Direct Activation of Second Messenger Pathways Mimics Cell Adhesion Molecule-dependent Neurite Outgrowth

Jane L. Saffell, Frank S. Walsh, and Patrick Doherty

Department of Experimental Pathology, UMDS, Guy's Hospital, London Bridge, London SE1 9RT, United Kingdom

Abstract. We present evidence that direct activation of neuronal second messenger pathways in PC12 cells by opening voltage-dependent calcium channels mimics cell adhesion molecule (CAM)-induced differentiation of these cells. PC12 cells were cultured on monolayers of control 3T3 cells or 3T3 cells expressing transfected N-cadherin in the presence of KCl or a calcium channel agonist Bay K 8644. Both potassium depolarization and agonist-induced activation of calcium channels promoted substantial neurite outgrowth from PC12 cells cultured on control 3T3 monolayers and increased neurite outgrowth from those cultured on N-cadherin–expressing 3T3 monolayers. The potassium-induced response could be inhibited by L- and N-type calcium channel antagonists and by kinase inhibitor K-252b but was unaffected by pertussis toxin. In contrast activators of protein kinase C did not stimulate neurite outgrowth, and the neurite outgrowth response induced by activation of protein kinase A was not inhibited by calcium channel antagonists or pertussis toxin. These studies support the postulate that CAM-induced neuronal differentiation involves a specific transmembrane signaling pathway and suggest that activation of this pathway after CAM binding may be more important for the neurite outgrowth response than CAM-dependent adhesion per se.

T HE innervation of target tissue by neurons during development or after injury requires the extension of axons and their guidance along precise pathways. Studies, largely using in vitro model systems, have suggested that a wide variety of stimulatory and inhibitory cues may contribute to growth and guidance. These include cell surface glycoproteins, diffusible factors, and constituents of the extracellular matrix (Doherty and Walsh, 1989; Strittmatter and Fishman, 1991; Bixby and Harris, 1991; Schwab, 1990). Four well-defined receptor systems present in neuronal growth cones have been shown to promote or support neurite outgrowth over a variety of cell types, including astrocytes, Schwann cells, and muscle cells. These are the integrins (Reichardt and Tomaselli, 1991) which are receptors for extracellular matrix components such as laminin; and the neural cell adhesion molecules (CAMs)[1] N-cadherin (Takeichi, 1988, 1991; Doherty et al., 1991a; Bixby and Jhabvala, 1990), neural cell adhesion molecule (NCAM) (Cunningham et al., 1987; Walsh and Doherty, 1991; Bixby et al., 1987), and L1 (Seilheimer and Schachner, 1988; Lagenaur and Lemmon, 1987). These CAMs mediate cell–cell interactions primarily by a homophilic interaction with products of the same gene expressed on the surface of other cells, although recent evidence suggests that two of these molecules can also function as heterophilic receptors for products of

related genes. For example, N-cadherin can bind to R-cadherin (Inuzuka et al., 1991) and L1 can function as a neurite outgrowth promoting receptor for the Axonin-1 glycoprotein (Kuhn et al., 1991).

When NIH 3T3 fibroblasts are transfected with cDNAs encoding NCAM and N-cadherin, the transgene product is able to promote neurite outgrowth from a variety of neurons cultured on monolayers of the transfected cells (Matsunaga et al., 1988; Doherty et al., 1990a,b). In addition, the rat pheochromocytoma cell line, PC12, which can be induced by nerve growth factor (NGF) and FGF to differentiate from its adrenal chromaffin cell phenotype to that of a sympathetic neuron (Greene and Tischler, 1976; Togaris et al., 1985), will assume the same morphological phenotype when cultured on 3T3 monolayers expressing transfected N-cadherin or NCAM. The neurite outgrowth induced by N-cadherin exceeded that elicited by NGF (Doherty et al., 1991b) and this study also showed that CAMs directly induce this change in PC12 cell morphology.

It is possible that CAM-induced neurite outgrowth simply depends on the increased adhesion resulting from direct homophilic binding. Alternatively, it may be primarily dependent on transmembrane signaling and second messengers. In this context, experimentally induced increases in the levels of intracellular calcium are known to affect neurite outgrowth and growth cone motility in a number of neuronal types (Kater and Mills, 1991). Recent studies have shown that blockage of the L- and N-type calcium channels or treatment with pertussis toxin substantially inhibits the ability of

---

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; NCAM, neural cell adhesion molecule; NGF, nerve growth factor.

663

substrate-associated N-cadherin and NCAM to promote neurite outgrowth from PC12 cells and primary neurons (Doherty et al., 1991*b*, 1992*a,b*). On the basis of these results we have postulated that CAMs primarily promote cell migration and neurite outgrowth via a G-protein–dependent activation of neuronal calcium channels and consequent influx of extracellular calcium, and that this is not simply related to their ability to support adhesion per se.

There is also other evidence that the ability of a molecule to promote cell migration and neurite outgrowth may be fundamentally different from its ability to support adhesion. For example, if the cytoplasmic domain of the α_2 integrin receptor subunit is replaced with that of the α_4 or α_5 subunit, the ability of the chimeric receptors to recognize laminin and collagen is not impaired. However, whereas receptors containing the α_2 or α_5 cytoplasmic domains promoted stable exertion of physical force to collagen or laminin substrata, the receptor containing the α_4 subunit cytoplasmic domain stimulated cell migration over the same substrata (Chan et al., 1992).

In a similar vein our own group has recently shown that use of the VASE exon (Doherty et al., 1992*a*) or exon 18 (Doherty et al., 1992*c*) which respectively modify the extracellular and cytoplasmic domains of NCAM, can both impair NCAM's ability to act as a substrate molecule that promotes neurite outgrowth without obviously impairing its ability to support adhesion. In addition, whereas polysialic acid on neuronal NCAM promotes NCAM-dependent neurite outgrowth (Doherty et al., 1990*a,b*) it can act as a general inhibitor of cell adhesion (Rutishauser et al., 1988).

