The contrasting roles of N-CAM and N-cadherin as neurite outgrowth-promoting molecules

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Summary

The neural cell adhesion molecule (N-CAM) is a prominent member of the immunoglobulin gene superfamily of recognition molecules. It operates in a calcium-independent manner to promote cell–cell adhesion. Alternative splicing of a single gene generates more than twenty N-CAM isoforms and these can be further modified by the differential addition of complex N- and O-linked carbohydrates. In contrast, N-cadherin is a major calcium-dependent adhesion molecule in the brain; it is not a member of the immunoglobulin gene superfamily and, as far as we know, exists as a single gene product with no evidence of differential post-translational modification. Both molecules are believed to operate through a homophilic binding mechanism and both are expressed at key developmental times in a number of tissues including the brain. Antibody perturbation experiments suggest that both of the above cell adhesion molecules (CAMs) can support neurite outgrowth over complex cellular substrata such as astrocytes and Schwann cells. In the present review we discuss the use of a molecular genetic approach to study the neurite outgrowth-promoting activity of these molecules. Using this approach we have found that both CAMs are potent inducers of neurite outgrowth from a variety of neurons. However, whereas a critical value of N-CAM expression is required for increased neurite outgrowth, with small increases above this value having substantial effects, N-cadherin promotes neurite outgrowth in a highly linear manner. In addition, whereas N-CAM promotes chick retinal ganglion cell (RGC) neurite outgrowth at E6 but not E11, N-cadherin does so throughout this developmental period. These studies show fundamental differences in neuronal responsiveness to CAMs, and suggest a more dynamic regulation for N-CAM-dependent neurite outgrowth than for N-cadherin-dependent neurite outgrowth.

Key words: N-CAM, N-cadherin, neurite outgrowth, cerebellar neurons, retinal–tectal projection, polysialic acid.

Introduction

Specific innervation of target regions can be achieved during development by long-range axonal growth along very precise anatomical pathways. Axonal growth at the molecular level can be both promoted and inhibited by several classes of soluble and bound molecules, including neurotransmitters (Cohen and Kater, 1989; Lipton *et al.* 1988; Lankford *et al.* 1988), extracellular matrix components (Rogers *et al.* 1983) and a wide variety of membrane-associated glycoproteins (Rutishauser *et al.* 1983; Bixby *et al.* 1987; Schwab, 1990; Matsunaga *et al.* 1988; Doherty *et al.* 1990*a*; Raper and Kapfhammer, 1990). Although interplay between these molecules has the potential to direct axonal growth, guidance has only been convincingly demonstrated *in vitro* when target and intermediate target tissues are placed in close apposition to growing axons (Lumsden and Davies, 1986; Teissier-Lavigne *et al.* 1988; Heffner *et al.* 1990). The chemotropic molecules have yet to be purified and characterised, and it remains unclear whether they promote as well as guide axonal growth. The generation of the complex neuronal networks in the CNS of higher vertebrates is likely to be dependent on a wide variety of growth-promoting, inhibitory, and tropic molecules acting in concert.

One of the fundamental pre-requisites for the developing nervous system is axonal growth *per se*. In this context, neurite outgrowth over a wide variety of cellular monolayers *in vitro* (e.g. Schwann cells, astrocytes, myotubes and fibroblasts) is largely dependent on neuronal expression of receptors for a limited number of cell- or substrate-associated molecules (for a review, see Doherty and Walsh, 1989). These receptors include some members of a large family of closely related heterodimers, collectively termed Integrins, which recognise and mediate neurite outgrowth over various extracellular matrix proteins including laminin, and receptors for the cell adhesion molecules N-CAM, N-cadherin and L1 (a.k.a. NILE, G4, 8D9 antigen and closely related to Ng CAM). These three CAMs share the property of homophilic binding, that is the receptor in the neurons and their ligands on opposing cell membranes are at the very least products of the same gene. For example, N-CAM on the neuron appears to interact directly with transfected N-CAM expressed by fibroblasts to promote neurite outgrowth (Doherty *et al.* 1990*b*). The observation that a cocktail of antibodies that block the function of Integrins and the above CAMs can, in general, completely inhibit neurite outgrowth over the above non-neuronal cells suggests that these same molecules may mediate a substantive amount of growth during development (see for example, Bixby *et al.* 1987; Tomaselli *et al.* 1988; Seilheimer and Schachner, 1988).

As all post-mitotic neurons constitutively express all of the above CAMs, it seems pertinent to ask how a limited number of CAMs might provide the positional information required for complex events like axonal growth and guidance. Variance could arise from changes in the level of

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CAM expression, post-translational modification of CAM structure and the expression of distinct isoforms of an individual CAM (Edelman, 1986). Although there is good evidence for both prevalence and post-translational modulation operating in simple adhesion models, the relationship between this and the complex cell–cell interactions that result in neurite outgrowth is at best tenuous (Doherty *et al.* 1990*b*). In the context of isoform diversity, the past 2–3 years have seen a fundamental change in our understanding of the level of complexity that can arise at the level of a single CAM gene as a result of alternative splicing, with N-CAM at the forefront of this research. There are four main classes of N-CAM that generally share a common extracellular binding domain and differ primarily in their carboxy-terminal domains. Two classes are transmembrane proteins with large ($180\times10^3 M_r$ isoform) or small ($140\times10^3 M_r$ isoform) cytoplasmic domains; one class is linked to the membrane *via* a glycosylphosphatidylinositol (GPI) anchor and the fourth may be directly secreted from the cell ($110–115\times10^3 M_r$ isoform) (Cunningham *et al.* 1987; Nybroe *et al.* 1988; Walsh, 1988). In addition, a series of recent studies have documented more subtle changes in the primary structure of the extracellular domain of N-CAM that also arise from alternative splicing of the gene (Dickson *et al.* 1987; Prediger *et al.* 1988; Small *et al.* 1988; Gower *et al.* 1988). The functional consequences of this diversity have yet to be determined.

