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Review

Signaling mechanisms of neurite outgrowth induced by the cell adhesion molecules NCAM and N-Cadherin

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Abstract. Formation of appropriate neural circuits depends on a complex interplay between extracellular guiding cues and intracellular signaling events that result in alterations of cytoskeletal dynamics and a neurite growth response. Surface-expressed cell adhesion molecules (CAMs) interact with the surroundings via the extracellular domain and bind to the cytoskeleton via their intracellular domain. In addition, several CAMs induce signaling events via direct interactions with intracellular proteins or via interactions with cell surface receptors. Thus, CAMs are obvious candidates for transmitting extracellular guidance cues to intracellular events and thereby regulating neurite outgrowth. In this review, we focus on two CAMs, the neural cell adhesion molecule (NCAM) and N-cadherin, and their ability to mediate signaling associated with a neurite outgrowth response. In particular, we will focus on direct interaction between NCAM and N-cadherin with a number of intracellular partners, as well as on their interaction with the fibroblast growth factor receptor (FGFR).

Keywords. Cell Adhesion Molecules, NCAM, N-Cadherin, neurite outgrowth, FGFR, intracellular signaling.

Introduction

The appropriate growth, guidance, and stabilization of axons and dendrites (collectively termed neurites in this review) during differentiation of neuronal precursor cells occur in a timely and ordered manner and involve a complex interplay between extracellular guiding cues and intracellular signaling events. In response to extracellular cues detected by a number of cell surface molecules, proteins inside the neuron become activated. Integration of cellular events, such as cell signaling, gene transcription, and modulation of the cytoskeleton, that are initiated by these proteins ultimately results in neurite guidance and elongation. Cell adhesion molecules (CAMs) are cell surface molecules grouped into four subclasses based on their structural characteristics: (*i*) cadherins (of which, more than 100 have been identified, and most are expressed by the brain [1, 2]), (*ii*) the integrin family, of which several are expressed in the brain, (*iii*) selectins (of which, three have been identified and found to be expressed on circulating cells), and (*iv*) the immunoglobulin (Ig) superfamily of CAMs, many of which have been identified in the brain. CAMs are responsible for mediating adhesion of cells to other

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cells and/or the extracellular matrix by engaging in *trans* interactions via their extracellular domains. CAMs usually are transmembrane proteins, and while the extracellular domain mediates adhesion, the intracellular domain interacts with the cytoskeleton. CAMs thereby provide a direct link between the extracellular growth/guiding cues and the intracellular scaffold that is responsible for morphology and growth.

Although CAMs originally were defined by their ability to attach cells to each other and to the extracellular matrix, many CAMs now are known to function also as signaling receptors that mediate neurite outgrowth [3]. Some CAM-mediated signaling is induced by direct interactions between CAM intracellular domains and a number of intracellular adapter and/or signaling molecules. Other CAM-mediated signaling is induced via interactions with a number of other cell surface receptors, such as growth factor receptors. Interestingly, different CAMs interact with different intracellular components and thus may regulate different aspects of the intracellular machinery that is responsible for mediating neurite growth. On the other hand, a number of CAMs have been identified as "ligands" for the same growth factor receptors, suggesting that although CAMs directly interact with different intracellular components, growth factor receptors may serve to integrate and modulate signaling from different CAMs.

In this review we will focus on two CAMs, the neural cell adhesion molecule (NCAM) from the Ig superfamily of CAMs and N-cadherin from the cadherin superfamily of CAMs, and their ability to mediate intracellular signaling associated with a neurite outgrowth response. In particular, we will focus on NCAM and N-cadherin and their direct interaction with a number of intracellular partners as well as on their interaction with the fibroblast growth factor receptor (FGFR).

NCAM-mediated adhesion and its role in neurite outgrowth

NCAM is a member of the Ig superfamily of CAMs. This family is characterized by a specific topology of their ectodomain, consisting of a variable number of Ig modules followed by a number of fibronectin type III (FN3) modules. The typical Ig structure consists of approximately 100 amino acid residues that are arranged in two anti-parallel β-sheets that form a sandwich, and the F3 modules have a tertiary structure similar to the Ig structure [4]. NCAM consists of five membrane-distal Ig modules (termed Ig I–V) and two membrane-proximal FN3 modules (termed FN3, I–II) (Fig. 1). NCAM is encoded by a single gene, the *NCAM1* gene, and alternative splicing of a number of exons in this gene gives rise to several different NCAM isoforms. The three major isoforms of NCAM are NCAM-120, NCAM-140, and NCAM-180, each named according to their apparent molecular weight. These three isoforms have similar ectodomains, but NCAM-120 lacks a transmembrane domain and is linked to the membrane via a glycosylphosphatidylinositol (GPI) anchor. The two transmembrane forms, NCAM-140 and NCAM-180, differ in the length of their intracellular domain, with NCAM-140 being shorter than NCAM-180 [5].

In addition to alternative splicing, NCAM also undergoes various posttranslational modifications. The most notable of these is the addition of polysialic acid (PSA), which is believed to have a profound effect on NCAM function. For example, addition of PSA to NCAM increases lateral diffusion of NCAM in the membrane at contact zones [6] and PSA is widely accepted to alter the adhesive properties of NCAM. Polysialylated NCAM is heavily hydrated because of the large negative charge of PSA. The large size of PSA, together with the hydration of the molecule and the ability of PSA to rotate about its glycosidic bonds, collectively result in PSA taking up considerable extracellular space when expressed [7]. This, in turn, likely decreases NCAM homophilic binding and perhaps favors NCAM interactions with heterophilic binding partners, such as FGFR, and promotes plasticity. In support for this function of PSA, the space between apposing cells is reduced when PSA is removed [7], and the adhesive properties of NCAM are increased [8]. Furthermore, the addition of PSA to NCAM has been reported to affect the adhesive properties of other CAMs. Thus, the adhesive properties of CAMs, such as C and N-cadherin, L1, and $\alpha_v\beta_1$ integrin, are reduced when Chinese hamster ovary (CHO) cells are transfected with polysialyl transferase (PST) to induce PSA-NCAM synthesis [9],– an effect that could be attributed to the steric respulsion that PSA on opposing membranes evokes [10]. In accordance with the de-adhesive effect of PSA, PSA downregulation in the adult is hypothesized to induce a change in NCAM function from a plasticity-promoting to a stability-promoting molecule, favoring stable cell-cell contacts over plastic changes such as neurite outgrowth. On the other hand, enzymatic removal of PSA from NCAM has also been demonstrated to result in increased Erk phosphorylation and initiation as well as outgrowth of neurites [11, 12], suggesting that the presence of PSA may not be plasticity promoting in all circumstances.