In the present study we have directly tested our postulate (see above) by determining if direct activation of the proposed second messenger pathway is sufficient to mimic CAM-dependent neurite outgrowth. Depolarizing concentration of KCl and the calcium channel agonist Bay K 8644 have each been added to cocultures of PC12 cells growing on confluent monolayers of control and N-cadherin–expressing 3T3 cells to directly activate voltage-dependent calcium channels by a mechanism independent of CAMs. Previous studies have shown that K^+ depolarization and Bay K 8644 promote the survival of primary neurons by increasing calcium flux through neuronal calcium channels (Collins and Lile, 1989; Collins et al., 1991; Koike et al., 1989).

The effect on PC12 cells of these agents in the presence and absence of L- and N-type calcium channel antagonists, pertussis toxin, and kinase inhibitor K-252b has been assessed by measuring neurite outgrowth. The results have shown that both Bay K 8644 and depolarization-induced calcium channel opening can result in a morphological response by PC12 cells which is indistinguishable from CAM-induced neurite outgrowth. This response can be inhibited by a combination of L- and N-type calcium channel antagonists and kinase inhibitor K-252b but not by pertussis toxin. These data are consistent with CAM-induced neuronal differentiation being dependent on activation of second messenger pathways rather than on adhesion per se.

Materials and Methods

Cell Culture

Parental and N-cadherin–transfected 3T3 cells were maintained on plastic culture dishes in DME containing 10% FCS. PC12 cells were grown on collagen-coated plastic culture dishes in defined SATO medium (Doherty et al., 1988). All cells were grown at 37°C in 8% CO_2. For establishment of cocultures, 3T3 cells were seeded at 8×10^4 cells per chamber of an eight-chamber slide (Lab-Tek, Naperville, IL) coated sequentially with poly-L-lysine and collagen (Doherty et al., 1990*b*), and allowed to form confluent monolayers overnight. PC12 cells were introduced onto the monolayer at a density of 1.5×10^3 cells per chamber after being trypsinized from the culture plate (0.1% trypsin wt/vol in PBS/0.02% EDTA), pelletted, resuspended in SATO medium and triturated (19-guage syringe needle) to achieve a single-cell suspension. Cocultures were maintained for 40–48 h in SATO medium in the presence of other reagents as indicated and then fixed for 30 min with 4% paraformaldehyde for staining as below.

Immunostaining

Fixed cultures were washed and nonspecific protein binding sites blocked with 0.5% gelatin (45 min, room temperature). PC12 cells were then visualized by the sequential application (60 min each, room temperature), after fixing as above, of the OX-7 mAb which recognizes the Thy 1 antigen (diluted 1:50) followed by biotinylated anti-mouse immunoglobulin and Texas red–conjugated streptavidin (both Amersham International [Amersham, UK], diluted 1:500). Cultures were then mounted and analyzed as below.

Image Analysis

Fluorescent images were detected using a low light–sensitive video camera (model 4722-5000; Cohu Inc., San Diego, CA) and analyzed using a Sight Systems Image Manager (Sight Systems, Newbury, England). Mounted cultures were scanned systematically over the whole slide area and the length of the longest neurite per PC12 cell was measured as previously described (Doherty et al., 1991*b*). To enable scoring of cells with no visible neurite it was necessary to assign them the minimum possible length of 2 μm; this did not significantly raise the mean.

Other Reagents

Pertussis toxin and K-252b were gifts from Drs. J. Kenimer and Y. Matsuda, respectively. Diltiazem and tetrodotoxin were purchased from Sigma Chemical Co. (St. Louis, MO), ω-conotoxin GVIA from Life Technologies, Inc. (Grand Island, NY) and Bay K 8644 from Calbiochem Corp. (La Jolla, CA). All channel antagonists/agonists, K-252b, and pertussis toxin were used at concentrations established from the literature to have maximal effects on their targets.

Results

Potassium Depolarization Mimics N-cadherin–induced Neurite Outgrowth

PC12 cells express N-cadherin and after 48 h the majority of PC12 cells grown on confluent monolayers of N-cadherin–expressing 3T3 cells extended one or more primary neurites, the extent of neurite outgrowth exceeding that elicited by a maximally active NGF concentration (50 ng/ml) over the same culture period (see Doherty et al., 1991*b*). In contrast, PC12 cells cultured on parental 3T3 monolayers showed virtually no neurite outgrowth (Fig. 1). The effect of elevated potassium (5–75 mM) on neurite outgrowth was tested for PC12 cells cultured on confluent monolayers of 3T3 cells. After 48 h the cocultures were fixed and stained using a Thy 1 antibody which binds specifically to PC12 cells. PC12 cells responded to increasing KCl concentration by extending neurites (Fig. 1) and this response is quantitated in Fig. 2. The mean length of the longest neurite per cell increased with increasing KCl concentration, reaching a maximum at ∼45 mM KCl. From a basal value of 13.9 ± 1.0 μm ($n = 133$) on parental 3T3 monolayers, the mean length of the longest neurite (which we also refer to as the mean neurite

length) rose by 130% to 31.9 ± 2.5 μm (n = 127) in the presence of 45 mM KCl. PC12 cells in a parallel experiment growing on N-cadherin–expressing monolayers had an almost identical mean neurite length of 31.5 ± 2.5 μm (n = 142).

Having ascertained from the dose–response curve that 40 mM KCl was near maximal for depolarization-induced neurite outgrowth, a number of corroborative experiments (n = 5–7) were conducted to further test the effect of 40 mM KCl on PC12 cells cultured on control and N-cadherin–expressing 3T3 monolayers. The results, showing mean neurite length as a percentage of basal growth on 3T3 monolayers and percentage of cells with a neurite >20 μm are summarized in Fig. 3. The mean neurite length of PC12 cells cultured on control 3T3 monolayers in the presence of 40 mM KCl was 282 ± 14% (n = 7) of basal growth which slightly surpassed the mean neurite length of PC12 cells cultured on N-cadherin–expressing monolayers which was 257 ± 18% (n = 5) of basal growth. Similarly, whereas <20% of PC12 cells extended a neurite >20 μm on control 3T3 monolayers, this rose to 60.3 ± 7.0% (n = 7) in the presence of 40 mM KCl which is again almost identical to the 61.8 ± 8.6% (n = 5) found on N-cadherin–expressing monolayers. Both these measurements show that potassium depolarization is able to fully mimic the N-cadherin–dependent neurite outgrowth response by PC12 cells. Data taken from Reber and Reuter (Fig. 1 A; 1991) shows 40 mM K⁺ to induce a sustained rise in PC12 cell calcium from ∼100 to 250 nM. A maximal response (to ∼300 nM) was apparent at 50–60 mM K⁺.

