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Protein Kinase Inhibitors Block Neurite Outgrowth from Explants of Goldfish Retina*

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A role for protein phosphorylation in the process of neurite outgrowth has been inferred from many studies of the effects of protein kinase inhibitors and activators on cultured neurotumor cells and primary neuronal cells from developing brain or ganglia. Here we re-examine this issue, using a culture system derived from a fully differentiated neuronal system undergoing axonal regeneration—the explanted goldfish retina following optic nerve crush. Of the relatively non-selective protein kinase inhibitors employed, H7, staurosporine and K_{252a} were found to block neurite outgrowth, whereas HA1004 had no effect, a result which appears to rule out a critical role for protein kinase A. The more selective protein kinase C inhibitors, sphingosine, calphostin C and Ro-31-8220 were all inhibitory, as was prolonged treatment with phorbol ester and the protein phosphatase inhibitor okadaic acid. These results are in support of a role for protein kinase C in axonal regrowth.

KEY WORDS: protein kinase; neurite outgrowth; goldfish retina; regeneration.

INTRODUCTION

Cultured neural cells, both primary and neurotumor-derived, have provided useful model systems for studies of nerve growth and regeneration. Much progress has been made in advancing our understanding of the various aspects of neurite outgrowth. Less well-understood are the factors which regulate this complex process. Since modulation of protein phosphorylation/dephosphorylation events represents a major cellular regulatory pathway, it is likely that both tyrosine and serine/threonine protein kinases are major players in the regulation of neurite outgrowth. Reports of the effects of protein kinase modulators on neurite outgrowth from cultured neural cells include inhibitory effects of phorbol esters (TPA) on mouse neuroblastoma cells (1,2), rat cerebellar granule cells (3) and chick sensory (4) or rat sympathetic (5) ganglia and stimulatory effects for human neuroblastoma cells (6–9), chick ciliary ganglia (10), and rat cortical neurons (11). Such results suggested that protein kinase C (PKC) plays a role in neurite outgrowth/differentiation. However, whether its effect is positive or negative was unclear, not only because of the opposite effects cited above, but also due to the fact that prolonged phorbol ester treatment down-regulates PKC (12). Studies of the effects of protein kinase inhibitors have been carried out with PC12 cells, a cell line which responds to nerve growth factor (NGF) by the production of neurites (13) and early activation of a cascade of tyrosine and serine/threonine phosphorylations (14), including activation of PKC (15). Staurosporine (at low concentrations; 16), K_{252a} (17–20), sphingosine (21) and calphostin C (20) all inhibit NGF- dependent neurite outgrowth from PC12 cells. Although low concentrations of TPA in the presence of NGF have been reported to increase neurite outgrowth in PC12 cells (21), down regulation of PKC by pretreatment with TPA had no effect (22). Staurosporine (at higher concentrations) is also reported to increase neurite outgrowth in PC12 cells (19,20), however this response appears to be indepen-

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dent of PKC (20). For SH-SY5Y human neuroblastoma cells, H7, staurosporine, sphingosine and K_{252a} all stimulate neurite outgrowth (9,23), a result in agreement with the ability of prolonged TPA treatment to cause differentiation of these cells (9,24). In addition, intracellular delivery of antibodies to specific isoforms of PKC induces morphological differentiation of SH-SY5Y cells (25). Support for the importance of protein phosphorylation state for neurite outgrowth also comes from studies of the protein phosphatase inhibitor, okadaic acid, which was found to be inhibitory for both PC12 cells (26) and neuroblastoma (27,28).

Primary neuronal cultures also vary in their responses to protein kinase inhibitors. In rat cortical neuron cultures, H7 treatment, as well as PKC down-regulation, potentiates the neurite outgrowth response to epidermal growth factor (11), whereas, in rat cerebellar granule cells, down-regulation of PKC, staurosporine and sphingosine treatment each inhibit neurite outgrowth (3). For chick ciliary ganglia grown on a laminin substratum, short-term TPA treatment enhances neurite outgrowth, while H7 and sphingosine inhibit (10), a result which was interpreted to reflect a possible role for PKC in signal transduction from the laminin receptor. PKC down-regulation in distal neurites of compartmented rat sympathetic neuronal cultures caused a slowing in the rate of NGF-dependent neurite outgrowth (5), however, H7 and staurosporine did not inhibit. Although sphingosine (5), calphostin C and chelerythrine (29), when applied to distal neurite compartments, were found to be inhibitory, inhibition was not prevented by PKC down-regulation. Some of the apparently anomalous results with these various culture systems may be due to the presence of multiple isoforms of PKC (30), which might differ in their sensitivity to inhibitors and their ability to be down-regulated by TPA treatment.

