MOLECULAR MECHANISMS OF NCAM FUNCTION

Anders M. Hinsby, Vladimir Berezin and Elisabeth Bock

Protein Laboratory, Institute of Molecular Pathology, University of Copenhagen, Panum Institute 6.2, Blegdamsvej 3, DK-2200, Denmark

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1. ABSTRACT

Neural cell adhesion molecule (NCAM) was originally characterised as a homophilic cell adhesion molecule (CAM) abundantly expressed in the nervous system. However, the last decade of research has challenged the traditional view and defined novel roles for NCAM. NCAM is now considered a signaling receptor that responds to both homophilic and heterophilic cues, as well as a mediator of cell-cell adhesion. This review describes NCAM function at the molecular level. We discuss recent models for extracellular ligand-interactions of NCAM, and the intracellular signaling cascade that follows to define cellular outcomes such as neurite outgrowth.

2. THE NCAM MOLECULE

NCAM belongs to the immunoglobulin superfamily of CAMs, which is characterised by extracellular immunoglobulin-like (Ig) and often fibronectin type 3 (F3) modules. From the N-terminal NCAM has five consecutive Ig modules (IgI through IgV) followed by two F3 modules (F3.I and F3.II) (see Figure 1). NCAM is encoded by a single-copy gene that spans more than 85 kilobases and contains at least 26 exons (1). The five Ig modules are encoded by exons 1-10 with the optional inclusion of the 30 bp VASE exon between exons 7 and 8 in Ig module IV. The two F3 modules are encoded by exons 11 to 14 with alternative splice variants generated by insertion of four small exons between F3.I and F3.II. The three major isoforms of NCAM: NCAM120, NCAM140 and NCAM180 (also termed NCAMC, NCAMB and NCAMA, respectively) differ by alternative exon usage downstream of exon 14. Exon 15 introduces a stop-codon yielding NCAM120 that lacks a cytoplasmic part, but is anchored to the membrane via a glycosylphosphatidylinositol (GPI) moiety. Omission of exon 15 gives rise to the transmembrane splice variants NCAM180 (exons 16-19) or NCAM140 (exons 16, 17 and 19) (2, 3) (see Figure 1 for overview).

2.1. Polysialylation of NCAM

Variations of the NCAM molecule can be introduced via post-translational modifications. Significantly, IgV contains two N-linked core glycosylations to which polysialic acid (PSA), i.e. long linear chains of α-2, 8-sialic acid, may be added (4, 5) by the actions of two polysialyl transferase enzymes (6). However, the PSA appears not to be restricted to N-linked glycosylations and recent evidence suggests additional O-linked PSA-modification site(s) in the F3 module region of NCAM (7). PSA is reduced by 85% in NCAM knock-out mice (8) and thus appears to be an almost unique feature of NCAM, with the remaining 15% generated mainly by auto-polysialylation of the transferases themselves (9). PSA is predominantly expressed during embryonic development, where it constitutes approximately 30% w/w of NCAM decreasing to 10% or less in the adult brain (10, 11). Removal of PSA increases NCAM-mediated adhesive properties *in vitro* (12), diminishes the neurite extension
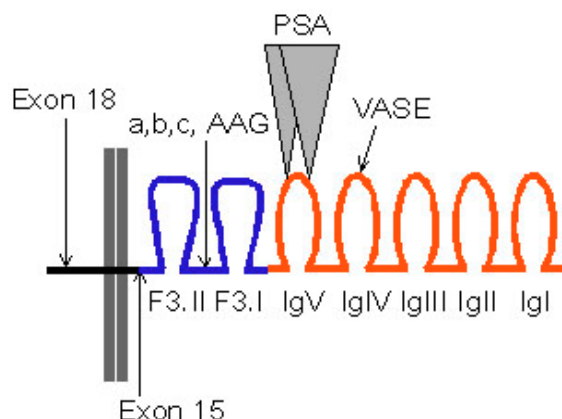
Figure 1. Schematic of the NCAM molecule. The extracellular part of NCAM consists of five Ig and two F3 modules. Arrows indicate localisation of insertion sites for exons used to generate alternative splice variants. The use of exon 15 generates the NCAM120 isoform, which lacks an intracellular protein domain and is attached to the membrane by a GPI anchor. Omission of exon 15 generates the two transmembrane isoforms NCAM140 (lacking exon 18, shown in figure) and NCAM180 (with exon 18). The carbohydrate PSA can be added by the action of polysialyl transferase enzymes predominantly at two sites in the fifth Ig module (IgV).

response to NCAM homophilic binding (13), and leads to a decrease (estimated at 10-15 nm in a model cell-line) in the distance between opposing cell membranes (14). Based on these findings it has been proposed that expression of PSA changes NCAM from a molecule that promotes stability to one that promotes plasticity (6, 15). This notion is supported by the finding that PSA-NCAM is located to areas in the adult brain that retain a high degree of plasticity such as the olfactory bulb and hippocampus (16), and by several studies that have pointed to a pivotal role of PSA in NCAM functions in the developing nervous system and in memory formation (see section 2.2). The molecular basis for this role remains to be determined, but it has been reported that due to the large size and charge of the hydrated PSA-moiety (14) it disrupts the adhesive properties of NCAM and other CAMs (N-Cadherin and L1-CAM) by steric and electrostatic inhibition of membrane apposition (17, 18). Conversely, PSA can positively modulate other NCAM interactions such as heterophilic association with the heparan sulphate proteoglycan Agrin (19, 15). Moreover, it was recently shown that PSA modulates BDNF signaling (20, 21). Enzymatic removal of PSA from NCAM on cortical neurons led to a decrease in the survival of these cells, but the effect was reversed by addition of exogenous BDNF. Furthermore, a reduction in the phosphorylation of the BDNF receptor (TrkB) was observed after PSA removal. These findings indicate that PSA positively modulates BDNF signaling and leads to an increase in the survival of cortical neurons (21). However, the mechanism by which PSA-NCAM modulates BDNF signaling remains to be elucidated.

From the above studies it appears that PSA has an anti-adhesive role, involving the inhibition of NCAM-

NCAM interactions between adhering cells, which facilitates cell migration and axon growth. On the other hand, PSA can positively regulate other protein interactions and modulate signaling processes. This latter role of PSA may explain *in vivo* studies showing that PSA is required for adhesion between neuronal precursor cells of the rostral migratory stream (RMS) (see section 2.2 for details).