In the developing brain, NCAM has been described as a modulator of migration, axon growth, axon fascicu-

lation and pathfinding [13]. In particular there is a high expression of PSA-NCAM during neuronal development and genetic ablation of PSA imposes dramatic effects on brain morphology and is lethal [14], indicating that PSA is vital for the normal function of NCAM during development. In contrast, NCAM null mice are viable and brain morphology is affected in only a few structures such as the olfactory bulb and the hippocampus [15, 16]. In the adult nervous system, NCAM expression is upregulated in regenerating neurites [17, 18], suggesting that NCAM may also be important during neuronal regeneration. The mechanisms by which NCAM binds in a homophilic manner to NCAM has been the subject of many studies, and the results have been conflicting [19]. Accordingly, based on biophysical evidence, NCAM has been suggested to engage in homophilic binding via two different interactions involving either IgI–IgII or all five Ig domains [20]. On the other hand, a recent hypothetical model suggets, based on X-ray crystallography data, that two NCAM molecules on the same cell form high-affinity interactions by bending over each other and forming a cross through reciprocal interactions between IgI and IgII. When these NCAM *cis* dimers meet NCAM *cis* dimers on apposing cells, they interact in *trans* by one of two mechanisms: (*i*) Ig module II and III of each dimer interact antiparallelly with IgIII and II, respectively, of the apposing dimer and thereby form a so-called "flat zipper," or (*ii*) Ig modules I, II, and III in one dimer interact with Ig module III, II, and I, respectively, of the apposing dimer and thereby form a so-called "compact zipper". Neither of these *trans* interactions are mutually exclusive, and a combination of the two zipper types could result in a complex two-dimensional cluster formation [19, 21]. However, a two-dimensional zipper most likely is only possible when PSA is not attached to NCAM. As mentioned above, PSA takes up considerable space; therefore, the NCAM de-adhesive effects observed with PSA may be attributable to interference with NCAM zipper formations [21].

A number of studies have shown that homophilic binding of NCAM triggers signaling events that ultimately result in cellular responses, such as differentiation, survival, or modulation of synaptic plasticity. These events are hypothesized to occur both via direct activation of downstream signaling cascades and via interactions with other cell surface receptors such as FGFR.

The tertiary structure of the cytoplasmic domain of NCAM has not yet been resolved. However, because of a high level of prolines and charged amino acids, the intracellular domain is hypothesized to contain only a few secondary structures (such as α-helixes and β-sheets) and most likely has an extended structure, suggesting that multiple protein interaction sites are possible [22]. Accordingly, ligand affinity chromatography studies recently identified a number of cytosolic proteins that interact with the intracellular part of NCAM [23, 24]. The vast majority of the interaction partners appear to be related to cytoskeleton activities, suggesting that NCAM could be a link between extracellular adhesion cues and cytoskeletal changes during neurite outgrowth. Although several interaction partners are shared between NCAM-140 and NCAM-180, a number of interaction partners are distinct for each isoform, indicating that NCAM-140 and NCAM-180 may have distinct functions.

Both NCAM-140 and NCAM-180 interact with the cytoskeletal proteins α-tubulin and β-tubulin [23], the two main components of microtubules. Because microtubules are central for neurite extension, this interaction links NCAM to neurite elongation. In contrast to NCAM-140, NCAM-180 affinity columns also pull down microtubule-associated protein-1A (MAP1A), which stabilizes the microtubule whereby polymerization and growth is promoted [25]. Thus, NCAM-180 may be a link between microtubules and MAP1A and mediate microtubule stability, whereas NCAM-140 may not have the same stability-promoting effect. Both NCAM-140 and NCAM-180 interact, however, with leucine-rich acidic nuclear protein (LANP, also known as pp32) [24], a protein hypothesized to interact with MAP1B and thereby prevent MAP1B from stabilizing the microtubule [26]. However, in the case of MAP1B, the stability-promoting effect has been reported to result in inhibition of neurite outgrowth [26]; therefore, sequestering MAP1B via LANP would further promote neurite outgrowth. Early studies also suggested that NCAM-180, but not NCAM-140, co-purifies with spectrin [27], a protein involved in linking cellular membranes to motor proteins as well as to the actin filament cytoskeletal system. All three NCAM isoforms were later shown to bind spectrin [28], but NCAM-140 has a significantly lower affinity for spectrin compared with NCAM-180. Furthermore, NCAM-120, without an intracellular domain, may co-precipitate with spectrin because of their common membrane localization in rafts. Thus, NCAM-180 is likely the main NCAM isoform associated with spectrin. Spectrin interacts with the growth-associated protein 43 (GAP-43), a protein that is highly expressed in growth cones and believed to be important for growth cone extension [29]. The finding that NCAM-180 and GAP-43 both interact with spectrin raised the possibility that spectrin may be a link between these two proteins. Along this line, it was demonstrated that NCAM stimulation induce GAP-43 phosphorylation. Further-