The studies thus far carried out involve either differentiating neurotumor cells or cultures of developing neurons. Axonal regeneration may recapitulate some of the cellular processes invoked in development or could utilize unique mechanisms not seen in differentiation. More specific insight into the role of protein kinases in regenerating axons might be gained by analysis of a fully differentiated neuronal system such as that provided by the goldfish retina following optic nerve crush. The latter treatment induces the retinal ganglion cells to grow new axons, a process which can be studied in vitro by explantation of post-crush retina (31,32). Enhanced phosphorylation of axonal proteins during goldfish optic nerve regeneration has been observed (33). Here we report that neurite outgrowth from goldfish retinal explants is inhibited by many of the protein kinase inhibitors described above, as well as by the novel, more selective PKC inhibitor, Ro-31-8220. The data are consistent with a role for PKC in axonal outgrowth during nerve regeneration.

EXPERIMENTAL PROCEDURE

Materials. Protein kinase inhibitors and activators were obtained as follows: H7 [1-(5-isoquinoline sulfonyl)-2- methylpiperazine] and HA1004 [N-(2-guanidinoethly)-5-isoquinolinesulfonamide] from Seikagaku America (Rockville, MD); staurosporine, sphingosine and phorbol-12-mysristate-13-acetate (TPA) from Sigma Chemical Co. (St. Louis, MO); K_{252a}, Calphostin C, and Ro-31-8220 {3-[1-(amidinothio) propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl) maleimide methane sulfonate from Calbiochem (LA Jolla, CA). The protein phosphatase inhibitor, okadaic acid, was also purchased from Calbiochem.

Explant Culture. Goldfish (Carassius auratus), 6–7 cm in body length were obtained from Grassy Forks Fisheries (Martinsville, IN). Explant cultures were prepared from goldfish retinas following intraorbital crush of the optic nerve (under tricaine anesthesia) as previously described (31,32). Briefly, retinas were removed at 7–21 days following optic nerve crush, chopped with a McIlwain tissue chopper into 500 μm squares, then placed onto polylysine-coated dishes (30 mm) and cultured in L-15 medium (Gibco) at 20–22° in a humid chamber. Each experimental group consisted of 5 dishes containing 9 explants in a 3 × 3 array. After 7 days in culture, the neurite growth index (NGI, a measure of neurite length and density; 32) was determined. Neurite outgrowth was also assayed by an automated program which utilized digitized video images of the explants (34). Both measures of neurite outgrowth yielded results which were in good agreement with each other. Unless otherwise stated, compounds to be tested for their effects on neurite outgrowth were added to the culture dishes at the time of explantation and were present throughout the 7 day culture period.

RESULTS

Neurite Outgrowth Measurements. Initial studies utilized isoquinoline sulfonyl protein kinase inhibitors: H7, which shows little selectivity for PKA vs. PKC and HA1004, a more potent inhibitor of PKA than of PKC (35,36). Whereas HA1004 was ineffective at the highest concentration tested (30 μM), H7 inhibited neurite outgrowth from goldfish retinal explants with an IC_{50} of approximately 5 μM (Fig. 1; Table I). This result suggests that PKA activity is not required for neurite outgrowth in this culture system. H7 was considered non-toxic to the retinal ganglion cells, since exposure of the explants to 30 μM H7 for the first 24 hours of culture followed by washout of the drug, had no effect on subsequent neurite outgrowth. Explants grown on a laminin substratum showed the same sensitivity to H7 as those on a polylysine substratum (data not shown). Two additional relatively non-selective protein kinase inhibitors,

