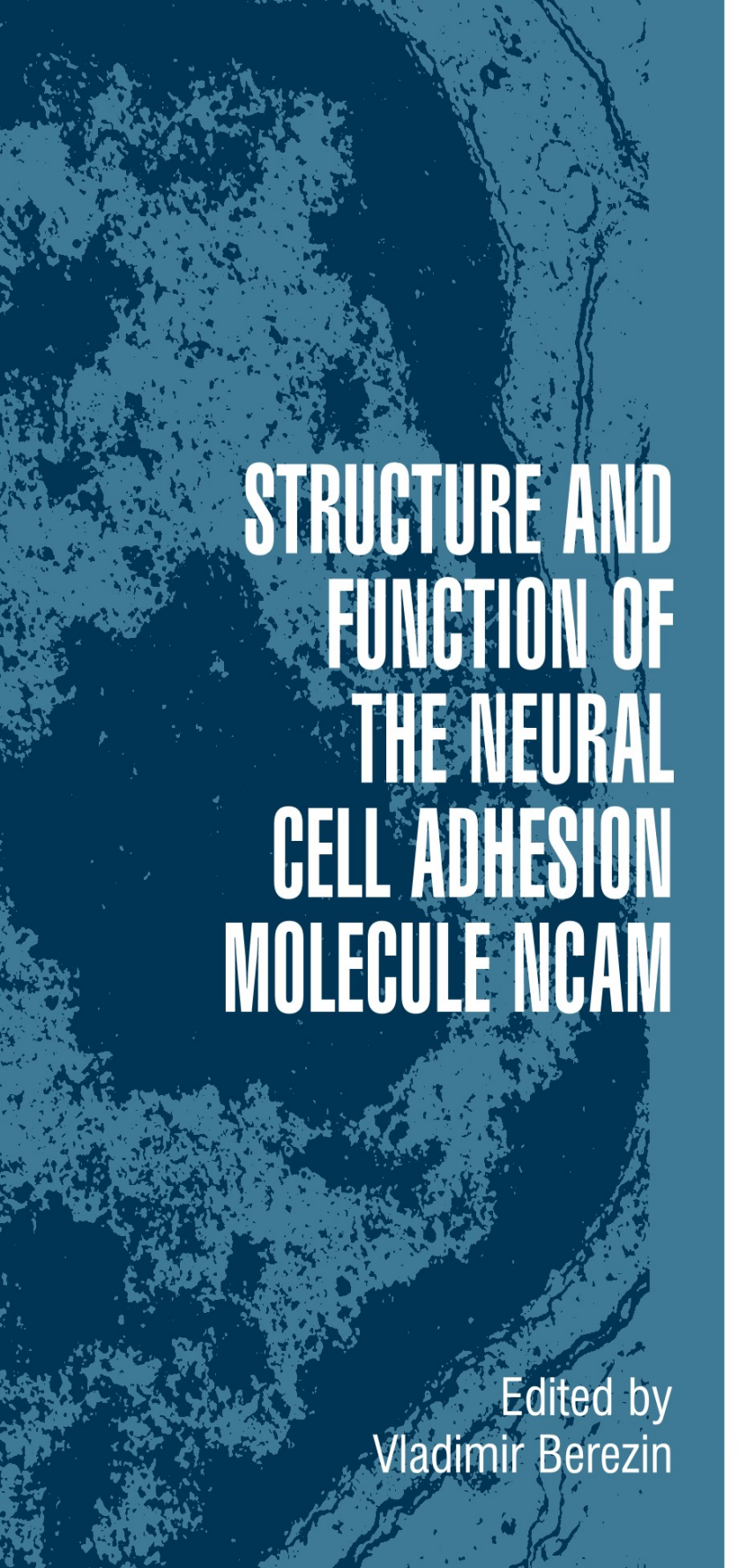

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STRUCTURE AND FUNCTION OF THE NEURAL CELL ADHESION MOLECULE NCAM

Edited by
Vladimir Berezin

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Structure and Function of the Neural Cell Adhesion Molecule NCAM

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Preface

Dr. Elisabeth Bock (Photo Keenpress)

This book contains review articles that produce a snapshot of recent developments in the field of the neural cell adhesion molecule NCAM. The chapters are grouped into sections reflecting various aspects of NCAM structure and function. The themes cover the structural basis of cell adhesion mediated by NCAM and NCAM interaction partners, NCAM-mediated signaling determinants of NCAM function under physiological conditions and in disease, and the therapeutic potential of NCAM mimetics.

Section 1, "Structure and Ligands of NCAM," introduces the reader to the structural basis of NCAM-mediated cell adhesion, discussing the current knowledge of extracellular and intracellular NCAM ligands and the structural basis of NCAM interactions with the fibroblast growth factor (FGF) receptor. Section 2, "NCAM and Polysialic Acid," focuses on NCAM polysialylation, discussing the structural and functional aspects of the most important posttranslational modifications of NCAM by the addition of a long linear homopolymer of sialic acid to the fifth Ig-like NCAM module. Section 3, "NCAM-mediated Signal Transduction," is devoted to signal

transduction mechanisms associated with NCAM-mediated adhesion, with a focus on signaling pathways involved in NCAM-mediated neurite outgrowth, the role of growth-associated proteins, signaling through lipid microdomains, and signaling crosstalk with the epidermal growth factor (EGF) receptor. Section 4, "NCAM Metabolism," focuses on current knowledge about NCAM biosynthesis and the generation and role of soluble NCAM. Section 5, "NCAM, Synapses, Emotions, and Memory," describes the role of NCAM in spine dynamics, synaptogenesis, and learning and memory. Section 6, "NCAM in Disease," covers the role of NCAM in neuropsychiatric disorders, neurodegenerative disorders, and cancer. Section 7, "Pharmacology of NCAM: NCAM Mimetics," reviews current achievements in the in vivo and in vitro pharmacology of small NCAM mimetic peptides. Section 8, "Orthologs and Paralogs of NCAM," discusses current knowledge about the structure and function of the NCAM ortholog Fasciclin II and the NCAM paralog NCAM2.

The concluding section is devoted to Professor Elisabeth Bock, to whom this book is dedicated. Prof. Bock in the early 1970s identified the neural cell adhesion molecule NCAM as brain specific protein D2 (published in *Journal of Neurochemistry*, 1974, vol. 23, pp. 879–880). She has dedicated her professional career to the characterization of this very important and intriguing molecule.

I would like to thank everyone who has contributed to this book by writing review articles on various aspects of NCAM structure and function, Kristine Immediato for her kind assistance in managing the manuscripts, and Michael Arends for his professional proofreading of a number of the manuscripts. I would also like to acknowledge the European Union-supported Integrated Project PROMEMORIA, which since 2005 has been a staunch supporter of NCAM-related research.

Denmark Vladimir Berezin

Contents

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Part I
Structure and Ligands of NCAM

Structural Biology of NCAM

Vladislav Soroka, Christina Kasper, and Flemming M. Poulsen

Introduction

The phenomenon of cell adhesion has been discovered by H. V. Wilson in 1907. He demonstrated species-specific cell aggregation using dissociated sponge cells [1]. In 1955, Townes and Holtfreter demonstrated type-specific sorting of cells harvested from the three germ layers of amphibian embryos [2]. During the following 20 years, adhesion of embryonic cells was studied intensively resulting in identification, isolation, and characterization of membrane-associated molecules capable of mediating adhesion between cells or between cells and the extracellular matrix (ECM).

Since then, CAMs have been the subjects of thousands of studies. Such high interest is not unexpected, considering that intercellular interactions play a crucial role in a broad range of biological and pathological processes, including cell migration, differentiation, embryogenesis, and function of the immune system. All of these phenomena depend on protein–protein recognition at the cell surface. Cell adhesion molecules are multifunctional proteins capable not only of mediating recognition and mechanical connections between cells serving as a "glue," but also of transmitting mechanical and chemical signals across the cell membrane. Most CAMs have cytoplasmic regions that allow communication through intracellular signaling pathways, providing access to the regulation of gene expression, cytoskeletal architecture, cell metabolism, cell cycle, and survival [3].

A full understanding of how CAMs mediate cellular adhesion requires quantitative functional assays to evaluate the strength of the binding interactions, as well as high-resolution three-dimensional structures to provide the atomic details of the interactions.

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NCAM Belongs to the Immunoglobulin Superfamily of CAMs

The immunoglobulin superfamily (IgSF) of CAMs includes transmembrane and membrane-anchored glycoproteins that are characterized by the presence of a variable number of Ig modules similar to the variable or constant Ig modules of antibodies [4, 5]. In the human genome, the IgSF is the largest family of proteins, currently comprising 765 genes [7]. Members of the IgSF include major histocompatibility complex class I and II molecules, proteins of the T cell receptor complex, other lymphocyte surface glycoproteins, virus receptors, tumor markers, growth factor receptors, and cell-surface glycoproteins primarily found in the nervous system [4]. IgSF molecules are the most diverse group of cell-surface receptors, with at least 65 proteins implicated in cell adhesion [3]. The extracellular parts of many IgSF molecules consist of multiple Ig-like modules connected like beads on a string. These are often followed by multiple copies of another building block module, the fibronectin type III (Fn3) module [8]. The extracellular part of CAMs of the IgSF may be composed exclusively of Ig-like modules, like in PECAM-1 or VCAM-1, or may contain both Ig-like modules and Fn3 modules, like in NCAM or L1 [3]. The total number of modules varies from 1 in P_0 to 17 in sialoadhesin [3, 9]. These molecules can be subdivided further by their mode of membrane anchorage into a glycosylphosphatidylinositol (GPI)-linked subgroup (e.g., F11, TAG-1, and BIG-1) and a transmembrane subgroup (e.g., neurofascin, neural-glial cell adhesion molecule (NgCAM), L1, and NCAM). The length of the cytoplasmic parts of the transmembrane members of the IgSF varies from 15 amino acid residues in neuromusculin to 557 in the nematode protein UNC-5 [3].

IgSF CAMs mediate Ca^{2+}-independent homophilic binding (i.e., between two or more CAMs originating from the same gene) and heterophilic binding (i.e., between two different CAMs, or between CAM and other molecules). Both types of interactions can take place on the surface of the same cell (i.e., *cis*-interactions) or between two apposing cell membranes (i.e., *trans*-interactions). Cell–cell binding activities are localized to *N*-terminal Ig modules 1–4, which are most accessible for a corresponding counter-receptor on an apposing cell. A protein–protein binding surface may comprise several modules, which possibly increases binding specificity and affinity.

Architecture of NCAM

NCAM was described originally as synaptic membrane protein D2 by Jørgensen and Bock and was later shown to mediate cell–cell adhesion [10, 11]. NCAM was the first CAM that was identified. The protein also was isolated and characterized by Brackenbury and Thiery in an independent study [12]. NCAM is expressed in neurons, glial cells, heart, and skeletal muscles. As many as 27 distinct NCAM isoforms can be generated through alternative RNA splicing [13]. The three major

NCAM isoforms are NCAM-180, NCAM-140, and NCAM-120, with molecular masses of 180, 140, and 120 kDa, respectively [13]. The extracellular parts of the three major NCAM isoforms are identical and comprise five *N*-terminal Ig modules followed by two Fn3 modules that are closest to the membrane. The transmembrane isoforms, NCAM-180 and NCAM-140, have intracellular parts of different lengths. The NCAM-120 isoform is linked to the membrane via a glycosyl-phosphatidylinositol (GPI) anchor with no intracellular residues. The intracellular parts of NCAM-140 and NCAM-180 isoforms consist of 120 and 385 residues, respectively [13]. Since only few short helices and strands can be predicted in both sequences, the cytoplasmic part of NCAM appears largely unfolded. NCAM has six possible *N*-linked glycosylation sites. One site is located in the third Ig module. Two sites are in the forth Ig module, and three sites are in the fifth Ig module. NCAM differs markedly from other glycosylated proteins by its ability to carry high levels of the negatively charged polysialic acid (PSA) that consists of homopolymers (more than 55 residues long) of α2-8 linked *N*-acetyl neuraminic acid residues [14]. The PSA chains are attached to the fifth Ig module at two of three *N*-linked glycosylation sites. The polysialylated form of NCAM has reduced homophilic binding capacity compared with the non-polysialylated form [15] and is expressed during embryonic development, in brain arias involved in neuronal plasticity, and by a number of invasive tumors. The latter is associated with a poor prognosis in cancer patients [16]. See the review by Gascon et al. [17] for more details regarding PSA-NCAM biology.

The Topology of Ig- and Fn3-homology Modules

A module can be defined as a subregion of a polypeptide chain that independently forms a tertiary structure fold. Loops connecting secondary structure elements and intermodular interfaces are relatively easy to change in an evolving system without significant distortions of the tertiary fold, thus providing the necessary flexibility in the creation of new binding surfaces during evolution. The Ig fold provides a stable platform that is resistant to proteolysis and upon which a diversity of sequences is displayed by variation of the amino acids.

The Ig-like modules are ellipsoid-like structures composed of 70–110 amino acids with dimensions of ~20 × 25 × 40 Å. The tertiary structure of the Ig module is formed by two anti-parallel β-sheets that are packed together face to faceand stabilized by a hydrophobic interior of the module (i.e., the hydrophobic core) formed by the side chains from both β-sheets. Each β-sheet consists of 3–5 β-strands designated by A, A′, B, C, C′, C″, D, E, F, and G in order of appearance in the sequence. The β-strands are 5–10 amino acid residues long and are connected by loops of varying lengths. The amide and carbonyl groups of the β-strand residues form numerous inter-strand hydrogen (H–) bonds that stabilize the β-sheets. A disulfide bond between two highly conserved cysteine residues in strands B and F connects the β-sheets and further stabilizes the overall structure. Some of these residues are

evolutionarily conserved and can be used to identify Ig modules that do not have a disulfide bond connecting the two β-sheets.

The β-strand number, configuration, and H-bond pattern define the topology of the Ig module. Thus Ig modules are divided into C1, C2, V, I1, and I2 sets (constant, variable, and intermediate, respectively) (Fig. 1). Every set clusters modules that are more similar to one another in sequence and structure than they are to any other module from a different set. The V- and C-sets are named based on their similarity to antibody variable (V) and constant (C) modules [4]. In 1994, a new topology of Ig modules was observed in telokin, the *C*-terminal module of myosin light chain kinase, and was defined as the intermediate (I) set [18]. The I-set has a topology intermediate between the V- and C-sets. The two β-sheets of the I-set module consist of A, B, E, and D strands in one sheet and A′, G, F, C, and C′ strands in the other sheet. The B and E strands in one sheet and G, F, and C strands in the other sheet are present in all types of Ig modules and form the core of the Ig fold.

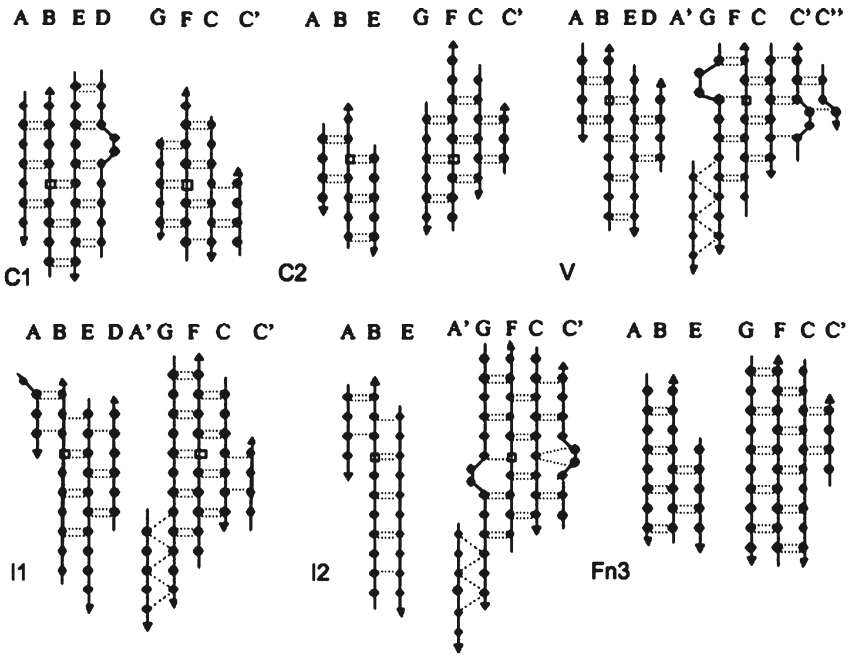

Fig. 1 Schematic presentation of the secondary structure of the IgSF and Fn3 modules. Each type of module is shown as two β-sheets. The β-strands are designated by letters A, A′, B, C, C′, C″, D, E, F, and G. *Solid circles* represent β-strand residues. *Dashed lines* represent interstrand backbone hydrogen bonds. The cysteines that form the intersheet disulfide bridge are shown as *open squares*. *Arrows* indicate the direction of the polypeptide chain. The A′ and G strands are parallel β-strands, and their hydrogen bond network therefore appears different from the others. Modified from Wang and Springer [67]

An important feature of the V- and I-set folds that distinguishes them from the C-set is that the A strand forms anti-parallel H-bonds with the B strand, and the A′ strand forms parallel H-bonds with the F strand. A kink between the A and A′ strands usually involves a *cis*-proline. The I-set has been further divided into the I1- and I2-sets (Fig. 1), based on the presence of a D strand in the I1-set that is absent in the I2-set [19]. This division is analogous to the division of the C-set into the C1- and C2-sets (Fig. 1). The I1-set also resembles the C1-set by the presence of a short C′ strand in some members. Because the I-set has features common to both V- and C-types, it has been hypothesized to be evolutionary the "oldest" type of Ig module [20, 21].

In many IgSF CAMs, several membrane-proximal Fn3 modules follow the Ig modules. The Fn3 module was identified originally as a repeating motif of ~90 amino acids in the ECM protein fibronectin [22]. This common structural motif has been predicted to occur in 165 human proteins [7]. The Fn3 module is similar in size and is topologically identical to the Ig C2-type module and has a typical β-sandwich fold with one β-sheet containing A, B, and E strands and the other containing G, F, C, and C′ strands (Fig. 1). The module does not have the conserved disulfide bridge that is observed in Ig domains, and the packing of the two β-sheets is stabilized by a number of conserved hydrophobic and aromatic residues. Although Ig modules and Fn3 modules exhibit a high degree of structural similarity, Fn3 modules have distinct hydrophobic cores and possess patterns of conserved residues that are different from the Ig modules [23].

Three-Dimensional Structure of NCAM Ig Modules

The three-dimensional structures of the three separate *N*-terminal Ig modules of NCAM were first determined by nuclear magnetic resonance (NMR) spectroscopy [24–26]. These were followed by crystal structures of the Ig1-2 and Ig1-2-3 fragments determined at 1.85 and 2.0 Å resolution, respectively [27, 28]. In the structure of Ig1-2-3, the Ig1 and Ig2 modules are positioned in an extended conformation with Ig3 oriented at an angle of ~45° to the Ig1–Ig2 axis (Fig. 2). Interestingly, due to only a one-residuelong linker, the G-strand of the Ig2 module continues uninterrupted into the A-strand of the Ig3 module. The overall solution structures of the individual Ig1, Ig2, and Ig3 modules are similar to the respective crystal structures. The three *N*-terminal Ig modules of rat NCAM adopt the topology of the I1-set in the IgSF, with the exception that the C′ strand and CD-loop are replaced by a heparin binding sequence (KHKGRDVILKKDVRFI) in Ig2.

In the crystal structures both the Ig1-2 and Ig1-2-3 fragments of rodent NCAM form dimers. Residues from the *C*-terminal part of the Ig1 module and from the *N*-terminal part of the Ig2 module together form a large binding surface. Despite forming a dimer, the Ig1 and Ig2 modules do have a limited degree of freedom. In the Ig1-2-3 fragment structure, the tilt angle between Ig1 and Ig2 is 11° and thereby differs by 13° compared with the average of 24° observed for the four copies of the

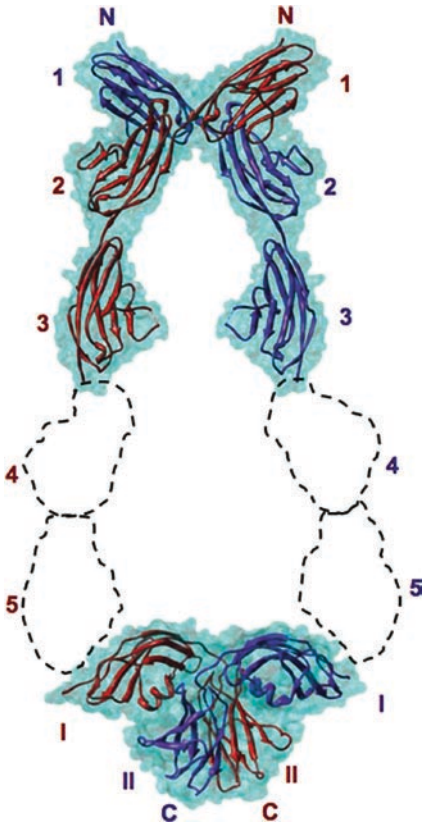

Fig. 2 Crystal structures of the rodent NCAM Ig1-2-3 and human NCAM Fn3-1-2 fragments shown as dimers (PDB codes 1QZ1 and 2VKW, respectively). Ribbon diagram with β-strands shown in red and purple. The *N*- and *C*-termini indicated. The likely overall shapes and sizes of the Ig4 and Ig5 modules are shown by *dashed line.*

Ig1-2 fragment [27]. This results in small variations of the surface area (from 1,525 to 1,613 Å^2) buried upon Ig1–Ig2 dimer formation. The most prominent feature of the Ig1–Ig2 interaction is the intercalation of two aromatic residues, Phe19 and Tyr65, of the Ig1 module into hydrophobic pockets formed by Ig2 residues. The Phe19Ser point mutation has been shown to completely abolish Ig1-2 dimerization in solution, thereby confirming that the Ig1–Ig2 interaction observed in the structures is biologically relevant [29]. The sequence of residues in Ig2 involved in binding to Ig1 in the dimer is localized to the FG-loop β-hairpin (GRILARGEINFK). Interestingly, this 12 residues long peptide in solution binds the NCAM Ig1 module with 12-fold greater affinity than the Ig2 module itself [30].

In addition to the Ig1–Ig2 interaction, the Ig1-2-3 crystal structure confirmed the previously observed interactions between Ig2 and Ig2 with a contact area of 958 Å^2 (calculated as water-inaccessible area) and revealed two novel interactions between

the Ig1-2-3 dimers [27]. In the first one, Ig2 interacts with Ig3 and *vice versa* with a contact area of 1,407 Å^2 per Ig1-2-3 dimer (Fig. 3a). A central element of this interaction is the intercalation of the side chain of Phe287 from Ig3 into a hydrophobic pocket formed by the side chains of Val145, Arg146, and Arg158 of Ig2 and Lys285 from Ig3. The Ig1-2-3 dimers form zipper-like arrays in the crystal *via* the Ig2-to-Ig3 interactions (Fig. 3a). In the second new interaction, Ig1 binds to Ig3 and *vice versa* with a contact area of 858 Å^2 per dimer (Fig. 3b). Arg198 and Asp249 form direct H-bonds to the backbone oxygen atoms of Ala81 and Glu82 and two salt bridges with Lys76, respectively. The conserved Phe36 and Phe221 are packed against Asp249 and Gln47, respectively. Together two Ig1-to-Ig3 interaction sites and one Ig2-to-Ig2 site make up a predominant contact between Ig1-2-3 dimers in the crystal (2,654 Å^2) forming the second array of Ig1-2-3 dimers (Fig. 3b, c) perpendicular to the Ig2-to-Ig3-mediated array (Fig. 3a).

To examine the biological significance of the observed Ig1-to-Ig3 and Ig2-to-Ig3 binding sites in the structure of NCAM Ig1-2-3, the authors tested the inhibitory

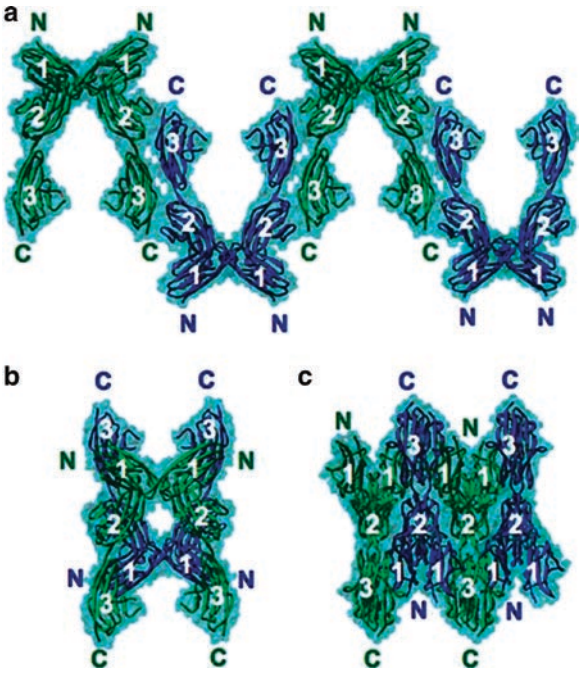

Fig. 3 Crystal structure of the Ig1-2-3 fragment of NCAM reveals two kinds of Ig1-2-3 arrays. (**a**) The Ig1-2 mediated *cis* dimers of the Ig1-2-3 fragment are shown in *blue* and *green* and form a "flat" zipper via an Ig2-to-Ig3 mediated *trans* interaction, reflecting an interaction between NCAM molecules on apposing cells. (**b** and **c**) The Ig1-2-3 fragment *cis* dimers also form a non-symmetrical "compact" zipper via Ig1-to-Ig3 and Ig2-to-Ig2 *trans* interactions. Four *cis* dimers shown in *blue* and *green* are held together by two Ig1-to-Ig3 interactions on one side and one Ig2-to-Ig2 interaction on the opposite side. The termini are denoted by *N* and *C*. The view in B and C are perpendicular to each other

effect of the recombinant Ig3 module on NCAM-mediated adhesion in a co-culture system of the NCAM-expressing PC12-E2 cells grown on a confluent monolayer of NCAM-expressing fibroblasts. Two Ig3 mutants containing mutations of the residues R198A, D249G, and E253A of the Ig1-to-Ig3 binding site, and K285A and F287A of the Ig2-to-Ig3 binding site, respectively, were not able to inhibit NCAM–NCAM binding. However, the wildtype Ig3 module indeed inhibited NCAM-mediated neurite outgrowth, thereby supporting the theory that both the Ig1-to-Ig3 and Ig2-to-Ig3 sites participate in NCAM–NCAM binding [28].

The three-dimensional structures of the Ig4 and Ig5 modules are not yet determined but both modules were predicted to adopt the I1-set fold [24]. Interestingly, the length of the Ig4 module can be increased by ten amino acid residues (KASWTRPEKQ) encoded by the variable alternatively spliced exon (VASE) inserted between the C′ and D strands. This modification is likely to add the C″ strand to Ig4 and thereby change the VASE-negative Ig4 module's fold from the I1- to V-set. Expression of VASE exon reduces NCAM-specific neurite outgrowth [31].

Three-Dimensional Structures of NCAM Fn3 Modules

The three-dimensional structures of the first (Fn3-1) and the second (Fn3-2) modules of NCAM have been determined by X-ray (1.8 Å) and NMR techniques, respectively [32, 33]. These were followed by the crystal structure of the tandem Fn3-1-2 fragment of human NCAM [34]. One prominent feature of the Fn3-1 module is a cluster of the negatively charged residues Asp497, Asp511, Glu512, and Glu514. Replacement of these residues with positively charged Arg residues eliminates polysialylation of a truncated NCAM protein consisting of Ig5, Fn3-1, the transmembrane region, and cytoplasmic tail [32]. Another highly unusual feature of the Fn3-1 module is an α-helix that links the D and E strands. Replacement of this eight-amino acid α-helix with an analogous linker from another Fn repeat shifts the site of polysialylation from the *N*-linked glycans of the Ig5 module to the *O*-linked glycans of Fn3-1. The α-helix appears to determine the orientation of the Fn3-1 module with respect to the Ig5 module. This has a decisive effect on NCAM polysialylation by polysialyltransferase [32].

In the structure of the tandem Fn3-1-2 fragment, the individual Fn3 modules are similar to the previously determined structures of the Fn3-1 and Fn3-2 modules (root mean square deviation of 0.5 Å for 100 Cα atoms of Fn3-1 and 1.3 Å for 92 Cα atoms of Fn3-2). A highly flexible linker connects the two modules and allows unusually sharp intermodular angle of ~80° (Fig. 2). The crystal structure of the Fn3-1-2 fragment also reveals a possible mechanism of dimerization. In the crystal, the Fn3-1-2 fragments form dimers with an extensive binding surface area of 3,040 Å^2. The first of the two main contacts responsible for dimer formation is the α-helix of Fn3-1 packed against the GFCD sheet of the Fn3-2 module. In the second contact, the two Fn3-2 modules interact via their A and G strands.

Although the Fn3-1-2 fragment eluted as a mixture of monomers and dimers in gel filtration chromatography, the authors did not claim that the observed dimerization is physiologically relevant [34]. *Cis*-dimerization of Fn3 modules was reported earlier for the axonin-1/TAG-1 cell adhesion molecule [35].

Surface plasmon resonance (SPR) experiments have demonstrated that the NCAM Fn3-1-2 fragment binds to a recombinant protein comprising the fibroblast growth factor 1 receptor (FGFR1) Ig modules 2 and 3 with a K_D value of 10 μM. The binding of the individual Fn3 modules to FGFR1 could not be detected, indicating that both Fn3 modules are necessary for obtaining reasonable binding affinity [33]. This result is corroborated by the finding that only very weak binding between the NCAM Fn3-2 module and the FGFR Ig3 module (but not the Ig2 module) was detected by NMR titration experiments [33]. The solution structure of the Fn3-2 module has led to the identification of the FGFR1 binding site in NCAM [33]. The site has been mapped to the *N*-terminal parts of the F and G strands that form a β-hairpin. A peptide, termed FGL (EVYVVAENQQGKSKA), corresponding to the loop connecting the F and G strands of the Fn3-2 module has been identified to bind and activate FGFR1 [33]. Two more peptides derived from NCAM Fn3 modules 1 and 2 have been shown to activate FGFR1. The FGF receptor activation motif (FRM) peptide (SIDRVEPYSSTAQ) from the AB loop of Fn3-1 stimulates FGFR-dependent neurite outgrowth [36]. Whereas, the BCL peptide (NLIKQDDGGSPIRHY) derived from the loop connecting the B and C strands of Fn3-2 binds and activates the FGFR [37]. The FRM peptide located at the C-terminus of Fn3-1 and the FGL and BCL peptides located at the *N*-terminus of the Fn3-2 module form a contiguous patch due to a sharp bend between the Fn3 modules. The patch becomes disrupted upon straightening of the Fn3 tandem [34]. Therefore, together the two NCAM Fn3 modules are likely to form a single FGFR binding site consisting of the *N*- and *C*-termini of Fn3 modules 2 and 1, respectively. Interestingly, the linker connecting Fn3-1 and Fn3-2 can be modified by insertions of several exons (e.g., AAG, a, b, and c). These range from the introduction of a single Lys residue (exon AAG) to the addition of 5, 16, and 14 amino acid residues at exons a, b, and c, respectively. Together exons a, b, and c encode 35 residues and form the muscle-specific domain [38]. These modifications are likely to have a regulatory role because neurons express NCAM isoforms with no additional exons between the Fn3 modules, whereas other cell types often express NCAM proteins containing one or more of these exons. The presence of the exons may partially or completely disrupts the binding of Fn3-1-2 to FGFR1.

Models of NCAM-Mediated Homophilic Binding

Several models of module arrangement in NCAM-mediated homophilic binding have been suggested. The earliest model suggested that homophilic adhesion is mediated primarily by the Ig3 module [39–41]. Data obtained from cell aggregation experiments were performed on mouse L-cells expressing chicken NCAM

constructs with deletions of different modules. These experiments were followed by point-mutational analysis and competitive inhibition using synthetic peptides that demonstrated an involvement of Ig3 in cell aggregation. The same group of researchers had mapped the epitopes of four monoclonal antibodies capable of inhibiting NCAM-mediated cell–cell adhesion to Ig3. The identified sequence, KYSFNYDGSELIIKKVDKSDE, from the D-E-strands in Ig3 of chicken NCAM, and a shorter peptide, KYSFNYDGSE, were shown to inhibit NCAM-mediated adhesion [40]. In the proposed model, Ig3 in one NCAM molecule binds to Ig3 in an NCAM molecule expressed on an apposing cell surface (Fig. 4a).

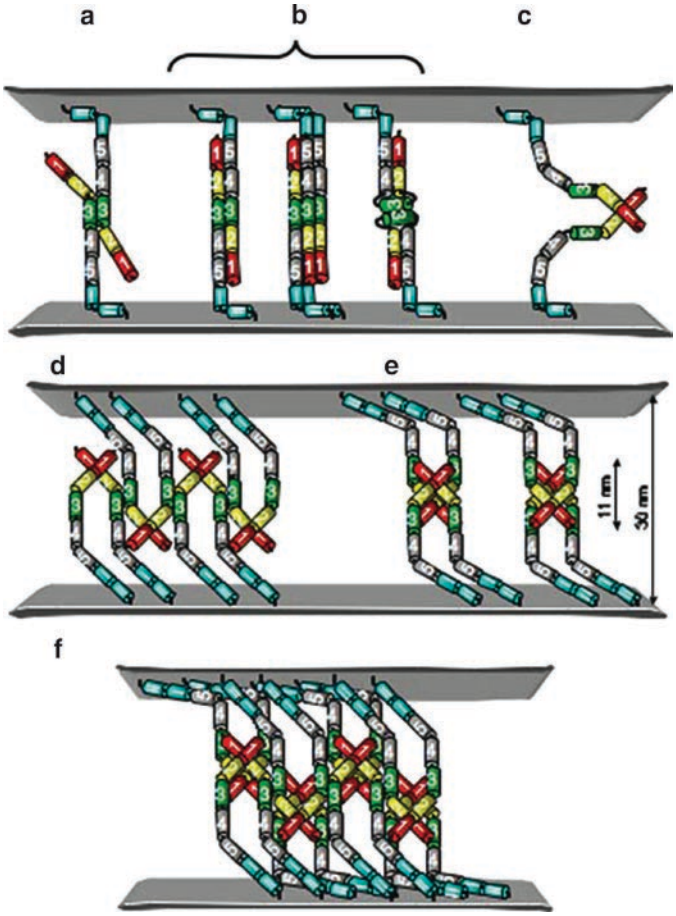

Fig. 4 Models of NCAM homophilic adhesion complex. NCAM molecules are shown attached to the cell membranes of two apposing cells. The individual Ig and FN3 modules schematically shown as cylinders. Ig1 is shown in *red*, Ig2 is *yellow*, Ig3 is *green*, Ig4 and Ig5 are *gray*. The two membrane-proximal FN3 modules are shown in cyan. The Ig and FnIII modules are numbered by Arabic and Roman numerals, respectively. The models based on: (**a**) cell aggregation experiments [39–41]; (**b**) microspheres aggregation experiments [42]. (**c**) SPR binding experiments [43]. (**d**–**f**) crystal structure of the NCAM Ig1-2-3 fragment [28]

The next three models are based on results from binding experiments in which microspheres were coated with individual NCAM modules produced as recombinant proteins in an *E. coli* expression system [42]. Aggregation of microspheres loaded with recombinant modules was monitored by a Coulter Counter and by fluorescence microscopy. In experiments examining combinations of modules, specific binding was demonstrated between Ig1 and Ig5 and between Ig2 and Ig4. Furthermore, microspheres coated with recombinant Ig3 exhibited strong self-aggregation. Two models of *trans*- and *cis*-interactions were proposed. The model of *trans* binding depicts two NCAM molecules on apposing cells aligned anti-parallel to each other so that Ig1 interacts with Ig5, Ig2 interacts with Ig4, and Ig3 interacts with apposing Ig3 (Fig. 4b). As an extension of this model, a possible formation of a tetrameric complex was suggested. In the tetramer, Ig3 was suggested to mediate *cis* dimerization of NCAM molecules on the same cell (Fig. 4b). According to yet another model suggested in the same publication, NCAM molecules might be folded into hairpin loops, in which Ig1 binds Ig5, and Ig2 binds Ig4, thus leaving Ig3 exposed for *trans* interactions (Fig. 4b).

All of these early models were based on classical cell or microspheres aggregation techniques (so-called indirect methods). When studying the low-affinity interactions, these methods are prone to artifacts attributable to the inherent tendency of microspheres to aggregate without any additional protein. The aggregation rate and the size of aggregates were clearly affected by temperature and agitation rate (Dr. V. Kiselyov, personal communication).

The very first direct measurements of binding between NCAM Ig modules employing the SPR method clearly demonstrated binding between the individual Ig1 and Ig2 modules. The K_D value for this interaction was estimated to be $55 \pm 16\,\mu M$, whereas the K_D value for the Ig1-2 double module binding was predicted to be 3.3 ± 1.8 nM [43]. Later, gel filtration experiments showed that a recombinant Ig1-2 fragment of rat NCAM formed a stable dimer in solution under physiological conditions [25]. This indicated a high affinity interaction that agreed well with the predicted 3.3 nM K_D value for this interaction. In contrast, a dissociation constant of only ~100 μM was reported for the Ig1-2 and Ig1-2-3 fragments of chicken NCAM [26, 44]. The interacting residues in Ig1 and Ig2 were mapped in a series of NMR titration experiments [25, 44]. The model of the Ig1-2 dimer was built based on both NMR and mutation studies [25]. The model was significantly corrected by the crystal structure of a recombinant protein consisting of the NCAM Ig1-2 modules [27]. The crystal structure for the first time provided a detailed picture of Ig1–Ig2 interactions and corrected both the tilt and twist angle between Ig1 and Ig2 in the NMR-based model. Although the crystal structure of the Ig1-2 dimer was an important step forward, determining whether it accounts for a *trans*- or *cis*-interaction was not possible. At the same time, no interactions between Ig3 modules of chicken NCAM in solution could be detected, although Ig3 was folded and clearly inhibited aggregation of synthetic lipid vesicles containing NCAM [26]. Moreover, the same group demonstrated that the Phe19Ser point mutation did not affect cell aggregation mediated by full-length NCAM, although it abolished dimerization of the Ig1-2-3 fragment in solution [26].

Further clues to the possible mechanism of homophilic binding came from the crystal structure of a fragment consisting of NCAM Ig1-2-3 modules [28]. The parallel interaction of the NCAM Ig1-2-3 molecules in the crystal mediated by the Ig1-to-Ig2 contact may reflect a *cis* interaction between NCAM molecules. The anti-parallel interactions mediated by the Ig1-to-Ig3, Ig2-to-Ig2, and Ig2-to-Ig3 contacts may reflect a *trans* interaction. Based on the crystal structure of the Ig1-2-3 fragment, the new model for NCAM homophilic adhesion postulated two zipper-like arrays of NCAM molecules (Fig. 4d–f). In the "compact" zipper formation, NCAM *cis* dimers originating from apposing cell membranes are arranged as arrays through Ig1-to-Ig3 and Ig2-to-Ig2 interactions (Fig. 4d). The "compact" zippers may form first because they allow larger distances between apposing cell membranes than the "flat" zippers. In the "flat" zipper (Fig. 4e), the Ig2-to-Ig3 interactions suggest a lateral association between the "compact" zippers of NCAM, thereby forming a double zipper adhesion complex (Fig. 4f). Since the biological significance of the Fn3-1-2 modules dimer is not clear, it is difficult to estimate how the Fn3-1-2 fragment dimerization can contribute to the overall picture of the homophilic binding mechanism of NCAM.

The hypothesis of a zipper-like mechanism of cell adhesion is not new and was first suggested for *N*-cadherins [45]. A similar zipper-like array of *trans*-interacting *cis*- homodimers was observed in the crystal structure of the junctional adhesion molecule [46]. A zipper-like mechanism of homophilic interactions also was suggested for axonin-1/TAG-1 [47], in which alternating molecules from apposed membranes form a linear zipper-like array. However, the double-zipper model proposed for NCAM differs from the previously described zippers.

We will try to evaluate whether the NCAM zipper model agrees with other results. One of the prominent features of NCAM is its ability to bind heparin and heparan sulfates. Interestingly, in the compact zipper formation observed in the Ig1-2-3 structure, the heparin binding sites (KHKGRDVILKKDVRFI) [48] form two nearly continuous belts on both sides of the zipper [28]. The heparin binding sites are solvent-exposed and, therefore, accessible for binding to heparin and heparan sulfate molecules, suggesting that NCAM may be engaged in homophilic and heterophilic interactions simultaneously. Kulahin et al. [49] arrived at a different conclusion after a series of NMR titration experiments. It was demonstrated that binding between Ig1 and Ig2 can be equally but only partially inhibited by heparin, chondroitin sulfate, and the heparin analog sucrose octasulfate (SOS). The latter is known to bind heparin-binding proteins. The experiments were performed with single Ig1 and Ig2 modules that are capable of only low-affinity interactions (K_D of 0.05–2.5 mM) [25, 43]. Attempts to inhibit the high-affinity dimerization of the Ig1-2 fragment by a 50-fold excess of SOS on a gel filtration column equilibrated with phosphate-buffered saline containing 1 mM SOS were unsuccessful. However, 1 mM SOS (10-fold excess) completely inhibited binding of Ig2 to immobilized heparin in the SPR experiments (V. Soroka, unpublished results).

In a thorough study using a bead aggregation assay, Atkins et al. [50] clearly demonstrated that Ig1-Ig2 binding can mediate aggregation of protein A-coated beads loaded with chimeric constructs containing various extracellular NCAM

modules linked to Fc fragments [50]. Unexpectedly, deletion of Ig5 profoundly inhibited (by ~70%) NCAM-mediated bead aggregation. Introduction of a four-residue-long insert (Ala–Ser–Ser–Gly) between Ig2 and Ig3, which disrupted orientation between the modules but left the individual modules intact, inhibited NCAM-mediated bead aggregation by 50%. The same insert introduced between Ig1 and Ig2 completely abrogated NCAM-mediated bead aggregation. Apparently, bead aggregation assays can detect only the relatively high-affinity interactions mediated by Ig1–Ig2 dimerization, whereas interaction between single Ig1 and Ig2 modules was not detectable. The same authors previously introduced a Phe19Ser mutation that abolished the Ig1–Ig2 fragment dimerization in solution but failed to abrogate NCAM-mediated adhesion between COS-7 cells [26], clearly demonstrating the discrepancies that exist between cell- and bead-based aggregation assays.

An increasing number of publications demonstrate that cell adhesion can be mediated by low-affinity interactions that are difficult to detect in solution, but can be observed in protein crystals. For example, the recombinantly expressed Ig1-4 fragment of the chicken axonin-1/TAG-1 CAM was monomeric during purification by gel filtration but formed zippers in the crystal structure. Interactions observed in crystals were successfully verified by extensive mutagenesis followed by cell aggregation assay in transfected wildtype J558L myeloma cells [47]. The structure of axonin's homolog human TAG-1 was shown recently to form dimers rather than zippers. Although the dimerization site overlapped with the one observed in the zipper formation, the two interaction models were mutually exclusive [51]. Interestingly, both interaction mechanisms agree with the mutagenesis experiments of Freigang et al. [47].

Surface Force Apparatus (SFA) and Atom Force Microscopy Experiments

An alternative to structure- and cell/bead aggregation-based approaches for determining the mechanism of NCAM-mediated cell adhesion is direct measurement of the forces generated between single NCAM molecules. Rupture forces between full-length NCAM extracellular parts revealed two binding events: a weaker interaction ($170 \pm 30\,\mu M$) was attributed to the Ig1-2 part, and a stronger ($100 \pm 60\,\mu M$) interaction was attributed to Ig3 [52, 53]. The measured affinities present an obvious contradiction. The Ig1-2 dimer was clearly detected in gel filtration and ultracentrifugation by two different groups, whereas the Ig3–Ig3 interaction that supposedly has higher affinity was not detected in any of these assays [26, 28]. Moreover, no Ig3-mediated dimerization of the Ig1-2-3 fragments in solution was observed in gel filtration or dynamic light scattering (DLS) experiments [28]. The NCAM constructs used in the SFA experiments regrettably have some serious flaws. The mutant that lacks the Ig1-2 modules also lacks 17 residues of the *N*-terminal part of the Ig3 module, and this will inevitably have a severe impact on the three-dimensional structure of the construct. Atkins et al. [26] previously reported that deletion of only four *N*-terminal residues of Ig3 resulted in an unstable Ig3 module that

aggregated in solution. The second mutant used in SFA experiments lacks Ig3. Unfortunately, this construct also lacks the A and A′ strands and nearly half of the B strand of Ig4 (a total of 18 residues). The construct also contains 17 residues belonging to the *N*-terminal part of Ig3 [52]. The effects of these deletions/insertions are difficult to determine and potentially compromise the conclusions of this work.

The structures of five of seven NCAM modules have been solved to date. Numerous unsuccessful attempts have been made to produce crystal structures of the NCAM Ig1-2-3-4 and Ig1-2-3-4-5 fragments, as well as solution structures of the Ig4 and Ig5 modules [54]. To our knowledge, only seven X-ray structures of an Ig module containing adhesion molecules have been determined that comprise three or more Ig modules: CD4 [55], hemolin [56], chicken axonin-1/TAG-1 [47], human TAG-1 [51], rodent NCAM [28], and human ICAM-1 [57]. The relatively modest number of structures of the multi-modular CAM fragments may reflect the increasing difficulties regarding protein production/crystallization and crystal quality. With regard to NCAM, the longer the crystallized fragment, the lower the diffraction resolution. The crystals of the single Fn3-1 module contained two copies per asymmetric unit (AU) and diffracted to 1.7 Å [32]. The Ig1-2 fragment produced crystals that had four copies of Ig1-2 per AU and diffracted to 1.85 Å [27]. The crystals of the Fn3-1-2 fragment had two protein molecules per AU. Despite a highly flexible linker connecting the two Fn modules, the crystals diffracted to 2.1 Å [34]. The Ig1-2-3 fragment diffracted to 2.0 Å but had only one molecule per AU [28]. The crystals of the Ig1-2-3-4 recombinant fragment of NCAM diffracted only to 4.0 Å resolution, whereas no diffraction was observed for the construct containing Ig1-2-3-4-5 modules [54].

Model of the Extracellular Part of NCAM

We attempted to model the entire extracellular part of NCAM. To connect the Ig1-2-3 and Fn3-1-2 fragments of NCAM (PDB codes 1QZ1 and 2VKW, respectively) we modeled Ig4 and Ig5 and their orientation using the crystal structure of Ig3-4-5 fragment of ICAM-1 (PDB code 2OZ4). The intermodular orientation of Ig5 and Fn3-1 is based on the intermodular geometry between the Ig and Fn3 modules observed in the crystal structure of eRPTPμ (PDB code 2V5Y).

Our homology modeling of NCAM produced a bent rod-like structure with the *N*- and *C*-terminal parts of respectively 17.6 and 8.5 nm positioned at approximately right angle (Fig. 5). The length of the N-terminal part of the modeled NCAM is very similar to the thickness of the NCAM artificial monolayer that was determined by the X-ray reflectivity (XR) measurements as 20.6 ± 0.7 nm [58]. Earlier, analyses of NCAM by electron microscopy have revealed a bent rod-like structure [59, 60]. The angle of the bend at the hinge region between the *N*-terminal (~18 nm) and *C*-terminal (~10 nm) parts varies considerably (50–140°) with an average value of 98° [60]. The recent crystal structure of NCAM Fn3-1-2 fragment revealed a sharp bend (~80°) between the two Fn modules [34]. Although the angle

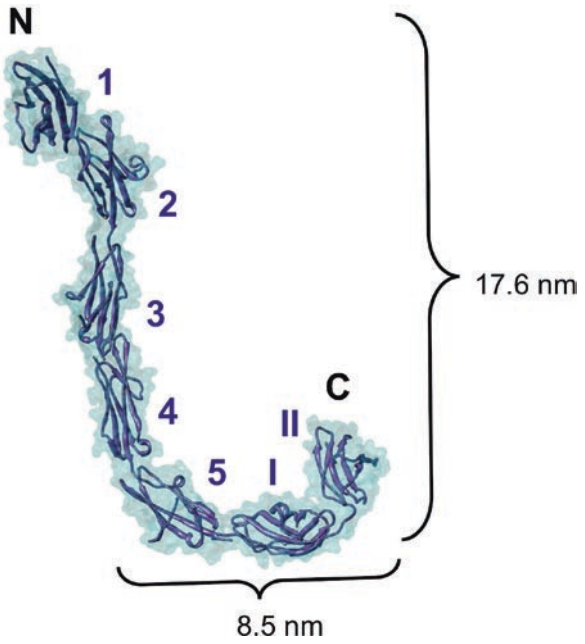

Fig. 5 Model of the extracellular part of NCAM. The model is based on the crystal structures of the Ig1-2-3 and Fn3-1-2 fragments of NCAM (PDB codes 1QZ1 and 2VKW, respectively). The crystal structure of the Ig3-4-5 fragment of ICAM-1 (PDB code 2OZ4) was used to model position and orientation of the Ig4 and Ig5 modules of NCAM. Orientation of the Ig5 and Fn3-1 modules is based on the intermodular geometry between the Ig and Fn3 modules in the crystal structure of eRPTPμ (PDB code 2V5Y)

of the bend between the two Fn3 modules is similar to the average value observed in the electron microscopy studies, it is unlikely that the Fn3-2 module followed by the 22 residues long juxtamembrane linker (TSAQPTAIPANGSPTSGLSTGA) can assume an extended and rigid conformation creating a 10 nm long *C*-terminal part of NCAM. Based on electron microscopy results and on an average length of ~4.3 nm (43 Å) for the Ig module and ~4.0 nm (40 Å) for the Fn3 module of NCAM [28, 34], the hinge region is most likely located after the Ig4 or Ig5 module. Probably, it is the sum of the Ig4–Ig5 and Ig5-Fn3-1 bends that defines the shape of NCAM. The distances observed in our model of the extracellular part of NCAM agree well with those observed in the electron microscopy and XR studies.