more, NCAM-mediated neurite outgrowth was inhibited in neurons from GAP-43 knockout mice [30], further supporting a functional relationship between NCAM and GAP-43. Interestingly, a recent study [31] demonstrated that transfection with dominant negative spectrin inhibits NCAM-induced neurite outgrowth only when GAP-43 is available. Transfection with dominant negative spectrin did not have an effect, however, on NCAM-stimulated neurite outgrowth in PC12-E2 cells not expressing GAP-43, indicating that NCAM not interacting with spectrin can induce neurite outgrowth via a spectrin/GAP-43-independent pathway. Because this pathway could be inhibited by transfecting cells with the cytoplasmic part of NCAM-140 (believed to act as an NCAM-140 inhibitor by competing with intact NCAM-140 for binding to NCAM-140 interaction partners), the spectrin/GAP-43-independent pathway most likely is mediated via NCAM-140. Thus, NCAM-140 and NCAM-180 appear to induce neurite outgrowth through two distinct pathways. When GAP-43 is not present, NCAM-stimulated neurite outgrowth is mediated by NCAM-140, whereas in the presence of GAP-43, NCAM-180 mediates neurite outgrowth spectrin- and GAP-43-dependently.

In addition to the interactions with various cytoskeletal components, the intracellular part of NCAM-140 and NCAM-180 also interacts with a number of signaling pathways. Buttner and colleagues [24] showed that phospholipase Cγ (PLCγ) and protein phosphatase 1 and 2 (PP1, PP2) interacted with NCAM140 and NCAM-180. However, the functional outcome of PLCγ interactions with NCAM has not yet been explored, although it could be speculated that NCAM participates in FGFR-induced signaling by binding to PLCγ and thereby bringing FGFR and PLCγ into close proximity to each other. In addition to PLCγ, PP1, and PP2, NCAM-180 also interacts with the proteins turned-on-after-division-64 (TOAD-64) and Rho kinase (ROCK) [24]. Both of these proteins are associated with neurite outgrowth [32, 33] and may be yet another mechanism, in addition to GAP-43, by which NCAM-180 modulates growth cone activity and neurite outgrowth. NCAM-140, but not NCAM-120 or NCAM-180, constitutively co-immunoprecipitates with the Src-related tyrosine kinase $p59^{fyn}$, and NCAM crosslinking with antibodies induces transient $p59^{fyn}$ phosphorylation [34]. In contrast, pharmacological inhibition of Src-family kinases abolishes NCAM-stimulated neurite outgrowth [35]. Although NCAM was reported to co-immunoprecipitate with $p59^{fyn}$, further investigations indicated that the interaction is not direct; rather, it is mediated via interactions of the protein tyrosine phosphatase-α (RPTP-α) with NCAM [36]. After activation of $p59^{fyn}$ by NCAM clustering, the focal adhesion kinase (FAK), a nonreceptor tyrosine kinase, is recruited to the NCAM-$p59^{fyn}$ complex and is phosphorylated [34]. Thus, NCAM-140 stimulation induces $p59^{fyn}$ activation with subsequent recruitment and activation of FAK, ultimately inducing neurite outgrowth. In conclusion, the pathway that NCAM-140 utilizes to induce neurite outgrowth conceivably involves RPTP-α and $p59^{fyn}$/FAK, whereas NCAM-180-induced neurite outgrowth involves spectrin, GAP-43, and possibly TOAD-64 and ROCK (Fig. 2). Finally, activation of most of the above mentioned pathways may result in activation of a number of different transcription factors and c-FOS, nuclear factor κB (NF-κB) and cyclic AMP response element-binding protein (CREB), are thought to be involved in NCAM mediated neurite outgrowth. Thus, NCAM stimulation results in increased expression of c-FOS [37] and activation of NF-κB [38, 39] and CREB [40].

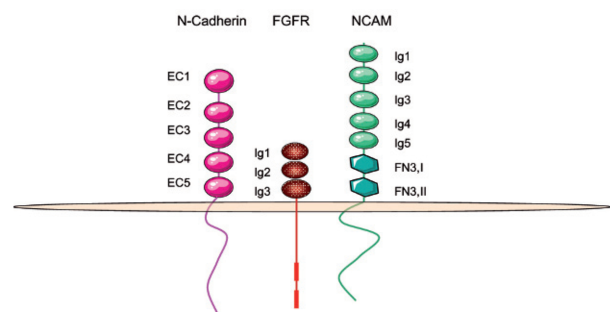

Figure 1. Diagram of the structure of N-Cadherin, FGFR and NCAM. N-Cadherin, FGFR and NCAM are single pass transmembrane proteins. Extracellularly N-Cadherin is composed of five Extracellular Cadherin (EC) domains. FGFR is composed of three Immunoglobulin (Ig) domains and NCAM is composed of five membrane distal Ig domains and two membrane proximal Fibronectin type III (FN3) domains.

NCAM signaling through FGFR

In addition to interactions with the aforementioned intracellular signaling partners following homophilic NCAM binding, NCAM heterophilic interactions with a number of extracellular interaction partners also have been suggested to be involved in NCAM-mediated neurite outgrowth [41]. Among these heterophilic interaction partners is FGFR. Early evidence for an NCAM-FGFR interaction came from studies showing that NCAM-mediated neurite outgrowth in PC-12 cells is inhibited by a tyrosine kinase inhibitor [42]. More precise evidence that the tyrosine kinase involved in NCAM-mediated neurite outgrowth was FGFR came from investigations demonstrating that NCAM-induced neurite outgrowth was inhibited by antibodies directed against FGFR

[30,35,42] and by transfection with a dominant negative kinase-deleted FGFR (Dn-FGFR) [43]. Deeper insight into how signaling by the different NCAM isoforms depends on FGFR signal transduction came from a study performed with CHO cells transfected with NCAM-120, NCAM-140, or NCAM-180 [44]. This study demonstrated that pharmacological inhibition of FGFR inhibited NCAM-180 signal transduction, whereas NCAM-140 signal transduction was less affected by FGFR inhibition, and NCAM-120 signaling was completely unaffected, indicating that NCAM-180 signaling is most dependent on FGFR in this system [44].

