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A developmentally regulated switch in neuronal responsiveness to NCAM and N-cadherin in the rat hippocampus

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Summary

Monolayers of control 3T3 fibroblasts and 3T3 cells expressing transfected NCAM or N-cadherin have been used as a culture substratum for rat hippocampal neurons. Both NCAM and N-cadherin are expressed in the hippocampus through embryonic day 17 (E17) to postnatal day 4 (PND4); however, whereas E17 neurons responded to transfected NCAM by extending considerably longer neurites, PND4 neurons responded very poorly. The converse was true for responsiveness to N-cadherin. These data demonstrate a switch in neuronal responsiveness to NCAM and N-cadherin in the developing hippocampus. NCAM-dependent neurite outgrowth from E17 neurons was largely dependent on the presence of α2-8-linked polysialic acid (PSA) on neuronal NCAM. NCAM-dependent neurite outgrowth could be fully inhibited by pertussis toxin or a combination of L- and N-type calcium channel antagonists thus providing direct evidence concerning the nature of the second messenger pathway activated in primary neurons by cell adhesion molecules (CAMs).

Key words: NCAM, N-cadherin, polysialic acid, neurite outgrowth, hippocampus, cell adhesion.

Introduction

During development, neurons extend axons over considerable distances in a very precise manner in order to synapse with their appropriate target tissue. A considerable amount of the positional information required for growth and possibly also guidance is likely to be read and interpreted by a combination of three distinct classes of receptors present on axonal growth cones. These include some members of a large family of αβ heterodimers called integrins that promote adhesion to and neurite outgrowth over extracellular matrix components such as laminin (Reichardt and Tomaselli, 1991), members of the immunoglobulin gene superfamily including the neural cell adhesion molecule NCAM (Williams, 1987; Cunningham et al., 1987; Walsh and Doherty, 1991) and finally members of the cadherin superfamily, in particular neural or N-cadherin (Takeichi, 1988; 1991). NCAM and N-cadherin differ from the integrins in that they operate via a homophilic rather than heterophilic binding mechanism; that is, they serve as both their own receptor and ligand (e.g. see Edelman et al., 1987; Doherty et al., 1991a; Takeichi, 1991). The importance of these molecules for axonal growth has been established by a large number of antibody perturbation experiments. For example, whereas neurite outgrowth over fibroblasts can be fully inhibited by antibodies that block the function of β1 integrins, a maximal block of neurite outgrowth over myoblasts, astrocytes and Schwann cells requires a cocktail of antibodies that block the function of β1 integrins, NCAM, N-cadherin and the L1 glycoprotein (Bixby et al., 1987; Tomaselli et al., 1988; Neugebauer et al., 1988; Seilheimer and Schachner, 1988; reviewed in Doherty and Walsh, 1989). In addition, purified laminin (Rogers et al., 1983), L1 (a.k.a. G4, 8D9 antigen and NgCAM) (Lagenaur and Lemmon, 1987) and N-cadherin (Bixby and Jhabvala, 1990) are all potent substrata for neurite outgrowth. Similarily when N-cadherin- and NCAM-deficient cells are transfected with complementary DNA (cDNA) for 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 variety of neuronal cell types (Matsunaga et al., 1988; Doherty et al., 1989, 1990a,b, 1991a).

Measurements on growth cone adhesion to various extracellular matrix components suggest that there is no simple correlation between adhesion and neurite outgrowth (Gundersen, 1987) and removal of polysialic acid (PSA) from neuronal NCAM increases its adhesive function (Hoffman and Edelman, 1983) but actually reduces NCAM-dependent neurite outgrowth (Doherty et al., 1990b). Although many of the molecules that can stimulate neurite outgrowth exist as lipid-anchored glycoproteins on the cell surface (e.g.

TAG1, Furley et al., 1990; NCAM, Doherty et al., 1990a), all of the known growth cone receptors that transduce recognition signals into increases in neurite outgrowth are transmembrane spanning glycoproteins with cytoplasmic domains. In the case of integrins and cadherins, the interaction of the cytoplasmic domain with the cytoskeleton has been extensively characterised (reviewed in Takeichi, 1991 and Reichardt and Tomaselli, 1991). It is also likely that the transmembrane spanning isoform of NCAM with a large cytoplasmic domain (the 180×10^3 M_r isoform) that is expressed mainly by neurons can also directly interact with the cytoskeleton (Pollerberg et al., 1987). These data suggest that transmembrane signalling may be an important consequence of the interaction of these receptors with their ligands.