2.2. NCAM function in the nervous system

NCAM is extensively expressed in the developing nervous system with a distinct pattern of spatiotemporal distribution (22, 2) that correlates with axon guidance and targeting (23, 24, 25, 26). Characteristic transcriptional and post-translational modifications regulate NCAM function during development. Thus, NCAM in the adult brain has a lower relative content of PSA, whereas expression of the optional VASE exon increases from less than 3% during development to 40% in the adult brain (27). *In vitro* studies have indicated that these changes convert NCAM from a molecule that induces plasticity (as reflected by promotion of neurite outgrowth) to one that mediates stability (by NCAM-NCAM adhesion) (12, 13, 28, 29). Interestingly, regions that retain a high degree of plasticity in the adult brain have been found to continue expressing PSA-NCAM (16) without the VASE exon (27). Together, these findings indicate that PSA-NCAM is involved in the structural modelling of the nervous system by promoting neuronal plasticity during morphogenesis. However, NCAM-deficient mice are viable, healthy and fertile, with only minor morphological abnormalities in the brain (30, 8) implying that other CAMs can compensate for NCAM deficiency. Albeit, a significant reduction in the size of the olfactory bulb (30, 8), defects in the fasciculation and pathfinding of hippocampal mossy fibers (31), and deficits in spatial learning (8) have been reported as a consequence of NCAM deficit. The reduced size of the olfactory bulb may be explained by deficits in the RMS from the anterior subventricular zone, which provides interneurons for the olfactory bulb postnatally (32). The migratory stream is formed by chains of neuronal precursor cells with extensive contacts migrating independently of glial cells (33). NCAM-deficient mice exhibit accumulation of neural precursors along this migratory pathway to the olfactory bulb (30, 34), and this condition is phenocopied by enzymatic removal of PSA both *in vitro* (34, 35) and *in vivo* (36). Intriguingly, removal of PSA leads to dispersion of the migratory chain of neural precursors into single cells, as observed in cultures of anterior ventricular zone explants (35). This latter finding surprisingly points to a physiological pro-adhesive function of PSA, which may be accomplished either by PSA working directly as a receptor (for an unknown cell-membrane component) or by PSA facilitating other protein-protein interactions.

The NCAM deficient mice also exhibit learning deficit (8) although it is unclear whether this is due to a developmental defect. Application of various learning paradigms in animal models has strengthened the notion of NCAM involvement in memory formation by establishing that: 1) NCAM polysialylation in the hippocampus (37, 38, 39), and NCAM synthesis (40, 41) increase after a learning task, and 2) memory formation can be abrogated by
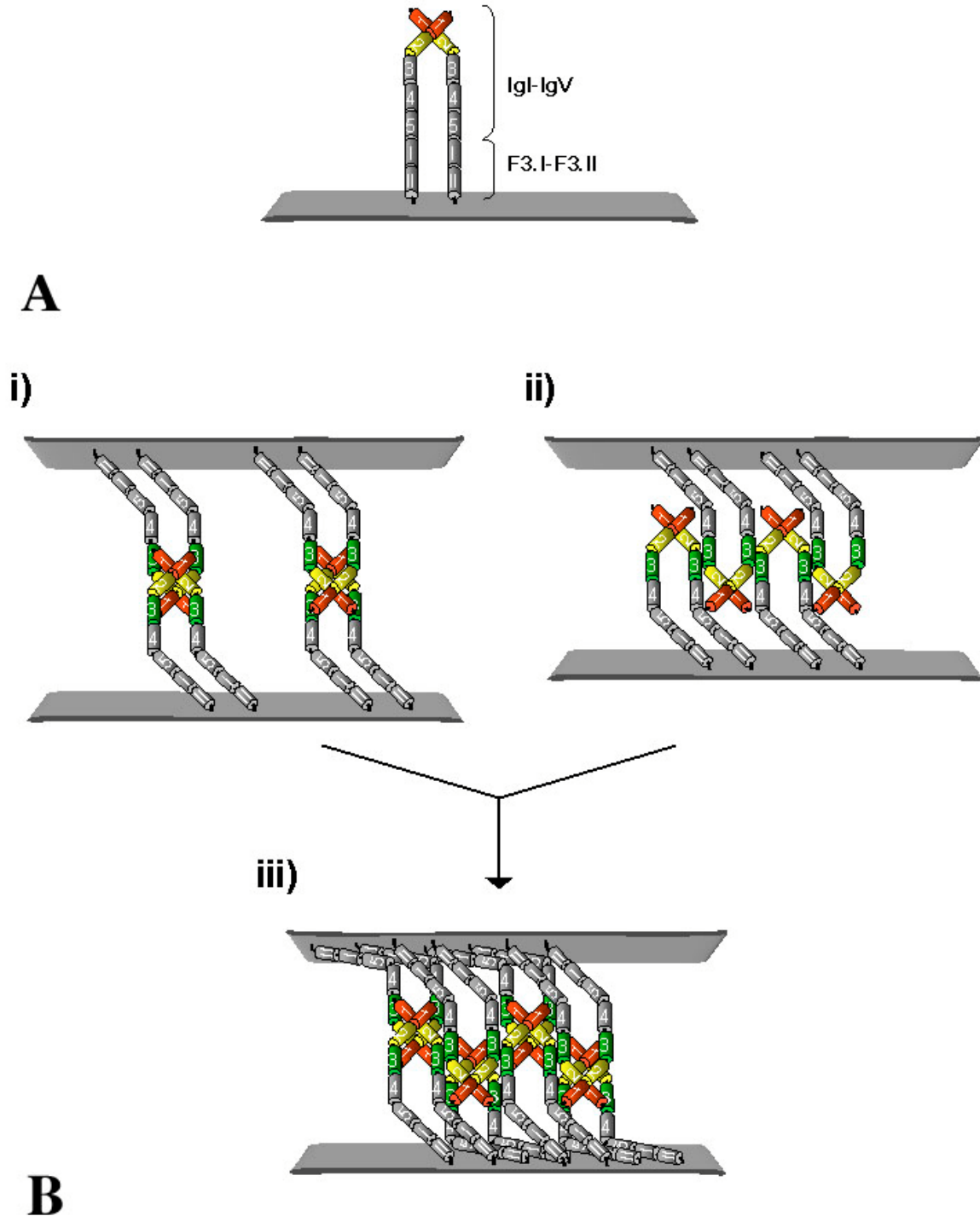
Figure 2. Model for homophilic NCAM binding. Crystallographic data from the combined IgI-IgIII module indicate cis- (i.e. between NCAM molecules on the same membrane) and trans- (i.e. between NCAM molecules on opposing membranes) interactions of NCAM homophilic binding. Modules engaged in NCAM-NCAM interactions are coloured. A) Depicts the cis-interactions between two NCAM molecules. Reciprocal interactions between IgI and IgII are observed. B) Depicts NCAM-NCAM interactions including trans-binding, which can be achieved by two mechanisms: i) Interactions between IgII and IgII, and IgIII and IgI, ii) Interactions between IgII and IgIII. The two sets of trans-interactions are arranged perpendicularly to each other. Note that ii) interactions require closer proximity between the opposing plasma membranes than i) interactions, if the angles between individual modules are unchanged. It has been speculated that i) represents an independent mode of NCAM homophilic binding, which can be combined with the interactions shown in ii) to accommodate a tight 'double zipper' mode of NCAM homophilic binding (see Figure 2B iii). This figure has been modified from (60).

intracranial injection of NCAM antibodies (42, 41) or enzymatic removal of PSA (43). Long term potentiation (LTP) is an enduring increase in the synaptic efficiency i.e. an augmentation of the post-synaptic response to a pre-synaptic action potential, and is considered to underlie synaptic plasticity involved in learning and memory storage. LTP is inhibited in hippocampal slice cultures from NCAM knock-out mice (44, 45) and from wild-type mice upon administration of NCAM antibodies or enzymatic removal of PSA (46, 47, 44). For more extensive recent reviews on the role of NCAM and polysialylation in the nervous system the reader is referred to references (48, 49, 50, 51).