A direct interaction between NCAM and FGFR was demonstrated only recently [45]. Both the first and second FN3 modules were shown by surface plasmon resonance (SPR) to be involved in binding to FGFR [46]. Furthermore, more detailed studies by NMR titration analysis of the second NCAM FN3 module revealed that the FGFR binding site is localized to a sequence corresponding to the F and G β-strands and the interconnecting loop in this module [45]. In FGFR, the Ig2–3 domain as well as the acidic box located in the linker between Ig1 and Ig2, is believed to be important for NCAM interactions [47, 48]

Investigations of the signaling events that are initiated downstream of NCAM-FGFR interactions are ongoing. However, inhibition of PLCγ inhibited NCAM-induced neurite outgrowth, suggesting that NCAM activates FGFR with subsequent activation of PLCγ. When this relationship was investigated further, the activation of FGFR and PLCγ by NCAM was shown to result in increased intracellular Ca^{2+} concentrations that were attributable to IP_3 generation by the action of PLCγ, followed by release of Ca^{2+} from intracellular Ca^{2+} stores, and to the action of arachidonic acid (AA) on nonselective cation channels in the cell membrane [49]. This increase in Ca^{2+}, on the other hand, may result in the activation of Ca^{2+}-dependent molecules, such as cytoskeletal proteins, and/or activation of Ca^{2+}-dependent transcription factors, such as CREB, resulting in new protein synthesis. Thus, NCAM appears to utilize FGFR-dependent PLCγ activation with subsequent IP_3 and AA generation to induce an increase in intracellular Ca^{2+} concentration that, in turn, results in neurite outgrowth. In addition to the intracellular increase in Ca^{2+}, activation of the PLCγ pathway has been shown to activate Protein Kinase C (PKC) via diacylglycerol (DAG). Accordingly, a number of pharmacological PKC inhibitors inhibit NCAM-mediated neurite outgrowth [35, 50], suggesting that NCAM-mediated FGFR-dependent neurite outgrowth also depends on PKC activation via the FGFR-PLCγ-DAG pathway. The functional importance of PKC activation was further elucidated by recent findings showing that FGFR receptor activation is needed for recruitment of PKC to the NCAM/spectrin complex, and this recruitment is necessary for NCAM-induced neurite outgrowth [28].

Thus, upon NCAM homophilic binding, FGFR appears to be activated and induce PLCγ activity, followed by increased intracellular Ca^{2+} and PKC activity. PKC, in turn, is recruited to NCAM-spectrin complexes. When PKC is in complex with NCAM and spectrin (particularly in lipid rafts), it likely encounters and acts on a number of substrate molecules, such as GAP-43, that are involved in neurite outgrowth. Increases in Ca^{2+} may concurrently modulate a number of molecules, and the actions of PKC and Ca^{2+} together may result in a neurite outgrowth response (Fig. 2).

Finally, a second FGFR-induced pathway leading to MAPK activation also is induced by NCAM activation. Thus, following transfection with dominant negative FGFR substrate 2 (FRS2) and/or ShcA, NCAM-induced FGFR-dependent neurite outgrowth is inhibited, indicating that both ShcA and FRS2 are necessary for NCAM-induced neurite outgrowth [48] (Fig. 2). In contrast, FGF2-induced neurite outgrowth was not dependent on ShcA activation but instead relied only on FRS2 activation, suggesting that in the same cell system, FGF ligands and NCAM induce differential FGFR responses. Furthermore, NCAM was shown to activate ShcA not only through FGFR but also through a Fyn-dependent pathway, indicating that NCAM itself initiates ShcA activation as well as induces FGFR-dependent ShcA activation.

In conclusion, NCAM-induced neurite outgrowth likely depends on at least two FGFR-mediated pathways – the PLCγ-PKC Ca^{2+} pathway and the FRS2/ShcA MAPK pathway – suggesting that NCAM interactions with FGFR induce the activation of several FGFR downstream pathways.

N-cadherin-mediated adhesion and its role in neurite outgrowth

The cadherins comprise a family of calcium-dependent CAMs characterized by an extracellular cadherin repeat sequence of approximately 110 amino acids that folds into a β-sandwich module. Most cadherins are single-pass transmembrane molecules with an intracellular domain that varies between different subfamilies [1]. Based on shared properties and structural similarities between different family members, cadherins can be further divided into subfamilies [1,51]. These subfamilies include the classical (type I) cadherins, type II cadherins, desmosomal cadherins, protocadherins, and atypical cadherins. The type I and

type II cadherins, for example, can be segregated based on the fact that type I cadherins contain a cell adhesion recognition motif (the HAV sequence) in the *N*-terminal cadherin repeat (EC1) domain (Fig. 1), whereas type II cadherins do not have this sequence. Furthermore, type I cadherins have one tryptophan in the EC1 domain, whereas type II contains two tryptophan [52]. In addition, subtle structural differences within each subfamily may result in different functions. For example, two atypical cadherins, Celsr2 and Celsr3, are both involved in neurite growth. Celsr2 stimulates and Celsr3 inhibits neurite growth [53]. These differences between two structurally similar proteins appear to be attributable to one single amino acid in the transmembrane part of the molecules. In Celsr2, a histidine is replaced with an arginine. This single amino acid substitution results in higher Ca^{2+} influx (via activation of PLC and IP3) to the cytoplasm upon homophilic binding of Celsr2 compared with Celsr3. The difference in Ca^{2+} flux, in turn, appears to induce activation of different second messengers, ultimately resulting in a differential growth response. Thus, Ca^{2+}/Calmodulin kinase II (CaMKII) is activated upon high Ca^{2+} influx (by Celsr2), whereas calcineurin is activated at lower Ca^{2+} influx (by Celsr3) [53].