Direct evidence that NCAM and N-cadherin can operate via G-protein-dependent opening of both N- and L-type calcium channels has recently been obtained by culturing PC12 cells on monolayers of control 3T3 fibroblasts or 3T3 fibroblasts expressing transfected chick N-cadherin or human NCAM. Both CAMs directly induced a change in the morphology of PC12 cells from an adrenal to a neuronal phenotype and this could be fully inhibited by pertussis toxin or a combination of L- and N-type calcium channel antagonists (Doherty et al., 1991b).

In the present study, we have cultured dissociated neurons from rat hippocampi on monolayers of control 3T3 fibroblasts or 3T3 fibroblasts that express physiological levels of transfected chick N-cadherin or human NCAM. When isolated at a stage when many of the neurons have just been generated, but are unlikely to have as yet initiated axonal growth (E17), the neurons responded well to transfected NCAM by increasing their rate of neuritic growth, but responded very poorly to N-cadherin. In contrast, when isolated at later stages in development (PND4), the neurons were considerably more responsive to N-cadherin than NCAM. The differential responsiveness of the early neurons was entirely dependent on the presence of α2-8-linked PSA on neuronal NCAM. The NCAM-dependent neurite outgrowth, but not the substantial amount of basal neurite outgrowth on control fibroblasts, was fully inhibited by pertussis toxin or a combination of N- and L-type calcium channel blockers. These data suggest that NCAM may be more important than N-cadherin for determining initial neurite outgrowth from hippocampal neurons, and that this is largely controlled by post-translational addition of α2-8-linked PSA to neuronal NCAM. Furthermore, these data provide the first evidence that NCAM-dependent neurite outgrowth from primary neurons involves the activation of second messenger pathways that are similar, if not identical, to those activated by CAMs in the PC12 cell line.

Materials and methods

Cell culture and determination of neurite lengths

Parental and CAM-transfected clones of mouse 3T3 fibroblasts were cultured as described previously (Doherty et al., 1991b). Monolayers for co-culture were established by seeding 80,000 cells per chamber in 8-chamber tissue-culture slides (Lab-Tek) that had been coated with polylysine followed by collagen. Rat hippocampi were dissected at E17 or PND4 and dissociated by enzymatic treatment with trypsin (0.05%) for 20 minutes at 37°C, followed by gentle trituration through a fire-polished glass pipette to obtain a single cell suspension. Cells were plated at 1500 (E17) or 2000 (PND4) per chamber onto confluent monolayers of control and transfected 3T3 cells. Co-cultures were maintained for 20 hours in serum-free chemically defined SATO medium (see Dohety et al., 1990a). Cultures were then fixed and processed for GAP43 immunoreactivity as described (Doherty et al., 1991a).

GAP43-labelled neurons were detected by fluorescence microscopy using a low-light-sensitive video camera (CGHV, model 4722-5000) and analysed using a Sight Systems Image Manager (Sight Systems, Newbury, England). Cultures were scanned in a systematic manner and the length of the longest GAP43-positive neurite measured. All neurons were included in the analysis (for details, see Doherty et al., 1990a).

Western blot analysis

Western blot analysis was carried out on extracts of E17 and PND4 hippocampi as described in Moore et al. (1987) and modified in Doherty et al. (1991a). The primary antibodies used were a 1:2000 dilution of a rabbit polyclonal antiserum raised to a glutathione transferase fusion protein containing 109 amino acids (314-423) of human N-cadherin extracellular domain sequence (Walsh et al., 1990) or a 1:1000 dilution of a rabbit polyclonal antiserum raised against affinity-purified mouse NCAM (Moore et al., 1987).

Perturbation of neurite outgrowth

Pertussis toxin was a gift from Dr J. Kenimer. It was present throughout the period of culture at 1 μg/ml (for other details, see Doherty et al., 1991b). Endo-N (used at a 1:500 dilution of a phage lysate) specifically removes α2-8-linked PSA from neuronal NCAM and has previously been shown to modulate NCAM-dependent neurite outgrowth in a highly specific manner (see Doherty et al., 1990b; 1991a). Diltiazem was purchased from Sigma and w-conotoxin GVIA from Cabiochem. All reagents were added as ×2 stocks 1-2 hours prior to addition of neurons and were maintained throughout the 20 hour period of co-culture. Control experiments showed that none of the above reagents affects NCAM expression in transfected 3T3 cells.