Perspectives

The challenge of revealing the mechanism of NCAM homophilic binding does not lie solely in demonstrating homophilic interactions in solution or in bead aggregation assays. Although identifying the Ig modules involved in homophilic interactions is clearly

informative, the real challenge is to understand the mechanism of NCAM-mediated adhesion at the atomic level. Due to their inherent flexibility, the mutimodular CAMs usually produce at best, poorly diffracting crystals. Fortunately, an alternative approach can be used to overcome disorder in the crystal packing. CAMs can be crystallized in complex with Fab fragments of a monoclonal antibody [61]. Fab fragments can either be produced by proteolysis of the monoclonal antibody or recombinantly by cloning the antibody gene from hybridoma into *E. coli*. As an alternative to hybridomas, antibodies to practically any protein can be obtained using phage display antibody libraries [62]. The high-affinity Fab fragment bound to a structural epitope is likely to form rigid complex, thereby reducing CAM's flexibility and providing additional polar surfaces for well-ordered crystal packing.

Some limited structural information can be provided by electron microscopy (EM) of the 2D crystals. Although electron crystallography has the potential of yielding structural information equivalent to X-ray diffraction, typical resolution obtained by EM is rarely better than 9 Å. But, even relatively modest resolution is sufficient to determine the overall shape of the molecule and reveal the binding mechanism of CAMs. 2D crystallization of soluble proteins is based on a specific interaction between the native or His-tagged protein and specific ligands coupled to lipid molecules incorporated into a lipid film [63]. Recently, successful attempts have been made to develop an automated system to screen the 2D crystallization trials [64].

The solution-based techniques also have a great potential in determining the structure of even large CAMs. With every major synchrotron facility dedicating at least one beamline entirely for the small angle X-ray scattering (SAXS) experiments, this technique is rapidly becoming available to a wider scientific community. The main advantages of SAXS are the relatively short time required for data collection as well as the ability to incorporate any sort of prior structural information [65]. These greatly compensate for the limited resolution of 15–10 Å typically achieved in SAXS-based models. SAXS has also demonstrated the power to discriminate between the alternative models of protein complexes.

NMR of multimodular proteins is rapidly becoming a viable alternative to protein crystallography. The impressive effectiveness of assignment of such proteins has been demonstrated by solving the structure of several fragments of the fibronectin consisting of two and three modules [66].

Since the "head-on" approach in structural analysis of the entire extracellular parts of multimodular CAMs succeeds only rarely, one should bypass the problem. Rather than adopting traditional "method-based" approach, the structural projects should be "problem-based" instead. At first, one should establish an efficient protein production procedure capable of delivering a reasonably large number of overlapping recombinant fragments (2–3 modules long) from several homologous CAMs. These fragments should be tested in a series of automated high-throughput crystallization screens. NMR can solve few important fragments that fail to yield diffracting crystals quickly. SAXS can be used to examine the large multimodular fragments preventing possible overinterpretations of the crystal packing artifacts. In case of NCAM, solution of the structures of several NCAM homologs, such as

mammalian NCAM-2, *Drosophila* Fasciclin-II, and *Aplysia* apCAM, might be considered. Thus, apart from determining the 3D structure of the entire extracellular part of NCAM researchers will create a coherent picture of evolution of NCAM proteins.

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Extracellular Protein Interactions Mediated by the Neural Cell Adhesion Molecule, NCAM: Heterophilic Interactions Between NCAM and Cell Adhesion Molecules, Extracellular Matrix Proteins, and Viruses

Janne Nielsen, Nikolaj Kulahin, and Peter S. Walmod

Abbreviations

CAH	Carbonic anhydrase
CAM	Cell adhesion molecule
CHL1	Close homolog of L1
CNS	Central nervous system
CSPG	Chondroitin sulfate proteoglycans
ECM	Extracellular matrix
FGFR	Fibroblast growth factor receptor
FnIII	Fibronectin type 3
GAG	Glycosaminoglycans
GDNF	Glial cell line-derived neurotrophic factor
GPI	Glycosylphosphatidylinositol
HB-GAM	Heparin-binding growth-associated molecule
HBD	Heparin binding domain
HNK-1	Human natural killer-1
HSPG	Heparan sulfate proteoglycans
Kd	Equilibrium dissociation constant
LRP	Laminin receptor precursor
MSD1	Muscle-specific domain 1
NCAM	Neural cell adhesion molecule
NMR	Nuclear magnetic resonance
PG	Proteoglycans
PI-PLC	Phosphatidylinositol-specific phospholipase C
PrP	Protease resistant protein
RPTP	Receptor protein tyrosine phosphatase
PSA	Polysialic acid

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RABV	Rabies virus
SOS	Sucrose octasulfate
SPR	Surface plasmon resonance
VASE	Variable alternative spliced exon

Introduction

The neural cell adhesion molecule (NCAM) is expressed in most tissues, including lung, stomach, kidney [1], heart [2–4], and muscles [5]. Furthermore, natural killer (NK) cells are divided into subgroups based on their expression, or lack of expression of NCAM [6, 7], and the protein also serves as a prognostic marker of certain cancers, including small-cell lung cancer, multiple myelomas, and CD4 (+)/CD56 (+) haematodermic neoplasms [8–10]. However, as the name implies, the NCAM protein is expressed in particularly large amounts in the nervous system, where it is involved in numerous processes, including neural development, regeneration, and learning and memory formation (for review, see [11]).

NCAM mediates cell–cell adhesion through homophilic *trans*-interactions and engages in numerous heterophilic intra- and extracellular protein interactions. This review focuses on the extracellular heterophilic protein interactions between NCAM and other cell adhesion molecules (CAMs), extracellular matrix (ECM) proteins, and the rabies virus (RABV). NCAM also forms extracellular interactions with growth factors and growth factor receptors, including fibroblast growth factor receptor (FGFR), glial cell line-derived neurotrophic factor (GDNF), and its co-receptor GDNF family receptor α (GFRα) [12–14]. However, these interactions are not discussed in this review; nor is the modulation of NCAM function through extracellular interactions with adenosine triphosphate (ATP) discussed. For recent reviews on these topics, the reader is referred to [11, 15–17].

NCAM Isoforms

NCAM is transcribed from a single gene, but as a result of alternative splicing, the protein exists in multiple isoforms. The three main isoforms of NCAM are referred to as NCAM-120, which is attached to the plasma membrane with a glycosylphosphatidylinositol (GPI) anchor, NCAM-140, and NCAM-180, both of which are class 1 transmembrane proteins with cytosolic tail regions of ~119 and 386 amino acids, respectively (UniProtKB/Swiss-Prot entry P13595-2 and P13595, respectively). In addition to the membrane-attached isoforms, NCAM also exists in soluble forms derived either from extracellular cleavage of the protein within the membrane-proximal, extracellular part of the protein by matrix metalloproteinases [18, 19] or by cleavage of the NCAM-120 GPI-anchor by phosphatidylinositol-specific phospholipase C (PI-PLC) [20, 21]. Furthermore, due to the insertion of a stop codon, alternative splicing can result in a secreted

isoform consisting of the ~655 N-terminal amino acids (UniProtKB/Swiss-Prot entry P13592-1) [22, 23].

The extracellular part of NCAM comprises approximately 700 amino acids (the exact size depends on the presence or absence of alternatively spliced regions) organized as five N-terminal Ig-homology modules (Ig1-5) followed by two membrane-proximal fibronectin type III-homology modules (FnIII1-2) [24]. This general composition of the extracellular region is modulated by the alternative splicing of six small exons within the *NCAM* transcript. Thus, the extracellular part of the protein optionally includes a 10 amino acid-long region termed VASE (variable alternative spliced exon) [25], three short regions (MSD1a, -b, and –c; consisting of 15, 48, and 42 amino acids, respectively) that together comprise the so-called muscle-specific domain 1 (MSD1) [4, 26, 27] and the one amino acid-long insert termed AAG. The VASE region is located in the middle of Ig4, whereas the MSD1a–c and AAG regions are located between the FnIII1 and FnIII2 modules [28]. Finally, as mentioned above, the inclusion of an exon introducing a stop codon between MSD1 and AAG leads to the formation of a secreted form of NCAM consisting of the extracellular region of the protein that is truncated between the FnIII1 and FnIII2 modules [23].

NCAM Glycosylation

Extracellular NCAM interactions are modulated and/or mediated by glycosylations of the protein. NCAM contains six consensus sequences for *N*-linked glycosylation (N-1–N-6). These sequences are highly conserved between species, and all can be glycosylated [29]. As shown in Fig. 1, the sites for *N*-linked glycosylation are confined to Ig3, Ig4, and Ig5. Ig1, Ig2, FnIII1, and FnIII2 are unglycosylated with the exception of an *O*-linked glycosylation within the alternatively spliced MSD1 region [30].

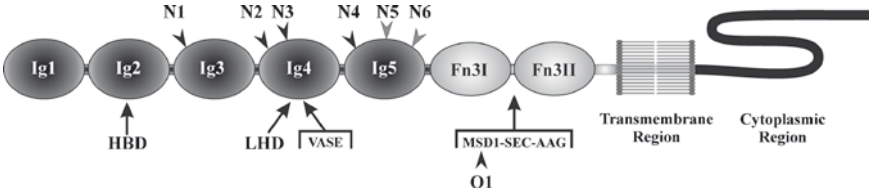

Fig. 1 Extracellular structure of NCAM. The figure illustrates the structure of the extracellular part of NCAM. Ig1-5 indicates immunoglobulin homology modules. FnIII1-2 indicates fibronectin type III homology modules. The extracellular region of NCAM can be varied by alternative splicing at the positions indicated by the framed text. The position of sites involved in heterophilic interactions described in the text are indicated by arrows. The positions of N-linked (Nx) and *O*-linked (Ox) glycosylation sites, numbered from the N-terminal, are indicated by arrowheads (black, non-PSA sites; gray, PSA sites). See text for details and references. VASE, variably alternative spliced exon; MSD1, muscle-specific domain 1; SEC, exon expressed by secreted NCAM; AAG, AAG exon; HBD, heparin binding domain; LHD, lectin homology domain

A unique feature of NCAM glycosylation, when compared with other CAMs, is the potential glycosylation with the unusual carbohydrate polysialic acid (PSA; homopolymers of α2-8-*N*-acetylneuraminic acid). This type of glycosylation is restricted to the N-5 and N-6 positions in the Ig5 module [31–33]. Because the individual PSA glycosylations consist of chains of at least 30 negatively charged sialic acid residues [32], they have strong effects on extracellular heterophilic and homophilic NCAM interactions. Accordingly, PSA acts as a modulator of NCAM-mediated cell adhesion and affects processes such as cell migration, neurite outgrowth, and synaptic plasticity and maturation related to learning and memory consolidation. For recent reviews of the function of the NCAM PSA-glycosylation, see [34–36].

NCAM also expresses carbohydrates with the human natural killer-1 (HNK-1) epitope (also referred to as L2 or CD57) [37, 38] that can be found at the N2–N6 glycosylation sites [31, 33] and at the *O*-linked glycosylation site in MSD1 [39]. HNK-1 also is expressed by other neural CAMs, including NCAM2 [40, 41], myelin-associated glycoprotein (MAG) [42], melanoma cell adhesion molecule (MCAM) [43], L1 [37], close homolog of L1 (CHL1) [44, 45], P0, and F3/contactin 1 [46]. One of the functions of HNK-1 in the nervous system has been speculated to be facilitation of the migration of neural crest cells [47].

Finally, NCAM also expresses a number of non-PSA/non-HNK-1 glycosylations. Some of these are specifically found on NCAM molecules expressed in the olfactory system, where they are suggested to be involved in the establishment of connections between the olfactory neuroepithelium and the olfactory bulb [48–51].

Extracellular Interactions between NCAM and other CAMs

Although most CAMs originally were recognized as CAMs based on their *trans*-homophilic interactions, many CAMs also engage in interactions with many extracellular, heterophilic binding partners. Particularly, different types of CAMs commonly interact with each other (for review, see [11]), creating additional opportunities for fine-tuning cellular responses mediated by CAMs and their binding partners. The following sections give an overview of the interactions between NCAM and the other CAMs.

Interactions between NCAM and the Prion Protein

The protease resistant protein (PrP) is a prion (short for proteinaceous infectious particle). The normal PrP protein is ubiquitously expressed and is denoted PrP^{C}, whereas a misfolded form of the protein gives rise to diseases such as Creutzfeldt-Jakob disease (in humans) and scrapie (in animals) and is referred to as PrP^{Sc} [52]. PrP^{C} is a ~200 amino acid-long GPI-anchored receptor. The normal type of the

protein is structurally composed of a highly flexible N-terminal region that includes the ~40 amino acid-long so-called octapeptide repeat region, followed by a globular membrane-proximal domain consisting of three α-helices, two β-strands, and two disulfide bridges [53].

PrP^C has been shown to bind, directly or indirectly, to several ECM molecules and membrane-localized proteins, including NCAM, laminin and vitronectin, the laminin receptor precursor (LRP), the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptor-2 (GluR2) subunit, the astroglial α2/β2-Na^+/K^+-ATPase, and the stress-inducible protein 1 (STI1) [54–60]. Because PrP^C interacts in *trans* with ECM components and CAMs (see below), it has been referred to as a CAM [60].

NCAM dose-dependently binds PrP^C directly, and cross-linking experiments followed by protein identification by mass spectrometry suggest that PrP^C interacts with all three main isoforms of NCAM. The PrP^C-NCAM interaction demonstrates 1:1 stoichiometry, which does not require the presence of *N*-linked glycosylation on NCAM and is not affected by the presence of PSA on NCAM [56, 58].

Peptide array analysis and binding experiments with recombinant PrP proteins suggest that the interaction between PrP^C and NCAM occurs between one of the α-helices in the membrane-proximal globular domain of PrP^C (around amino acid residues 144–154) and the NCAM Ig5, FnIII1, and FnIII2 modules [56]. The large apparent contact region for PrP^C on NCAM may be explained by the fact that PrP^C presumably interacts with NCAM via both *cis*- and *trans*-interactions [58]. However, the exact interaction sites for the suggested NCAM-PrP^C *cis*- and *trans*-interactions have not been identified.

The biological function of the interaction between NCAM and the prion protein is unclear. *In vivo* studies in mice that were inoculated with prions for the induction of experimental scrapie demonstrated that both wildtype and NCAM knockout animals were sensitive to the treatment, developing pathological symptoms such as astrogliosis and deposition of prion protein [56]. Thus, interactions between PrP^C and NCAM appear to be involved in biological processes in the nervous system unrelated to the diseases attributed to PrP^{Sc}.

PrP is a GPI-anchored protein and is predominantly localized in lipid rafts. Consequently, interactions between PrP^C and NCAM increase the fraction of NCAM in lipid rafts. This is evident from investigations of brain homogenates from PrP knockout mice that contain more NCAM than wildtype animals, but have ~25% less NCAM-140 and NCAM-180 localized in lipid rafts and growth cones. Likewise, PrP knockout mice express more of the Src-family non-receptor tyrosine kinase Fyn than wildtype animals, but contain ~25% less activated Fyn in lipid rafts. These observations are supported by *in vitro* studies demonstrating that the interactions between NCAM and PrP^C organize a fraction of NCAM molecules in lipid rafts, where they initiate Fyn-dependent induction of neurite outgrowth [58].

NCAM is well documented to mediate neurite outgrowth through several pathways, including signaling through FGFR, Fyn-FAK, or heterotrimeric G-proteins [11, 16]. Furthermore, the different NCAM isoforms can signal through different pathways.

For instance, growth-associated protein-43 (GAP-43) was shown to be involved in neurite outgrowth mediated through NCAM-180 interactions with the actin-binding protein spectrin, but not through NCAM-140 signaling through Fyn [61]. The transmembrane isoforms of NCAM can be palmitoylated [62], and this post-translational modification serves to direct the protein to lipid rafts. In addition, NCAM-140 is directed to lipid rafts as a result of interactions with the receptor protein tyrosine phosphatase-α (RPTPα). Abrogating or preventing palmitoylation or interactions with RPTPα prevents NCAM-140 from being associated with lipid rafts and also inhibits neurite outgrowth mediated through NCAM-140 and Fyn [63, 64]. The NCAM-PrP^C interactions described above suggest that PrP^C, together with the palmitoylations and RPTPα interactions of NCAM, contributes to the promotion of Fyn-mediated signaling by NCAM-140 localized in lipid rafts.

Whereas, PrP^{Sc} is well known to induce neurodegeneration, PrP^C has been shown to stimulate neurite outgrowth and neuronal cell survival and to promote formation and consolidation of memory [59, 65–67]. However, antibody-mediated dimerization of PrP^C is sufficient to induce apoptosis [68]. Furthermore, neuronal apoptosis induced by cellular exposure to recombinant PrP amyloid fibrils is facilitated by binding of the fibrils to PrP^C [69]. Thus, extracellular PrP^C interactions can promote neuronal cell survival and differentiation, as well as apoptosis. However, the extent to which these processes are modulated by NCAM remains to be determined. Likewise, the role of PrP^C in NCAM-mediated processes remains to be clarified.

Interactions between NCAM and TAG-1

TAG-1 (also known as axonin-1 and contactin-2) is an Ig superfamily CAM belonging to the contactin family. TAG-1 is composed of six N-terminal Ig modules followed by four membrane-proximal FnIII modules and exists in both secreted and GPI-anchored forms. The protein is located on neurons and is important for neuronal migration and axon guidance [70, 71].

The first four N-terminal TAG-1 Ig modules form a horse-shoe-type structure that has been shown to engage in homophilic *cis*- and *trans*-interactions resulting in the clustering of TAG-1 molecules in multimeric complexes or zipper-like structures [72–74].

TAG-1 binds to NCAM with an equilibrium dissociation constant (Kd) around 1 nM [75] and also forms heterophilic interactions with other Ig-CAMs, including another member of the contactin family, F3, and two members from the L1 family, L1 and NrCAM [76–79]. In addition, TAG-1 binds to β1-containing integrins [80] and to a range of ECM molecules, including phosphacan, neurocan, laminin, and certain types of collagen (Fig. 2) [75].

TAG-1 binds to L1 and NrCAM through *cis*-interactions, and heterophilic *cis*-dimerization of TAG-1 and NrCAM has been suggested to facilitate subsequent

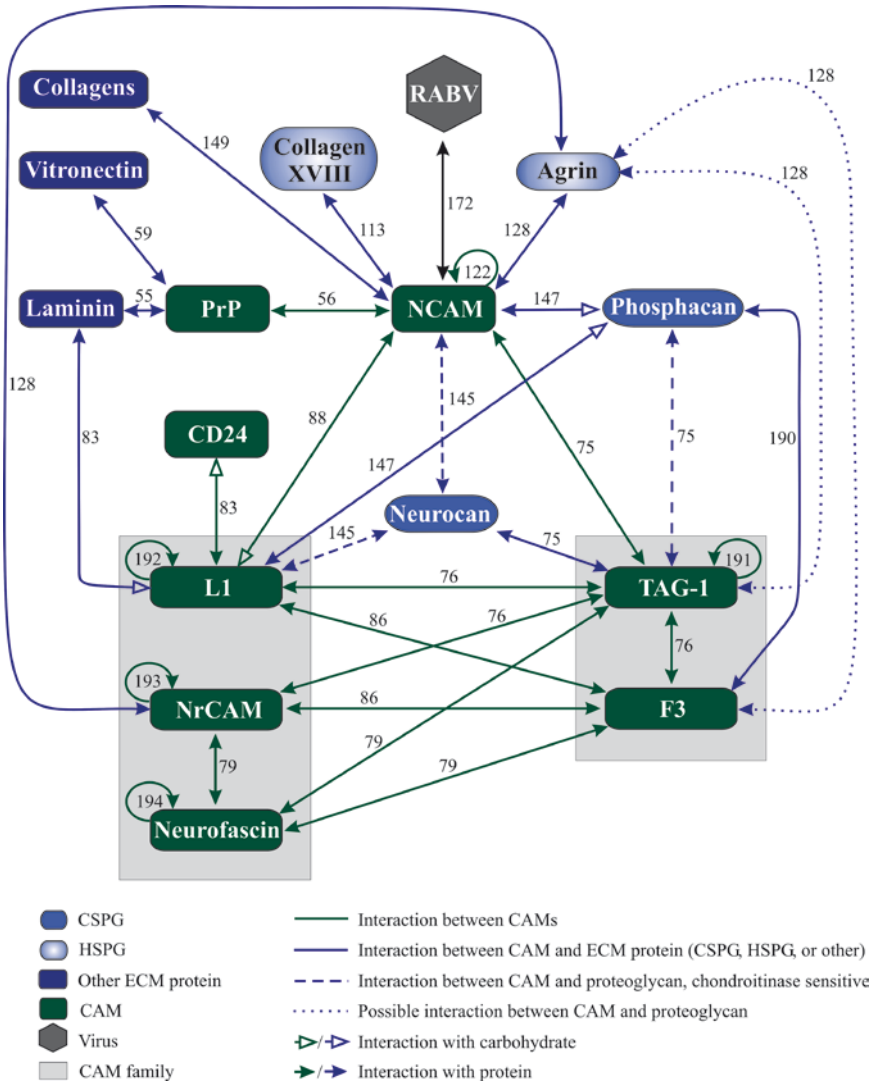

Fig. 2 Interactions between CAMs and ECM molecules. The figure provides a schematic overview of the interactions between the CAMs and ECM molecules described in the text. Interactions occurring via amino acids are indicated by solid arrows. Interactions occurring via carbohydrates are indicated by open arrows. Light gray shading indicates CAMs belonging to the same family. Numbers indicate references describing the individual interactions. See text for further details and references

trans-interactions between the dimers [77, 81]. It is unknown whether NCAM and TAG-1 interact in *cis* and/or *trans*, which NCAM and TAG-1 modules engage in the interaction and the biological function of the interaction.

Interactions between NCAM and L1

L1 is one of four CAMs belonging to the L1 family of the Ig superfamily of CAMs (the remaining three members are NrCAM, neurofascin, and CHL1). The protein is extracellularly composed of six Ig modules followed by five membrane-proximal FnIII modules. Similar to the N-terminal of TAG-1, the four N-terminal L1 Ig modules are able to adopt a horseshoe-like structure [82], but the protein also may exist in an outstretched form (for review, see [83]). L1 forms *cis*-interactions with NCAM, the contactins TAG-1 and F3, and FGFR and can interact in *trans* with the CAM CD24 (nectadrin, heat stable antigen) [77, 84–87].

The interaction between NCAM and L1 is mediated by a lectin-like sequence in the NCAM Ig4 module that binds to carbohydrates expressed by L1 [88]. The interaction has been reported to induce L1 phosphorylation [89], and the exposure of neuronal cell cultures to a peptide corresponding to the NCAM L1 binding sequence abrogates the NCAM-L1 interaction and inhibits neurite outgrowth in cultures of cerebellar neurons [88]. The NCAM-L1 interaction has synergistic effects on L1-mediated cell aggregation and adhesion, a phenomenon referred to as "assisted homophilic L1–L1 *trans*-binding" [90, 91], and has been suggested to require NCAM dimerization [92].

CD24 is a 27 amino acid-long, GPI-anchored, heavily glycosylated protein that is classified as a CAM because of its interaction with P-selectin. CD24 is upregulated in a wide range of tumors, including tumors in the central nervous system (CNS) (for review, see [93]). The interaction between CD24 and L1 is mediated by carbohydrates expressed by CD24, and *trans*-interactions between CD24 and L1 can induce L1-mediated neurite outgrowth [94]. L1 can bind up to five CD24 molecules and although no direct interaction between NCAM and CD24 has been reported, the *trans*-interactions between L1 and CD24 appear to be able to co-exist with L1–NCAM *cis*-interactions, resulting in NCAM–L1–CD24 complexes [85, 95, 96]. L1–CD24 complexes have been reported to be excluded from lipid rafts [94], suggesting that NCAM molecules engaged in NCAM–L1–CD24 complexes also are located outside lipid rafts. CD24 and NCAM co-localize in areas with differentiating postmitotic neurons [97], but whether the presence of CD24 can modulate NCAM signaling has not been investigated.

Because both NCAM and L1 bind TAG-1, complexes of NCAM, L1, and TAG-1 may exist. Alternatively, NCAM–L1 interactions may reduce interactions between NCAM and TAG-1, or between L1 and TAG-1. However, the interplay between the respective interactions between NCAM, L1, and TAG-1, and their biological functions, remains to be investigated.

As mentioned above, PrP is able to recruit NCAM-140 and NCAM-180 to lipid rafts. However, the presence or absence or PrP does not affect the L1 fraction in lipid rafts [58], suggesting that PrP–NCAM interactions are unable to modulate the L1 membrane distribution. However, whether PrP–NCAM interactions reduce the degree of NCAM–L1 interactions is not known.

A schematic overview of some of the extracellular protein interactions observed for the aforementioned CAMs, including interactions with other ECM components, is shown in Fig. 2.

Interactions between NCAM and Extracellular Matrix Proteins

In addition to mediating adhesion between adjacent cells, NCAM also mediates interactions between cells and the ECM. One element of the ECM is an interlocking network of fibrous proteins and sulfated carbohydrate polymers termed as glycosaminoglycans (GAGs). GAGs are usually attached to core proteins, thereby forming carbohydrate–protein complexes termed as proteoglycans (PGs). Depending on their monosaccharide composition, GAGs are divided into heparan sulfates and chondroitin sulfates with the corresponding proteoglycans termed heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs), respectively [98]. Proteoglycans are expressed in almost all tissues, including the developing brain, where they exhibit a highly regulated spatiotemporal expression pattern (for review, see [98]). Proteoglycans interact with a broad array of molecules such as CAMs, growth factors, and other ECM proteins. The following section describes how NCAM interacts with proteoglycans and how these interactions affect NCAM-mediated functions.

In addition to proteoglycans, some of the major components of the ECM include collagen, laminin, and fibronectin. NCAM has been reported to interact with some of these proteins, but the interactions have not been well characterized and therefore will be described only briefly.

Structural Basis for Interactions between NCAM and Heparan Sulfate Proteoglycans

NCAM binds heparin [99]. However, because heparin is produced by mast cells and released into the vasculature only upon tissue injury [100], it is unlikely to interact with NCAM *in vivo* to any major extent. Heparin is structurally similar to heparan sulfate polymers but more highly sulfated and is with a less complex sulfation pattern [98], and even though HSPGs are considered more physiologically relevant NCAM ligands, binding of heparin to NCAM is generally accepted to mimic interactions between heparan sulfate and NCAM, and thereby interactions between HSPGs and NCAM.

An interaction between NCAM and heparin was first demonstrated in 1985. A 170 kDa CAM, denoted C_1H_3, bound heparin. Furthermore, embryonic chick retinal cells adhered to a C_1H_3 substrate in such a manner that it could be inhibited by heparan sulfate [99]. C_1H_3 was subsequently shown to be identical to NCAM [101], and the heparin binding domain was found to be localized within the NCAM Ig1 and Ig2 modules [102]. Using synthetic peptides, Cole and Akeson [103] characterized the NCAM heparin binding domain (HBD) and showed that it consisted of two basic regions between residues 150 and 166 within the Ig2 module (UniProtKB/Swiss-Prot entry P135969) [103]. Recombinant NCAM proteins mutated within the putative HBD exhibit reduced binding to heparin, and adhesion of chick retinal cells to a substrate composed of these NCAM mutants is substantially reduced,

supporting the importance of the identified HBD for interactions between NCAM and heparin [104]. In agreement with the existence of an HBD within the NCAM Ig2 module, surface plasmon resonance (SPR) studies have demonstrated a direct interaction between this module and heparin [105]. Furthermore, the adhesion of neurons to immobilized Ig2 modules can be reduced by the addition of heparin [106]. Recently, the HBD of NCAM was studied by nuclear magnetic resonance (NMR) spectroscopy. The results from this study further support the importance of the previously identified amino acid residues within the NCAM Ig2 module for NCAM–heparin interactions, but also show that the heparin binding region of NCAM covers a larger area of the Ig2 module and includes more residues than previously suggested [107]. Importantly, the identified HBD in Ig2 overlaps with the homophilic binding site for the NCAM Ig1 module, indicating that heparan sulfates can modulate homophilic NCAM interactions, and vice versa (discussed below). Scatchard plot analysis of the interaction between NCAM and heparin indicates that only one functional binding site for heparin exists within NCAM [108].

The affinity of the interaction between NCAM and heparin is generally reported to be in the intermediate nanomolar range. Thus, NCAM isolated from brains of newborn rats was found to bind heparin with a Kd of ~50 nM [108], whereas another study reported a Kd of ~100 nM [109]. In contrast, the affinity of the interaction between the NCAM Ig2 module and the chemical heparin analog sucrose octasulfate (SOS) was found by NMR titration to be around 3 mM [107]. However, the reported affinity of the interaction between the NCAM Ig1 and Ig2 modules also is substantially lower when determined by NMR titration (Kd ~2.5 mM) [110] compared with determinations performed by SPR analysis (Kd ~55 μM) [105].

Interestingly, NMR studies indicate that the affinity of the NCAM–heparin interaction is of the same magnitude as the affinity of homophilic NCAM interactions [107, 110]. Consistent with this notion, the affinity for homophilic interactions between NCAM molecules isolated from adult rat brain was shown by a solid-phase binding assay to be ~70 nM [111], which is very similar to the ~50 nM found for the NCAM–heparin interaction by a similar assay [108]. Noteworthy, the affinity of homophilic NCAM interactions is ~30 times lower for NCAM isolated from brains of newborn rats (Kd ~2 μM) [111]when compared with the NCAM isolated from brains of adult rats (Kd ~70 nM) [108]. This is probably attributable to the glycosylation of NCAM with PSA, which is much more prominent in newborn rats than in adult rats and is known to reduce the strength of homophilic NCAM interactions [35, 36]. In contrast, the affinity of NCAM–heparin interactions is not affected by the presence of PSA [109]. Thus, these studies indicate that when NCAM is highly polysialylated (e.g., during the early stages of nervous system development), the affinity of NCAM–heparin interactions is stronger than the affinity of homophilic NCAM interactions. In contrast, when NCAM is not glycosylated with PSA (the predominant form of NCAM in the adult nervous system), NCAM seems to have a largely similar affinity for heparin and other non-PSA NCAM molecules.

Biological Significance of Interactions between NCAM and Heparan Sulfate Proteoglycans

As described above, the structural basis for the interactions between NCAM and HSPGs has been characterized in detail. However, the biological significance of such interactions is less clear. Several studies have shown that HSPGs are functional heterophilic ligands for NCAM and interactions between NCAM and HSPGs facilitate the attachment of cells to substrates *in vitro*. The interaction can occur both between cell surface-localized HSPGs and culture surface-bound NCAM molecules [99, 101, 103, 104] and vice versa [112–115].

Interactions between cells and substrates are prerequisites for several events occurring during the development of the nervous system such as cell migration and neurite outgrowth. Consequently, the main function of NCAM–HSPG interactions could be to facilitate such processes. Consistent with this hypothesis, heparin can abrogate NCAM-mediated inhibition of cell migration [116], and HSPGs also appear to be involved in NCAM synaptogenic activity [117]. Interestingly, a dendrimeric peptide largely corresponding to the NCAM HBD identified by Cole and Akeson [103] induces neurite outgrowth from cerebellar neurons independently of whether the peptide serves as a substrate or is in solution [107], suggesting that HSPGs not only serve as a substrate for NCAM, but also may modulate NCAM-mediated intracellular signaling.

Modulation of Homophilic NCAM Interactions by Heparan Sulfate Proteoglycans

In addition to acting as heterophilic ligands for NCAM, HSPGs also possibly modulate homophilic NCAM interactions. However, the published data on this subject are somewhat conflicting. Moran and Bock [111] found that homophilic NCAM interactions are not affected by heparin and structural data obtained by X-ray crystallography indicated that the HBD identified by Cole and Akeson [103] is spatially separated from the homophilic binding site in the NCAM Ig2 module [118]. These observations suggest, therefore, that NCAM–HSPG interactions and homophilic NCAM interactions can co-exist. However, as mentioned above, more recent NMR studies indicate that the HBD in NCAM covers a larger area on NCAM than the originally proposed and partially overlaps the binding site for homophilic NCAM interactions [107]. Likewise, SPR data show that heparin inhibits homophilic interactions mediated between the NCAM Ig1 and Ig2 modules [107] and heparin has been found to partially inhibit cell–cell adhesion mediated by homophilic NCAM interactions [119].

Trans-homophilic NCAM interactions occurring in co-cultures of NCAM-expressing neurons growing on top of a layer of NCAM-expressing cells induce neurite outgrowth [120]. In such cell cultures, the addition of heparin inhibits neurite outgrowth [107], suggesting that interactions between NCAM and HSPGs might modulate homophilic NCAM interactions. Interestingly, neurite outgrowth induced

by *trans*-homophilic NCAM interactions also has been found to be inhibited by the removal of heparan sulfate chains by heparinase treatment, indicating that HSPGs are necessary for the induction of NCAM-mediated neurite outgrowth. The latter observation prompted the hypothesis that NCAM–HSPG interactions can modulate the strength of homophilic NCAM interactions and also have the potential to induce neurite outgrowth via membrane-bound HSPGs expressed on neurons [107].

Neurite outgrowth induced by homophilic NCAM interactions does not occur unless cellular NCAM expression reaches a certain level [121]. This NCAM expression threshold may be explained by the fact that neurite outgrowth induced by homophilic NCAM interactions appears to require the clustering of NCAM molecules, which subsequently increases the local concentrations of the heterophilic signal-transducing NCAM binding partners (e.g., FGFR) (for review, see [15]). Interactions between NCAM molecules located on opposing cell membranes seem to be essential for this clustering [122]. However, if the clustering of NCAM molecules becomes too pronounced, the heterophilic binding partners, in principle, might be excluded from the complexes, and neurite outgrowth will not be induced. NCAM polysialylation is speculated to inhibit the tight clustering of NCAM molecules, possibly explaining how PSA can promote neurite outgrowth induced by homophilic NCAM interactions [15].

Interactions between NCAM and HSPGs may similarly modulate NCAM clustering by reducing homophilic NCAM interactions. This could occur if HSPGs and NCAM compete for the same binding site, as suggested by the overlap of the HBD and the homophilic NCAM binding site described above [107]. Alternatively, the binding of HSPG may sterically hinder homophilic NCAM interactions. Regardless of the mechanism, interactions between NCAM and HSPGs may prevent tight clustering of NCAM molecules and thereby allow neurite outgrowth induced by homophilic NCAM interactions.

Interestingly, NCAM polysialylation appears to modulate the strength of NCAM–HSPG interactions. Thus, removal of PSA from NCAM-expressing cells reduces adhesion of the cells to an HSPG substrate [115]. It was undetermined whether PSA bound directly to HSPGs; however, the authors did suggest a model where the removal of PSA promotes a tight clustering of NCAM molecules, thereby leading to an inhibition of interactions between NCAM and HSPGs. Thus, both PSA and HSPGs appear to serve as modulators of homophilic NCAM interactions, thereby fine-tuning the outcome of such interactions. Additionally, NCAM–HSPG interactions likely promote cell–substrate adhesion and neurite outgrowth independently of homophilic NCAM interactions.

The Heparan Sulfate Proteoglycans Agrin and Collagen XVIII are NCAM Ligands

HSPGs comprise a highly diverse group of molecules [98]. Understanding the biological role of the interactions between NCAM and HSPGs requires identifying the specific HSPGs with which NCAM interacts. The inhibition of NCAM–HSPG-mediated cell–substrate adhesion and neurite outgrowth by the addition of soluble heparin or by heparinase treatment indicates that NCAM interacts with the heparan sulfate chains of HSPGs.

Thus far, two specific HSPGs, agrin and collagen XVIII, have been identified to be HSPG ligands for NCAM, but additional NCAM-binding HSPGs possibly exist [113, 114].

The HSPG agrin is known primarily as a key regulator of synaptogenesis at neuromuscular junctions (for reviews, see [123, 124]). However, agrin also is expressed in the brain [112], primarily during development, where it has been suggested to play a role in axonal growth, establishment of the blood–brain barrier, and possibly synaptogenesis (for review, see [123]).

Agrin is produced by neurons and glial cells [112, 113, 125, 126] and exists in both membrane-bound and secreted forms [112, 126]. Agrin is a large multimodular protein with three heparan sulfate chains [126]. It interacts with a number of molecules that, in addition to NCAM, includes (1) the CAMs α-dystroglycan [127], NrCAM [128], and αv-containing integrins [129], (2) the receptor protein tyrosine phosphatase RPTPσ [130], (3) the ECM molecules laminin-1, laminin-2 (merosin), laminin-4 [131], tenascin, and thrombospondin, and (4) the growth factors FGF-2 [125], heparin-binding growth-associated molecule (HB-GAM) (pleitrophin) [132] and neuregulins [133]. Finally, agrin also has been shown to bind to β-amyloid and to accelerate the formation of β-amyloid fibrils [134], suggesting that it might play a role in the development or progression of Alzheimer's disease.

NCAM and agrin can be co-purified from extracts of chick embryonic brain [125, 135], and NCAM-expressing cells adhere better to an agrin substrate than cells not expressing NCAM [113], suggesting that the two proteins bind directly or indirectly. Soluble agrin reduces the adhesion of cells from chicken brain to a substrate consisting of peptides corresponding to NCAM HBD [112], and SPR experiments have shown that a peptide corresponding to NCAM HBD binds directly to agrin [125], further supporting that agrin is a ligand for NCAM.

The heparan sulfate chains of agrin are necessary for the adhesion of NCAM-expressing cells to an agrin substrate, suggesting that NCAM interacts with the heparan sulfate chains of agrin [113]. However, the core protein of agrin also is necessary for NCAM-agrin interactions [113], possibly because the core protein serves to position the heparan sulfate chains appropriately for interactions with NCAM. Interestingly, the affinity of the interaction between agrin and the NCAM HBD peptide has been determined to be ~650 nM [125], which is somewhat lower than that found for the NCAM–heparin interaction [108, 109]. One explanation for these differences in affinities may be that the heparan sulfate chains of agrin are less sulfated than heparin, and therefore less negatively charged.

Another HSPG known to interact with NCAM is collagen XVIII, the antigen for the monoclonal antibody 6C4 [113]. Collagen XVIII is a large protein consisting of alternating collagenous and non-collagenous repeats (for review, see [136]). It is a constituent of most basement membranes [137], and proteolytic cleavage of its C-terminal end yields a product, denoted endostatin, that is primarily known for its anti-angiogenic activity (for reviews, see [136, 138]). Collagen XVIII interacts with several molecules, including the ECM proteins laminin-1, perlecan [139], nidogen-1 and -2 [140], glypican-1 and -4 [141], fibulin-1 and -2 [139], the CAM α5β1 integrin [142], and RPTPσ [130].

NCAM-collagen XVIII interactions are sensitive to heparinase treatment [113, 114], indicating that NCAM interacts with the heparan sulfate chains of the molecule.

These heparan sulfate chains are located N-terminally with respect to endostatin [143], suggesting that NCAM does not interact with the endostatin part of collagen XVIII.

The physiological role of the N-terminal part of collagen XVIII is not well understood, but the heparan sulfate chains appear to be important for anchoring the protein to other constituents of basement membranes [143].

NCAM-expressing cells adhere to crude fractions of proteoglycans devoid of agrin and collagen XVIII in a heparinase-sensitive manner, suggesting that NCAM, in addition to its interactions with agrin and collagen XVIII, interacts with at least one additional HSPG [113, 114].

Structural Basis for NCAM Interactions with Chondroitin Sulfate Proteoglycans

NCAM also interacts with the other major class of proteoglycans, the CSPGs [114, 144–148]. Results from NMR studies show that the chondroitin sulfate binding site of NCAM coincides with the extended heparin binding site in the Ig2 module [107]. Correspondingly, chondroitin sulfate competitively inhibits the binding of NCAM to heparin in solid-phase binding assays [108, 149]. However, chondroitin sulfate inhibits binding to heparin less efficiently than free heparin, indicating that chondroitin sulfate may have a lower affinity for NCAM than heparin. Conversely, results from other studies indicate that chondroitin sulfate does not interact with NCAM. Thus, binding of cells to a substrate consisting of NCAM or the NCAM HBD peptide was inhibited by free heparin or heparan sulfate, but not by chondroitin sulfate [99, 103, 150]. This apparent inconsistency may be explained partly by the use of different chondroitin sulfates in different studies. For instance, one study [150] used chondroitin sulfate A, whereas another study [107] used chondroitin sulfate C.

Similar to the extended heparin binding site identified by Kulahin et al. [107], the NCAM chondroitin binding site overlaps with the homophilic binding site for the NCAM Ig1 module, and results from NMR studies show that chondroitin sulfate interferes with NCAM Ig1–Ig2 interactions [107]. In accordance with these observations, the addition of soluble CSPGs inhibits the aggregation of NCAM-coated beads [144].

The Chondroitin Sulfate Proteoglycans Neurocan and Phosphacan are Heterophilic NCAM Ligands

Two CSPGs, neurocan and phosphacan have been shown to interact with NCAM. Neurocan belongs to the lectican family of CSPGs and is expressed exclusively in neural tissue. It is secreted by neurons and is one of the most abundant CSPGs in the developing brain (for reviews, see [98, 151]). Neurocan consists of globular N- and

C-terminal domains connected by a central flexible filament to which two to three chondroitin sulfate chains are attached [152]. Neurocan interacts with many other molecules, that, in addition to NCAM, include (1) the CAMs L1 [144, 145] and TAG-1 [75], (2) the ECM molecules tenascin-C [153], tenascin-R [154], hyaluronan [152], and heparin [155], (3) the growth factors FGF-2, HB-GAM, and amphoterin [154], and (4) the cell-surface glycosyltransferase GalNacPTase [156].

Neurocan binds directly to NCAM with an affinity in the nanomolar range. One study determined a Kd of ~1 nM [145] and another, ~25 nM [148]. The binding of neurocan to NCAM is reduced by the presence of free chondroitin sulfate and by chondroitinase treatment, indicating that NCAM interacts with the chondroitin sulfate chains of neurocan [145, 148]. However, neurocan-induced inhibition of aggregation of NCAM-coated beads is only minimally sensitive to chondroitinase treatment [144], and SPR studies have shown that NCAM binds to chondroitinase-treated neurocan [148], supporting the hypothesis that the core protein also interacts with NCAM. Nevertheless, intact neurocan binds NCAM more efficiently than the core protein alone [148]. The N- and C-terminals, as well as the central part of neurocan, bind to NCAM, indicating that neurocan contains more than one binding site for NCAM [148].

The CSPG phosphacan is a secreted splice variant of RPTPζ/β. RPTPζ/β exists in two isoforms, long and short, and phosphacan corresponds to the entire extracellular domain of the long phosphatase isoform. Phosphacan and both phosphatase isoforms consist of an N-terminal carbonic anhydrase (CAH) domain and an FnIII module followed by a flexible spacer region. The short RPTPζ/β isoform differs from the long isoform by the exclusion of 860 amino acids within the spacer region. The attachment sites for chondroitin sulfate chains are primarily found within this sequence; thus, phosphacan and the long RPTPζ/β isoform are CSPGs, whereas the short isoform has been termed a part-time proteoglycan.

The expression of phosphacan is confined to neural tissue, where it is produced by astrocytes (for reviews, see [98, 151]). Phosphacan interacts with a number of molecules, including (1) the CAMs NCAM, L1 [144, 146], TAG-1, and NrCAM [75], (2) the ECM molecules tenascin-C [153] and tenascin-R [154], and (3) the growth factors FGF-2 [157], HB-GAM [158], and amphoterin [154]. Additionally, a recombinant protein containing the CAH and FnIII domains of RPTPζ/β binds to the CAM contactin 1 [159], and RPTPζ/β has been shown to bind to midkine, a growth factor related to HB-GAM [160], suggesting that phosphacan also interacts with these two molecules.

NCAM and phosphacan interact directly with each other with an affinity in the low nanomolar range [146], and the interaction inhibits the aggregation of NCAM-coated beads [144]. The interaction is only minimally affected by chondroitinase treatment, indicating that NCAM primarily interacts with parts of phosphacan other than the chondroitin chains [146]. Specifically, *N*-linked complex-type oligosaccharides attached at two phosphacan positions have been shown to mediate the binding to NCAM [147], prompting the hypothesis that the lectin-homology domain in the fourth NCAM Ig module [88] may be the binding site for phosphacan [147].

The aforementioned studies indicate that the molecular elements responsible for NCAM–neurocan and NCAM–phosphacan interactions are different. Whereas the former interaction relies both on the chondroitin sulfate chains and the core protein [144, 145, 148], the latter interaction appears to be independent of the chondroitin sulfate chains of the CSPG [146, 147]. Moreover, *N*-linked glycosylations of the core protein are involved in NCAM–phosphacan interactions, but not in NCAM–neurocan interactions [147]. As described above, a chondroitin sulfate binding site has been identified in the NCAM Ig2 module [107], but the aforementioned findings indicate that this site alone cannot account for NCAM–CSPG interactions and that additional binding sites for neurocan and phosphacan exist within NCAM.

Biological Significance of NCAM Interactions with Chondroitin Sulfate Proteoglycans

The biological significance of the interactions between NCAM and CSPGs is not well understood. Generally, CSPGs have been considered to form barriers to neuronal migration and neurite extension. However, CSPGs cannot be classified exclusively as inhibitory for migration and neurite extension because many studies have demonstrated that they are permissive for these processes. The effects of CSPGs on cell migration and neurite outgrowth appear to rely on the context within which these molecules are expressed (for reviews, see [98, 151, 161, 162]).

As mentioned above, both neurocan and phosphacan have been found to inhibit the aggregation of NCAM-coated beads [144–146], indicating that the interaction of these molecules with NCAM interferes with homophilic NCAM interactions and thereby potentially with NCAM-dependent cell adhesion and neurite outgrowth. However, chondroitinase treatment inhibits neurite outgrowth induced by homophilic NCAM interactions [107], demonstrating that the presence of chondroitin sulfate augments the ability of homophilic NCAM interactions to promote neurite outgrowth. The reason for this could be that CSPG–NCAM interactions prevent NCAM molecules from clustering too densely, as described above for HSPGs. NCAM–CSPG interactions, in principle, also can modulate other heterophilic NCAM interactions. The lectin-homology domain that is suggested to be a binding site for the *N*-linked oligosaccharides of phosphacan, for example, is involved in interactions between L1 and NCAM [88], suggesting that the binding of phosphacan to NCAM can competitively inhibit NCAM–L1 interactions. This could lead to a reduction of L1-mediated neurite outgrowth because interactions between L1 and NCAM were shown to be important for this process [90, 91], and phosphacan does indeed inhibit neurite outgrowth in neurons grown on an L1 substrate [146]. However, phosphacan also interacts with L1 [146, 147], making interpretations difficult. Another of the heterophilic ligands for NCAM, TAG-1 (see above) also binds to both phosphacan and neurocan [75]. These four molecules very likely influence the interactions between one another (see Fig. 2). Biological responses based on interactions between these molecules, therefore,

will reflect the local balance between them, providing a mechanism for fine-tuning processes such as cell adhesion, migration, and neurite extension. Because phosphacan is identical to the extracellular part of the long isoform of RPTPζ/β, a reasonable expectation is that these two molecules have a number of biologically relevant interaction partners in common, such as tenascin, which is known to interact with RPTPζ/β [163]. In addition, NCAM likely interacts with RPTPζ/β, suggesting that this phosphatase could be involved in NCAM-induced signaling. Alternatively, NCAM and other phosphacan binding molecules may modulate RPTPζ/β function by sequestering RPTPζ/β ligands in the ECM or by sterically blocking ligands from binding to RPTPζ/β.

NCAM Interactions with Collagen

Collagens are fibrous structural proteins that constitute a major part of the ECM. NCAM interacts with several types of collagens. One of these, collagen XVIII, was described above because of the importance of the heparan sulfate chains for its interaction with NCAM.

A possible interaction between NCAM and collagen was originally suggested after co-localization of NCAM and interstitial collagen fibers of the endoneurium was observed [164, 165]. Later, NCAM was shown to bind directly to several types of collagen (type I–VI and IX) [149, 166]. The affinities for the respective interactions are in the low nanomolar range (Kd ~2–20 nM). Scatchard plot analysis indicates that collagen types I, II, III, and V each contain a single NCAM binding site [166]. The structural basis for NCAM–collagen interactions is not known, but the interaction can be inhibited by heparin or chondroitin sulfate [149]. One interpretation of this observation is that collagen binds to the heparin binding site in the NCAM Ig2 module. Alternatively, the collagen binding site in NCAM is in close proximity to the heparin binding site, and consequently, the simultaneous binding of heparin/chondroitin sulfate and collagen is not sterically possible. However, demonstrating a direct interaction between collagen and the NCAM Ig1 or Ig2 modules by SPR analysis has not been possible [105].