Although the 'blocking antibody' approach has been successful in identifying molecules that support neurite outgrowth, more direct assays are required to address questions concerning the relationship between the level of CAM expression and neuronal response, the effects of post-translational modifications on function, the relative efficacy of different isoforms of a single CAM and finally the post-recognition events that underly signal transduction and neurite outgrowth. There are three problems that limit the classical biochemical approach to the study of CAMs: firstly, the ability to purify and coat substrata with molecules in an active form; secondly, the ability to separate out different isoforms of individual CAMs and thirdly, the fact that these molecules may behave quite differently outside their natural membrane environment. These difficulties can be overcome by a molecular genetic approach. Cloned complementary DNA encoding unique isoforms of individual CAMs can be transfected into cells that do not normally express that molecule. The functional consequences of expression of the 'transgenic' molecule can be evaluated using a variety of bioassays. In the present report we discuss the use of this strategy for evaluating and contrasting the role of N-CAM and N-cadherin as neurite outgrowth promoting molecules.

The neural cell adhesion molecule; generation of molecular complexity

Western blot analysis and immunoprecipitation studies have demonstrated that N-CAM exists in multiple forms, with only a part of this diversity explicable by differential post-translational modification (for a review see Nybroe *et al.* 1988). A series of studies on both individual cDNA clones (Murray *et al.* 1984; Goridis *et al.* 1985; Hemperly *et al.* 1986; Barthels *et al.* 1987; Small *et al.* 1988; Barton *et al.* 1988; Gower *et al.* 1988) and genomic clones (e.g. Owens *et al.* 1987; Barbas *et al.* 1988; Thompson *et al.* 1989) has resulted in an essentially complete picture of the N-CAM gene, the mRNAs derived from the gene and the protein products encoded by the gene (for a review see Walsh and Doherty, 1991). All N-CAM-related mRNAs are derived from a single locus on human chromosome 11q23 (Nguyen *et al.* 1986; Walsh *et al.* 1986) and a syntenic region on mouse chromosome 9 (D'Eustachio *et al.* 1985). In addition to the 5′ regulatory region, a total of 26 exons have been identified. Alternative splicing of these exons generates four main size classes of N-CAM that differ in their mode of association with the membrane and/or the length of their cytoplasmic tails (see Fig. 1). All of these isoforms use exons 1–10 which encode a region of five immunoglobulin-like domains that constitute the major extracellular part of the protein (a total of 480 or so amino acids). Likewise, all isoforms utilise exons 11 and 12, the latter of which encodes a region that shows partial homology with the type III repeats of fibronectin. Differential usage of a 239 base pair exon, named SEC, situated between exons 12 and 13, results in the synthesis of soluble N-CAM. This exon prematurely closes the reading frame of the protein and generates a product that is directly secreted from cells. The SEC isoform has been studied primarily in cultures of human muscle, and based on Western blot analysis is likely to represent a minor class of N-CAM relative to the membrane-associated isoforms. The remaining N-CAMs utilise exons 13 and 14. The sequence encoded by exon 13 is similar to that of exon 12 in that it shares homology with the fibronectin type III repeat. The attachment of N-CAM to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor is determined by the use of exon 15 and is associated with the generation of N-CAM proteins of $120–125\times10^3 M_r$. This exon encodes a 27 amino acid carboxy-terminal region, that in common with other GPI-anchored proteins is probably cleaved prior to its attachment to the lipid anchor, although the exact site of cleavage has not yet been identified. Use of exon 16 (rather than 15) is associated with the synthesis of a 70 amino acid sequence with an α helical conformation over about 20 amino acids that is compatible with a transmembrane-spanning domain. Usage of the three remaining exons (16–19) generates an N-CAM isoform of $180\times10^3 M_r$ that may be directly linked to the cytoskeleton and is expressed in a highly restricted manner essentially by post-mitotic neurons (Pollerberg *et al.* 1985, 1986). Failure to use exon 18 results in the expression of a $140\times10^3 M_r$ transmembrane isoform that does not constitutively associate with the cytoskeleton.

In addition to the above classes of N-CAM, subtle differences have also been identified in the primary structures of the extracellular domains. A series of 3 small exons of 15, 48 and 42 base pairs have been identified between exon 12 and the SEC exon described above. The region is used as a block exclusively by muscle resulting in the terminology MSD1a–c. However, various combinations of the exons can be found in brain mRNAs. The region is distinctive in that the MSD1a exon encodes a string of proline residues and MSD1b and c encode a region rich in serine and threonine. The latter act as a specific site of O-linked carbohydrate attachment in muscle. Another exon in the region between the SEC exon and exon 13 is extremely small and inserts only 3 base pairs (AAG) in some cDNAs. This exon changes an Arg to Gln-Gly in the primary structure of those N-CAM isoforms that use it.