Results and discussion

NCAM and N-cadherin expression in the hippocampus

During development NCAM generally shifts from a PSA-rich (embryonic) to a PSA-poor (adult) form (Rothbard et al., 1982). This change may reflect a loss of plasticity and establishment of relatively stable synapses (Hoffman and Edelman, 1983; Doherty et al., 1990b). Highly sialylated NCAM is expressed in a restricted manner in the adult brain, for example in the olfactory bulb, nerve and neuroepithelium, a region conspicuous for continued neurogenesis throughout life (Chuong and Edelman, 1984; Graziadei and Monti-Graziadei, 1978). In addition blockade of neuromuscular activity, which results in defasciculation and axonal sprouting, is also associated with a re-expression of PSA on neuronal NCAM (Landmesser et al., 1990). In the

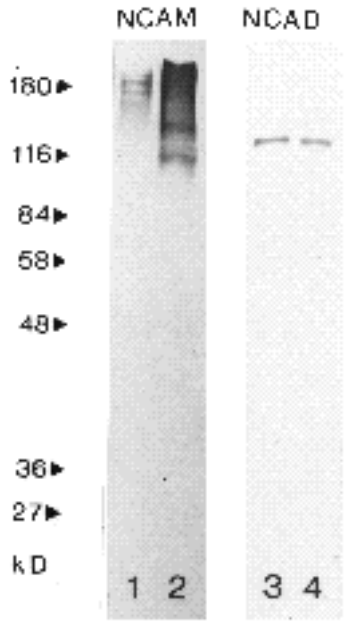

Fig. 1. Western blot analysis of NCAM and N-cadherin in the developing hippocampus. Extracts of E17 (lanes 1 and 3) or PND4 hippocampi (lanes 2 and 4) were subjected to gel electrophoresis and Western blot analysis for NCAM immunoreactivity (lanes 1 and 2) or N-cadherin immunoreactivity (lanes 3 and 4). A total of 40 μg protein was added to lanes 1 and 2 and 10 μg to lanes 3 and 4.

present study, a western blot analysis of NCAM and N-cadherin expression was conducted on E17 and PND4 rat hippocampi. At E17, NCAM blotted as a broad smear between 140 and $200\times10^3 M_r$ characteristic of the embryonic form (Fig. 1, lane 1). The intensity of NCAM staining was considerably greater at PND4, with the smear ranging from 110 to $>200\times10^3 M_r$; however, discrete bands were also apparent at 140 and $120\times10^3 M_r$ (Fig. 1, lane 2). This is indicative of a partial reduction in the extent of NCAM sialylation; however, a monoclonal antibody to α2-8-linked PSA shows expression of the embryonic form of NCAM in the rat hippocampus late into development (Aaron and Chesselet, 1989). In contrast to NCAM, N-cadherin immunoreactivty is observed as a single band with if anything a reduction in intensity over the E17 to PND4 period (Fig. 1, lanes 3 and 4).

Morphology of E17 hippocampal neurons on control and transfected 3T3 cells

Culture of a number of primary neurons on monolayers of transfected NCAM or N-cadherin expressing 3T3 cells is associated with direct induction of neurite outgrowth by the transgene product (Doherty et al., 1989; 1990a,b; 1991a). In the case of NCAM, the response can be inhibited by removal of transfected NCAM from the monolayer, or by antibodies that bind exclusively to neuronal NCAM but not by antibodies that block the function of L1/G4, N-cadherin or β1-integrins. The N-cadherin response can be inhibited by antibodies that block N-cadherin function, but not antibodies to NCAM, L1/G4 or β1-integrins (Doherty et al., 1991a).

In the present study, E17 hippocampal neurons were cultured for 20 hours on monolayers of control 3T3 cells or 3T3 cells expressing physiological levels of human NCAM or chick N-cadherin (e.g. see Doherty et al., 1991a). The neurons attached well to monolayers of control 3T3 cells and many established a clear polarity even after this short period of culture. Representative examples of GAP43-stained neurons are shown in Fig. 2 A-C. Neuronal polarity was characterised by one neuritic process, presumably the axon, being longer than the other processes (e.g. see Dotti et al., 1988). At this early stage of culture, however, polarity was not associated with a loss of GAP43 immunoreactivity from the presumptive dendritic processes (Goslin et al., 1988). Axons on NCAM-expressing monolayers were clearly longer than those on control 3T3 monolayers (Fig. 2 D-F) but by simple visual inspection there was no obvious difference between axons of neurons on control and N-cadherin-expressing monolayers (not shown). The length of the longest GAP43-positive process per cell was measured from 80-100 neurons in each of three independent experiments with the results summarised in Fig. 3. Overall, culture on N-cadherin-expressing monolayers was associated with a small but highly significant ($P < 0.005$) increase in axonal length of on average 23%. In contrast, axons were approximately twice as long on NCAM-expressing monolayers as compared with control monolayers. These observations contrast with those on PND6 rat cerebellar neurons, E6-E12 chick retinal ganglion cells (Doherty et al., 1991a) and PC12 cells (Doherty et al., 1991b) which are all equally or more responsive to N-cadherin than NCAM. One possible reason for this difference may be the early developmental age of the hippocampal neurons.