In the brain, cadherins are differentially expressed, depending on developmental stage and cell type. Because cadherins are hypothesized to bind preferentially to similar cadherins (e.g., N-cadherin to N-cadherin), expression of specific cadherins in different cell types may provide important molecular cues for shaping both anatomically distinct brain structures and functional neuronal circuits [54]. In addition to this role, cadherins are also important regulators of neurite growth during development [55] and regeneration [18]. One of the best-studied cadherins involved in neurite growth is N-cadherin.

N-cadherin belongs to the classical subfamily of cadherins and is structurally composed of five extracellular cadherin modules (Fig. 1), a single-pass transmembrane domain, and an intracellular domain that can interact with catenins. Between each of the five extracellular cadherin modules are Ca^{2+} binding sites, and the binding of Ca^{2+} to cadherin rigidifies the structure and is essential for cadherin function.

Similar to NCAM, N-cadherin mediates adhesion via homophilic *cis* and *trans* interactions, and N-cadherin likely first engages in *cis* interactions to subsequently engage in *trans*-homophilic interactions [56] (see also [57]). Unlike NCAM, however, the homophilic interaction and the transition from *cis* dimers to *trans* interactions were shown for another classical cadherin, E-cadherin, to be regulated by extracellular Ca^{2+} concentration. Thus, E-cadherin engaged in *cis* dimers only in the presence of Ca^{2+}, and *trans* interactions were not initiated before the Ca^{2+} concentrations increased to 1–2 mM [58]. N-cadherin and E-cadherin appear to be very similar at the high-resolution structural level, and a similar Ca^{2+} regulating mechanism likely applies to N-cadherin.

Studies investigating the mechanism by which N-cadherin engages in *cis* and *trans* homophilic interactions have provided somewhat conflicting results [56]. However, the minimum unit for N-cadherin homophilic binding is the outermost two extracellular cadherin (EC1–2) domains [59] and structural investigations suggest that a tryptophan in the EC1 domain is important for homophilic interactions [60]. This tryptophan is part of a flexible strand region, and mediates EC1-EC1 homophilic binding through a mechanism in which the side chain of tryptophan in one cadherin molecule inserts into a hydrophobic pocket in the EC1 domain of another cadherin molecule, forming a "strand dimer" formation. This interaction was speculated to be a *cis* interaction [60], but as crystal structure analysis of other classical cadherin constructs showed the formation of the strand dimer in *trans* [61, 62] it is now speculated that the strand-dimer reflects both *cis* and *trans* interactions [56, 57]. In addition to the EC1-EC1 interaction sites, biophysical analysis of E-cadherin and C-cadherin has provided evidence for a role of the remaining four EC domains of classical cadherins in homophilic binding [57]. Accordingly, cadherins have been suggested to interact in *trans* via three mutually exclusive interactions that involve bonds between the outermost EC domains (EC1), bonds between the first three EC domains (EC1-EC3), or bonds between all five EC domains [57]. The bonds between the inner domains, however, also have been suggested to reflect *cis* interactions that optimally position the EC1 domain for *trans* interactions [63]. In the case of N-cadherin, cell adhesion measured by cell aggregation has been demonstrated by a truncation approach to be increased when EC3–5 is included [59], suggesting that although the minimum unit for N-cadherin-mediated adhesion is EC1–2, EC3–5 also may be important for optimal adhesion. However, exactly how N-cadherin interacts in *cis* and in *trans* remains to be clarified. Importantly, regardless of the mode of interaction, to mediate strong and functional adhesion, cadherins must cluster, and the clustering is regulated by intracellular signaling molecules such as the GTPases Rac1 and RhoA [64,65]. In addition, the cadherin cytosolic domain and linkage to the cytoskeleton are required, suggesting a link between cadherin *trans* homophilic interactions, the cytosolic domain,

and coupling to the cytoskeleton and cellular signaling [66].

The cadherin cytosolic domain, together with the EC1 domain, is the most highly conserved domain between different classical cadherins [67], and important intracellular interaction partners are shared between the different classical cadherins. Accordingly, classical cadherins directly interact with β-catenin (or γ-catenin/plakoglobin) and p120 catenin [68] (Fig. 2), which in the case of E-cadherin have been demonstrated to bind to the *C*-terminal part and the juxtamembrane part of the E-cadherin cytoplasmic domain, respectively [69, 70]. The binding of catenins to cadherin likely is regulated by phosphorylation of both catenins and cadherins by a number of different kinases, such as Fyn, Src, Fer [71, 72], GSK-3β, and casein kinase II (CKII) [73].

β-catenin interacts with α-catenin, and α-catenin binds to F-actin. Thus, a prevailing theory posited that cell adhesion is strengthened through a stable cadherin/β-catenin/α-catenin/F-actin interaction. However, recent studies have suggested that α-catenin does not interact with cadherin and actin simultaneously [74, 75], indicating that the intracellular domain may strengthen adhesion via mechanisms other than stable contact between N-cadherin and the cytoskeleton. In line with this, Drees and coworkers found that free α-catenin compete with actin-related protein 2/3 (Arp2/3) in binding to actin. Arp2/3 promotes actin polymerization and branching and is related to the actin meshwork at lamellipodia of growth cones, whereas α-catenin has been shown to interact with α-actinin [76], a molecule that binds to actin bundles associated with strong cell-cell adhesion. An alternative explanation for the strengthening effect of the cytoplasmic domain on adhesion could be that the increased concentration of α-catenin following clustering of cadherins results in an increased local concentration of free α-catenin that may then compete with Arp2/3 for binding to actin and thereby promote the formation of actin bundles and strong cell-cell adhesion over growth cone extension [73, 74].