The remaining region of alternative splicing is within the fourth immunoglobulin-like domain encoded by exons 7 and 8. The alternative usage of a 30 base pair exon

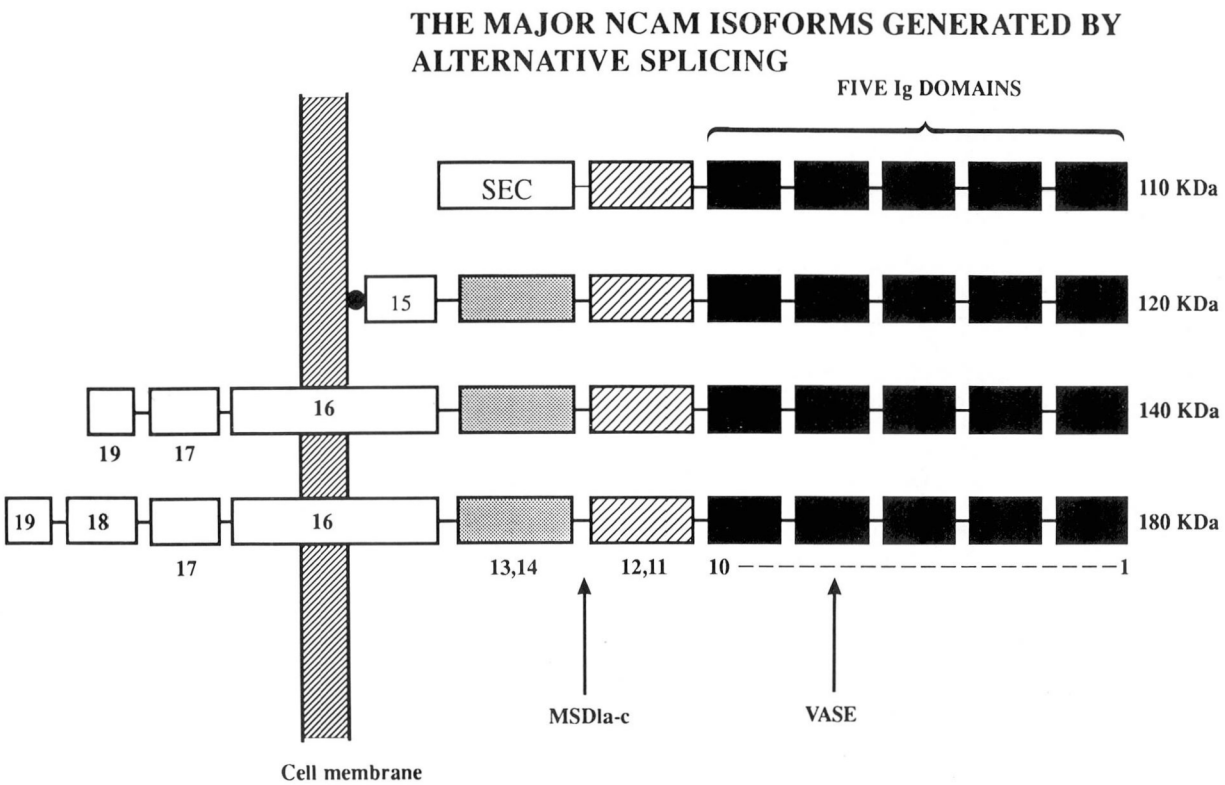

Fig. 1. A schematic diagram of the four main classes of N-CAM protein: for details see text.

results in the insertion of a 10 amino acid polypeptide. The exon has recently been termed VASE and its usage increases dramatically at later stages of brain development (Small and Akeson, 1990).

Alternative splicing of the N-CAM gene can generate at least 20 isoforms. These isoforms can be further modified by the addition of N- and/or O-linked carbohydrate. In this context N-CAM is unique in the nervous system in that it can carry several chains of up to 200 α 2-8 linked sialic acid residues (see Rutishauser *et al.* 1988). As mentioned above, the MSD1 region is a site of O-linked carbohydrate attachment found exclusively in skeletal muscle where it is expressed in a developmentally regulated manner.

It is beyond the scope of the present article to describe the very extensive changes in the patterns of N-CAM isoforms that occur during development. However, the following generalisations are worthy of note. In the context of neurite outgrowth, polysialic acid (PSA)-rich N-CAM is expressed during times of plasticity whereas N-CAM with a much reduced PSA content is expressed in the mature nervous system (e.g. Schlosshauer *et al.* 1984). Similarly, usage of the VASE exon increases as the brain develops (Small and Akeson, 1990). Neurons initially express the 140×10^3 M_r class of N-CAM isoform and only later also start to express the 180×10^3 M_r class. Tissues such as muscle and glial cells like astrocytes and Schwann cells express the 120×10^3 M_r and 140×10^3 M_r class of isoforms (Nybroe *et al.* 1988). The expression of N-CAM by Schwann cells and muscle is also regulated in a manner consistent with a role in promoting axonal growth. For example, whereas N-CAM expression in both peripheral nerve and muscle is down-regulated in the adult it is re-expressed following denervation suggesting a role in nerve regeneration and muscle reinnervation (Moore and Walsh, 1986; Martini and Schachner, 1988). Direct evidence for differential functions of the various N-CAM isoforms has yet to be obtained; however, removal of PSA from N-CAM potentiates its function as an adhesion molecule in simple liposome aggregation assays (Hoffman and Edelman, 1983).