A major attraction of hippocampal neurons is the obvious polarity they exhibit in vitro (see above). In this context, we could find no evidence for NCAM differentially affecting axonal versus dendritic growth; for example, in one experiment, the length of the longest neurite (presumably the axon) was increased by 93%, whereas the length of the second longest process was increased by 86% (these values are relative to their own controls and on 3T3 monolayers the longest process was on average 2.25 times longer than the second longest process). Culture on NCAM-expressing monolayers was also not associated with major effects on the number of primary neurites per cell (3.2±0.21 neurites per cell on control monolayers and 3.54±0.19 on NCAM-expressing monolayers; both values are the mean ±s.d. from three independent experiments). A monoclonal antibody that has previously been shown to specifically block NCAM function completely inhibited the above responses to NCAM without significantly affecting neurite outgrowth on control or N-cadherin-expressing 3T3 cells (data not shown).

PSA is a major and highly specific regulator of NCAM-dependent neurite outgrowth

As discussed above, dramatic changes in the amount of NCAM-associated PSA occur during development, with the pattern of expression suggesting that the highly sialylated

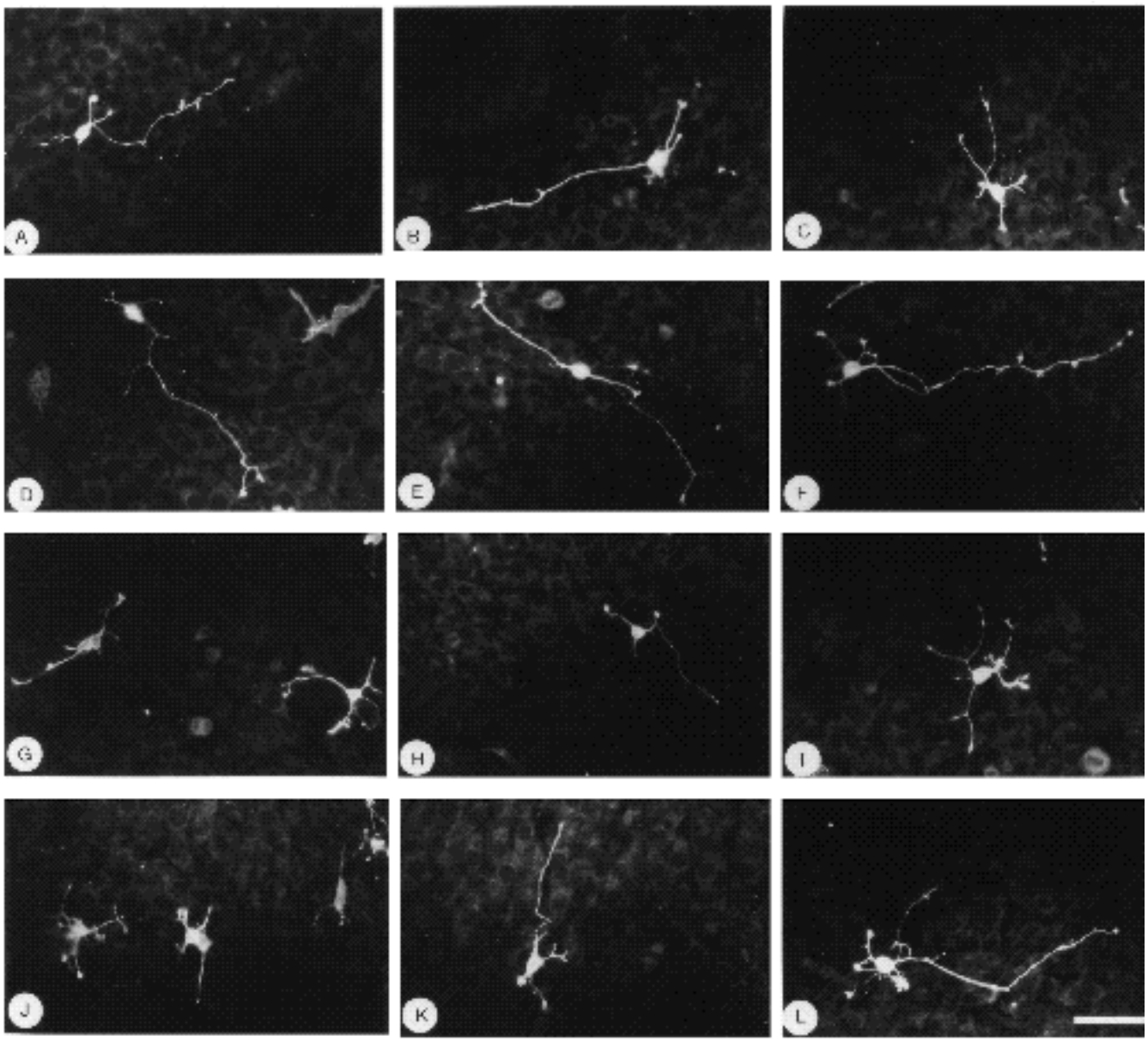

Fig. 2. Morphology of E17 hippocampal neurons on control and transfected 3T3 cells. E17 hippocampal neurons were cultured on control (A-C) or NCAM expressing (D-L) monolayers for 20 hours before being fixed and stained for GAP43 immunoreactivity. The effects of pertussis toxin (G-I) or a combination of diltiazem and ω-conotoxin (J-L) on neurite outgrowth on the NCAM-expressing monolayers is shown. For details see text and Table 1. Scale bar is 50 μm.

form of NCAM might function in morphoregulatory events requiring plasticity such as cell migration and/or axonal growth. In contrast, the form carrying less PSA might promote and maintain the formation of more stable connections between cells. These generalisations are supported by the observation that removal of sialic acid from embryonic