Collagen binds directly to heparin [149]; thus, an alternative interpretation of the observed NCAM–collagen–heparin interactions is that the binding sites on collagen for NCAM and heparin overlap or are located close to one another, thereby preventing collagen from interacting with NCAM and heparin simultaneously.

The biological role of NCAM–collagen interactions has not been investigated in detail, but the interactions provide a mechanism by which cells attach to their surroundings and provide a basis by which cells affect the structure of the ECM and vice versa. Notably, NCAM is able to modify collagen fibrillogenesis (i.e., the diameter of collagen type I fibers is increased in the presence of NCAM) [166]. However, the physiological importance of the effect of NCAM on collagen fiber diameter has not been investigated. An increased diameter of collagen fibers possibly provides the tissue with greater resistance to mechanical stress, which is

important in muscles, for example, where NCAM is found at sites believed to be important for intramuscular force transmission [167].

NCAM and Laminin

Laminin is a major component of basement membranes and is known to promote cell migration and neurite outgrowth (for review, see [168]). NCAM does express, as previously mentioned, the carbohydrate epitope HNK-1 [37, 38], and this epitope has been found to be important for adhesion of neural cells to laminin, suggesting the possibility that NCAM interacts directly with laminin, but no direct binding between NCAM and laminin has been demonstrated [149]. However, laminin interacts directly with heparin [109] and HSPGs, including agrin [125]. Thus, NCAM and laminin are likely to constitute parts of the same extracellular networks.

Extracellular Interactions between NCAM and Viruses

Common to each of the heterophilic NCAM interactions described above is their ability to mediate and modulate interactions between cells and their surroundings. These interactions appear to have evolved to allow the fine-tuning of cellular adhesion and related processes. However, NCAM also is exploited for interactions unrelated to adhesion and is one of three identified receptors for RABV (the other two receptors are the nicotinic acetylcholine receptor and the neurotrophin receptor p75; for review, see [169]).

In infected individuals, RABV is predominantly present in neural tissues and saliva, and consequently, the virus typically is transmitted through saliva into bite wounds or mucous membranes. After infection, the virus enters the peripheral nervous system, through which it is transported to the CNS by retrograde axonal transport (see [169, 170]). If untreated, the result of RABV infection is encephalitis and, ultimately, death, and the virus is estimated annually to cause 55,000 human deaths worldwide, predominantly in Africa and Asia [171].

RABV is a *Lyssavirus* belonging to the *Rhabdoviridae* family. The virus is an RNA virus with a negative-stranded RNA genome encapsulated in an envelope consisting of lipids and two proteins, G and M, of which only G is exposed on the surface. RABV infection occurs when the G protein of the virion binds to a cell surface-bound receptor and subsequently is endocytosed. Upon endocytosis, the viral membranes fuse with endosomal membranes, thereby releasing the viral ribonucleoprotein (including the viral genetic material and polymerase) into the cytosol of the infected cell where viral replication occurs [170].

A number of *in vitro* studies suggest that NCAM is a receptor for RABV. First, studies with cell lines and neurons from NCAM knockout mice show a direct correlation between the amount of NCAM expressed by cells and the degree of virus

infection following incubation with RABV [172, 173]. Second, incubation with soluble NCAM leads to a dose-dependent decrease in RABV infection. Third, incubation with RABV leads to a rapid decrease in the amount of NCAM at the cell surface, suggesting that NCAM is internalized subsequent to binding RABV. Incubation with heparan sulfate reduces the degree of RABV infection *in vitro* [172], indicating that RABV may bind NCAM near the HBD in the Ig1 or Ig2 module, but no studies regarding the specific interaction sites for RABV on NCAM have been conducted.

The transmembrane NCAM-140 and NCAM-180 isoforms appear to be equally able to mediate RABV infection [172]. The virus also can bind to the GPI-anchored NCAM-120 isoform. However, HEp-2 cells stably expressing NCAM-120 are even less efficient for RABV replication than cells of the same type not expressing NCAM [173].

In vivo studies have shown that NCAM knockout mice live longer after infection with RABV than do wildtype animals. Furthermore, the lack of NCAM expression appears to cause a more restricted degree of RABV infection in the CNS, including a reduced degree of infection in cortical regions [172]. These observations suggest that NCAM is important for RABV infection *in vivo*.

NCAM is not the only CAM of the Ig superfamily that serves as a virus receptor. For instance, adenovirus 2 and 5 and coxsackie virus B3 and B4 bind to the coxsackie virus and adenovirus receptor (CXADR/CAR) [174]. Herpes simplex virus (HSV)-1 and -2, bovine herpes virus (BHV)-2, and pseudorabies virus bind to nectin-1 and/or nectin-2 [175, 176], and polio virus binds to the necl-5/poliovirus receptor (PVR) [177]. The affinities of homophilic *trans*-interactions formed by CAMs are generally weak when compared with certain heterophilic extracellular interactions, such as interactions between growth factors and their receptors. Consequently, a virus may easily “hitchhike” a CAM as a virus receptor because the binding with which it needs to compete is weak. In contrast, a virus would have more difficulty utilizing, for example, a growth factor receptor for entry because the virus would have to compete with a high-affinity ligand. This may in part explain why so many viruses utilize CAMs as receptors for cell entry, a hypothesis described in detail by Wang [178].

Concluding Remarks

NCAM interacts with numerous molecules outside and inside the cell. This review has focused on the heterophilic extracellular interactions between NCAM and other CAMs and between NCAM and molecules of the ECM.

The ability of the described binding partners of NCAM to engage in multiple interactions appears as a recurring theme throughout the review. This suggests that NCAM often appears in complexes consisting of multiple CAMs and ECM constituents. The prevalence and biological significance of such potential complexes are poorly understood and should be addressed in future studies.

As discussed above, the binding of NCAM to a given heterophilic partner likely modulates other NCAM interactions, both homophilic and heterophilic, and comparable reactions can be envisioned for most of the heterophilic binding partners of NCAM. Interestingly, the potential modulation of individual protein interactions through the formation of heterophilic protein complexes creates an opportunity for regulating interactions between two molecules with constant expression patterns by varying the expression of a third interaction partner common to the two molecules.

The structural basis for some of the aforementioned interactions has been determined, whereas it is still unresolved for others. To further our understanding of the complex interplay between CAMs and the ECM, future studies should determine the structural basis for such interactions. This would be an essential first step in deciphering the significance of the regulatory mechanisms posed by the potential complexes delineated above.

The spatiotemporal expression pattern of NCAM and many of its heterophilic interaction partners indicates that these molecules are particularly important for the development of the nervous system. The migration of neural cells and the extension of neurites are both fundamental processes for a proper formation of the nervous system, and the biological significance of the heterophilic NCAM interactions with regard to these processes has been emphasized above. Importantly, the relationship between the adhesion and speed of migrating cells is known to exhibit a bell-shaped response with maximal speed at intermediate adhesion strength [179, 180]. The extension of neurites requires some of the same biomechanical processes as those required for cell migration, including the protrusion of lamellipodia/growth cones followed by anchorage of the protrusions to the ECM and extension and reorganization of cytoskeletal elements. Consequently, neurite outgrowth also has been speculated to demonstrate a bell-shaped response with respect to increasing adhesion strength. If the adhesion is too strong or too weak, neurite extension will not occur. Consequently, a given molecule may either promote or inhibit cell migration or neurite outgrowth depending on the context. The molecule may enhance the processes if it serves to decrease the adhesion strength of a very strong interaction, whereas it may inhibit the processes if it serves to increase the adhesion strength of a very weak interaction [145]. Thus, the specific combinations of CAMs and ECM molecules that determine the adhesion strength of a given cell contribute to the degree of migration/neurite outgrowth. In this context, the potential regulation of interactions of individual molecules by multimolecular complexes formed by NCAM and its heterophilic binding partners is highly interesting because it most likely affects the adhesive properties of the individual molecules, and thus the strength of cell adhesion. Consequently, dissecting how NCAM-mediated adhesion is affected by changes in the molecular context ought to be the focus of future studies. Likewise, conducting investigations of the effects of NCAM on cell adhesion mediated by other CAMs will be important, especially because NCAM, through its potential polysialylation, can inhibit adhesion mediated by other CAMs [36, 181, 182].

The regulation of different cellular processes, such as migration and neurite outgrowth, has generally been believed to be governed by chemical signals elicited

by the binding of growth factors to their receptors and the subsequent activation of signal transduction cascades. However, mechanical forces evidently provide signals affecting cellular actions. Importantly, interactions between cells and the ECM can affect gene transcription and enzyme activity through transmembrane receptors that are mechanically coupled to the cytoskeleton (for recent reviews, see [183–185]). The heterophilic interactions of NCAM with several ECM constituents, and its coupling to the cytoskeleton through spectrin [186], suggest that NCAM could be important for relaying mechano-physical information to the cell. Future studies in this area will be fundamental for understanding the mechanisms by which intracellular signaling is elicited by heterophilic and homophilic NCAM interactions.

Finally, the exploitation of NCAM as a receptor by RABV illustrates that CAMs sometimes participate in biological processes unrelated to their main functions. RABV utilizes the ability of NCAM to be endocytosed, a process that has been the topic of only a few studies [187, 188]. The engagement of NCAM in complexes with heterophilic binding partners appears to affect the ability of RABV to bind to NCAM, as suggested by the reduced infection of NCAM-expressing cells by the addition of heparan sulfate [172]. As mentioned earlier, this effect of heparan sulfate suggests that RABV binds the NCAM Ig1 and/or Ig2 modules. However, extracellular interactions mediated by NCAM *in vivo* may not only affect the accessibility of RABV to NCAM, but also interfere with the endocytosis of the molecule. Endocytosis of NCAM is an important process not only for RABV infection but also for learning and memory consolidation [189]. The process appears to be stimulated by NCAM mono-ubiquitylation [188]. However, it is unclear how the extracellular interactions of NCAM are reduced so that endocytosis may occur. Determining how this switch between adhesion and endocytosis is achieved and regulated will be an interesting topic for future study.

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Intracelluar Ligands of NCAM

Bettina Büttner and Rüdiger Horstkorte

Introduction

Neural cell adhesion molecule (NCAM) (for review see: [1–3]) was first identified and characterized more than 30 years ago [4, 5]. For understanding how NCAM is implicated in its various functions such as neurite outgrowth, cell migration, stabilization of cell–cell contacts, synapse formation, learning and memory, it is necessary to analyze the adjacencies of the molecule outside and inside the cell. The identification of direct binding ligands or indirect associating molecules of NCAM can enlighten the signal transduction pathways of NCAM and give information on putative cross-talks with other signaling pathways. Furthermore, information on its interaction with the cytoskeleton helps to understand the specific functions of the different intracellular domains of NCAM.

Two of the three major isoforms of NCAM are transmembrane glycoproteins with large carboxy-terminal intracellular domains of different length resulting from alternative splicing of one single gene [6–8]. The intracellular domain of rat NCAM 140 has a length of 119 amino acids, whereas the intracellular part of NCAM 180 consists of an additional insert of 271 amino acids, resulting in a total intracellular length of 390 amino acids (Fig. 1). These large domains imply that they are implicated in the various functions of NCAM and can be the point of contacts for intracellular interactions. In particular, the presence of an additional insert in the intracellular domain of NCAM 180 suggests that the two transmembrane isoforms might have different functions.

In the following session, we will chronologically present the known intracellular binding proteins of NCAM.

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Fig. 1 Complete intracellular domain of rat-NCAM (*Black*: NCAM 140, *Red*: NCAM 180-specific insert). The sequence is divided into 4 constructs (*blue*), which were used for immunoprecipitation experiments. All potential phosphorylation sites (analyzed by NetPhos) are *underlined*

Spectrin, the First Identified Intracellular NCAM-Associated Protein

The first intracellular protein, demonstrated to bind to NCAM, was published by Pollerberg et al. [9]. Earlier studies already demonstrated that NCAM 180 appears later during development compared to NCAM 140, when mitosis and neuron migration has been completed and cells are morphologically differentiated [10, 11]. Furthermore, NCAM 180 was shown to have a reduced lateral mobility within the plasma membrane when compared with NCAM 140 [12]. These facts suggested a role of NCAM 180 in stabilizing of cell–cell contacts in differentiated cells by interacting with the cytoskeleton. By analyzing this in more detail by immunofluorescence using neuroblastoma and cerebellar cells, it was demonstrated that the NCAM 180 isoform is predominantly expressed at sites of cell–cell contacts of cell bodies and of growth cones contacting other cells [9, 12]. At these sites, cytoskeleton proteins such as brain spectrin and actin are also accumulated. In further experiments, brain spectrin and ankyrin but no other cytoskeleton proteins could be co-purified with NCAM 180 by immunoaffinity chromatography using adult mouse brain protein fractions. Furthermore, Pollerberg et al. [9] showed in a solid-phase radioligand-binding assaythat brain spectrin specifically binds to NCAM 180 but not to NCAM 140 or NCAM 120. The co-localization of brain spectrin and NCAM 180 and the association between these molecules indicate that spectrin directs the accumulation of NCAM 180 at specific sites of the cell membrane, but it is also possible that NCAM 180 direct the accumulation of spectrin in NCAM-rich areas. However, ankyrin could not be demonstrated to co-localize with NCAM 180. Therefore, it was speculated that only a very minor portion of ankyrin is associated with NCAM 180.

More than 10 years later, more recent studies revealed that not only NCAM 180 can bind spectrin but also NCAM 140, however, with lower efficiency than NCAM 180 [13,14]. In lipid rafts, even NCAM 120 is associated with spectrin [13].

Functional spectrin consists of two subunits building a heterodimer of one α and one β-subunit or a homodimer of two equal subunits [15]. Spectrin could be co-immunoprecipitated with NCAM from brain homogenate [13, 14] and vice versa also NCAM 120, NCAM 140 and NCAM 180 co-immunoprecipitated with spectrin from brain or NCAM-transfected CHO cells. ELISA-based protein ligand-binding assays with immobilized intracellular domains of NCAM 140 or NCAM 180 indicated that both isoforms directly bind the NH2-terminal region of isolated β1-spectrin, however only NCAM 180 interacts with the complete spectrin α1β1 [13]. The observation, that 13% of the total membrane-bound β1-spectrin is monomeric, suggests that also the NCAM-interaction with the isolated β1-subunit might play a role in vivo. The interaction of β1-spectrin with NCAM 120, which lacks an intracellular domain, is only possible within lipid rafts, where GPI-anchored NCAM 120 is mainly localized. NCAM 140- and NCAM 180-spectrin complexes can be found in both in rafts and raft-free membrane areas.

Spectrin's Function in Recruiting PKCβ

Activated PKCβ is known to interact with the pleckstrin homology domain of β1-2 spectrin [16] and plays a role in NCAM-mediated neurite outgrowth [17]. Therefore, spectrin was suggested to function as a molecular bridge between NCAM and PKCβ.

Extracellular activation of NCAM causes a recruitment of β1spectrin to NCAM 140/180 and a redistribution of NCAM–spectrin complexes into lipid rafts. The redistribution of NCAM–spectrin-complexes into lipid rafts correlates with an increased level of PKCβ within lipid rafts. Inhibition of the FGF-receptor, which is also involved in NCAM-mediated promotion of neurite outgrowth, inhibits the recruitment of the PKCβ to lipid rafts and abolishes its association with NCAM 140/180. Furthermore the formation of the NCAM-spectrin–PKCβ complex had also been shown to be important for NCAM-mediated neurite outgrowth, since the disruption of the complex abolishes NCAM-induced neurite outgrowth. These observations indicate an indirect association of NCAM 140 or NCAM 180 with activated PKCβ via spectrin in dependence of FGF receptor activation and are the basis of a novel model, where spectrin has the important role to form a scaffold to which signaling molecules such as the PKCβ become recruited.

Spectrin's Function in Recruiting NMDA Receptor and CaMKIIα

Recently, it was demonstrated that the NCAM–spectrin interaction is also involved in the formation of a postsynaptic signaling-complex [18]. After clustering of NCAM, not only spectrin but also the NMDA-receptor, CamKIIα, PSD95 or

α-actinin were accumulated in postsynaptic densities. Spectrin binds to the intracellular domain of the NMDA-receptor [19] and it could be demonstrated that the NMDA-receptor as well the CamKIIα and spectrin is co-immunoprecipitated together with NCAM using brain homogenate [18]. It was already speculated that the presence of NCAM is necessary in NMDA-receptor-dependent LTPs since in NCAM-deficient mice, LTPs were impaired [20, 21]. Also, the disruption of the spectrin-NCAM-scaffold in NCAM deficient mice prevents redistribution of the CaMKIIa and impairs NMDA-dependent LTP formation.

NCAM Interacts with the Tyrosine-Kinase Fyn

Besides binding and activating the FGF-receptor, NCAM 140, but not NCAM 180 activates the MAP-kinase pathway through Fyn and FAK. A small fraction of membrane-bound NCAM 140 (nearly 3%), but not NCAM 180, was shown to be constitutively associated with the lipid raft-associated nonreceptor tyrosine kinase Fyn [22]. Homophilic NCAM-binding results in the recruitment of the focal adhesion kinase FAK to the NCAM 140-fyn-complex, leading to activation of the MAP kinases ERK1/2 and phosphorylation of the transcription factor CREB [23]. The activation through Fyn and FAK and the resulting induction of neurite outgrowth are dependent on the localization of NCAM 140 within lipid rafts, whereas the activation of the FGF-receptor is mediated by NCAM molecules localized outside of lipid raft compartments.

A direct binding of Fyn to NCAM could not been proved yet. However, the activation of Fyn depends on its dephosphorylation by the receptor protein tyrosine phosphatase α (RPTPα) [24, 25]. RPTPα is present in high concentration in brain and recently a direct interaction of the RPTPα with NCAM 140 and with lower affinity to NCAM 180 was demonstrated [26]. In agreement with these data, in RPTPα-deficient mice, NCAM-Fyn interaction and NCAM-mediated neurite outgrowth is abolished [26].

NCAM Binds Not Only Spectrin, But Also Several Other Major Cytoskeletal Proteins

The observation that the over-expression of the intracellular domain of NCAM 180 in dominant-negative experiments leads to increased neurite outgrowth was explained by destabilization of NCAM-cytoskeleton interactions and indicated that especially NCAM 180 is associated with more cytoskeleton proteins and not only with spectrin [27]. Therefore, nearly 20 years after the identification of spectrin, we used ligand affinity chromatography of cytoskeleton-enriched fractions of rat brain and tried to identify novel binding proteins of NCAM 140 or NCAM 180.

We expressed the intracellular domains of rat NCAM 140 (NCAM cyt 140), NCAM 180 (NCAM cyt 180) and a control protein, purified them by immunoaffinity chromatography and immobilized them on CNBr-activated-Sepharose columns. Eluted proteins of the NCAM cyt 180-column were cut out of the gel, digested with trypsin and analyzed by peptide mass fingerprinting using MALDI-TOF MS. The following proteins could be identified: α- and β-tubulin, microtubule-associated protein MAP 1A, α-actinin 1, β-actin and surprisingly the rhoA-binding kinase α (ROKα), which might be associated to one of the cytoskeletal proteins [28]. All the eluates of the affinity chromatographies (empty columns, NCAM cyt 140- or NCAM cyt 180-columns and control protein-columns) were further analyzed by Western blot. We could identify all of the proteins identified by peptide mass fingerprinting in the eluates of the NCAM cyt 180 columns and reproduce the data obtained by MALDI-TOF MS. In addition, the eluates of the NCAM cyt 140 columns were also analyzed. Only α- and β-tubulin and α-actinin 1 were present in the eluate of the NCAM cyt 140 column, whereas microtubule-associated protein MAP 1A, β-actin and ROKα could not be detected [28]. None of these proteins was detected in eluates of the respective empty- or control columns [28]. Furthermore, we tested the presence of spectrin and tropomyosin by Western blot analysis. We could detect spectrin in the eluates of the NCAM cyt 140 and the NCAM cyt 180 columns, whereas tropomyosin was exclusively present in the eluate of the NCAM cyt 180-column [14]. In addition, spectrin could be co-immunoprecipitated together with NCAM from rat brain [14].

As mentioned before, spectrin is supposed to link NCAM to actin-filaments, which are composed of β-actin units. α-Actinin is an actin-binding protein, which mediates the bundling of actin-filaments, whereas tropomyosin is a small protein, which is responsible for the stabilization of the actin filaments. From this the question might arise, why β-actin and tropomyosin are present only in the NCAM 180-column eluate and not in the NCAM 140 column eluate, whereas the actin-binding proteins spectrin and α-actinin are present in both eluates. The following explanations are possible: First and most likely, NCAM 180, but not NCAM 140, interacts directly with β-actin and tropomyosin as well as via spectrin. Second, NCAM 140 interacts only with monomers of spectrin and α-actinin that are not associated with actin-filaments. Alternatively, NCAM 140-associated spectrin and α-actinin are in conformations that are not able to bind to actin-filaments. This explanation agrees with the observation by Leshchyns'ka et al. [13] that NCAM 140 only interacts with the spectrin β1-subunit and not with the complete spectrin, what might explain, that NCAM 140 does not associate with actin (filaments).

In a following study, it could be shown that α- and β-tubulin interact with the membrane-proximal domain of NCAM 140 and NCAM 180, whereas the ROKα interacts with the C-terminal part (amino acid residues 958–1,090) of the NCAM-180-specific insert [29]. The latter observation is in agreement with the identification of ROKα in the eluate of the NCAM 180 column and not in the eluate of the NCAM 140 column. ROKα was additionally demonstrated to co-immunoprecipitate with NCAM from mouse brain solubilisate, which supports the association also under more in vivo conditions [14]. The interaction with α- and β-tubulin reveals

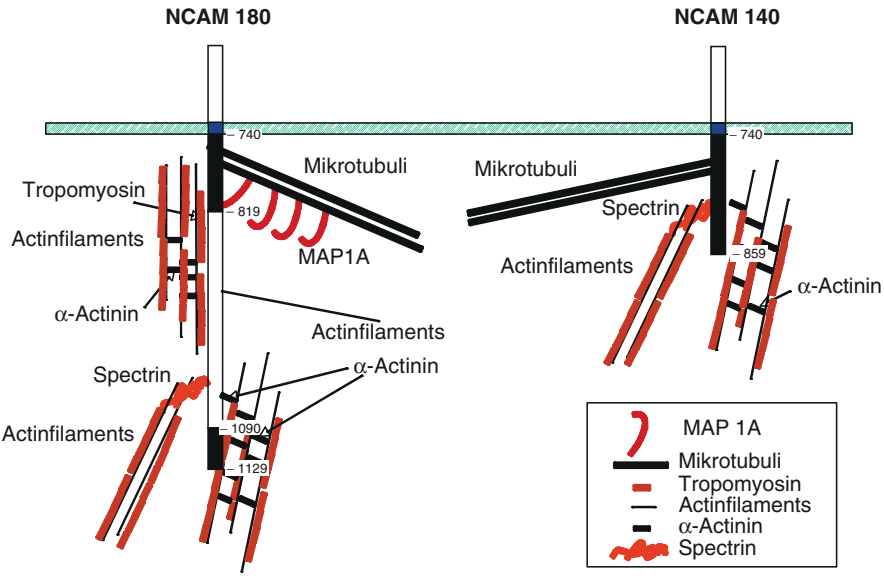

Fig. 2 Interaction of NCAM with cytoskeleton proteins. Note that only spectrin was shown to bind directly to NCAM

for the first time that NCAM is not only linked with the actin cytoskeleton but also to the microtubule-system. Figure 2 summarizes known interactions between NCAM and cytoskeletal proteins.

Interaction with Signaling Molecules

Using again the ligand affinity approach – now focusing more on cytosolic and not cytoskeleton proteins – we recently identified further novel proteins binding to the intracellular domains of NCAM [29]. This time, cytosolic-enriched protein fractions of rat brain were passed over the NCAM cyt 140 or NCAM cyt 180 columns. Proteins eluted from the NCAM cyt 180 column were again cut out of the gel, subjected to in-gel digestion with trypsin and analyzed by MALDI-TOF-MS. We were able to identify LANP (leucine-rich acidic nuclear protein), TOAD-64 (turned on after division-64), and syndapin in the NCAM cyt 180 column eluates by peptide mass finger printing and by Western blot analysis. Whereas LANP and syndapin could also be demonstrated to associate with NCAM 140, TOAD-64 interacts specifically with the NCAM 180 isoform [29].

Furthermore, we tested the presence of three other proteins in the eluates: phospholipase Cγ (PLCγ), which is implicated in NCAM-mediated signal transduction, and the serine/threonine phosphatases PP1 and PP2A since LANP is known to be a regulator of these phosphatases [30, 31]. We could detect all three proteins in the

eluate of the NCAM cyt 140 as well as of the NCAM cyt 180 column by Western blot analysis.

The interaction of PLCγ, LANP, TOAD-64, syndapin, PP1 or PP2A to NCAM was analyzed in more detail. Five fragments of the intracellular domain of NCAM, which comprise different parts of the complete intracellular domain of rat NCAM 140 and NCAM 180 were expressed and purified. All five NCAM fragments, representing together the complete sequence of both NCAM cyt 140 and 180, were further used to perform pull-down assays with cytosolic-enriched protein fractions of rat brain. PLCγ, TOAD-64, syndapin, PP1 and PP2A associate with NCAM cyt fragment 1, which represents the membrane close domain of 80 amino acids of both NCAM cyt 140 and NCAM cyt 180 (encoded by exon 16 and 17). LANP interacts with the N-terminal NCAM cyt fragment 5 (39 amino acids long), which represents the C-terminal part of NCAM 180 and NCAM 140 (encoded by exon 19) [29].

LANP has been described as a heat-stable inhibitor of the protein phosphatase PP2A [31] and positively regulates the activation of PP1 [30]. The interaction of PP1 and PP2A with the intracellular domains of NCAM indicates a possible dephosphorylation of serine and threonine residues of NCAM 140 and NCAM 180 by these phosphatases. The intracellular domain of NCAM 140 contains 5 putative serine and 6 putative threonine phosphorylation sites while NCAM 180 contains 28 putative serine and 21 putative threonine phosphorylation sites. First hints that NCAM could be dephosphorylated by protein phosphates came from experiments, where NCAM (phosphorylated by GSK-3) was dephosphorylated after incubation with PP2A [32]. We localized the binding of PP1 and PP2A to the first N-terminal 80 amino acids of the cytosolic domain of NCAM 140 and NCAM 180. This fragment contains two putative serine and three putative threonine phosphorylation sites, which could be dephosphorylated by PP1 or PP2A by regulating the phosphorylation of NCAM.

NCAM 180 might also be phosphorylated by the serine/threonine kinase ROK α, since the ROKα binding region within the NCAM 180-specific insert (NCAM cyt fragment 3) contains nine predicted serine and six predicted threonine phosphorylation sites.

Turned on after division-64 (TOAD-64) belongs to the TUC (TOAD/Ulip/CRMP)-family of proteins. TOAD-64 is highly up-regulated during early postnatal neuronal development [33] and is implicated in signaling events in growth cones of neurites. It is a cytoplasmic protein and can be associated with plasma membranes [34]. Surprisingly, TOAD-64 only associates with NCAM 180 although NCAM 140 is the active isoform for the stimulation of neurite outgrowth. The mapping studies suggested its association with a sequence of 80 amino acids close to membrane. However, this sequence is present in both NCAM-isoforms. An explanation could be that this membrane-proximal domain has another conformation in the NCAM 180- than in the NCAM 140-isoform due to the presence of the following additional insert of 267 amino acids, which might allow specifically an association with TOAD-64.

Syndapin, a protein highly enriched in brain [35], was shown to interact with the N-terminal 80 amino acids of the intracellular domain of NCAM 140 and NCAM 180. Qualman et al. showed that syndapin I associates with the GTPase dynamin I

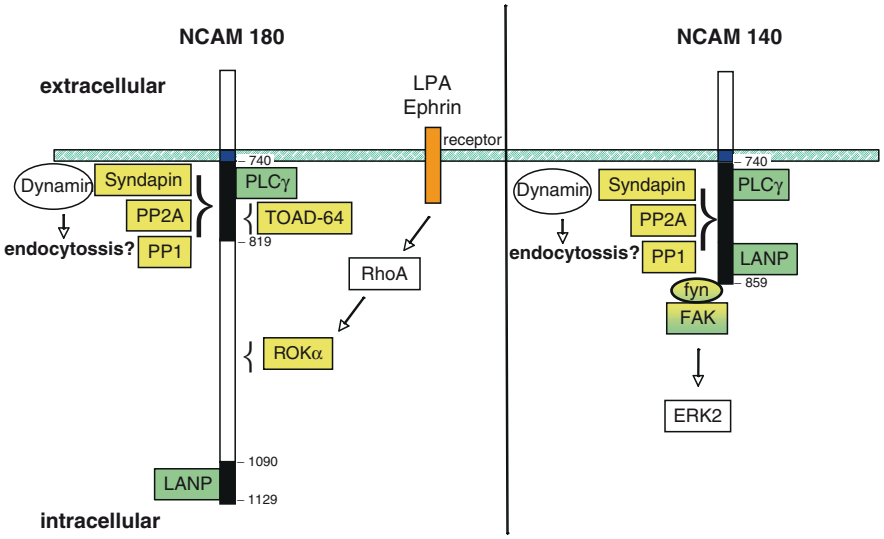

Fig. 3 Interaction of NCAM with signaling molecules. The intracellular domains of NCAM 140 and NCAM 180 are shown. Proteins, which bind direct to NCAM are indicated by *green* boxes. Proteins, which are not known to bind directly or indirectly to NCAM are indicated by *yellow* boxes. Numbers refer to the amino acids number of the NCAM sequence

and plays a role in clathrin-mediated endocytosis of synaptic vesicles [36]. Minana et al. (2001) demonstrated that internalized NCAM colocalizes with clathrin and α-adaptin of the adaptor complex AP-2, as well as with transferrin, which is used as a marker of early endosomes [37]. Very recently, it was demonstrated that NCAM is endocytosed via the clathrin-pathway [38]. The interaction of NCAM with syndapin indicates that syndapin is involved in the clathrin-mediated endocytosis of NCAM. Figure 3 summarizes the interactions between NCAM and signaling molecules described above.

Direct Binding of LANP and PLCγ to NCAM

LANP interacts directly with the intracellular domains of both NCAM 140 and NCAM 180. This was demonstrated by a GST-LANP pull-down assay with the purified recombinant intracellular domains of NCAM [29]. LANP, a member of the leucine rich protein family, also known as pp32 (phosphoprotein with a molecular weight of 32 kDa) was first identified in cerebellum from rat [39–41]. The N-terminal domain of the protein contains many leucine-rich repeats, which are supposed to mediate protein–protein interactions. LANP is upregulated in the central nervous system during the early stage of postnatal development. In undifferentiated neuronal cells, LANP is predominantly present in the nucleus, but during neuritogenesis

it translocates into cytoplasm [42]. There, it interacts with the light chain of free microtubule-associated protein 1B (MAP 1B), thereby inhibiting the binding rate of MAP 1B to microtubules, probably leading to improved flexibility of the neuronal cytoskeleton and facilitation of neurite outgrowth [42]. Since we identified MAP 1A as a binding partner of NCAM 180 and LANP has been demonstrated to interact not only with MAP 1B [42] but also with other members of the microtubule associated protein family such as *tau*, MAP 2 or MAP4 [43,44], it is possible that LANP might be a linker protein between NCAM 180 and MAP 1A.

We also investigated whether the interaction of the PLCγ with NCAM is directed or mediated by intermediate proteins and which domains of the PLCγ are implicated in direct interaction. For this, we performed GST-pull-down assays with GST-PLCγ fragments derived from human PLCγ comprising different structural and functional domains of PLCγ: the PH-domain, the N-terminal SH2-domain, the C-terminal SH2-domain and both SH2-domains in one construct. For the verification of direct interaction with some of the GST-PLCγ fragments, purified recombinant NCAM fusion proteins were used in the pull-down-experiments. As a negative control fusion protein of the intracellular domain of CEACAM, a transmembrane protein of the immunoglobulin superfamily was used. The intracellular domains of NCAM 140 or NCAM 180 directly interact with the PH-domain and the C-terminal SH2 domain of PLCγ. Both isoforms have a stronger affinity to the C-SH2 domain than to the PH-domain. A small amount of NCAM 180 also interacts with the N-terminal SH2 domain [29].

PLCγ could also be shown to co-immunoprecipitate with NCAM from mouse brain homogenates, which verifies also an interaction of PLCγ with NCAM under more in vivo conditions [29].

MyoNAP, a Novel NCAM-Binding Molecule in Avians

Very recently, Kim and Hirayama searched for highly expressed transcripts during myogenesis in quails [45]. One of the transcripts turned out to induce neurite-like processes after transfection in myoblasts. It could be demonstrated that the process formation depends on intact microtubules. A yeast-two-hybrid assay revealed that this protein directly binds the C-terminal domain of avian NCAM. Therefore, the yet unknown protein was named MyoNAP (Myogenesis-related and NCAM-associated protein). The MyoNAP-NCAM interaction could be shown to be necessary for the formation of MyoNAP-induced neurite like protrusions in quail myoblasts.

Conclusion

Taken together, there are up to now five proteins identified to directly bind to the intracellular domain of NCAM: spectrin, RPTPα, LANP, PLCγ and MyoNAP. The binding affinity and/or quantity to NCAM of these proteins partly depend on

the NCAM isoforms. Whether the other intracellular associated NCAM molecules presented here are directly or indirectly associated with NCAM has to be proven in the future.

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NCAM and the FGF-Receptor

Vladislav V. Kiselyov

Structural Biology of NCAM

In mammals, there are two related neural cell adhesion molecules, termed NCAM1 [1, 2] and NCAM2 [3], which are encoded by two different genes. The two molecules belong to the immunoglobulin (Ig) superfamily and have similar domain structure: they consist extracellularly of five Ig-like modules, two fibronectin type III (F3) modules and intracellularly of a cytoplasmic part of varying length. Not much is known about the NCAM2 function, structure and signalling and in the following, only NCAM1 will be considered, which for simplicity is termed NCAM.

NCAM is expressed as three major isoforms, which arise due to alternative splicing of a single gene consisting of at least 26 exons [4–9]. Two of the isoforms are transmembrane proteins, NCAM-A and NCAM-B, with apparent molecular weights of 180 kD and 140 kD, respectively (as determined by SDS-PAGE) while the third isoform, NCAM-C (with an apparent molecular weight of 120 kD), is glycosylphosphatidylinositol (GPI)-anchored. The structures of the first three Ig modules and the two F3 modules have been determined by X-ray crystallography and nuclear magnetic resonance (NMR) analysis [10–16] while the structures of the fourth and fifth Ig modules, as well as of the cytoplasmic part are still unknown. The Ig fold of the first three N-terminal fold is of the intermediate type and the other two Ig modules are also predicted to be of this type [10]. The extracellular part of NCAM is encoded by exons 1–14 (two exons per module) and is similar for the three major isoforms. The two transmembrane isoforms are in addition encoded by exons 16–19 for NCAM-A and exons 16, 17, 19 for NCAM-B. The NCAM-C isoform, which is GPI-anchored, additionally contains only exon 15. The extracellular part of NCAM may also contain additional short sequences such as the 30-basepair exon VASE (Variable Alternatively Spliced Exon), which is inserted in

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the middle of the fourth Ig module between exons 7 and 8, and exons a, b, c, AAG, inserted between the first and second F3 modules of NCAM. NCAM molecules are post-translationally modified by attachment of long chains of polysialic acid (PSA) to the fifth Ig module [17, 18] and the first F3 module [16].

NCAM Functions

NCAM is involved in the regulation of cell adhesion and neurite outgrowth [19–22] and plays a major role during development of the nervous system. NCAM also regulates synaptic plasticity, learning and memory consolidation [23–27]. During embryonic development, NCAM is heavily polysialilated and is widely expressed in the whole organism. However, in the adult it is mainly found in tissues of neural origin; the amount of NCAM polysialylation is markedly decreased [28, 29], whereas expression of exon VASE is increased [30], and the polysialilated form of NCAM (without exon VASE) is only expressed in areas which retain a high degree of plasticity such as the hippocampus and the olfactory bulb [31], indicating that a decrease in polysialylation together with an increase in expression of the VASE exon changes NCAM from a plasticity promoting molecule to a molecule that generates stability [20, 25].

NCAM regulates cell adhesion and neurite outgrowth by means of homophilic binding (NCAM self binding) and subsequent activation of intracellular signaling. The major signaling partner of NCAM is the fibroblast growth factor (FGF) receptor [15, 32]. NCAM is also known to activate non-receptor tyrosine kinases Fyn and FAK [33]. The NCAM mediated signaling results in activation of the mitogen-activated protein kinase (MAPK) pathway [34, 35]. At present, it is unclear whether the MAPK pathway is activated via the FGF-receptor or the Fyn/FAK complex (or maybe both).

NCAM is also involved in heterophilic interactions with other molecules, such as heparin, heparan/chondroitin sulfates [36–40], various types of collagen [40, 41], L1 [42], TAG-1/axonin-1 [43], neurocan [44], phosphacan [45], agrin [46], glial cell line derived neurotrophic factor (GDNF), GDNF family receptor α [47], ATP [15, 48, 49], glucocorticoid receptor [50], cellular prion protein [51], brain-derived neurotrophic factor (BDNF) [52], platelet-derived growth factor (PDGF) [53], rabies virus [54], spectrin [55] and various cytoskeletal components [56].

This review focuses on the structural basis for the FGF-receptor activation by NCAM. For review of the signaling pathways activated by NCAM, see [57–60].

Mechanism of the NCAM Homophilic Binding

The mechanism of the NCAM mediated homophilic binding has been the subject of much controversy. Several alternative models have been proposed, which involve binding between multiple NCAM modules in various configurations (see Fig. 1).

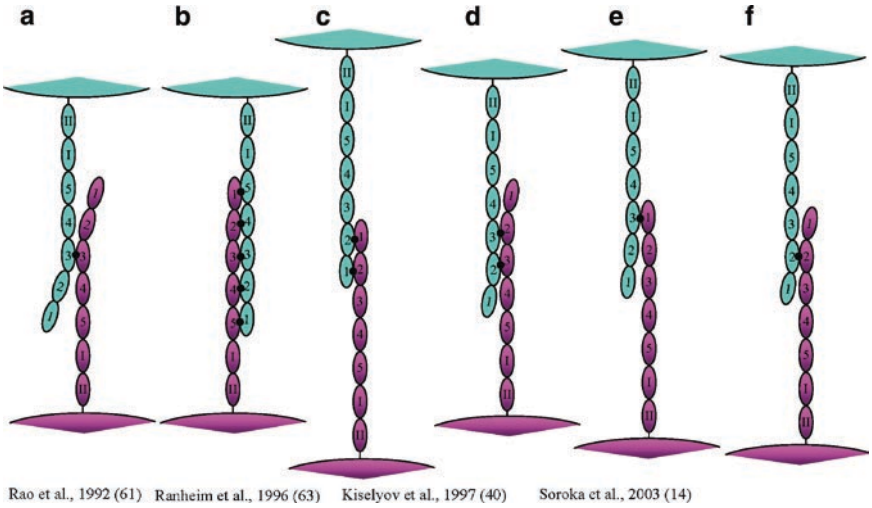

Fig. 1 Models for the NCAM homophilic binding proposed by different researchers. The contact site between the interacting modules is indicated by a *black dot*

In aggregation experiments with various truncated forms of NCAM, it was established that the third Ig module of NCAM was important for the NCAM homophilic binding, and the model in which the third Ig module binds to itself (shown in Fig. 1a) was suggested [61, 62]. In experiments with micro-beads coated with the various modules of NCAM, the binding of the third Ig module to itself was confirmed, and it was also found that the first module bound to the fifth, and the second – to the fourth [63], which allowed to modify the above model (see Fig. 1b). However, neither of these interactions could be detected by means of surface plasmon resonance (SPR) analysis [40]. Furthermore, the self-binding of the third Ig module could not be detected by one of the most sensitive methods currently available – NMR [13]. It should be noted that the third Ig module used in the NMR study [13] was properly folded (as judged by the NMR structure determination of the module) meaning that the absence of binding was not due to the module mis-folding. Using SPR experiments, binding between the first and second Ig modules of NCAM was detected and a model shown in Fig. 1c was suggested [40]. This model was later confirmed in multiple NMR and crystallography experiments [11, 12, 14, 64]. Recently, the crystal structure of the first three Ig modules of NCAM was determined [14]. This structure confirmed interaction between the first and second Ig modules of NCAM (Fig. 1c) and also demonstrated the importance of the third Ig module for the homophilic binding. However, the third Ig module was found to make a contact with the second Ig module in an anti-parallel way (Fig. 1d) and with the first Ig module (Fig. 1e). A contact between two second Ig modules of NCAM was also shown (see Fig. 1f). Combination of the interactions shown in Fig. 1c with those in Fig. 1d leads to a one-dimensional "zipper" formation of NCAM molecules [14]. Another one-dimensional "zipper" can be made if one combines contacts

shown in Fig. 1e, f [14]. These two types of "zippers" are not mutually exclusive and their combination can lead to a two-dimensional "zipper" array of NCAM molecules. For review of these interactions, see [65]. As shown in Fig. 1, the membrane separations spanned by the adhesive complexes are different for the various models. Using surface force measurements, the distance dependence of the attractive and repulsive forces for the membrane-bound NCAM extracellular modules was determined [66]. The NCAM fragment consisting of all five Ig modules of NCAM exhibited adhesion at two different distances: 29 nm and 18 nm. The first distance corresponds to the anti-parallel interaction of the first and second Ig modules (Fig. 1C), whereas the second – to the models in which an overlap of approximately all five Ig modules occurs (see Fig. 1a, b and d). In order to discriminate between the models involving self-binding of the third module (Fig. 1a, b) and the anti-parallel binding of the second and third module (Fig. 1d), the first and the second Ig modules were deleted from the extracellular NCAM domain, and this construct appeared to adhere at the membrane separation corresponding to the full overlap of the Ig modules [66]. This seems to support the models involving self-binding of the third module (Fig. 1a, b). However when deleting the first and second Ig modules from the extracellular NCAM domain, amino acids 28–219 were also deleted [66], and thus the whole A β-strand (amino acids 209–215) and the loop region between the A and A' β-strands (residues 216–218) were deleted, according to the NMR and crystal structures of the module [13, 14]. It was demonstrated by NMR [13] that the third Ig module, when lacking only a part of the A β-strand (residues 209–213), was unfolded. This means that the deletion construct used in the surface force measurements [66] most likely contained an unfolded third Ig module, and thus the self-binding of this construct cannot be used as evidence in support of the models shown in Fig. 1a, b.

Thus, it appears that multiple inter-module interactions are involved in the NCAM homophilic binding. Despite numerous studies, only the anti-parallel binding of the first and second Ig modules can be considered firmly established (Fig. 1c). This interaction has been detected in many experimental set-ups employing SPR, NMR, X-ray diffraction and surface force measurements. There is a lot of evidence showing that all of the other contacts shown in Fig. 1 may also be relevant for the NCAM homophilic binding, but the direct evidence (such as binding between the individual modules with estimation of the interaction's affinity) is still lacking.

Interaction of NCAM with the FGF-Receptor

The FGF-receptor family consists of four closely related receptor tyrosine kinases (FGF-receptor 1–4), which regulate a multitude of cellular process via interactions with FGFs (FGF1–FGF23) [67, 93]. The prototypical FGF-receptor consists of three Ig modules, a transmembrane domain and a cytoplasmic tyrosine kinase domain. The Ig1 and Ig2 modules are separated by a very long linker (20 to 30 amino acids, depending on the isoform), which contains a stretch of acidic residues, termed the

acid box. FGF binding to the FGF-receptor results in the receptor dimerization [68, 69] leading to auto-phosphorylation of the receptor tyrosine kinase domains. Regulation of the ligand binding to the FGF-receptor is primarily achieved by alternative splicing of the receptor, leading to the receptor isoforms lacking the first Ig module [67, 94]. The triple-Ig module FGF-receptor isoforms have lower affinity for FGFs when compared with the double-Ig module receptor [70, 71]. The structure of the first Ig module has recently been determined by NMR [72] and the module's inhibitory effect appears to be due to the intra-molecular binding of the module to the receptor's second Ig module in the area of the second module's binding sites for FGF [73]. For review of the FGF-receptor activation by FGFs, see [74].

The FGF-receptor can also be activated by cell adhesion molecules such as NCAM, L1 and N-cadherin [32, 58, 59, 75]. It is believed that these cell adhesion molecules interact directly with the FGF-receptor. However, a direct binding to the FGF-receptor has only been demonstrated for NCAM [15, 76]. Using SPR, it was shown that a recombinant protein consisting of the combined first and second F3 modules of NCAM bound to a recombinant protein consisting of the combined second and third Ig modules of the FGF-receptor1 [15] and FGF-receptor2 [76] with a Kd value of approximately 10 μM. However, binding of the individual F3 modules of NCAM to the FGF-receptor1 fragment could not be detected by SPR indicating that both F3 modules of NCAM are required for an efficient binding, and a model shown in Fig. 2a was suggested [15]. When using a more sensitive method such as NMR, a weak binding between amino acids located in the FG loop region of the second F3 module of NCAM and the FGF-receptor1 could be shown [15]. A peptide corresponding to the FG loop region was shown to stimulate the FGF-receptor1 phosphorylation [15], induce neurite outgrowth and neuronal survival in primary rat neurons [77], protect hippocampal neurons from ischemic insult [78], promote early postnatal sensorimotor development and enhance social memory

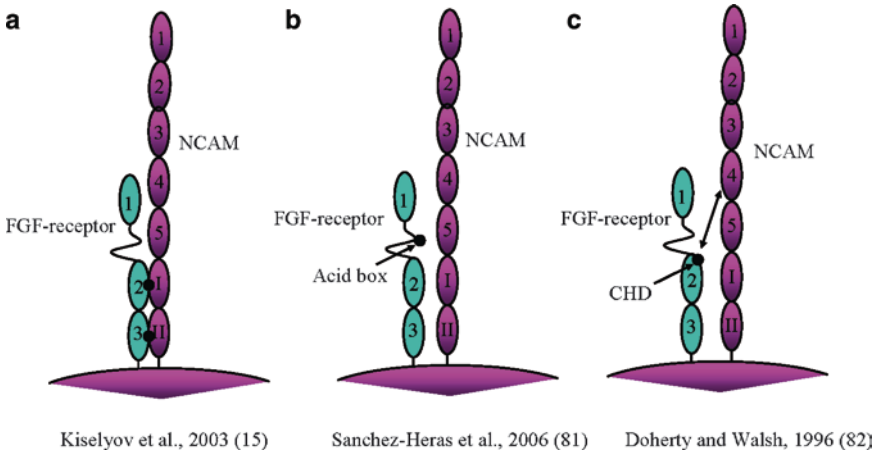

Fig. 2 Models for the interaction between NCAM and the FGF-receptor proposed by different researchers. The contact site between the interacting modules is indicated by a *black dot*

retention [79], and reduce neuropathological signs and cognitive impairment induced by beta-amyloid 25–35 peptide [80], supporting the notion that the FG loop region of the second F3 module of NCAM is important for activation of NCAM signaling. Affinity of the NCAM binding to the FGF-receptor (with a Kd value of 10 μM) may seem rather low in comparison with that of the FGF binding to the FGF-receptor (with a Kd value of 10–100 μM). However, in view of a very high concentration of NCAM in the membrane, approximately 85% of the FGF-receptor molecules are estimated to be involved in a transient binding to NCAM [15]. Recently, it has been shown by immunoprecipitation that the acid box region of the FGF-receptor is necessary for binding to NCAM, N-cadherin and L1 (see Fig. 2b) while the FGF-receptor's Ig modules appear not to be required for the binding [81], which seems to contradict the SPR and NMR experiments showing importance of the second and third Ig modules of the FGF-receptor for binding to NCAM [15, 76]. It should be noted that this immunoprecipitation experiment [81] employed a detergent triton X-100 (at a concentration of 1%) for cell lysis and subsequent washing of the complex between NCAM and the FGF-receptor. Therefore, the reported absence of binding of the second and third Ig modules of the FGF-receptor to NCAM could be due to the denaturation effect of the employed detergent. At present, it is not clear whether or not the acid box of the FGF-receptor is involved in a direct binding to NCAM. Another area of the FGF-receptor previously suggested to be involved in binding to NCAM, L1 and N-cadherin is the CHD (Cell adhesion molecule Homology Domain) region located in the second Ig module of the FGF-receptor [82]. CHD shares sequence homology with functional motifs present in NCAM (exon VASE in the fourth Ig module) and N-cadherin (HAV motif), which formed the basis for the model shown in Fig. 2c [82]. However, recent data [81] indicate that the CHD region is not necessary for the interaction with NCAM.