In the following we will describe and discuss what is known about the molecular mechanisms underlying the functions of NCAM in the nervous system.

3. A CLOSE-UP LOOK AT NCAM'S HOMOPHILIC BINDING ACTIVITY

Initial cell aggregation studies using chicken NCAM-deletion mutants strongly indicated the involvement of a deca-peptide sequence in NCAM IgIII (52, 53) in homophilic binding, presumably via binding to itself (54). However, subsequent interaction studies in solution using recombinant NCAM modules have not been able to confirm IgIII's binding to itself (55, 56). Instead analysis by surface plasmon resonance (55) and titration by NMR spectroscopy (57, 58) have revealed interactions between IgI and IgII. These interactions were confirmed by crystallographic data from the combined IgI-IgII modules, which led to a model of a reciprocal cross-shaped binding mode between two IgI-IgII modules (59). The apparent discrepancy between the cell based studies (IgIII-IgIII binding) and the molecular binding studies in solution (IgI-IgII binding) was partially reconciled when the structure of the combined IgI-III modules of NCAM recently was resolved by x-ray crystallography (60). Here, two major groups of interactions were distinguished: parallel and anti-parallel to the long axis (N- to C-terminal) of the molecule. The anti-parallel interactions observed are considered to constitute trans-interactions (i.e. binding between NCAMs on opposing membranes) and consist of two sets of interactions. One set of binding sites involves IgII-IgII and IgI-IgIII interactions and the other set consists of IgII-IgIII interactions. It has been proposed that NCAM binding in trans is initiated by IgII-IgII and IgI-IgIII interactions (see Figure 2B i), which can subsequently be tightened by IgII-IgIII trans-interactions with other NCAM molecules (see Figure 2B ii), thus forming a 'double zipper' complex (see Figure 2B iii). In this crystallography based model, the deca-peptide motif previously proposed to mediate NCAM IgIII homophilic binding is an integral part of the IgIII-IgI interface of trans-interactions.

Interestingly, the model proposed by Soroka et al. (60) also accounts for NCAM-NCAM binding in cis (i.e. binding between NCAMs on the same membrane). Interactions parallel to the long axis are considered to be cis-interactions and are observed between IgI and IgII (see Figure 2A). Cis-homophilic binding between NCAM

molecules may participate in accumulating NCAM on the cell surface as observed for NCAM180 in post-synaptic membranes (61) and at locations of cell-cell contact including contact sites between growth cones and other cells (62). In a functional context it is noteworthy that intracellular signaling can be initiated by cross-linking NCAM on the membrane with specific antibodies (e.g. (63, 64)), thus providing an indication that NCAM-NCAM cis-interactions are an integral part of NCAM function in signaling processes. In a subsequent section (section 4.1) we will outline a possible mechanism for triggering of intracellular signaling by NCAM clustering on the cell surface.

Other studies have indicated a tight homophilic NCAM trans interaction, which is mediated by reciprocal binding between all five Ig modules (IgI-IgV, IgII-IgIV and IgIII-IgIII) (65, 66). However, none of these Ig module interactions have so far been confirmed by structural studies (55, 56, 60). Nonetheless, it is likely that NCAM homophilic binding can be achieved by more than one mechanism. The PSA-modification may constitute one way to switch between different modes of trans-interactions. In the absence of PSA a tighter and more stable configuration may be favoured (e.g. the 'double' zipper proposed by Soroka et al. (60), see Figure 2B iii) confering a role for NCAM binding in stabilising cell-cell contacts. Conversely, in the presence of PSA a looser configuration (e.g. Figure 2B i), or perhaps complete abrogation, of NCAM homophilic trans-interaction may modulate the adhesive and signaling properties of NCAM thereby promoting neurite extension and plasticity. PSA-NCAM engages in several interactions different from those of NCAM without PSA, as indicated by the previously mentioned pro-adhesive role of PSA in neuronal precursor cells of the RMS and the PSA dependent interaction with agrin. The tight 'double-zipper' NCAM binding mode does probably not permit cis-interactions with heterophilic ligands due to steric hindrance induced by NCAM clustering. Thus, PSA-mediated abrogation of this binding mode may allow NCAM to engage in cis-interactions with heterophilic binding partners.

4. NCAM IN HETEROPHILIC INTERACTIONS

Although originally characterised as a homophilic binding protein, a great number of studies have reported on heterophilic NCAM interactions. It is beyond the scope of this review to discuss each ligand in detail, but a comprehensive list of reported heterophilic NCAM ligands is presented in Table 1. The fibroblast growth factor receptor-1 (FGFR-1) has been functionally characterised as a co-factor for NCAM signaling to neurite outgrowth in PC12 cells and primary neurons (see section 5.2), and a recent structural study has provided a model for direct NCAM/FGFR-1 interaction (67). Another recent study has defined glial-cell-line derived neurotrophic factor (GDNF) as a heterophilic ligand of NCAM. The NCAM/GDNF interaction is functionally relevant, since it leads to neurite outrowth in primary neurons. Interestingly, GDNF/NCAM signaling appears to be independent of homophilic NCAM binding in trans, and provides convincing evidence that

Molecular mechanisms of NCAM function

Table 1. Heterophilic ligands of NCAM

Ligand	Observed features of interaction	Reference
Glial derived neurotrophic factor (GDNF)-GDNF family receptor - α	Initiating intracellular signaling via NCAM leading to neurite outgrowth or cell migration	68
Fibroblast growth factor receptor-1 (FGFR-1)	Initiating intracellular signaling involved in NCAM mediated neurite outgrowth.	72, 67
Phosphacan/protein-tyrosine phosphatase-ζ/β (Chondroitin sulfate proteoglycan)	Inhibition of NCAM homophilic binding, Modulation of neuronal adhesion and neurite extension.	170, 171
Neurocan (Chondroitin sulfate proteoglycan)	Inhibition of NCAM homophilic binding, Modulation of neuronal adhesion and neurite extension	172, 173
Agrin/Heparan sulfate proteoglycans	Interaction with extracellular matrix	174, 175, 176, 19, 15, 55
Collagens	Binding possibly via heparin Interaction with the extracellular matrix.	177, 55
L1 CAM	Enhancing L1 homophilic binding Modulation of L1 function	178, 179, 180
Tag-1/Axonin-1	-	181
Prion protein (PrP)	-	182
Rabies virus	NCAM expression increases susceptibility to rabies virus infection.	183
ATP	Modulation of FGFR binding and neurite outgrowth	81, 82, 83

homophilic binding in some cases is not required for NCAM function as a signaling receptor (68). These two heterophilic NCAM interactions of special interest will be described in the following.