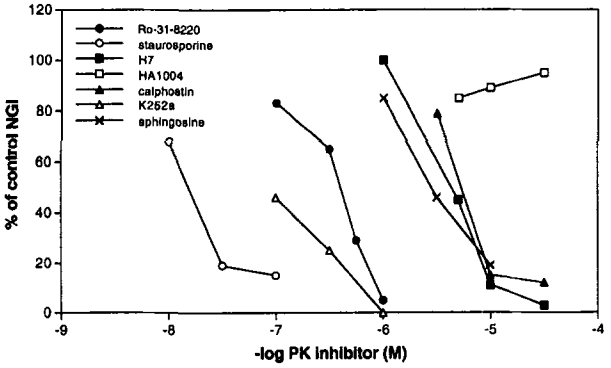

Fig. 1. Effect of protein kinase inhibitors on neurite outgrowth from explants of goldfish retina. Retinal explants were grown for 7 days in the presence of the indicated concentration of protein kinase inhibitors, then the nerve growth index (NGI) was determined. Data are expressed as percent of control NGI, which averaged 2.8 ± 0.2 (mean ± SEM, n = 20). Each point represents data from 45 explants from one of 2 or 3 experiments which gave similar results.

Table I. Summary of Protein Kinase Inhibitor Effects on Neurite Outgrowth

Inhibitor	Retinal Explant $IC_{50}(\mu M)$	PKC/PKA $IC_{50}(\mu M)^a$	Inhibits Interaction With	Reference
H7	5	6/3	ATP	35, 36
HA1004	—	40/2	ATP	35, 36
Staurosporine	0.02	0.007/0.007	ATP	19, 37
K_{252a}	0.1	0.025/0.018	ATP	19, 38
Ro-31-8220	0.5	0.01/0.9	ATP	39, 40
Sphingosine	3	2.8/—	DAG	41
Calphostin C	6	0.05/50	DAG	42, 43

[a] in vitro.

staurosporine and K_{252a}, were both found to be potent blockers of neurite outgrowth (Fig 1; Table I). Similar relative potencies of these two compounds have been observed for neurite outgrowth inhibition in PC12 cells (16,18). Washout of either of these two inhibitors after 24 hour exposure resulted in only partial (30–50%) relief of the neurite outgrowth inhibition, suggesting either some irreversible toxicity or inefficiency of the washout procedure. In order to further explore the possible role of PKC, the effects of three relatively selective PKC inhibitors were determined (Fig. 1, Table I). Sphingosine, Ro-31-8220 and calphostin C were all effective inhibitors of neurite outgrowth from goldfish retinal explants. In addition, incubation of retinal explants for 7 days with 1 μM TPA, a treatment which would be expected to cause down-regulation of protein kinase C, results in 70% inhibition of neurite outgrowth (data not shown). Okadaic acid, a protein phosphatase inhibitor (44), resulted in a dose-dependent inhibition of neurite outgrowth (data not shown), with an IC_{50} of 5 nM suggesting that type 1 protein phosphatase activity may be required for neurite outgrowth.

Morphological Studies. The agents examined in this study may be acting on one or more of three stages of axonal regrowth: initiation, elongation or maintenance. In order to explore this issue, goldfish retinal explants were grown for 4 days in control medium, at which time protein kinase or phosphatase inhibitors were added and both the extent of neurite outgrowth and neurite morphology were compared to that of control explants after an additional 3 days in vitro (Fig. 2). Addition of staurosporine, H7, sphingosine and okadaic acid, at 75–90% of their maximally effective doses, completely prevented any further neurite outgrowth. In contrast, K_{252a} treatment resulted in an apparent 20% increase in neurite outgrowth ($p < 0.01$), with enhanced fasciculation evident (Fig. 2e). When viewed under phase contrast (Fig. 3), neurites exposed to H7, sphingosine and okadaic acid exhibited signs of deterioration, including swelling and increased numbers of enlarged varicosities. In some of the sphingosine-treated explants, proximal regions of neurites were absent. The morphology of staurosporine-treated neurites did not differ markedly from controls.

