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Calcium Influx into Neurons Can Solely Account for Cell Contact-dependent Neurite Outgrowth Stimulated by Transfected L1

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Abstract. We have used monolayers of control 3T3 cells and 3T3 cells expressing transfected human L1 as a culture substrate for rat PC12 cells and rat cerebellar neurons. PC12 cells and cerebellar neurons extended longer neurites on human L1 expressing cells. Neurons isolated from the cerebellum at postnatal day 9 responded equally as well as those isolated at postnatal day 1–4, and this contrasts with the failure of these older neurons to respond to the transfected human neural cell adhesion molecule (NCAM). Human L1-dependent neurite outgrowth could be blocked by antibodies that bound to rat L1 and, additionally, the response could be fully inhibited by pertussis toxin and substantially inhibited by antagonists of L- and N-type calcium channels. Calcium influx into neurons induced by K^+ depolarization fully mimics the L1 response. Furthermore, we show that L1- and K^+-dependent neurite outgrowth can be specifically inhibited by a reduction in extracellular calcium to 0.25 μM, and by pretreatment of cerebellar neurons with the intracellular calcium chelator BAPTA/AM. In contrast, the response was not inhibited by heparin or by removal of polysialic acid from neuronal NCAM both of which substantially inhibit NCAM-dependent neurite outgrowth. These data demonstrate that whereas NCAM and L1 promote neurite outgrowth via activation of a common CAM-specific second messenger pathway in neurons, neuronal responsiveness to NCAM and L1 is not coordinately regulated via posttranslational processing of NCAM. The fact that NCAM- and L1-dependent neurite outgrowth, but not adhesion, are calcium dependent provides further evidence that adhesion per se does not directly contribute to neurite outgrowth.

AXONAL and dendritic growth and arborization are central to the development and regeneration of the nervous system. Both processes are likely to depend upon the functional interplay between a vast array of environmental cues provided by components of the extracellular matrix, as well as by molecules present on the surface and secreted by cells with which the neuronal growth cone comes into contact (reviewed in Doherty and Walsh, 1989; Bixby and Harris, 1991; Lumsden and Cohen, 1991).

Over the last decade a very large number of extracellular matrix and integral membrane glycoproteins that mediate contact-dependent axonal growth have been identified. Prominent among the neuronal growth cone receptor systems that recognize and transduce positive growth signals are members of three gene families, namely the integrins (Reichart and Tomaselli, 1991; Hynes, 1992), the Ig gene superfamily (Williams, 1987; Walsh and Doherty, 1991; Rathjen and Jessell, 1991), and the cadherins (Takeichi, 1991). Evidence from antibody perturbation experiments has shown that when neurons extend neurites over complex cellular substrata (e.g., astrocytes, myoblasts, or Schwann cells), a cocktail of antibodies that block the function of β1-integrins, the neural cell adhesion molecule (NCAM)[1] and L1 cell adhesion molecules (CAMs) (both Ig superfamily members), and N-cadherin are often required for a maximal inhibition of neurite outgrowth (e.g., see Bixby et al., 1987, 1988). These studies suggest that contact-dependent growth of axons requires the integration of signals arising from a number of receptor–ligand interactions.

N-cadherin and L1 in neurons can promote neurite outgrowth following their homophilic binding to products of the same gene purified and coated to a tissue culture substratum (Lemmon et al., 1989; Bixby and Zhang, 1990). L1 in neurons can also promote neurite outgrowth following heterophilic binding to a distinct but related gene product called Axonin-1 (Kuhn et al., 1991). Similarly, when N-cadherin- and NCAM-deficient cells are transfected with cDNAs encoding these molecules, expression of the transgene can be correlated with an increase in the ability of the transfected cell to promote neurite outgrowth from a wide variety of neuronal cell types (Matsunaga et al., 1988; Doherty et al.,

1. *Abbreviations used in this paper:* NCAM, neural cell adhesion molecule; PSA, polysialic acid.

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1989, 1990*a*, 1991*a,b*). In the case of NCAM, neurite outgrowth was shown to be dependent on NCAM in both the neuron and substratum supporting a homophilic binding mechanism (Doherty et al., 1990*b*). The use of a transfection-based strategy to study CAMs offers a number of advantages over more conventional methods of biochemical purification and coating to a substratum. For example, in the latter case the coated molecule is often required to support both adhesion and consequently neurite outgrowth, and it remains unclear how these distinct functions are related (e.g., see Doherty et al., 1992*a*). In transfection models, the control substratum (i.e., untransfected cells) can be selected for its ability to support adhesion per se, allowing the neurite outgrowth promoting activity of the transfected CAM to be studied on its own. More importantly, facets of function relating to the lateral diffusion of CAMs in membranes and/or their ability to interact with cytoskeletal elements in a cellular substratum are obviously lost when the CAM is studied as a purified molecule. In this context, recent results have shown that NCAM isoforms that differ only in the size of their cytoplasmic domain (as a consequence of natural alternative splicing of the NCAM gene) differ considerably in their ability to promote neurite outgrowth and that this most likely relates to NCAMs lateral diffusion properties in the cellular substratum (Doherty et al., 1992*b*).

The cDNAs encoding mouse (Moos et al., 1988), rat (Miura et al., 1991), and human L1 (Hlavin and Lemmon, 1991; Reid and Hemperly, 1992) have all been isolated and characterized. These cDNAs all encode proteins of ~1,260 amino acids that share ~85% identity between human and mouse. An alternatively spliced exon that encodes a four-amino acid peptide in the cytoplasmic domain was also identified in rat and human cDNAs. In the present study we have transfected mouse NIH-3T3 fibroblasts with a plasmid vector containing the full coding sequence of human L1. Stable clones expressing human L1 have been isolated and characterized for their ability to promote neurite outgrowth from rat PC12 pheochromocytoma cells (see Greene and Tischler, 1976; Doherty et al., 1991b) and rat cerebellar granule cells. Previous studies have suggested that a *cis*-interaction between L1 and NCAM in the same membrane may result in the formation of a potent receptor complex that can then interact better than L1 on its own for *trans* binding to L1 on a second membrane (Kadmon et al., 1990*a,b*). A similar functional interplay between NCAM and L1, that is primarily controlled by long chains of α2-8–linked polysialic acid (PSA) on NCAM, has been suggested to be important for establishment of the correct innervation pattern in the chick hindlimb (Landmesser et al., 1990). In the present study we address three important questions relating to L1 and NCAM function in neurons. Firstly, does L1 induce neurite outgrowth via activation of the same neuronal second messenger pathway as NCAM and does this depend on the flux of extracellular calcium into neurons? Secondly, do neurons undergo a similar age-dependent loss of responsiveness to L1 as they do for NCAM-dependent neurite outgrowth? Finally, is NCAM function required for L1-dependent neurite outgrowth and is the latter directly modulated by the presence of PSA on neuronal NCAM? Our results clearly show that L1 and NCAM can promote neurite outgrowth via activation of a common neuronal CAM-specific second messenger pathway, and that direct activation of the pathway is sufficient to fully mimic the response. In contrast, factors that operate to modulate NCAM-dependent neurite outgrowth, such as alternative splicing and reduced expression of PSA on neuronal NCAM, do not directly impinge on L1's ability to promote neurite outgrowth. Furthermore, we provide novel data to support the postulate that CAM-dependent activation of second messengers is solely responsible for the neurite outgrowth response.