NCAM greatly increases its adhesive properties (Hoffman and Edelman, 1983). The α2-8-linked PSA that is present predominantly, if not exclusively, on NCAM can be specifically removed by treatment with endoneuraminidase N (endo-N) (Rutishauser et al., 1985; 1988; Doherty et al., 1990b). We have previously found that removal of PSA from dissociated E6 chick retinal ganglion cells inhibits NCAM-dependent neurite outgrowth by approximately 35% on a clone of transfected 3T3 cells that expresses a similar level of NCAM to that used here (Doherty et al., 1990b). In the present study, E17 rat hippocampal neurons were cultured for 20 hours on control and on NCAM- and N-cadherin-expressing 3T3 cells in the presence of a maximally active concentration of endo-N and axonal length determined as above (Fig. 4). There was no significant effect of endo-N on neurite outgrowth over control 3T3 cells or 3T3 cells expressing N-cadherin. In control medium, neurites were on average 102% longer on NCAM transfectants than on control 3T3 cells; in the presence of endo-N, this difference was reduced to 20% (Fig. 4).

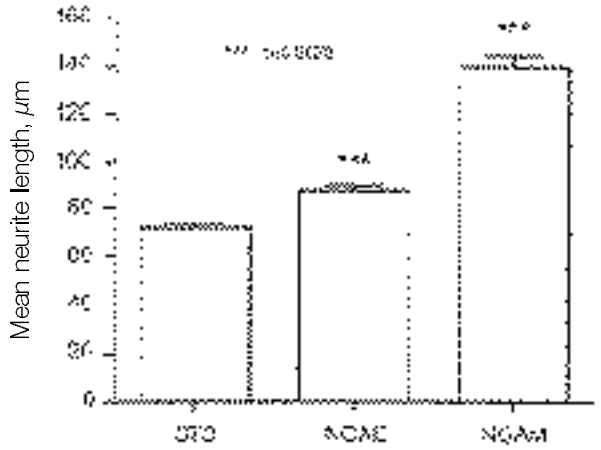

Fig. 3. Relative length of E17 axons on control and transfected 3T3 cells. E17 hippocampal neurons were cultured on monolayers of control 3T3 cells or 3T3 cells expressing human NCAM or chick N-cadherin. After 20 hours the cultures were fixed and the length of the longest GAP43-positive neurite measured from 80-100 neurons sampled from replicate cultures in each of three independent experiments. The results show the mean±s.e.m. for these three experiments

Although a small response to NCAM remained, this was no greater than the N-cadherin response. The much greater contribution of PSA to hippocampal neurite outgrowth relative to that previously measured for chick retinal ganglion cells may again reflect the very early developmental age of the hippocampal neurons. The lack of effect of endo-N on growth over control 3T3 cells and N-cadherin-expressing 3T3 cells confirms our previous observation that PSA on neuronal NCAM does not act as a direct modulator of integrin or N-cadherin function (Doherty et al., 1991a).