4.1. NCAM interaction with the FGFR-1

The FGFRs are prototypical receptor tyrosine kinases composed of a ligand-binding extracellular part consisting of three consecutive Ig modules connected via a single membrane-spanning domain to an intracellular portion that contains a tyrosine kinase domain (69). Binding of FGF family members, which are high-affinity FGFR ligands, activates the tyrosine kinase and results in the phosphorylation of a number of regulatory tyrosine residues, thus creating binding sites for the protein tyrosine binding (PTB) or Src Homology 2 (SH2) domains of downstream effector molecules (70, 71).

At least some NCAM functions require activation of FGFRs (e.g. (72, 73)). Cross-talk between CAMs and growth factor receptors is not a novel concept. A symbiotic relationship between signaling by several growth factor receptors and integrins appears to exist (74). Integrin-mediated adhesion can lead to enhancement of, and even ligand-independent, phosphorylation of growth factor receptors such as the FGFR and epidermal growth factor receptor (EGFR) (75). The mechanism is debated, but recent findings indicate that an intracellular complex is formed, which contains the docking protein p130CAS and the non-receptor tyrosine kinase (NRTK) c-Src that are required for integrin triggered phosphorylation of the EGFR (76).

In the case of NCAM, recent evidence suggests a more direct mechanism of FGFR engagement. Treatment of cells with a soluble recombinant version of the NCAM F3.II module induces phosphorylation of the FGFR-1, and direct binding of the F3 modules of NCAM to the two membrane proximal Ig modules of the FGFR-1 is observed by surface plasmon resonance analysis and NMR spectroscopy (67). Moreover, the F3 modules are not involved in homophilic NCAM interactions (66, 65), and it thus appears that NCAM (via the F3 modules) may serve as a ligand of the FGFR-1 (67). Another putative point of interaction has previously been suggested at the far N-terminal end of the FGFR IgII module where a so-called CAM homology domain (CHD) is located. The CHD is a linear 20 amino acid stretch that consists of a series of three short sequences with homology to sequences found in NCAM, L1-CAM and N-cadherin, respectively. Antibodies against the CHD block neurite outgrowth mediated by these three CAMs, and a peptide corresponding to the NCAM homology region specifically abrogates NCAM-mediated neurite outgrowth (77). However, evidence for NCAM interaction with the CHD of the FGFR has not been presented, and according to a structural study of the FGFR-1 the CHD is partially inaccessible to ligands (78). Although the issue is not resolved, a possible explanation is that the CHD does not directly mediate CAM-FGFR interaction, but rather serves some regulatory function.

Despite the ability of a soluble version of the NCAM F3.II module to induce FGFR phosphorylation, activation mediated by NCAM *in situ* probably still differs from soluble growth factor stimulation. Recent articles indicate that growth factor receptors may be stabilised in the plasma membrane by interactions with integrins (76) or N-cadherin (79). The FGFR-1 is known to auto-activate upon overexpression (e.g. (80)) and NCAM-induced stabilisation and clustering of the FGFR on the plasma membrane may increase the level and local concentration of the receptor, thus leading to activation of the kinase. NCAM-mediated FGFR activation may thus direct a spatiotemporally specific FGFR response, differing from the growth factor induced activation by modulating the stability and localisation of the receptor.

4.2. Janus-like ATP regulation of NCAM function

An ecto-ATPase activity has been located to

immunoprecipitates of NCAM from synaptosomes of rat brain homogenates and NCAM-transfected L-fibroblasts (81, 82). It is associated with all three major isoforms of NCAM, including the GPI-anchored NCAM120, thus indicating an extracellular location of the ATPase. Verification has been provided by use of a competitive traceable ATP analogue, FSBA, which inhibits the NCAM associated ATPase activity and documents binding sites for ATP in the extracellular part of NCAM (82). The function of ATP in NCAM actions appears to bifurcate. ATP and non-hydrolysable ATP-analogues interfere with NCAM-mediated adhesion, and hereby inhibit aggregation and neurite outgrowth induced by NCAM homophilic binding. Conversely, induction of neurite outgrowth is observed in neuronal cells non-permissive of NCAM interactions in trans, upon treatment with ATP, but not non-hydrolysable analogues (83). This latter effect can be inhibited by antibodies to NCAM and the FGFR, which points to a role for ATP hydrolysis in the NCAM-mediated activation of the FGFR. This idea is strengthened by the location of an ATP binding motif in the FGFR binding part of the second F3 module of NCAM, and surface plasmon resonance analysis revealed that ATP can fully abrogate F3 FGFR interaction (67). These findings suggest a Janus-like role of ATP in NCAM function. On the one hand ATP binding inhibits NCAM homophilic interaction and subsequent activation, but on the other hand NCAM-mediated FGFR activation may be aided by ATP hydrolysis.

4.3. NCAM is a receptor for GDNF

GDNF and members of the GDNF family ligands (GFLs) have pronounced protective effects on neuronal subpopulations, such as dopaminergic neurons. GFLs convey signaling across the cell-membrane through high-affinity binding to soluble or GPI-linked GDNF family receptor α (GFRα) proteins, which subsequently form a complex with the Ret receptor tyrosine kinase (84). However, recently it has been shown that GDNF signaling in the absence of Ret can be initiated by an interaction with NCAM140 (68). NCAM140 cross-links with GDNF and the binding is promoted by GFRα. GDNF induces the activation of NCAM-associated NRTK Fyn in Schwann cells and primary neurons. Moreover, GDNF treatment leads to migration of Schwann cells and neurite outgrowth in hippocampal and cortical neurons in a manner that depends on Fyn and NCAM, but not Ret or FGFR activation. Interestingly, it appears that GFRα expression ablates NCAM homophilic adhesion. It has been suggested that GFRα forms a complex with NCAM on the cell surface to increase affinity for GDNF binding while interrupting NCAM-NCAM interactions. The study by Paratcha *et al.* (68) provides evidence for a new role of NCAM as a signaling receptor that functions independently of homophilic binding.