In addition to the role of strengthening cadherin-mediated adhesion, β-catenin and p120 catenin also are important for cadherin-mediated neurite growth. With regard to the role of β-catenin in axonal outgrowth, evidence has suggested both a stimulatory and an inhibitory effect of β-catenin. Accordingly, transfecting *Xenopus* retinal ganglion cells (RGC) with an N-cadherin construct lacking most of the extracellular domain (N-cad[intra]) results in inhibition of axonal initiation and axonal elongation [77], suggesting an important role for the intracellular domain in neurite outgrowth. However, when RGCs were transfected with a construct encompassing the β-catenin binding site of N-cadherin, no inhibitory effect was observed [77]. This construct specifically inhibited N-cadherin/β-catenin interactions by competing with endogenous N-cadherin for β-catenin binding, suggesting that β-catenin is not involved in N-cadherin-mediated axon elongation. A construct containing the membrane-proximal part of the intracellular domain, however, inhibited axonal growth to an extent similar to N-cad(intra), suggesting that this site may be important for initiation and elongation of RGC axons. A second study on the role of β-catenin in neurite outgrowth in PC12 cells suggested that increasing the levels of β-catenin results in inhibition of neurite outgrowth induced by Nerve Growth Factor (NGF) [78]. Accumulated cytosolic β-catenin may translocate to the nucleus and induce transcription via interactions with transcription factors of the Tcf/Lef family. The inhibitory effect was partially attributable to this transcriptional function. However, β-catenin also associated with adenomatous polyposis coli (APC) protein, which binds microtubules and is important for microtubule assembly and bundling during axonal and dendritic growth, suggesting that effects on the cytoskeleton also may be involved in β-catenin-mediated growth inhibition [78]. Unphosphorylated APC has high affinity for microtubules and lower affinity for β-catenin [79], favoring APC binding to microtubules when free β-catenin levels are low (e.g., when β-catenin is bound to N-cadherin). In contrast, APC phosphorylation induced by activated GSK-3β increases the affinity of APC for β-catenin. When APC is phosphorylated or β-catenin levels are increased, APC may bind β-catenin rather than microtubules, resulting in inhibition of growth. Thus, N-cadherin may be regarded as a growth-promoting adhesion molecule that functions to sequester β-catenin and thereby prevent interactions with APC. In contrast to the findings in the aforementioned studies, increased β-catenin levels in a neuroblastoma cell line (NB-1) were shown to correlate with increased neurite extension [80]. Furthermore, Yu and Malenka [81] demonstrated that overexpression of β-catenin in hippocampal cells induced an increased overall length of neurite branches, suggesting that β-catenin is indeed involved in N-cadherin-mediated axonal growth. One reason for the discrepancies between the studies showing inhibition and stimulation, respectively, could be that the involvement of N-cadherin and β-catenin in outgrowth is context-specific. In the study showing outgrowth inhibition by β-catenin, only cells that were not involved in cell-cell contact were studied, implying that N-cadherin does not engage in *trans* homophilic interactions. In this instance, N-cadherin possibly functions as a

sequestor of β-catenin, whereby interactions with APC are prevented and microtubule assembly and bundling take place. In contrast, cells engaging in cell-cell contacts were examined in the study that demonstrated a stimulatory effect of β-catenin on axonal outgrowth, suggesting that when N-cadherin interacts in *trans*, β-catenin may function as a growth-promoting protein. Regardless, the exact role of β-catenin in cadherin-mediated axonal outgrowth remains to be clarified.

Similar to β-catenin, p120 is also involved in N-cadherin-stimulated neurite outgrowth, and N-cadherin is proposed to function as a regulator of cytosolic p120 concentration. p120 was first shown to interact with N-cadherin in a number of fibroblast cell lines and this interaction correlated with an unusual dendritic-like morphology of the fibroblasts [82]. The expression of the unusual phenotype was subsequently correlated with decreased expression of actin stress fibers and focal adhesions, and the ability of p120 to activate the GTPases Rac1 and Cdc42 via direct interactions with the guanine exchange factor vav2 as well as to inactivate the GTPase RhoA [83]. Interestingly, ROCK, which may be involved in NCAM-mediated neurite outgrowth, is a downstream effector of RhoA and constitutively active ROCK suppresses the effect on morphology induced by p120 [83], indicating that ROCK may be important for both NCAM- and cadherin-regulated processes. Although these findings are related to fibroblasts, activation of both Rac1 and Cdc42 are known to induce neurite outgrowth, whereas RhoA activation decreases neurite outgrowth [84–87]. Hence it is possible that p120 may have a growth-promoting effect in neurons. Indeed, a p120 isoform, δ-catenin, stimulates neurite outgrowth in PC12 cells and hippocampal neurons [88]. In this situation, growth depends on δ-catenin interactions with cortactin and inhibition of RhoA and ROCK. Cdc42 and Rac1, in contrast, were dispensable for δ-catenin-induced neurite outgrowth. Interestingly, cortactin interacts with the Arp2/3 complex to induce actin nucleation, suggesting that δ-catenin and α-catenin may have opposite roles. Furthermore, tyrosine phosphorylation by Src kinases negatively regulates the interaction between δ-catenin and cortactin, whereas it has no effect on δ-catenin/N-cadherin interactions, suggesting that Src kinase phosphorylation of δ-catenin inhibits neurite outgrowth [88]. Importantly, as the growth-promoting effect of p120 can be inhibited by increasing the concentration of cadherin containing the juxtamembrane domain, it is possible that one function of cadherin, as in the case of β-catenin, is to serve as a buffer that regulates cytosolic levels of p120. High cadherin concentrations maintain low cytosolic p120 concentrations and prevent inappropriate growth. Low cadherin concentrations and/or high p120 concentrations increase the cytosolic proportion of p120 and stimulate growth [83]. Furthermore, at sites with high levels of cadherin there will be a store of p120 that is readily available for release from cadherin to stimulate growth, and this release could be stimulated/inhibited by the aforementioned phosphorylation of p120 and cadherin by various kinases, which may modify the binding of p120 to cadherin and thereby modify p120-induced neurite growth.