The cell adhesion molecule N-cadherin

A number of recent studies have indicated that a family of calcium-dependent cell adhesion molecules called the cadherins play a critical role in cell–cell interactions (Takeichi, 1991). To date three members of this family have been identified and are named based on their main tissue of expression. These are N-cadherin, expressed in the nervous system and muscle, E-cadherin in liver and P-cadherin in placenta (Takeichi, 1991). It has been assumed that these cadherins are functionally important due to their regulated expression during development (Takeichi, 1991) and by inference from blocking antibody studies using tissue culture cells (Neugebauer *et al.* 1988) and various *in vivo* perturbation assays (Takeichi, 1991). However, with the recent cloning of the three presently known members of the cadherin family and the development of assays based on gene transfer, new information is being generated on the molecular specificity of cadherin binding. The cadherins in general are transmembrane proteins with a relative molecular mass of about 124 000 and appear to be the products of different genes. N-cadherin has been cloned from chicken (Hatta *et al.* 1988), *Xenopus* (Detrick *et al.* 1990), mouse (Mitayani *et al.* 1989) and human (Walsh *et al.* 1990) tissues. There is extensive sequence similarity between individual cadherins in different species and between different cadherins. For instance, the cytoplasmic domain of N-cadherin in the

chicken is 99% similar to that of human N-cadherin (Walsh *et al.* 1990). Overall, chicken and human N-cadherin are 91% similar. Between different family members the greatest degree of similarity is found in the cytoplasmic domain, perhaps reflecting a highly conserved function for this whole region. The amino-terminal domain also appears to show at least 50% sequence similarity between different cadherins. The availability of full length cDNA clones for a number of different cadherins has allowed the development of model assay systems based on gene transfer. These have identified some of the main factors associated with cadherin-mediated adhesion. Firstly, cadherin mediated adhesion is homophilic (Nose *et al.* 1986) and the observed increase in calcium-dependent adhesion is due to the transfected cadherin. Secondly, in terms of extracellular recognition, cadherins do not participate in heterotypic interactions which means that cadherins bind only to themselves and not to other family members or other proteins. This phenomenon has been used to show that cells can sort out, based on the cadherins they express (Nose *et al.* 1986) and additionally the level of cadherin is an important parameter (Friedlander *et al.* 1989). A start has been made in defining important structural regions in the cadherins. An amino-terminal region of 113 amino acids appears to be involved in mediating adhesive interactions and this is also the site where adhesion-blocking antibodies bind (Nose *et al.* 1987). A common cell adhesion recognition sequence has been found in this region and the tripeptide-HAV appears to be the minimum structure (Nose *et al.* 1990; Blaschuk *et al.* 1990). Interestingly this site appears to be common to all three cadherin family members and synthetic peptides modelled around the HAV peptide act as general perturbants of all cadherin-mediated interactions (Blaschuk *et al.* 1990). The other important sites in N-cadherin that specify homophilic interaction have not yet been defined.

Transfection strategy for studying CAM function

As discussed above (see Introduction) there are several limitations to either an antibody perturbation or classical biochemical approach to the study of CAM function, especially (as is the case with N-CAM) if several closely related isoforms of the one CAM exist. Molecular genetic approaches based on transfection with specific cDNA constructs allow for the generation of large numbers of cell clones that stably express individual CAMs at varying levels at the cell surface. The strategy is relatively simple. Firstly the CAM of interest is cloned from an appropriate cDNA library. The respective cDNA is then sub-cloned into a vector that usually contains a promoter region to drive expression and a 'selection' gene. In our own studies we have used a vector that contains the β-actin promoter and neomycin resistance gene (Gunning *et al.* 1987; Gower *et al.* 1988). Following standard transfection protocol, cells that stably incorporate the vector into their genome can be selected by antibiotic resistance, cloned and characterised for expression of the 'transgene' product. Using such a strategy, Edelman *et al.* (1987) described the expression of three major isoforms of chick N-CAM (the 180, 140 and $120\times10^3 M_r$ isoforms illustrated in Fig. 1) in L-cells and/or COS cells. These workers reported an increase in intracellular adhesion between transfected cells, providing additional support for the postulate that N-CAM can mediate cell–cell adhesion *via* a homophilic binding mechanism. Similarly, Matsunaga *et al.* (1988) have shown that monolayer cultures of Neuro 2a or L-cells expressing chicken N-cadherin *via* gene transfer promote neurite outgrowth from explant cultures of embryonic chick retina.

We have produced a large number of NIH-3T3 cell clones expressing five distinct N-CAM isoforms, specifically the 140 and $120\times10^3 M_r$ isoforms plus and minus the MSD1 region, and the secreted isoform that also contained the MSD1 region (Gower *et al.* 1988; Doherty *et al.* 1989). In addition we have produced a number of 3T3 clones expressing a 5-fold range of levels of the only known isoform of chicken N-cadherin (Doherty *et al.* 1991). These transfected cells have been tested for their ability to support neurite outgrowth from a variety of neurons using a very simple experimental paradigm. Monolayers of parental and transfected 3T3 cells are established and dissociated neurons are seeded onto these at low density for a 20–24 h period. The cultures are then fixed and stained using antibodies that recognise neuron-specific markers (e.g. the PSA expressed exclusively on neuronal N-CAM, neurofilament protein or GAP-43). The cultures are then examined by video-microscopy and the lengths of neuronal processes measured using standard computer-assisted morphometric analysis (see for example, Doherty *et al.* 1989, 1990*a*,*b*).