DISCUSSION

Axonal growth is a complex process, requiring alterations in gene expression, synthesis and assembly of cytoskeleton, synthesis and insertion of new surface membrane, formation and movement of growth cones, communication between the cell body and growing axon, interaction between growing fibers, and recognition of target tissue. Thus there are many possible sites for regulation of axonal growth by protein phosphorylation. Among the effects of modulation of protein phosphorylation state are regulation of the catalytic activity of proteins and of the efficiency of protein-protein or protein-nucleic acid interactions. Although the process of axonal growth is common to both the developing nervous system and neurons capable of axon regeneration following injury, each might not be expected to respond identically to perturbation of protein phosphorylation. In order to assess the role of protein phosphorylation during axonal growth in a regenerating system, we have examined the effect of a series of protein kinase and phosphatase inhibitors on neurite outgrowth from goldfish retina explants, which, following a conditioning lesion of the optic nerve in vivo, become primed to display vigorous neurite outgrowth in vitro.

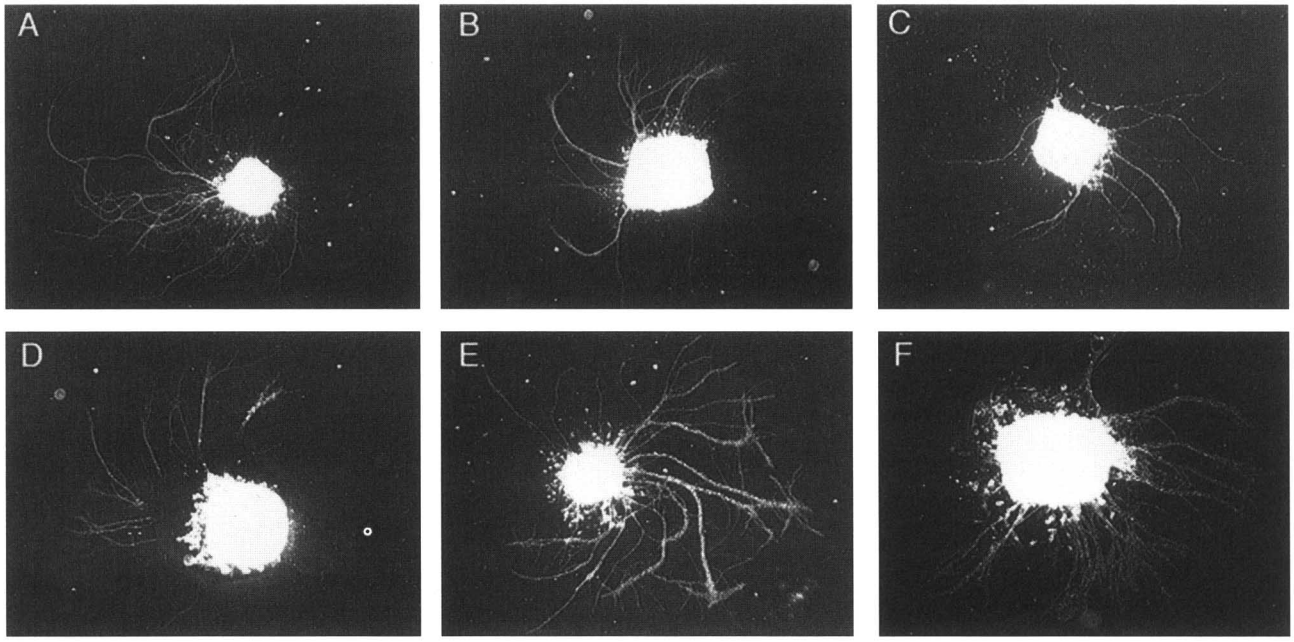

Fig. 2. Effect of protein kinase inhibitors on outgrowth and appearance of existing neurites. Dark field images of retinal explants (500 μm on a side) grown for 4 days in control medium, then exposed to the following conditions for 3 additional days: A, control; B, 100 nM staurosporine; C, 30 μM H7; D, 10 μM sphingosine; E, 300 nM K_{252a}, and F, 10 nM okadaic acid.