KCl could also elicit a neurite outgrowth response from NGF primed (but not naive) PC12 cells cultured on a collagen-coated substratum. Potassium depolarization did not appear to stimulate the release of polypeptide growth factors from the monolayer cells as dialyzed conditioned media from the latter had no neurite growth promoting activity (J. Saffell, unpublished results). Thus we conclude that in the above cocultures KCl probably acts directly on the PC12 cells.

The effect of depolarization was also tested for PC12 cells growing on N-cadherin–expressing monolayers (Fig. 1, G and H). The mean length of the longest neurite rose from 257 ± 18% (n = 5) of basal growth to 410 ± 25% (n = 6) in the presence of 40 mM KCl and the percentage of cells with a neurite >20 μm from 61.8 ± 8.6 (n = 3) to 82.3 ± 5.5 (n = 6) % (Fig. 3). These data show that the effects of depolarization and N-cadherin–mediated neurite outgrowth are partially additive, suggesting that neither factor alone elicits a maximal response.

Calcium Channel Agonist Bay K 8644 also Mimics the N-cadherin Response

Bay K 8644 is a calcium channel agonist which acts by reducing the level of depolarization required for calcium channel activation. In combination with KCl it allows the calcium channel to open in the presence of lower potassium concentrations than would otherwise be effective, i.e., it shifts the KCl-neurite outgrowth dose response curve to the left but has little effect on its own (see Koike et al., 1989; Collins et al., 1991). The effect of Bay K 8644 on neurite outgrowth was determined by culturing PC12 cells on confluent control 3T3 monolayers in the presence of a maximal concentration of Bay K 8644 (5 μM) and/or a concentra-

tion of KCl sub-optimal for neurite outgrowth (20 mM). The results of a representative experiment are shown in Fig. 4. On its own Bay K 8644 had no significant effect on neurite outgrowth. However, in the presence of 20 mM KCl Bay K 8644 increased the mean length of the longest neurite by 85%, from 14.5 ± 1.1 (n = 173) to 26.91 ± 1.7 (n = 192) μm, (P < 0.0005). In this experiment 20 mM KCl, alone was able to slightly increase neurite outgrowth to 138% of the control. The Bay K 8644 response was identical to the maximal KCl depolarization response measured in sister cultures (data not shown).

The effect of Bay K 8644 on neurite outgrowth from PC12 cells cultured on N-cadherin–expressing monolayers was also determined. In the absence of KCl, Bay K 8644 again had no significant effect on neurite outgrowth. Thus N-cadherin is unlikely to stimulate growth via a voltage-dependent mechanism (see below). In this experiment 20 mM KCl on its own increased the mean length of the longest neurite from 28.15 ± 2.1 (n = 166) to 42.7 ± 3.5 (n = 143) μm, a rise of 52%. In the presence of 20 mM KCl, Bay K 8644 induces an additional, albeit small, significant increase in neurite outgrowth to 51.42 ± 3.13 μm (n = 129) (P < 0.05). This suggests that neither agonist-induced nor N-cadherin–induced neurite outgrowth promoting effects are maximal.

Effects of Pertussis Toxin, Calcium Channel Antagonists and Kinase Inhibitor K-252b on Potassium-induced Neurite Outgrowth

PC12 cells were cultured on control 3T3 monolayers for 48 h in the presence of 40 mM KCl and each of the following: pertussis toxin (1 μg/ml), diltiazem (10 μM), ω-conotoxin (0.25 μM), a mixture of diltiazem and ω-conotoxin, and the kinase inhibitor K-252b (100 ng/ml), to test the effects of these perturbants of CAM-induced neurite outgrowth (Doherty et al., 1991b) on the KCl depolarization-induced response. The results of a representative experiment are shown in Fig. 5, where neurite length is expressed as a percentage of the KCl-induced response on control 3T3 monolayers.