A threshold effect of the major N-CAM isoforms of N-CAM on neurite outgrowth

Neurons freshly dissociated from post-natal day 7 rat cerebellum were cultured on monolayers of parental 3T3 cells and monolayers from 11 independent stable clones of 3T3 cells expressing one of the five N-CAM isoforms described above. After 20–21 h the co-cultures were carefully fixed and a variety of morphometric parameters measured, including the total neuritic output per granule cell, the length of the longest neurite per cell, the number of branch points per neurite and finally the number of neurites per cell. These parameters were plotted as a function of the level of human N-CAM (irrespective of isoform type) expressed on the surface of the transfected cells. N-CAM affected the first three parameters but had no effect on the number of neurites per cell (see Doherty *et al.* 1990*a*). In each instance, the cell clone expressing the highest level of N-CAM had the greatest effect, increasing both the length of the longest neurite per cell and the total sum of neurites by almost 2.5 times relative to what was not insubstantial growth on parental (untransfected) 3T3 cells. Similarly the number of branches per neurite was increased by a factor of 3. In contrast all three parameters were unaffected when neurons were grown on monolayers that synthesised the secreted N-CAM isoform or when phosphatidylinositol-specific phospholipase C was included in the media of GPI-linked N-CAM transfectants. This enzyme quickly removes GPI-linked, but not transmembrane linked, N-CAM from the transfected cell monolayers. As expected, the enzyme had no effect on the ability of the monolayers expressing transmembrane N-CAM isoforms to support neurite outgrowth. These data demonstrated, in an unequivocal manner, that provided N-CAM is expressed in its natural membrane environment it is capable of directly promoting neurite outgrowth. An interesting observation was the highly co-operative nature of all three of the above responses (shown schematically in Fig. 2; for details, see Doherty *et al.*

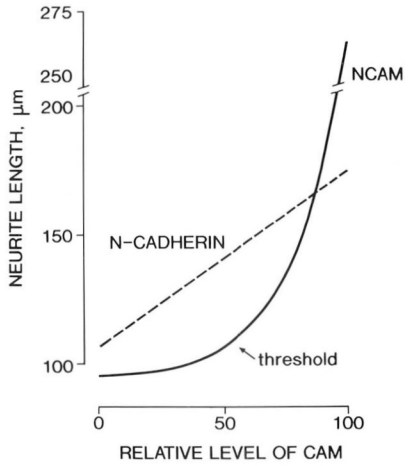

Fig. 2. The relationship between CAM expression and neurite outgrowth. Cerebellar neurons were grown on monolayers of 3T3 cells expressing various levels of human N-CAM or chick N-cadherin. The results show a schematic diagram of mean neurite length plotted as a function of N-CAM or N-cadherin level. N-CAM-dependent neurite outgrowth was highly co-operative whereas N-cadherin dependent neurite outgrowth was linear. For details see Doherty *et al.* (1990*b*) and Doherty *et al.* (1991).

1990*a*). Several cell clones, irrespective of isoform type, that expressed at least half of the level of N-CAM as the most effective clone had little or no effect on any of the above parameters. Thus a discrete threshold value of N-CAM is required for the expression of this function; above this value relatively small changes in N-CAM expression can have substantial effects on neurite outgrowth. For example, in the case of the longest neurite per cell, a 40 % increase in N-CAM expression resulted in a 250 % increase in the magnitude of response. For all three parameters measured, there was a clear relationship between the level of N-CAM expression and the magnitude of response. None of the data points, irrespective of isoform type, de*via*ted substantially from the simple curve illustrated in Fig. 2. Thus the chief determinant of neurite outgrowth was the relative level of N-CAM expression; lipid-linkage conferred no advantage over the transmembrane spanning N-CAMs and the O-glycosylated MSD1 region had no obvious effect on function in this simple model.

A fundamental difference between N-CAM- and N-cadherin-dependent neurite outgrowth

Rat cerebellar granule cells were next cultured on monolayers of 3T3 cells expressing a five-fold range of chick N-cadherin, also introduced *via* gene transfer. Control experiments showed this range to be similar to that present on non-neuronal cells isolated from the developing chick brain (Doherty *et al.* 1991). In contrast to N-CAM, all of the N-cadherin-expressing clones had positive effects on neurite outgrowth. These data confirm and extend the findings of Matsunaga *et al.* (1988) who demonstrated that monolayer cultures of Neuro 2a or L-cells expressing chicken N-cadherin *via* gene transfer promote neurite outgrowth from explant cultures of embryonic day 8 chick retina. Also, it has recently been shown that when substrate-bound, purified N-cadherin can support neurite outgrowth from dissociate cultures of chicken ciliary ganglia (Bixby and Zhang, 1990). The magnitude of the neuronal response increased in a highly linear manner as a function of the relative level of N-cadherin expression, and this is shown schematically in Fig. 2 (for details see Doherty *et al.* 1991). The linear nature of the N-cadherin response contrasts with the highly co-operative relationship previously shown for N-CAM-dependent neurite outgrowth. At their simplest, these data demonstrate that neurons are more able to sense and respond differentially (in terms of both absolute growth and changes in growth rate) to relatively small variations in N-CAM expression compared with N-cadherin expression. This is one reason why we would suggest that N-CAM-dependent neurite outgrowth may be regulated in a more dynamic manner than N-cadherin dependent neurite outgrowth. The differences in the nature of the dose–response curves may underly the more widespread role that N-cadherin appears to have in supporting neurite outgrowth over N-cadherin- and N-CAM-positive cell monolayers (e.g. myotubes, astrocytes and Schwann cells) compared with N-CAM (see below).