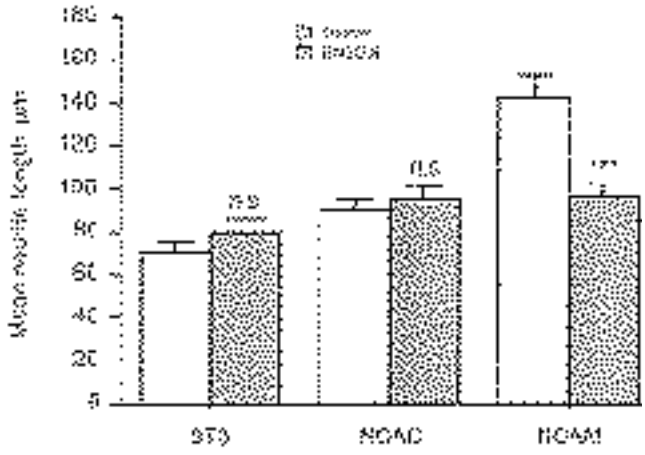

Fig. 4. NCAM-dependent neurite outgrowth is inhibited by endo-N. E17 hippocampal nerons were cultured on monolayers of control 3T3 cells or 3T3 cells expressing human NCAM or chick N-cadherin in SATO media (blank bars) or SATO media supplemented with endo-N at a 1:500 dilution of a phage stock (stippled bars). The results show absolute neurite lengths and each value is the mean±s.e.m. from 80-100 neurons sampled from replicate cultures.

This highly specific enhancement of NCAM-dependent neurite outgrowth by PSA contrasts with the more general effects that this has on adhesion phenomena. For example, PSA on NCAM inhibits NCAM-dependent adhesion (Hoffman and Edelman, 1983) and also inhibits neuronal attachment to laminin and polyornithine (Acheson et al., 1991). However, in the same study, removal of PSA had no effect on laminin-dependent neurite outgrowth. Generalisations on the modulatory role of PSA on receptor function cannot therefore be extended from simple adhesion studies to more complex physiological responses (i.e. neurite outgrowth) that are mediated by the same receptors. In more complex models where axons have a choice between growing in a fasciculated form (axon-axon interaction) or growing on an alternative substratum, endo-N can affect (perhaps indirectly) growth on collagen (Rutishauser et al., 1985) and L1-dependent growth in vivo (Landmesser et al., 1990). In any case, the results of the present study demonstrate that the differential responsiveness of early neurons to NCAM and N-cadherin can be entirely accounted for by post-translational processing of NCAM and support the general postulate that in its highly sialylated form NCAM may promote plasticity in general and neurite outgrowth in particular.

Developmental changes in neuronal responsiveness to NCAM and N-cadherin

We have tested whether the ability of hippocampal neurons to respond to NCAM and N-cadherin in the monolayer changes during development. Fig. 5 summarises results from two independent experiments; PND4 hippocampal neurons respond very poorly to NCAM in the monolayer with increases in neurite lengths of on average 22% and

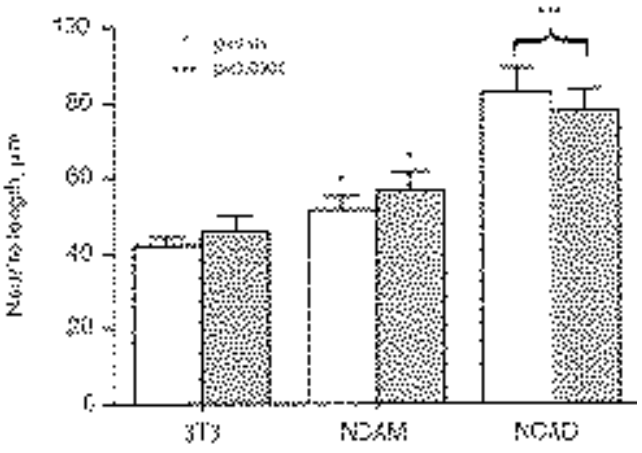

Fig. 5. Relative length of PND4 axons on control and transfected 3T3 cells. PND4 hippocampal neurons were cultured on monolayers of control 3T3 cells or 3T3 cells expressing human NCAM or chick N-cadherin. After 20 hours the cultures were fixed and the length of the longest GAP43-positive neurite measured from 80-100 neurons sampled from replicate cultures in two independent experiments. The results show the mean±s.e.m. for each experiment. Statistical analysis was conducted for each experiment independently and refers to a comparison between growth on control and transfected cells in experiment one (blank bars) and experiment two (stippled bars).