The ability of pertussis toxin (at 1 μg/ml), which inactivates G proteins of the Gi/Go families by ADP-ribosylation of the α subunit, to inhibit KCl- (40 mM) dependent outgrowth was determined in two experiments. In both instances pertussis toxin had only marginal inhibitory effects on the K⁺ response (e.g., see Fig. 5). This contrasts with the complete inhibition of CAM-induced neurite outgrowth and provides direct evidence for a pertussis toxin–sensitive heterotrimeric G protein–modulating CAM function upstream of calcium channel opening. Diltiazem, which blocks L-type calcium channels, significantly inhibited the KCl response in three experiments by, on average, 66 ± 7.3%. In addition ω-conotoxin, which blocks N-type calcium channels inhibited the response in sister cultures by 28 ± 9.1% (both values mean ± SEM for three experiments (see Fig. 5 for an individual experiment). In general, when diltiazem and ω-conotoxin were present together there was a slightly greater inhibition, e.g., see Fig 5. However, in a total of three experiments a residual KCl response of 30 ± 8.4% (mean ± SEM) remained after treatment with both diltiazem and ω-conotoxin. Reber and Reuter (1991) have previously shown that KCl-dependent influx of calcium into PC12 cells can be substantially inhibited by L-type calcium channel antagonists with a small increase in the magnitude of this block
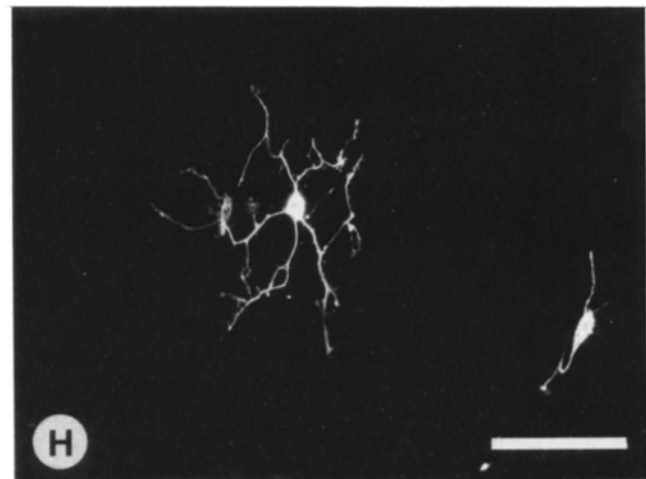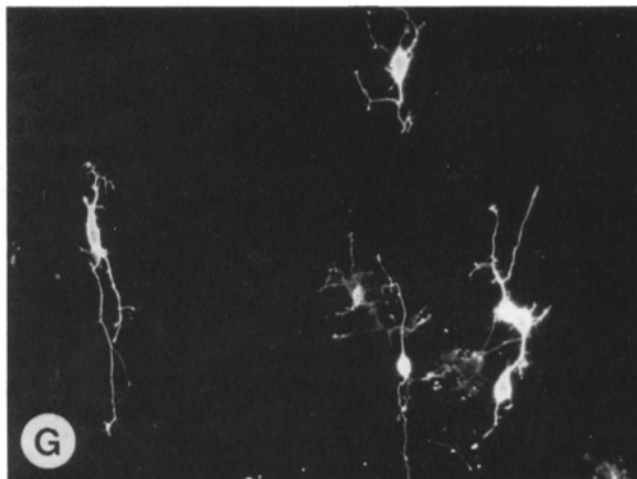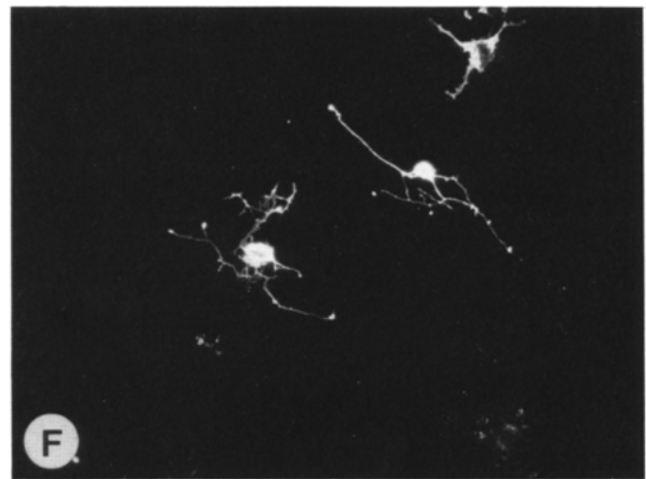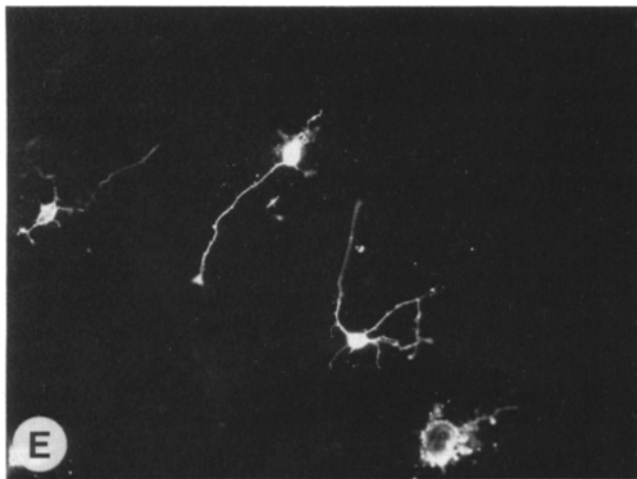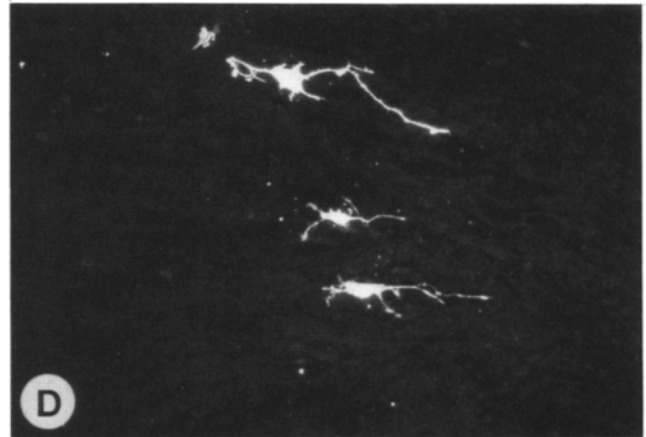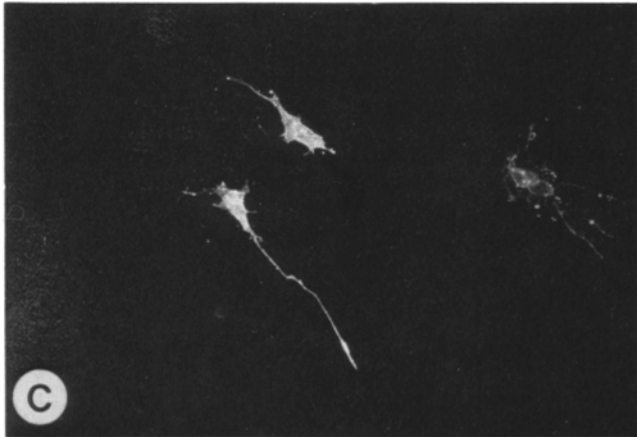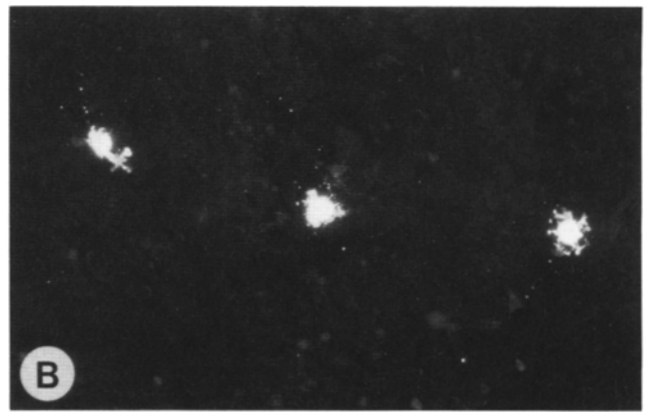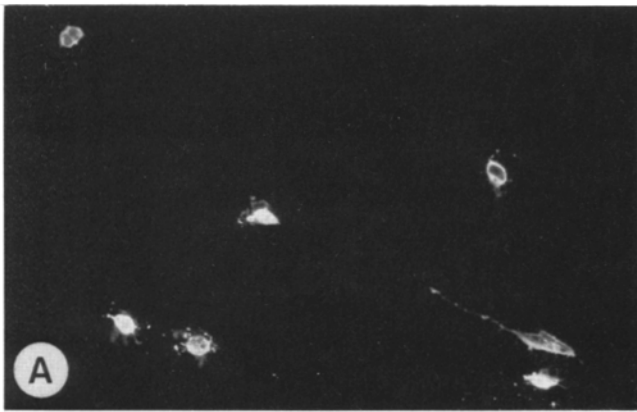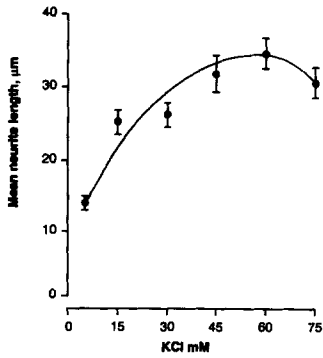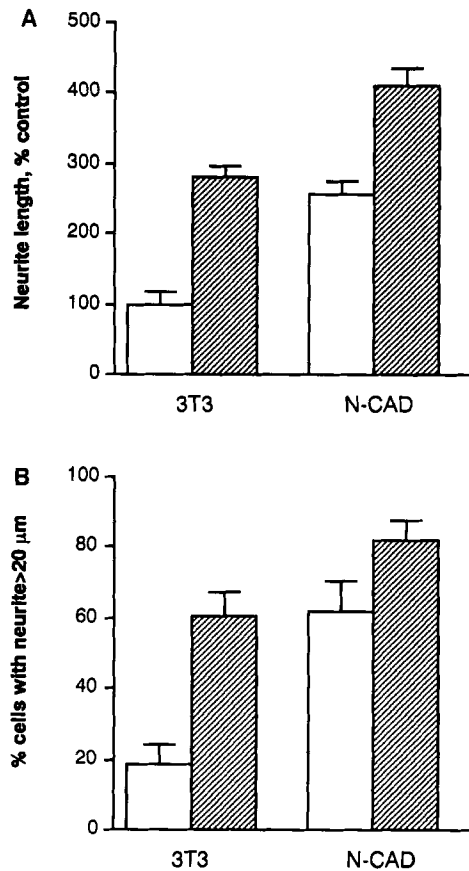
Figure 2. Neurite outgrowth from PC12 cells on control 3T3 monolayers increases with KCl concentration. PC12 cells were grown on confluent monolayers of 3T3 cells for 48 h in the presence of KCl (5–75) mM before being fixed, stained, and analyzed to determine the mean length of the longest neurite. Each value is the mean of 100–150 ± SEM PC12 cells sampled from replicate cultures.