Of the seven protein kinase inhibitors tested, all but one, HA1004, inhibited neurite outgrowth when added to goldfish retinal explants at the time of explantation. Comparison of the dose-response curves for H7 and HA1004 with their known $IC_{50's}$ for PKC and PKA in vitro appeared to rule out a role for PKA. The $IC_{50's}$ for the non-selective inhibitors staurosporine and K_{252a} were somewhat higher than those reported for PKC in vitro, while that for sphingosine, a PKC-selective inhibitor was consistent with its reported in vitro effects. For Ro-31-8220 and calphostin C, $IC_{50's}$ substantially higher than those reported for PKC (but still less than for PKA) were observed. It is not known what intracellular concentration of these agents is attained when they are added to an intact cultured cell system. An IC_{50} similar to that reported here for Ro-31-8220 was found for inhibition of rabbit smooth muscle cell proliferation (45). In addition, it has been reported that the relative affinities of calphostin C, staurosporine and Ro-31-8220 vary depending on whether PKC is in the cytosolic or the membrane compartment (46), with the latter two inhibiting membrane-bound PKC more avidly and the former more effective against the cytosolic form. The data with the relatively PKC-selective inhibitors, sphingosine, calphostin C and Ro-31-8220 are consistent with a role for PKC in retinal explant neurite outgrowth. However, both sphingosine and calphostin C inhibit neurite outgrowth from PC12 cells in the absence of any effects of TPA treatment (20–22). Such a result may call into question the selectivity of these two agents, but it remains possible that they may be acting in PC12 cells on isoforms of PKC which are resistant to TPA-induced down-regulation. Ro-31-8220 has not, to our knowledge, been previously used for studies of neurite outgrowth. Given the lack of specificity of the effects of many of these agents, no firm conclusions can be drawn based on the actions of any one of them, but taken together, the data support a role for PKC in axonal regrowth from goldfish retinal ganglion cells, but, with the exception of PKA, do not exclude a role for other protein kinases. A study of the effect of kinase inhibitors on growth cone motility in explant cultures of goldfish retina found no effect of calphostin C (1 μM), but observed a reversible growth cone arrest with H7 (at 200 but not 100 μM). The use of additional more selective inhibitors pointed to a role for myosin light-chain kinase (47). In addition, we have observed inhibitory effects of the protein phosphatase inhibitor, okadaic acid, a result also found for PC12 cells and SH-SY5Y neuroblastoma (26,27). It may be that cycles of phosphorylation/dephosphorylation are important for the axonal growth process. Whether these inhibitors are affecting basal or stimulated protein phosphorylation is not known. The nature of any such putative stimulus is open to speculation, but it is not, as

