The changing role of NCAM as a neurite outgrowth-promoting molecule during development

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Axonal growth, guidance and ultimately synapse formation are controlled by interactions of the neuronal growth cone with molecules present in its local microenvironment. At present we know of four well-defined receptor systems present on neuronal growth cones that recognize and integrate the growth promoting information present in the extracellular matrix and on the surface of other cells. These are the integrin receptors and the neuronal cell adhesion molecules (CAMs) NCAM, N-cadherin and L1 [1, 2]. Whereas integrins are receptors for extracellular matrix molecules such as laminin and collagen, the above CAMs primarily recognize and bind to products of the same gene expressed on the surface of other cells, i.e. they act as homophilic binding molecules. There is growing evidence that in addition to supporting adhesion per se, these CAMs are involved in promoting axonal growth and possibly also contribute to pathway finding during development. For example, cell types normally deficient in NCAM and N-cadherin but transfected with cDNA encoding these molecules are able to promote neurite outgrowth from a variety of neurons cultured on them [1, 3]. In addition the rat pheochromocytoma cell line (PC12), which assumes the morphological phenotype of a sympathetic neuron in the presence of nerve growth factor and fibroblast growth factor [6, 7] adopts the same phenotype when cultured on these CAM-expressing monolayers [8].

Studies on rat cerebellar granule cells show that there is a difference in the relationship between the level of expression of NCAM and N-cadherin and their ability to promote neurite outgrowth. Whereas for N-cadherin the increase in neurite outgrowth is linearly proportional to the relative level of expression of the molecule in the monolayer [9], the response to increasing NCAM levels is highly cooperative so that beyond a threshold, small changes in NCAM expression levels can substantially increase neurite outgrowth [4, 9]. In addition to this difference in response to NCAM and N-cadherin levels, neuronal responsiveness to these CAMs changes with developmental age; older neurons become less responsive to NCAM whereas responsiveness to N-cadherin stays the same or increases. This trend is demonstrated by chick retinal ganglion cells taken at embryonic day 6 (E6) and E11 [5] and also by rat hippocampal neurons taken at E17 and post-natal day 4 (P. Doherty, unpublished work). In both cases, the greater responsiveness of the younger neurons to NCAM was not attributable to higher levels of expression of the molecule in their growth cones, but could be substantially reduced by enzymic removal of α2-8-linked polysialic acid (PSA) from neuronal NCAM. This suggests that PSA expressed on NCAM is a positive modulator of NCAM-dependent axonal growth during development. Recent studies have shown that NCAM-dependent neurite outgrowth may also be modulated by alternative splicing in the extracellular domain. Neurite outgrowth is inhibited when rat cerebellar neurons are cultured on monolayers expressing an isoform of NCAM in which the variable alternatively spliced exon (VASE) encoding a 10 amino acid insert into the fourth immunoglobulin domain [10] has been expressed although they remain highly-responsive to transfected N-cadherin (P. Doherty, unpublished work). The temporal expression of VASE is consistent with a putative role as a negative modulator of synaptic plasticity as the proportion of VASE-containing NCAM transcripts rises from ~3% of all NCAM transcripts early in development when neurons would be extending axons to ~50% in the adult CNS when stable synapses are well established [10].

CAMs share two fundamental functional properties: their ability to promote adhesion between cells and their ability to promote axonal growth. It has been widely accepted that these functions are directly related, adhesive interactions mediated by these molecules directly resulting in neurite outgrowth. Evidence is growing, however, to suggest that this may not be so. For example, enzymic removal of α2-8-linked PSA from neuronal NCAM reduces its ability to induce neurite outgrowth from chick RGCs, rat hippocampal neurons and rat cerebellar neurons (see earlier) although it increases its ability to promote adhesion [11]. Recent studies using the PC12 cell line [8], rat

Abbreviations used: CAMs, cell adhesion molecules; PSA, polysialic acid; VASE, variable alternatively spliced exon.

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hippocampal neurons and rat cerebellar neurons (P. Doherty, unpublished work) cultured on monolayers transfected with cDNA encoding NCAM and N-cadherin show that CAM-dependent neurite outgrowth can be completely blocked by a combination of L- and N-type Ca\(^{2+}\)-channel antagonists (Dihydropyridine and \(\omega\)-conotoxin respectively) or by pertussis toxin which acts for ADP-ribosylating certain G-proteins to render them inactive. Background integrin-dependent and NGF-induced neurite outgrowth from PC12 cells are not affected by these treatments. A variety of inhibitors of protein kinase A and C failed to inhibit CAM-dependent neurite outgrowth from PC12 cells, suggesting that G-proteins do not activate Ca\(^{2+}\) channels via changes in these second messenger pathways. However, the kinase inhibitor K-252b was found to inhibit CAM responses, although it also inhibited basal neurite outgrowth on control 3T3 monolayers [8]. These results suggest that CAM-dependent neurite outgrowth is specifically mediated by a transmembrane signalling pathway involving a G-protein-dependent activation of L- and N-type Ca\(^{2+}\) channels. This is the first direct evidence that CAM-mediated neurite outgrowth may depend upon activation of neuronal second messenger pathways and not adhesion per se.