Materials and Methods

Plasmid Construction

Full-length human L1 cDNA (Reid and Hemperly, 1991) was subcloned from pBluescript into expression vectors pJ4Ω (Morgenstern and Land, 1990) and pCDNA1 (Invitrogen) under the control of Mo MuLV LTR and CMV promoters, respectively. The L1 cDNA was removed from pBluescript using ClaI and XbaI (the latter site was end-repaired using the Klenow fragment of DNA polymerase 1) for ligation into ClaI and SmaI cut pJ4Ω or NotI and XhoI for ligation into pCDNA1 cut with XmaIII and XhoI. The integrity of the inserted L1 cDNA was checked by partial sequence and restriction analyses.

Transfection

Cotransfection of human L1 with the selectable plasmid pHβAP-1-neo (Doherty et al., 1991*b*) at a ratio of 20:1 was performed using the calcium phosphate transfection protocol provided with the CellPhect Transfection Kit (Pharmacia Fine Chemicals, Piscataway, NJ). 3T3 cells were grown for 24 h to a density of 1×10^4 cells per 60-mm petri dish before addition of the calcium phosphate–treated DNAs. Cells were cultured for 16 h at 37°C in complete media before transfer to 100/150-mm petri dishes containing DME, 10% FCS, 2 mM glutamate, and 0.5 mg/ml G418. After 10–14 d in culture, G418-resistant colonies were isolated and characterized for L1 expression.

Characterization of Transfected Cells

Control and G418-resistant clones were characterized for expression of L1 by immunocytochemistry and Western blotting using the 5G3 monoclonal antibody (Mujoo et al., 1986; Wolff et al., 1988) and the Neuro4 mAb. For the generation of the latter antibody, Balb/c mice were immunized with an adult human brain glycoprotein fraction. After fusion with p3x63Ag8.653 cells and selection in HAT, the Neuro4 antibody was selected by immunoblotting of 200/190- and 140-kD bands in crude membrane fractions. Cultures were processed for immunocytochemistry by sequential incubation with 5G3 or Neuro4 (both at 1:500 dilution of ascites), biotinylated anti–mouse Ig and Texas red streptavidin (Amersham International, Amersham, UK) (both diluted 1:500) as previously described (Doherty et al., 1991*a*). Western blotting of whole cell extracts of control and transfected 3T3 cells and PC12 cells was carried out essentially as previously described using the primary antibodies at a 1:200 dilution and the ECL Western blotting reagents from Amersham International (Moore et al., 1987; Doherty et al., 1991*a*). The relative level of human L1 on the various clones of transfected cells was determined by measuring the binding of a saturating concentration of Neuro4 by standard enzyme-linked immunoadsorbent assay (Doherty et al., 1990*a*). Results obtained with 5G3 were no different from those obtained with Neuro4 and examples of the latter only are shown throughout. PC12 cells co-cultured on monolayers of control and transfected 3T3 cells (see below) were also immunostained with purified Ig fraction of a rabbit antiserum raised against mouse L1 (Rathjen and Schachner, 1984), using biotinylated anti–rabbit Ig and Texas-red streptavidin as above.

Cell Culture and Neurite Outgrowth

The neurite-outgrowth promoting activity of transfected human L1 was determined as previously described for transfected NCAM and N-cadherin (Doherty et al., 1991*a*, 1992*a–c*). In brief, rat cerebellar neurons isolated at PND 1–9 or naive and primed PC12 cells (see Greene, 1984) were cultured for 16–24 h on confluent monolayers of parental 3T3 cells or clones of 3T3 cells expressing human L1. Co-cultures were established by seeding ~1,000 PC12 cells or ~2,000 cerebellar neurons onto 3T3 cell monolayers established in individual chambers of eight-chamber Lab-Tek slides. The co-culture media was SATO supplemented with 2% FCS (Doherty et al., 1992*a*). In some experiments the levels of calcium and magnesium were

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changed by direct supplementation of calcium/magnesium-free DME as indicated (see text). The average length of the longest neurite on PC12 cells and cerebellar neurons was determined using a Sight Systems Image Manager (Sight Systems, Newbury, England) as previously described (Doherty et al., 1991*a*).

Other Reagents

Pertussis toxin and K-252b were gifts from Dr. J. Kenimer and Dr. Y. Matsuda. Heparin, diltiazem, and verapamil were from Sigma Chemical Co. (St. Louis, MO). Nifedipine was from Life Technologies Ltd. (Grand Island, NY), ω-conotoxin MVIIA was from Peninsula Laboratories (Liverpool, UK). Endo-N was a kind gift from Dr. J. Roth and the monovalent Fab fraction of antimouse L1 was generously donated by Dr. Fritz Rathjen. All of the reagents were used as previously described (see Doherty et al., 1990*b*, 1991*a*) at concentrations established to block their respective targets and also shown to have no nonspecific effects on neurite outgrowth. BAPTA/AM was obtained from Calbiochem Novabiochem (UK) Ltd. (Nottingham, UK). There was no difference in neuronal cell numbers on control and transfected monolayers in the presence and absence of any of these agents (our unpublished observations, but see Doherty et al., 1991).

Results

Expression and Characterization of Human L1 in 3T3 Cells

NIH-3T3 cells were transfected with one of two distinct plasmids containing the full coding sequence of human L1 and clones selected that were resistant to G418 (0.5 mg/ml). These clones were initially characterized for cell surface expression of human L1 using the well characterized 5G3 mAb (Mujoo et al., 1986) that reacts specifically with human L1 (Wolff et al., 1988) and the Neuro 4 mAb that also reacts specifically with human L1 (J. Hemperly, unpublished observations; this study). Parental 3T3 cells showed weak to negative intracellular staining with both antibodies (not shown, but see Fig. 1 *B*), whereas a number of clones of transfected cells showed bright positive staining over the entire cell surface, again with both antibodies (e.g., see Fig. 1 *A*). Specific binding of antibodies to transfected cells was confirmed by quantitative enzyme-linked immunoabsorbent assay and a number of clones that expressed similar levels of human L1 were thus identified and expanded for further study (data not shown). The presence of the antigen on the cell surface was confirmed by the ability of both antibodies to stain live cells (data not shown). There were no obvious differences between cells transfected with the two plasmids.