seen on addition of ω-conotoxin. Interestingly, a combination of L- and N-type channel blockers could not fully inhibit K^+-dependent increases in intracellular calcium and this was particularly prominent at the growth cone. Thus, the effects of KCl and calcium channel antagonists on neurite outgrowth correlate well with their ability to modulate the level of intracellular calcium in PC12 cells via the activation and antagonism of L- and N-type calcium channels.

K-252b is a broad-specificity kinase inhibitor which has been shown to reduce CAM-induced neurite outgrowth to below basal levels on control 3T3 monolayers (Doherty et al., 1991b). In this study, KCl depolarization-induced neurite outgrowth was similarly reduced to below basal levels by K-252b, indicating that if it is acting by inhibiting a kinase, this kinase is present downstream of calcium entry into PC12 cells.

Calcium Channel Activation Does Not Contribute to the Neurite Outgrowth Response Induced by Activation of Protein Kinase A

The above results have clearly shown that activation of calcium channels can mimic CAM-dependent neurite outgrowth. We have also tested if this pathway can be activated by other agents. Phorbol esters at concentrations that activate protein kinase C did not induce neurite outgrowth from PC12 cells cultured on monolayers of 3T3 cells (our own unpublished observation). However cholera toxin, which increases intracellular cAMP levels with concomitant activation of protein kinase A, is a potent inducer of neurite outgrowth in this coculture model (Doherty et al., 1991b). In the present study we have tested the ability of pertussis toxin and calcium channel antagonists to block this response with the results summarized in Table I. The results show very clearly that a pertussis toxin-sensitive G protein and L- and N-type calcium channels are not involved in this pathway. The response to cholera toxin could however be partially blocked by Rp-cAMP (a competitive inhibitor of protein kinase A) and K-252b (our own unpublished observations).
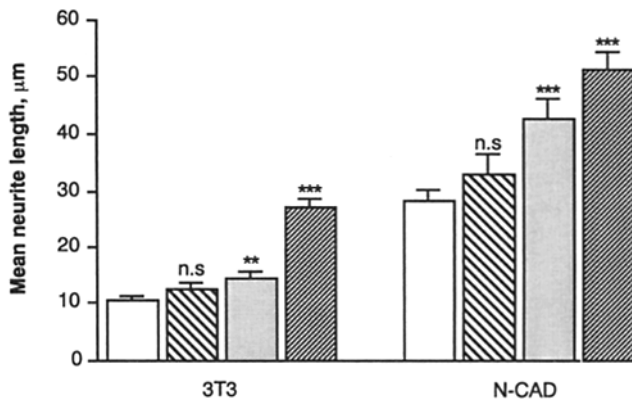
Figure 3. Depolarizing concentrations of KCl increase outgrowth from PC12 cells on control and N-cadherin–expressing 3T3 monolayers. PC12 cells were cultured on control or N-cadherin-transfected 3T3 monolayers in the presence or absence of 40 mM KCl for 48 h before being fixed and stained. The results show mean length of the longest neurite normalized to the control growth on 3T3 monolayers (A) and the percentage of cells with a neurite >20 μm (B). Each value is the mean ± SEM pooled for 5–7 independent experiments. (□) control; (▨) 40 mM KCl.