The reason(s) for the qualitative difference between N-CAM- and N-cadherin-dependent neurite outgrowth are not known. There are at least two possibilities; firstly, whereas N-cadherin is constitutively linked to the cytoskeleton, the 140 and $120\times10^3 M_r$ classes of N-CAM are not (see above). Linkage to the cytoskeleton will restrict lateral mobility in the membrane and the latter may be essential for co-operative binding. The second possibility is that transduction of the recognition signals *per se* may differ for these molecules. Recent studies on the responsiveness of a simple neuronal cell line to CAMs suggest the latter to be less likely. N-CAM- and N-cadherin-dependent morphological differentiation of the PC12 cell line can be inhibited by the same pharmacological reagents, suggesting that a common set of intracellular second messengers induce the morphological response (P. Doherty and F. S. Walsh, unpublished observations). The first of the two possibilities is testable by expressing N-cadherin deletion mutants that do not link to the cytoskeleton in 3T3 cells and by testing the ability of the $180\times10^3 M_r$ class of N-CAM isoforms (that do link to the cytoskeleton) for their ability to promote neurite outgrowth.

Developmental changes in neuronal responsiveness to CAMs

If CAMs have an instructive rather than permissive role in axonal growth then all neurons would not be expected to respond in a similar manner to an identical set of recognition cues present in the local microenvironment. In an extensive series of studies a variety of neurons were cultured on several cellular monolayers and 'blocking' antibodies to CAMs and β1-class Integrins tested for their ability to inhibit neurite outgrowth (see for example, Bixby *et al.* 1987, 1988; Tomaselli *et al.* 1988; Seilheimer and Schachner, 1988; Neugebauer *et al.* 1988; reviewed by Doherty and Walsh, 1989). Three important points emerged: firstly, four classes of recognition molecules expressed by neurons (N-CAM, N-cadherin, L1 and β1 class Integrins) mediated a substantial amount of neurite outgrowth over a variety of complex cellular substrata, with, for example, fibroblasts stimulating neurite outgrowth solely *via* neuronal Integrin receptors whereas Schwann cell-mediated neurite outgrowth involved all four classes of receptor. Secondly, individual neuronal

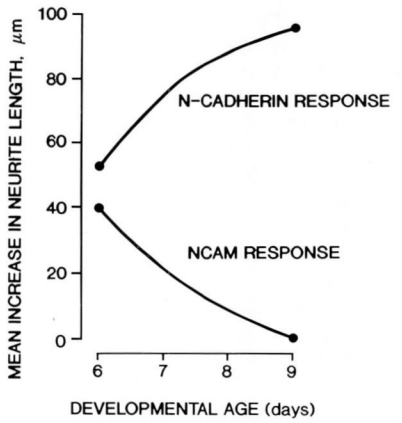

Fig. 3. An overview of the developmental profile of chick retinal ganglion cell responsiveness to N-CAM and N-cadherin expressed in 3T3 cells following gene transfer. Cultures of E6–E9 retinae were established on confluent monolayers of control 3T3 cells, or 3T3 cells expressing transfected N-CAM or N-cadherin. After 24 h the cultures were fixed and the length of the longest neurofilament-positive process was determined. The results show the absolute increase in neurite lengths on transfected cells relative to control 3T3 cells. Basal growth on the latter was 40–50 μm. For details see Doherty *et al.* (1991).

populations responded in a characteristic manner to an identical set of recognition glycoproteins present on one cell type. For example, chick ciliary ganglion neurons utilised Integrins and N-cadherin to extend neurites on astrocytes whereas retinal neurons utilised Integrins, N-cadherin and N-CAM. Finally, neuronal responsiveness to a fixed set of recognition cues was found to change as a function of developmental age; with a notable example being a down-regulation of Integrin receptor function in chick ciliary ganglion neurons over the E8–E14 period (see also chapter by Cohen in this volume).

In order to understand further the role of N-CAM and N-cadherin as neurite outgrowth-promoting molecules, we have directly compared the developmental profile of chick retinal ganglion cell (RGC) responsiveness to a fixed level of human N-CAM and chick N-cadherin expressed *via* gene transfer in 3T3 cells. During development of the retino–tectal map in the chick, most RGCs are extending axons at E6 and have reached the target at E11. At E6, RGCs responded to both N-CAM and N-cadherin by extending longer neurites (Doherty *et al.* 1991). Neurite outgrowth induced by transfected human N-CAM could be fully inhibited by antibodies that bound exclusively to the chick N-CAM expressed by the RGCs, but not by antibodies that bind to other growth cone receptors that mediate neurite outgrowth (e.g. the G4/L1 glycoprotein, N-cadherin and the β1-Integrin family). These data provide substantive evidence for a homophilic binding mechanism directly mediating N-CAM-dependent neurite outgrowth. Similarly, the N-cadherin response could only be perturbed by antibodies to N-cadherin. The ability of RGCs to respond to a given level of N-CAM expression was rapidly lost over the period between E6 and E11. In contrast, the older neurons readily extend neurites in response to transfected N-cadherin (shown schematically in Fig. 3; for details see Doherty *et al.* 1991). These data reinforce the concept that neuronal responsiveness to CAMs can change quite dramatically during development.