Human L1 was further characterized by immunoblotting. Both mAbs recognized a doublet band at ~150–160 kD in extracts of transfected 3T3 cells but failed to show any specific binding to parental 3T3 cells (Fig. 1 *C*). This is unlikely to relate to species-specific activity of the antibodies as the Neuro4 mAb bound to previously reported bands at 190/200 and 140 kD in rat PC12 cells and cerebellar neurons (not shown). Furthermore, a Fab fraction of a rabbit antiserum raised against mouse L1 (Rathjen and Schachner, 1984) showed very strong staining of rat PC12 cells with only low level background staining to 3T3 cells in co-culture (Fig. 1 *B*). This antibody did not however recognize human L1 in the above transfectants. This result was confirmed by quantitative enzyme-linked immunoabsorbent assay; in three independent experiments there was no significant difference in the binding of this antibody to control and human L1 expressing 3T3 cells. These data show that parental 3T3 cells express negligible levels of endogenous L1, and that human L1 expressed in transfected cells exists as a doublet of 150–160 kD. For a comparison, human neuroblastoma cells express L1 as a diffuse component ranging from 200 to 215 kD with additional bands at ~150 kD. Removal of N-linked carbohydrates from the larger human L1 bands shifts the molecular mass to a 150–165-kD doublet (Wolff et al., 1988). Thus, L1 expressed in 3T3 cells runs at a similar molecular mass to L1 in human neuroblastoma cells but fails to show the same degree of heterogeneity, probably due to a more limited pattern of posttranslational processing. Similar results have been found with 3T3 cells transfected with NCAM (Doherty et al., 1989).

Neurite Outgrowth on Monolayers of Control 3T3 Cells and 3T3 Cells Expressing Human L1

In our initial experiments, we cultured naive PC12 cells for 20–48 h on confluent monolayers of control 3T3 cells and 3T3 expressing human L1. In a typical experiment there was no obvious morphological response at 20 h, but a significant enhancement of neurite outgrowth was clearly apparent by 48 h. For example, in one experiment the mean length of the longest PC12 cell neurite was 35.6 ± 2 μm on L1 transfectants as compared to 18.0 ± 1 μm on parental 3T3 cells ($P < 0.005$, each value the mean ± SEM of ~120 PC12 cells) with the percentage of these neurites >20 μm in length increasing from 40 to 79%. Thus, L1 appears to stimulate neurite outgrowth to a similar extent as transfected NCAM and N-cadherin (Doherty et al., 1991*a*) and there was no obvious difference in the morphology of PC12 cells on monolayers expressing these individual CAMs (data not shown).

To try to obtain a more rapid response from PC12 cells we initially cultured them for 3–6 d in NGF (~50 ng/ml) before culturing them on monolayers of control and transfected 3T3 cells (in the presence of NGF antibodies to neutralize any residual NGF). Fig. 2 shows the mean length of the longest PC12 cell neurite after 16 h of culture on monolayers of control 3T3 cells as compared to three individual clones of transfected 3T3 cells that express similar levels of L1. In each case the length of the longest neurite was significantly greater on L1 expressing cells ($P < 0.005$). Primed PC12 cells also showed a more rapid response to transfected NCAM and N-cadherin (see below) and this phenomenon may relate at least in part to NGF-induced increases in L1 (aKa NILE), NCAM, and N-cadherin in PC12 cells (McGuire et al., 1978; Mann et al., 1989; Doherty et al., 1991*a*).

As the three L1 expressing clones (which vary in their level of expression by <15%; data not shown) promote neurite outgrowth by a similar extent, we have focused our attention on clone 1. Also, control experiments with PC12 cells showed that the most substantial benefit of pretreatment with NGF was over a 3–4 day period, and this was therefore used in all subsequent experiments. The overall effect of human L1 on neurite outgrowth from primed PC12 cells, determined in the five independent consecutive experiments, is shown in Fig. 3. There was a highly significant 92% increase in the mean length of the longest neurite, and the percentage of cells with a neurite >40 μm increased by a factor of 2.6 from 24 to 63%. In parallel experiments, transfected NCAM and N-cadherin increased the length of the longest neurite by 102 ± 12 (3)% and 84 ± 21 (3)%, respectively, (both values mean ± SEM for the given number of independent experi-

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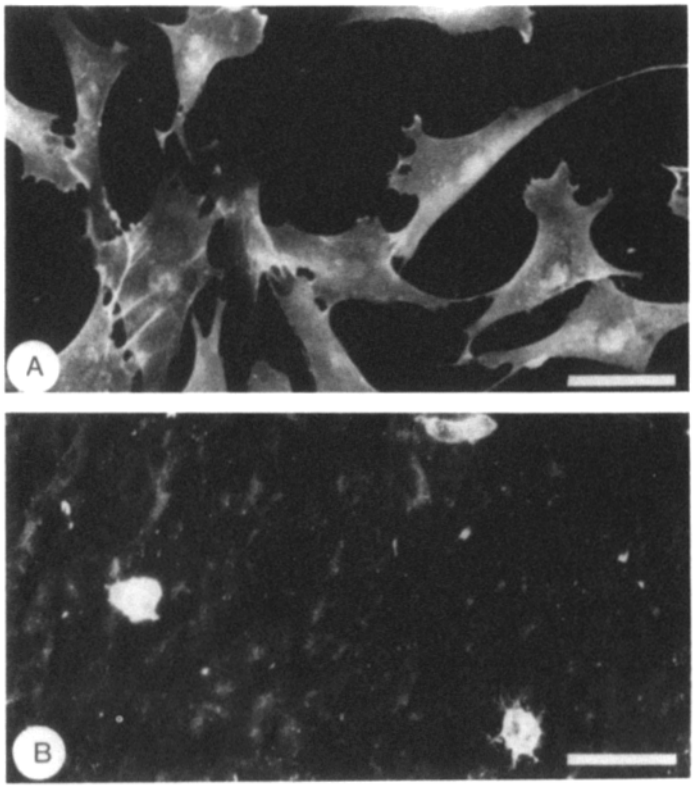