Mechanism of the FGF-Receptor Activation by NCAM

As mentioned above, approximately 85% of the FGF-receptor molecules are estimated to be bound to NCAM if the mechanism of interaction between NCAM and the FGF-receptor is according to the model shown in Fig. 2a [15]. It should be noted that the FGF-receptor binding to NCAM (according to this model) is not expected to influence significantly the receptor activation by FGF due to the much higher affinity (approximately 1000-fold) of the FGF binding compared to that of NCAM. In order to activate the FGF-receptor, the FGF-receptor binding to NCAM should also lead to the receptor dimerization. Furthermore, the FGF-receptor is believed to be activated by NCAM only when NCAM is involved in the homophilic binding (mediating cell–cell adhesion). If the mechanism of the NCAM homophilic binding is according to any of the models shown in Fig. 1, then it is not clear how the FGF-receptor binding to NCAM could lead to the receptor dimerization. However, combination of the models shown in Fig. 1c,d and in Fig. 1e, f leads to two kinds of one-dimensional "zipper" formations. The two one-dimensional "zippers"

can be combined to form a tightly packed two-dimensional array of NCAM molecules, as seen in the crystal structure of the first three Ig modules of NCAM [14, 65]. The peptides corresponding to the interactions shown in Fig. 1d–f inhibit the NCAM homophilic binding and also inhibit the NCAM-mediated neurite outgrowth [14]. This suggests that NCAM clustering achieved by the formation of one-dimensional and/or two-dimensional "zipper" formations of NCAM molecules may be a possible mechanism for the FGF-receptor activation by the NCAM homophilic binding [65]. NCAM clustering is expected to cluster the FGF-receptor molecules and therefore increase the local concentration of the receptor. It has previously been suggested that the FGF-receptor could also be dimerized through a direct receptor-receptor binding in the absence of FGF [83, 84], explaining the background level of the FGF-receptor activation in the absence of FGF. This notion is supported by the fact that in the crystal structure of the FGF-receptor1 in complex with FGF2 [68], the receptor molecules are found to be involved in a direct interaction. The increase in the local concentration of the FGF-receptor is expected to shift the equilibrium between the monomeric and dimeric receptors toward the receptor dimer, which is supported by the fact that over expression of the FGF-receptor1 results in a considerable increase in the background level of the receptor activation [85]. Thus, the "zipper" model of the NCAM homophilic binding allows explaining the FGF-receptor activation by NCAM. Furthermore, this model also provides a possible explanation for the effect of PSA on NCAM signaling. During development (and in the adult brain in areas retaining high degree of plasticity), NCAM is heavily polysialilated. Removal of PSA from NCAM inhibits the NCAM stimulated neurite outgrowth [86] and increases the adhesive properties of NCAM [87, 88]. Since the hydrated volume of the PSA-moiety is approximately three times larger than the size of the NCAM molecule [89], it is obvious that the tightly packed two-dimensional "zipper" cannot be formed when NCAM is heavily polysialilated because there is very little room between NCAM molecules in this "zipper." However, it is reasonable to speculate that the two types of one-dimensional "zippers" may still be formed when NCAM is polysialilated because in this case there is sufficient room for the flexible PSA chains to the side of the "zippers." Since these two "zippers" contain smaller number of stabilizing contacts per NCAM molecule than the tight two-deminsional "zipper,", the latter is expected to produce stronger adhesion than the other two types of one-dimensional "zippers." On the other hand, NCAM molecules in the tight "zipper" probably cannot interact with the FGF-receptor due to the lack of room between the tightly packed NCAM molecules, whereas it is reasonable to assume that the polysialilated NCAM molecules in one-dimensional "zippers" are accessible for interaction with the receptor because the receptor molecules can now get to the "NCAM-zipper" from the side. One can conclude therefore that expression of non-polysialilated NCAM is expected to result in a stronger homophilic binding, but weaker (if any) activation of the FGF-receptor; while polysialilated NCAM molecules probably have a reduced capacity for the homophilic binding, but can interact and activate the FGF-receptor. Thus, expression of PSA by NCAM may function as a "switch" regulating whether NCAM is involved in adhesion or signaling. Another molecule which may

function as a "switch" in the regulation of the FGF-receptor activation by NCAM is adenosine triphosphate (ATP), which is one of the most abundant neurotransmitters in the nervous system [90, 91]. NCAM has been shown extracellularly to bind and hydrolyze ATP [48, 49]. The role of this ATPase activity is little understood. However, ATP has been shown to inhibit interaction between NCAM and the FGF-receptor1 [15] and to inhibit the NCAM mediated neurite outgrowth [15, 92]. Using NMR, it was demonstrated that ATP binds to the nucleotide binding motif of NCAM (A^{686}ENQQGKS^{693}) located in the FG loop of the second F3 module, which is the same area shown to be involved in binding to the FGF-receptor1; and by SPR, it was shown that ATP could inhibit the binding of NCAM to the FGF-receptor1 with an inhibition constant of 0.3 mM [15]. In the extracellular environment in the brain, ATP is found in sufficiently high concentrations (in the mM range) for inhibition of the NCAM binding to the FGF-receptor only in the vicinity of synapses. One may therefore hypothesize that in the absence of ATP, the FGF-receptor activation by NCAM provides a stimulating environment for growth cone extension. However, when the growth cone reaches its target and a new synaptic contact is formed, release of ATP may switch NCAM from the signaling mode to a mode promoting adhesion.

Conclusion

Despite numerous studies, the mechanisms of both the NCAM homophilic binding and the NCAM binding to the FGF-receptor are still controversial. It appears that the multiple inter-module contacts are involved in both interactions. The affinities corresponding to these contacts are probably rather low, which is probably the reason why some of these interactions can be detected in one experimental set-up (favoring these interactions), but not in another. Recent structural data [14, 15] allow making a speculative model [65] of the FGF-receptor activation by NCAM which, as shown above, is the only one that can explain from the structural point of view how interaction between NCAM and the FGF-receptor could lead to the receptor dimerization; why the interaction between NCAM and the FGF-receptor activates the receptor *only* when NCAM is involved in the homophilic binding; and how PSA could regulate the strength of the NCAM homophilic binding and the signaling properties of NCAM. Further study is required to come to the conclusion whether this model is true or not.

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The Role of ATP in the Regulation of NCAM Function

Martin V. Hübschmann and Galina Skladchikova

Introduction

The neural cell adhesion molecule (NCAM) is a membrane glycoprotein that mediates both cell-cell and cell-matrix adhesion through homophilic and heterophilic interactions. It is widely expressed in the nervous system, both during development and in the adult organism. In addition to mediating cell adhesion, NCAM extracellular binding can initiate signaling processes and thereby affect diverse biological processes such as axonal growth and fasciculation, cell migration, and synaptic plasticity (reviewed in [1, 2]). Early studies showed that activation of the fibroblast growth factor receptor (FGFR) is necessary for the neuritogenic signaling of NCAM. FGFR antibodies, as well as a peptide derived from FGFR can inhibit NCAM-induced neurite outgrowth [3]. It has also been shown that stimulating the PC12 neuronal cell line with soluble NCAM-Fc chimeras leads to an increased FGFR phosphorylation, and cerebellar neurons expressing a dominant negative form of FGFR are unable to respond to NCAM stimulation [4]. More recently, a direct interaction between NCAM and FGFR has been demonstrated and an NCAM-derived peptide corresponding to the FGFR binding site of NCAM also promotes the activation of FGFR [5].

NCAM belongs to the immunoglobulin superfamily of cell adhesion molecules, characterized by the immunoglobulin (Ig) module. Alternative splicing and post-translational modifications lead to the generation of a number of distinct NCAM forms, all arising from a single gene. Three main isoforms, NCAM-120, NCAM-140 and NCAM-180 that have been named after their apparent molecular weight, are produced by alternative splicing. NCAM-140 and NCAM-180 are single span transmembrane proteins while NCAM-120 is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. For all three main isoforms, the extra-

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cellular part of NCAM consists of five N-terminal Ig modules followed by two fibronectin type III (F3) modules. The second (membrane proximal) F3 module contains the sequence AENQQGKS, homologous to the Walker motif A (G/AXXXXGKT/S, where X denotes any amino acid), which is a common nucleotide binding motif [6].

ATP as a Signaling Molecule in the Nervous System

ATP acts as an extracellular signaling molecule both in the central and the peripheral nervous system. It is often released from neural cells as a co-transmitter together with other transmitters such as acetylcholine, noradrenaline, GABA or glutamate [7–10], but can also be released alone [11]. In addition, glial cells have been shown to release ATP by channel-mediated mechanisms [12, 13]. Once released, extracellular ATP is rapidly degraded by a number of ecto-nucleotidases. Ecto-nucleotidases constitute a heterogeneous group of nucleotide-hydrolyzing enzymes that are generally membrane bound, but some ecto-nucleotidases may be cleaved and released extracellularly giving rise to soluble forms [14]. Extracellular ATP is initially converted by ecto-nucleotidases to ADP or AMP, which subsequently is further hydrolyzed to adenosine that is finally cleared from the extracellular space by cellular reuptake. The ecto-nucleotidase activity in rat hippocampus is found to be regulated in different learning paradigms, and has thus been suggested to play an active role in memory formation [15–17].

Extracellular ATP activates purinergic receptors, also called purinoceptors, which are classified as P1 receptors or P2 receptors based on their ligand specificity. P1 receptors are activated by the nucleoside adenosine and P2 receptors by ATP, ADP, UTP, UDP or nucleotide sugars [18]. The P2 receptors are further subdivided in two types, namely P2X receptors that are ligand gated ion channels and P2Y receptors that are G-protein coupled receptors. By activation of P2X receptors, ATP causes postsynaptic influx of Ca^{2+} and Na^{+}, thus acting as a fast excitatory neurotransmitter. In addition to mediating fast neurotransmission, ATP can induce long term structural and functional changes in the nervous system. Neural stem cell proliferation can be stimulated by ATP [19, 20], and ATP enhances neurite outgrowth from PC12 cells, neuroblastoma cells and hippocampal neurons [21–23], but has also been shown to inhibit motor axon outgrowth from rat neural tube explant cultures [24]. In the hippocampus, ATP has further been implicated in both long-term potentiation (LTP) and long-term depression (LTD) [25–27], two principal forms of synaptic plasticity that are considered to be essential in learning and memory.

In addition to activating purinoceptors, extracellular ATP is the substrate of ecto-protein kinases. Inhibiting extracellular protein kinase activity has been shown to block neurite outgrowth from PC12 cells, to inhibit synapse formation between cerebral cortical neurons and to block ATP-induced LTP in guinea-pig hippocampal slices [28–30]. NCAM antibodies inhibit phosphorylation of specific proteins on

the external surface of NG108-15 cells, a neuronal cell line [31]. This implies that the extracellular part of NCAM is a direct target of ecto-protein kinases, but the functional significance of this phosphorylation remains unclear.

NCAM ecto-ATPase Activity

Ecto-nucleotidases are characterized by being activated by high concentrations of either Ca^{2+} or Mg^{2+}. A major fraction of the (Ca^{2+}–Mg^{2+})-dependent ATPase activity of rat brain microsomes can be solubilized with the detergent CHAPS, retaining 85% of the initial enzyme activity. Similarly, the majority of microsomal NCAM is solubilized by this procedure, and immunoisolation of the solubilized NCAM leads to co-isolation of ATPase activity, indicating that NCAM either possesses intrinsic ATPase activity or is tightly associated with an ATPase [32]. Contrary to these findings, the majority of rat brain microsomal Mg^{2+}-ATPase activity does not coelute with NCAM when purified by anion exchange chromatography [33]. Furthermore, an anti-peptide antibody targeting a sequence in the 67 kDa rabbit skeletal muscle transverse tubule ecto-Mg^{2+}-ATPase detects a band of ~67 kDa in immunoblots of solubilized rat brain microsomes, and this band appears in the same anion exchange chromatography fractions at the peak Mg^{2+}-ATPase activity, demonstrating that NCAM is not the major ecto-ATPase in the brain [33].

Further experiments studying the association of a minor fraction of total brain ecto-ATPase activity with NCAM were performed using fibroblasts transfected with either the transmembrane NCAM-140 isoform or the lipid-anchored NCAM-120 isoform. In both cases, immunoisolation of NCAM led to co-isolation of ATPase activity [34]. These findings indicated that the ATPase activity was associated with the extracellular part of NCAM, which was further verified by experiments examining soluble forms of NCAM released from rat brain synaptosomes by either spontaneous proteolytic cleavage or by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). In these experiments, NCAM spontaneously released from synaptosomes by proteolysis co-eluted with the major peak of ATPase activity when subjected to three different chromatographic procedures. Analysis of the ATPase activity released from synaptosomes by PI-PLC showed that almost 90% of this activity could be removed from the supernatant by NCAM immunoadsorption. Covalent modification of ATP-binding sites on synaptosomes with the ATP analog 5′-fluorosulfonylbenzoyl adenosine (FSBA) followed by NCAM immunoisolation inhibited the NCAM immunoisolated ATPase activity, and subsequent immunoblotting showed FSBA-modification of all three major NCAM isoforms [34]. In conclusion, these data indicate that NCAM has an intrinsic ATPase activity, located in the extracellular part of the protein. The functional significance of this NCAM ecto-ATPase activity is not well understood. Obviously, it contributes to lowering the ATP concentration in the extracellular compartment, which is a prerequisite for ATP functioning as an extracellular signaling molecule, but the rate of ATP hydrolysis catalyzed by NCAM is relatively low, suggesting that

ATP removal by NCAM might not be physiologically important. The enzymatic activity might also cause cyclic conformational changes in NCAM that affect other aspects of NCAM function. Both heterophilic and homophilic NCAM interactions could be influenced by such conformational changes, possibly resulting in activity-dependent modifications of NCAM-mediated adhesion and signaling processes.

NCAM–FGFR Interaction and ATP

The FGFR consists of an extracellular region containing three Ig modules, a single transmembrane helix and an intracellular tyrosine kinase domain. A recombinant protein consisting of FGFR Ig modules 2 and 3 has been shown to interact directly with NCAM F3 modules 1 and 2 by surface plasmon resonance (SPR) [5]. Using nuclear magnetic resonance (NMR), the structure of the NCAM F3 module 2 was determined and by ^{15}N-labeling this module, perturbation of specific amino acid residues could be detected when adding FGFR Ig module 3. The perturbed residues were N^{688}, Q^{690}, G^{691} and K^{692}, indicating that these residues either take part in, or are located in the vicinity of the binding site between these two modules [5]. The perturbed residues overlap with the Walker motif A in NCAM, making it plausible that ATP could interfere with the NCAM-FGFR interaction. Indeed, addition of ATP completely abolished the above described binding of NCAM F3 module 1 and 2 to FGFR Ig module, as observed by SPR [5].

In the presence of ATP, perturbation of two residues in the NCAM F3 module 2, Y^{683} and V^{684}, was also detected by NMR, verifying the suggested direct binding of ATP to NCAM [5]. In a binding model proposed by Kiselyov et al. [5], the side chain of Y^{683} interacts hydrophobically with the adenosine moiety of ATPwhile the K^{692} of the Walker motif A together with the adjacent K^{694} form ionic bonds with the triphosphate moiety of ATP (Fig. 1). Further evidence that the NCAM extracellular binding sites for ATP and for FGFR overlap came from SPR experiments using a 15 amino acid peptide derived from the NCAM sequence comprising the Walker motif A. The peptide, E^{681}-A^{695}, corresponds to the loop between the F and G β strands of the NCAM F3 module 2 and is thus termed the FG loop peptide. A dendrimeric form of the FG loop peptide was shown to directly bind immobilized FGFR Ig modules 2 and 3, and addition of ATP inhibited the binding by 70%, showing that ATP interferes with NCAM–FGFR interaction by binding to the same site on NCAM as FGFR [5].

ATP and NCAM Ectodomain Shedding

It has long been known that NCAM exists both as membrane bound and soluble protein. Several NCAM forms have been detected as soluble proteins in brain, cerebrospinal fluid (CSF), plasma, and in conditioned medium of cultured neurons

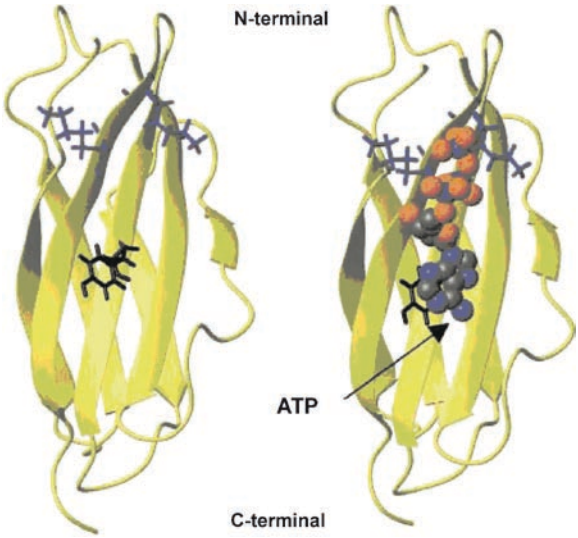

Fig. 1 Ribbon representation of NCAM F3 module 2 illustrating the ATP binding model proposed by Kiselyov et al. [5]. K^{692} and K^{694} are colored blue, Y^{683} is colored black. On the right, the module is shown in complex with ATP

[35–37]. An indication that soluble NCAM plays an important role in development came from a study by Rabinowitz et al. [38]. In this study, all membrane-associated forms of NCAM were replaced with a soluble, secreted form consisting of the extracellular region of NCAM. This mutation resulted in dominant embryonic lethality with abnormalities in neural tube and somite formation apparent from embryonic day 8.5. The same lethal phenotype was observed in embryos derived from embryonic stem cells homozygous for the targeted NCAM mutation, demonstrating that the phenotype is not caused by a homophilic NCAM interaction with endogenous membrane bound NCAM. A number of heterophilic binding partners of NCAM have been identified including the closely related adhesion molecule L1 [39], the glial cell line-derived neurotrophic factor (GDNF) [40], FGFR [5] and extracellular matrix components such as heparin and heparan sulfate proteoglycans [41, 42]. Soluble NCAM interacting with one or more of these binding partners might have a profound impact on essential cell adhesion and/or signaling processes, thus affecting both development and function of the mature nervous system.

Abnormal levels of soluble NCAM have been linked to neuropsychiatric diseases including recurrent unipolar major depression, bipolar disorder and schizophrenia [43, 44]. In schizophrenic patients, increased levels of soluble NCAM have been detected in hippocampus, prefrontal cortex and in CSF [45–48]. A key morphological characteristic of schizophrenia is ventricular enlargement [49] and this characteristic was found to be positively correlated with soluble NCAM levels in CSF [48]. To study the effects of soluble NCAM overproduction in the brain, transgenic mice have been generated that express the soluble extracellular region of

NCAM under control of the neuron-specific enolase promoter [50]. This promoter is inactive until late in neuronal differentiation, thus circumventing the embryonic malformations observed by Rabinowitz et al. [38]. These transgenic mice show a reduced synaptic connectivity of GABAergic interneurons and behavioral deficits similar to those observed in other rodent schizophrenia models [50]. Aberrant GABAergic neurotransmission has been implicated in schizophrenia by both animal models and clinical studies (reviewed in [51, 52]), and it is tempting to speculate that deregulation of NCAM shedding plays a causative role in schizophrenia.

Several mechanisms for generating soluble NCAM have been described, including the secretion of a truncated NCAM protein generated by alternative splicing [53], cleavage of the NCAM-120 GPI anchor [37] and extracellular proteolytic cleavage of membrane bound NCAM by the tissue plasminogen activator-plasmin system [54] or by metalloproteinases [55–57]. Metalloproteinase-mediated NCAM ectodomain release can be induced by ATP, both in primary rat hippocampal neurons and in fibroblastoid L-cells stably transfected with any of the three main NCAM isoforms. ATP-induced NCAM shedding occurs even in L-cells transfected with a mutated NCAM construct in which the three residues (Y^{683}, K^{692} and K^{694} in NCAM F3 module 2) implicated in ATP binding are substituted with alanine [56]. This indicates that the observed shedding does not involve direct ATP-NCAM binding. In contrast to the ATP-induced NCAM shedding observed in cultures of L-cells and hippocampal neurons, spontaneous release of NCAM from rat brain synaptosomes was shown to be inhibited by ATP [34]. However, spontaneous NCAM release from synaptosomes was observed in an EDTA-containing buffer, making it likely that the involved release mechanism is metalloproteinase independent as metalloproteinases are inhibited by EDTA.

NCAM ectodomain shedding has not only been studied in relation to mental and neurological disorders. Cell migration is known to be affected by NCAM expression in several neuronal cell types, and metalloproteinase-dependent shedding of NCAM-140 has been shown to promote cell migration toward extracellular matrix proteins [55]. NCAM-dependent neurite outgrowth can be both promoted and obstructed by NCAM shedding, depending on experimental conditions [56–58]. The significance of NCAM shedding in the regulation of neural plasticity has also been studied in vivo. After induction of LTP in the dentate gyrus of anesthetized rats, a significant increase in soluble NCAM concentration has been detected in hippocampal perfusates [59]. ATP is released after LTP induction in rat hippocampal slices [60] and ATP-induced NCAM shedding could be one of the mechanisms that facilitate the structural remodeling taking place after LTP induction.

ATP and NCAM-Mediated Neurite Outgrowth

The wiring of the nervous system during embryonic development is a highly coordinated process that requires accurate guidance of migrating cells and extending axons. Cell adhesion molecules, including NCAM, have been shown to play an

essential role in these events (reviewed in [61–63]). A key feature of neuronal differentiation is neurite sprouting. Upon homophilic NCAM binding, intracellular signaling is triggered that ultimately leads to neurite extension. This has been shown for a number of neuronal cell types co-cultured with fibroblasts genetically engineered to express NCAM. In this model system, the neuronal cells grown in co-culture with fibroblasts expressing NCAM exhibit significantly enhanced neurite outgrowth in comparison with neuronal cells grown in co-culture with fibroblasts that do not express NCAM. Addition of ATP in the co-culture medium results in a dose-dependent reduction of NCAM-induced neurite outgrowth to the level of control neurite outgrowth (Fig. 2). The inhibitory effect of ATP can be abrogated by adding the ATP-hydrolyzing enzyme apyrase together with ATP. Interestingly, the non-hydrolysable ATP analog AMP-PCP also inhibits NCAM-induced neurite outgrowth with a dose response similar to ATP [23]. These results indicate that ATP itself, not breakdown products hereof, affects NCAM-induced neurite outgrowth, and that ATP hydrolysis is not necessary for the observed inhibition.

As discussed above, NCAM binds directly to and signals through FGFR. The neuritogenic effect of NCAM can be mimicked by adding soluble NCAM F3 module 2, which contains the FGFR binding site, to hippocampal neurons in culture

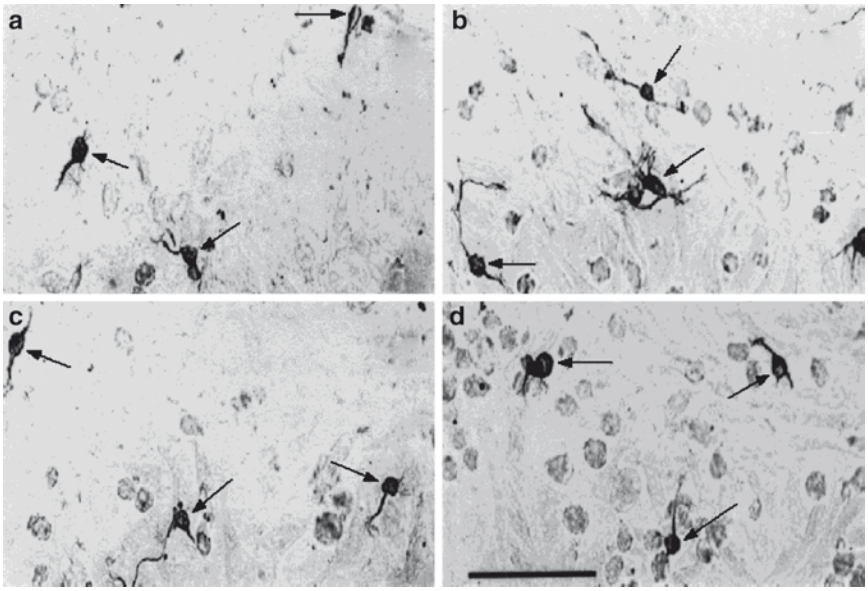

Fig. 2 ATP blocks NCAM-induced neurite outgrowth. Rat hippocampal neurons (indicated by *arrows*) grown on a monolayer of control fibroblasts (**a**, **c**) or fibroblasts expressing NCAM (**b**, **d**), immunostained for GAP-43. NCAM promotes neuritogenesis in the absence (**a**, **b**) but not in the presence (**c**, **d**) of 2.5 mM ATP. Scale bar = 50 μm. From Skladchikova et al. [23]

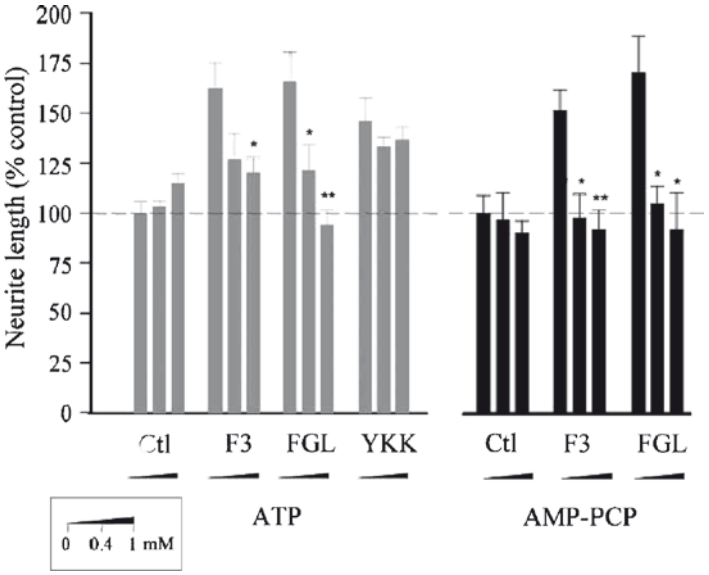

Fig. 3 Effects of ATP and AMP-PCP on NCAM-specific neurite outgrowth. Rat hippocampal neurons were stimulated with either recombinant NCAM F3 module 2 (F3), FG loop peptide (FGL), or FG loop peptide with alanine substitutions at Y^{683}, K^{692} and K^{694} (YKK). After 24 h stimulation in the presence or absence of ATP/AMP-PCP, neurons were fixed and stained in order to quantify neurite outgrowth. ATP dose dependently inhibits the neuritogenic effect of NCAM F3 module 2, and of the FG loop peptide. In contrast, ATP has no inhibitory effect on neurite outgrowth induced by the FG loop peptide having alanine substitutions at the residues involved in ATP binding. *$p<0.05$, ** $p<0.01$. From Kiselyov et al. [5]

(Fig. 3). The neurite outgrowth induced by the F3 module 2 can be inhibited by FGFR antibodies, by ATP, and by AMP-PCP [5]. Similarly, the FG loop peptide also induces neurite outgrowth from hippocampal neurons and this effect can also be inhibited by ATP and AMP-PCP. When the three residues in the FG loop peptide implicated in ATP binding (Y^{683}, K^{692} and K^{694}) are substituted with alanine, the peptide retains neuritogenic potential, but the inhibitory effect of ATP can no longer be detected (Fig. 3). Taken together, these results suggest that ATP inhibits NCAM-induced neuritogenesis by interfering with the direct NCAM-FGFR interaction.

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Part II
NCAM and Polysialic Acid

Polysialylation of NCAM

Herbert Hildebrandt, Martina Mühlenhoff, and Rita Gerardy-Schahn

Introduction

The precise orchestration of cell adhesion and cell communication is indispensable for development, and particularly important for nervous system wiring and plasticity. Among the numerous cell adhesion molecules of the immunoglobulin superfamily dedicated to this task, the neural cell adhesion molecule NCAM stands out as a developmentally regulated switch in its pattern of glycosylation, which fundamentally alters its biophysical properties and, as a consequence, its binding abilities. The glycan responsible for the conversion of different NCAM isoforms from an interactive to an anti-adhesive state is a linear hompolymer of α2,8-linked *N*-acetylneuraminic acids called polysialic acid (polySia,[1] Fig. 1). The large negatively charged and highly hydrated structure can extend beyond the protein core and double the hydrodynamic radius of the extracellular part of NCAM, thereby increasing the intermembrane space and disrupting the adhesive properties of NCAM and other cell adhesion molecules [1–3]. As highlighted by several recent reviews, polySia is a prominent regulator of neural cell migration and differentiation during nervous system development, and tightly associated with neurogenesis and synaptic plasticity in the adult brain [4–8]. Here, we briefly review the patterns of polySia expression during ontogenesis and its potential role in tumor progression before discussing its regulation by the two polysialyltransferases ST8SiaII and ST8SiaIV, as well as the latter's the individual and combined impact of these enzymes on NCAM polysialylation during brain development.

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[1] The most commonly used abbreviation for polysialic acid in neuroscience is PSA, but in tumor biology, PSA stands for prostate specific antigen. To avoid confusion, we therefore prefer to use polySia to abbreviate polysialic acid.

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Developmental Regulation of NCAM Polysialylation

As first described 25 years ago, the hallmark of NCAM polysialylation is its regulation during development [9, 10]. Although essentially confined to the nervous system development and plasticity, polySia is transiently expressed in mesodermal and endodermal derivatives during organogenesis [11, 12]. NCAM does not carry polySia during the time of its first appearance on embryonic day 8–8.5 in the mouse, but shortly thereafter, polysialyated NCAM becomes predominant, reaching its maximum in the perinatal phase [13–15]. As shown by Western blot analyses of whole brain lysates, polySia expression keeps pace with the rapid increase in brain weight until day 9 of postnatal development, and almost all of the NCAM is polysialylated [16]. Subsequently, polySia drops rapidly, by approximately 70% within 1 week, accompanied by the first occurrence of polySia-free NCAM-140 and NCAM-180, the two transmembrane isoforms (see Fig. 1) that are the major

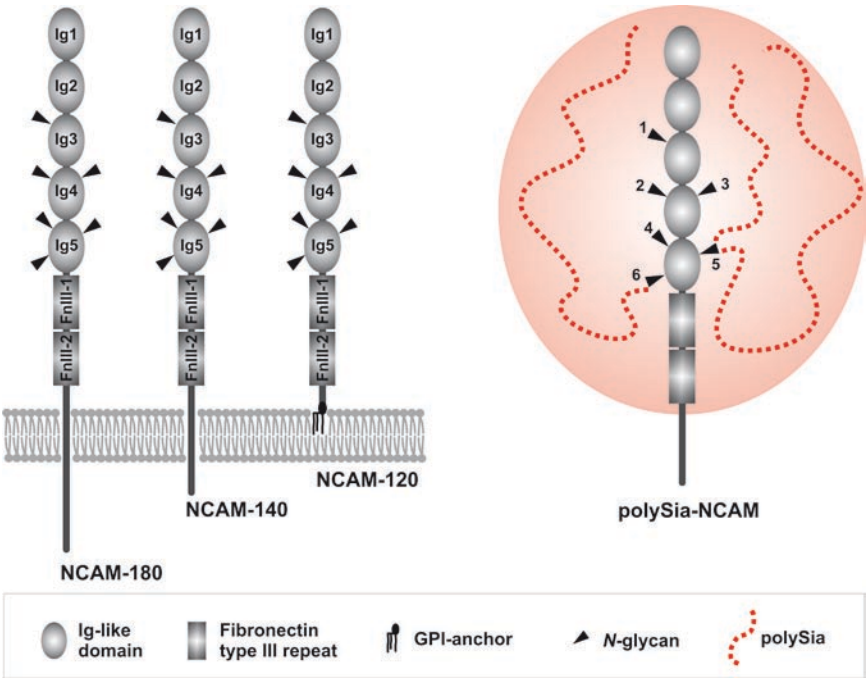

Fig. 1 Scheme of the three major NCAM isoforms (*left*) and the polysialylated form of NCAM (*right*). The extracellular part of NCAM is composed of five immunoglobulin(Ig)-like domains and two fibronectin type III (FnIII) repeats. NCAM-180 and NCAM-140 are transmembrane proteins which differ in the length of their intracellular part, whereas NCAM-120 is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. NCAM is a glycoprotein containing 6 *N*-glycosylation sites. In the polysialylated form of NCAM (polySia-NCAM), the *N*-glycans located at the 5th and 6th *N*-glycosylation site are modified by one or more polySia chains. The hydrodynamic radius of polySia is depicted as a shaded sphere

polySia carriers in mouse brain. By contrast, glycosylphosphatidylinisotol-anchored NCAM-120, the characteristic isoform of mature oligodendrocytes, is devoid of polySia during its massive upregulation in the early postnatal brain, which is associated with the onset of myelination [16]. Thus, the time-course of polySia downregulation and the dramatic increase of polySia-free NCAM coincide with the completion of major morphogenetic events within the first 3 weeks of postnatal brain development. However, as reviewed in great detail elsewhere, the expression of polysialylated NCAM persists into adulthood and is maintained at sites of ongoing neurogenesis or plasticity [5, 17]. In the absence of any known polySia-specific degrading enzyme to modulate polySia on the surface of vertebrate cells, the state of NCAM polysialylation depends predominantly on the biosynthetic pathway. This is underscored by the observation that the largely overlapping expression patterns of the polysialyltransferases ST8SiaII and ST8SiaIV are closely correlated with polySia immunoreactivity (see [18] for review). In addition to transcriptional control, polysialylation of NCAM is likely to be regulated by nontranscriptional mechanisms. As shown in the developing chick, polySia synthesis depends on calcium from intracellular compartments [19] and based on pharmacological and correlative studies, a protein kinase C-dependent regulation of polysialyltransferase activity has been suggested [20, 21]. Moreover, experiments with cultured neurons and insulin secreting β-cells indicate the possibility of a rapid mobilization of polysialylated NCAM to the cell surface by an activity- and calcium-dependent mechanism suggesting regulation of an exocytotic pathway [22]. Similarly, regulated exocytosis may contribute to the activity-dependent modulation of polySia required for hippocampal synaptic plasticity [23].

Re-expression of Polysia in Tumors

Although polySia is diminished in the majority of tissues during development, various tumors are known to re-express polySia [24–26]. Among the polySia-positive tumors are small cell and non-small cell lung carcinomas, multiple myeloma, Schwann cell tumors, pituitary tumors, Wilms' tumor, rhabdomyosarcoma and neuroblastoma [27–36]. A comparative study carried out with isogenic cell lines expressing NCAM with or without polySia identified polySia as a modulator of the malignant potential of small cell lung carcinoma [37]. The occurrence of polySia seems to facilitate the detachment of tumor cells from the primary tumor and presumably promotes the invasion and metastatic potential of these tumors [36–40]. As indicated by results obtained in vitro, polySia supports the undifferentiated state of tumor cells [41, 42]. In patients with neuroblastoma or rhabdomyosarcoma, high polySia serum levels have been correlated with poor prognosis and polySia itself as well as the transcript level of the polysialyltransferase ST8SiaII were suggested as molecular markers to monitor metastatic neuroblastoma [31, 32, 43, 44]. On the other hand, nonpolysialylated NCAM suppresses tumor progression in xenografted tumor cells and correlates inversely with malignancy [45–47]. Thus, polySia

represents an oncodevelopmental antigen, which significantly contributes to tumor growth and metastasis.

PolySia Biosynthesis

Biosynthesis of polySia is catalyzed by two Golgi resident enzymes, the polysialyltransferases ST8SiaII and ST8SiaIV (formerly named STX and PST, respectively, Fig. 2) [48–51]. Both enzymes show 59% identity on the amino acid sequence level and share the typical features of eukaryotic sialyltransferases. They are type II transmembrane glycoproteins with a short *N*-terminal cytoplasmic tail, a transmembrane domain, a stem region, and a large C-terminal catalytic domain that resides in the lumen of the Golgi apparatus. The catalytic domain includes three consensus sequences called sialylmotifs L, S, and VS that are found in all mammalian sialyltransferases and are involved in substrate binding [52, 53]. Although ST8SiaII and ST8SiaIV are typical members of the sialyltransferase family, they are unique with respect to their catalytic ability to synthesize polySia, i.e. α2,8-linked sialic acid

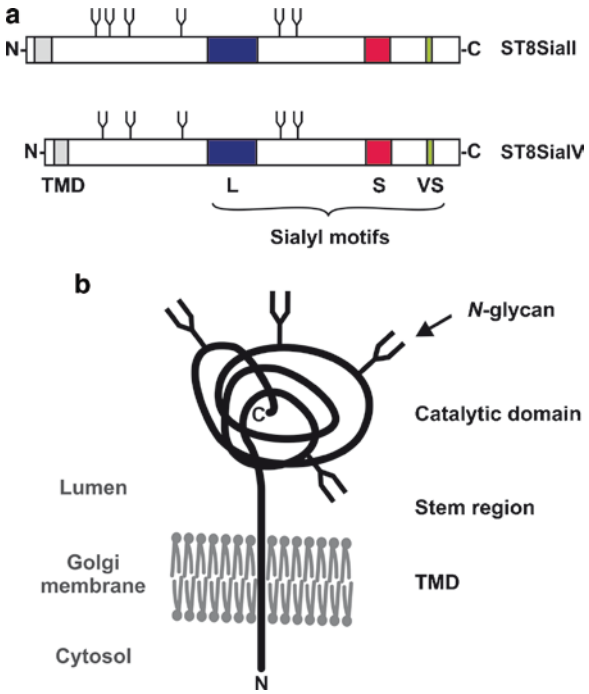

Fig. 2 (**a**) Schematic representation of the polysialyltransferases ST8SiaII and ST8SiaIV showing the transmembrane domain (TMD) and the sialylmotifs large (L), small (S), and very small (VS) of the catalytic domain. The relative positions of the *N*-glycans are indicated by Y-shaped symbols. (**b**) Type II transmembrane topology of polysialyltransferases

polymers which can exceed 50 residues [15, 54–56]. In accordance with this, only sialic acid oligomers with ≤7 residues were observed in ST8SiaII/ST8SiaIV double deficient mice [15]. Since no mammalian sialyltransferase has been crystallized so far, insight in the structural and/or mechanistic differences between mono-, oligo-, and polysialyltransferases is missing.

In contrast to most glycosyltransferases, which modify glycan structures irrespective of the carrier protein, the polysialyltransferases ST8SiaII and ST8SiaIV are highly selective for NCAM, which is by far the predominant polySia acceptor. Besides NCAM, a limited number of other polysialylated proteins have been described including the α-subunit of the voltage-gated sodium channel in rat brain [57], the scavenger receptor CD36 in human milk [58], neuropilin-2 on human dendritic cells [59], and the polysialyltransferases themselves, which can polysialylate their own *N*-glycans in a process termed autopolysialylation [60–62]. However, the complete loss of polySia in the brain of ST8SiaII/ST8SiaIV double deficient mice indicates that in the central nervous system polysialylation of potential alternative acceptor molecules also depends on ST8SiaII and ST8SiaIV activity [63]. Future studies are required to elucidate the enzyme responsible for polysialylation of these molecules. With regard to NCAM, both enzymes ST8SiaII and ST8SiaIV have been shown to catalyze the transfer of multiple α2,8-linked sialic acid residues to terminally α2,3- or α2,6-sialylated galactose residues that are bound in α1,4-linkage to *N*-acetyl glucosamine [64, 65]. Although NCAM carries six *N*-glycosylation sites, the addition of polySia is restricted to *N*-glycans at the 5th and 6th site which are located in the 5th Ig-like domain (Fig. 1) [66–68]. Structural analysis of polysialylated *N*-glycans of NCAM revealed complex structures with a high degree of heterogeneity. PolySia was found on di-, tri-, and tetraantennary glycans that were, in part, additionally modified by fucose, sulfate and uronic acid residues [67–71]. These findings indicate that the pronounced acceptor specificity of polysialyltransferases is not mediated by the recognition of a particular glycan structure, but by the specific interaction with the NCAM protein core. Crystallization of the first FNIII domain of NCAM revealed a unique acidic surface patch and a novel α-helix between β-strand 4 and 5, two structural motifs essential to allow polysialylation of the *N*-glycans in the adjacent 5th Ig-like domain [72]. In accordance with a specific enzyme-acceptor protein interaction, in vitro studies demonstrated that both ST8SiaII and ST8SiaIV polysialylate *N*-glycans attached to NCAM with a much higher efficiency than isolated *N*-glycans released from NCAM [73, 74]. The majority of polysialylated NCAM glycans in perinatal mouse brain was found to carry two polySia chains [56]. However, incomplete diantennary *N*-glycans with only one polySia polymer as well as a small proportion of glycans that appeared to carry three or even four chains were also observed [56]. This highlights that the number of polySia chains per NCAM molecule can vary. At this level, heterogeneity depends not only on the polysialyltransferases but also on the glycosylation machinery, which determines the number of polySia acceptor sites on NCAM, i.e. the number of terminally sialylated antennae provided by the *N*-glycans at site five and six (Fig. 3).

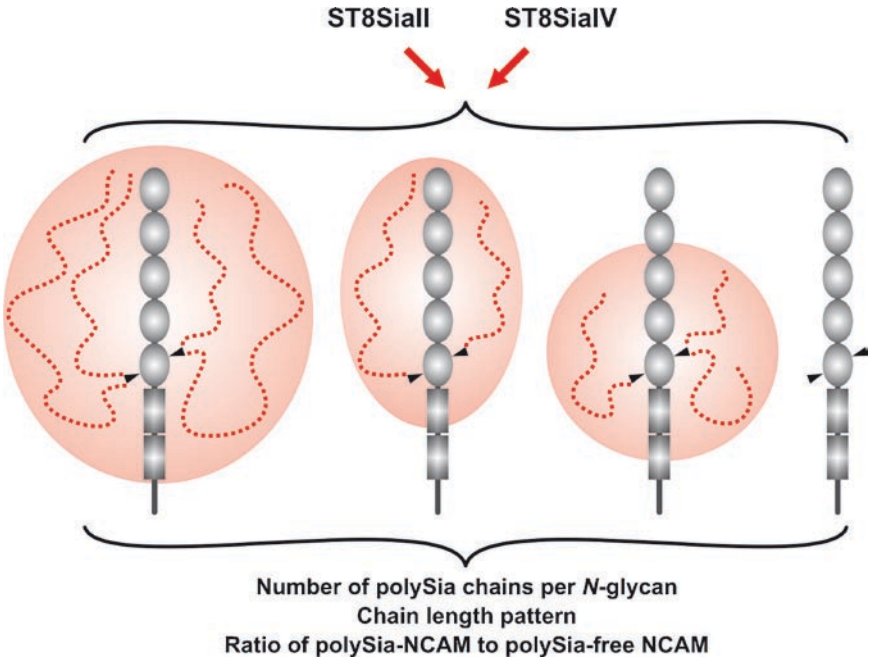

Fig. 3 Potential for variability in NCAM polysialylation. The polysialylation pattern of NCAM is defined by the interplay of ST8SiaII and ST8SiaIV and can vary with respect to the number of polySia chains per NCAM, the length of each individual polySia chain, and the ratio of polysialylated to polySia-free NCAM

Transfection and in vitro experiments unequivocally demonstrated that ST8SiaII and ST8SiaIV are individually able to synthesize polySia on NCAM [48–51, 64, 73], provoking the question why NCAM polysialylation is mediated by two enzymes. In vitro analyzes using soluble polysialyltransferases lacking their transmembrane domain revealed distinct differences between ST8SiaII and ST8SiaIV. Under the in vitro conditions used, ST8SiaII produced shorter polySia chains than ST8SiaIV and appeared to be less efficient in NCAM polysialylation [75, 76]. If both enzymes worked together, a synergistic effect was observed, yielding higher numbers of polySia chains and a higher degree of polymerization [65, 75]. Using *N*-glycosylation site mutants of NCAM, Angata et al. observed that ST8SiaIV strongly preferred the sixth over the fifth *N*-glycosylation site, whereas this preference was only moderate for ST8SiaII [65].

To understand the impact of ST8SiaII and ST8SiaIV in vivo, genetic mouse models lacking either ST8SiaII or ST8SiaIV were generated, which show only partial loss of polySia [77, 78]. The biochemical analysis of the polySia pattern in perinatal brain of polysialyltransferase-deficient mice revealed striking differences in the ability of the two enzymes to polysialyate the complete NCAM pool and highlighted that, in contrast to the in vitro findings, ST8SiaII but not ST8SiaIV is

much more efficient in NCAM polysialylation. Whereas in wild-type and ST8SiaIV-null mice almost all NCAM is kept in the polysialylated state at postnatal day one, 45% of the brain NCAM was found polySia-free in ST8SiaII-deficient mice [15]. The quality of the polysialylated NCAM, however, was remarkably similar in all three genotypes [56]. Independent of the enzyme setting, *N*-glycosyation sites 5 and 6 were almost completely polysialylated and the same set of heterogeneous *N*-glycans served as polySia acceptors, excluding differential glycan acceptor specificities for ST8SiaII and ST8SiaIV. In vivo, ST8SiaII and ST8SiaIV are both able to synthesize polySia chains with up to 90 sialic acid residues [56]. However, at the fifth *N*-glycosylation site, loss of either enzyme resulted in slight alterations of the chain length pattern and the highest amount of long polySia chains was found in the presence of both enzymes. Thus, in line with the in vitro findings a synergistic action in the synthesis of polySia with a high degree of polymerization was observed, although in vivo, this effect is restricted to the fifth *N*-glycosylation site [56]. A comprehensive study of the NCAM polysialylation pattern in perinatal brain of mice with variant allelic combinations of ST8SiaII and ST8SiaIV demonstrated that alterations in the expression of the two polysialyltransferases affect the total amount of polySia, the chain length distribution, the ratio of polysialylated to polySia-free NCAM, and the amount of polySia per NCAM molecule [15]. Thus, the degree of NCAM polysialylation can be precisely adjusted by alterations in the ST8SiaII and ST8SiaIV level (Fig. 3).

Although the data are not consistent in all details, there is a close correlation between polySia immunoreactivity and the combined mRNA expression of polysialyltransferases [14, 33, 79–82]. Despite considerable overlap, there are marked differences in tissue- and time-specific mRNA expression patterns suggesting an independent regulation of ST8SiaII and ST8SiaIV at the transcriptional level. Most notably, ST8SiaII is predominant during embryonic development, while ST8SiaIV is the major polysialyltransferase of the adult brain [16, 79, 81, 82]. In contrast to earlier Northern blot analyzes indicating manifold higher ST8SiaII levels in the embryonic and perinatal phase [14, 81, 82], recent studies using real-time quantitative RT-PCR determined that the ST8SiaII transcript level in perinatal mouse brain is less than twofold higher than the level of ST8SiaIV [15, 16]. The factors responsible for the joint but sometimes distinct regulation of the two polysialyltranserases are largely unknown. Initial investigations of the proximal promoter regions of the polysialyltransferases provided first evidence for specific regulatory elements [83–85]. As shown in human tumor cells two drugs, retinoic acid and valproic acid, are able to differentially affect polysialyltransferase mRNA levels [86, 87] and elevated polySia levels due to overexpression of the developmentally regulated transcription factor Pax3 could be assigned to a specific increase of ST8SiaII mRNA [88]. However, the data available so far are not sufficient to explain how the spatial and temporal expression patterns of ST8SiaII and ST8SiaIV are regulated.

In contrast to the mouse system, expression analysis, gene-targeted knockdown experiments, and in vitro catalytic assays indicate that in the developing and adult zebrafish ST8SiaII is the major, if not the only enzyme capable of performing NCAM polysialylation [89]. The evolutionary divergence of ST8SiaIV in bony-fish

supports the assumed functional loss of ST8SiaIV [89]. Despite the very low expression levels of ST8SiaIV reported by Marx et al. [89], a recent study describes partially overlapping expression domains of ST8SiaII and ST8SiaIV throughout the mature zebrafish brain [90]. Evidently, the function of ST8SiaIV in zebrafish remains to be elucidated.

Phenotype of Polysia-deficient Mice

The finding that, at least in mammals, both polysialyltransferases can partially compensate for each other is reflected by the mild but distinct phenotypes of mice lacking only one polysialyltransferase [77, 78]. In perfect agreement with the predominance of ST8SiaII during embryonic and early postnatal development, ST8SiaII-deficient mice display neurodevelopmental defects manifesting in the aberrant topology of hippocampal mossy fiber projections [78]. In contrast, and consistent with the prevalent expression of ST8SiaIV in the adult, the lack of ST8SiaIV gives rise to markedly impaired synaptic plasticity in the CA1 subregion of the hippocampus without detectable morphological defects [77]. In extension of this finding, a prominent role of polySia in regulating ionotropic receptor functions involved in long-term potentiation and memory formation was unraveled [91–94].