PSA as a specific modulator of N-CAM function

N-cadherin appears to have a more widespread role than N-CAM in supporting neurite outgrowth over N-CAM/N-cadherin positive cell monolayers. For example chick ciliary ganglion neurons are responsive to N-CAM on muscle (Bixby *et al.* 1987) but not N-CAM on Schwann cells (Bixby *et al.* 1988) or astrocytes (Tomaselli *et al.* 1988), whereas in each instance the same neurons all respond to N-cadherin. As discussed above, these data may be explained by the fundamental differences in the nature of the respective dose–response curves for N-CAM and N-cadherin. However, the down-regulation of RGC responsiveness to N-CAM (discussed above) demonstrates that factors other than the level of N-CAM in the monolayer can modulate N-CAM function. As the older RGCs readily respond to N-cadherin one or a combination of several mechanisms may account for the loss of responsiveness to N-CAM. These include a reduction in neuronal N-CAM expression, a change in the pattern of N-CAM isoforms expressed by RGCs or developmentally controlled changes in the post-translational processing of RGC N-CAM. The first possibility can be excluded as a general mechanism as we are unable to demonstrate substantial changes in the level of N-CAM expression on RGC growth cones between E6 and E9 in the chick (Doherty *et al.* 1991).

During development, N-CAM generally shifts from a PSA-rich to poor form with the retina being no exception (Schlosshauer *et al.* 1984). Within the developing chick retina the expression of polysialic acid on N-CAM is also spatially restricted. Whereas antibodies that recognise the N-CAM polypeptide stain all of the developing retinal layers at E6, PSA expression is primarily found in the RGC layer and the optic fibre layer (Doherty *et al.* 1991). In primary cultures of E6 retina the RGCs stain strongly for α2-8 linked PSA and this can be specifically removed from N-CAM by endoneuraminidase N (Rutishauser *et al.* 1988). Whereas the removal of PSA from N-CAM increases its adhesive properties in simple aggregation assays (Hoffman and Edelman, 1983), we found that it reduces N-CAM-dependent neurite outgrowth. This is shown schematically in Fig. 4 for RGC growth on two independent clones of 3T3 cells expressing moderate and higher levels of the 120×10^3 M_r N-CAM isoform. Removal of PSA very clearly shifts the dose–response curve to the right; i.e. N-CAM dependent neurite outgrowth is significantly inhibited. In contrast, removal of polysialic acid from neuronal N-CAM had no effect on the ability of the same neurons to respond to N-cadherin (Doherty *et al.* 1991). These data suggest that N-CAM does not promote neurite outgrowth *via* a simple adhesion-based mechanism. This contention is supported by our recent observation that N-CAM- and N-cadherin-dependent morphological differentiation of PC12 cells is likely to involve activation of second messenger pathways (see above).

The strategy of utilising a post-translational mechanism for modulating CAM function is likely to be complex. In the case of N-CAM, the addition of PSA seems to improve its ability to act as a neurite outgrowth-promoting molecule, yet at the same time reduces its ability to promote adhesion *per se*. One advantage of this strategy is that a loss of polysialic acid on neurons during development may act as an instructive signal for neurons to reduce the rate of axonal growth at or near their target tissue, at the same time, making N-CAM available in its highly adhesive state to mediate or modulate other contact-dependent responses. Such a strategy may be

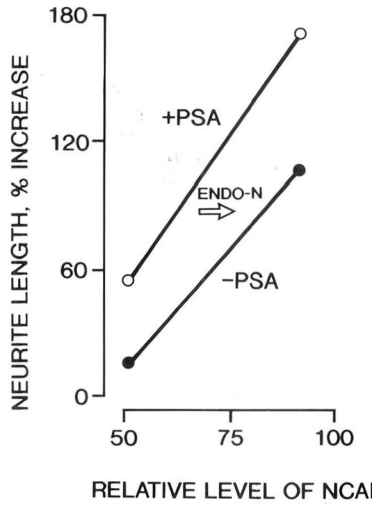

Fig. 4. PSA modulates N-CAM dependent neurite outgrowth. E6 retinal neurons were cultured on monolayers of control 3T3 cells or 3T3 cells expressing GPI-linked N-CAM (clones A8 and A10, see Doherty *et al.* 1990*b*). Growth media was supplemented with endo-N and after 24 h the cultures were fixed and the length of the longest neurofilament-positive process determined for RGCs. The results show the percentage increase in mean neurite length for RGCs cultured on N-CAM-expressing monolayers relative to control monolayer.

reinforced by other changes such as isoform switching, although at present have no direct evidence to support this.

Synergism between CAMs

When purified N-CAM and L1 are coated to a nitrocellulose substratum there is an apparent enhancement of L1-dependent adhesion and neurite outgrowth from mouse cerebellar neurons (Kadmon *et al.* 1990). In order to determine if synergism is restricted to these two CAMs we have cultured rat cerebellar neurons of 3T3 cells that express ~20 units of human N-CAM together with 60 units of chick N-cadherin (both values are directly related to the curves shown in Fig. 3; for details see Doherty *et al.* 1991). This level of N-CAM is at least 50% below the threshold value for neurite outgrowth. In the presence of antibodies or synthetic peptides that block N-cadherin function, neuronal growth on the co-transfected clone was indistinguishable from that found on untransfected 3T3 cells. In addition enzymatic removal of the sub-threshold N-CAM by PI-PLC has no effect on neurite outgrowth. These data demonstrate unequivocally that in the absence of N-cadherin function, as expected, the sub-threshold level of N-CAM does not promote neurite outgrowth. However when N-cadherin is functional the otherwise sub-threshold level of N-CAM can also contribute to neurite outgrowth. For example the co-transfected cells promoted neurite outgrowth to a similar extent as a clone that expressed no N-CAM but almost twice as much N-cadherin (Doherty *et al.* 1991). That this was directly attributable to N-CAM was demonstrated by the ability of both heparin (which in this model specifically inhibits N-CAM function) and PI-PLC to inhibit significantly and specifically neurite outgrowth on the co-transfected cell. Therefore in addition to changes in the level of N-CAM expression, changes in N-CAM isoforms and changes in post-translational processing of N-CAM, a fourth parameter can modulate N-CAM dependent neurite outgrowth, that is expression of an accessory CAM that in this instance was N-cadherin.