5. NCAM: THE INSIDE STORY

It has been more than a decade since the notion of NCAM as a signaling receptor first emerged (85, 86, 87). NCAM signaling has primarily been studied in assay systems, which analyse neurite extension of primary neurons or the neuronal cell line PC12 in response to

homophilic NCAM trans interactions. For this purpose the co-culture system where neurons or PC12 cells are grown on top of a cellular monolayer of NCAM-positive or -negative fibroblasts (88) is widely used. Alternatively, NCAM signaling can be initiated by treatment of cells with soluble NCAM molecules, specific antibodies, or more recently by the application of NCAM mimicking peptides (89). Discrepancies between studies exist and are most likely a consequence of cell- and stimulus-specific events.

5.1. NCAM signaling in neurite outgrowth

Development of the nervous system involves the process of neuronal wiring, which involves axonal projections extending over long distances to reach specific target cells. The cellular reactions to neurotrophic factors in the developing nervous system include interconnected events, such as neuronal differentiation, survival, and initiation, protrusion and consolidation of neuritic processes. The growth cone at the leading edge of extending axons governs the pathfinding process by continously responding to extracellular cues that may be either attractive or repulsive. Transmembrane receptors on the growth cone bind to ligands encompassing secreted proteins, such as neurotrophins (e.g. nerve growth factor (NGF)), and cell-attached guidance cues including NCAM, to promote elongation and guidance of neurites. The motility of the growth cone owes to cytoskeletal dynamics involving constant assembly and breakdown of directed actin filaments and microtubuli, which are primary downstream targets in regulation of growth cone advance or collapse (90, 91).

In vitro, a simplified version of neurite extension can be studied in neurite outgrowth assays, like the co-culture set-up described above, in which neurons are grown on a cellular substrate. NCAM, lacking endogenous intracellular enzymatic activity, has been shown to promote neurite outgrowth by recruitment of tyrosine kinases, which leads to activation of two principal branches of signaling either initiated by the FGFR or the NRTK Fyn, respectively. In addition, it has recently been found that GDNF and its receptor GFR can bind to and functionally activate NCAM (leading to neurite outgrowth and migration) in the absence of FGFR-1 and in a manner independent of homophilic NCAM binding (68). NCAM signaling, predominantly in the functional context of neurite outgrowth, will be described in the following sections. A schematic representation of NCAM-mediated signaling events is shown in Figure 3.

5.2. NCAM signaling via the FGFR

A link between NCAM and FGFR signaling has been established by the identification of a shared set of effector proteins that are necessary for neurite outgrowth in cerebellar neurons mediated by FGF, as well as NCAM, L1-CAM and N-cadherin (92, 93, 94). Furthermore, expression of a dominant negative FGFR-1 in cerebellar neurons and PC12 cells abrogated the neuritogenic effect of these three CAMs (72, 95). By the use of pharmacological inhibitors these hallmark studies provided an initial model of NCAM signaling: Activation of the FGFR leads to
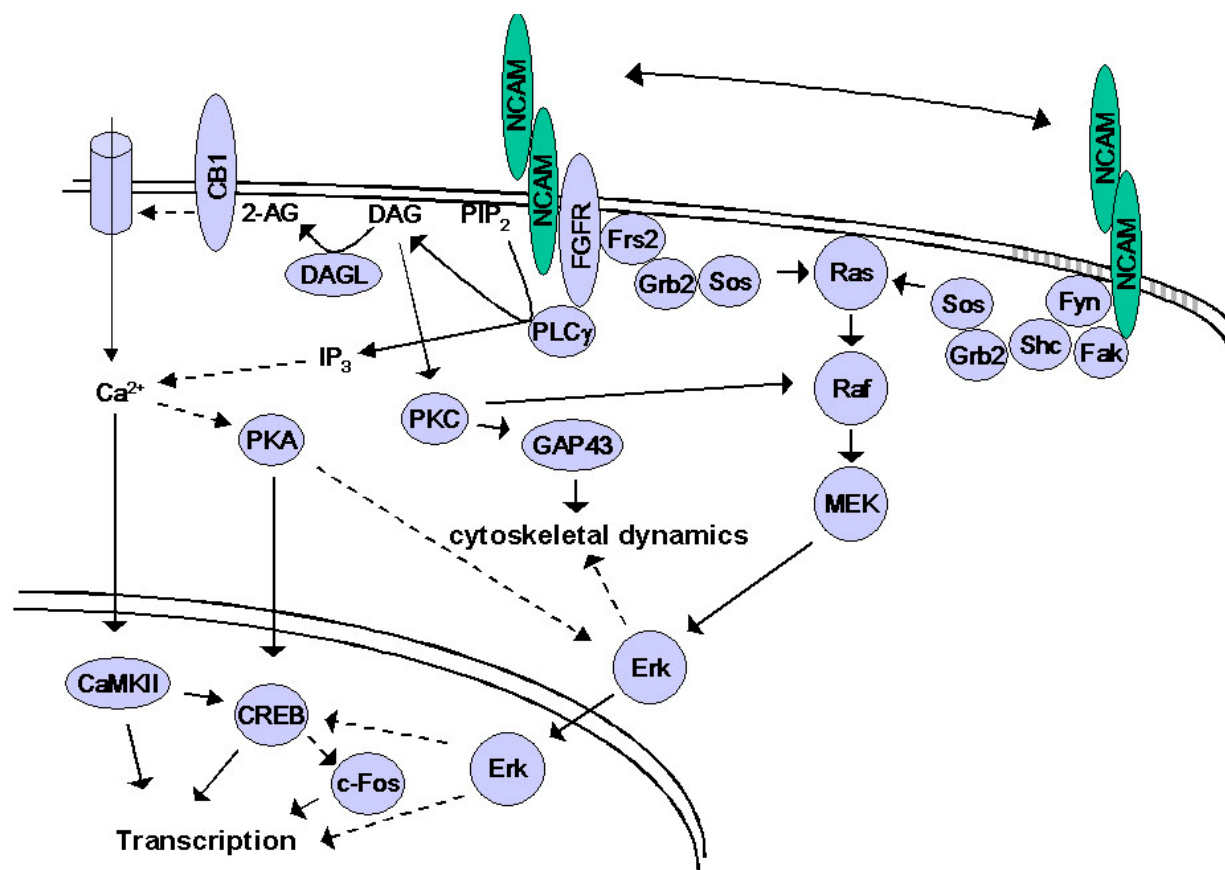
Figure 3. NCAM-mediated signaling to neurite outgrowth. NCAM initiates two principal pathways of signaling via tyrosine kinases Fyn, which is associated with lipid rafts, and FGFR. The diagram illustrates events from NCAM activation to transcription and/or changes in cytoskeletal dynamics. Dotted arrows (- - ->) indicate that the interaction between the indicated proteins is not direct or that the pathway has not been characterised at the molecular level. See text for details, alternative mechanisms of signal transduction and abbreviations. (IIIIII) indicates a lipid raft microdomain. PIP2, PKC and GAP43 are all believed to localise to lipid rafts. However, this is not reflected in the diagram.