Figure 1. L1 immunoreactivity in transfected 3T3 cells and PC12 cells. (*a*) A culture of transfected 3T3 cells was fixed with 4% paraformaldehyde and stained with the Neuro4 mAb (1:500 dilution). Positive staining was found over the entire surface of the cells. (*b*) A co-culture of naive PC12 cells on a confluent monolayer of control 3T3 was fixed with paraformaldehyde and stained with rabbit antibodies raised against mouse L1. Note the bright positive staining on the PC12 cells and the failure of the antibody to bind to control 3T3 cells. (*c*) The Neuro4 mAb recognized major bands at ~150–160 kD in Western blots of SDS extracts of transfected 3T3 cells (lane *1*), but failed to bind to any bands in untransfected 3T3 cells (lane *2*). Bars, 50 μm.

ments). Thus, over a ~16-h period of co-culture all three CAMs promote neurite outgrowth from primed PC12 cells by a similar extent. Transfected NCAM and N-cadherin can also promote neurite outgrowth from a variety of primary neurons including rat cerebellar neurons (e.g., see Doherty et al., 1992*a*). In the present study cerebellar neurons isolated at PND 1, 2, 3, 4, and 9 were cultured for ~24 h on confluent monolayers of control and human L1 expressing 3T3 cells before being fixed and the average length of the longest GAP-43 positive neurite was determined for each cell. Expression of human L1 was associated, in each of five independent experiments, with a significant ($P < 0.005$) neurite outgrowth promoting response. There was no evidence for a differential response between PND1 and PND9 and the pooled results from the five experiments are shown in Fig. 3 alongside those for primed PC12 cells. At PND9 the response to L1 (an increase in mean length from 32.1 ± 2.0 μm to 72.0 ± 4.7 μm, and in the percentage of cells with a neurite longer than 40 μm from 24.6 to 75.6%) was slightly greater than the average response. The same neurons rapidly lose their ability to respond to transfected NCAM over the PND6-PND8 period (Doherty et al., 1992*a*,*b*). Therefore neuronal responsiveness to NCAM and L1, in terms of neurite outgrowth, are not co-ordinately regulated.

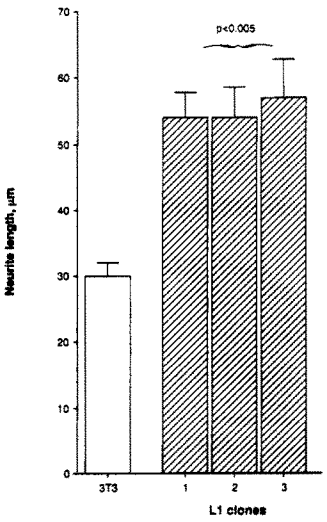

Figure 2. The length of the longest PC12 cell neurite on control and human L1-expressing 3T3 cells. Primed PC12 cells (6 d pretreatment with 50 ng/ml NGF) were cultured on monolayers of control 3T3 cells or three independent clones of transfected 3T3 cells expressing human L1. Co-cultures were fixed after 16 h and the length of the longest neurite on Thy-1 stained PC12 cells was determined. Each value is the mean + 1 SEM of 120–150 PC12 cells sampled in replicate cultures. Statistical significance was determined for the difference in means for growth on control as compared to transfected 3T3 cells by the *t* test.

Antibodies to Neuronal L1 Block Human L1-dependent Neurite Outgrowth

To show unequivocally that the increased neurite outgrowth on L1 transfected cells was indeed dependent on L1 function, a Fab fraction of an anti-mouse L1 rabbit antiserum was added to cultures of both primed PC12 cells and rat cerebellar neurons co-cultured on control and human L1 expressing 3T3 cells. This antibody bound avidly to rat L1 (see Fig. 1 *B*) but did not show any significant binding to control 3T3 cells (Fig. 1 *B*) and 3T3 cells expressing human L1 (see above). The results of a typical experiment are shown in Fig. 4. This antibody completely inhibited the human L1-associated response from both PC12 cells and rat cerebellar neurons. In a total of three independent experiments (two with PC12 cells, one with cerebellar neurons) the L1 response was inhibited by 92.3 ± 9.3% (mean ± SEM). The specificity of the antibody reagent has been established by showing that it does not inhibit neurite outgrowth over control 3T3 monolayers nor does it inhibit NCAM or N-cadherin-dependent

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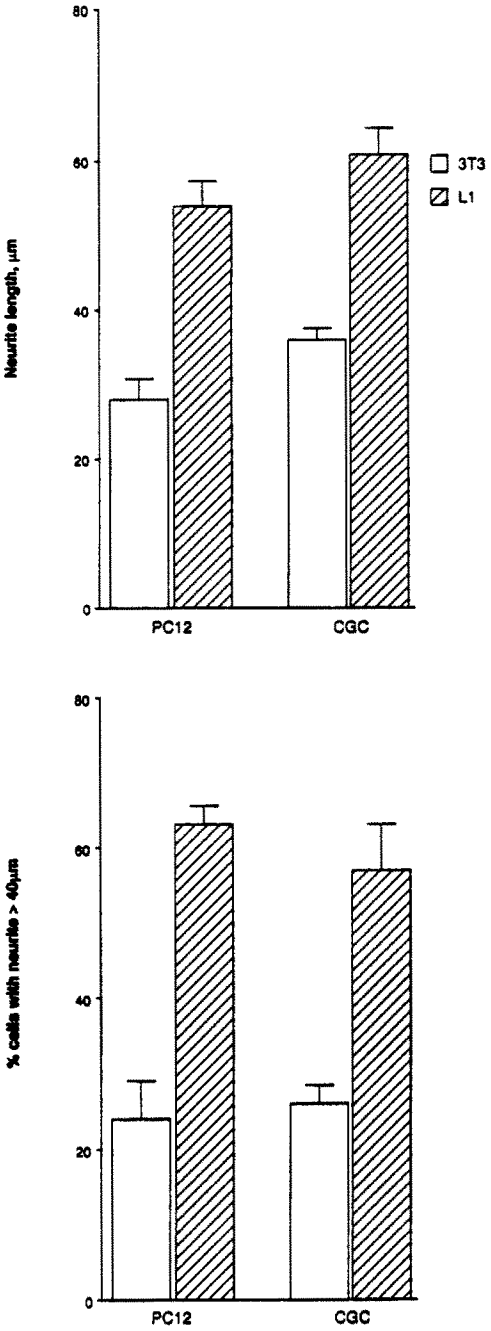

Figure 3. Neurite outgrowth from PC12 cells and cerebellar neurons on control and human L1-expressing 3T3 cells. Primed PC12 cells and cerebellar granule cells (*CGC*) were cultured on monolayers of control 3T3 cells or 3T3 cells expressing human L1. After ~16 h PC12 cell co-cultures were fixed and stained for Thy-1 and after ~24 h the cerebellar co-cultures were fixed and stained for GAP-43. The length of the longest neurite on each cell was determined and the results show mean neurite length (*top*) or the percentage of cells with a neurite >40 μm in length (*bottom*). Each value is the mean (+1 SEM) for five independent consecutive experiments.

neurite outgrowth (Doherty et al., 1991*a*). As the antibody bound exclusively to L1 in the neuron these data provide substantive evidence that a homophilic binding of rat L1 to human L1 underlies the above response (see also Lemmon et al., 1989).