Discussion

CAMs such as N-cadherin and NCAM share at least two fundamental functional properties: their ability to promote adhesion and their ability to promote neurite outgrowth (Bixby et al., 1987; Doherty and Walsh, 1989). One possibility is that these functions are directly related, i.e., neurite outgrowth is a direct consequence of the adhesive interactions mediated by these molecules. An alternative possibility is that they are fundamentally different and in some ways contradictory functions that are not directly related. For example, in the case of NCAM, at some stages of development this molecule promotes plasticity (e.g., cell migration and

Figure 1. PC12 cells extend neurites on N-cadherin–expressing 3T3 monolayers and on control 3T3 monolayers in the presence of 40 mM KCl. PC12 cells were grown for 48 h on a substratum of confluent 3T3 cells in the absence (A and B) or presence (E and F) of 40 mM KCl, or on a substratum of N-cadherin–expressing cells in the absence (C and D) or presence (G and H) of 40 mM KCl. PC12 cells were specifically stained using mouse anti–rat Thy1 mAb. Bar, 50 μm.

Figure 4. Bay K 8644 increases neurite outgrowth from PC12 cells on control and N-cadherin–expressing 3T3 monolayers. PC12 cells were cultured on control (□) and N-cadherin-expressing 3T3 mono-layers in the presence of Bay K 8644 (■), 5 μM; KCl (▨), 20 mM; or both (▨) for 48 h before being fixed and stained. Each value is the mean length of the longest neurite ± SEM of 100–200 PC12 cells sampled from replicate cultures. *** P < 0.0005; ** P < 0.0025; and n.s. not statistically different. Statistical analysis compared growth on either 3T3 or N-cadherin monolayers in control media against growth on the same monolayers in test media, i.e., the presence of Bay K 8644, KCl, or both.

axonal growth), whereas at other stages of development NCAM may be involved in stabilizing connections (axon fascicles and synapses) between cells (Hoffman and Edelman, 1983; Rutishauser et al., 1988). Whereas the former undoubtedly requires homophilic binding and the consequent activation of second messenger pathways (Doherty et al.,
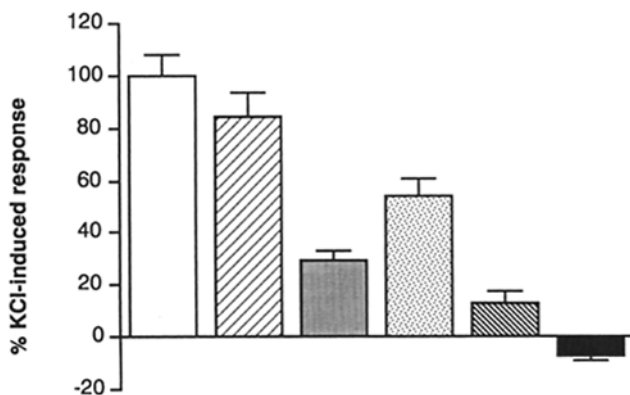
Figure 5. The effects of a variety of perturbants on KCl-induced neurite outgrowth. PC12 cells were cultured on 3T3 monolayers in the presence of 40 mM KCl plus each of the following: pertussis toxin (▨) (1 μg/ml), diltiazem (▨) (10 μM), ω-conotoxin (▨) (0.25 μM), diltiazem + ω-conotoxin (■), and K-252b (■) (100 ng/ml). Results are expressed as % KCl-induced response where 100% response is the difference in mean length of the longest neurite for PC12 cells growing on 3T3 monolayers in the absence and presence of 40 mM KCl. None of these agents had any significant effect on PC12 cell morphology on 3T3 monolayers in control media, i.e., no KCl (Doherty et al., 1991b). Each value is the mean ± SEM of 120–170 PC12 cells sampled from replicate cultures. The average length of the longest neurite in the absence and presence of 40 mM KCl was 11.3 ± 0.7 (164) and 35.3 + 2.0 (162) μm, respectively. Both values mean ± SEM (n).

Table I. Effects of Pertussis Toxin and Calcium Channel Antagonists on Neurite Outgrowth Induced by Cholera Toxin

Media	Mean length of the longest neurite	Percent cells with a neurite >20 μm
	μm	
(a) Control	15.1 ± 0.8 (165)	26
(b) Cholera toxin	67.7 ± 4.0 (128)***	88
+ Diltiazem	72.5 ± 4.3 (145)[n.s.]	88
+ ω-conotoxin	74.9 ± 4.3 (143)[n.s.]	94
+ Pertussis toxin	61.4 ± 3.4 (164)[n.s.]	89

PC12 cells were grown for 24 h on a substratum of confluent 3T3 cells in (a) control media or (b) media containing cholera toxin (1 ng/ml) further supplemented with Ditiazem (10 μM), ω-conotoxin (0.25 μM) or pertussis toxin (1 μg/ml). The results show the mean length of the longest neurite per cell ± SEM for the given number of PC12 cells sampled from replicate cultures. The percentage of these neurites >20 μm in length is also shown.
*** P < 0.0005 is compared with growth in the absence of cholera toxin.
[n.s.] Not statistically different from growth in media supplemented with cholera toxin alone.

1991b), the latter may depend only on the adhesive forces generated by such binding. Evidence that different isoforms of NCAM can differentially affect adhesion and neurite outgrowth has recently been obtained (Doherty et al., 1992a,c).

We have recently provided evidence that CAMs directly induce neurite outgrowth by triggering the activation of second messenger pathways in neurons (Doherty et al., 1991b, 1992a, b). Our results showed that pertussis toxin or a combination of L- and N-type calcium channel antagonists could fully block NCAM and N-cadherin–dependent neurite outgrowth from PC12 cells and both hippocampal and cerebellar neurons. We interpreted these data as supporting the involvement of a CAM-induced, G protein–dependent activation of both L- and N-type calcium channels. In the present study we have tested whether, by simply activating this pathway, we can mimic CAM-dependent neurite outgrowth. In this context Collins et al., (1991) have recently shown that 40 mM KCl can fully mimic the survival effects of trophic factors on chick ciliary ganglion neurons and that this is a direct result of activation of voltage-gated calcium channels. In the present study, KCl-induced a dose-dependent neurite outgrowth response from PC12 cells which, at its maximal level (∼40 mM KCl) was indistinguishable from that induced by transfected N-cadherin. In combination N-cadherin and KCl effects were only partially additive suggesting that a saturable response had not been reached by either factor.

