and the cytoskeleton of the growth cone

Cytoskeletal organization in the growth cone

Changes in growth cone morphology and motility are produced by changes in the organization of its cytoskeleton, which is composed of microtubules and microfilaments (Yamada *et al.* 1970, 1971; Luduena and Wessells, 1973; Letourneau *et al.* 1986; Forscher and Smith, 1988). The microtubules extend from the neurite shaft and splay into the centre part of the growth cone. They have ocasionally been observed to extend to peripheral parts of the growth cone or to enter filopodia. The actin cytoskeleton is composed of two distinct microfilament subpopulations: one forms a meshwork of relatively short randomly oriented filaments in the lamellipodia, the second is composed of parallel bundles of up to a dozen filaments that radiate from the leading edge of the lamellipodia and extend into the filopodia (Yamada *et al.* 1970, 1971; Luduena and Wessells, 1973; Letourneau and Ressler, 1983; Forscher and Smith, 1988; Lewis and Bridgman, 1992). Disruption of this cytoskeletal organization with cytoskeletal drugs such as cytochalasin, nocodazole and taxol results in alterations of growth cone morphology and motility (Yamada *et al.* 1970, 1971; Letourneau and Ressler, 1984; Marsh and Letourneau, 1984; Letourneau *et al.* 1986; Forscher and Smith, 1988; Neely and Gesemann, 1994). In particular, disruption of the microfilaments by cytochalasin results in loss of filopodia, retraction of lamellipodia and cessation of growth cone motility (Yamada *et al.* 1970, 1971; Marsh and Letourneau, 1984; Bentley and Toroian-Raymond, 1986; Neely and Gesemann, 1994), the same response that is observed after electrical stimulation and/or elevation of intracellular calcium concentration (Cohan and Kater, 1986; Fields *et al.* 1990; Grumbacher-Reinert and Nicholls, 1992; Neely, 1993).

Cytoskeletal organization in growth cones after calcium influx

Growth cones of leech neurones collapse after an increase in intracellular calcium concentration due to activity, depolarization or treatment with a calcium ionophore (Grumbacher-Reinert and Nicholls, 1992; Neely, 1993; Neely and Gesemann, 1994). These changes are accompanied by a loss of microfilaments from the peripheral areas of growth cones, while the organization of microtubules is unaffected (Fig. 3). Pre-incubation of the neurones with phalloidin, a microfilament stabilizing agent, inhibits calcium-induced growth cone collapse (Neely and Gesemann, 1994). In DRG growth cones, elevated intracellular calcium concentrations cause disruption of microfilaments and destabilization of microtubules (Lankford and Letourneau, 1989). Interestingly, the loss of microfilaments and collapse of DRG growth cones caused by a membrane-associated collapsing factor is not associated with an increase in cytosolic calcium concentration (Fan *et al.* 1993).