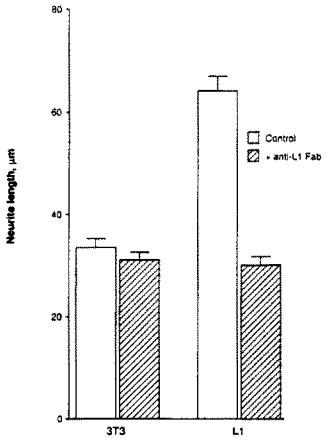

Figure 4. Antibodies to neuronal L1 block human L1-dependent neurite outgrowth. Primed PC12 cells were cultured on monolayers of control and human L1 expressing on 3T3 cells in control media or media supplemented with a monovalent Fab fraction of a rabbit antiserum to mouse L1 (at 250 μg/ml). This antibody bound to the PC12 cells but not the monolayers (see text). After ~16 h the cultures were fixed and the length of the longest neurite on each PC12 cell was determined. Each value is the mean + 1 SEM for 120–150 PC12 cells sampled in replicate cultures.

Pertussis Toxin Blocks the L1 Response

Pertussis toxin ribosylates the α subunit of heterotrimeric G proteins of the Gi/Go families and thereby inhibits their function. We have previously shown that pertussis toxin can block NCAM- and N-cadherin–dependent neurite outgrowth from PC12 cells and that pretreatment of PC12 cells is sufficient for maximal inhibition (Doherty et al., 1991*a*). In the present study, pertussis toxin was added to PC12 cells and cerebellar neurons were cultured on control and human L1 expressing 3T3 cells with the result from the latter shown in Fig. 5. Pertussis toxin completely abolished the response to L1 without affecting basal (presumably integrin dependent) neurite outgrowth over control 3T3 cells. Results pooled from a total of four independent experiments (three with PC12 cells, one with cerebellar neurons) showed pertussis toxin to block the L1 response by 91.5 ± 8.1%. In the presence of pertussis toxin, neurite outgrowth on control 3T3 monolayers was 102.7 ± 4.0% of that found in the absence of toxin (both values mean ± SEM). The target for pertussis toxin was a neuronal rather than 3T3 cell G-protein as demonstrated by the fact that pretreatment of neurons but not pretreatment of monolayers was sufficient for maximal inhibition of the response (data not shown).

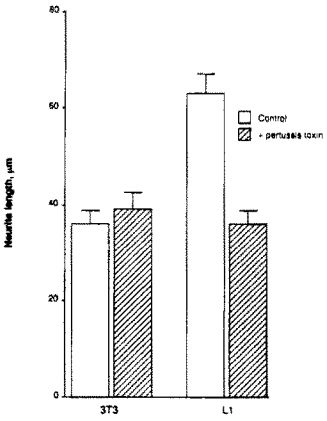

Figure 5. Pertussis toxin inhibits the L1 response. Cerebellar neurons were cultured on monolayers of control and human L1 expressing 3T3 cells for ~24 h in control media or media supplemented with pertussis toxin (500 ng/ml). The results show the mean length of the longest neurite per cell and each value is the mean + 1 SEM for 120–150 neurons sampled in replicate culture.

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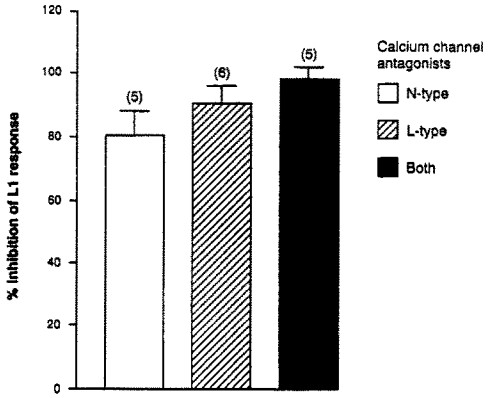

Figure 6. The effect of calcium channel antagonists on the L1 response. Primed PC12 cells and cerebellar neurons were cultured on monolayers of control 3T3 cells or 3T3 cells expressing human L1 in the presence and absence of antagonists of N-type calcium channels (ω-conotoxin at 0.25 μM), L-type calcium channels (diltiazem, verapamil, or nifedipine, all at 10 μM) or a combination of both (for details, see text). The percentage increase in mean neurite length on L1 expressing 3T3 cells as compared to control 3T3 cells was determined in each instance and the results show the ability of calcium channel antagonists to inhibit this response. Each value is the mean + 1 SEM for the given number of independent experiments. None of these agents significantly affected neurite outgrowth over control 3T3 cell monolayers (Doherty et al., 1991*b*). For example, in five experiments a combination of ω-conotoxin and an L-type channel antagonist reduced growth on parental 3T3 cells by 6.6 ± 5.1% (mean ± SEM).

L- and N-type Calcium Channel Antagonists Inhibit L1-dependent Neurite Outgrowth

Verapamil, diltiazem, and nifedipine specifically block L-type calcium channels in cells, whereas ω-conotoxin blocks N-type calcium channels (e.g., see Discussion). In the present study these reagents were tested for their ability to block the L1 response in both PC12 cells and cerebellar neurons. There was no significant difference in results obtained with each of the individual L-type channel antagonists (each tested twice) and no major difference in the results obtained with PC12 cells and cerebellar neurons. The results have therefore been pooled and are summarized in Fig. 6. From these data it can be seen that blocking L- or N-type calcium channels on their own was sufficient to inhibit the L1 response by 91 ± 5.2% (n = 6) and 80.8 ± 8.0% (n = 5), respectively. When both were blocked the response was inhibited by 99.4 ± 3.8% (n = 5). Again control experiments confirmed previous results by demonstrating that these agents do not modulate neurite outgrowth over parental 3T3 cells (see Fig. 6 legend).