If this is indeed the case, it should be possible to promote neurite outgrowth by activating the postulated transmembrane signalling pathway independently of CAMs. To this end, we recently conducted experiments on PC12 cells in which a depolarizing concentration of KCl (40 mM) known to open the voltage-activated L- and N-type Ca\(^{2+}\) channels [12] was tested for its ability to promote neurite outgrowth from PC12 cells cultured on control untransfected 3T3 monolayers (J. L. Saffell, F. S. Walsh & P. Doherty, unpublished work). The results show that neurite outgrowth comparable with the CAM-dependent response is elicited under such conditions and can be completely blocked by a combination of L- and N-type Ca\(^{2+}\)-channel antagonists. The Ca\(^{2+}\) channel agonist Bay K8644 which acts by reducing the level of depolarization required to open Ca\(^{2+}\) channels was similarly able to mimic the CAM-dependent response. These results imply that activation of the postulated neurite outgrowth-promoting pathway using mechanisms independent of CAMs to open Ca\(^{2+}\) channels is sufficient to trigger the full morphological response without the need for CAM binding. By comparing the effect of pertussis toxin and kinase inhibitor K-252b on K\(^{+}\)-depolarization-mediated and CAM-mediated neurite outgrowth, it is possible to determine whether their targets lie upstream or downstream of Ca\(^{2+}\)-channel opening in the postulated transmembrane signalling pathway. In contrast to its effect on the CAM-dependent response, pertussis toxin has a negligible effect on the K\(^{+}\)-depolarization-induced response, indicating that its target G-protein does indeed lie upstream of the Ca\(^{2+}\) channel, the position expected for a G-protein postulated to signal between homophilically bound CAM and Ca\(^{2+}\) channel. K-252b, however, fully inhibits the K\(^{+}\)-depolarization-induced response, which suggests that its site of action is downstream of Ca\(^{2+}\) entry. Collectively, these results not only support the CAM-binding Ca\(^{2+}\)-influx transmembrane signalling postulate, but also suggest that for the neurite outgrowth response, the ability of CAMs to open Ca\(^{2+}\) channels may be more important than their ability to support adhesion per se.

The above results lead us to the view that the adhesion-promoting and neurite outgrowth-promoting properties of CAMs are fundamentally different and in some ways contradictory functions that are not necessarily directly related. In the case of NCAM our postulate is that at some stages of development this molecule promotes plasticity (i.e. cell migration and axonal growth) via activation of neuronal second messenger pathways and that this is unrelated to adhesion per se, whereas at other stages of development it might be involved in stabilizing connections between cells (e.g. axon bundles and synapses) via direct adhesive interactions that do not require activation of second messenger pathways. In other words the function of NCAM in the neuron changes from one of promoting neurite outgrowth to one that may promote adhesion and this is accomplished by a combination of both post-translational changes (e.g. loss of PSA) and isoform switching (e.g. inclusion of the VASE exon).

Glycoproteins modulate changes in synaptic connectivity in memory formation
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Introduction
It is generally agreed that memory formation must involve the making of new synapses or the modification of those already existing within the brain. Glycoproteins, such as neural cell adhesion molecules (N-CAMS) and integrins, are major components of synaptic membranes and are thus likely to be important in the regulation of plastic changes.

McCabe and Rose [1] showed increased \(^{11}H\)fucose incorporation into tissue slices taken from the brains of day-old-chicks trained on a one-trial passive avoidance learning task. This effect was abolished if the birds were trained but then rendered amnesic [2]. Increased fucokinase activity could be measured within 1 h of training the chicks [3] and it was shown that the newly synthesized glycoproteins were destined for the synaptic membrane [4].

Experimental results and discussion
All our investigations were based on the one-trial passive avoidance learning paradigm. One-day-old chicks were placed in pairs in pens under conditions of controlled temperature and illumination. After a period of equilibration, chicks were injected intracerebrally with radiolabelled sugar, amnesic agent or antiserum. The birds were then trained using a small chrome bead dipped in either methylanthranilate (M chicks) or, in the case of controls, water (W chicks) according to the method described by Lössner & Rose [3]. On testing between 30 min and 24 h later, over 80% of W chicks pecked at a dry chrome bead while over 75% of M chicks avoided it.

To identify glycoproteins involved in memory formation, we studied the incorporation of radiolabelled fucose into two specific forebrain loci, the intermediate medial hyperstriatum ventrale (IMHV) and lobeus parolfactorius (LPO), at 6 h and 24 h after training. We used a double-labelling procedure in which incorporation into brains from chicks trained on the M bead was from \(^{14}C\)fucose and that into brains of control chicks trained on the W bead was from \(^{11}H\)fucose [5]. At both times, training produced region-specific changes in fucose incorporation into both pre- and post-synaptic glycoproteins. At 6 h there was a relative decrease in labelling of both the synaptic plasma membranes (SPMs) and post-synaptic densities (PSDs) of the right IMHV of M chicks, a decrease which persisted in PSDs until 24 h. There was a slight decrease in uptake into the right LPO SPMs at 6 h but this effect had been reversed by 24 h. At 24 h, there was a 64% and 24% increase in incorporation into SPMs and PSDs, respectively, in the left LPO. These results are summarized in Table 1.

When LPO glycoprotein components were analysed by SDS/PAGE, at 6 h after training there was a reduced fucosylation of 150–180 kDa pre-synaptic glycoproteins and an increased fucosylation of a 41 kDa pre-synaptic glycoprotein. At 24 h, a left LPO pre-synaptic component of molecular mass 50 kDa showed the greatest training-induced increase in fucosylation. Also, post-synaptic glycoproteins of molecular masses 150–180 kDa, 100–120 kDa and 33 kDa showed increased labelling. It is noteworthy that the first two sets of molecular masses quoted are similar to those of the N-CAMS. These results supported our hypothesis that memory formation involves the strengthening of connections between pre- and post-synaptic

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Abbreviations used: N-CAM; neural cell adhesion molecules; M chicks, chicks trained using methylanthranilate; W chicks, control chicks trained using water; IMHV, intermediate medial hyperstriatum ventrale; LPO, lobeus parolfactorius; SPMs, synaptic plasma membranes; PSDs, post-synaptic densities; 2-dGal, 2-deoxy-galactose.