Concluding remarks

When N-cadherin- and N-CAM-deficient cells are transfected with cDNA for these molecules, the expression of the transgenic product at the cell surface can be correlated with an increase in the ability of the transfected cell to promote neurite outgrowth from a variety of primary neurons. However there are fundamental differences in the relationship between the level of expression of the transfected molecules and their ability to promote neurite outgrowth. Whereas N-CAM dependent neurite outgrowth is highly co-operative and exhibits a clear threshold effect, N-cadherin-dependent neurite outgrowth is essentially linear. In addition, neurons can show opposing temporal changes to N-CAM and N-cadherin with chick RGCs responding to N-cadherin through E6–E11, whereas responsiveness to a fixed level of N-CAM was rapidly lost over the E6–E9 period of development. The observation that neurons can respond to small changes in N-CAM expression in a more dynamic fashion than to small changes in N-cadherin expression and that they undergo more pronounced changes in responsiveness as a function of their developmental age suggests that N-CAM may play a more instructive (relative to permissive) role in determining axonal growth during embryogenesis.

N-CAM and N-cadherin differ dramatically in the extent of diversity that arises from their single genes. Whereas N-cadherin exists as a single invariant gene product there are considerably more than 20 N-CAM isoforms and these can be differentially glycosylated. Glycosylation and, more specifically, the addition of α 2-8 linked polymers of sialic acid to N-CAM improves its ability to act as a neurite outgrowth-promoting molecule, yet at the same time reduces its ability to promote adhesion. These data reinforce the concept that there is no simple correlation between adhesion and neurite outgrowth. We would suggest that recognition and signal transduction are more important than adhesion, although the latter is an obvious prerequisite for neurite outgrowth.

The functional consequences of N-CAM diversity in terms of polypeptide structure remain to be elucidated. However we have not found any difference in the ability of the GPI-linked and the 140×10^3 M_r transmembrane N-CAM to provide a recognition signal to neurons suggesting that the differences may lie in transduction of a recognition signal. One possibility is that GPI anchors are utilised when a cell has simply to provide recognition or positional information to a second cell whereas transmembrane molecules might be required for a cell to actively respond (by migration and/or neurite outgrowth) to such information (for a review see Walsh and Doherty, 1991). The O-glycosylated MSD1 region is exclusively expressed by lipid-linked N-CAM isoforms in muscle; we found no evidence that this region directly modulates N-CAM–N-CAM binding. A generalised function of O-linked carbohydrate is to linearise polypeptide structures (Jentoft, 1990) and based on this we would propose that the MSD1 region may act as a 'spacer unit' for myotube N-CAM (Fig. 5).

Our experiments have only directly tested for functional differences in the 120 and 140×10^3 M_r classes of N-CAM

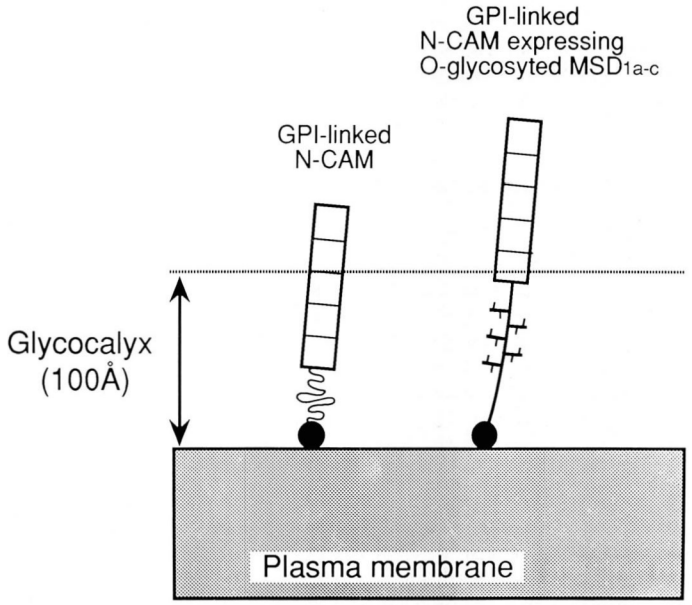

Fig. 5. A model illustrating the possible function of the O-glycosylated MSD1 region of skeletal muscle N-CAM (the 125×10^3 M_r isoform). Attachment of O-linked carbohydrate may serve to linearise the polypeptide structure of N-CAM in the region of the MSD1 polypeptide. As such the immunoglobulin domains of N-CAM may be extended out of the glycocalyx where it could function more readily in cell–cell interactions. This model has been adapted from that of Jentoft (1990).

isoforms and before we can reach final conclusions as to the ability of the different isoforms to modulate recognition these studies will have to be extended to include the 180×10^3 M_r N-CAM isoform that constitutively associates to the cytoskeleton and to the VASE exon which is alternatively spliced within the immunoglobulin region. In addition, testing for differences in the ability of N-CAM to transduce a recognition signal into a complex cellular response (e.g. neurite outgrowth) will require the transfection of all of the isoforms into neurons.

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