Reducing Extracellular Calcium or Preloading Neurons with a Calcium Chelator Inhibits L1-dependent Neurite Outgrowth

The experiments with calcium channel antagonists suggest that an influx of extracellular calcium into neurons is required for CAM-dependent neurite outgrowth. To test this more directly, cerebellar neurons were cultured on monolayers of control and human L1 expressing 3T3 cells in the presence of varying levels of extracellular calcium. Fig. 7 *A* shows that basal neurite outgrowth (and hence cell viability) is not affected by reducing extracellular calcium to 0.25 mM

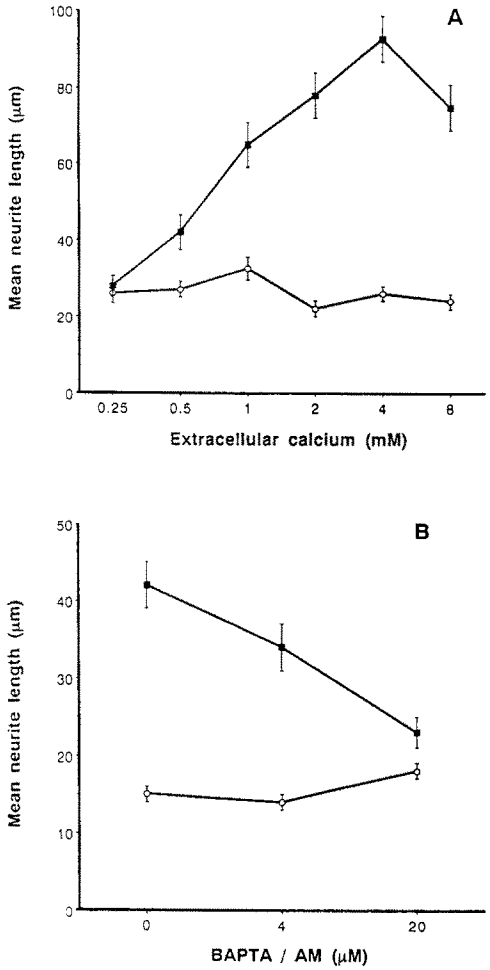

Figure 7. L1-dependent neurite outgrowth is abolished by reducing extracellular calcium or preloading neurons with a calcium ion chelator. (*a*) Cerebellar neurons were cultured under conditions of varied extracellular calcium (0.25, 0.5, 1, 2, 4, and 8 mM) in the presence of a constant magnesium concentration (0.5 mM) on monolayers of control 3T3 cells (○) or 3T3 cells expressing human L1 (■). After ~24 h co-cultures were fixed and stained for GAP-43. The length of the longest neurite on each cell was determined and the results show mean neurite length. (*b*) Cerebellar neurons were preloaded with calcium chelating agent BAPTA/AM (0, 4, and 20 μM in SATO 2% FCS containing 4 mM Ca^{2+} and 0.5 mM Mg^{2+}) for 2 h at 37°C. Co-cultures (parental 3T3, ○, and human L1, ■) were established after a 70-fold dilution of neurons. Control experiments showed that the residual BAPTA/AM (<0.3 μM) had no effect on neurite outgrowth. After ~16 h co-cultures were fixed and stained for GAP-43. The results show the mean length of the longest neurite per cell. For both *a* and *b*, each value is the mean ± SEM for 120–150 neurons sampled in replicate cultures.

or increasing it to 8 mM. In contrast, L1-dependent neurite outgrowth was absolutely dependent on the extracellular calcium concentration being >0.25 mM, with the response peaking at 4 mM. Identical results were obtained for NCAM-dependent neurite outgrowth (data not shown).

Additional supporting evidence for calcium influx into the neurons underlying the response was obtained by preloading the neurons with BAPTA/AM (e.g., see Koike et al., 1989). This calcium chelator is membrane-permeant and enters the cell where it is sequestered by hydrolysis of its acetoxymethyl ester group. Once inside the cell it will chelate and

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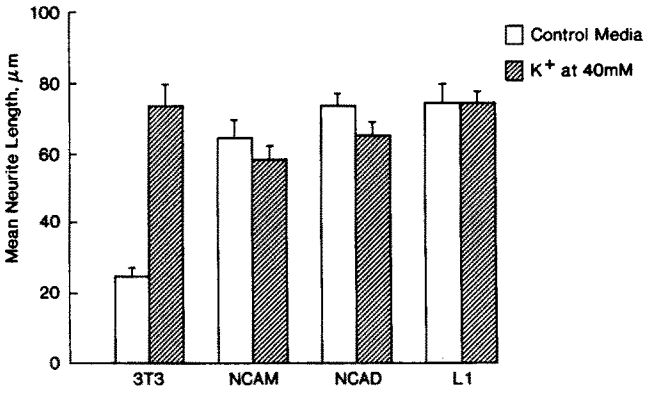

Figure 8. K^+ depolarization mimics CAM-dependent neurite outgrowth by cerebellar neurons (PND4). Cerebellar neurons were cultured on monolayers of control 3T3 cells and 3T3 cells expressing either NCAM, NCAD, or human L1, in the presence or absence of elevated extracellular potassium (40 mM). Co-cultures were fixed and stained for GAP-43 after ~24 h. The results show the mean neurite length of the longest neurite per cell. Each value is the mean ± SEM for 120–150 neurons sampled in replicate cultures.

thereby attenuate changes in intracellular calcium. The results in Fig. 7 *B* show that BAPTA/AM pretreated neurons can extend axons as normal on parental 3T3 cells. In contrast, L1-dependent neurite outgrowth was significantly inhibited by pretreatment with 4 μM BAPTA/AM and fully inhibited by 20 μM BAPTA/AM. Koike et al. (1989) have previously shown that pretreatment of sympathetic neurons with 20 μM BAPTA/AM or withdrawal of extracellular calcium can also block K^+ depolarization dependent survival, over a 48-h culture period.

Calcium Influx into Neurons Fully Mimics CAM-dependent Neurite Outgrowth

Second messenger pathway activation by K^+ depolarization has previously been shown to mimic the NCAM and N-cadherin response of PC12 cells (Saffell et al., 1992). In this study, potassium depolarization induced significant neurite outgrowth from cerebellar neurons cultured on monolayers of 3T3 fibroblasts. The effect of potassium (5–100 mM) was dose dependent with an optimal response found at a concentration of 40 mM. The response was comparable but not additive to CAM-induced neurite outgrowth, indicating the induction of a common pathway (Fig. 8). The potassium-induced response could be inhibited by reduction of extracellular calcium to 0.25 mM, N- or L-type calcium channel blockers either on their own (not shown) or in combination, and treatment with the calcium chelator BAPTA/AM. Pertussis toxin did not inhibit potassium-induced neurite outgrowth from cerebellar neurons (Table I). It has previously been shown that pertussis toxin did not inhibit the potassium response of PC12 cells (Saffell et al., 1992). These data confirm that potassium-induced neurite outgrowth is also dependent on calcium influx through N- and L-type calcium channels. The failure of a combination of N- and L-type calcium channel inhibitors to fully block the response, most likely reflects the fact that potassium depolarization–induced increases in intracellular calcium can be substantially, but not completely inhibited by these antagonists (Reber et al., 1992).