Since NCAM is the major carrier of polySia in mammalian brain development, mice with genetic ablation of NCAM are almost completely devoid of polySia [95]. While the overall brain architecture of these mice is surprisingly normal, two major morphological aberrations have been described and extensively studied. One is a dramatic reduction in the size of the olfactory bulbs caused by a migration deficit of newly born olfactory bulb interneurons derived from the subventricular zone [96–99]. The other is a defective lamination of mossy fibers projecting from the dentate gyrus to the CA3 subfield of Ammon's horn [96, 100, 101]. Both phenotypic traits must be explained by the loss of polySia and not NCAM because they could be copied by enzymatic removal of the sugar polymer leaving the NCAM protein backbone unaltered [97, 101]. It was therefore not surprising that the malformations found in $NCAM^{-/-}$ animals also develop in the polySia-deficient $ST8SiaII^{-/-}$ $ST8SiaIV^{-/-}$ double knockout mice [63]. Since polySia impairs not only NCAM but also other cell surface interactions [1–3], the prevailing view is that the structural deficits shared by polysialyltransferase-deficient and NCAM-knockout mice are due to aberrant, NCAM-independent cell surface interactions induced by the absence of polySia [8]. In the particularly well-studied case of precursor migration toward the olfactory bulb, the lack of polySia in $NCAM^{-/-}$ mice disturbs not only contacts within the chains of migrating cells but also the interactions with their stationary environment, which is dramatically altered due to a massive astrogliosis [99]. A recent report on polysialyltransferase double-knockout mice confirmed that defective chain migration in the absence of polySia is associated with altered morphology of astroglia [102]. Interestingly, this study describes more widespread deficits of precursor cell migration during cerebral cortex development together with a

general up-regulation of the astrocytic marker GFAP in the forebrain of ST8SiaII$^{-/-}$ ST8SiaIV$^{-/-}$ mice and reveals that the absence of polySia promotes PDGF-induced differentiation of astroglia in vitro.

Considering the prominent role assigned to polysialylation of NCAM during developmental plasticity (see [8] for a recent review), the overall mild phenotype of mice lacking polySia due to NCAM deficiency was puzzling. Only after polySia could be ablated independent from NCAM the vital role of polysialylation became apparent. Beyond the recapitulation of major defects in brain morphology as described for NCAM deficient mice, the block of polysialic acid biosynthesis in ST8SiaII$^{-/-}$ ST8SiaIV$^{-/-}$ double knockout animals leads to additional, far more severe defects [63]. Although born overtly normal and at the expected Mendelian ratio, the polysialyltransferase-deficient mice suffer from drastic growth retardation in the early postnatal phase and more than 80% of these mice die during the first 4 weeks. With regard to brain development ST8SiaII$^{-/-}$ ST8SiaIV$^{-/-}$ animals are characterized by a high incidence of hydrocephalus and severe malformations of a specific set of brain fiber tracts. Remarkably, all of these defects found in the ST8SiaII$^{-/-}$ ST8SiaIV$^{-/-}$ but not in the NCAM$^{-/-}$ mice are rescued by the additional ablation of NCAM demonstrating that the untimely appearance of "naked," i.e. polySia-free NCAM causes the fatal developmental phenotype [7, 63]. These findings prove that the abundant expression of polySia during development is an essential control mechanism to specifically regulate NCAM interactions.

Future Directions

Future work will aim at dissecting the different molecular and cellular mechanisms underlying the changes induced by regulating polysialylation of NCAM in vivo and at making use of this knowledge in novel experimental and therapeutic approaches. Particularly interesting is the recent progress made in animal models of peripheral and central nervous system repair by applying polySia via engineered overexpression of polysialyltransferases as well as in steering the migratory capacity of Schwann cells and embryonic stem cell-derived glial precursors [103–110]. Complementary, first studies indicate that purified or synthetic polySia may prove useful as a biocompatible, and bioresorbable material for nerve tissue engineering [111–114]. Further challenges involve testing if enzymatic degradation of polySia by target-oriented application of phage-derived endosialidases [115–117], manipulation of endogenous polysialyltransferase activity and/or the use of NCAM or polySia peptide mimetics [118–120] have the potential to affect tumor development or to trigger endogenous brain repair processes.

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Structural Basis for the Polysialylation of the Neural Cell Adhesion Molecule

Karen J. Colley

Introduction

Polysialic Acid: An Important Regulator of NCAM-dependent and NCAM-independent Adhesion

NCAM is an immunoglobulin super family protein that consists of five immunoglobulin (Ig) domains, two fibronectin type III (FN_{III}) repeats, and a transmembrane (TM) region plus cytoplasmic tail (NCAM140 and NCAM180) or a glycosyl-phosphatidylinositol (GPI) anchor (Fig. 1) [3]. Studies by Nelson et al. [4] have demonstrated that of NCAM's six N-glycosylation sites, sites 5 and 6 on the fifth Ig domain (Ig5) carry the bulk of NCAM's polysialic acid (polySia) (Fig. 1). Other work has demonstrated that the presence of long chains of polySia on the N-glycans of NCAM negatively modulates NCAM-dependent and NCAM-independent cell interactions [5,6]. More recently, elegant biophysical studies by Leckband, Rutishauser and colleagues [1] have quantitatively demonstrated the negative effects polySia has on both NCAM-mediated and cadherin-mediated adhesion processes.

The expression of polySia is developmentally regulated [2]. Polysialylated NCAM is highly expressed in the embryo and neonate, and exhibits a decreased and more restricted expression in the adult animal, where it is found in areas of the adult brain that require morphofunctional plasticity [7–9]. During development, polySia promotes axon guidance and targeting, and the separation and migration of cells (reviewed in [7–10]). The presence of surface polySia also allows cells to exhibit reversible interactions needed for cell movement (for example [11]). In adult animals, continued polySia expression is observed in specific areas of the brain, such as the hippocampus, the suprachiasmatic nucleus, and on olfactory bulb precursors that persist in their ability to generate neurons and show physiological

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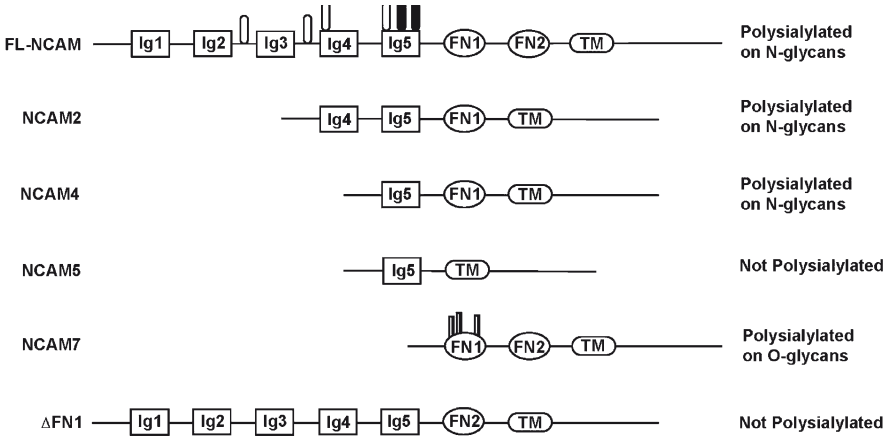

Fig. 1 NCAM and Domain Deletion Mutants. Shown are full length NCAM (FL-NCAM), NCAM2 (Ig4-Ig5-FN1-TM-Tail), NCAM4 (Ig5-FN1-TM-Tail), NCAM5 (Ig5-TM-Tail), NCAM7 (FN1-FN2-TM-Tail) and ΔFN1 (Ig1-Ig2-Ig3-Ig4-Ig5-FN2-TM-Tail). Ig, immunoglobulin domain; FN, fibronectin type three repeat; TM, transmembrane region. White elongated ovals represent N-glycans and black elongated ovals represent polysialylated N-glycans [1,2]. Sites of O-polysialylation in the NCAM7 protein (Thr 505, Thr 516 and Thr 553) are indicated by gray rectangles. The ability of each protein to be polysialylated by coexpressed polySTs, and the predominant type of glycan polysialylated are indicated

or morphological plasticity [12–15]. PolySia is also reexpressed during axon regeneration, and in various muscle diseases involving dystrophy and denervation [9,16,17]. High levels of polySia are also observed on the surface of some cancer cells, such as neuroblastomas, medulloblastomas, gliomas, small cell lung carcinomas, Wilms' tumor, and in other pediatric tumors, where its presence is frequently correlated with the cancer's highly metastatic behavior [9,16–25].

Recent studies using polysialyltransferase (polyST) and NCAM knockout mice have revealed the critical role of polySia in brain development. Mice lacking NCAM exhibit no obvious developmental defects, but do show a reduction in the size of the olfactory bulb, as well as deficits in spatial learning [26]. Mice lacking either ST8Sia IV/PST or ST8Sia II/STX exhibit a partial loss of polySia that results in compromised long term potentiation, long term depression, axon guidance, and synapse formation in the hippocampus [27,28]. However, mice lacking both polySTs and, therefore, all polySia, exhibit severe defects in brain development, fail to thrive, and die within 1 month after birth [29,30]. They also have smaller olfactory bulbs, as was observed in NCAM knockout mice, due to a decrease in neuroblast migration to the olfactory bulb in the absence of polySia. Strikingly, elimination of NCAM in the double polyST knockout mice reversed the developmental defects observed when polySia alone was eliminated, suggesting that the regulated polysialylation of NCAM glycans and the resulting decrease in cell adhesion is critical for brain development and animal survival [30].

Polysialylation of NCAM: A Protein Specific Modification

In mammals, polySia is found as homopolymers of α2, 8-linked Neu5Ac capping terminal α2, 3- or α2, 6-linked sialic acid on the N-linked and O-linked glycans of a small group of proteins. The polySTs, ST8Sia IV/PST and ST8Sia II/STX, are responsible for the polysialylation of mammalian proteins [31]. The proteins specifically modified by polySia include NCAM [2,32], the α subunit of the voltage dependent sodium channel [2,32–34], the two polySTs [35], a form of the scavenger receptor, CD36, found in milk [36], and neuropilin-2 found on dendritic cells [37]. Interestingly, the polySia on CD36 and neuropilin-2 is found exclusively on O-glycans [36,37]. The limited number of polysialylated proteins in mammalian cells suggests that the process of polysialylation is protein-specific. In other words, it requires that the polySTs recognize specific features of their glycoprotein substrates, and this allows the polymerization of polySia chains on the glycans of these substrates.

In most cases, the types of glycosyltransferases expressed by a cell and their levels of expression will determine structures of glycans found on proteins and lipids [38]. General variations in glycan structures may be related to a protein's folding pattern or subunit interactions, which may enhance or limit access to particular glycosylation enzymes [39]. However, in some instances, a protein's sequence and/or structure may be recognized by a particular enzyme, and this interaction will increase the efficiency of a glycosylation event. Examples of such protein-specific glycosylation events include the biosynthesis of the mannose 6-phosphate recognition marker on lysosomal enzymes (mediates their lysosomal targeting) [40], the biosynthesis of the GalNAc-4-SO_4 structure on N-glycans of pituitary glycoprotein hormones (mediates their clearance from the circulation) [41], the biosynthesis of $Glc_1Man_9GlcNAc_2$ glycans on misfolded glycoproteins in the endoplasmic reticulum (allows chaperone interactions) [42,43], and the addition of GlcNAc to O-fucose on epidermal growth factor repeats by Fringe (modifies Notch ligand interactions and signaling) [44,45].

In the case of mannose 6-phosphate and GalNAc-4-SO_4 biosynthesis, enzyme recognition is mediated by specific protein features. GalNAc-4-SO_4 is found at the termini of the N-glycans modifying pituitary glycoprotein hormones, and regulates the circulatory half-lives of these proteins by mediating their internalization by a hepatic receptor and their subsequent degradation in lysosomes [41]. Recognition of the glycoprotein hormones by a specific N-acetyl-galactosaminyltransferase requires a cluster of basic residues in an α-helix positioned 6-9 amino acids amino terminal to the hormone's N-glycan [46,47]. Mannose-6-phosphate residues on the N-linked glycans of lysosomal enzymes are required for their recognition by specific receptors in the *trans* Golgi network and subsequent trafficking to lysosomes [48,49]. The N-acetylglucosamine-1-phosphotransferase that catalyzes the first step in the two-step biosynthesis of mannose 6-phosphate requires maintenance of lysosomal enzyme tertiary structure and a signal patch that includes specific lysine residues [40,50–53].

Early studies first suggested the possibility that the polySTs recognize specific features of the NCAM protein. Kojima et al. [54] found that while ST8Sia II/STX could polysialylate a variety of glycoprotein substrates under in vitro conditions, NCAM served as a 1500-fold better substrate than the next best substrate, fetuin. Angata et al. [55] demonstrated that both polySTs could add polySia to glycan substrates, but that the polysialylation of glycans attached to the NCAM protein was much more efficient. In addition, using catalytically active, but unpolysialylated forms of polySTs (sites of autopolysialylation mutated), we demonstrated that no endogenous proteins in COS-1 cells are polysialylated when these enzymes are expressed, and that polySia is only detected when NCAM is coexpressed with the mutant enzymes [56,57]. Taken together, these results suggest that the polySTs must recognize some protein features of NCAM and their other substrates for efficient polysialylation of substrate glycans. Our work, described below, confirms that NCAM polysialylation, like the examples above, exhibits specific protein sequence and structural requirements and, thus, is a protein-specific glycosylation event.

NCAM Domains Required for Polysialylation

Early studies by Nelson et al. [4] demonstrated that a truncated NCAM protein consisting of the Ig4, Ig5, and the first FN_{III} repeat (FN1) fused to the TM region, and cytoplasmic tail was polysialylated by the endogenous polyST activity of F11 rat/mouse hybrid cells. In this truncated NCAM protein, Ig4 could be partially replaced by another Ig domain from the L1 cell adhesion molecule, but FN1 was absolutely required and could not be deleted or replaced by the second FN repeat (FN2) of L1. They proposed that the polySTs recognize and bind all three NCAM domains (Ig4, Ig5, FN1) simultaneously. We reevaluated the NCAM domain requirements for polysialylation using the two polySTs that were not available to these researchers and a series of domain deletion mutants [58] (see Fig. 1). We found that a protein possessing Ig4, Ig5 and FN1 (NCAM2) was efficiently polysialylated by coexpressed polySTs, in agreement with the earlier work of Nelson et al. [4]. However, we also observed that a shorter protein possessing just Ig5 and FN1 (NCAM4) was efficiently polysialylated, suggesting that Ig4 was not necessary for recognition and polysialylation [58]. Other work by Fujimoto et al. [59] agreed with our findings. The most important observation in our study was that, while NCAM4 possessing both the Ig5 and FN1 domains was polysialylated by co-expressed polySTs, a protein containing only the Ig5 domain (NCAM5) was not polysialylated [58]. This comparison suggested that NCAM FN1 was necessary for the polysialylation of the N-glycans on the adjacent Ig5 domain. Additional experiments with soluble NCAM proteins showed that membrane association was not necessary for their polysialylation, but did in some cases enhance the level of polysialylation [58]. This enhancement is likely the result of optimum positioning and concentration of the membrane-associated polyST and NCAM proteins on the lumenal face of the Golgi membrane. Taken together, these results suggest a model

in which the polySTs recognize features of NCAM FN1, and this "docking" positions them for polysialylation of the N-glycans on the adjacent Ig5 domain.

Fibronectin type I, II and III repeats were first identified in fibronectin [60] and were subsequently found in many other proteins [61]. The FN_{III} repeat is the largest of the three (~90 amino acids) and the most common. It is estimated to be present in approximately 2% of all mammalian proteins and has been identified in neural cell adhesion molecules, receptor protein kinases and phosphatases, adhesive matrix proteins, cytokine receptors, cytoplasmic muscle proteins, and prokaryotic carbohydrate cleavage enzymes [61]. The sequence homology for different FN_{III} repeats is relatively low (<20%), while the sequence homology for the same (similarly positioned) FN_{III} repeat in the same protein across different species is high (80–90%) [62]. In contrast to their low sequence homology, FN_{III} repeats exhibit high structural homology and fold into a β sandwich structure with one three-strand and one four-strand anti-parallel β sheet (Fig. 2) [63].

To address the question of whether the structure and/or sequence of NCAM FN1 was important for its role in polysialylation, we asked whether any other FN repeat could fulfill the requirement for NCAM FN1 in polysialylation. We deleted FN1 from the full-length NCAM (FL-NCAM) sequence, effectively placing FN2 in its position (Fig. 1). A test of the expression and trafficking of the ΔFN1 protein showed that it was well expressed, and efficiently left the endoplasmic reticulum and trafficked to the cell surface, indicating that it was not grossly misfolded. We observed no polysialylation of ΔFN1 by ST8Sia IV/PST or ST8Sia II/STX following expression in COS-1 cells. These results suggested that specific features of NCAM FN1 are critical for polyST recognition and NCAM polysialylation [64].

The Unique β Sandwich Structure of NCAM FN1: The Role of an Acidic Surface Patch and Novel α-Helix in NCAM Polysialylation

Our next challenge was to determine the features of NCAM FN1 required for polyST recognition and NCAM polysialylation. A comparison of NCAM FN1 and FN2 amino acid sequences demonstrated that they are only ~25% identical. Nevertheless, because they were FN_{III} repeats, we predicted that they are likely to have similar structures. We modeled the structure of human NCAM FN1 based on the known structure of rat NCAM FN2 (see Fig. 2 for rat NCAM FN2 structure). What was immediately apparent was the presence of an acidic surface patch found on FN1, but missing from FN2 [64]. In collaboration with Drs. Arnon Lavie and Nikolina Sekulic, we obtained the X-ray crystal structure of human NCAM FN1 (Fig. 2 and 3) [65]. We found that NCAM FN1 did indeed fold into a β sandwich structure, but that it had two unique features. We confirmed the presence of the acidic surface patch formed from Asp 497, Asp 511, Glu 512 and Glu 514 (Fig. 3) on one face of the β sandwich. We also found a novel, eight amino acid α-helix

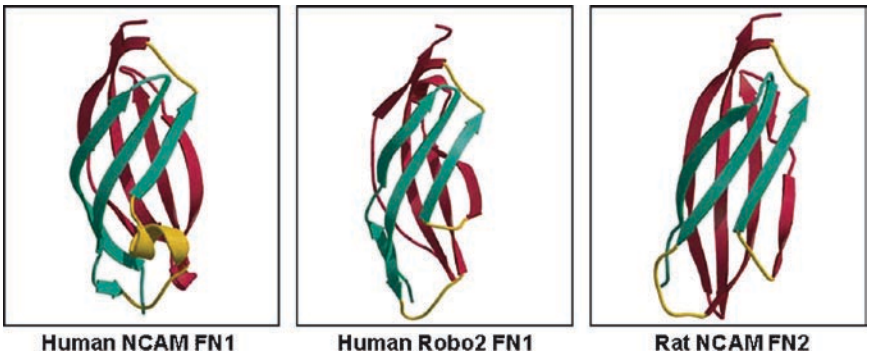

Fig. 2 Comparison of human NCAM FN1 structure to other fibronectin type III repeats. Structural representations of human NCAM FN1 (Protein Data Bank ID 2HAZ), human Robo2 FN1 (Protein Data Bank ID 1UEM), and rat NCAM FN2 (Protein Data Bank ID 1LWR). Note the absence of the α-helix from the latter two structures. In the Δhelix-TT proteins, the eight amino acids of the helix are replaced by two threonines as found in the corresponding region of Robo2 FN1

linking the fourth and fifth strands of the NCAM FN1 β sandwich, which has not been observed in any other FN_{III} repeat structure solved to date (Fig. 3) [65].

To evaluate the role of the acidic patch in NCAM polysialylation, we replaced the three core amino acids of this patch, Asp 511, Glu 512 and Glu 514, with both arginine and alanine residues in FL-NCAM, and in the truncated mutant NCAM4, which possesses the Ig5 and FN1 domains [64] (Fig. 3). Replacing the acidic residues with arginine eliminated the polysialylation of FL-NCAM and NCAM4 by the polySTs without detectably compromising their expression, folding, or trafficking [64]. Interestingly, replacing the acidic residues with alanine dramatically decreased NCAM4 polysialylation, but did not substantially impact FL-NCAM polysialylation [64]. From these and other results, we predicted that the acidic patch was likely to be a small part of a larger polyST interaction domain. We hypothesized that NCAM4 is not optimally positioned vis à vis the membrane-associated polySTs because it is missing the FN2 domain, and, as such, may not make as many contacts with the polySTs as FL-NCAM. If this is the case, then even replacing the NCAM4 acidic patch residues with neutral alanine residues may disrupt an already compromised interaction between enzyme and substrate [64].

Naturally, we considered the possibility that the FN1 α-helix may be part of an extended polyST recognition region on FN1, and we sought to replace the amino acids of the α-helix to test this idea. The Robo2 FN1 NMR structures were used for the molecular replacement solution of the NCAM FN1 structure (see Fig. 2 for Robo2 FN1 structure) [65]. In Robo2 FN1, two threonine residues link the fourth and fifth strands of the β sandwich. Overlay of the structures of human NCAM FN1 and Robo2 FN1 demonstrated that the eight amino acid α-helix in NCAM FN1 spanned the same distance as these two threonine residues [65]. Therefore, we rationalized that replacing the α-helix with two threonine residues would not

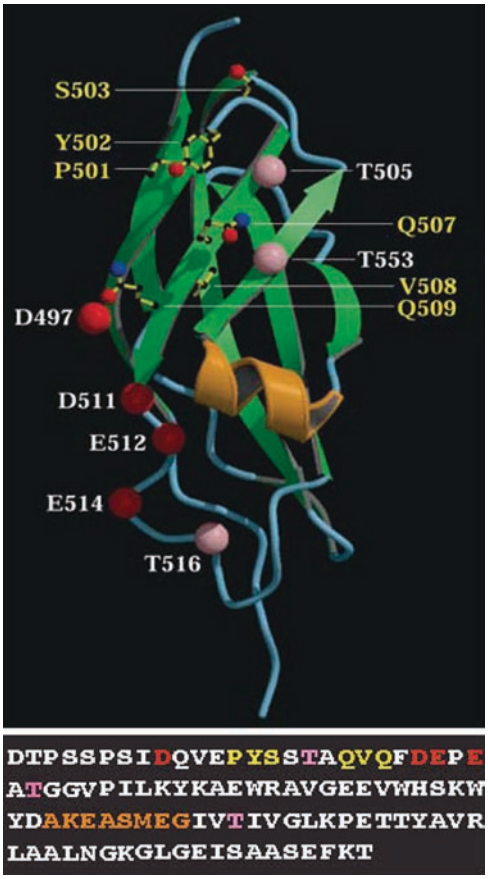

Fig. 3 Location of NCAM FN1 acidic patch, α-helix, Pro_{501}-Tyr_{502}-Ser_{503}, Gln_{507}-Val_{508}-Gln_{509} and sites of O-glycan polysialylation. Acidic patch residues Asp497, Asp511, Glu512 and Glu514 are indicated in red in the sequence and by large red balls in the structural representation (D497, D511, E512 and E514). The eight amino acids of the α-helix are indicated in orange in both the structural representation and the sequence. The side chains of $Pro_{501}Tyr_{502}Ser_{503}$ and $Gln_{507}Val_{508}Gln_{509}$ (P501, Y502, S503 and Q507, V508, Q509) are shown in yellow on strands β1 and β2 in the structural representation, with the small red balls representing the oxygens of the side chains of Tyr_{502}, Ser_{503}, Gln_{507} and Gln_{509}. In the sequence, these residues are indicated in yellow. The three threonine residues that are modified with O-linked polysialylated glycans in the Δhelix proteins are represented by large pink balls (T505, T516, and T553) in the structural representation and shown in pink in the sequence

substantially alter the strand arrangement and overall structure of NCAM FN1. We generated a Δhelix-TT protein and found that it was expressed well and trafficked to the cell surface just like FL-NCAM [65]. We were initially disappointed to find that the Δhelix-TT protein was as efficiently polysialylated as FL-NCAM when coexpressed with ST8Sia IV/PST, suggesting that the α-helix did not play a role in polyST recognition. However, additional analyzes with the enzyme peptide

N-glycosidase F (PNGase F) that specifically cleaves N-linked, but not O-linked glycans, led to a surprising finding-that the polySia on the Δhelix-TT protein was modifying O-linked glycans and not N-linked glycans [65]. One possibility was that the two, newly added threonine residues were serving as sites for O-linked glycosylation, and that these O-glycans were being polysialylated. However, a replacement of the helix with two alanine residues revealed that this was unlikely. The Δhelix-AA protein, like the Δhelix-TT protein, was polysialylated on O-linked glycans [65]. These results suggested that while the α-helix is not necessary for polyST recognition, it is important for the positioning of Ig5 N-glycans for polysialylation.

Are Interdomain Interactions Critical for Polysialylation of the Ig5 N-glycans?

The presence of polySia on O-glycans has now been observed on a muscle specific domain of an NCAM splice variant [66,67], the CD36 scavenger receptor [36], and most recently on neuropilin-2 [37]. In addition, early work in our laboratory had also detected polySia on O-glycans on unidentified cancer cell proteins [68], on FL-NCAM (small proportion of total polySia) [58], and on a domain deletion mutant, NCAM7 (all polySia), after coexpression with ST8SiaIV/PST in COS-1 cells [58]. NCAM7 consists of FN1, FN2, the TM region and cytoplasmic tail of NCAM140, and was originally intended to be a negative control (Fig. 1) [58]. Treatment of immunoprecipitated NCAM7 with PNGase F, followed by immunoblotting with the OL28 anti-polySia antibody, demonstrated that this protein is polysialylated on O-glycans. Considering these previous results along with the O-polysialylation of the Δhelix proteins, we wondered whether the FN1 α-helix is necessary for an interaction between Ig5 and FN1 that allows the correct positioning of Ig5 N-glycans for polysialylation by the polyST docked on FN1. If this is the case, then one would predict that this interaction would not occur in the absence of the α-helix, that the Ig5 N-glycans would not be polysialylated, and instead O-glycans in the FN1-FN2 region might be polysialylated (see models in Fig. 4). Initial experiments supported this premise. First, we found that O-glycans in the FN1 domain are polysialylated in the Δhelix proteins [65]. Using a prediction program designed by Igor Almedia and Rafel Torres, Jr. (University of Texas, El Paso), we identified three threonine residues that were likely sites of O-glycosylation (Fig. 3). Replacement of these threonines in the Δhelix-AA protein abolished polysialylation, thus demonstrating that the polysialylated O-glycans of the Δhelix proteins are found in FN1 [65]. Second, we found that replacement of the acidic patch residues in either Δhelix-AA or NCAM7 proteins substantially decreases or eliminates polysialylation of the O-glycans in these proteins, suggesting that the polySTs interact with the same site or overlapping sites on FN1 for both N- and O-glycan polysialylation [65]. In sum, our results indicate that the FN1 α-helix is not necessary for polyST recognition, but does play an important role in positioning

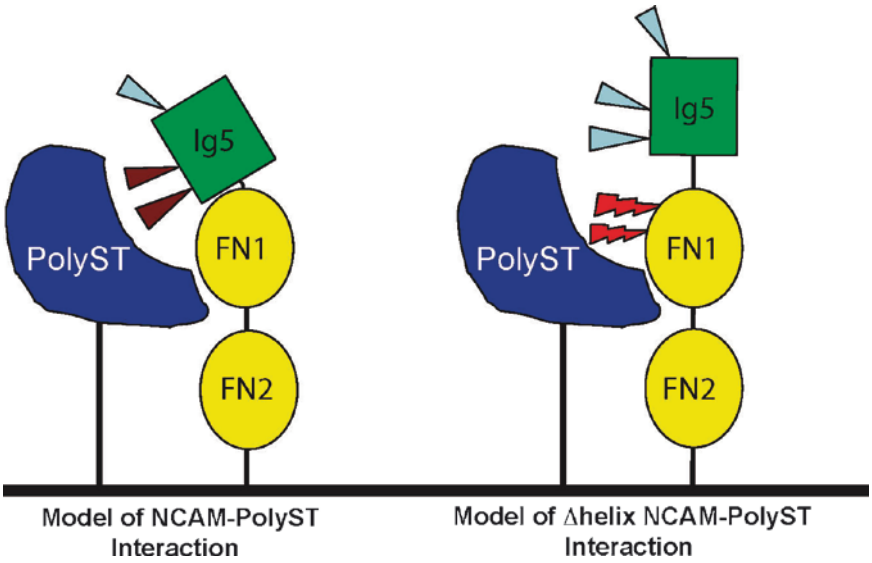

Fig. 4 Model of possible Ig5-FN1 interactions and changes upon elimination of the α-helix. We predict that the polyST recognizes and docks on the FN1 repeat of NCAM, and an interaction between the Ig5 and FN1 domains requiring the α-helix in FN1 positions the Ig5 N-glycans for polysialylation by the "docked" polyST (model on left). If the α-helix is replaced, the Ig5-FN1 interaction no longer occurs and the Ig5 N-glycans (dark red triangles) are moved away to reveal O-glycans in the FN1 repeat (red lightning bolts) that are then polysialylated by the "docked" polyST

the Ig5 N-glycans for polysialylation. Solid evidence for an interaction between Ig5 and FN1 requires additional experiments, however, it is intriguing that rotary shadowing electron microscopy detects a bend in the NCAM molecule that is predicted to be in the vicinity of the Ig5-FN1 region [69,70].

Reconstitution of PolyST Recognition and Polysialylation of an Unpolysialylated NCAM-OCAM Chimeric Protein

Up until this point, we had identified amino acids and structural features of NCAM FN1 necessary for NCAM polysialylation. Recently, we decided to determine whether these sequences were also sufficient for NCAM polysialylation. To do this, we generated a chimeric protein in which NCAM FN1 was replaced by the analogous FN1 repeat of the olfactory cell adhesion molecule, OCAM. OCAM is an NCAM family member that is expressed postnatally in the brain, olfactory epithelium, and retina [71–73]. OCAM is 45% identical to NCAM, has the same domain structure, and possesses the consensus glycosylation sites corresponding to the fourth, fifth and sixth N-glycans found in NCAM's Ig5 domain [73].

Despite these similarities, OCAM is not polysialylated [73]. The FN1 repeats of NCAM and OCAM are 37% identical, making the OCAM FN1 domain an excellent background for inserting NCAM FN1 sequences. Notably, comparison of the known structure of human NCAM FN1 with a modeled structure of OCAM FN1 reveals that OCAM FN1 lacks the acidic surface patch and α-helix observed in the NCAM FN1 structure. Initially, we inserted the four acidic patch residues and the β4-α-helix-β5-region from NCAM FN1 into the OCAM FN1 of the NCAM-OCAM chimera, and found that these sequences were sufficient for recognition and low levels of chimera polysialylation (data not shown). We were able to increase the level of polysialylation by adding six additional amino acids, $Pro_{501}Tyr_{502}Ser_{503}$ and $Gln_{507}Val_{508}Gln_{509}$, from NCAM FN1 (Fig. 3 and Fig. 5). These residues were

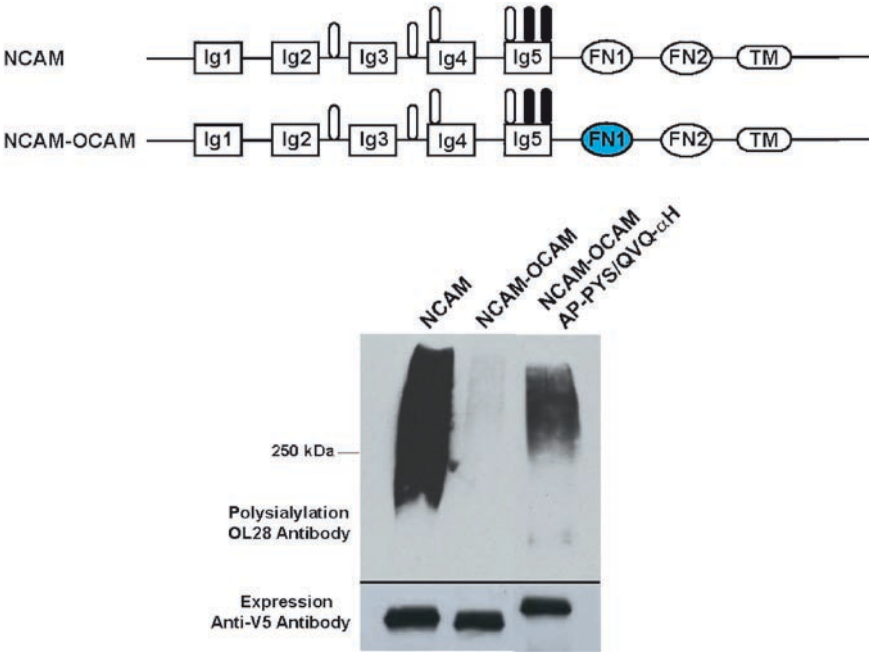

Fig. 5 Reconstitution of polysialylation of an NCAM-OCAM chimera by insertion of NCAM FN1 sequences. Top, schematic representation of FL-NCAM and the NCAM-OCAM chimera. The OCAM FN1 is indicated in turquoise. Bottom, a comparison of the polysialylation of FL-NCAM, NCAM-OCAM and NCAM-OCAM including specific NCAM FN1 sequences. FL-NCAM, the NCAM-OCAM chimera and the NCAM-OCAM chimera containing the NCAM FN1 acidic patch residues, $Pro_{501}Tyr_{502}Ser_{503}$ and $Gln_{507}Val_{508}Gln_{509}$, and the β4 strand-α-helix-β5 strand sequences (NCAM-OCAM AP-PYS/QVQ-αH) were coexpressed with ST8Sia IV/PST in COS-1 cells. NCAM proteins were immunoprecipitated using an antibody against the V5 epitope tag and subjected to immunoblotting using the anti-polySia antibody, OL28 (Polysialylation, OL28 Antibody). NCAM protein expression was determined by immunoblotting sample aliquots with anti-V5 antibody (Expression, Anti-V5 Antibody) (see Methods in references [62,63])

chosen because they were near the acidic patch region on strands 1 and 2 of the β sandwich and were some of the most divergent between the NCAM and OCAM FN1 domain sequences. Currently, we are determining which FN1 sequences are absolutely required for polyST recognition and which glycans are polysialylated in the modified NCAM-OCAM chimeras.

A Model of PolyST-NCAM Interaction

Our results suggest a model of polyST-NCAM interaction that depends upon two protein-protein interactions. One that involves the "docking" of the polyST on the NCAM FN1 domain, and a second between the Ig5 and FN1 domains that positions the Ig5 N-glycans for polysialylation. We predict that the polyST-FN1 interaction is the basis for the protein specificity of polysialylation, but also may be required for the polymerization of the polySia chains on the NCAM glycans. In fact, the affinity of this interaction could either be envisioned to dictate the length of the polySia chains polymerized or the number of NCAM proteins in a population polysialylated. Interestingly, preliminary co-immunoprecipitation experiments in our laboratory suggest that the polySTs bind directly to NCAM and that ST8Sia II/STX has a greater affinity for NCAM than does ST8Sia IV/PST (B. E. Close and K. J. Colley, unpublished observations). Recent in vivo work by Galuska et al. [74,75] demonstrates that the two polySTs synthesize polySia chains of the same average length, but that they differentially modify the two major attachment sites in NCAM, and that ST8Sia II/STX more efficiently polysialylates an entire population of NCAM molecules. This work suggests that potential differences in the affinities of the two polySTs for NCAM have a greater impact on the glycans modified and the efficiency of the polysialylation process, and less impact on polySia chain length per se.

Future Directions

Several additional questions must be addressed in order to understand the mechanism of protein polysialylation. We have reconstituted polyST recognition and polysialylation in the NCAM-OCAM chimera by inserting several different sequences from NCAM FN1; however, the contribution of each is not understood, nor is the identity of the glycans polysialylated. Our model predicts that the polySTs directly bind NCAM and our preliminary data suggest this is so. However, it is not known which of the FN1 residues required for NCAM polysialylation are also required for polyST-NCAM binding. In addition, our data suggest that the Ig5 and FN1 domains may interact to position the Ig5 N-glycans for polysialylation. Additional structural analysis and binding studies using these two domains should provide the answer to this question and reveal whether polyST binding is necessary

to induce an Ig5-FN1 interaction. An unexplored area concerns the contribution of protein-glycan interactions to the initial engagement of the polyST and NCAM, and/or to the chain elongation process. It is also not known how other polyST substrates are recognized by the polySTs and whether similar amino acids and structural features mediate these interactions. Notably, none of these other proteins contain FN_{III} repeats. Finally, once the requirements for NCAM recognition by the polySTs are defined, it would be useful to design inhibitors of the polyST-NCAM interaction and NCAM polysialylation.

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The Role of PSA-NCAM in Adult Neurogenesis

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Introduction

A fundamental feature of neural circuits is the continuous activity-dependent plasticity of synaptic connections. This lifelong self-reorganization process most likely involves a spectrum of modifications including the molecular remodeling of synapses, leading to their strengthening/silencing, the formation of new synapses, and the destabilization of previously established contacts [1–3]. A fascinating, newly recognized form of adult plasticity consists of the recruitment of newly generated neurons into functional circuits. In the mammalian brain, this process of "rejuvenation" of existing circuits occurs in two discrete regions, the subventricular zone of the lateral ventricle (SVZ) [4] and the subgranular zone of dentate gyrus (SGZ) in the hippocampus [5]. The exact physiological relevance of postnatal or adult neurogenesis is not yet clear. Neurogenesis in the dentate gyrus was shown to be changed by enriched environments, exercise, hippocampus-dependent learning tasks, stress, and pathological conditions [6–9]. Consequently, it has been proposed that integration of new neurons into functional circuits might be involved in learning and memory processes [10]. Neurogenesis in the SVZ and recruitment of new neurons into olfactory circuits was suggested to be important for olfactory sensory discrimination [11].

Neurogenesis in the adult brain recapitulates the complete process of neuronal development, including proliferation, neuronal fate specification, differentiation,

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migration, synaptic integration and survival of new neurons. Neural stem/progenitor cells located within the SVZ give rise to neuroblasts that migrate, first, tangentially via the rostral migratory stream (RMS), and then radially into the olfactory bulb. These new neurons differentiate into two types of interneuron: granule cells and periglomerular cells [11,12]. Stem cells in the SGZ of the dentate gyrus, generate neuroblasts that migrate into the inner granule cell layer and differentiate into new granule cells [5]. A critical feature of adult neurogenesis is that stem cell proliferation and cell migration are spatially restricted to a specific permissive environment where intercellular interactions play a critical role.

In adults, neural stem cells and their progeny are localized in specific niches [13–15]. By definition, the stem cell niche is an anatomical and functional unit that provides a specific microenvironment where stem cells replenish themselves through self-renewal and give rise to different progenies through asymmetric divisions. The cellular composition of the niche in the two germinal regions has been extensively studied and characterized [12,16–18]. In both structures, stem cells have been identified as glial fibrillary acidic protein (GFAP+) and nestin+ positive astrocytes (type B cells in the SVZ and radially oriented astrocytes in the SGZ). These cells show self-renewal through slow symmetric division and give rise to transit-amplifying cells (type C cells in the SVZ and type D1 cells in the SGZ) that, in turn, differentiate into doublecortin-positive committed neuroblasts (type A cells in the SVZ and D2/D3 cells in the SGZ) [13]. A particularly interesting feature of the neurogenic niche is the intimate association of neural precursor cells (NPCs) with microcapillaries in both the SVZ and the SGZ. Endothelial cells and the specialized basal lamina seem to provide attachment for NPCs and generate signals for the self-renewal and priming of stem cells for the production of neurons [13,19].

Remarkable progress has been made in identifying key molecules mediating interactions between stem cells, immature neurons and the local environment [20]. Among these, isoforms of the neural cell adhesion molecule (NCAM) carrying the large carbohydrate polymer, polysialic acid (PSA), are of particular interest. NCAM is a member of the immunoglobulin superfamily that is involved in cell surface recognition, and can promote cell adhesion through a homophylic Ca^{2+}-independent binding mechanism [21]. NCAM is traditionally viewed as mediator of cell–cell interactions establishing a physical anchorage of cells to their environment. However, the attachment of the PSA chain to the NCAM protein core provides unique properties to the molecule. PSA has a large hydrated volume and high negative charge density, and therefore, is well placed to attenuate adhesion forces, and to negatively regulate overall cell surface interactions [22,23]. Here, we review the expression pattern and role of PSA-NCAM in adult neurogenesis.

PSA-NCAM Expression in Adult Neurogenic Sites

NCAM and PSA-NCAM expression is a characteristic feature of the postnatal neurogenic niches [12,24,25]. The available evidence indicates that in the SVZ, PSA-NCAM is not expressed by type B stem cells or other astrocytes, though

these cells are NCAM positive [26]. Similarly, type C transit amplifying cells are negative for PSA-NCAM. On the other hand, type A, committed neuronal precursor cells, are strongly stained for this adhesion molecule [12,27]. The situation is similar in the SGZ niche, where radial astrocytes (primary progenitor or stem cells) as well as horizontal astrocytes do not appear to express PSA-NCAM [18,28]. The primary progenitor cell in the SGZ gives rise to D1 cells, which divides to generate D2 cells. This later extends processes and becomes a D3 cell, which eventually matures into a new granule neuron. It is of interest that, in contrast to type C cells in the SVZ, D1 proliferating transit amplifying cells as well as D2 and D3 cells express PSA-NCAM [18]. However, D1 cells differ in many respects from SVZ type C cells. D1 cells appear to divide only once, and to express doublecortin which is not expressed by C cells [18]. Therefore, D1 cells may be more like SVZ young neuroblasts (type A cells), which express PSA-NCAM and doublecortin. This contention received strong support from a recent study demonstrating that most of the proliferating cells in the SGZ niche express the neuronal marker Hu [28]. Taken together, PSA-NCAM expression in the postnatal neurogenic niche seems to correlate to the developmental stage when newborn neurons start to extend processes, detach from the proliferative niche and migrate. Interestingly, proliferative cells in the SGZ, including GFAP positive radial astrocytic stem cells express N-cadherin/catenin that is progressively downregulated in neuroblasts that withdraw from the niche [28]. Thus, the balance between PSA and N-cadherin/-catenin expressions may regulate anchoring and dynamic cellular arrangements in the niche [28].

Migratory newborn neuroblasts remain highly positive for PSA-NCAM in the olfactory system, as well as in the dentate gyrus. However, after having reached their final location, newborn neurons progressively downregulate PSA-NCAM during dendritic growth and synaptic integration [25].

Putative Functions of PSA-NCAM in Adult Neurogenesis

Neuronal Precursor Migration

The functional significance of PSA on NCAM in adult neurogenic zones is incompletely understood. Genetic deletion of the NCAM molecule ($NCAM^{-/-}$) results in about 30% decrease in the size of the OB, while the overall brain size is reduced by about 10% [29,30]. These defects can be duplicated by the injection of Endo-N, an enzyme that specifically cleaves the PSA moiety associated with NCAM, suggesting that the observed phenotypical changes in the $NCAM^{-/-}$ animal are primarily related to the absence of the PSA chain itself [31]. Parallel to the reduction of OB size, an increased number of neuronal precursors are observed in the SVZ-RMS of $NCAM^{-/-}$ animals compared to wild type (WT) littermates [31,32]. It has been suggested that this accumulation of neuroblasts in the SVZ-RMS is the result of impaired chain migration of these cells toward the OB [31,33]. To our knowledge, no defects in the migration of newly generated hippocampal neurons have been

reported in the absence of PSA, most likely, because of the short distance covered by these cells. Importantly, several lines of evidences demonstrate that in the SVZ, migrating capabilities of the NCAM molecule reside on PSA tails: (i) migrating cells expressing NCAM exhibited high PSA contents [12,34,35]; (ii) functional blocking of PSA residues (by enzymatic removal or by neutralizing antibodies) without alteration of the protein core was sufficient to disrupt migration [31,34,36,37]; and, (iii) simultaneous genetic deletion of the two enzymes responsible of PSA synthesis (polysialyltransferases ST8SiaII and ST8SiaIV) led to gross abnormalities in both radial and tangential neuronal migration during development [38].

The mechanism by which PSA-NCAM might influence neuroblast migration remains unknown. It has been proposed that PSA at the cell surface is required for weak adhesive interactions that would allow cell motility [31]. However, evidence suggests that PSA-NCAM may not be required for cell motility. Instead, PSA-NCAM seems to be critical for the efficiency of migration. For example, newly generated OB precursors express high levels of PSA-NCAM and migrate to the OB using a very effective strategy known as chain migration [25,39]. Albeit less efficiently, neuroblasts devoid of PSA are still able to migrate [40]. However, they are no longer capable of forming chains, resulting in an accumulation of the migrating neurons in the SVZ [31,33,40]. Moreover, NCAM deficient cells were shown to accurately migrate when transplanted into a wild type environment, whereas wild type cells into the mutant SVZ exhibited an NCAM knockout behavior suggesting that PSA effects on SVZ-migrating neuroblasts might be modulated by the environment [33]. In order to explain these observations, it was postulated that neuroblasts use other cells in the chains as a substrate for migration and, consequently, it is the presence/absence of chains more than the PSA complement which determines whether a transplanted cell would properly migrate or not. Interestingly, migrating neuroblasts derived from NCAM knockout animals actually formed chains when cultured in high matrigel concentrations as substrate [40], indicating again that environmental cues might override the absence of PSA.

One attractive hypothesis reconciling all these observations is that PSA would be involved in regulating directed migration towards guidance cues. A recent work focusing on oligodendrocyte progenitor cells (OPCs) migration gave further support to this hypothesis [41]. Using a direct-viewing chemotaxis chamber, Zhang and colleagues provide new insights into the functional consequences of PSA removal in motile neural cells. They showed that basic locomotion (chemokinesis) of OPCs was unaffected by the absence of PSA. In contrast, directional migration (chemotaxis) towards concentration gradients of Platelet-Derived Growth Factor (PDGF) was severely impaired. Interestingly, this effect was dose-dependent, as OPCs migrated towards high concentrations of PDGF independently of PSA. These findings support the contention that PSA-NCAM is part of the regulatory network required for the adequate sensing of environmental cues conferring, thus, the competence to be directionally guided by the tiny concentrations of these factors found in the extracellular milieu. Although this hypothesis is consistent with the observation that a large number of immature neurons in the RMS of $NCAM^{-/-}$ animals show altered orientation [42], it has not been confirmed in vivo yet.

How PSA-NCAM enhances, at the molecular level, the sensitivity to attractive/repellent cues remains unknown. Work in different experimental models has demonstrated that PSA increases the responses to BDNF [43], glutamate [44,45], CNTF [46] and PDGF [41]. In addition, PSA appears to be required for FGFR activation [47]. Thus, PSA might endow NCAM with the ability to interact and/or activate a number of receptors on the cell membrane. Indeed, it has been postulated that PSA, by disrupting NCAM-NCAM homophilic interactions, might promote a switch in NCAM functions from adhesion to a signaling state [48]. Alternatively, reduced adhesion per se in the presence of PSA could modulate membrane signaling.

Survival of Newly Generated Neurons

The role of PSA-NCAM in survival of newly generated neurons has been addressed in an in vitro model of neurogenesis [49]. It has been demonstrated that removal of the polysialic tail of NCAM by Endo-N dramatically decreases the number of newly generated neurons. Similar results were obtained when PSA was blocked by a specific antibody and in cultures prepared from the NCAM$^{-/-}$ mice [49]. Using pulse-chase labeling of neuronal progenitors with the proliferation marker BrdU, it was possible to differentiate between two distinct, nonetheless closely related, events of neurogenesis, namely the mitotic activity per se and the early survival of newly generated neurons [49]. The evidence indicates that the lack of PSA-NCAM or NCAM does not influence mitotic activity, but rather it increases early cell death of newly generated immature neurons. These observations are consistent with previous data demonstrating that interfering with PSA-NCAM affects the survival of neurons in dissociated [43] and organotypic cultures [46]. Most importantly, it has been recently shown that apoptosis is increased nearly threefold in the SVZ and RMS of NCAM knockout animals versus wild types [42]. The enhanced rate of apoptotic cell death was cell specific, since it occurred in the population of migrating neuroblasts (PSA+NCAM+) but not in GFAP positive astrocytes (PSA-NCAM+), supporting the hypothesis that PSA-NCAM and not NCAM is important for cell survival. This hypothesis receives further support from in vitro experiments demonstrating that enzymatic removal or antibody blocking of PSA produce a significant increase in TUNEL labeling [42]. These results are also in agreement with recent data indicating that loss of PSA induces massive neuronal apoptosis in the developing brain [38]. Whether PSA is also a prosurvival factor in hippocampal neurogenesis has not been examined in vivo. Nonetheless, indirect evidence suggests that PSA can prevent glutamate-induced cell death of hippocampal neurons in vitro arguing for this hypothesis [44].

How might PSA-NCAM have influenced the survival of newly generated neurons? One possibility is that BDNF signaling through Trk B receptors was impaired in the absence of PSA-NCAM [43,50]. Indeed, the survival promoting effects of BDNF were significantly less in Endo-N treated and NCAM$^{-/-}$ cultures than in control preparations [42]. Another, very interesting possibility is that PSA-NCAM

influences the low affinity neurotrophin receptor p75 signaling. The first evidence for this is related to the fact that SVZ derived neurons in culture express p75, TrkB, TrkC but not TrkA, the high affinity receptor for NGF [51]. In vitro, NGF significantly increased apoptosis in cells lacking PSA. Given that TrkA is not expressed by SVZ-derived neurons, this effect is mediated by p75. In agreement with these results, pharmacological blockade of p75, but not Trk receptors, prevented neuronal cell death induced by the removal of PSA. It has also been demonstrated that the inhibition of two well-established pro-apoptotic cascades downstream of p75, ceramide and c-Jun N-terminal kinase [52–56], completely prevented neuronal cell death induced by the absence of PSA in control as well as NGF treated cultures. Together, these data raised the possibility that the removal of PSA from NCAM induced an enhanced activation of p75 signaling pathways. In agreement with this idea, it was found that both in vivo and in vitro, immature neurons lacking PSA-NCAM express significantly higher levels of p75 than control cells. However, definitive evidence for this hypothesis would require further experiments involving the use of p75 knockout animals and function blocking antibodies. The fact that PSA removal did not affect TrkB or TrkC expression illustrates the specificity of this effect.