production of diacylglycerol (DAG) via phospholipase Cγ (PLCγ) mediated hydrolysis of phosphoinositol 4, 5-bisphosphate (PIP2) in the plasma membrane (72). DAG lipase (DAGL) converts DAG to arachidonic acid, which presumably mediates N- and L-type calcium channel activation with concommitant Ca^{2+} influx (87, 93, 94). A recent report suggests that it is arachidonyl glycerol (2-AG) generated by DAGL, rather than arachidonic acid, that mediates FGF and CAM signaling. 2-AG is a ligand of endocannabinoid receptors (96), and Williams *et al* (97) has shown that inhibition of the CB1 cannabinoid receptor effectively blocks FGF and N-cadherin-mediated neurite extension in cerebellar neurons. Moreover, cerebellar neurons extend neurites in response to treatment with CB1 agonists in a manner dependent on N- and L-type calcium channel opening (97). However, it is still unclear how CB1 mediates Ca^{2+} influx. NCAM signaling also mobilises Ca^{2+} from intracellular stores (98), most likely by the PLCγ-mediated release of inositol 1, 4, 5-trisphosphate (IP_3) from the membrane and the IP_3-dependent activation of intracellular Ca^{2+} channels (99).

Considerable evidence for a role of cytosolic Ca^{2+} in growth cone behaviour exists (100, 101). The precise mechanism(s) of action(s) remains to be fully elucidated and is complicated by the fact that a cytosolic Ca^{2+} increase can result in both growth cone collapse and advancement. The final response might depend on the amplitude, frequency, duration, and/or spatial distribution of intracellular Ca^{2+} concentration changes (102, 103, 104).

Ca^{2+} as a second messenger can advance an intracellular signal by activation of a plethora of downstream effector proteins including protein kinase C (PKC), Ca^{2+}-calmodulin kinase II (CaMKII) and protein kinase A (PKA). The two former by direct actions and the latter via activation of Ca^{2+}/calmodulin sensitive adenylyl cyclases (AC) (105). As will be elaborated on elsewhere in this review, PKC, CaMKII and PKA have all been assigned roles in NCAM signaling.

PKC, a serine/threonine kinase, has recently been added to the list of NCAM effector proteins. Abrogation of PKC activation or recruitment inhibits NCAM-mediated neurite outgrowth in PC12 cells (73) and hippocampal neurons (108). In the latter study it was reported that upon NCAM activation (by crosslinking with NCAM antibodies) $PKC\beta_2$ is redistributed to NCAM

clusters in lipid rafts via the scaffolding protein spectrin that concommitantly binds directly to transmembrane NCAM isoforms and $PKC\beta_2$ (108). Most PKC isoforms are activated by DAG (109), which is generated by the enzymatic activity of PLC. In accordance, it has been reported that PKC recruitment to NCAM clusters depends on FGFR activation (108), which is directly upstream of $PLC\gamma$ in the NCAM signaling cascade. For an overview see Figure 3.

5.3. NCAM signaling via Fyn

Another branch of NCAM signaling is initiated by the interaction with Fyn. Fyn belongs to the Src family of NRTKs and is highly expressed in neural tissue including differentiating neurons (110). A role of Fyn in NCAM-mediated neurite outgrowth was established when cerebellar and dorsal root ganglion neurons from Fyn knock-out mice were shown to exhibit diminished neurite outgrowth when cultured on NCAM140 transfected fibroblasts compared to wild type neurons (111). By using immunoprecipitation assays, NCAM120 and NCAM140, but not NCAM180, have been found to be constitutively associated with Fyn (112, 63). Moreover, upon clustering of NCAM140 on the cell surface by antibody cross-linking or after treatment with soluble recombinant NCAM, an increase in the phosphorylation of Fyn, as well as the recruitment and phosphorylation of another NRTK, the focal adhesion kinase (FAK), was observed (63). The functional relevance of these two tyrosine kinases has been studied in PC12 cells where inhibition of either Fyn or FAK leads to abrogation of NCAM-mediated neurite outgrowth (73). After activation of Fyn and FAK by NCAM, they induce further downstream signaling resulting in activation of Erk MAPKs (see following section). Considering the multiple functions of FAK in integrin function, where it acts as a molecular signaling platform and directly interacts with other signaling and cytoskeletal cell components, which are involved in cell survival and migration, as well as mitogenic responses (113, 114), there is undoubtedly still much to be learned about the role of FAK in NCAM-mediated signaling.

5.4. NCAM signaling pathways converge on Erk1 and Erk2 MAPK activation

In PC12 cells, NCAM requires both FGFR and Fyn signaling for neuritogenic activity (73), and both of these pathways can lead to Erk MAPK activation. Some evidence points to transient versus sustained Erk MAPK activation as decisive in determining a mitogenic versus a differentiation (neurite outgrowth) response in PC12 cells (115). Accordingly, a sustained NCAM-mediated activation of Erk MAPKs has been observed in PC12 cells and hippocampal neurons (73). Activation of Erk MAPKs commences at the cell membrane by activation of the small GTP binding protein Ras. From Ras, the signal progresses through the sequential activation of serine/threonine kinases Raf and MAPK kinase (MEK) leading to phosphorylation of Erk MAPKs, which subsequently translocate to the nucleus and induce expression of target genes (70). In PC12 cells, activation of MEK, the kinase immediately upstream of MAPKs Erk1 and Erk2, rescues an inhibition of the Fyn, as well

as the FGFR, signaling pathways. Conversely, MEK inhibition eliminates NCAM-mediated neurite outgrowth, and it thus appears that the FGFR and the Fyn signaling pathways converge at Erk MAPKs (73). How does NCAM act through Fyn and the FGFR to trigger Ras-MAPK signaling? The docking protein FGFR substrate-2 (Frs2) (116) is a major substrate of the FGFR and its activation is required for FGF-stimulated sustained MAPK signaling in PC12 cells (117). Another docking protein, ShcA, has been shown to contribute to Fyn/FAK mediated MAPK activation in integrin signaling (118). Frs2 (117) and Shc (119) connect with the Ras-MAPK pathway by recruiting the adaptor protein Grb2, which associates with the GTP exchange factor SOS to induce Ras activation (70). It has recently been shown that ShcA and Frs2 are both necessary for NCAM mediated neurite outgrowth in PC12 cells, and ShcA is phosphorylated in cerebellar neurons after treatment with an NCAM mimicking peptide (120). In addition, MAPK activation by NCAM might also be achieved by the cytoplasmic Ca^{2+} increase, which induces the activation of MAPK in PC12 cells (121, 122), and/or by PKC, which can activate Raf (123). See Figure 3 for overview of NCAM signaling to the Ras-MAPK pathway.