Table 1. Effects of Various Treatments on Neurite Outgrowth Induced by K^+ Depolarization

Media	Mean neurite length
	μm
Control*	34.5 ± 3.0 (123)
K^+ at 40 mM‡	78.7 ± 5.6 (119)*
(i) Low calcium	37.8 ± 4.0 (123)‡
(ii) + Diltiazem and ω-conotoxin	42.8 ± 4.4 (116)‡
(iii) + BAPTA/AM	33.9 ± 3.0 (134)‡
(iv) + Pertussis toxin	77.5 ± 4.0 (130)§

Cerebellar neurons (PND4) were grown for 24 h on confluent monolayers of 3T3 cells in (*a*) control media, or (*b*) media supplemented with K^+ at 40 mM in the presence of reduced extracellular calcium (0.25 mM), diltiazem (10 μM) and ω-conotoxin (0.25 μM), BAPTA/AM (20 μM), or pertussis toxin (1 μg/ml). None of the treatments (i–iv) had any significant effect on neurite outgrowth on 3T3 monolayers in control media (see Figs. 5–7). The results show the mean neurite length of the longest neurite per cell ± SEM for the given numbers of cerebellar neurons sampled from replicate cultures.
* Significantly different from growth in absence of K^+ ($P < 0.0005$).
‡ Significant inhibition of K^+ response ($P < 0.0005$).
§ Nonsignificant difference from growth in presence of K+ ($P < 0.25$).

Agents That Perturb NCAM Function Do Not Directly Modulate L1-dependent Neurite Outgrowth

Heparin binds to the second Ig domain of NCAM and blocks its function by either sterically hindering homophilic binding and/or preventing NCAM interactions with heparin-sulphate–containing proteoglycans (Cole and Glaser, 1986; Cole and Akeson, 1989). Heparin (250 μg/ml) completely blocks NCAM-dependent neurite outgrowth (Doherty et al., 1990*a*,*b*). In the present study, in the absence of heparin, L1 increased the length of the longest PC12 cell neurite by 91 ± 8.9%, whereas in its presence neurite length was increased by 75 ± 11% (both values mean ± SEM for measurements made on ~120 PC12 cells). Thus heparin does not block L1 function.

The α2-8-linked PSA that is present predominantly, if not exclusively, on NCAM can be specifically removed by endoneuraminidase N (endo N) (Rutishauser et al., 1988; Doherty et al., 1990*b*). Removal of PSA from neuronal NCAM substantially inhibits NCAM-dependent neurite outgrowth over NCAM-transfected cells. At PND4, cerebellar neurons are particularly sensitive to removal of PSA (Doherty et al., 1992*a*). In the present study, a maximally active concentration of endo N was added to these neurons growing on parental and L1 expressing 3T3 cells. As previously reported, endo N had no effect on basal neurite outgrowth (38.0 ± 3.0 μm as compared with 36.0 ± 2.7 μm), nor did endo N affect the enhanced growth apparent on L1 transfectants (67.3 ± 4.3 μm as compared to 62.9 ± 4.0 μm; both sets of values are the mean ± SEM for ~150 neurons measured in the presence and absence of endo N, respectively). Thus, the ability of neuronal L1 to bind to L1 in the substratum and transduce the recognition event into a cellular response is not directly modulated by the presence of PSA on neurons.

Discussion

Antibodies to L1 have been reported to inhibit granule cell migration (Lindner et al., 1983) and perturb fiber outgrowth

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(Fischer et al., 1986) in microexplants of the developing cerebellum. In addition, antibodies to L1 can induce defasciculation of axon bundles (Rathjen, 1988) and reduce neurite outgrowth along other neurites (Chang et al., 1987) and over the surface of Schwann cells (Seilheimer and Schachner, 1988; Bixby et al., 1988). In addition to NCAM, the expression of L1 and the immunologically related Ng CAM is reduced on both axons and Schwann cells after fiber tract formation, but all three molecules are upregulated after injury to the peripheral nervous system (Daniloff et al., 1986; Martini and Schachner, 1988). All of these data suggest that L1 may play an important role in fibre tract formation.

In the present study, we have expressed human L1 in mouse NIH-3T3 fibroblasts. These cells have been shown to express negligible amounts of endogenous L1 by immunocytochemistry, immunoblotting, and by antibody perturbation. Expression of human L1 was associated with an enhanced ability of the transfected cells to promote neurite outgrowth from naive and primed PC12 cells and from rat cerebellar neurons isolated over the PND1-PND9 period of development. These responses could be fully inhibited by antibodies that specifically bind to and block the function of rat L1. These data provide substantive evidence that the human L1 promotes neurite outgrowth by directly binding to neuronal L1 (see also Lemmon et al., 1989).

The ability of PC12 cells and primary neurons to respond to transfected NCAM and N-cadherin by increasing neurite outgrowth is dependent upon the activation of a common second messenger pathway in the neurons (Doherty et al., 1991*a*, 1992*a–c*). Activation of this pathway can be inhibited by pertussis toxin, and the main trigger for the response appears to be the opening of both N- and L-type calcium channels. Evidence for this comes from both the above perturbation studies, and also from more recent studies that demonstrate that direct activation of calcium channels can fully mimic the CAM response (Saffell et al., 1992).

In the present study, we have provided the first evidence that L1-dependent neurite outgrowth from PC12 cells and primary neurons involves activation of this (or a very similar) pathway. The L1 response could be fully inhibited by pertussis toxin or a combination of L- and N-type calcium channel antagonists. An unexpected observation was that L- or N-type antagonists could substantially (80–90%) inhibit the L1 response on their own. Similar results have now also been observed in a limited number of experiments for NCAM/N-cadherin–dependent neurite outgrowth from the same neurons. This contrasts with previous studies on naive PC12 cells (Doherty et al., 1991*a*) and on hippocampal neurons (Doherty et al., 1992c) where an inhibition of NCAM-dependent neurite outgrowth by $>\sim$60% required the addition of both N- and L-type antagonists. The likeliest explanation of the current data is that a threshold level of calcium is required for the response, and that in some instances flux through both types of calcium channel is required to reach this value (see also Kater and Mills, 1991). Thus, regulation at the level of calcium influx could contribute to the previously reported threshold effect of NCAM on neurite outgrowth (Doherty et al., 1990*a*) and also for the synergism between cotransfected NCAM and N-cadherin in promoting neurite outgrowth (Doherty et al., 1991*b*). A greater than maximal activation of a single pathway would also readily explain the redundancy of individual CAMs apparent in some antibody perturbation studies (see Bixby et al., 1987). Direct evidence for a calcium influx into the neurons mediating L1-dependent neurite outgrowth was obtained by showing that reduction in extracellular calcium, or pre-loading neurons with a calcium chelator, specifically abolished this response.