Neuronal Precursor Differentiation

Highly polysialated isoforms of NCAM are present in neurons at the time of neuritogenesis. This could limit the level of NCAM homophilic interactions and, consequently, neurite outgrowth. In agreement with this expression profile, major defects in fasciculation and lamination of mossy fibers in the hippocampus has been described in the NCAM deficient animal [57]. Since similar deficits are also observed in the ST8SiaII/ST8SiaIV double knockout, it appears that PSA contribute to proper axonal growth of hippocampal granule cells [58].

More recent data suggest that the absence of PSA from the surface of neuroblasts may induce differentiation of newly generated neurons. According to a recent report, application of Endo-N into the rostral migratory zone results in a large number of multiple processes bearing cells [59]. This premature differentiation of neuroblasts in the absence of PSA has also been detected in the NCAM knockout animals [48] and is consistent with in vitro results showing an accelerated neurite outgrowth from neuroblastoma cells after removing PSA [60,61]. Intriguingly, Petradis et al. found that differentiation required activation of MAP kinase through p59fyn and hypothesized that, in the absence of PSA, enhanced NCAM homophylic interactions might account for this effect. However, results obtained in the NCAM$^{-/-}$ RMS argue against this possibility and suggest that additional pathways might be activated in the absence of PSA. In vitro studies provide one potential explanation to these observations. Indeed, it has been shown that PSA removal results in the rapid upregulation of p75 receptor [42]. Since p75 signaling contributes to early dendritic growth of new olfactory neurons [51], these data open the intriguing

possibility that progressive decrease in PSA contents might contribute to the arrest of migration and the initiation of neurite growth.

At later stages, neurite development seems to depend specifically on BDNF-TrkB [51]. In contrast, p75 stimulation leads to neuronal death [48]. These dual effects of p75 have been long identified [62] and are likely to be related to the complement of Trk receptors expressed at each maturational stage.

Concluding Remarks

Recent evidence suggests that newly generated neurons might endow the postnatal brain with new levels of plasticity [11]. Interestingly, PSA-NCAM appears to be tightly involved in regulating migration, survival and neurite maturation of new neurons [29,31,33,51]. The mechanism by which PSA-NCAM affects these processes is not yet clear. Influencing p75 neurotrophin receptor expression and signaling might play a role in these processes. It is intriguing that p75 receptors have been implicated in cell death as well as in neurite outgrowth [48,51]. It is possible that by limiting p75 expression, PSA-NCAM protects newborn neurons from being dependent on trophic support, restrains neurite outgrowth and promotes migration before integration into functional circuits. The progressive downregulation of PSA-NCAM and the increase in p75 expression after neurons reached their appropriate place would contribute to the adequate maturation and synaptic integration of new neurons (Fig. 1).

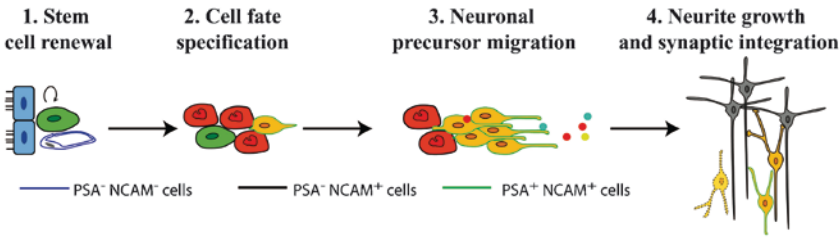

Fig. 1 Proposed model of PSA functions in adult neurogenesis. Schematic representation of PSA-NCAM expression in the sequential steps of adult neurogenesis. (a). Neural stem cells (*green cells*) require a complex local microenvironment (neuro-vascular niche) for self renewal. The niche, represented here by blood vessels (in white) and ependymal cells (*in blue*), contains NCAM but is essentially devoid of PSA. NCAM does not seem to be involved neither in stem cell maintenance nor in niche physiology. (b) When stem cells divide asymmetrically, they give rise to transient-amplifying (*red*) cells that will differentiate into committed neuroblasts (*in yellow*). Similar to stem cells, transient-amplifying cells express nonsialylated NCAM that might participate in cell fate choice. (**c**) After specification, neuroblasts detach from the niche and migrate to their final location. PSA-NCAM is strongly expressed in migratory neuroblast where it might be essential for the adequate sensing of tiny concentrations of environmental cues (*colored circles*) required for efficient migration. (d) In the olfactory bulb, progressive downregulation of PSA results in a parallel increase of p75 that initially promotes neurite outgrowth and then the apoptotic elimination of misplaced/nonconnected neurons (*dashed cell*)

Numerous questions remain. For example, does PSA-NCAM play a role in the synaptic integration of new neurons? How is the polysialylation state of NCAM regulated during this process? Do the activation of new neurons and the physiological network activity regulate PSA-NCAM expression? If yes, how? Could PSA engineering promote the generation and integration of new neurons? Answering these questions represents the next challenge for future research. More importantly, with the recent association of PSA-NCAM with a number of psychiatric diseases [63–65], this information will be invaluable in the development of therapeutic treatment for these disorders.

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Use of PSA-NCAM in Repair of the Central Nervous System

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Introduction

Polysialic Acid and Global Regulation of Cell Interactions

Polysialic acid (PSA) is a long linear homopolymer of alpha-2,8-linked sialic acid. In vertebrates, this glycan is added to the fifth Ig-like domain of the neural cell adhesion molecule (NCAM) (Fig. 1a). While the regulation of PSA expression is complex and remains poorly understood, the addition of the polymer to NCAM is known to be carried out by two Golgi polysialyltransferases, ST8SiaII (STX) and ST8SiaIV (PST). Either enzyme is capable of producing long PSA chains [1, 2].

A fundamental property of this long, hydrated homopolymer is its enormous excluded volume that prevents close contacts between the membranes of neighboring cells [3] (Fig. 1b). Recent biophysical tests using lipid bilayers containing adhesion molecules and PSA have confirmed that this polymer increases molecular repulsion and attenuates transbinding between surface molecules [4]. The fact that PSA can affect cell interactions, both in vitro [4, 5] and in vivo, [6] that do not necessarily depend directly on NCAM's intrinsic activity, emphasizes that PSA provides for a global regulation of cell interactions (Fig. 1b).

These steric effects of PSA at the cell surface provide an attractive and consistent mechanism for explaining the diverse biological roles of PSA during development and in the adult, as described below. Nevertheless, other modes for PSA function, such as cell-matrix interaction and effects on neurotrophins, are possible and might contribute as well. See [7].

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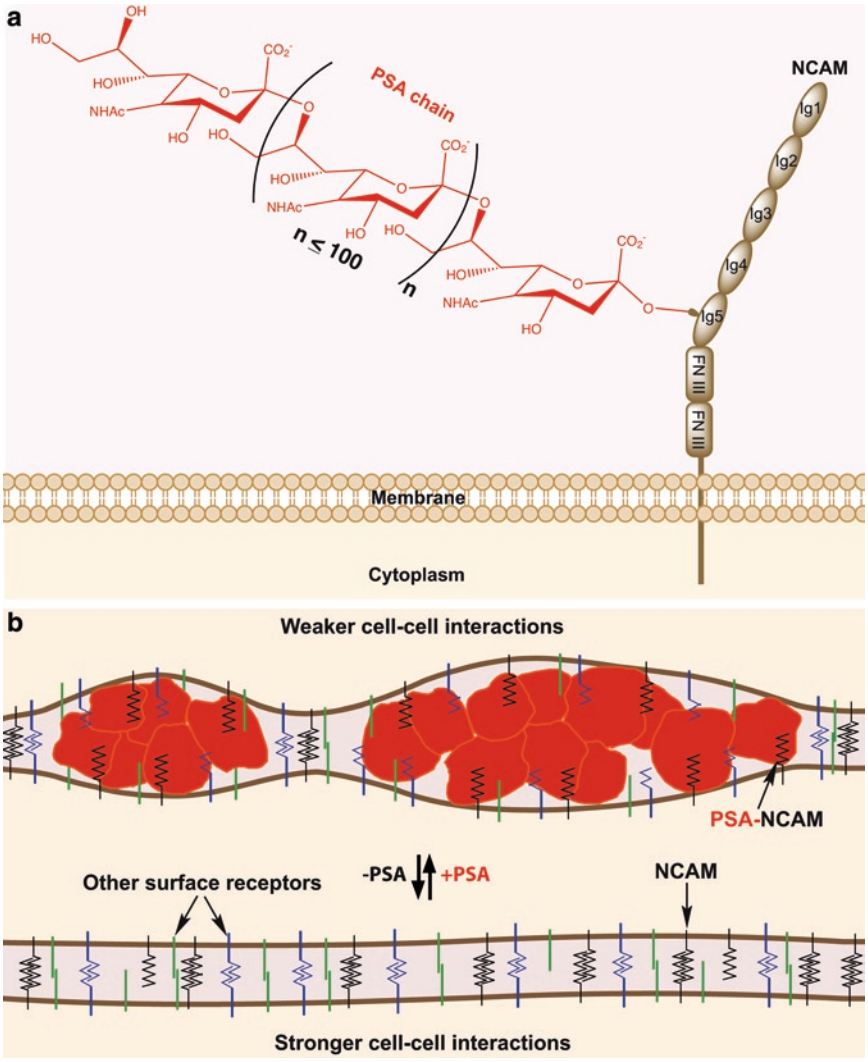

Fig. 1 PSA-NCAM and global regulation of cell-cell interactions. (**a**) Drawing of a PSA chain attached to the fifth Ig-like domain of NCAM at the cell surface. (**b**) Representation of cell-cell interactions in the presence and absence of PSA (red). Note that PSA's large excluded volume restricts close contact between membranes of neighboring cells. Although PSA is only attached to NCAM, its effect on membrane-membrane apposition results in a global regulation of *trans* interactions mediated not only by NCAM but also by other cell-contact-dependent receptors (green and blue). FN: fibronectin domain, Ig: immunoglobulin domain, NCAM: neural cell-adhesion molecule

PSA-Induced Tissue Plasticity

PSA's modulation of cell interactions has the ability to enhance tissue plasticity by facilitating changes in cell position and shape. This is particularly useful in the developing brain where PSA is abundantly expressed on both neural and non-neural precursors.

At the end of development, PSA expression is downregulated except in certain brain regions that continue to undergo morphological and/or physiological changes during adulthood. See [7, 8]. As a prelude to a review of PSA in tissue repair, the following discussion focuses on neural precursor migration and establishment of neuronal connections.

PSA Promotes Precursor Cell Migration

The best documented example of PSA function in cell migration is that of progenitors born in the subventricular zone (SVZ). These cells express high levels of PSA and migrate along the rostral migratory stream (RMS) to replenish the interneurons of the olfactory bulb. When PSA is removed experimentally, the SVZ cells are unable to move away from the germinal zone, resulting in a smaller olfactory bulb [9–15]. PSA has been suggested to facilitate cycles of adhesion and de-adhesion that are required when these cells move forward in a stream, using each other as a substrate [12, 16]. PSA expression has also been shown to facilitate migration of newborn hippocampal granule cells [17], as well as that of other neural precursors [15, 18].

PSA Facilitates Development of Neuronal Projections

Neurons that produce long axonal tracts express high levels of PSA. In some instances, PSA allows these axons to defasciculate, e.g., projections of spinal motoneurons in the plexus region, where they are instructed by local cues to form multiple nerve branches [19, 20]. In other situations, PSA can help axons ignore inappropriate interactions with their environment [21], e.g., it can shield growing axons from forming synapses in pretarget regions of the optic tract/tectum and optimize navigation of their growth cones [22]. In addition, PSA enhances arborization and target innervation, in that its removal reduces the length and number of retinotectal branches [22]. A similar role is observed in the developing corticospinal tract where PSA facilitates initiation of sprouting and allows branching of collaterals during innervation of the spinal cord [23].

Use of PSA Gain-of-Function to Promote Adult Tissue Repair

In general, the adult mammalian CNS is refractory to structural change and thus, is unable to reverse damage produced by injury or disease. On the other hand, the engineered overexpression of PSA has the potential for producing favorable conditions for repair in tissues. There are two basic strategies by which PSA can be used

in this context: (1) unleash endogenous repair mechanisms by directly reducing the inhibitory effects of the CNS environment, and (2) enhance the effectiveness of transplanted cells that have been designed to either compensate for deficiencies or support the repair process. To provide for PSA expression, synthesis of the glycopolymer is induced by introduction of the polysialyltransferase gene. The construct is delivered, usually using a virus, either directly into the damaged tissue, or into the exogenous cells prior to transplantation.

In using PSA to promote repair, the effects of the treatment on normal tissue must also be considered. That is, will PSA expression also result in alteration of healthy tissue? Fortunately, PSA by itself does not dictate the nature of the change that occurs in developing tissues, but rather exerts a permissive action that allows the forces that are driving change to override stabilizing cell interactions such as adhesion [7]. Therefore, if healthy tissue is not trying to reorganize its structure, such deleterious effects may well be minimal. While this seems to be the case in studies in which PSA expression is induced in host tissue [24, 25], this possibility will need to be evaluated in the context of each application.

The following proof-of-principle studies illustrate each of the expression strategies and delivery options described above, as applied to repair situations that require axon outgrowth or cell migration.

Regeneration of Damaged CNS Axons

Severed CNS axons are unable to regrow and reinnervate their targets (Fig. 2a). This deficiency is typically not the result of an intrinsic incapacity of CNS axons to resume elongation, but instead reflects a nonpermissiveness of the CNS environment in general, and of the scar tissue at lesion sites, in particular [26–30]. The dense astrocytic scar not only constitutes a physical obstacle to axonal growth cone, but also contains growth inhibitory signals on astrocytes, oligodendrocytes and in the extracellular matrix [28, 29, 31].

Engineered expression of PSA on scar astrocytes. For a brief period after lesion, the CNS can actually be supportive of axonal sprouting. This short-lived regenerative attempt coincides with a transient upregulation of PSA on astrocytes, and axons with more persistent sprouting tend to be in close apposition with a subpopulation of astrocytes that can prolong their PSA expression [32, 33]. This relationship between PSA and enhanced axon response to injury is strengthened by the fact that PSA is upregulated at the site of a peripheral nerve lesion, where it has been shown to promote selective motor axon targeting, collateral sprouting, terminal arborization as well as withdrawal of misprojections [34].

Overexpression of PSA on lesion astrocytes for an extended period has the potential of both loosening physical barriers imposed by the dense scar tissue, and attenuating inhibitory interactions with growth cones, thereby sustaining the regenerative process beyond the abortive sprouting phase. To achieve this sustained expression at the site of a spinal cord injury, the HIV(ALSV-A) virus/GFAP-TVA

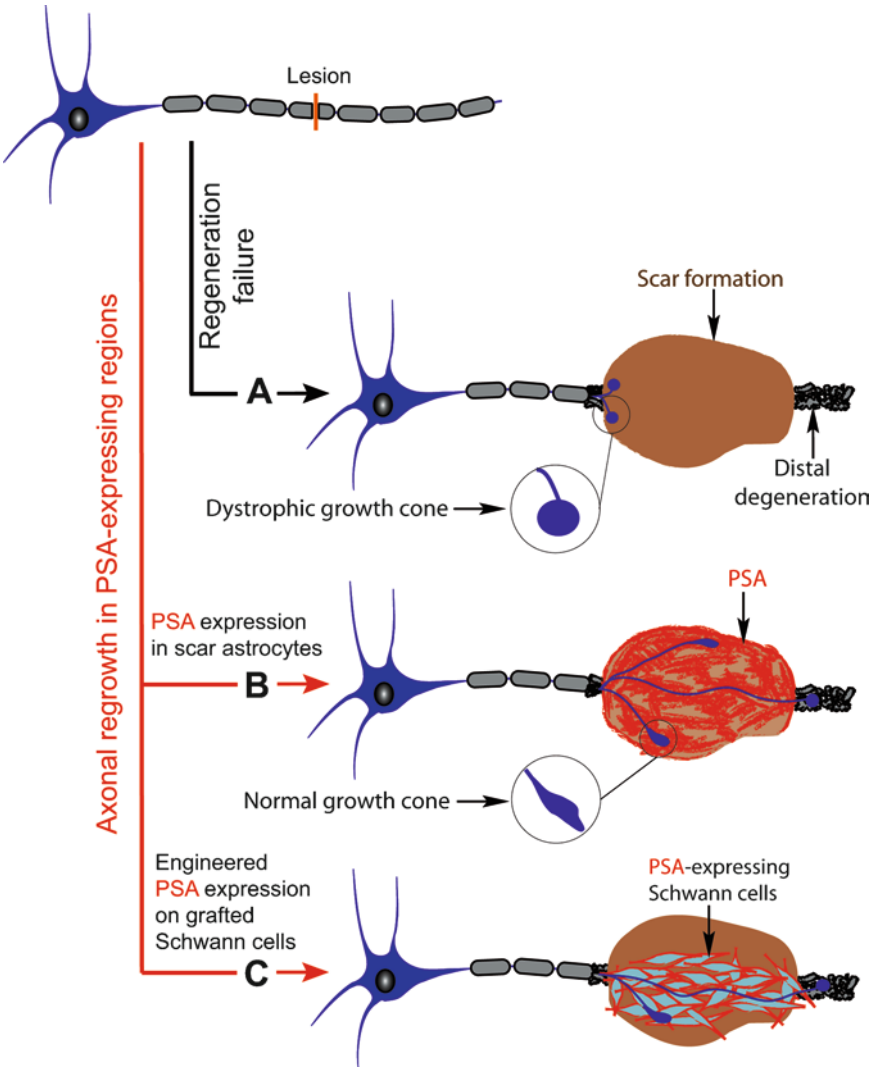

Fig. 2 Engineered PSA expression promotes CNS axon regeneration. The drawing illustrates regeneration of CNS axons through a lesion site. The scar tissue constitutes an inhibitory environment for axonal regeneration (**a**) however local overexpression of PSA on scar astrocytes renders the tissue permissive to axonal regrowth (**b**) [24, 25, 37, 38]. In addition, the engineered expression of PSA on grafted Schwann cells appears to enhance their regeneration promoting effect (**c**) [37, 42]. Note that, in the presence of PSA, torpedo-shaped growth cones replace the dystrophic ones associated with growth inhibition

transgenic system [35, 36] was used to selectively infect astrocytes with the gene encoding the PST (Sia8IV) polysialyltransferase. A sustainable high level of PSA expression was observed on these cells for over 6 weeks, and nearly 10% of lesioned, corticospinal fibers were able to regrow completely through the injury site

and into the segment of the spinal cord distal to the lesion (Fig. 2b). Across the lesion site, axons grew in close proximity with PSA-expressing astrocytes, while swollen dystrophic growth cones, indicative of abortive growth [26, 31], were found in PSA-poor areas. However, after exiting the PSA-expressing scar, the regenerating axons were unable to continue growing in the distal stump (Fig. 2b), probably because of the inhibitory nature of this new, PSA-negative environment [24, 25].

A similar approach was used to augment the sprouting response of Purkinjie cells in lesioned cerebellum [37], and the growth of sensory axons into a spinal lesion cavity, with some axons growing across the cavity and extending into the rostral cord [38]. In this study, regeneration of Purkinje axons was additionally stimulated by GAP-43 and L1 over-expression, and regrowth of spinal sensory fibers was prepotentiated by a peripheral nerve-conditioning lesion. Although the viral gene delivery system used in these studies was not engineered to specifically target astrocytes, the PSA expression obtained included large numbers of these cells [37, 38]. However, it is possible that the contribution of PSA in these studies also reflects other cell types.

Transplantation of Schwann cells engineered to express PSA. Schwann cells are known to support peripheral nerve regeneration and their transplantation has been shown to promote regeneration of lesioned CNS axons as well. They produce stimulating factors and provide both a substrate for regrowth and ensheathment of regenerating axons [39–41].

On the basis of PSA's role during development, engineering PSA expression into Schwann cells prior to transplantation might be expected to affect their axon growth promoting capabilities. When Schwann cells were transformed in vitro with a PST lentiviral vector and then transplanted into a cerebellar lesion in the GAP-43 and L1-enhanced mice described above, their ability to support regeneration of lesioned Purkinje axons increased about nine times [37] (Fig. 2c). Grafting of PSA-engineered Schwann cells into a spinal contusion injury also facilitated elongation of serotoninergic fibers [42]. In this study, PSA expression levels were decreased after the first week after transplantation, at which time a stimulation of axonal remyelination by both host and transplanted Schwann cells as well as by resident oligodendrocytes was obtained. An improvement in functional recovery was also claimed to result from the transplantation.

Delivery of Neural Progenitors to a Brain Injury

Creation of a pathway for endogenous progenitors. Several studies have shown that SVZ neural progenitors, in addition to their massive migration to the olfactory bulb, can also populate lesioned areas of the adult neocortex and striatum [43–46]. Although the recruited cells can differentiate and integrate into the damaged tissue [44, 47], their numbers are too small to provide significant restoration of function.

As discussed above, newly generated PSA-expressing SVZ progenitors migrate along the RMS in a manner that suggests that they use each other as a substrate. Thus, it is possible that expression of PSA in the cortical environment could also improve the recruitment of SVZ progenitors. To test this hypothesis, PSA expression was induced in cortical astrocytes along a needle-track pathway extending from the SVZ to a cortical lesion, using the HIV(ALSV-A) virus/GFAP-TVA transgenic system described above, (Fig. 3a) [25].

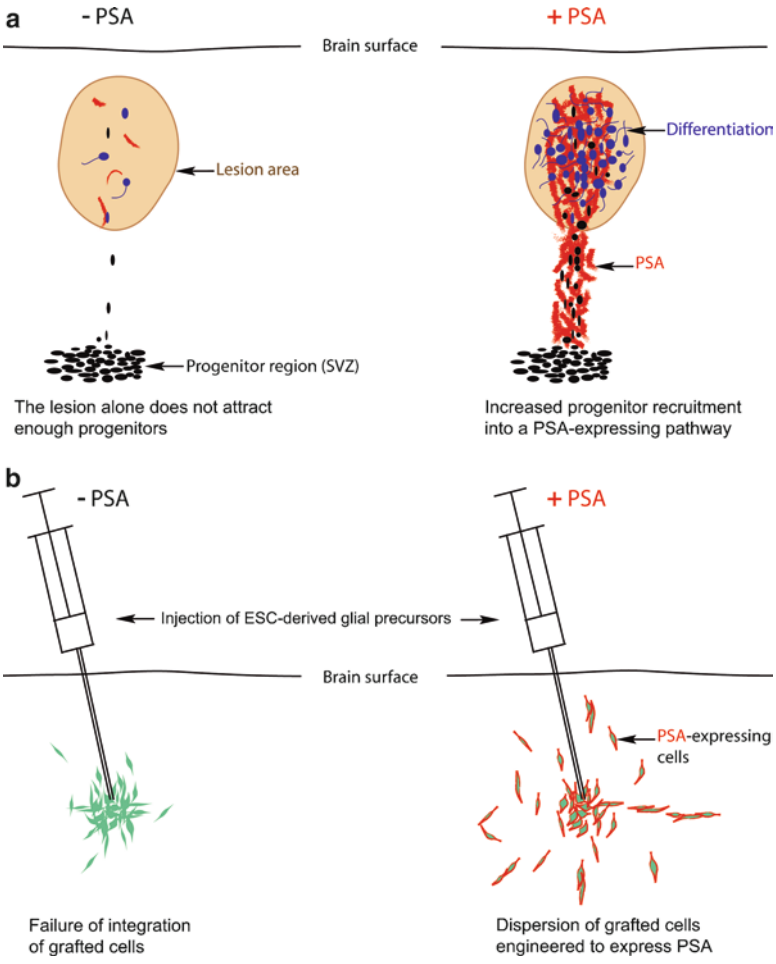

Fig. 3 PSA overexpression enhances brain cell therapy. (**a**) The creation of a PSA-expressing path between the SVZ and a lesion site facilitates recruitment of progenitors to replenish the damaged area [25]. The recruited cells are then able to differentiate in an environment-dependent manner. (**b**) Overexpression of PSA on grafted ESC-derived glial progenitors facilitates their dispersion into the host tissue [48]

The PSA pathway by itself was able to increase migration by four-fold over controls, but for only a short distance into the corpus callosum. With introduction of a large cortical lesion, however, there was a six-fold increase in migrating cells and they extended all along the pathway as well as laterally into the lesion (Fig. 3a). The SVZ cells in the pathway displayed leading processes characteristic of migrating cells.

If the cells that have been recruited are to be useful for repair, they will need to differentiate in a region-appropriate manner. In this respect, it is encouraging that the SVZ cells recruited into the corpus callosum became mostly oligodendrocytes, a cell type necessary for remyelination, whereas those that repopulated the cerebral cortex primarily adopted a neuronal fate [25].

Transplantation of progenitor cells engineered to express PSA. In a replacement therapy using transplanted cells, the grafted cells not only need to be steered toward an appropriate fate, but they also have to be able to migrate to and within the injury site. Expression of PSA in ES cell-derived glial progenitors using the STX polysialyltransferase gene was found to enhance their migratory potential after transplantation in adult rodent striatum [48]. While the cells increased their overall dispersion, (Fig. 3b), they also showed a pronounced tendency to travel towards the SVZ germinal zone, sometimes forming clusters in subependymal niches. This preferential migration toward the SVZ is reminiscent of the switch from a dorsolateral to a ventral route that PSA-expressing mouse neural precursors undergo when grafted in the developing chick neural crest [49]. After migration, the transplanted PSA-expressing glial progenitors were able to differentiate into both astrocytes and oligodendrocytes [48].

Summary and Prospects

The studies described here provide an encouraging beginning to the adaptation of PSA biology to tissue repair therapeutics. Induction of PSA expression has been shown to be possible both in vitro and in vivo, with persistence adequate for at least some aspects of CNS repair. The results obtained appear to be consistent with the roles observed for PSA in normal development, which aids considerably in the choice and design of therapeutic strategies. With positive effects being observed for both cell migration and axon outgrowth, with apparent retention of differentiation capabilities, the approach would appear to be quite versatile.

From these effects alone, one can already conceive of applications in which PSA engineering might contribute to aspects of treatment for a variety of CNS conditions. For example, local overexpression in the CNS tissue can potentially increase migration of endogenous progenitors into stroke lesions, while PSA engineering in ESC-derived dopaminergic grafts may allow them to disperse and integrate well into Parkinsonian host tissue.

That having been said, there is obviously a long way to go. Significant challenges are still to be met in order to optimize the PSA delivery and expression protocols.

Moreover, these will need to be adapted to meet the requirements of specific clinical situations. To this end, nonviral methods for engineering PSA in tissues should be explored, including the use of small molecules to alter PSA biosynthesis, direct PSA synthesis at the cell surface, and the use of PSA-mimetics [50].

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Part III
NCAM-Mediated Signal Transduction

Signaling Pathways Involved in NCAM-Induced Neurite Outgrowth

Dorte Kornerup Ditlevsen and Kateryna Kolkova

Introduction

The neural cell adhesion molecule (NCAM) is an immunoglobulin superfamily member that plays an important role in the developing nervous system. In the adult brain, NCAM is involved in synaptic plasticity, including the processes of learning and memory formation. Moreover, NCAM has been linked to human brain disorders such as Alzheimer's disease [1], bipolar disorder [2, 3], and schizophrenia [4].

Two genes encoding the related NCAM proteins, NCAM1 and NCAM2, have been identified. NCAM2 was discovered relatively late (1997) [5] compared with the discovery of NCAM1 (1974) [6]. The present review will focus on NCAM1, which will be referred to simply as NCAM. NCAM is expressed in three major isoforms that are products of alternative splicing of a single gene product on the surface of most neural cells. The isoforms have been named according to their apparent molecular size. The two largest isoforms, NCAM-140 and NCAM-180, are transmembrane, and only differ in the size of their cytoplasmic domain, whereas the NCAM-120 extracellular domain is attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor. NCAM-180 is expressed in neurons, NCAM-120 is expressed in glia, and NCAM-140 is expressed in both cell types [7]. The NCAM ectodomain consists of two membrane-proximal fibronectin type III (F3) modules followed by five immunoglobulin-like (Ig) modules. NCAM is the dominant carrier of polysialic acid (PSA) that is attached posttranslationally to the fifth Ig module. The expression of PSA on NCAM decreases markedly during development. In the adult brain, PSA is confined to areas that retain a high degree of plasticity, such as the olfactory bulbs and the dentate gyrus of the hippocampus [8].

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NCAM mediates cell adhesion by a homophilic binding mechanism (i.e., NCAM on one cell binds to NCAM on an opposing cell). Importantly, NCAM does not function merely as glue that adheres cells together. In contrast, upon homophilic binding, NCAM initiates the formation of intracellular membrane-proximal signaling complexes, thereby activating a complex network of signal transduction. Notably, also heterophilic NCAM interactions have been demonstrated to regulate cellular events such as differentiation, proliferation and survival [9–11]. NCAM interacts with a number of heterophilic binding partners such as fibroblast growth factor receptor (FGFR), the cell adhesion molecule, L1, and a number of components of the extracellular matrix, and has, in addition, recently been suggested to regulate signaling via the receptors for several growth factors; brain-derived neurotrophic factor (BDNF; [12]), glial cell-line derived neurotrophic factor (GDNF; [13]), platelet-derived growth factor (PDGF; [14]), and epidermal growth factor receptor [15].

The precise mechanism of homophilic NCAM binding remains controversial. However, when the structure of the combined Ig1-3 modules recently was solved by X-ray crystallography [16], a new model was proposed to reconcile many of the previous discrepancies. This model suggests that NCAM engages in *cis* dimers in the absence of opposing NCAM-expressing cells. These *cis* dimers bind in *trans* to existing *cis* dimers on opposing cells in two different ways. In the flat zipper formation, Ig2 and Ig3 bind in an antiparallel manner to Ig2 and Ig3 of an opposing NCAM dimer, whereas in the compact zipper formation, all three outermost Ig domains are engaged in antiparallel binding (for explanation see [17]). In combination, the two zipper formations may result in a tightly packed two-dimensional zipper which, due to the limited space, is only possible in the absence of PSA. When PSA is attached to NCAM, only one-dimensional zippers are hypothesized to form, favoring interactions with heterophilic partners. In the absence of PSA, in contrast, tight two-dimensional zippers are hypothesized to favor homophilic adhesion over heterophilic interactions (for explanation see [17]). This hypothesis is in agreement with reports suggesting that the expression of PSA is a plasticity-promoting phenomenon [18].

Here, we review the available data on NCAM-mediated signal transduction gained in the last two decades, focusing primarily on the signaling events underlying NCAM-mediated neurite outgrowth, although the signaling events involved in NCAM-mediated neuronal survival [19] and astrocyte proliferation [20] also have been studied.

Different approaches are used for NCAM stimulation in vitro. In the so-called co-culture setup, neurons or neuronal cell lines are grown on top of a confluent monolayer of fibroblasts transfected to express NCAM, to allow homophilic binding between NCAM expressed on the neuron and NCAM expressed on the fibroblasts [21, 22]. Other approaches used for the purpose of stimulating NCAM include cross-linking of NCAM molecules with antibodies against NCAM [23, 24], the addition of recombinant proteins comprising parts of NCAM fused to the Fc portion of IgG [25, 26], or small synthetic peptides mimicking NCAM binding [27, 28].

The MAPK Pathway

The mitogen-activated protein kinases (MAPKs), extracellular regulated kinases ERK1 and 2 are central players in NCAM signaling. Increased ERK1 and ERK2 phosphorylation following NCAM stimulation using NCAM antibodies, or NCAM-Fc proteins in neuroblastoma cells, and cerebellar granule neurons (CGN), was originally demonstrated by Schmid and colleagues [24]. These results were confirmed by Kolkova and colleagues [21] in rat pheochromocytoma (PC12) cells and hippocampal neurons grown in coculture with NCAM-negative or NCAM-positive fibroblasts. Moreover, MAPK enzymatic activity has been demonstrated to increase upon NCAM stimulation in correlation with the increased phosphorylation of these kinases [24]. The importance of MAPKs in neurite outgrowth has been demonstrated by inhibition of the upstream activator MAP and ERK kinase (MEK) inhibiting NCAM-induced neurite outgrowth in CGN [24] and PC12 cells [21]. Inhibition of MEK also inhibited NCAM-mediated neuronal survival in dopaminergic neurons [19].

Raf is activated by Ras, and is a well known activator of MEK. Expression of a dominant negative version of Ras or Raf has been demonstrated to inhibit NCAM-mediated neurite outgrowth from PC12 cells. Conversely, expression of constitutively active versions of these two signaling molecules resulted in neurite outgrowth in the absence of NCAM homophilic interactions, thus resembling the effect of NCAM stimulation [21]. Moreover, expression of dominant negative Ras inhibited NCAM-mediated ERK phosphorylation in B35 and COS cells [24]. At least two possible routes exist for the signal going from NCAM to the Ras-MAPK pathway: one through the nonreceptor tyrosine kinase Fyn, and the other through FGFR. Both routes are involved in NCAM signaling and will be discussed in the sections that follow.

Fibroblast Growth Factor Receptor

FGFR is an important extracellular NCAM interaction partner. FGFR involvement was suggested originally in 1994 when inhibitory antibodies against FGFR were found to block NCAM-mediated neurite outgrowth in CGNs [22]. The question of whether NCAM interacts directly with FGFR or through intermediate binding partners was answered in 2003 when data demonstrating direct binding between the extracellular modules of NCAM and FGFR were presented [29]. In the 2003 study, a recombinant protein consisting of the two NCAM fibronectin modules was shown to bind to a recombinant protein consisting of the second and third FGFR Ig modules using surface plasmon resonance (SPR). Moreover, weak binding was detected by nuclear magnetic resonance (NMR) between the second NCAM fibronectin module and the third FGFR Ig module. In addition, a peptide derived from the second NCAM F3 module, termed FGL, and a peptide derived from the first F3

module, termed FRM, was demonstrated to induce FGFR phosphorylation, neurite outgrowth, and neuronal survival in primary rat neurons [29–31]. How binding of NCAM to FGFR results in activation of FGFR is still unclear, but based on the available data, regarding NCAM homophilic binding discussed above, and given that FGFR is activated by dimerization resulting in *trans*-phosphorylation, the following mechanism may be proposed. NCAM is hypothesized to be present at the cell surface as *cis*-dimers in the absence of NCAM *trans*-homophilic binding. An estimated 85% of the FGFR molecules in neuronal plasma membranes will be bound, at any given time, to an NCAM dimer (for explanation see 29). Approximately 1,000-fold more NCAM molecules than FGFR molecules exist in the neuronal plasma membrane. Therefore, two FGFR molecules are unlikely to be bound to the same NCAM dimer. However, when NCAM engages in *trans* homophilic binding, NCAM clustering will increase the concentration of NCAM-associated FGFR molecules, resulting in a higher probability of FGFR dimerization and activation [17].

Further evidence supporting an important role of FGFR in NCAM-mediated signaling has been provided by studies on NCAM-mediated neurite outgrowth. Expression of dominant negative FGFR in PC12 cells or CGNs abolished NCAM-mediated neurite outgrowth [28, 32]. Use of inhibitory antibodies to FGFR or a pharmacological inhibitor of FGFR likewise reduced NCAM-induced neurite outgrowth from PC12 cells [21], CGNs [22], and hippocampal neurons [33]. FGFR also is involved in another cellular response to NCAM signaling, neuronal survival. Inhibition of FGFR abolished NCAM-mediated neuronal survival in rat dopaminergic neurons induced to undergo apoptosis [19].

NCAM does not merely mimic the actions of fibroblast growth factors (FGFs) on FGFR. The “acid box” region of FGFR has been demonstrated to be dispensable for FGF function but required for NCAM interaction with FGFR [34], suggesting that NCAM interacts with a region of the receptor which is not required for FGF binding. Moreover, diverging activation patterns of adaptor molecules following activation of FGFR by FGF2 compared with activation of FGFR by NCAM has been demonstrated, whereas FGFR substrate-2 (FRS2) and Grb2 play important roles in both NCAM and FGF2 signaling, the docking protein ShcA was shown to be involved in NCAM-mediated neurite outgrowth but not in FGF2-mediated neurite outgrowth [35]. Thus, FGFR mediates activation of a different pathway when stimulated through NCAM than when stimulated by FGF2.

FGFR has long been assumed to be a mediator of the signal from NCAM to the MAPK pathway, and this assumption has been based on knowledge of FGF signaling, but inhibition of FGFR only recently has been demonstrated to abolish ERK phosphorylation induced by the NCAM mimetic C3d (unpublished data, [36]). C3d is the dendrimeric version of a synthetic peptide which, in the absence of NCAM homophilic binding, elicits cellular responses similar to the responses resulting from NCAM homophilic binding [37]. In Chinese hamster ovary (CHO) cells transfected to express the individual NCAM isoforms alone, ERK phosphorylation induced by NCAM antibodies has been found to depend on FGFR when NCAM-180 is expressed alone, but not when NCAM-140 is expressed alone.

In neuroblastoma cells expressing all three isoforms, inhibition of FGFR had no effect on NCAM-mediated ERK phosphorylation [26]. The authors suggested on the basis of these results that NCAM-180 signals via FGFR, whereas NCAM-140 does not predominantly signal via FGFR. Therefore, when FGFR is inhibited in the presence of NCAM-140, alternative signaling pathways are employed for ERK activation.

This proposal contrasts with the data mentioned above that demonstrate a requirement for FGFR in NCAM-mediated ERK phosphorylation in rat CGNs (unpublished data, [36]) that express both NCAM-140 and NCAM-180 [7]. This apparent discrepancy may result from the use of primary neurons as opposed to cell lines. Nonneuronal cells are likely to provide a different environment of interaction partners compared with primary neurons, possibly resulting in a different cellular response. Moreover, the expression of individual NCAM isoforms creates an artificial situation where the possibility of NCAM isoform heterodimer formation is prevented.

In conclusion, FGFR plays an important role in NCAM-mediated neurite outgrowth and neuronal survival and is necessary for NCAM-induced activation of the MAPK pathway, at least for some isoforms of NCAM.

Nonreceptor Tyrosine Kinases, Fyn and FAK

Members of the Src-family of nonreceptor tyrosine kinases are highly expressed in neural tissue and concentrated in membranes of neuronal growth cones in patterns similar to cell adhesion molecules [38]. The involvement of Src-family kinases in NCAM signaling was tested initially by growing sensory neurons and CGNs from mice carrying null mutations of the *Fyn*, *Src*, or *Yes* genes. Using this approach, NCAM-mediated neurite outgrowth was inhibited in Fyn^- but not in Src^- or Yes^- neurons, suggesting that Fyn is an important component in NCAM signaling [39]. Fyn was later found, by co-immunoprecipitation, to constitutively associate with NCAM-140 but not with NCAM-120 and NCAM-180 from the mouse cerebellum. Furthermore, NCAM clustering by antibodies or treatment of B35 cells with an NCAM-Fc fusion protein was reported to result in Fyn phosphorylation [23]. Further confirmation of the role of Fyn in NCAM-mediated signaling came when inhibition of Src-family kinases was demonstrated to abolish NCAM-mediated neurite outgrowth [21] and neuronal survival [19]. Additionally, activated Fyn is reduced in brains of NCAM-deficient mice [40].

The mechanism for the association of NCAM with Fyn recently has been suggested to depend on binding of a third interaction partner, the receptor protein tyrosine phosphatase-α (RPTPα) that, like NCAM and Fyn, is highly expressed in neurons and growth cones and is known to activate Src-family kinases [41]. This suggestion was based on several lines of evidence. First, Fyn is not co-immunoprecipitated with, and not activated by, NCAM in RPTPα-deficient neurons. Second, NCAM partially co-localizes with RPTPα, and clustering of NCAM with antibodies increases this colocalization, which was confirmed by immunoprecipitation.

Third, NCAM-mediated neurite outgrowth is abolished in RPTPα-deficient hippocampal neurons as well as by expression of dominant negative mutants of RPTPα in wildtype hippocampal neurons [40]. Interestingly, NCAM-140 was found to be the most potent RPTPα-binding isoform, which is in agreement with the findings of Beggs and colleagues described above, that NCAM-140 constitutively associates with Fyn, whereas NCAM-180 and NCAM-120 do not [23].

Following activation of Fyn, another nonreceptor tyrosine kinase is recruited and activated by phosphorylation. Focal adhesion kinase (FAK) has been shown to coimmunoprecipitate with Fyn and NCAM in COS, and B35 cells upon NCAM stimulation [23]. Accordingly, NCAM-mediated neurite outgrowth has been reported to be abolished in PC12 cells that express a dominant negative FAK [21]. The MAPK pathway is hypothesized to be activated downstream of Fyn and FAK, based on experiments by Schmid and colleagues [24], showing that expression of dominant negative versions of FAK in B35 and COS cells resulted in decreased NCAM-mediated ERK phosphorylation. This hypothesis was confirmed by more recent experiments demonstrating that inhibition of Src-family kinases decreased NCAM-mediated ERK phosphorylation in CGNs (unpublished data, [36]). In conclusion, NCAM is constitutively associated with Fyn via RPTPα. Upon NCAM stimulation, FAK is recruited and activated by phosphorylation. FAK, in turn, activates the MAPK pathway, thus resulting in neurite outgrowth and neuronal survival. Consequently, NCAM signals to the MAPK pathway via two separate pathways, one pathway initiated by binding to FGFR and the other initiated by recruitment of FAK to the Fyn-RPTPα complex.

Rafts and Cytoskeletal Components

Signaling through different membrane-proximal complexes likely is regulated by localization to raft or nonraft membrane areas. Lipid rafts are hypothesized to dynamically compartmentalize the membrane by functioning as platforms for specific classes of proteins, thereby regulating the likelihood that specific proteins will encounter each other, depending on their localization within or outside of the rafts [42]. The association of NCAM with lipid rafts has been suggested by several groups. Initially, before the advent of the raft concept, NCAM-120 was found not to detach from the membrane by urea washing, indicating a strong membrane attachment of this isoform [43]. Disagreement exists about whether all three isoforms are raft-associated. Only NCAM-120 and NCAM-140 were found in the raft fraction of isolated rat growth cones [44], whereas all three isoforms were found in the raft fractions of mouse brain homogenates, neuroblastoma cells [26], and rat synaptosomal membranes [33]. The apparent discrepancy likely reflects differences in raft localization of the NCAM isoforms between the different cell types employed.

NCAM-120 is assumed to be associated with lipid rafts via its GPI anchor, similar to many other GPI-linked proteins. NCAM-140 and NCAM-180 have four

palmitoylation sites in the membrane-proximal part of the cytoplasmic domain. When a mutant NCAM-140 lacking these sites was expressed in CHO cells, the raft association was lost, indicating the importance of these sites in NCAM raft association [26].

The localization of the transmembrane isoforms appears to be dynamically regulated. Thus, NCAM is redistributed into rafts upon NCAM clustering [40, 45], and this redistribution has been suggested to depend on the prion protein (PrP). PrP has been demonstrated to interact directly with NCAM and to be involved in stabilization of NCAM in lipid rafts. Thus, the amount of NCAM-140 and NCAM-180 in lipid rafts is reduced in brains of $PrP^{-/-}$ mice compared with wildtype mice, and coexpression of PrP and NCAM-140 in CHO cells was found to enhance targeting of NCAM-140 to lipid rafts [46].

Several findings suggest that NCAM distribution between raft and nonraft areas is an important mechanism regulating the formation of membrane-proximal signaling complexes. Src-family kinases, such as Fyn, are believed to be raft associated [42], and exclusion of NCAM-140 from rafts by mutation of the palmitoylation sites abolishes NCAM-140-mediated FAK activation [26]. Santuccione and colleagues demonstrated that NCAM-mediated Fyn activation was inhibited in neurons from $PrP^{-/-}$ mice where NCAM localization to lipid rafts was reduced [46]. FGFR appears to be absent from lipid rafts [26, 47], suggesting that the two pathways resulting in MAPK activation (FGFR and Fyn/FAK) discussed above are spatially separated.

NCAM is linked to the cytoskeleton via spectrin, and spectrin is associated with lipid rafts. Spectrin associates with membrane lipids and with a variety of adaptor proteins, thereby acting as a scaffolding protein for the organization of microdomains of the membrane, and linking the membrane to the cytoskeleton [48]. An early study demonstrated that spectrin binds specifically to NCAM-180 [49], and other isoforms were not found to associate with spectrin. Later, however, all three isoforms were found to associate with spectrin in immunoprecipitates, and the intracellular domains of NCAM-140 and NCAM-180 were found to bind directly to spectrin [45]. NCAM-140 displayed a significantly lower affinity for spectrin than NCAM-180, and this may explain why the interaction between NCAM-140 and spectrin was not detected in the earlier study. Raft association of both proteins possibly explains co-immunoprecipitation of NCAM-120 with spectrin, despite the lack of an intracellular domain in NCAM-120 [45]. Alternatively, NCAM-120 may take part in a heterodimer with NCAM-140 or NCAM-180, and thereby associate with spectrin. However, no experimental evidence has demonstrated that heterodimers are formed. NCAM clustering results in recruitment of spectrin and redistribution of the NCAM-spectrin complexes to lipid rafts [26], and spectrin is necessary for NCAM-mediated neurite outgrowth [33]. Spectrin also has been shown to be involved in NCAM-mediated assembly and remodeling of the postsynaptic signaling complex [50].

Growth associated protein-43 (GAP-43) is another raft-associated protein that binds to the cytoskeleton, and is involved in NCAM signaling. GAP-43 is enriched in growth cones of neurons where it participates in the dynamic regulation of the

cytoskeleton that is required for the motility of the growth cone by binding to filamentous actin [51]. Although GAP-43, originally, was found to be necessary for NCAM-induced neurite outgrowth in CGNs [52], GAP-43 is not in all cases a prerequisite for NCAM-induced neurite outgrowth. Thus, NCAM has been found to induce neurite outgrowth in PC12 cells that do not express GAP-43 [33]. However, when PC12 cells were transfected to express GAP-43, or when hippocampal neurons were transfected to overexpress GAP-43, neurite outgrowth was increased both in NCAM-stimulated and nonstimulated cells [33].

GAP-43 has been shown following NCAM homophilic binding to be phosphorylated at ser41, and this phosphorylation correlated with NCAM-mediated neurite outgrowth [52]. Importantly, mutation of this or other phosphorylation sites in GAP-43 to prevent phosphorylation abolishes NCAM-mediated neurite outgrowth [33]. Moreover, GAP-43 recently has been suggested to function as a switch between signaling mediated by the two transmembrane NCAM isoforms. This hypothesis states that in the presence of GAP-43, NCAM-mediated signaling mainly depends on NCAM-180, whereas in the absence of GAP-43, NCAM-mediated signaling mainly depends on NCAM-140 [33]. Several lines of evidence favor this model. First, the effect on NCAM-mediated neurite outgrowth in PC12 cells and hippocampal neurons of exogenously expressing the cytoplasmic domain of the two isoforms, where the cytoplasmic domain is assumed to block intracellular signaling by competing for the intracellular signaling partners, depends on the presence of GAP-43. Thus, NCAM-mediated neurite outgrowth was abolished by expression of the cytoplasmic domain of NCAM-180, but not NCAM-140, in cells expressing GAP-43 (hippocampal neurons and transfected PC12 cells), whereas the opposite results were obtained in cells not expressing GAP-43 [33]. Second, spectrin, which as described above predominantly associates with the NCAM-180 isoform, was found only to be necessary for NCAM-mediated neurite outgrowth in the presence of GAP-43. Third, the NCAM-180 isoform was enriched in GAP-43-containing rafts [33]. Altogether, these results suggest that in the presence of GAP-43, NCAM predominantly induces neurite outgrowth through an NCAM-180-spectrin-GAP-43 complex, whereas in the absence of GAP-43, neurite outgrowth is induced via NCAM-140, possibly through RPTPα and Fyn, which have a stronger association with NCAM-140 than NCAM-180.

Inconsistencies exist, however, between the “switch” hypothesis and earlier reports. First, Fyn in association with NCAM-140 was shown to be indispensable for NCAM-mediated neurite outgrowth in GAP-43-expressing neurons [39], suggesting that NCAM-mediated neurite outgrowth is not entirely dependent on NCAM-180 in GAP-43-expressing cells. Second, when NCAM-negative neurons were transfected with one NCAM isoform at a time, only NCAM-140 induced neurite outgrowth [26], questioning the ability of NCAM-180 to induce neurite outgrowth. However, all interpretations of the roles of different NCAM isoforms were based on an assumption that these isoforms are expressed on the plasma membrane as individual entities. NCAM has been suggested by X-ray crystallography to be expressed as dimers. The formation of heterophilic dimers of different NCAM isoforms, although obscure, may explain the discrepancies described above.

To summarize, the spatial separation of NCAM isoforms and interaction partners into different subdomains of the membrane is greatly important for NCAM-mediated signaling, and differences exist in the signaling pathways activated by the two transmembrane NCAM isoforms.