Table 1. *Effects of pertussis toxin and calcium channel antagonists on neurite outgrowth from E17 hippocampal neurons*

Monolayer	3T3	NCAM
(1) Control	100±9.4 (86)	193±13 (84)
Pertussis toxin	108±10.8 (82)*	102±9.5 (83)*,†
(2) Control	100±6.1 (79)	187±10 (76)
Diltiazem	106±6.2 (109)*	143±9.3 (107)†
ω-conotoxin	92±6.2 (84)*	138±6.9 (95)†
Diltiazem+ω-conotoxin	96±6.2 (81)*	108±6.9 (97)*,†

E17 hippocampal neurons were cultured on monolayers of control 3T3 cells or 3T3 cells expressing transfected NCAM in media containing pertussis toxin (1 μg/ml), diltiazem (10 μM), ω-conotoxin (0.25 μM) or ω-conotoxin and diltiazem. The results show the mean length of the longest GAP43 neurite per cell as a percentage of that measured on control 3T3 cells. Each value is the mean ± s.e.m. for the given number of neurons sampled from replicate cultures. The control value for neurite outgrowth was 68.3 μm and 77.4 μm in experiments 1 and 2.

*not statistically different from control 3T3 cells in SATO media.

†$P < 0.025$ as compared to NCAM-expressing cells in SATO media.

25%. In contrast, N-cadherin induced a very robust response and increased neurite lengths by 95% and 70% in the same experiments. Loss of responsiveness to NCAM cannot therefore be attributed to a general inability of the neurons to respond to CAMs. In more recent experiments, we have also found that the ability of NCAM (in the monolayer) to stimulate neurite outgrowth from rat cerebellar granule cells is lost over the PND6-PND8 period independently of changes in their ability to respond to N-cadherin. Furthermore, removal of PSA from responsive neurons also inhibited NCAM-dependent neurite outgrowth by 70-80% (Doherty et al., 1992). Thus the original observation that chick retinal ganglion cells lose their ability to respond to NCAM during development can be extended to two quite different neuronal populations and may therefore be considered as a general feature of development. Although the removal of PSA from neurons clearly inhibits NCAM-dependent neurite outgrowth, a similar loss of this function is found in NCAM isoforms that contain the product of the alternatively spliced VASE exon (Doherty et al., 1992). In the developing cerebellum there is a dramatic increase in use of the VASE exon over the PND6-8 period and this, rather than loss of PSA, may be the primary reason for loss of NCAM-dependent neurite outgrowth (our unpublished observations). Similar studies are currently being undertaken to establish a more detailed picture of the pattern of alternative splicing of the NCAM gene in the developing hippocampus. In addition to the studies on NCAM, the observation that hippocampal neurons respond considerably better to N-cadherin at PND4 than at E17 demonstrates developmental regulation of neuronal responsiveness to a second CAM.

NCAM-dependent neurite outgrowth is inhibited by pertussis toxin and calcium channel antagonists

Pertussis toxin ribosylates and thereby interferes with the function of several types of G-protein. The fact that pertussis toxin can inhibit CAM-antibody-triggered calcium influx into PC12 cells implicates a G-protein in this process (Schuch et al., 1989). Pre-treatment of PC12 cells with pertussis toxin can also fully inhibit NCAM- and N-cadherin-dependent morphological differentiation of PC12 cells, but has no effect on NGF/integrin-dependent differentiation (Doherty et al., 1991b). In the present study, pertussis toxin had no effect on the substantial basal differentiation of E17 hippocampal neurons on control 3T3 fibroblasts (Table 1); these latter cells have previously been shown to support integrin-dependent but not CAM (L1, NCAM, N-cadherin)-dependent neurite outgrowth (Doherty et al., 1991a). Pertussis toxin completely inhibited the NCAM-dependent component of neurite outgrowth from hippocampal neurons (Fig. 2 G-I; Table 1). These data provide the first direct evidence for G-protein involvement in CAM-induced neurite outgrowth from primary neurons. The data also provide additional evidence that integrins do not utilise the same signal transduction mechanism as CAMs for promoting neurite outgrowth (Bixby and Jhabvala, 1990; Doherty et al., 1991b). We have also recently confirmed that N-cadherin-dependent neurite outgrowth from primary neurons is also inhibited by pertussis toxin (our unpublished observations).

The possibility that the major target of the pertussis-toxin-sensitive G-protein is neuronal calcium channels is supported by both indirect and direct evidence with the PC12 pheochromocytoma cell line (Schuch et al., 1989; Doherty et al., 1991b). The molecular basis of calcium channel heterogeneity in primary neurons is complex. In brief, calcium channels can be classified on both electrophysiological and pharmacological grounds. A general consensus exists that two main classes of pharmacological reagents, the dihydropyridines and ω-conotoxin, block L-type and N-type channels, respectively (e.g. see Plummer et al., 1989). However, both classes of channel share at least some common molecular features in that both can be blocked by ω-aga-1a toxin (Scott et al., 1990).