A very important question is whether the above perturbants block a relatively specific CAM activated pathway or whether they simply block steps that are common to a variety of pathways that lead to neurite outgrowth. Our own published studies have shown that integrin dependent neurite outgrowth from PC12 cells and primary neurons is not inhibited by pertussis toxin and calcium channel antagonists. Likewise NGF dependent neurite outgrowth from PC12 cells is also not affected (Doherty et al., 1991*a*, 1992*a–c*). More recently these inhibitors have been shown to have no effect on neurite outgrowth stimulated by agents that operate by increasing the level of intracellular cAMP in PC12 cells (Saffell et al., 1992). Thus, to date, the only molecules that activate this pathway are NCAM, N-cadherin and L1, suggesting that this is indeed a CAM specific pathway for neurite outgrowth. That there are undoubtedly convergent steps downstream of calcium channel activation is demonstrated by the ability of K-252b, a general kinase inhibitor, to inhibit all of the above pathways that lead to neurite outgrowth (our own unpublished observations, see also Doherty et al., 1991*a*).

Recent studies on transfected NCAM suggest that lateral diffusion in the substratum may be important for activation of the above pathway in neurons (Doherty et al., 1992*b*). The fact that at least three CAMs can activate the same pathway raises the possibility of an 'adaptor' molecule that can interact with several CAMs and also with the effector molecule(s). However in this context it should be noted that various CAMs are directly and/or indirectly associated with each other; for example antibodies to NCAM can co-cluster L1 (Kadmon et al., 1990*b*) and L1 and Axonin-1 co-localize to patches on cell somas and neurites (Kuhn et al., 1991). Thus the adaptor molecule could conceivably be one of the above CAMs. In addition, local hot spots of calcium channels have been described in the growth cone membrane, and these are associated with areas of morphological change (Silver et al., 1990). Thus the possibility that CAMs could directly activate calcium channels by co-clustering them should also be considered, although the fact that pertussis toxin can block the response clearly suggests that other molecules are involved.

Purified CAMs coated to an otherwise inert substratum do not appear to promote neurite outgrowth via activation of the above second messenger pathway (P. Sonderegger and J. Bixby, individual personal communications). In these studies CAM dependent adhesion per se may be sufficiently permissive to allow for neurite outgrowth. Failure to activate the pathway may be directly related to the fact that the CAMs are immobilized on the substratum. Diffusional entrapment of adhesion molecules into transient clusters on one membrane may be dependent on similar events in the apposing membrane and this has been evoked as a mechanism for activation of second messenger pathways in lymphocytes (Singer, 1992). We would suggest that similar models, possibly including co-clustering of an adaptor or effector molecule, may account for activation of the CAM specific second messenger pathway in neurons.

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In contrast to neurite outgrowth, cell adhesion is associated with the formation of stable adhesion plaques and this most probably involves linkage of CAMs to the underlying cytoskeleton (e.g., see Nagafuchi and Takeichi, 1988). It has been suggested that PSA on neuronal NCAM can act as a global modulator of CAM function by sterically hindering membrane apposition and thereby modulating *trans*-binding of a variety of CAMs and in particular L1 (Rutishauser et al., 1988; Landmesser et al., 1990). NCAM and L1 may be physically associated in the same membrane (Simon et al., 1991), and it is also conceivable that PSA could modulate L1 function via this *cis*-interaction. In the present study we have shown that removal of PSA from neuronal NCAM has no direct effect on L1 function as a neurite outgrowth promoting molecule. This completes a series of studies in which we have previously shown that this treatment can inhibit NCAM-dependent neurite outgrowth by up to 80% (Doherty et al., 1992*a*), but has no effect on integrin or N-cadherin–dependent neurite outgrowth. Thus in terms of neurite outgrowth, but not adhesion (Acheson et al., 1991) PSA can be considered as a highly specific modulator of NCAM function. PSA may operate by favoring the formation of transient rather than stable clusters of NCAM via charge repulsion and/or steric hinderance and this may favor neurite outgrowth at the expense of adhesion. As CAMs can clearly interact to promote neurite outgrowth, a direct modulation of NCAM function would in some systems be expected to indirectly modulate the function of other molecules and in particular L1.

During development, cerebellar neurons lose their ability to respond to NCAM over a very short period (PND6–PND8), and this most probably relates to increased expression of NCAM isoforms containing the product of VASE exon (Doherty et al., 1992*a* and our own unpublished observations). The fact that the same neurons remain highly responsive to L1 demonstrates that neuronal responsiveness to NCAM and L1 is not co-ordinately regulated. In addition, the above data show that two independent mechanisms that down regulate NCAM-dependent neurite outgrowth, i.e., loss of PSA and use of the VASE exon, do not directly impinge on L1's ability to promote neurite outgrowth.

Finally, in the present study we have shown that NCAM- and L1-dependent neurite outgrowth can be dissociated from NCAM/L1–dependent adhesion as relatively modest reductions in extracellular calcium inhibit the former but not the latter (e.g., see Miura et al., 1992). The reduction in calcium did not impair neurite outgrowth per se as this was unaffected on control 3T3 monolayers (see also Campenot and Draker, 1989). It follows that CAM-dependent adhesion does not directly contribute to the neurite outgrowth response. Rather, CAM-dependent neurite outgrowth would appear to be absolutely dependent on the ability of NCAM and L1 to provide a recognition signal that is transduced into a cellular response via the activation of a CAM-specific second messenger pathway in neurons (Doherty and Walsh, 1992). The fact that L1-dependent neurite outgrowth can be fully inhibited by reduction of the level of extracellular calcium or by calcium channel blockers or pretreatment of neurons with a calcium chelating agent indicates that calcium influx into neurons is the key step in the L1-dependent response. Direct stimulation of calcium influx into neurons can, in the absence of any presumptive adhesion step, fully mimic the cell-contact–dependent neurite outgrowth response stimulated by L1, NCAM, and N-cadherin. We would therefore conclude that activation of this second messenger pathway is likely to be solely responsible for the neurite outgrowth promoting activity of a large number of CAMs.

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