Intracellular Ca^{2+} and Activation of CaMKII

NCAM-induced signaling has been demonstrated to involve an increase in cytoplasmic Ca^{2+} concentration. Early studies demonstrated that antagonists of L- and N-type Ca^{2+} channels can inhibit NCAM-dependent neurite outgrowth [53]. Binding of antibodies to NCAM resulted in opening of Ca^{2+} channels in a pertussis toxin-dependent manner followed by an increase in intracellular Ca^{2+} [54]. Supporting the involvement of Ca^{2+} in NCAM signaling, Saffell and colleagues demonstrated that direct activation of Ca^{2+} channels can mimic NCAM-dependent neurite outgrowth [55]. By means of single-cell Ca^{2+} imaging, later studies showed that the synthetic peptide, C3d, mimicking NCAM effects on neurons induces an increase in intracellular Ca^{2+} in primary hippocampal neurons and PC12 cells [56]. In Ca^{2+}-free conditions, the increase in Ca^{2+} was significantly diminished, although a small transient increase in intracellular Ca^{2+} occurred, indicating mobilization of Ca^{2+} from internal stores [56]. Recently, Kiryushko and colleagues demonstrated that another synthetic NCAM mimetic known to trigger NCAM signaling cascades induced Ca^{2+} influx in primary hippocampal neurons [57]. This Ca^{2+} influx was significantly inhibited by blockers of both L-type and T-type, but not N-type, voltage-gated calcium channels. The discrepant results regarding the types of channels involved may reflect the employment of different tools in the studies (i.e., physiological NCAM homophilic binding *vs.* NCAM mimetic).

A possible signaling cascade responsible for NCAM-mediated activation of Ca^{2+} channels was suggested in 1994. Activation of FGFR leads to activation of phospholipase Cγ (PLCγ), which in turn generates diacylglycerol (DAG). Arachidonic acid (AA) is released from DAG by DAG lipase, and AA activates Ca^{2+} channels [58]. This suggestion was based on data demonstrating activation of neurite outgrowth by AA nonadditively mimicking the effects of NCAM in CGNs. AA-mediated neurite outgrowth was inhibited by Ca^{2+} channel inhibitors, and NCAM-mediated neurite outgrowth was blocked by inhibition of DAG lipase [58]. The importance of this pathway for NCAM-mediated Ca^{2+} channel activation was confirmed by later reports demonstrating that a peptide inhibitor of PLCγ inhibits NCAM-mediated neurite outgrowth [32], and that a PLC inhibitor and a DAG lipase inhibitor decreased both neurite outgrowth and the increase in intracellular Ca^{2+} induced by NCAM mimetic peptides, C3d and P2d, both of which mimic hemophilic NCAM binding [19, 21, 57]. Importantly, inhibition of PLC or DAG lipase had no effect on AA-induced neurite outgrowth, confirming that PLC and DAG lipase are positioned upstream of AA in neurite outgrowth induction [21, 22].

Interestingly, although inhibition of FGFR decreases Ca^{2+} entry following NCAM stimulation, the inhibition is not complete, whereas inhibition by a general inhibitor of tyrosine kinases resulted in complete inhibition [57]. This suggests that another tyrosine kinase, in addition to FGFR, is involved in NCAM-mediated Ca^{2+} entry and this was found to be Src-family kinases [57].

The regulation of growth cone guidance is mediated by the most abundant protein in the mammalian brain, Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) [59–61]. CaMKII has been implicated in NCAM-mediated neurite outgrowth by use of a CaMKII inhibitor [62]. A recent study by Ditlevsen and colleagues confirmed the importance of CaMKII for NCAM-dependent neurite outgrowth from dopaminergic neurons stimulated with the C3d peptide employing the same inhibitor, but they also found, interestingly, that both CaMKII and PLC were dispensable for another endpoint of NCAM signaling, neuronal survival, in the same cell type using the same means of NCAM stimulation [19]. CaMKII activity is modulated by binding to the signaling mediator calmodulin that is activated upon Ca^{2+} binding. NCAM is hypothesized to activate CaMKII through the FGFR-PLC-DAG-AA-Ca^{2+} pathway. In support of this assumption, neurite outgrowth induced by stimulating Ca^{2+} influx or by the addition of AA or FGF2 was inhibited by the CaMKII inhibitor [62].

PKC

The role of protein kinase C (PKC) was established by use of several PKC inhibitors and stimulators in both PC12 cells and primary CGNs [21, 63]. Moreover, homophilic NCAM binding has been demonstrated to result in increased autophosphorylation of PKC [63]. Application of PKC inhibitors led to inhibition of NCAM-dependent neurite outgrowth, whereas the PKC stimulator phorbol myristate acetate (PMA) mimicked NCAM-induced neurite outgrowth. Furthermore, PMA rescued inhibition of FGFR, PLC, Fyn, FAK, and Ras, but not MEK. These results suggested that PKC represents a point of convergence for NCAM signaling through FGFR and MAPK pathways. PKC is downstream of FGFR and PLC, and can activate Raf to create a link to the MAPK pathway [18]. In contrast to this theory, we recently found that inhibition of PKC or PLC had no effect on NCAM-mediated ERK phosphorylation (unpublished data, [36]), suggesting that this pathway is dispensable for activation of the MAPK pathway following NCAM activation.

The PKC family consists of 11 isoforms activated by Ca^{2+} and/or DAG. One of these isoforms, PKCβ2, was found to be associated with spectrin and thereby with NCAM, and this association was found to be important for NCAM-mediated neurite outgrowth [45]. The importance of PKCβ2 was supported by Kolkova and colleagues [63] who demonstrated that a specific inhibitor of the PKCβI and PKCβII isoforms (in addition to expression of dominant negative forms of PKCα, PKCβI, or PKCβII in PC12 cells) abolished the NCAM-specific increase in neurite

outgrowth from CGNs. Moreover, PKCε also has been demonstrated to play an important role in NCAM signaling in CGNs [63].

cAMP and PKA

A role for protein kinase A (PKA) was originally dismissed by Doherty et al. [53] based on experiments employing a competitive PKA inhibitor that showed no inhibitory effect on NCAM-dependent neurite outgrowth. However, a later study employing the same and another PKA inhibitor found that PKA is necessary for NCAM-mediated neurite outgrowth [64]. Production of cyclic adenosine monophosphate (cAMP) results from the activity of the adenylyl cyclase family of enzymes that are regulated by intracellular Ca^{2+} change, heterotrimeric G-proteins, and PKC (for review see [65]). Activation of G-proteins has been suggested to be a part of NCAM-dependent neurite outgrowth [53, 66]. As discussed above, Ca^{2+} and PKC are well known participants in NCAM signaling. Interestingly, however, pertussis toxin-sensitive G-proteins, but not PKA or PKC, recently were found to be necessary for NCAM-mediated ERK phosphorylation (unpublished data, [36]).

Nevertheless, G-protein or Ca^{2+}-dependent activation of adenylyl cyclases may link NCAM with cAMP and PKA, with possible regulation by PKC activation.

The cGMP Pathway

Nitric oxide (NO) is synthesized by nitric oxide synthase (NOS) and is involved in several processes regulated by NCAM, including synaptic plasticity [67, 68], neurite outgrowth [69], and neuronal survival [70]. The major physiological target of NO is the soluble form of the enzyme guanosine cyclase (GC) that synthesizes cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). cGMP in turn activates a number of downstream targets, including protein kinase G (PKG). The NOS-NO-GC-cGMP-PKG pathway recently has been demonstrated to be involved in two biological endpoints of NCAM signaling, neurite outgrowth and neuronal survival [71]. First, inhibition of GC resulted in inhibition of NCAM-mediated neurite outgrowth from two kinds of primary neurons and also resulted in inhibition of NCAM-mediated survival of dopaminergic neurons induced to undergo apoptosis. Second, inhibition of NOS or PKG likewise reduced NCAM-mediated neurite outgrowth in hippocampal neurons. Third, inhibition of NOS was rescued by a cGMP analog. Altogether, these data demonstrate that the cGMP pathway is required for NCAM-mediated neurite outgrowth and survival. The mechanism for NCAM-mediated activation of NOS has not yet been determined, but several possible links exist within the signaling molecules known to be involved in NCAM signaling. An important regulator of NOS activity is free cytosolic Ca^{2+} [72]. Moreover, NOS is known to be regulated by phosphorylation by PKA, PKC, or CamKII [73].

PI3K and Akt

Phosphatidylinositide 3-kinase (PI3K) has been implicated mostly in signaling pathways involved in cell survival and has been demonstrated to play an important role in NCAM-mediated neurite outgrowth and neuronal survival [74]. The major downstream target of PI3K is the kinase Akt that is activated following binding to the lipid products of PI3K. Akt phosphorylation is increased upon NCAM stimulation with the NCAM mimetic peptides, C3d, P2d and FGLd [30, 74, 75], and this activation of Akt was shown to depend on PI3K. The mechanism of NCAM activation of PI3K, and thereby Akt, has been addressed only recently. PI3K is known from other systems to be activated by FGFR via the docking protein Gab1 [76] by binding to phosphorylated FAK [77], Ras, or G-proteins [78], thus making all of these signaling molecules likely candidates for mediating the signal from NCAM to PI3K and Akt. Nevertheless, on the basis of studies employing a phosphospecific antibody cell-based ELISA method, we found that inhibition of FGFR, Fyn (upstream of FAK and Ras), or G-proteins had no specific effect on NCAM-mediated Akt phosphorylation. Instead, we found that NCAM-mediated Akt phosphorylation was inhibited by inhibiting guanylyl cyclase which generates cGMP (unpublished data, [36]). We therefore suggest that NCAM activates Akt through the cGMP-pathway and PI3K.

CREB

The transcription factor cAMP response element-binding protein (CREB) has been shown to be activated by phosphorylation at ser133 following clustering in neuroblastoma cells [24], PC12 cells, and hippocampal neurons [64]. This activation appears to be important for NCAM-mediated neurite outgrowth. Expression of dominant negative CREB in PC12 cells has been demonstrated to abolish NCAM-induced neurite outgrowth in PC12 cells [64].

CREB has been presumed to constitute a point of convergence for the many signaling pathways activated by NCAM, and this presumption is based on the knowledge that many of the kinases described above (e.g., PKA, ERK, CaMKII, PKG, and Akt) phosphorylate CREB in other systems [79]. Nevertheless, evidence has only been published in support of ERK being located upstream of CREB in NCAM-mediated signaling. An inhibitory effect on CREB phosphorylation was found when the ERK activator MEK was inhibited [24]. We recently addressed the hypothesis that CREB constitutes a point of convergence in NCAM signaling by testing inhibition of a number of signaling molecules known to be involved in NCAM signaling. Confirming the results of Schmid and colleagues, we found that inhibition of MEK decreased NCAM-mediated CREB phosphorylation (unpublished data, [36]). Interestingly, inhibition of PKA,

CaMKII, PKG, or Akt had no effect on NCAM-mediated CREB phosphorylation; therefore, CREB is likely not a point of convergence in NCAM signaling (unpublished data, [36]). The pathways known to be involved in NCAM-mediated neurite outgrowth, but apparently not involved in CREB activation, may exert their effect on neurite outgrowth through other transcription factors. Alternatively, the system may be redundant, suggesting that inhibition of one pathway will be rescued by another.

Nuclear factor κB (NFκB) and c-*fos* are two other transcription factors known to be involved in NCAM signaling. NFκB has been shown to depend on PLC, PKC, and CaMKII in astrocytes [80], and c-*fos* is regulated by CREB [64].

Concluding Remarks

A complex network of signaling pathways is required to transmit the signal of NCAM stimulation at the cell surface to the changes in cytoskeletal composition and gene expression required for the neurite outgrowth response. Overlapping, but not identical, signaling pathways are required for another cellular endpoint of NCAM signaling, neuronal survival [19]. A simplified schematic illustrating the signaling pathways required for NCAM-mediated neurite outgrowth is shown in Fig. 1. The cell adhesion molecule L1 also has been shown to mediate both neurite outgrowth and neuronal survival. L1 signals to these two endpoints via overlapping but distinct pathways. Interestingly, whereas PLC and CamKII are dispensable for NCAM-mediated neuronal survival [19], FGFR, cAMP, and PKA are dispensable for L1-mediated neuronal survival [81].

The MAPK pathway is central not only to NCAM signaling but also to the neuritogenic and other effects of other CAMs and growth factors such as *N*-cadherin, L1, and FGF [82]. Moreover, MAPK activation is pivotal to the very different cellular responses induced by nerve growth factor (NGF) and epidermal growth factor (EGF) in PC12 cells, where NGF induces neurite outgrowth and EGF induces proliferation. Both factors induce MAPK activation, but the kinetics appear to confer the specificity of the signal. Thus, MAPK activation induced by EGF is transient, whereas NGF induces sustained MAPK activation [83]. In accordance, heterophilic NCAM stimulation has been demonstrated to induce prolonged MAPK activation (4 h) and promote differentiation [11]. The duration of the individual signaling molecule phosphorylations induced by homophilic NCAM interaction is not known and will be an interesting topic for future research.

Although a number of signaling pathways are known to be involved in NCAM signaling, little is known regarding the relative roles of the individual pathways. Future studies will seek to differentiate the signaling pathways, possibly by developing NCAM mimetics that activate only single pathways, or mimetics that activate only single isoforms of NCAM, to address some of the unresolved issues discussed in this review.

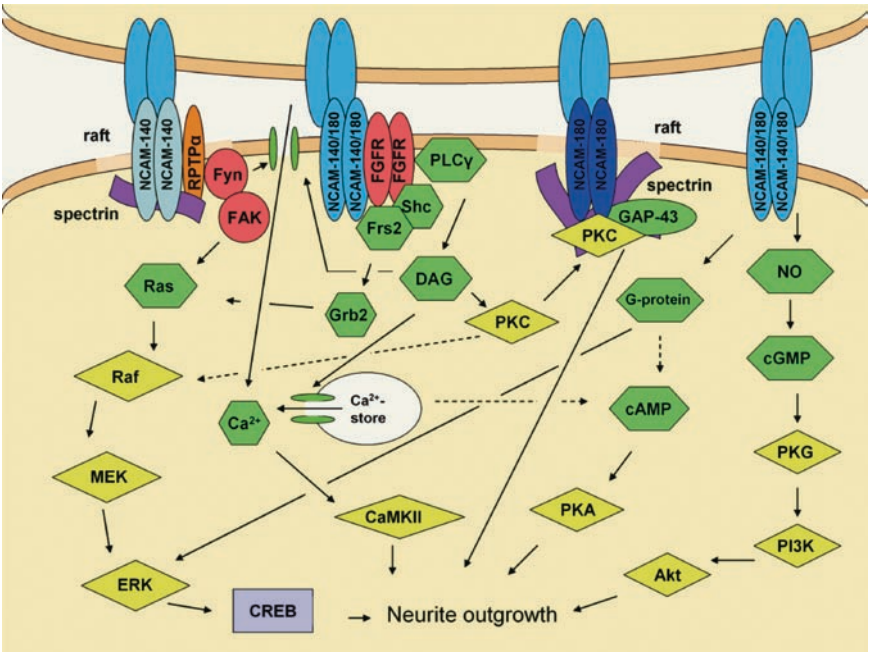

Fig. 1 A schematic illustrating the signaling pathways involved in NCAM-mediated neurite outgrowth. NCAM is shown as *blue ovals*, tyrosine kinases as *pink circles*, other kinases as *yellow rhombus*, spectrin as *purple streamers*, RPTPα as an *orange oval*, CREB as a *gray square*, and other factors as *green hexagons*. *Dashed arrows* indicate putative relationships. For simplicity, RPTPα, and thereby Fyn and FAK, are only depicted in association with NCAM-140 even though it also binds with lower affinity to NCAM-180. Likewise, PKC is only depicted in association with NCAM-180 even though PKC also binds NCAM-140 (see text for details). Finally, only homodimers are depicted, even though heterodimers composed of NCAM-140 and NCAM-180 theoretically also exist

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Role of the Growth-Associated Protein GAP-43 in NCAM-Mediated Neurite Outgrowth

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Introduction

During development, the neural cell adhesion molecule (NCAM) and growth-associated protein-43 (GAP-43) play key roles in neurite outgrowth and growth cone navigation [1–3]. These processes are vital for axonal growth and formation of highly organized neuronal nets in the brain. In adult organisms, NCAM and GAP-43 participate in the reorganization of synaptic contacts and learning and memory formation [4–6]. Enhanced synthesis of these proteins occurs during development and axonal regeneration. They are localized in detergent-resistant plasma membrane domains (rafts) [7–11]. NCAM was shown to deliver external signals to GAP-43 in growth cones, stimulating both GAP-43 phosphorylation on S41 and neurite outgrowth [1]. Therefore, numerous data suggest functional interdependence between NCAM and GAP-43. In this chapter, we discuss the interactions between GAP-43 and NCAM isoforms in the process of neurite outgrowth.

Role of NCAM in the Nervous System

NCAM appears early in embryonic development during the blastoderm stage [12]. During development, NCAM levels peak at the late embryonic/early postnatal stage and then rapidly decrease [13–16]. Several reports suggest an involvement of NCAM in regenerative processes (for review see [17]). NCAM is expressed in three major

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isoforms (120 kDa, 140 kDa, and 180 kDa) generated by alternative splicing of mRNA transcribed from a single gene. NCAM-180 and NCAM-140 are transmembrane proteins, whereas NCAM-120 is linked to the plasma membrane via a lipid anchor. The formation of different NCAM isoforms is temporally and cell-specifically regulated. NCAM-120 is expressed mainly in glial cells of adult animals [18–20]. NCAM-140 is expressed in neurons, glia [19, 21], and tissues of non-neural origin, such as skeletal muscle [22], cardiac muscle [23, 24], smooth muscle [25], and kidneys [26]. NCAM-180 is present mainly in neurons. Expression of NCAM-180 also has been detected in astrocytes [19, 21, 27], adult epididymis [28], heart [23], pancreatic islets [29], and embryonic muscle [30]. Mice lacking NCAM-180 have been shown to have defects in the development of the olfactory bulb [31] and in circadian rhythms [32].

In contrast to the other two NCAM isoforms, NCAM-180 is not synthesized in the proliferating neuronal precursors and becomes detectable only at later stages of development [33]. These in situ observations are supported by in vitro experiments, in which neuroblastoma cells induced to differentiate with dimethyl sulfoxide or laminine shifted NCAM synthesis toward predominately NCAM-180 [34, 35]. Importantly, neuroblastoma growth cones that establish contacts with target cells contain mainly NCAM-180, whereas NCAM-140 has been found mainly in growth cones at the time they were still in the process of the target search [35, 36]. Moreover, Sytnyk et al. [37] reported that NCAM-180 plays a role in synapse stabilization and rapidly accumulates at sites of contact between axonal growth cones and postsynaptic cells in hippocampal cultures. During aging, a significant selective downregulation of NCAM-180 was observed [38]. However, NCAM-180 synthesis remained significant in some brain regions, such as the hippocampus [39] and olfactory bulb [40], where neuritogenesis, sprouting and synaptic remodeling occur continually.

NCAM mediates neurite outgrowth through several signaling pathways. One pathway depends on the nonreceptor protein-tyrosine kinase Fyn and the focal adhesion kinase (FAK). Another pathway involves signaling mediated by the fibroblast growth factor receptor (FGFR). A third pathway involves signaling through pertussis toxin-sensitive heterotrimeric G-proteins (for review, see [41]). Signaling through the Fyn–FAK pathway is known to be mediated by the NCAM-140 isoform, whereas signaling through FGFR can be mediated by both NCAM-140 and NCAM-180 [42, 43]. Additionally, NCAM has been demonstrated to interact with the main cytoskeleton elements. Both NCAM-140 and NCAM-180 have been shown to associate with α- and β-tubulin [44], and NCAM-180 binds brain spectrin [36], which in turn interacts with multiple targets, including filamentous actin, Na^+, K^+-ATPase, the voltage-gated Na^+ channel, and acidic phospholipids [4, 45]. Therefore, NCAM can directly affect the assembly, disassembly, and stabilization of cytoskeletal elements and neurite outgrowth [46].

Role of GAP-43 in Remodeling of the Actin Cytoskeleton and Neurite Outgrowth

GAP-43 is abundant in growth cones during development. In adulthood, GAP-43 expression is relatively low, with the exception of neurons that are hypothesized to be involved in synaptic remodeling, such as in the associative (cerebral) cortex,

hippocampus, and olfactory bulb [47]. GAP-43 is reexpressed in neurons that have damaged axons [48]. When nervous system tissue architecture is disrupted, GAP-43 expression is increased, not only in neurons, but also in glial cells in situ [47]. In mature neurons, GAP-43 is a presynaptic protein that is localized only in axons, whereas dendrites have no GAP-43 [49–51]. However, at the initial stage of neurite growth, GAP-43 was found in all neurites [47]. GAP-43 knockout mice show disrupted cortical maps, possibly attributable to defective axonal guidance [52, 53]. Conversely, transgenic mice overexpressing GAP-43 show enhanced neurite sprouting in the adult nervous system [54, 55] and improved learning ability [6]. The high correlation between extension of neurite processes and increased GAP-43 synthesis has made GAP-43 a tool often used to mark neurite outgrowth.

The neuritogenic function of GAP-43 depends on its ability to bind to the plasma membrane [56, 57] (for discussion, see [10, 58]). This ability is provided by the presence of cysteines C3 and C4 that are dynamically palmitoylated [59–63]. Rat-1 and 3T3 fibroblasts and PC12E2 cells transfected with plasmids encoding GAP-43 mutated at the C3C4 site display cytosolic localization of this protein and are morphologically indistinguishable from untransfected controls, while expression of a plasmid construct encoding wild-type GAP-43 induces formation of filopodial extensions during spreading [10, 64, 65].

Another important post-translational modification of GAP-43 – phosphorylation by protein kinase C (PKC) on S41 [47, 66–68] – is considered to be very important for neuronal signal transduction. In particular, phosphorylation-dephosphorylation on S41 regulates the interaction of GAP-43 with the actin cytoskeleton. S41-phosphorylated GAP-43 stabilizes actin filaments and promotes neurite outgrowth. In contrast, unphosphorylated GAP-43 may reduce actin filament length [3, 69]. At the same time, phosphorylation on S41 prevents GAP-43 from binding to the lipid membrane [70, 71] and interacting with calmodulin [72]. This modification also prevents GAP-43 from site-specific proteolysis by μ-calpain [73]. Dephosphorylation of S41 restores the susceptibility of the GAP-43 molecule to these interactions. One of the after-effects may be colocalization of dephosphorylated GAP-43 with a disorganized cytoskeleton in the collapsed area of axonal endings [3, 69]. Additionally, the interaction of dephosphorylated GAP-43 (or its *N*-terminal fragment formed by μ-calpain) with heterotrimeric GTPase G_0 (G_0 protein) may result in activation of signal pathways leading to cytoskeleton destruction and growth cone collapse [56, 74, 75].

Involvement of GAP-43 in Neuronal Adhesion and NCAM-Mediated Neurite Outgrowth

Evidence suggests an involvement of GAP-43 in neuronal adhesion. When dorsal root ganglia (DRG) neurons are depleted of GAP-43 by antisense oligonucleotide treatment, they lose the ability to extend neurites on poly-L-ornithine-coated surfaces and fail to accomplish NGF-induced spreading [76]. However, when GAP-43-depleted DRG neurons are grown on laminin-coated substrates, they

extend long and thin neurites bearing growth cones that adhere poorly to the substrate [77]. In contrast, PC12 cells, which do not express GAP-43 (PC12-B subclone), exhibit reduced adhesion to uncoated plastic culture surfaces and are easily dislodged from culture plates [78]. When these cells are transfected with GAP-43 cDNA, their attachment to untreated substrates increases substantially [79].

A role for GAP-43 in neuronal adhesion and neurite outgrowth was suggested in a model of GAP-43 functionality in NCAM-induced neurite outgrowth [1]. NCAM was unable to stimulate neurite outgrowth in mouse cerebellar granule cells depleted of the GAP-43 gene by homologous recombination, while integrin-mediated neurite outgrowth was unaffected by GAP-43 gene deletion. Similarly, primary hippocampal neurons expressing GAP-43 mutated at C3C4 do not respond to NCAM stimulation [10]. GAP-43 mutated at C3C4, in contrast to wild-type GAP-43, is suggested to be unable to attach to the cytoplasmic membrane. Thus, GAP-43 association with the membrane may be important for NCAM-induced neuritogenesis.

Role of GAP-43 Phosphorylation in NCAM-Mediated Neurite Outgrowth

The role of GAP-43 phosphorylation in the regulation of the cytoskeleton is described above (for details, see "Role of GAP-43 in Remodeling of the Actin Cytoskeleton and Neurite Outgrowth" in this chapter). NCAM-stimulated neurite outgrowth has been shown to be associated with increased GAP-43 phosphorylation on S41 in cerebellar granule cells and hippocampal primary neurons [1, 10]. Meiri et al. [1] demonstrated that both neurite outgrowth and GAP-43 phosphorylation in isolated growth cones are stimulated by the first three immunoglobulin (Ig) modules of NCAM-soluble chimera as efficiently as by the whole molecule. Similarly, peptide P2 (GRILARGEINFK) corresponding to the 12-amino acid sequence localized in the F and G β-strands and interconnecting loop of the second NCAM Ig module [80] also is capable of inducing both GAP-43 phosphorylation on S41 and neurite outgrowth in primary hippocampal neurons [10]. In contrast, mutations on S41 in GAP-43 led to abrogation of NCAM-stimulated neurite outgrowth in hippocampal neurons [10]. Thus, GAP-43 phosphorylation on S41 is required for NCAM-stimulated, GAP-43-mediated neurite outgrowth.

Other sites in GAP-43 (S191 and S192) have been shown to be phosphorylated by casein kinase II (CKII) in vitro [81, 82] and in isolated growth cones [83]. However, this effect has not yet been shown in vivo [68, 84]. Korshunova et al. [10] found that expression of GAP-43 mutated at S191 and S192 in primary hippocampal neurons downregulates both NCAM-mediated and GAP-43-dependent neurite outgrowth. These results suggest that phosphorylation of GAP-43 on S191 and S192 may be important for NCAM-induced neuritogenesis.

FGFR Function Is Required for NCAM-Stimulated GAP-43 Phosphorylation

FGFR activation is involved in the signaling pathway underlying neurite outgrowth stimulated by NCAM [1] (for review, see [41]). Neurite outgrowth stimulated by NCAM-mediated activation of FGFR was shown to be associated with increased GAP-43 phosphorylation on S41 [1]. FGFR activation by NCAM initiates the sequential activation of phospholipase Cγ (PLCγ) and diacylglycerol lipase (DAG lipase) that results in the release of arachidonic acid (AA) that, in turn, activates voltage-gated calcium channels (VGCC). Local increase in submembrane calcium contributes to activation of PKC, which phosphorylates membrane-associated GAP-43 on S41 [1, 71].

GAP-43 and PKC were found to be within the same lipid raft platform of the plasma membrane [7, 85]. The integrity of lipid rafts was shown to be mandatory for GAP-43 phosphorylation by PKC [85]. Moreover, after phosphorylation by PKC, GAP-43 was suggested to shift from raft to nonraft membranes [85]. However, according to Tejero-Diez et al. [71], S41 phosphorylation is highest in cytoskeleton-associated GAP-43 and lowest in membrane-associated GAP-43. The authors assume a continuous cycling of GAP-43 between intracellular compartments depending on its phosphorylation. S41-phosphorylated GAP-43 molecules were proposed to translocate to the actin cytoskeleton and stabilize newly formed adhesive complexes that are essential for neurite outgrowth [71]. Because the data are controversial, determining where GAP-43 phosphorylation occurs and where phosphorylated GAP-43 is localized needs to be clarified.

PSA-NCAM and GAP-43 Are Coexpressed as Plasticity-Promoting Molecules: Possible Signaling Mechanisms Linking PSA-NCAM to GAP-43 Phosphorylation

NCAM can be glycosylated with polysialic acid (PSA; large homopolymers of the negatively charged molecule α-2,8-sialic acid) on its extracellular domain [86]. The length of the PSA chain (i.e., the number of sialic acid residues) is developmentally regulated. During the late embryonic and early postnatal periods, highly polysialylated NCAM, so-called embryonic NCAM (PSA-NCAM), is abundant throughout the nervous system and associated with morphogenetic changes, such as cell migration, synaptogenesis, and axonal growth. With the establishment of neuronal connections, PSA-NCAM, similar to GAP-43 (for details, see “Role of GAP-43 in Remodeling of the Actin Cytoskeleton and Neurite Outgrowth” in this chapter), rapidly decreases [87]. Importantly, coexpression of PSA-NCAM and GAP-43 have been reported in regions of adult brain structures that display a high degree of structural plasticity [88–90]. Thus, both PSA-NCAM and GAP-43 emerge as plasticity-promoting molecules in the adult nervous system.

NCAM/PSA-NCAM most likely activates tyrosine kinase receptors, such as FGFR, through the induction of dimerization and clustering of receptor molecules [90, 91]. Strong adhesion mediated by nonpolysialylated NCAM through the formation of tight two-dimensional zipper-like adhesion clusters may hamper interactions between NCAM and FGFR [91]. In contrast, the formation of loose clusters of one-dimensional zippers in the presence of PSA favors the association between NCAM and FGFR [91]. Thus, the expression of PSA on NCAM may switch NCAM functions from adhesion to signaling. Consistent with this hypothesis, PSA expression appears to be required for NCAM-induced FGFR signaling [92]. Consequently, FGFR activation was shown to be associated with increased GAP-43 phosphorylation on S41 [1] (for details, see "FGFR Function Is Required for NCAM-Stimulated GAP-43 Phosphorylation" in this chapter). This hypothesis, however, has not been substantiated experimentally, and further studies are needed to elucidate the signaling mechanisms linking PSA-NCAM with GAP-43-phosphorylation.

Differential Role of NCAM Isoforms in GAP-43-Mediated Neurite Outgrowth

By comparing manifestations of GAP-43 and different NCAM isoforms in the organism during development, one might suggest that NCAM-180 is the most likely candidate for interactions with GAP-43. The earliest expression of GAP-43 and NCAM-180 is detected in postmitotic neurons. Both proteins are actively synthesized during development and significantly downregulated in adulthood. Both GAP-43 and NCAM-180 regulate specific mechanisms that control the growth of axons and dendrites, and the establishment of synaptic contacts plays a role in stabilizing newly established synapses. In mature neurons, synthesis of GAP-43 and NCAM-180 is relatively low, with the exception of neurons of associative brain areas (e.g., hippocampus and olfactory bulb) that are involved in synaptic remodeling [39, 40, 47]. NCAM-120 and NCAM-140 synthesis correlates with GAP-43 synthesis to a much lesser degree (for details, see "Role of NCAM in the Nervous System" and "Role of GAP-43 in Remodeling of the Actin Cytoskeleton and Neurite Outgrowth" in this chapter).

NCAM has been shown to be enriched in special microdomains of the plasma membrane, termed lipid rafts, some of which containing GAP-43 [9]. Numerous data suggest that rafts are heterogeneous with regard to lipid and protein composition [9, 58]. Analysis of rafts isolated by immunoprecipitation and containing GAP-43 has revealed that these rafts also are enriched in the NCAM-180 isoform, whereas NCAM-140 predominated in rafts not containing GAP-43 [10]. Thus, GAP-43 was suggested to be involved in NCAM-180-, rather than NCAM-140-mediated neurite outgrowth. This suggestion was confirmed by the observation that in GAP-43-negative PC12E2 cells, NCAM-induced neurite outgrowth depended solely on the function of NCAM-140 [93], whereas in PC12E2 cells transfected

with plasmids carrying the GAP-43 gene, NCAM-induced neurite extension depended on NCAM-180 [10]. This result was reproduced in hippocampal neurons possessing the endogenous GAP-43 gene. In this system, expression of dominant negative NCAM-180, but not dominant negative NCAM-140, abrogated NCAM-stimulated neuritogenesis [10]. These findings appear to contradict the results obtained by Niethammer et al. [11], in which cultured hippocampal neurons of NCAM-deficient mice were transfected with one of the NCAM isoforms and stimulated with the extracellular part of NCAM. Only NCAM-140-expressing neurons exhibited increased neurite outgrowth. The authors concluded that NCAM-140, but not NCAM-180, could serve as a homophylic receptor that induces neuritogenesis. However, only one NCAM isoform at a time was transfected into NCAM-negative neurons, leaving unanswered the question whether the presence of NCAM-140 might be important for NCAM-180 function. Importantly, the level of NCAM-140 expression in cultured hippocampal neurons is significantly higher than that of NCAM-180 [94]. In an experimental system employed by Korshunova et al. [10], transfection of hippocampal neurons with dominant negative NCAM-140 was insufficient for completely "switching off" NCAM-140-mediated signaling. Therefore, although the results obtained by Korshunova et al. [10] strongly suggest the importance of NCAM-180 for NCAM-induced neurite outgrowth in the presence of GAP-43, they do not exclude a neuritogenic role for NCAM-140.

Functional Complex of NCAM-180 with GAP-43 and Spectrin

Spectrin is an actin-binding protein that links membrane domains to the actin cytoskeleton. Pollerberg et al. [35, 36] reported that NCAM-180, but not NCAM-140 or NCAM-120, is bound specifically to brain spectrin. Leshchyns'ka et al. [95] found that all three NCAM isoforms coprecipitated with spectrin, but of the three, NCAM-180 was much more potent in precipitating spectrin. GAP-43 was suggested to interact also with brain spectrin [96]. Additionally, GAP-43 controls the dynamics of the actin cytoskeleton and neurite outgrowth [56, 97]. Korshunova et al. [10] subsequently demonstrated that spectrin may be involved in NCAM-mediated neurite outgrowth in the presence, but not absence, of GAP-43. Thus, NCAM-180 was proposed to be linked to the spectrin-actin network, and GAP-43 appears to regulate this connection. These data together suggest the existence of a functional NCAM-180/spectrin/GAP-43 complex that can transmit NCAM-initiated signals. Korshunova et al. [10] proposed a model in which GAP-43 acts as a "switch" between NCAM-140- and NCAM-180-induced signaling (Fig. 1). According to this model, both NCAM isoforms employ FGFR. In the presence of GAP-43, NCAM-induced neurite outgrowth is crucially dependent on the NCAM-180/spectrin/GAP-43 complex (Fig. 1a). This complex induces an FGFR-dependent signal cascade that leads to PKC activation as a consequence of the successive activities of PLCγ and DAG lipase. One of the prominent outputs of this cascade is GAP-43 phosphorylation by PKC, leading to stabilization of the actin cytoskeleton

and neurite outgrowth. In the absence of GAP-43, the NCAM-140/Fyn/FAK signaling pathway is involved in neurite outgrowth (Fig. 1b).

Open Question: Fyn/RPTPα Association with NCAM-180/Spectrin/GAP-43 Complex

NCAM-induced morphogenic effects depend on activation of Src family tyrosine kinases, particularly activation of Fyn [98]. NCAM-dependent neurite outgrowth is impaired in neurons from Fyn-deficient mice [99] and is abolished by inhibitors of Src kinase family members [43, 100, 101]. Fyn is constitutively associated with NCAM-140, but does not associate significantly with NCAM-180 [42]. He and Meiri [9] reported that GAP-43 and Fyn were found in different membrane raft fractions. Similar results were obtained by Botto et al. [7]. The authors isolated subsets of rafts from cerebellar granule cells and found that one of the subsets contained GAP-43 and PKC, but not Fyn. Thus, one could conclude that Fyn is not involved in NCAM-180/GAP-43-mediated neurite outgrowth. However, Bodrikov et al. [102] demonstrated that the cytoplasmic part of both NCAM-140 and NCAM-180 can be bound to Fyn through receptor protein-tyrosine phosphatase α (RPTPα), a known activator of Fyn. Indeed, Kolkova et al. [43] found that Fyn participates in NCAM-140-dependent neurite outgrowth (Fig. 1b). With regard to the possible interplay between NCAM-180 and RPTPα, the affinity of the NCAM-180–RPTPα interaction was reported to be significantly lower than that of the NCAM-140–RPTPα interaction [102]. This fact may explain the faint co-precipitation of NCAM-180 with Fyn that was demonstrated in earlier studies [42]. Thus, the NCAM-180–Fyn interaction, though not strong, may play a role in NCAM-180-mediated neurite outgrowth. The issue of whether phosphorylated Fyn and phosphorylated GAP-43 and/or NCAM-180 are localized in different raft fractions will need to be clarified further.

NCAM and Growth-Associated Proteins BASP1 and MARCKS

GAP-43 is a member of the family of neuronal growth-associated proteins that also includes brain acid soluble protein-1 (BASP1; also known as CAP-23 and NAP-22) and myristoylated alanine-rich C-kinase substrate (MARCKS). Although these proteins do not share noticeable sequence homologies, they have a number of properties in common [47, 65, 103–105]. All three proteins are characterized by high hydrophilicity, and all are subjected to N-terminal fatty acylation resulting in linkage to the plasma membrane preferentially in lipid rafts [8, 97, 106, 107]. All three proteins also contain a basic domain (termed an effector domain) that binds acidic phospholipids, calmodulin, and actin filaments. These interactions can be modulated

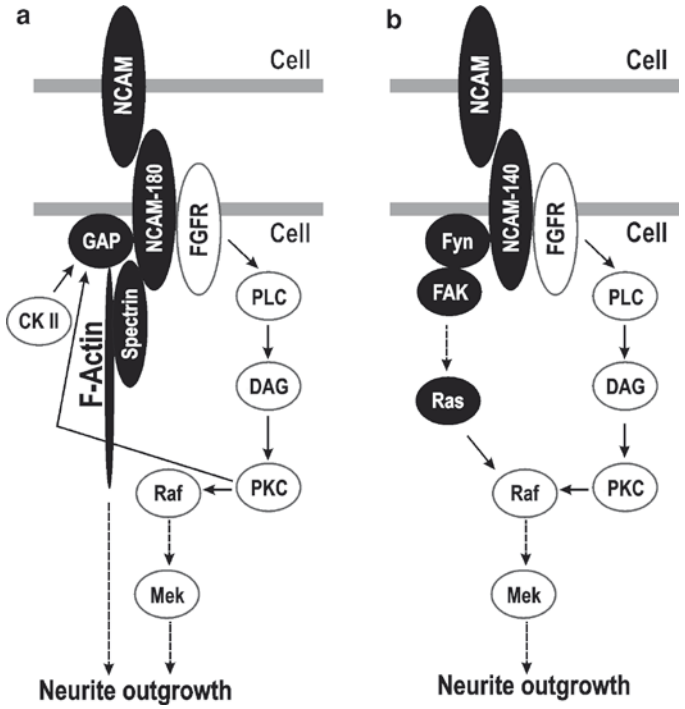

Fig. 1 Schematic representation of the functional relationship between GAP-43 and the two NCAM isoforms, NCAM-180 and NCAM-140. (**a**) In the presence of GAP-43, neurite outgrowth is mediated through the NCAM-180/spectrin/GAP-43 complex (modified from [10]). (**b**) In the absence of GAP-43, neurite outgrowth is mediated through the NCAM-140/Fyn/FAK complex (modified from [43]). FAK, focal adhesion kinase; Fyn, p59 fyn kinase; FGFR, fibroblast growth factor receptor; DAG, diacylglycerol; PKC, protein kinase C; CKII, casein kinase II; Mek, mitogen-activated protein kinase

by PKC-mediated phosphorylation within the effector domain [2, 47, 70, 108]. Numerous data implicate a role for BASP1 and MARCKS in regulating actin cytoskeleton dynamics and neurite outgrowth. However, no information has been provided regarding their participation in NCAM-triggered pathways. Recently, Korshunova et al. [109] found that BASP1 is not involved in NCAM-induced signaling.

Further investigation of NCAM, in particular NCAM-180, interactions with growth-associated proteins in axon endings will be useful for elucidating the participation of NCAM in the processes of neuronal net formation and learning and memory.

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The Neural Cell Adhesion Molecule NCAM and Lipid Rafts

Gro Klitgaard Povlsen and Dorte Kornerup Ditlevsen

Introduction

The neural cell adhesion molecule, NCAM, plays an important role in a number of processes in the central nervous system such as development of the nervous system, regeneration, synaptic plasticity, and memory formation.

NCAM belongs to the immunoglobulin-like superfamily of cell adhesion molecules and it is expressed in three major isoforms named according to their apparent molecular size, NCAM-120, NCAM-140 and NCAM-180. The two largest isoforms are transmembrane and differ structurally only in the size of their intracellular domain, whereas NCAM-120 lacks the intracellular and transmembrane domains and is linked to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor. NCAM-180 is mainly expressed in neurons, whereas NCAM-120 is expressed mainly in glia. NCAM-140 is expressed in both neurons and glia [50].

As the name implies, NCAM is involved in cell adhesion. It has become clear over the years, however, that NCAM not only functions to adhere cells to each other, but also that NCAM when involved in adherence is capable of inducing intracellular signalling resulting in different cellular outcomes including neurite outgrowth (reviewed in [16]), neuronal survival [16] and inhibition of astrocyte proliferation [39]. Thus, when NCAM on one cell engages in homophilic binding with NCAM expressed on an opposing cell, a complex network of intracellular signalling molecules is activated.

Different approaches have been employed for the study of NCAM-mediated signalling. Evaluation of neurite outgrowth from neurons or neuronal cell lines after stimulation of NCAM is widely used [18, 33]. NCAM can be stimulated by (1) growing the neurons or neuronal cell line on top of a confluent layer of fibroblasts expressing NCAM (or not expressing NCAM as control), thereby allowing homophilic NCAM binding [19, 33], (2) by addition of the extracellular domain of NCAM fused to the Fc portion of human Ig [11, 49], (3) by addition of cross-linking

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anti-NCAM antibodies [3, 41] or (4) by addition of peptides mimicking NCAM homophilic binding (4; 16). By using these different ways of NCAM stimulation, a complex network of intracellular signalling events has been revealed, as recently reviewed in [16]. Briefly, stimulation of NCAM can induce binding to and activation of the fibroblast growth factor (FGF) receptor [27, 73] which in turn results in an increase in intracellular Ca^{2+} through activation of phospholipase C, diacylglycerol and arachidonic acid [32, 72]. NCAM also activates the Src-family kinase member, Fyn [3] through receptor protein tyrosine phosphatase α (RPTPα) [5] resulting in activation of the mitogen-activated protein kinase pathway [33, 64]. Moreover, protein kinase A [29], protein kinase C (PKC) [34], Ca^{2+}/calmodulin-dependent kinase [74], cAMP response element binding protein [64], spectrin [55], growth associated protein-43 (GAP-43) [24], the cGMP pathway [16] and the phosphatidylinositide-3-kinase (PI3K) pathway [17] have all been demonstrated to be involved in NCAM-mediated signalling.

An increasing number of studies point to an importance of lipid rafts in the physical segregation of different signalling molecules which are activated following NCAM stimulation and this involvement of lipid rafts in NCAM signalling is the focus of this review.

Lipid Rafts

The existence of microdomains in the plasma membrane, termed lipid rafts, has been a subject of much interest during the last two decades. Lipid rafts are proposed to be membrane domains which form ordered phases enriched in cholesterol and spingolipids. They are composed predominantly of saturated hydro-carbon chains allowing a tight intercalation with cholesterol, which is in contrast to the surrounding disordered matrix of unsaturated and therefore kinked lipids. These specialised lipid assemblies are believed to function as platforms for specific classes of proteins including GPI-anchored proteins, transmembrane proteins and doubly acylated proteins such as Src-family kinases and the α-subunit of heterotrimeric G-proteins [65]. By their concentration of only certain classes of proteins, lipid rafts dynamically compartmentalise the membrane.

Although a definite proof (or disproof) of the existence of lipid rafts in living cells has not yet been obtained, and although debate is still ongoing [46], the concept of lipid rafts is generally accepted [2, 20, 43, 66, 67]. Evidence in support of the physical existence of lipid rafts has come mainly from two different experimental methodologies. First, studies on model membranes have demonstrated that lipid mixtures mimicking the composition of plasma membranes segregate into liquid-ordered and liquid-disordered phases (reviewed in [43]). Second, treatment of cells or isolated plasma membranes with the non-ionic detergent Triton X-100 at 4°C reveals insoluble membrane material enriched in cholesterol and sphingolipids which separates from the soluble material in a density gradient. Since these detergent-resistant membranes (DRMs) are enriched in cholesterol and sphingolipids

and contain proteins believed to be located to rafts, detection of a protein or protein complex in DRMs is often taken as an indication that these proteins are associated with lipid rafts. Moreover, GPI-anchored proteins acquire detergent resistance as they move through the secretory pathway and become associated with raft lipids [6].

A subset of lipid rafts, termed caveolae, can be distinguished by their characteristic morphology of flask-shaped invaginations of the plasma membrane. Caveolae are named after their expression of the protein caveolin which binds cholesterol. Caveolae, unlike other lipid rafts, can be detected by electron microscopy because of their unique morphology resembling the Greek letter omega. Although morphologically defined caveolae cannot be detected in cells of neuronal origin, resent reports have demonstrated that caveolin-1 and caveolin-2 are expressed in the brain [9, 28]. Interestingly, a recent study showed that although expression of caveolin in neurons does not result in the formation of actual caveolae, it seems to be involved in the regulation of neurotrophin signalling and synaptogenesis [25], suggesting that in the brain, caveolin may have other or additional functions than its involvement in microdomain formation.

In many cases, lipid rafts are reported to have no clear morphological characteristics. Such rafts are often referred to as non-caveolae lipid rafts. Due to this lack of morphological characteristics, non-caveolae lipid rafts have been operationally identified by their insolubility in cold non-ionic detergents (as for example Triton X-100 and Triton X-114) which solubilise non-raft membranes [44]. It should be noted, however, that even though DRMs probably contain raft material, a number of non-physiological rearrangements of membrane components are likely to occur during the process of solubilisation [46], and therefore DRMs and lipid rafts should not be equated. Some biological processes legitimately induce raft clustering, but distinguishing between this and artifactual aggregation of raft components is challenging in experiments relying solely on detergent extraction of a cell population. Extraction of live cells on cover slips limits the extent of aggregation that can occur [44]. In addition to studies of the physical existence of lipid rafts and isolation of the structures, the functionality of lipid rafts has also been studied in various ways. One frequently employed method to study the functional implications of lipid rafts is depletion of cellular cholesterol, which results in disruption of lipid rafts structures in the membrane [69]. If the functions and/or interactions of a protein are influenced by cholesterol depletion, it is often taken as an indication that this protein is dependent on lipid rafts for certain functions or interactions with other molecules. In order to more directly visualise lipid rafts in intact cells, the size of which is below the resolution limit of light microscopy, different approaches have been taken including fluorescence resonance energy transfer (FRET) and molecular cross-linking of raft components [2]. However, even by the combined use of these various methods, a clear consensus regarding the size, shape, stability and location of lipid rafts in intact cells has not yet emerged.

Lipid rafts are reported to be involved in important cellular events including membrane trafficking [23] and intracellular signalling [66]. In both cases, the

compartmentalisation achieved by the presence of lipid rafts constitutes the advantage that signalling molecules or trafficking components can be included or excluded to different extents, thereby either increasing the likelihood of interactions or in contrast separate molecules thereby decreasing the likelihood of interactions. It is believed that the raft association of a given protein can vary depending on oligomerisation or interaction with other proteins [66]. Moreover, the clustering of individual lipid rafts may introduce the associated proteins to a new environment of for example adaptors and kinases involved in signal transduction.

Although most studies on the role of lipid rafts has been performed on non-neuronal cells a growing amount of data point to an importance of lipid rafts in neuronal functions such as cell adhesion, axon guidance [30], synaptic transmission and neurotrophic factor signalling [70]. Moreover, lipid rafts have been suggested to be involved in the pathogenesis of several neurodegenerative diseases such as Alzheimer's disease, Prion diseases and Parkinson's disease [1].

Localisation of NCAM Inside and Outside Lipid Rafts

Several studies have investigated the distribution of NCAM between different microdomains in the plasma membrane as well as the functional dynamics of this NCAM distribution pattern. Even before the discovery of functionally distinct membrane microdomains and before introduction of the term "lipid rafts," it was reported by the group of Elisabeth Bock that the NCAM-120 isoform exhibited a very strong membrane attachment, making it resistant to membrane detachment by urea washing [51], an observation that constitutes an early indication of the association of NCAM-120 with detergent-resistant microdomains.

Later, sucrose density gradient centrifugations have been employed to study the distribution of NCAM between rafts and non-raft fractions, using raft fractions derived from different neuronal preparations. In rafts prepared from either whole mouse brain homogenates or from neuroblastoma cells, all three NCAM isoforms were detected [49], whereas raft fractions from isolated growth cones have been reported to contain only NCAM-120 and NCAM-140, not NCAM-180 [24]. Finally, rafts prepared from isolated forebrain synaptosomes were reported to contain all three NCAM isoforms [35]. The discrepancy between these studies may be the result of different experimental protocols, or may reflect differences in the raft localisation of the NCAM isoforms in neuronal growth cones [24], synaptosomes [35], or other neuronal regions or may result from contaminating glial membranes [49].