The involvement of both L- and N-type calcium channels in the KCl-induced response is demonstrated by the observation that both L- and N-type calcium channel antagonists could inhibit the response. However, whereas diltiazem and ω-conotoxin each block PC12 cell CAM-dependent neurite outgrowth by ∼50%, diltiazem was slightly more effective than ω-conotoxin at blocking the KCl-induced response. This may indicate that the degree of depolarization achieved by 40 mM KCl preferentially activates the L-type calcium channels so that a greater proportion of influxing calcium enters the cell through L-type rather than N-type channels. Direct evidence for this has been obtained by measuring the ability of nifedipine and ω-conotoxin to block K⁺ depolarization induced increases in intracellular free calcium in PC12 cells (Reber and Reuter, 1991).

Further evidence that simple opening of calcium channels is sufficient to promote morphological differentiation is shown by the effect on neurite outgrowth of the calcium channel agonist Bay K 8644 (Koike et al., 1989) which acts by reducing the level of depolarization required for calcium channel activation. At a sub-optimal KCl concentration (20 mM), Bay K 8644 was able to increase neurite outgrowth from PC12 cells growing on control monolayers to the level of the N-cadherin–mediated response. The latter response could also be enhanced by the agonist.

These results imply that activation of the postulated neurite outgrowth-promoting pathway by using mechanisms independent of CAMs is sufficient to trigger the full morphological response. Not only does this support the CAM binding calcium influx transmembrane signaling postulate, but also suggests that the ability of CAMs to open calcium channels is more important for the neurite outgrowth response than their ability to support adhesion per se. This is consistent with the evidence from previous studies that although removal of α2-8-linked polysialic acid from NCAM increases its ability to promote adhesion (Hoffman and Edelman, 1983), it actually reduces its ability to induce neurite outgrowth from a variety of neuronal types, including chick retinal ganglion cells (Doherty et al., 1990b) and rat cerebellar neurons (Doherty et al., 1992a) and rat hippocampal neurons (Doherty et al., 1992c). In the light of these results it might appear possible that CAM-dependent neurite outgrowth itself involves a depolarization stage. For example, a CAM-induced opening of sodium channels would result in depolarization leading in turn to opening of the voltage activated calcium channels. However, such a mechanism is unlikely because we have found that the sodium channel blocker tetrodotoxin has no effect on CAM-dependent neurite outgrowth (our own unpublished observation) and in contrast to the potassium depolarization response, the N-cadherin response is not potentiated by Bay K 8644.

The present study has shown that pertussis toxin has little effect on KCl-induced neurite outgrowth, indicating that there is no pertussis toxin-sensitive G protein involved in the pathway downstream of calcium entry through calcium channels. This is strong evidence that the G protein inactivated by pertussis toxin to prevent CAM-mediated neurite outgrowth is located upstream of the calcium channel which would be the expected position for a G protein postulated to signal between homophilically bound CAMs and calcium channels. It also provides additional evidence that pertussis toxin does not block a very general step involved in neurite outgrowth per se (Doherty et al., 1991b) Similarly, pertussis toxin did not block cholera toxin-dependent neurite outgrowth.

K-252b is a broad specificity kinase inhibitor. In a recent study we showed that it can fully inhibit both CAM-induced and basal neurite outgrowth from PC12 cells cultured on 3T3 monolayers, suggesting that an unidentified kinase may be involved in CAM-dependent neurite outgrowth (Doherty et al., 1991b). However, it remained to be determined whether this kinase was acting upstream or downstream of calcium channel activation. In the present study K-252b fully inhibited both KCl-induced and basal neurite outgrowth from PC12 cells, implying that if it inhibits a single kinase, this is positioned downstream of calcium entry and is not involved in G protein activation of calcium channels. In addi-

tion, we have found that K-252b can also inhibit NGF, FGF, and cholera toxin–dependent neurite outgrowth from PC12 cells cultured on 3T3 monolayers suggesting that it can act at a common step shared by several pathways.

In this context there appear to be at least four pathways that can lead to PC12 cell neurite outgrowth: an integrin-dependent pathway; a pathway activated by soluble growth factors such as NGF and FGF; a pathway activated by agents that increase cAMP levels; and a common pathway activated by a variety of CAMs including NCAM, N-cadherin, and more recently L1 (E. Williams, P. Doherty, G. Turner, R. A. Reid, J. Hemperly, and F. S. Walsh, manuscript submitted for publication). Whereas K-252b can inhibit all of the above pathways suggesting a late convergent step, a pertussis toxin sensitive G protein and activation of neuronal calcium channels appear at the moment to be totally exclusive to the CAM pathway (also see Doherty et al., 1991b).

The neurite outgrowth response resulting from KCl-induced opening of calcium channels was essentially identical to the N-cadherin response. The KCl concentration optimal for neurite outgrowth from PC12 cells, 40 mM, is also optimal for promotion of survival of a range of neuronal types (Koike et al., 1989; Collins and Lile, 1989; Collins et al., 1991). Thus, both survival and neurite outgrowth are promoted by a potassium depolarization–induced activation of calcium channels. Ernsberger et al. (1989) has shown that the survival of early embryonic chick sympathetic neurons is dependent on the nature of the culture substratum rather than the availability of NGF. It is therefore possible that, alongside other environmental influences which may affect intracellular calcium, such as afferent electrical input, CAM-mediated calcium influx could contribute to survival of neurons in vivo.

References

Bixby, J. L., and W. A. Harris. 1991. Molecular mechanisms of axon growth and guidance. *Annu. Rev. Cell Biol.* 7:117–159.

Bixby, J. L., and P. Jhabvala. 1990. Extracellular matrix molecules induce neurite outgrowth through different mechanisms. *J. Cell Biol.* 111:2725–2732.

Bixby, J. L., R. S. Pratt, J. Lillien, and L. F. Reichardt. 1987. Neurite outgrowth on muscle cell surfaces involves extracellular matrix receptors as well as Ca²⁺-dependent and independent cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 84:2555–2559.

Chan, B. M. C, P. D. Kassner, J. A. Schiro, H. R. Bayers, T. S. Kupper, and M. E. Hemler. 1992. Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. *Cell.* 68:1051–1060

Collins, F., and J. D. Lile. 1989. The role of dihydropyridine-sensitive voltage gated calcium channels in potassium mediated neuronal survival. *Brain Res.* 502:99–108.