To summarize, N-cadherin may act as a buffer that controls the cytosolic concentration of two molecules, β-catenin and p120. N-cadherin thereby regulates intracellular signaling via GTPases and cytoskeleton-associated proteins, leading to stimulation of neurite outgrowth or induction of stable cell-cell contacts, possibly depending on the concentration of cytosolic catenins and the availability of intracellular signaling molecules. The interaction between catenins and N-cadherin most likely is regulated by a number of different kinases and phosphatases, providing fine-tuned and complex regulation of N-cadherin-mediated neurite outgrowth. In addition to a function as buffer, N-Cadherin may also more directly activate GTPases, as has been shown to occur for E-Cadherin when E-Cadherin engages in homophilic interactions [89].

N-cadherin signaling through FGFR

Adding to the complexity of N-cadherin involvement in neurite growth, evidence suggests that N-cadherins also may engage in interactions with other molecules expressed at the membrane surface, such as FGFR. Thus, interfering with FGFR function in cerebellar granule neurons (CGN) by pharmacological inhibition of FGFR [90] or by transfecting CGNs or RGCs with DnFGFR [43, 91, 92] inhibits N-cadherin induced neurite outgrowth. FGFR co-immunoprecipitates with N-cadherin [47, 92, 93], and this precipitation appears to depend on the formation of N-cadherin clusters [92]. Importantly, however, FGFR was not found at points of cell-cell contacts [47], suggesting that, similar to NCAM [19], N-cadherin does not interact with FGFR when engaged in tight *trans* homophilic interactions. In accordance with this hypothesis, soluble forms of N-cadherin stimulate N-cadherin-mediated neurite outgrowth [92], indicating that cell-cell contacts are not necessary for N-cadherin-induced neurite outgrowth. N-cadherin homophilic interactions, however, appear to be necessary for N-cadherin-induced FGFR activation. Monomeric cyclic peptides that are hypothesized to bind to the EC1

site that is responsible for homophilic interactions, thereby interfering with N-cadherin homophilic binding, inhibit N-cadherin-induced neurite outgrowth [94, 95]. Conversely, the same N-cadherin peptides in dimeric form promote neurite outgrowth, presumably by promoting dimerization of N-cadherin molecules (each peptide sequence binds one *N*-cadherin) and this effect can be blocked by inhibiting N-cadherin interactions with FGFR [96], suggesting that N-cadherin-induced FGFR-dependent neurite outgrowth is initiated by N-cadherin homophilic binding. However, of note, it can not be ruled out that the dimeric peptide act by inducing heterodimerisation of N-Cadherin and FGFR rather than homodimerisation of N-Cadherin.

Although FGFR co-immunoprecipitates with N-cadherin, a direct interaction between N-cadherin and FGFR has not been reported. However, using antibodies directed against specific N-cadherin domains or peptides derived from specific parts of N-cadherin [90] or using deletion analysis [47,93], potential extracellular binding sites have been identified in both N-cadherin and FGFR. Using these approaches, the fourth EC (EC4) domain of N-cadherin was identified as important for FGFR interactions, whereas a stretch of amino acids that encompasses the acidic box in the linker region between Ig1 and Ig2 of FGFR was identified as important for N-cadherin binding [47, 90].

Similar to NCAM, N-cadherin-induced FGFR-dependent neurite outgrowth involves PLCγ as treatment with a peptide that competes with PLCγ for binding to FGFR inhibited N-cadherin-induced neurite outgrowth in rat cerebellar neurons [43]. Furthermore, pharmacological inhibition of DAG lipase, which converts DAG to AA downstream of PLCγ activation, likewise inhibited N-cadherin-induced neurite outgrowth in *Xenopus* RGCs [91]. Activation of the PLCγ pathway may result in activation of CaMKII via AA-induced Ca^{2+} influx and in *Xenopus* RGCs, pharmacological inhibition of CaMKII inhibited N-cadherin-induced neurite outgrowth [91]. Notably, CaMKII may phosphorylate a number of intracellular molecules, among which is the TOAD-64 protein that interacts with NCAM. Interestingly, the interaction between N-cadherin and FGFR in a number of cancer cell lines is associated with FGF2-induced sustained MAPK phosphorylation [93], suggesting that N-cadherin potentiates FGF2-induced signaling. This sustained MAPK activation is correlated with a significantly decreased internalization and increased half-life of FGFR, indicating that N-cadherin may stabilize FGFR expression at the cell surface [93]. Although these effects have been shown in cancer cells and not with regard to neurite outgrowth, NCAM sustained MAPK activation has been demonstrated to result in neurite outgrowth [28] and similarly NGF, a known stimulator of neurite outgrowth, induces sustained MAPK activation [97]. Process growth in astrocytes, in contrast, has been suggested to depend on transient MAPK activation [98]. Thus, it is possible that modulation of FGF2-induced MAPK phosphorylation may have an important role in N-cadherin-induced neurite outgrowth. Furthermore, FGFR1 bound to FGF2 can translocate to the nucleus where it serves as a regulator of transcription [99]. Stabilization of FGFR at the cell surface by N-cadherin likewise could affect FGFR-mediated neurite outgrowth.

NCAM- vs. N-cadherin-mediated neurite outgrowth

Both NCAM and N-cadherin are involved in the stimulation of neurite outgrowth and they exert this effect by using a number of intracellular molecules (Fig. 2). NCAM-induced neurite outgrowth involves an RPTPα-p59^{fyn}-FAK pathway and a spectrin-GAP-43 pathway. N-cadherin-induced neurite outgrowth involves catenin pathways. Many of these pathways likely result in activation of common downstream targets involved in neurite outgrowth. In addition, both NCAM and N-cadherin employ a common pathway that involves FGFR activation for stimulation of neurite outgrowth. In this regard, it is interesting that an NCAM-dependent complex of NCAM, FGFR4, N-Cadherin and cortactin has been demonstrated in pancreatic β-tumor cells [100], suggesting that NCAM and N-Cadherin may act in concert to modulate FGFR signaling. Also, it should be mentioned that it has recently been demonstrated that Cadherin-11 and the Ig family member, L1, which is a prominent inducer of neurite outgrowth [3], also interact with FGFR and induce neurite outgrowth [48, 101], suggesting that a number of different CAMs may signal via interactions with FGFR.