In PC12 cells, verapamil and diltiazem, which block dihydropyridine-sensitive or L-type calcium channels, fully inhibited CAM-antibody-triggered increases in intracellular calcium (Schuch et al., 1989). However, a combination of diltiazem and ω-conotoxin was required for a maximal inhibition of CAM-induced morphological differentiation of PC12 cells (Doherty et al., 1991b). In the present study, neither diltiazem nor ω-conotoxin inhibited E17 hippocampal neurite outgrowth over 3T3 fibroblasts, again supporting other observations that calcium fluxes are not involved in integrin-dependent neurite outgrowth and also demonstrating that these agents have no non-specific effects on neurite outgrowth (see also Campenot and Draker, 1989; Doherty et al., 1991b). Maximally active concentrations of diltiazem and ω-conotoxin (see Doherty et al., 1991b) on their own blocked NCAM-dependent neurite outgrowth from E17 hippocampal neurons by approximately 50% (Table 1). Their effects were additive and neurite outgrowth on NCAM transfectants in the presence of both agents was indistinguishable from that found on control 3T3 cells (Fig. 2 J-L, Table 1). A combination of diltiazem and ω-conotoxin also blocked NCAM- and N-cadherin-stimulated neurite outgrowth from rat cerebellar granule cells (Doherty et al., 1992). Based on our current understanding of the pharmacological properties of calcium channels, these data sup-

port our hypothesis that some of the morphoregulatory activities of cell adhesion molecules can be accounted for by G-protein-dependent activation of both N- and L-type neuronal calcium channels (Doherty et al., 1991b). In support we have recently found that transfected human L1 can also promote neurite outgrowth from PC12 cells and primary neurons and that this response can also be fully inhibited by pertussis toxin and calcium channel antagonists (our unpublished observations).

The exact nature of the biochemical changes induced in growth cones by calcium remains to be determined. However, it is interesting that K^+-induced depolarisation of PC12 cells cultured on control 3T3 monolayers induces a similar morphological response to that induced by transfected NCAM and N-cadherin and that this can also be substantially inhibited by a combination of L- and N-type calcium channel blockers, but is not inhibited by pertussis toxin (J. Saffell, P. Doherty and F.S. Walsh, unpublished observations). These data suggest that changes in calcium are sufficient to promote the morphological response and also provide direct evidence for pertussis toxin acting upstream of calcium channel activation. It is also interesting that in addition to modulating growth cone motility (see Kater and Mills, 1991) the turning behaviour of growth cones induced by NGF (Gundersen and Barrett, 1980), or by discrete electric fields (McCaig, 1989), may also be accounted for by calcium gradients within growth cones, the former due to release of calcium from intracellular stores and the latter dependent on calcium channel activation. Growth cone motility can be best explained by a set-point hypothesis (Kater and Mills, 1991) whereby optimal growth occurs at a given concentration of free calcium, both increasing and decreasing calcium around this level can inhibit growth cone motility and neurite extension. In this context, it will be very interesting to determine if the reduced neurite outgrowth that is associated with removal of PSA from neuronal NCAM is due to a reduction in transmembrane signalling or, alternatively, a substantial increase in signalling (i.e. a very large increase in growth cone calcium). The fact that removal of neuronal PSA does not inhibit neurite outgrowth on NCAM transfectants to below the basal level found on control 3T3 monolayers favours the former possibility.

Conclusions

Hippocampal neurons show developmental changes in their responsiveness to NCAM and N-cadherin. At E17, the neurons responded well to NCAM but not N-cadherin with the differential response being entirely dependent upon the presence of PSA on neuronal NCAM. In contrast, at PND4, the neurons responded better to N-cadherin and very poorly to NCAM. An age-dependent reduction in responsiveness to NCAM, but not N-cadherin, was also found for rat cerebellar granule cells and has also previously been reported for chick retinal ganglion neurons. These data suggest that loss of responsiveness to NCAM may reflect a general phenomenon that occurs at differing times for differing neurons during development of the CNS. This loss may be attributable to a time-dependent reduction in the amount of α2-8-linked PSA on neuronal NCAM and/or increased use of the VASE exon. Direct evidence that NCAM-dependent neurite outgrowth involves both a G-protein and the activation of both N- and L-type neuronal calcium channels has also been obtained; these and other data suggest that the CAM-activated pathway promoting neurite outgrowth in primary neurons is similar, if not identical, to that previously reported to be activated during CAM-dependent differentiation of PC12 cells.

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