A hypothesis for the mechanism of calcium-induced microfilament disruption

Intracellular calcium concentrations after depolarization

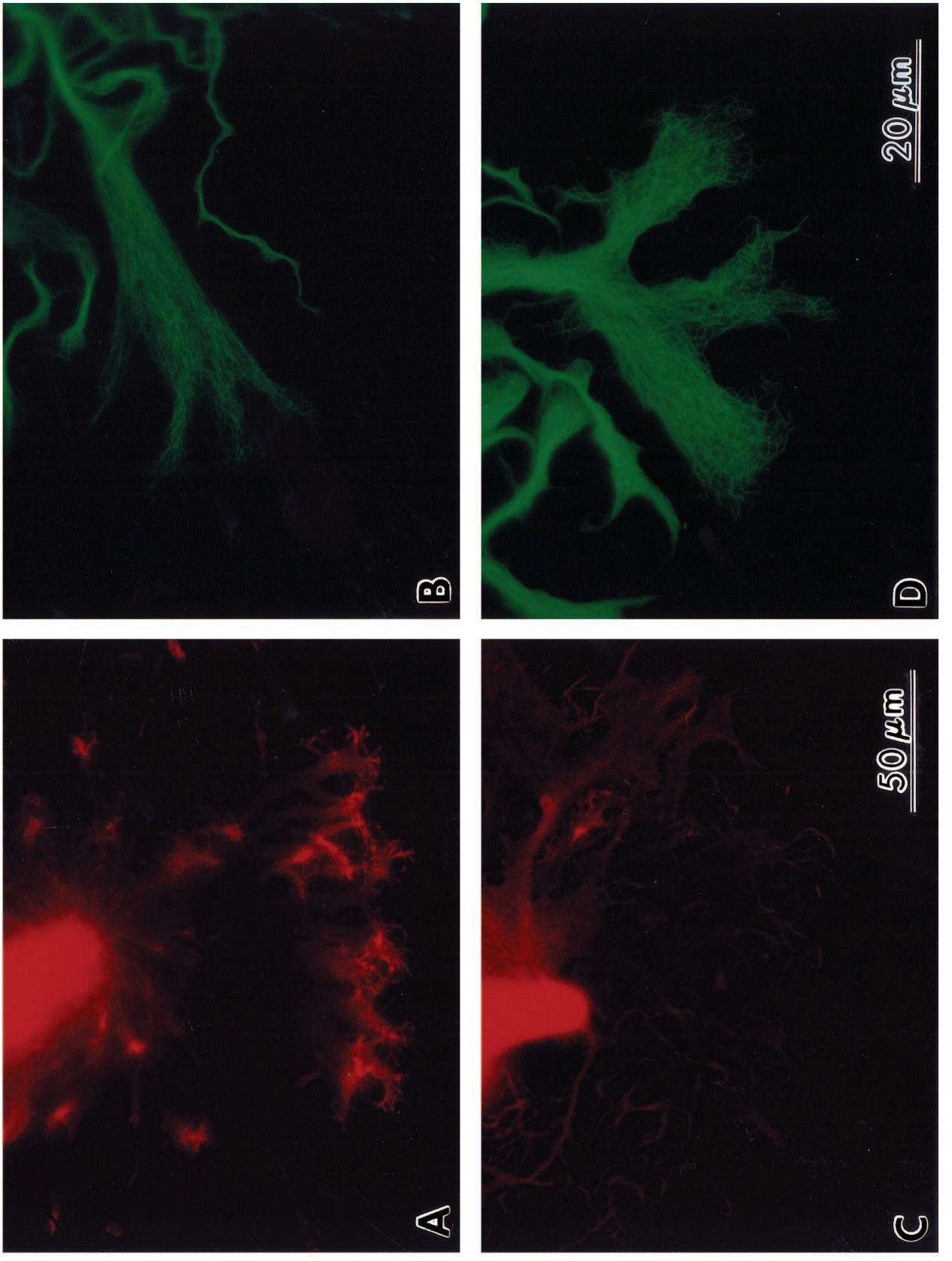
How could calcium cause the disruption of microfilaments? Intracellular calcium concentrations in growth cones are in the range 100–250 nmol l^{-1}, reaching 200–500 nmol l^{-1} after depolarization (Connor, 1986; Cohan *et al.* 1987; Lipscombe *et al.* 1988; Ishida *et al.* 1991; Garyantes and Regehr, 1992), and are therefore lower than the micromolar concentration required to activate proteins controlling cell functions (Baker and Knight, 1981; Manalan and Klee, 1984). Some studies using calcium-sensitive dyes, however, have shown higher intracellular calcium concentrations in spatially restricted areas ('calcium hotspots') of depolarized growth cones (Bolsover and Spector, 1986; Kater *et al.* 1988; Smith and Augustine, 1988; Tank *et al.* 1988; Regehr *et al.* 1989). Patch-clamp, toxin-binding and freeze-fracture studies demonstrate that calcium channels are grouped in clusters on growth cones (Pumplin *et al.* 1981; Fox *et al.* 1987; Lipscombe *et al.* 1988; Jones *et al.* 1989). Silver *et al.* (1990) have shown that calcium channel clustering can cause such 'calcium hotspots' with an average diameter of 7 μm and a calcium concentration of up to 1 μmol l^{-1}. Growth cones contain between 1–5 such 'hotspots' which are often located at the base of filopodia and coincide with the site of greatest outgrowth of neuroblastoma growth cone margins. These studies therefore suggest that local intracellular calcium concentrations may be high enough to activate many calcium-dependent proteins.

The structure of the microfilaments is regulated by the microfilament-associated proteins. Calcium could affect the organization of the microfilaments through modification of these proteins by calcium-dependent second-messenger cascades (Nairn *et al.* 1985; Aderem, 1992; Schulman *et al.* 1992). Alternatively, calcium might act directly on microfilament-associated proteins whose activity is calcium-dependent (Stossel *et al.* 1985; Vandekerckhove, 1990; Weeds and Maciver, 1993). Here we focus on the role of calcium-binding microfilament-associated proteins.

Is gelsolin a mediator of calcium-induced microfilament disruption?