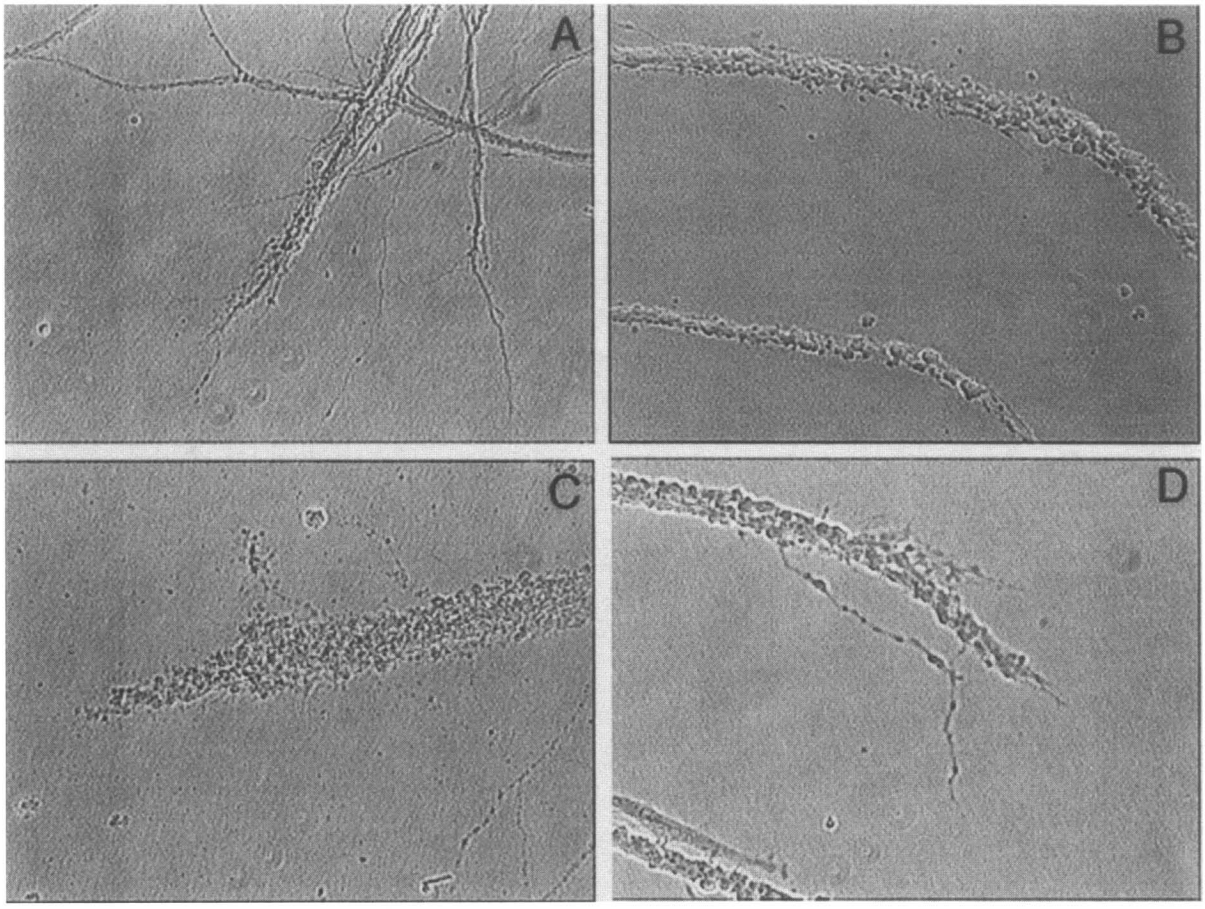

Fig. 3. Neurite morphology. Phase contrast images (20 × objective) of neurites from retinal explants grown for 4 days in control medium, then exposed to the following conditions for 3 additional days: A, control; B, 30 μM H7; C, 10 μM sphingosine, and D, 10 nM okadaic acid.

suggested in the chick ciliary ganglion cell study (10), due to signal transduction at the laminin receptor, since the goldfish retinal explants were grown on polylysine-coated dishes. While growth on laminin or a laminin/polylysine substratum enhances the rate of neurite outgrowth from retinal explants (48), it does not confer any additional sensitivity to the PKC inhibitor, H7. Neither is there convincing evidence that the goldfish retinal ganglion cells respond to known trophic factors.

In order to examine elongation and maintenance of neurites apart from the initiation process, five of these inhibitors were added to explants after 4 days of neurite outgrowth. Staurosporine, H7, sphingosine, and okadaic acid blocked further neurite outgrowth, a result which indicates that the protein phosphorylation state is important for neurite elongation. However, K_{252a} appeared to increase neurite outgrowth and fasciculation, suggesting that its inhibitory effect when added at the time of explantation may be due to an action on neurite initiation. Of possible relevance is a report that the K_{252a}-induced neurite outgrowth in SH-SY5Y neuroblastoma cells is accompanied by increased tyrosine phosphorylation and activity of the focal adhesion protein tyrosine kinase (23). Such a postulated action of K_{252a} in goldfish retinal explants could contribute to the enhanced fasciculation observed with this agent. Of the four agents which blocked elongation of existing neurites, H7, sphingosine and okadaic acid treatment resulted in degenerative changes in the appearance of the neurites, while the morphology of staurosporine-treated neurites showed no visible differences from control explants. A similar effect of sphingosine and lack of effect of staurosporine was reported for distal neurites of compartmented cultures of rat sympathetic neurons (5). H7 is also reported to alter the actin cytoskeleton of cultured cells in a PKC-independent manner (49). Although non-specific toxic actions should be considered, it seems unlikely that such effects could account for the deterio-

ration caused by all three compounds, each of which acts by distinct mechanisms to perturb the cellular protein phosphorylation state. It may be that staurosporine caused subtle or gradual changes that were not apparent by light microscopy or may be acting by a mechanism other than protein kinase inhibition. The effects of H7, sphingosine and okadaic acid imply that axon integrity, in addition to elongation, may be influenced by modulation of the balance between protein kinase and phosphatase activities.

The results of this study complement and extend those obtained from studies of neurotumor cells and primary cultures of developing neurons to include axons from regenerating mature neurons among the population whose growth is sensitive to perturbation of protein phosphorylation. They also suggest that this sensitivity is not a reflection of the differentiation state of the neuron or of a response to trophic factor but instead is an intrinsic property of the axonal growth process.

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