Collins, F., M. F. Schmidt, P. B. Guthrie, and S. B. Kater. 1991. Sustained increase in intracellular calcium promotes neuronal survival. *J. Neurosci.* 4:1281–1288.

Cunningham, B. A., J. J. Hemperly, B. A. Murray, E. A. Prediger, R. Brackenbury, and G. M. Edelman. 1987. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation and alternative splicing. *Science.* 236:799–806.

Doherty, P., and F. S. Walsh. 1989. Neurite guidance molecules. *Curr. Opin. Cell Biol.* 1:1102–1106.

Doherty, P., D. A. Mann, and F. S. Walsh. 1988. Comparison of the effects of NGF, activators of protein kinase C, and a calcium ionophore on the expression of Thy-1 and NCAM in PC12 cell cultures. *J. Cell Biol.* 107:333–340.

Doherty, P., M. Fruns, P. Seaton, G. Dickson, C. H. Barton, T. A. Sears, and F. S. Walsh. 1990a. A threshold effect of the major isoforms of NCAM on

neurite outgrowth. *Nature (Lond.)*. 343:464–466.

Doherty, P., J. Cohen, and F. S. Walsh. 1990b. Neurite outgrowth in response to transfected NCAM changes during development and is modulated by polysialic acid. *Neuron.* 5:209–219.

Doherty, P., L. H. Rowett, S. E. Moore, D. A. Mann, and F. S. Walsh. 1991a. Neurite outgrowth in response to transfected NCAM and N-cadherin reveals fundamental differences in neuronal responsiveness to CAMs. *Neuron.* 6:247–258.

Doherty, P., S. V. Ashton, S. E. Moore, and F. S. Walsh. 1991b. Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G protein-dependent activation of L- and N-type neuronal Ca²⁺ channels. *Cell.* 67:21–33.

Doherty, P., C. E. C. Kitty-Moolenaar, S. V. Ashton, R. J. A. M. Michalides, and F. S. Walsh. 1992a. The VASE exon down regulates the neurite outgrowth promoting activity of NCAM. *Nature (Lond.)*. 356:791–793.

Doherty, P., S. D. Skaper, S. E. Moore, A. Leon, and F. S. Walsh. 1992b. A developmentally regulated switch in neuronal responsiveness to NCAM and N-cadherin in the rat hippocampus. *Development (Camb.)*. In press.

Doherty, P., G. Rimon, D. A. Mann, and F. S. Walsh. 1992c. Modification of the cytoplasmic domain by use of exon 18 reduces NCAMs ability to act as a substrate for neurite outgrowth. *J. Neurochem.* In press.

Ernsberger, V., D. Edgar, and H. Rohrer. 1989. The survival of early chick sympathetic neurons in vitro is dependent on a suitable substrate but independent of NGF. *Dev. Biol.* 135:250–262.

Gallo, V., A. Kingsbury, R. Balazs, and O. S. Jorgensen. 1987. The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. *J. Neurosci.* 7:2203–2213.

Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:424–428.

Hoffman, S., and G. M. Edelman. 1983. Kinetics of neuronal binding by E and A forms of neural cell adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 80:5762–5766.

Inuzuka, H., S. Miyatani, and M. Takeichi. 1991. R-cadherin: a novel Ca²⁺-dependent cell-cell adhesion molecule expressed in the retina. *Neuron.* 7:69–79.

Kater, S. B., and L. R. Mills. 1991. Regulation of growth cone behavior by calcium. *J. Neurosci.* 11:891–899.

Koike, T., D. P. Martin, and E. M. Johnson. 1989. Role of calcium channels in the ability of membrane depolarization to prevent neuronal death induced by trophic-factor deprivation: evidence that levels of internal calcium determine nerve growth factor dependence of sympathetic ganglion cells. *Proc. Natl. Acad. Sci. USA.* 86:7421–6425.

Kuhn, LT. B., E. T. Stoeckli, M. A. Condrau, F. G. Rathjen, and P. Sonderegger. 1991. Neurite outgrowth on immobilized axonin-1 is mediated by a heterophilic interaction with Ll(G4). *J. Cell Biol.* 115:1113–1126.

Lagenauer, C., and V. Lemmon. 1987. A L1-like molecule, 809 antigen, is a potent substrate for neurite extension. *Proc. Natl. Acad. Sci. USA.* 84: 7753–7757.

Matsunaga, M., K. Hatta, A. Nagafuchi, and M. Takeichi. 1988. Guidance of optic nerve fibres by N-cadherin adhesion molecules. *Nature (Lond.)*. 334:62–64.

Reber, B. F. X., and H. Reuter. 1991. Dependence of cytosolic calcium in differentiating rat pheochromocytoma cells on calcium channels and intracellular stores. *J. Physiol.* 435:145–162.

Reichardt, L. F., and K. J. Tomaselli. 1991. Extracellular matrix molecules and their receptors: functions in neural development. *Ann. Rev. Neurosci.* 14:531–570.

Rutishauser, V., A. Acheson, A. Hall, D. Mann, J. Sunshine. 1988. The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science (Wash. DC).* 240:53–57.

Schwab, M. E. 1990. Myelin-associated inhibitors of neurite growth and regeneration in the CNS. *Trends Neurosci.* 13:452–456.

Seilheimer, B., and M. Schachner. 1988. Studies of adhesion molecules mediating interactions between cells of peripheral nervous system indicate a major role for L1 in mediating sensory neurons growth on Schwann cells in culture. *J. Cell Biol.* 107:341–351.

Strittmatter, S. M., and M. C. Fishman. 1991. The neuronal growth cone as a specialized transduction system. *Bioessays.* 13:127–134.

Takeichi, M. 1988. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development (Camb.)*. 102:639–655.

Takeichi, M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science (Lond.)*. 251:1451–1455.

Togaris, A., G. Dickens, H. Kozuya, and G. Guroff. 1985. The effect of fibroblast growth factor on PC12 cells. *J. Neurosci.* 5:307–316.

Walsh, F. S., and P. Doherty. 1991. NCAM gene structure and function. *Semin. Neurosci.* 3:271–284.