Two types of proteins could be involved in the calcium-induced loss of peripheral microfilaments and growth cone collapse: actin filament associated motors (such as myosin) or filament severing proteins (such as gelsolin) (Sobue, 1993; Weeds and Maciver, 1993; Cramer *et al.* 1994). The disappearance of the microfilaments in the peripheral areas of the growth cone is not due to their contraction, but to an actual loss (Neely and Gesemann, 1994). This suggests the involvement of a microfilament-severing protein rather than a microfilament-associated motor protein.

Gelsolin is a protein that severs actin filaments when it is activated by calcium. Biochemical studies have shown that gelsolin remains tightly bound to the fragments after cleaving, preventing repolymerization (Yin *et al.* 1980, 1981*b*). Subsequent binding of gelsolin to polyphosphoinositides (phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate) results in dissociation of the fragments from gelsolin, and the freed filament


A
B
C
D
50 μm
20 μm

fragments then act as nuclei for rapid actin reassembly (Janmey and Stossel, 1987, 1989; Janmey *et al.* 1987).

Ultrastructural analysis of the distribution of gelsolin has shown that it is mostly in the cytoplasm in resting macrophages and platelets. Upon activation of these cells, gelsolin is redistributed to the plasma membrane associated with the ends of short actin filaments (Hartwig *et al.* 1989). Presumably binding of gelsolin to phosphatidylinositol phosphates in the membrane causes the release of the filament fragments and a burst of actin polymerization at the membrane–cytoskeleton interface.

The role of gelsolin in cell motility has been analyzed in living fibroblasts and macrophages. Increasing the content of gelsolin in cultured fibroblasts by gene transfection enhances their motility (Cunningham *et al.* 1991). Injection of a constitutively severing form of gelsolin into fibroblasts or macrophages induces loss of stress fibres, inhibits membrane ruffling and causes these cells to become rounded (Cooper *et al.* 1987).

Gelsolin is expressed in many different tissues including the nervous system (Yin *et al.* 1981*a*). In the rat central and peripheral nervous systems, it is enriched in oligodendrocytes and Schwann cells (Tanaka and Sobue, 1994). In the rat retina, gelsolin occurs in glial cells and neurones, the photoreceptors and ganglion cells (Legrand *et al.* 1991). In culture, gelsolin occurs in sympathetic neurones and co-localizes with the microfilaments of the peripheral areas of the growth cone and the filopodia in differentiated PC12 cells, DRG neurones (Tanaka *et al.* 1993) and leech neurones (M. D. Neely and E. Macaluso, unpublished observation). The high concentration of gelsolin in the filopodia of leech neurones is interesting in correlation with a recent publication reporting that isolated filopodia respond to membrane depolarization with an increase in intracellular calcium concentration and a decrease in length (Davenport *et al.* 1993). Gelsolin's severing activity, intracellular distribution, effect on cell motility and abundance in neuronal growth cones suggest it as a possible effector of the calcium-induced disruption of microfilaments.

Conclusion

In this review, we have compared the effect of neural activity on neurones *in vivo* and in culture. In neurones in culture, changes in growth cone motility after electrical stimulation are accompanied by an influx of calcium through voltage-sensitive calcium channels. The effects of electrical activity and increases in intracellular calcium concentration on growth cone morphology are not the same for all neurones. Some growth cones collapse, some show greater motility and others do not respond at all, depending on the type of neurone, the type of neurite (axon *versus* dendrite) and environmental factors. These findings suggest that there are several different signals and signal transduction mechanisms that ultimately result in alterations of the cytoskeletal structure and growth cone motility. The nature of these signals and signal transduction pathways, and the mechanism by which the growth cone integrates all the information with which it is presented, remain to be elucidated. Gelsolin is one candidate that could couple altered levels of electrical activity to reorganizations of the cytoskeleton and therefore to changes in growth cone motility.

Fig. 3. Raised intracellular calcium concentration leads to disruption of microfilaments in leech neurones. The cytoskeleton of leech neurones growing on the plant lectin Concanavalin A was analyzed by immunofluorescence. In control cells, microfilaments (stained with Rhodamine phalloidin) are abundant in the peripheral areas of the neurites (A), whereas the microtubules (stained with an anti-tubulin antibody) are concentrated in the central parts of the growth cone, with some microtubules extending to more peripheral regions (B). Exposure of leech neurones to the calcium ionophore A23187 (which increases the intracellular calcium concentration) leads to a dramatic loss of microfilaments in the neurites (C). The distribution of the microtubules, however, is not changed by this treatment (D). Scale bars: A, C, 50 μm; B, D, 20 μm (reproduced with permission, after Neely and Gesemann, 1994).

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