5.5. NCAM signaling from lipid rafts

Lipids are not homogenously distributed in the plasma membrane of eukaryotic cells. It is believed that lipid microdomains, so-called lipid rafts, exist. These domains are characterised by their resistance to detergent solubilisation, although their existence *in vivo* is still being debated (124). Lipid rafts are enriched in sphingolipids and cholesterol and they accumulate specific proteins, thereby increasing the local concentration of signaling components, such as GPI-linked receptors and palmitoylated and/or myristoylated proteins including Fyn (125, 126). In addition, the rafts may also create a microenvironment that is locally protected from negative regulators of signaling, and they are thus perceived as platforms for several transmembrane signaling events (127). As described in the previous paragraphs, NCAM-mediated neurite outgrowth requires the activation of at least two different intracellular signaling pathways (one initiated by Fyn and the other by FGFR). The recruitment and activation of either of these pathways appear to rely on the localisation of NCAM in the plasma membrane. Fyn is localised to lipid rafts, while the FGFR appears to be excluded from these microdomains (128), and thus the two initiating effectors of NCAM signaling are localised in distinct plasma membrane sub-compartments.

Investigations have indicated that NCAM120 is highly enriched in lipid rafts (112) where also minor fractions of NCAM140 and NCAM180 can be found (129). Cells transfected individually with NCAM120, NCAM140 or NCAM180 and treated with soluble NCAM molecules display Erk MAPK phosphorylation by all three isoforms. An inhibitor of the FGFR blocks MAPK phosphorylation induced by NCAM180 whereas NCAM120 and NCAM140 stimulate MAPK independently of the FGFR. However, a point mutated NCAM140 version that is dissociated from lipid rafts

does rely on FGFR activation for MAPK phosphorylation (129). Moreover, only NCAM120 (112) and NCAM140 (63, 129) are capable of activating the Fyn-FAK pathway and only when they are located in lipid rafts. Interestingly, NCAM has also been found to co-localise and co-immunoprecipitate with caveolin, a raft-associated ingredient of the caveolae membrane invaginations, in detergent resistant fractions of isolated growth cones (130). Caveolin has previously been identified to convey integrin-mediated activation of Fyn (118, 131). It is possible that caveolin also participates in Fyn activation by NCAM. At least, the cytoplasmic part of NCAM is unlikely to be involved, given the fact that NCAM120 is sufficient for Fyn recruitment and activation (129). In conclusion, Fyn-FAK signaling from lipid rafts and FGFR signaling from non-raft plasma membrane areas both participate in linking NCAM adhesion to MAPK activation.

5.6. NCAM regulation of cytoskeletal dynamics

Neurite outgrowth relies on the continuous assembly, disassembly and stabilisation of cytoskeletal actin filaments and microtubuli (90). It is thus not surprising that signaling by most neuritogenic receptors is tightly linked to cytoskeletal dynamics, and often involves recruitment and activation of Rho family members, which include small GTPases such as Rac1 and Cdc42 (91). So far, there have been no reports providing information on the role of Rho family GTPases in NCAM-mediated neurite outgrowth. However, another link to actin cytoskeletal dynamics has been provided by GAP-43, which is a highly expressed protein in the developing nervous system and is enriched in growth cones. GAP-43 binds to and modifies filamentous actin and thus participates in cytoskeletal reorganisation and growth cone motility (132, 133). Serine 41 in GAP43 is a well documented PKC phosphorylation site (133), which supports growth cone advancement presumably by promoting actin polymerisation (134, 135). NCAM-mediated neurite outgrowth in cerebellar neurons has been found to be associated with phosphorylation of Ser41 in GAP-43 in an FGFR dependent way, and GAP-43 deficient cells do not extend neurites in response to NCAM and FGF (136). Thus, phosphorylation of GAP-43 downstream of FGFR-PKC activation is supposedly a key target in the NCAM-mediated regulation of cytoskeletal elements.

NCAM may also influence the structure of the cytoskeleton by the activation of Erk MAPKs, which are associated with microtubuli and phosphorylate microtubuli-associated proteins (137). In addition, Erk MAPKs activate myosin light chain (MLC) kinase with a concommitant increase in MLC phosphorylation and subsequent actomyosin-mediated contraction that leads to cell migration (138). Actomyosin-mediated contraction also promotes neurite growth cone advancement once actin filaments are coupled to a receptor complex that attaches the growth cone to a non-compliant substrate. Evidence for this mechanism was recently presented in a study using apCAM (the Aplysia homolog of NCAM) as adhesion receptor (139). Conversely, a retrograde flow of actin can occur by the same mechanism, when actin filaments are not stabilised by a substrate-bound receptor complex, and results in growth cone retraction (140).

5.7. NCAM signaling to transcriptional activation

The transcription factor CREB activates genes containing regulatory CRE elements such as the immediate early gene c-Fos, and CREB is crucial to memory formation in mammals (141) as well as in the invertebrates: Drosophila (142, 143) and the sea slug Aplysia (144, 145, 146). Both CREB and c-Fos have been found to be pivotal for NCAM-mediated neurite outgrowth in PC12 cells (107). Erk MAPK signaling can mediate CREB activation (147), and in accordance, CREB activation induced by NCAM clustering on cerebellar neurons has been shown to rely on MAPK signaling (64). However, signaling from NCAM to CREB may also proceed through PKA, since pharmacological inhibition of PKA has been shown to inhibit neurite outgrowth by NCAM in PC12 cells, and activation of neither PKA nor MAPK can rescue inhibition of NCAM-mediated neurite outgrowth by expression of a dominant negative CREB in PC12 cells (107). These findings suggest that PKA and MAPK signaling converge on CREB activation in NCAM signaling. Such a model is supported by a report showing that the concerted efforts of PKA and Erk MAPK mediate CREB activation in PC12 cells stimulated by Ca^{2+}-influx (148). Impey *et al.* (148) propose that Ca^{2+} stimulates cAMP production to activate PKA, which in turn is required for nuclear translocation of MAPK. Possibly, PKA also induces additional MAPK activation through the Ras-related small GTP-binding protein Rap1 ((149), but see also (150)). Additional mechanisms for CREB regulation by NCAM may exist. Using a specific kinase inhibitor it was shown that CaMKII is important for NCAM-mediated neurite outgrowth in cerebellar neurons (106), and CaMKII as well as PKA can directly phosphorylate CREB to regulate its activity (147).