NCAM and N-cadherin (and other CAMs) are dynamically expressed in the growing tip of neurites [102–104] and the strength of adhesion at the neurite tip is highly dynamic. A relationship has been suggested to exist between growth rate and adhesion stability, such that tight adhesion correlates with slow growth and loose adhesion correlates with fast growth [102]. Although this could be interpreted simply as a mechanical function of CAMs (e.g., tight adhesion prevents the tip from elongating), research over the past decade has established that many CAMs function as sensors of the environment that, upon extracellular cues (such as loose adhesion), induce intracellular signaling to promote neurite growth. For example, in

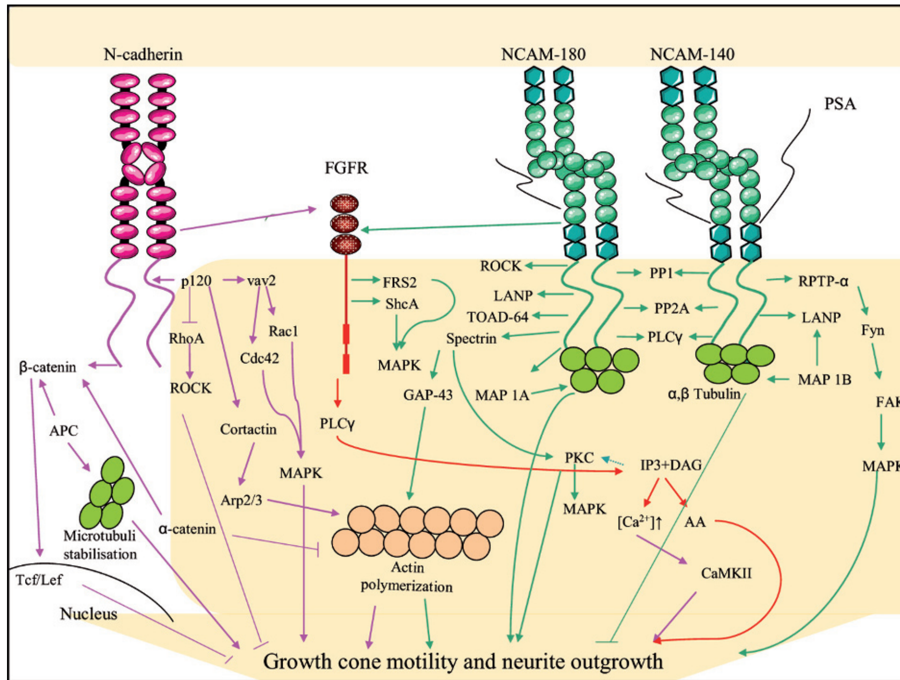

Figure 2. NCAM and *N*-cadherin intracellular interactions and signaling in neurite outgrowth. NCAM and *N*-cadherin have distinct and common cellular interaction partners and modulate neurite outgrowth via a number of different signaling pathways (see text for details). Blue and purple arrows indicate distinct *N*-cadherin and NCAM-induced intracellular events respectively. Red arrows indicate shared *N*-cadherin- and NCAM-induced FGFR-mediated intracellular signaling.

the case of NCAM- and N-cadherin-induced FGFR-dependent neurite outgrowth, loose homophilic interactions appear to be a prerequisite for interactions with, and stimulation of, FGFR and FGFR-induced pathways. Interestingly, growing neurites particularly express the polysialylated form of NCAM [103, 104]. PSA modulates the adhesive strength not only of NCAM but also of other cell adhesion molecules, including N-cadherin and thus PSA-NCAM may promote neurite outgrowth by loosening the adhesion not only of NCAM but also perhaps of N-cadherin. In addition it is also possible, that the adhesion of CAMs provides the growing axon with a "molecular clutch" to which growth via retrograde F-actin transport can be linked as recently described for N-Cadherin [105] Exactly how (or if) NCAM- and N-cadherin-induced signaling via RPTPα-p59^{fyn}-FAK, spectrin-GAP-43, and catenins is regulated by extracellular homophilic interactions is not known. Clustering of NCAM and N-cadherin at specific subcompartments may be necessary to increase the local concentration of the intracellular interaction partners whereby signaling can be induced. For example, in the case of p59^{fyn}-FAK activation by NCAM, clustering of NCAM by antibodies promotes NCAM-RPTPα interactions and the translocation of the complex to lipid rafts where p59^{fyn} is mainly localized [36]. In this instance, NCAM clustering results in an increased local concentration of RPTPα in lipid rafts that can then activate p59^{fyn}. Stimulation of NCAM has been similarly demonstrated to result in the redistribution of the NCAM-spectrin-PKC complex into lipid rafts where PKC may interact with growth-promoting molecules that are associated with lipid rafts, such as GAP-43. This latter redistribution is dependent on FGFR activation, suggesting that FGFR is first activated, and then the complex formation and redistribution takes place. Interestingly, FGFR has not been found in lipid rafts [106], suggesting that NCAM does not interact simultaneously with FGFR, spectrin or p59$^{fyn.}$ However, N-cadherin and NCAM that are not localized to lipid rafts may simultaneously stimulate FGFR-dependent neurite outgrowth and cross-talk and integration of all of the pathways, likely result in a strong neurite outgrowth-promoting effect.

Finally, when the neurite reaches an appropriate target expressing NCAM and/or *N*-cadherin, stable *trans* N-cadherin and NCAM interactions occur. This, in turn, will prevent translocation of the CAMs to specific subcompartments, exclude FGFR from the clusters, and cease neurite outgrowth signaling.

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