5.8. NCAM signaling in glial cells

The above-described signaling studies were performed assuming that NCAM acts as a promoter of neurite outgrowth in developing or regenerating neurons and is involved in synaptic plasticity events. Another side of the story relates to the supporting cells in the nervous system: the glial cells, which express NCAM120 and NCAM140 (151). A number of studies have provided evidence that the NCAM homophilic interaction inhibits astrocyte proliferation *in vitro* and *in vivo* (152). The signaling pathway for this effect appears to differ from NCAM signaling in neurons and PC12 cells, inasmuch as it has been shown to be independent of FGFR activation (153). In fact, FGF2 induces the proliferation of astrocytes, and this effect is partially reversed by NCAM homophilic binding by decreasing MAPK phosphorylation (154). Inhibition of MAPK as well as astrocyte proliferation by NCAM is reversed by glucocorticoid antagonists, and NCAM homophilic binding induces transcription from a reporter construct, which is controlled by a glucocorticoid response element (154, 155). These findings indicate that the glucocorticoid receptor (GR) participates in NCAM signaling in astrocytes. Studies in astrocytes have also revealed an NCAM-mediated activation of the transcription factor NFκB (156). This signaling pathway involves

enzymes such as PLCγ and CaMKII, but not the FGFR (153) or GR (156). However, NFκB activation is not involved in the NCAM-mediated inhibition of astrocyte proliferation (156), and thus the NCAM-related function of this transcription factor in astrocytes remains to be discovered. Finally, NCAM activation of Fyn has been reported, as previously mentioned, to mediate GDNF-stimulated Schwann cell migration (68).

5.9. Phylogenetic aspects of NCAM signaling

At neuromuscular junctions (NMJ) of Drosophila the NCAM homologue fasciclin II (FasII) has a central role in activity-dependent synaptic plasticity. FasII serves to stabilise newly formed synaptic structures (157). However, a local decrease in FasII levels on the presynaptic membrane is required for synaptic remodeling (158), and can be achieved by cAMP-Drosophila PKA (dPKA) (158) and Drosophila Ras-MAPK (159) signaling. Dynamic regulation of FasII membrane localisation can also be achieved by synaptic clustering with the PSD-95 family member Discs-Large (DLG) protein, which forms a ternary complex with FasII and the potassium channel Shaker (160). Synaptic localisation of DLG is modulated by phosphorylation mediated by Drosophila CaMKII (161), and it has been proposed that phosphorylation of DLG leads to dispersion of DLG, FasII and Shaker from the synapse. While FasII downregulation is required for structural changes involved in NMJ synaptic plasticity, dPKA signaling leading to Drosophila CREB (dCREB) activation promotes functional plasticity by increasing neurotransmitter release (162). Thus, dPKA controls structural and functional requirements of synaptic plasticity in the Drosophila NMJ, by regulating synaptic FasII levels and CREB activation, respectively.

In the sea slug Aplysia, downregulation of apCAM, the homologue of NCAM and FasII, is correlated with long-term facilitation of sensory and motor neurons participating in the gill-withdrawal reflex - a presumed model of long-term memory formation. Both cAMP (163) and Aplysia MAPK (apMAPK) (164) have been implicated in events leading to endocytosis of apCAM and Aplysia CREB (apCREB) activation. The mechanism of apCAM downregulation involves the sequential activation of: Adenylyl cyclase --> PKA --> MAPK. ApMAPK phosphorylates apCAM, which hereby is targeted for endocytosis and degradation (165).

The Drosophila and Aplysia studies have established an activity dependent inside-out signaling mechanism, which regulates surface membrane levels and/or localisation of NCAM homologues. It remains to be elucidated if such a dynamic regulation of NCAM levels exists in higher organisms, and plays a role in synaptic plasticity. However, sequence homology between FasII/apCAM and NCAM is restricted to the extracellular portion, which means that functional properties of the cytosolic part of NCAM may not be conserved. Moreover, a PSA modification of NCAM-type has not been observed in invertebrate species (6), and invertebrate NCAM homologues thus appear to mediate more stable cell contacts. In contrast, the PSA modification is pivotal to

NCAM's role in plasticity in vertebrates. As a consequence, the range of NCAM functions appears to have expanded in vertebrates, and therefore comparisons to NCAM molecules in arthropod model systems such as Aplysia and Drosophila are less straight-forward.

6. CONCLUDING REMARKS AND PERSPECTIVES

Multiple signaling pathways are activated upon NCAM homophilic binding leading to the extension of neurites. At a first glance there appears to be a significant degree of redundancy. However, signaling pathways should probably not be regarded as strictly hierarchical and separate linear entities, but rather a complex or network of proteins, which converge at specific points or 'hot spots'. One example of signal integration was described above with Erk MAPKs as a point of convergence for different branches of NCAM signaling. Furthermore, in some cases the amplitude of the overall signal and not that of the individual proteins is pivotal whether a specific response is triggered or not (e.g. 166, 167, 168). Due to the lack of endogenous intracellular enzymatic activity of NCAM itself and the low affinity for the FGFR (67), the NCAM-mediated activation of individual pathway components is probably much weaker than the activation achieved by a diffusible growth factor with high affinity for the FGFR. Therefore to reach the threshold signal amplitude at these points of convergence, several signaling pathways probably have to be activated in parallel. The requirement for multiple pathways also allows for extensive cross-talk and makes sense in the context of growth cone guidance, which relies on responding to the coordinated input from a diverse array of receptors for guidance cues.

Since some of these seemingly redundant signaling pathways may vary their activity over time, future studies on NCAM signaling will certainly have to consider the temporal aspects of the signaling process as a parameter. For instance, there is strong evidence that a sustained Erk MAPK activation in PC12 cells induces a differentiation response, whereas transient activation leads to proliferation. Furthermore, after NGF stimulation of PC12 cells, activation of Ras and Rap GTP binding proteins appear to distributed in time and this has been proposed to account for the sustained MAPK response leading to differentiation (169).

A novel role for NCAM in GDNF and TrkB signaling is emerging, and will clearly be a focus point for future research. NCAM signaling studies have so far almost exclusively analysed signaling initiated by homophilic interactions that involves FGFR and Fyn activation. It now appears that heterophilic ligand binding in itself can lead to NCAM signaling (as established for the GDNF/NCAM interaction (68)), and future experiments will have to define how NCAM is activated by heterophilic ligand binding. NCAM does not contain intrinsic enzymatic activity (at least on the cytosolic side), and it is still unclear how it initiates a signaling cascade. One mode of signal initiation relies on FGFR-1 activation, which can be accomplished by direct binding, as described in this review. However,

GDNF activation of NCAM leading to neurite outgrowth does not involve the FGFR-1, and thus a relevant issue is to delineate how GDNF/NCAM signaling compensates for the lack of FGFR-1.

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Send correspondence to: Prof Elisabeth Bock, Protein Laboratory, Institute of Molecular Pathology, University of Copenhagen, Panum Institute 6.2, Blegdamsvej 3, DK-2200, Denmark, Tel: 453-532-7300, Fax: 453-536-0116, E-mail: Bock@plab.ku.dk