The exact nature of the DRMs domains harbouring NCAM has not been investigated in detail. However, colocalisation of NCAM (detected by an antibody against the extracellular part, which therefore does not distinguish between the individual NCAM isoforms) with the lipid raft marker GM1 has been reported [49]. In another study, it was reported that NCAM-140 coimmunoprecipitates with caveolin in rafts from isolated growth cones, whereas

NCAM-120 does not [24], indicating that although morphologically defined caveolae do not exist in neurons, caveolin is present in a fraction of lipid rafts in neuronal membranes, and that NCAM-140 is associated with this caveolin-containing raft fraction, whereas NCAM-120 is associated with other non-caveolin-containing rafts.

In summary, all three NCAM isoforms appear to be, at least partially and at least in some cell types, localised to lipid rafts. However, the three major NCAM isoforms exhibit differences in the degree of raft association and also in the biochemical factors linking the molecules to the raft domains. Thus, whereas the glial isoform NCAM-120 is almost exclusively raft-associated [24, 37, 49, 51], NCAM-140 and NCAM-180 are found in both raft and non-raft fractions [41, 49]. Moreover, whereas NCAM-120 is thought to be constitutively raft-associated, the raft-association of NCAM-140 and NCAM-180 appears to be dynamically regulated. Thus NCAM distributed throughout the cell membrane redistributes specifically to lipid raft areas upon clustering of NCAM by means of NCAM antibodies [5, 41].

As regards the biochemical factors associating NCAM specifically with raft domains, NCAM-120 is assumed to be linked to lipid rafts by means of its GPI anchor, as is the case for many other GPI-linked proteins [65]. In the case of NCAM-140 and NCAM-180, it has been suggested that these isoforms are targeted to lipid rafts by palmitoylation of four residues in the intracellular juxtamembrane region present in both NCAM-140 and NCAM-180 [42]. This was supported by the finding that an NCAM-140 deletion mutant lacking these four palmitoylation sites exhibited strongly reduced raft association compared to wild-type NCAM-140 [49].

As mentioned, NCAM-140 and NCAM-180 redistribute to lipid rafts upon induction of NCAM clustering with anti-NCAM antibodies [5, 41, 63]. Recently, the prion protein (PrP) has been suggested to play a role in this dynamic redistribution of NCAM into lipid rafts. PrP is a GPI-linked protein that throughout most of its life cycle remains raft-associated [69], and has been shown to interact with NCAM in neuronal plasma membranes [63]. Furthermore, PrP has been shown to colocalise with NCAM in DRMs along neurites on cultured hippocampal neurons [63]. The hypothesis that PrP may be involved in recruiting NCAM to lipid rafts upon NCAM stimulation is supported by the findings that (a) the amounts of NCAM-140 and NCAM-180 in lipid rafts are significantly reduced in brains from $PrP^{-/-}$ mice, (b) treatment of neurons with soluble recombinant PrP-Fc protein induces redistribution of NCAM to lipid rafts and (c) overexpression of PrP increases the amount of NCAM-140 found in lipid rafts in Chinese hamster ovary (CHO) cells [63].

Thus, the dynamic redistribution of NCAM-140 and NCAM-180 into lipid rafts upon stimulation of these NCAM isoforms is probably dependent both on palmitoylation of their intracellular domains and on dynamic interactions with raft-associated interaction partners such as PrP. Another raft-associated protein that may be of importance to raft localisation of NCAM is GAP-43 that colocalises with NCAM-180 in rafts [24, 35] (also see later).

Localisation of NCAM Signalling Partners in Lipid Rafts and the Importance of Lipid Rafts for NCAM Signalling

As mentioned earlier, NCAM interacts with several transmembrane and intracellular signalling molecules and thereby induces a complex network of intracellular events upon NCAM homophilic binding. In the following, we will discuss the lipid raft association of some of the most central of these NCAM signalling partners, and the importance of lipid rafts for NCAM-mediated signalling via these partners.

The FGF Receptor

In a study from 2000, Davy and coworkers demonstrated that despite the fact that FGF-2 induces a compartmentalised signalling response within DRMs in the neuroblastoma cell line LAN-1, the FGF receptors in these cells do not appear to be localised to lipid rafts, and the distribution of FGF receptors between raft and non-raft areas does not change upon stimulation with FGF-2 [13]. Therefore, the authors suggested that the compartmentalised signalling response to FGF-2 in these cells is not the result of FGF receptor activation in rafts, but perhaps to a mechanism involving raft-associated heparan sulphate proteoglycans (HSPG) binding to FGF-2, since HSPGs are known to be membrane-bound low-affinity receptors for FGFs, and have been shown to be able to mediate FGF-2-signal in the absence of FGFRs [13].

In conclusion, the presence of FGF receptors in rafts has, to our knowledge, not been demonstrated, and although FGFs may be able to induce raft-mediated signalling responses in neuronal cells, this does probably not occur via activation of FGF receptors. Thus, NCAM-mediated signalling via FGF receptors probably occurs outside lipid rafts. This conclusion was supported by the work of Niethammer and Coworkers (49), who demonstrated that whereas stimulation of wild-type NCAM (by treatment with NCAM antibodies) induced activation of ERK independently of the concomitant treatment with a pharmacological FGF receptor inhibitor, ERK activation induced by stimulation of a raft-excluded NCAM-140 palmitoylation site mutant was completely abolished by FGF receptor inhibition [49]. These findings support the conclusion that raft-associated NCAM-140 conveys signalling to the ERK pathway independently of the FGF receptor (presumably via activation of the Fyn/FAK complex, see below), whereas NCAM-140 localised outside lipid rafts induces ERK activation via activation of the FGF receptor, likewise located in non-raft areas of the membrane. See Fig. 1.

*Fyn Kinase and RPTP*α

Fyn kinase is a member of the Src family of non-receptor tyrosine kinases. Several studies in different cell types, including neurons, have shown that Fyn is found mainly in lipid raft areas of the plasma membrane [21, 37, 49, 71]. NCAM-140 has

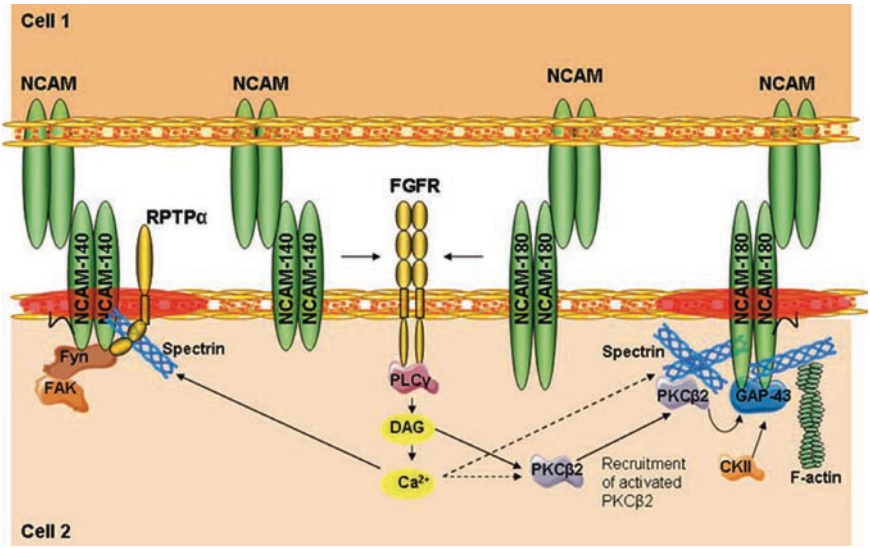

Fig. 1 Schematic illustration of NCAM-140- and NCAM-180-mediated signalling complexes at the plasma membrane inside and outside lipid rafts. See text for references and details. This figure was modified from [16]

been observed in a complex with Fyn, whereas Fyn does not associate significantly with NCAM-180 or NCAM-120 [3]. However, in oligodendrocytes, Fyn is also associated with NCAM-120 in isolated lipid rafts [37], and in tumour cells NCAM is associated with the Src kinase [10]. Thus, the specificity of NCAM's interactions with Src kinase family members appears to be cell-type-dependent and determined by unknown molecular mechanisms.

Various lines of evidence suggest that NCAM-mediated Fyn activation exclusively takes place in lipid raft areas of the membrane, meaning that only raft-associated NCAM can induce activation of Fyn. First, NCAM colocalises with Fyn in lipid rafts. Second, in transfected CHO cells, it has been shown that NCAM-140-mediated FAK activation is abolished upon disruption of lipid raft structures by cholesterol depletion and upon exclusion of NCAM-140 from lipid rafts by mutation of the intracellular palmitoylation sites [49]. Finally, in PrP negative neurons, which exhibit a considerably lower degree of NCAM redistribution to lipid rafts upon NCAM stimulation than wild-type cells, stimulation of NCAM did not induce Fyn activation, unlike in wild-type cells where NCAM stimulation induced strong Fyn/FAK activation [63], further supporting the conclusion that Fyn activation is an important component of NCAM-mediated signalling in rafts, but not in non-raft areas of the membrane. See also Fig. 1.

The molecular mechanism behind NCAM-140-mediated Fyn activation in lipid rafts was recently investigated [5]. The authors demonstrated that the intracellular domains of NCAM-140 and the transmembrane receptor protein tyrosine phosphatase RPTPα interact directly. Like Fyn and NCAM, RPTPα is particularly

abundant in the brain [31, 38], accumulates in growth cones [26] and is involved in neural cell migration and neurite outgrowth [53, 68, 75]. In support of a role of RPTPα in the NCAM–Fyn interaction, it was furthermore shown that the level of Fyn associated with NCAM correlates with the ability of NCAM-associated RPTPα to bind to Fyn, and that the NCAM–Fyn complex is disrupted in RPTP-deficient neurons, indicating that RPTP is a linker molecule between NCAM and Fyn. Finally, NCAM-mediated Fyn activation is abolished in RPTPα-deficient neurons [5], suggesting that RPTPα is a major activator of Fyn downstream of NCAM stimulation. This corresponds with the well-known function of RPTPα as a general activator of Src family tyrosine kinases in other signalling systems [57, 76].

In contrast to the mainly raft-associated Fyn, only 4–8% of all RPTPα molecules were found in lipid rafts of the brain [5]. Accordingly, in hippocampal neurons extracted with Triton X-100 to isolate lipid rafts, detergent-insoluble clusters of RPTPα only partially overlapped with the lipid raft marker GM1 [5]. Thus, RPTPα and Fyn appear to be segregated in non-raft and raft fractions, respectively, in the plasma membrane of unstimulated neurons.

But if Fyn and RPTPα are segregated in different fractions of the membrane, then how does the formation of an NCAM-140–RPTPα–Fyn complex in lipid rafts come into place? Answering this question, [5] demonstrated that NCAM stimulation (by means of treatment with anti-NCAM antibodies) increased the level of RPTPα in NCAM clusters as well as increasing the level of the lipid raft marker GM1 in detergent-insoluble clusters of RPTPα. Furthermore, it was shown that RPTPα levels are reduced by 60% in lipid rafts from NCAM-deficient neurons [5]. Together, these findings indicate that NCAM stimulation promotes NCAM–RPTPα complex formation and that NCAM recruits RPTPα to lipid rafts upon NCAM stimulation, followed by RPTPα-mediated activation of Fyn (see also Fig. 1). The segregation of RPTPα and Fyn to different subdomains of the plasma membrane and the dynamic redistribution of RPTPα to lipid rafts upon NCAM stimulation thus provides an additional level of regulation of NCAM-mediated Fyn activation.

Spectrin

It should be clear from the above that lipid rafts are important players in the control of both the nature and the strength of NCAM-mediated intracellular signals. In addition to their role in signal transduction processes, lipid rafts can also function as local organisers of the cytoskeleton, and the cytoskeleton itself may in turn form a scaffold on which signalling complexes are assembled [44]. In line with this, it has become clear that interactions of NCAM with cytoskeletal components are of critical importance for the formation of NCAM-associated signalling complexes. The importance of lipid rafts for the interactions between NCAM and cytoskeletal components in NCAM-mediated signalling complexes will be discussed in the following.

Spectrin is one of the major cytoskeletal proteins in close association with the cytoplasmic surface of the plasma membrane. It links cellular membranes to motor proteins as well as to all major cytoskeletal filament systems [15]. Due to its ability to interact simultaneously with integral membrane proteins, cytosolic proteins and certain phospholipids, either directly or through adaptor proteins, spectrin creates a multifunctional scaffold at the membrane interface on which macromolecular complexes, such as those found in lipid rafts, consisting of membrane proteins, cytoplasmic signalling molecules and structural elements, are organised [14, 15].

Although spectrin has been reported to be localised in lipid rafts in erythroid [61] and non-erythroid cells [41, 48], its presence in rafts is still regarded controversial [47, 62]. The reason for this controversy is that spectrin coisolates with the cholesterol-enriched fraction of membranes depending on the concentration of the detergent, pH and concentration of the protein in the sample [12, 36, 47]. Altogether, spectrin appears to be distributed to both lipid rafts and non-raft fractions of the membrane, and the actual pattern of distribution is cell-type-specific and probably also dynamically regulated within a given cell type.

That NCAM interacts with brain spectrin has been demonstrated in a number of studies [54, 55]. Whereas some studies have shown that the interaction with spectrin is specific for the NCAM-180 isoform [54, 55] and that expression of a dominant negative spectrin specifically disrupts NCAM-180-mediated neurite outgrowth [35], another study demonstrated that all three major NCAM isoforms coimmunoprecipitate with β1 spectrin, and that recombinant proteins comprising the intracellular parts of both NCAM-140 and NCAM-180 were able to interact with β1 spectrin [41]. However, in the latter study, the intracellular part of NCAM-140 exhibited significantly lower affinity for β1 spectrin than NCAM-180, which may explain why no interaction between NCAM-140 and spectrin was detected in earlier studies, and may indicate that the interaction between NCAM-180 and spectrin is the more physiologically relevant interaction. The fact that NCAM-120 coprecipitated with spectrin despite the lack of an intracellular domain in NCAM-120 may be due to the high concentration of NCAM-120 in lipid rafts and the ability of spectrin to interact with acidic lipids in rafts, resulting in coprecipitation of NCAM-120 and spectrin, because of their common association with these lipids rather than a direct molecular interaction. Another possible explanation is that NCAM-120 coprecipitates with spectrin due to involvement of NCAM-120 in heterodimeric complexes with other NCAM isoforms.

As regards the importance of lipid rafts for the association of NCAM with spectrin, it has been shown that spectrin colocalises with NCAM in both raft and non-raft areas of the membrane, and upon NCAM stimulation spectrin co-redistributes with NCAM to lipid rafts [41]. However, it is not clear from these experiments whether this co-redistribution is a result of NCAM-mediated recruitment of spectrin to rafts, spectrin-mediated recruitment of NCAM to rafts or possibly a two-way regulation where both molecules mutually promote their recruitment into lipid rafts. What has become clear in recent years, however, is that spectrin is of central importance for the assembly of NCAM-associated signalling complexes in lipid rafts by linking NCAM to several intracellular signalling molecules.

Thus, two recent studies have shown that both PKCβ2 and RPTPα are linked to the cytoplasmic part of NCAM and recruited to lipid rafts upon NCAM stimulation via interactions with spectrin [5, 41].

As discussed above, RPTPα was found to associate most potently with NCAM-140, and to be responsible for NCAM-140-mediated activation of Fyn kinase in lipid rafts [41]. Increased intracellular Ca^{2+} was shown to enhance formation of the RPTPα/NCAM complex, and co-redistribution of NCAM and RPTPα to lipid rafts upon NCAM stimulation was shown to depend on the FGF receptor-mediated increase in intracellular Ca^{2+} that is seen upon NCAM stimulation [41]. These findings indicate that although RPTPα associates constitutively with NCAM-140 in the absence of NCAM stimulation, NCAM-mediated FGF receptor stimulation and the resulting increase in intracellular Ca^{2+} strengthens the RPTPα/NCAM complex. Since cross-linking of spectrin molecules is a Ca^{2+}-dependent process, and since spectrin was shown to interact not only with NCAM-140 but also with RPTPα, the authors hypothesise that the Ca^{2+}-induced strengthening of the RPTPα/NCAM complex occurs via spectrin-mediated cross-linking of the molecules, and that this cross-linking facilitates redistribution of the NCAM-140/RPTPα/spectrin complex into lipid rafts [41].

PKCβ2 was found to associate most profoundly with the NCAM-180 isoform. NCAM stimulation was shown to induce recruitment of activated PKCβ2 together with spectrin into lipid rafts, and this redistribution was dependent upon FGF receptor activity [5]. Since PKC is known to be activated downstream of NCAM-mediated FGF receptor activation [33, 34, 58], these findings suggest that NCAM stimulation and the resulting FGF receptor activation lead to PKCβ2 activation, formation of an NCAM-180/spectrin/PKCβ2 complex, and recruitment of this complex into lipid rafts. Accordingly, co-immunoprecipitation of NCAM and PKCβ2 and redistribution of PKCβ2 into rafts upon NCAM stimulation were abolished upon transfection of neurons with a dominant-negative spectrin construct lacking the pleckstrin-homology domain previously demonstrated to bind to PKCβ2 [60], but retaining the NCAM-binding site [5].

In conclusion, spectrin appears to play a role in both NCAM-140- and NCAM-180-associated signalling complexes, but whereas spectrin is essentially for formation of the NCAM-180-containing complex with PKCβ2 in rafts, spectrin appears to have a non-essential but facilitating role for formation of the NCAM-140-containing complex with RPTPα and Fyn in rafts. See Fig. 1.

Growth-Associated Protein-43

Growth-Associated Protein-43 (GAP-43) is a presynaptic protein, thought to play a critical role in axonal growth. It is highly expressed in neurons in the developing nervous system and is enriched in growth cones [24, 40]. In isolated growth cones, GAP-43 has been shown to be concentrated in lipid rafts, and is often referred to as a conventional marker of DRMs or rafts [24, 40]. GAP-43 is localised to the

inner surface of the plasma membrane, and it has been shown to interact with a number of cytoskeletal proteins. Thus, GAP-43 binds to and modifies filamentous actin, and thereby participates in the cytoskeletal dynamics involved in growth cone motility [22, 52]. Furthermore, GAP-43 has been shown to interact with brain spectrin [59]. GAP-43 may, by binding to spectrin and filamentous actin, provide essential attachment sites for cytoskeletal elements, and form a multicomponent submembraneous complex essential in the organisation of the plasma membrane [59].

Although all three NCAM isoforms were found in GAP-43-containing lipid rafts immunoprecipitated from synaptosomal raft fractions, NCAM-180 was found to be selectively enriched over the other two isoforms in GAP-43-containing rafts [35]. This indicates a specific functional relationship between GAP-43 and NCAM-180 in lipid rafts. However, in another study, it was shown that GAP-43 colocalises in and co-immunoprecipitates from lipid rafts of isolated growth cones with NCAM-120 and NCAM-140, and that GAP-43 is activated in NCAM-140-containing lipid rafts, indicating GAP-43-NCAM-140 cosignalling in lipid raft complexes [24]. However, it must be noted that the latter study does not contain any clear report as to whether NCAM-180 was also co-immunoprecipitated with GAP-43 from the GAP-43-containing rafts, since the presented immunoblots do not allow evaluation of bands of the size of 180 kDa. Furthermore, the apparent disrepancies between the two mentioned studies can be due to differences in the isoform specificity of the GAP-43–NCAM interaction in lipid rafts between synaptosomes [35] and growth cones [24].

GAP-43 has been shown to play a role in NCAM-mediated neuritogenesis downstream of activation of PKC [35, 45]. Since GAP-43 binds to and modifies filamentous actin, thereby participating in the cytoskeletal dynamics involved in growth cone motility [22, 52], GAP-43 probably functions in NCAM-mediated neuritogenesis as a linker between NCAM and the dynamic actin-mediated cell shape changes associated with neurite outgrowth. Furthermore, it appears from recent studies that GAP-43 plays an important role in regulating the assembly of specific NCAM-associated signalling complexes in lipid rafts, thereby contributing to the specificity of signalling via the different NCAM isoforms [24, 35].

Thus, in a recent study, GAP-43 was suggested to act as a switch between NCAM-140- and NCAM-180-mediated signalling in lipid rafts [35]. This conclusion was based on several observations. First, studies in neurons and PC12 cells with and without GAP-43 expression indicated that in the presence of GAP-43, NCAM-mediated signalling depends mainly on NCAM-180, whereas it depends mainly on NCAM-140 in the absence of GAP-43. Second, spectrin was found to be of critical importance for NCAM-mediated signalling only in cells expressing GAP-43, indicating that GAP-43 expression causes NCAM-mediated signalling to depend mainly on NCAM-180-spectrin-containing signalling complexes. Taken together, the data presented in this study suggest that in the absence of GAP-43, NCAM-mediated signalling in rafts may depend mostly on the NCAM-140 isoform (probably via NCAM-140–Fyn signalling in rafts), whereas in the presence of GAP-43, NCAM signals primarily through an NCAM-180–spectrin–GAP43 complex in rafts, probably also involving PKC and some of its downstream signalling

partners [35] (see Fig. 1). However, it is important to note that apart from the segregated signalling by the two transmembrane NCAM isoforms in lipid rafts, both isoforms also signals via the FGF receptor outside lipid rafts, and this FGF receptor-mediated signalling does not appear to be regulated by GAP-43.

In summary, NCAM-mediated signalling in lipid rafts should be viewed as several distinct NCAM isoform-specific signalling pathways that are segregated by means of compartmentalisation in membrane microdomains, and that GAP-43 plays an important role in this segregation. This conclusion was further supported by a study in which isolated growth cones were extracted with the detergent Triton X-114, and separated into five distinct subpopulations of detergent-resistant microdomains. Subsequently, it was shown that activated Fyn and activated GAP-43 were found in two distinct raft subpopulations, indicating that the NCAM-140–Fyn and NCAM-180–PKC–GAP-43 signalling pathways are segregated by their differential distribution in raft subsets in growth cones [24].

Conclusions and Perspectives

In conclusion, membrane microdomains in growth cones or in synapses or elsewhere in neuronal cells form signalling scaffolds that respond to NCAM stimulation by initiation of functionally and physically distinct signalling responses.

In particular, it is becoming clear that the two transmembrane isoforms, NCAM-140 and NCAM-180, form distinct signalling complexes in lipid rafts, which are probably important determinants of the known functional differences between these isoforms, see e.g. [7, 8, 49, 56]. It also appears that these differences are at least partially determined by the differential association of these isoforms with Fyn, spectrin and GAP-43 and their segregation into functionally distinct subtypes of rafts. Figure 1 summarises the herein discussed characteristics of NCAM-140 and NCAM-180-associated signalling complexes in lipid rafts.

Thus, as in many other areas of cell biology, understanding the subcellular spatial specificity of membrane proximal and intracellular signalling events is becoming increasingly important within the field of NCAM research. In the nearest future, deeper insight into the subcellular compartmentalisation of NCAM into membrane microdomains, will probably contribute significantly to our understanding of the regulation of NCAM functions including functional characteristics of the different NCAM isoforms, and may explain cell-type-dependent specificities in NCAM-mediated signalling.

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The Neural Cell Adhesion Molecule and Epidermal Growth Factor Receptor: Signaling Crosstalk

Gro Klitgaard Povlsen

Introduction

Neural cell adhesion molecule (NCAM) was first described as a synaptic membrane protein termed the D2 antigen [1]. It was originally characterized as a cell adhesion molecule (CAM) abundantly expressed in the nervous system. However, the past decade of research has widened the picture of the functional roles of NCAM dramatically. It is now clear that NCAM is a multifunctional molecule exerting a broad range of physiological functions, not only in the brain [2,3] but also in other tissues, including a number of NCAM-expressing human tumor types [4]. In the developing nervous system, NCAM has been implicated in numerous processes, including growth, guidance, and migration of neural crest cells and neurons, axon bundling, formation of neuromuscular junctions, myoblast fusion, and neurogenesis [5–9]. In the adult nervous system, NCAM plays important roles in learning and memory formation [10]. In neural cells in vitro, NCAM homophilic binding has been shown to induce neurite outgrowth [2], promote neuronal survival [11,12] and neurogenesis [13], and inhibit proliferation of astrocytes and neural progenitors [14,15]. The role of NCAM in tumor cells has been less investigated, but NCAM definitely appears to play important roles in the regulation of tumor cell proliferation and migration [16,17].

A single gene encodes NCAM, but alternative splicing yields several isoforms. The three major isoforms are NCAM-120, NCAM-140, and NCAM-180, named after their molecular weights. NCAM-120 is mainly expressed on the surface of glial cells and is attached to the membrane via a glycosylphosphatidyl inositol anchor. NCAM-140 and NCAM-180 are transmembrane isoforms expressed on glial cells and neurons and mainly on neurons, respectively.

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At the molecular/cellular level, the functions of NCAM include (1) mediation of cell–cell adhesion by NCAM–NCAM homophilic binding, (2) activation of intracellular signaling pathways via formation of NCAM-associated subplasmalemmal signaling complexes and via activation of the fibroblast growth factor (FGF) receptor, (3) regulation of cytoskeletal function and coupling of membrane proteins to the cytoskeleton, and (4) regulation of growth factor signaling by modulation of the signals induced by FGF [18], glial-cell line-derived neurotrophic factor (GDNF) [19], brain-derived neurotrophic factor (BDNF) [20], platelet-derived growth factor (PDGF) [21], and epidermal growth factor (EGF) [22]. The three major NCAM isoforms have been shown to differ in their capabilities to activate different intracellular signals, cytoskeletal changes, and downstream cellular endpoints [22–24].

The functional interaction of NCAM with the FGF receptor has been known for more than a decade [25], and the underlying molecular mechanism has been shown to involve a direct interaction between the extracellular parts of NCAM and the FGF receptor, leading to activation of the FGF receptor kinase [26,27]. In contrast, the functional interaction between NCAM and the EGF receptor, which is the topic of the remainder of this review, has only been investigated in a few studies and appears to be indirect in nature, involving intracellular intermediates between the two molecules.

Interactions Between NCAMs and the EGF Receptor in Drosophila

The peripheral nervous system of the adult *Drosophila melanogaster* fruit fly has been used for genetic analyses of the molecular mechanisms of axon guidance in vivo due to its accessibility for genetic manipulation and mutant screens. One molecule found to be important for axon guidance processes in *Drosophila* is the *Drosophila* NCAM homolog, termed Fasciclin II (FasII) [28]. Interestingly, the axon guidance abnormalities resulting from genetic deletion of FasII in *Drosophila* has been shown recently to be rescued, to a wide extent, by ectopic expression of human NCAM-140 in *Drosophila*, indicating a high degree of evolutionary conservation of the functions of FasII/NCAM-140 in axon guidance [29].

Recently, the *Drosophila* EGF receptor (termed DER) has been demonstrated to be involved in the functions of FasII in wing sensory axon guidance [29]. Expression of a constitutively active DER in transgenic flies has been shown to yield a characteristic phenotype of smaller wings and wing axon misguidance. A strikingly similar axon misguidance phenotype was observed upon overexpression of FasII and, interestingly, upon ectopic overexpression of human NCAM-140 in the flies. Furthermore, expression of a mutant DER with 50% reduction in kinase activity suppressed the phenotypes caused by overexpression of FasII and human NCAM-140. Together, these findings suggest that FasII and human NCAM-140 ectopically expressed in *Drosophila* can activate DER, and that this activation of DER is important for the normal function of these CAMs in wing sensory axon guidance.

By a similar genetic analysis, the *Drosophila* homolog of the FGF receptor (termed Heartless) has been demonstrated to be required for the axon guidance functions of FasII and human NCAM-140 expressed in *Drosophila* [29].

Another molecule that has proven to be critical for axon guidance processes in *Drosophila* is the *Drosophila* homolog of the NCAM L1, termed Neuroglian (Nrg) [28]. Axon guidance and synapse formation abnormalities resulting from deletion or mutation of Nrg in *Drosophila* can be rescued, to a wide extent, by ectopic expression of human L1 in *Drosophila*, indicating a high degree of evolutionary conservation of the axon guidance and synapse formation functions of Nrg/L1 [29,30]. Similar genetic analyses as described above for FasII have been performed for Nrg and have shown that Nrg also functions in axon guidance via activation of both Heartless and DER [29,31]. Furthermore, it has been shown in Drosophila S2 cells that cell adhesion mediated by exogenously expressed human L1 resulted in activation of exogenously expressed human EGF receptor tyrosine kinase at sites of cell–cell contacts, and that EGF receptors and L1 from these cells co-immunoprecipitated, indicating a physical interaction [32].

In summary, in *Drosophila*, FasII and Nrg not only exert their effects on axon guidance and synapse formation via activation of Heartless, similar to the role of the FGF receptor in NCAM- and L1-mediated neurite outgrowth in mammalian neurons, but also via activation of DER, the *Drosophila* homolog of the mammalian EGF receptor. Furthermore, the findings from experiments with human NCAM and L1 molecules ectopically expressed in *Drosophila* suggest that the functional interactions between FasII/Nrg and DER may be evolutionary conserved and also play a role in the functions of NCAM/L1 in the mammalian nervous system.

The Mammalian EGF Receptor: Regulation and Role in the Nervous System

Unlike DER, the mammalian EGF receptor is a member of a family of homologous receptor tyrosine kinases termed the ErbB receptor family. This family contains, apart from the EGF receptor (another name for which is ErbB1), three receptors termed ErbB2, ErbB3, and ErbB4. The ErbB receptors are activated by different subsets of a large family of EGF-like ligands, which includes EGF and transforming growth factor-α (TGFα) as specific ligands for the EGF receptor, and a subfamily of ligands termed the neuregulins that are specific for ErbB3 and ErbB4. The mammalian EGF receptor is widely expressed in the central nervous system, including expression in the cerebral cortex, cerebellum, and hippocampus [33].

The mammalian EGF receptor has been demonstrated to play a pivotal role in neurogenesis in vivo and in vitro [34,35]. EGF receptor ligands also have been proposed to have neurotransmitter-like or neuromodulatory effects in the brain [36], and several studies have suggested that EGF regulates synaptic plasticity [35]. In cultured neurons, EGF receptor ligands have been demonstrated to function as neurotrophic and neuroprotective factors, enhancing the survival of a variety of

cultured postmitotic neurons [37–39]. Although different studies have reported a function of the EGF receptor as either a promoter or an inhibitor of neurite outgrowth in cultured neurons [40–42], to our knowledge no involvement of the mammalian EGF receptor in axon guidance processes in vivo has been demonstrated.

The functions of the four different mammalian ErbB receptors in the nervous system are very divergent, and often opposing [33,43]. Specifically, there are marked differences in the functions of the EGF receptor and its ligands, on the one hand, and ErbB3 and ErbB4 and their ligands (neuregulins), on the other hand. For example, in cerebellar granular neurons (CGNs), neuregulins stimulate neurite outgrowth [44], whereas the EGF receptor and its ligands inhibit neurite outgrowth [41]. Interestingly, as opposed to the aforementioned lack of reports on the involvement of the EGF receptor and its ligands in axon guidance in the mammalian peripheral nervous system, neuregulins have been reported in several studies to play important roles in such processes [45,46].

Tight regulation of EGF receptor expression, activity, and downstream signaling is vitally important for maintaining normal cell growth and development, and dysregulated EGF receptor function has pathological consequences as exemplified by the numerous human cancers characterized by EGF receptor dysregulation [47]. Positive regulation of the mammalian EGF receptor occurs by transactivation of the receptor by a wide variety of factors (collectively called EGF receptor transactivators) in addition to its cognate ligands [48,49]. Transient inhibition of EGF receptor activity is obtained by negative feedback loops, such as negative regulatory kinases, protein phosphatases, and molecules interfering with ligand binding, whereas more robust signal termination occurs when the EGF receptor is removed from the cell surface by endocytosis and targeted for degradation in lysosomes [50,51], a process termed EGF receptor downregulation.

Ligand-induced EGF receptor downregulation has been shown to involve ubiquitination of the EGF receptor by the RING-type E3 ubiquitin ligase c-Cbl. This ubiquitination is not in the form of polyubiquitin chains, which normally target proteins for proteasomal degradation, but rather consists of single ubiquitins added to multiple lysine residues in the cytoplasmic part of the receptor (termed multiple monoubiquitination) [52]. Monoubiquitination of the EGF receptor promotes EGF receptor endocytosis and serves as a “tag” that targets the EGF receptor for sorting to the lysosomal degradation pathway instead of being recycled back to the cell surface [50,52]. As opposed to ligand-induced EGF receptor downregulation, the mechanisms controlling the constitutive ligand-independent EGF receptor turnover that fine-tunes the basal level of EGF receptors in a cell has been the subject of only few investigations [53].

ErbB2, ErbB3, and ErbB4 all have been shown to be much more slowly downregulated upon stimulation with their respective ligands, or in the case of ErbB2 upon stimulation with a ligand of its heterodimerization ErbB receptor partner, than is the case for the EGF receptor [54–57]. Furthermore, in studies where ErbB2-4 downregulation upon ligand stimulation has been reported, it has been shown to occur by very different mechanisms than the c-Cbl-mediated ubiquitination and downregulation known from ErbB1. For example, slow ligand-induced

downregulation, as well as constitutive turnover of ErbB4, appears to involve protease-mediated cleavage of the receptor in the intramembranous or juxtamembranous domains, leading to shedding of the extracellular part and internalization of the intracellular part [58–60]. In summary, unlike DER, the mammalian EGF receptor is a part of a family of four receptors, the ErbB receptors, that despite their structural homology have been shown to be very different, with regard both to their roles in the nervous system (and in other tissues, which are not discussed here) and to the mechanisms of regulation of the individual ErbB receptors.

Crosstalk Between Neuronal CAMs and EGF Receptor Signaling in Mammalian Cells

Recently, the first study was published investigating the putative interaction between NCAM and the mammalian EGF receptor [22]. This study employed HEK293 cells either overexpressing the human EGF receptor alone or in combination with one of the two neuronal transmembrane NCAM isoforms. In this setup, the authors showed that NCAM-180 functions as a negative regulator of the EGF receptor by lowering the amount of EGF receptors in the cells in the absence of ligand and by accelerating ligand-induced EGF receptor downregulation [22]. NCAM-180, therefore, has the ability to lower the responsiveness of cells toward EGF receptor ligands and suppress ligand-independent, basal EGF receptor activity probably induced by some of the many identified EGF receptor transactivators. In contrast, expression of NCAM-140 did not influence either the level of EGF receptors in the cells or EGF-induced EGF receptor phosphorylation in these cells. Furthermore, NCAM-180 was shown to have no effect on another member of the mammalian EGF receptor family, ErbB4.

The Povlsen et al. study (22) also investigated the mechanism underlying NCAM-180-mediated EGF receptor downregulation. NCAM-180 expression was shown to stimulate EGF receptor ubiquitination, and NCAM-180-mediated EGF receptor downregulation was dependent on the activity of both lysosomal proteases and the proteasome [22]. These results indicate that NCAM-180-mediated EGF receptor downregulation occurs via the well-known pathway of EGF receptor ubiquitination, internalization, and lysosomal degradation. The finding that proteasomes also appear to be necessary for this downregulation, although the EGF receptor is not a direct target for proteasomal degradation [61,62] may be explained by the findings in two recent studies showing that although lysosomes are responsible for the actual EGF receptor degradation, proteasomes play important indirect roles in EGF receptor sorting and degradation [62,63].

The authors also investigated the importance of different extracellular and intracellular domains of NCAM for the functional interaction between NCAM and the EGF receptor. First, the authors detected no direct interaction between NCAM and the EGF receptor, either by surface plasmon resonance analysis employing proteins comprising the extracellular parts of the two molecules, or by co-immunoprecipitation.

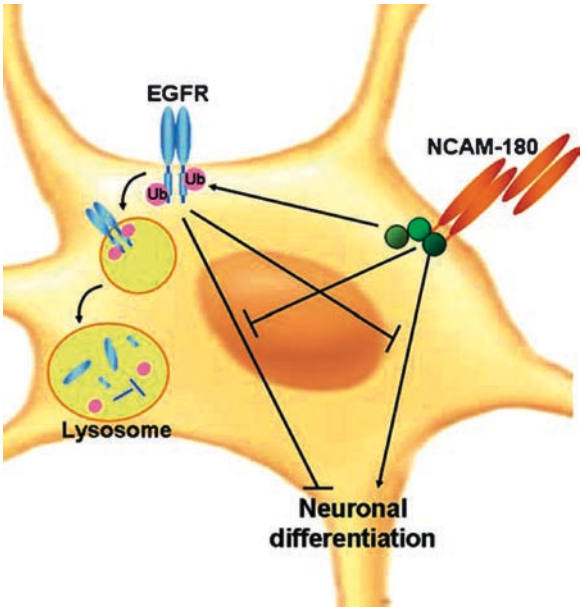

Fig. 1 Schematic summary of the functional interaction between NCAM and the EGF receptor in the regulation of neurite outgrowth and the proposed mechanism behind this interplay involving NCAM-180-mediated ubiquitination and degradation of the EGF receptor. NCAM-180 molecules involved in homophilic interactions are depicted in orange, the EGF receptor in blue, and NCAM-180 cytoplasmic interaction partners in green. Stump lines indicate functional inhibition, arrows indicate functional induction. Ub = ubiquitin molecules conjugated to the cytoplasmic part of the EGF receptor

Second, by challenging the NCAM-expressing HEK293 cells with a peptide that breaks NCAM homophilic binding and a cDNA construct that competes for intracellular interaction partners of NCAM-180, NCAM-180-mediated EGF receptor downregulation was shown to be induced by NCAM clustering and to require intact interactions of NCAM-180 with its intracellular interaction partners [22]. Moreover, the authors demonstrated that NCAM-180-mediated EGF receptor downregulation does not appear to be dependent upon NCAM-induced FGF receptor activation [22]. These findings are schematically summarized in Fig. 1.

To address the physiological role of the functional interaction between NCAM and the EGF receptor in neurons, the role of the EGF receptor in NCAM-mediated neurite outgrowth has been studied [22]. NCAM homophilic binding and EGF receptor activity were shown to have counteracting effects on neurite outgrowth in cerebellar granular neurons. NCAM homophilic binding induced neurite outgrowth, whereas the activated EGF receptor inhibited neurite outgrowth in these neurons [22]. Interestingly, the authors showed that treatment of neurons not engaged in NCAM homophilic binding with a pharmacological EGF receptor inhibitor induced a neuritogenic response, whereas the same inhibitor had no effect on neurite length in neurons engaged in NCAM homophilic binding [22]. These results indicate that

NCAM homophilic binding uncouples the inhibitory function of the EGF receptor in neurite outgrowth. Moreover, direct stimulation of the EGF receptor by different ligands specifically inhibited NCAM-mediated neurite outgrowth [22]. Together, these findings suggest that downregulation of anti-neuritogenic EGF receptor-mediated signals is one of the mechanisms underlying the neuritogenic response to NCAM homophilic binding. This functional interaction between NCAM and the EGF receptor in the regulation of neurite outgrowth is schematically summarized in Fig. 1.

The finding that NCAM-180 functions as a negative regulator of the human EGF receptor is in apparent discrepancy with the findings in *Drosophila*. However, the *Drosophila* study only included human NCAM-140, not NCAM-180, which was identified as the NCAM isoform capable of regulating the human EGF receptor. The *Drosophila* NCAM homolog FasII has a short cytoplasmic domain of the same length as the NCAM-140 cytoplasmic domain, so the possibility exists that whereas FasII can be regarded as a homolog of NCAM-140, a true homolog of NCAM-180 is not present in *Drosophila*.

Moreover, as discussed above, the EGF receptor has been subject to a strong evolutionary divergence from the single DER in *Drosophila* to the four ErbB family members in mammals [64]. Thus, whereas DER promotes axon outgrowth similarly to FasII [29], the functions of the mammalian EGF receptor have evolved to include an anti-neuritogenic effect in CGNs, which is in clear contrast to the neuritogenic effect of mammalian NCAM. Thus, although an interaction between NCAM and the EGF receptor has been evolutionary conserved, the effects of NCAM on the different ErbB receptors are diverse and may have changed significantly in comparison with the effect of FasII on DER as a result of the evolutionary divergence of these receptors.

With regard to the related NCAM L1, its functional interaction with the mammalian EGF receptor has been investigated, and this interaction appears to be more in accordance with the earlier findings regarding the interplay between the *Drosophila* L1 homolog Nrg and DER. Thus, cell adhesion mediated by L1 was shown in Drosophila S2 cells expressing human L1 and human EGF receptors to result in activation of the EGF receptor kinase at cell contact sites. Furthermore, L1 and EGF receptors from these cells were shown to co-immunoprecipitate, indicating a direct physical association between the two molecules. Together with the findings from the genetic studies in *Drosophila* [29,31], these findings support the hypothesis that L1 and its *Drosophila* homolog Nrg function as adhesion-induced transactivators of the EGF receptor and DER, respectively.

Conclusions and Perspectives

The demonstration of a functional interaction between human NCAM-180 and the EGF receptor links NCAM to a growing list of proteins that help desensitize EGF receptor signaling. Another recently discovered negative regulator of the human EGF receptor is LRIG1, the human homolog of the *Drosophila* Kekkon-1 protein.

LRIG1 is a transmembrane molecule possessing 15 leucine-rich repeat domains and three Ig domains in its extracellular part [65]. Due to its structural similarity with adhesion molecules of the Ig and leucine-rich repeat families, LRIG1 is considered to be a putative CAM. LRIG1 was shown to negatively regulate the EGF receptor by enhancing EGF receptor ubiquitination and degradation [66,67]. The underlying mechanism was demonstrated to include formation of a complex of LRIG1 and the EGF receptor, recruitment of c-Cbl to this complex via binding of c-Cbl to the cytoplasmic part of LRIG1, and c-Cbl-mediated ubiquitination of both LRIG1 and EGF receptor leading to their concomitant degradation [66].

Several other negative regulators of the human EGF receptor are CAMs. One example is E-cadherin, which binds directly to the EGF receptor and adhesion-dependently inhibits ligand-dependent EGF receptor activation, EGF receptor mobility in the membrane, and EGF receptor ligand-binding affinity [68,69]. Also in *Drosophila*, adhesion molecules have been identified as negative EGF receptor regulators. One such molecule is Echinoid, a homophilic adhesion molecule with six Ig modules and one fibronectin type III module in its extracellular part that has been shown to suppress DER signaling via its cytoplasmic domain [70]. Interestingly, the *Drosophila* L1 homolog Nrg appears to function either as a positive or a negative regulator of DER, depending on the cellular context and coexpression of other DER regulators [31,32]. Because any molecule functioning as a negative EGF receptor regulator is a potential tumor suppressor – whereas all EGF receptor transactivators are potential oncogenes – further understanding of the role of CAMs as a new class of EGF receptor regulators will be important for the understanding of the many types of human cancers involving overexpression of the EGF receptor. Finally, the recent findings on the NCAM–EGF receptor interaction also add a new perspective to the understanding of NCAM-mediated regulation of intracellular signaling and its importance in the regulation of neurite outgrowth.

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Part IV
NCAM Metabolism

Biosynthesis of NCAM

Kateryna Kolkova

Introduction

The neural cell adhesion molecule (NCAM) is one of the most studied and well-characterized cell adhesion molecules. It is implicated in various morphogenetic processes during development, such as proliferation, migration, differentiation, and synapse formation. NCAM comprises a group of closely related membrane glycoproteins belonging to the immunoglobulin (Ig) superfamily. NCAM is encoded by a single gene that, due to alternative splicing, gives rise to 27 different NCAM polypeptides. During biosynthesis, NCAM becomes modified posttranslationally by glycosylation, sulfation, phosphorylation, and palmitoylation, allowing even greater diversity of NCAM polypeptides. Therefore, multiple NCAM isoforms usually are grouped into three classes according to their approximate molecular weights of 180 kD, 140 kD, and 120 kD in the deglycosylated state. NCAM-A and NCAM-B are transmembrane isoforms, whereas NCAM-C is attached to the plasma membrane via a glycosyl phosphatidylinositol (GPI) tail. This review focuses on the NCAM biosynthesis in neural tissues, with an emphasis on the NCAM gene transcription, alternative splicing, biosynthesis, posttranslational modification, transport to the cell periphery, and expression in the brain and other organs during development.

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Transcription of NCAM Gene

NCAM is produced from a single gene that has been mapped to chromosome 9 in mice [1] and to band q23 on chromosome 11 in man [2, 3]. The NCAM gene is transcribed from a single promoter that does not contain a typical TATA box [4]. The NCAM promoter represents a single regulatory unit in genomic DNA, mapped to a 5 kb restriction-endonuclease-*Hin*dIII fragment that controls the expression of all major RNA size classes [5]. The initiation of RNA transcription occurs at several sites that are used indiscriminately by cells and tissues that express differentially processed NCAM transcripts. Hirsch et al. [6] have identified eight DNA regions interacting with nuclear proteins that are responsible for both the promotion and inhibition of transcription within the functionally important 840-bp region upstream of the main transcription site. One of these sites, site *g*, was localized to the segment containing a repressor element. Two other segments with maximal promoter activity represent an Sp1 factor binding site and a binding site for a factor with nuclear factor I activity. Three adjacent binding sites contain ATTA motifs that resemble the core recognition sequence of *Antennapedia* homeodomain proteins [6]. During development, the NCAM expression is controlled by several transcriptional regulators. Activity of the NCAM promoter is controlled by homeobox (Hox) B9 and HoxB8 proteins, in which the HoxB9 expression activates promoter activity, whereas the HoxB8 expression inhibits promoter activity [7]. HoxB9 and HoxB8 proteins interact with homeodomain binding sites that have TAAT sequences within a 47-base-pair DNA element in the NCAM promoter [7]. The HoxC6-encoded homeoprotein binds to a DNA sequence in the NCAM promoter (CCTAATTATTAA) that has been designated as homeodomain binding site I (HBS-I) [8]. It has been demonstrated that the mouse NCAM promoter is modulated by cut-like homeobox and paired-like homeobox 2 gene products [9]. Moreover, activity of the promoter is regulated by paired box (Pax) protein 8, which binds to the NCAM promoter through two TGCTCC motifs that resemble paired domain binding sites (PBS), PBS-1 and PBS-2 [10]. HBS and PBS in the NCAM promoter are necessary for proper patterning of NCAM expression during neural development. Transgenic mice carrying an NCAM promoter-reporter construct with HBS mutations showed altered expression patterns in the spinal cord [11]. To examine the role of the PBS in vivo, Holst et al. [12] have produced transgenic mice expressing the *lacZ* gene under the control of a wild-type NCAM promoter or an NCAM promoter containing PBS mutations. Their results indicate that PBS plays a key role in the regulation of the NCAM gene expression during neural development.

Alternative Splicing of NCAM Gene

Three major membrane-associated or transmembrane NCAM isoforms differ mainly in the way their *C*-terminal region is generated from a single copy gene by alternative polyadenylation and alternative splicing at the 3′ end of the NCAM mRNA [13–15].

The organization of NCAM gene exons is highly correlated with the NCAM domain structure. The NCAM gene contains 24 exons. Exons 1–14 are present in all NCAM mRNA, whereas the remaining exons are subjected to alternative. Exons 15–19 in the 3′ end generate the four NCAM mRNA classes (7.4, 6.7, 5.2, and 2.9 kb) that encode NCAM isoforms varying in membrane attachment and cytoplasmic domains. The 7.4 and 6.7 kb NCAM mRNAs contain exons 1–14, 16, 17, and 19, but lack exon 15. The 7.4 kb mRNA contains alternatively spliced exon 18, encoding an 801-nt fragment located within the cytoplasmic NCAM domain [16], whereas the 6.7 kb mRNA lacks exon 18. Both the 7.4 and 6.7 kb mRNAs encode two transmembrane NCAM isoforms, NCAM-180 and NCAM-140. These two isoforms differ in the size of their cytoplasmic domain. Exon 15 encodes an amino acid sequence unique to NCAM-120 [17].

The 5.2 and 2.9 kb NCAM mRNAs differ in the length of their 3′ noncoding regions [15]. Both of these mRNAs contain exons 1–15, and both encode the NCAM-120 isoform attached to the membrane via a GPI anchor [18]. However, Gower et al. [19] demonstrated that the 5.2 kb mRNA species from the skeletal muscle and brain incorporate an SEC exon that encodes a truncated form of NCAM that lacks the hydrophobic domain necessary for plasma membrane interactions. Another variable alternatively spliced exon (VASE) is 30 bp long and is inserted between exons 7 and 8 [20]. VASE has been identified in rat, mouse, and human cDNA clones [21–23]. An additional site of differential RNA processing, in which small alternatively spliced exons can be inserted, is located between exons 12 and 13 and is named α [22]. Three alternatively spliced exons comprising 15, 48, and 42 nucleotides can be inserted between exons 12 and 13 in various combinations to create NCAM muscle-specific domain 1 (MSD1), which is expressed in differentiated myotube cells [24]. The shortest exon inserted at the α splicing site consists of only the AAG trinucleotide [22]. AAG is not encoded by the alternative exons 15, 48, or 42, or constitutive exon 13, but may be inserted at the ends of these exons creating additional diversity of NCAM isoforms [25]. The AAG exon also can be inserted between constitutive exons 13 and 14 [25]. Analysis of individual NCAM transcripts from postnatal day 1 rat heart identified 27 NCAM isoforms created by alternative splicing at the exon 7–8 junction, exon 12–13 junction, and at the 3′ end [26].

Biosynthesis and Intracellular Transport of NCAM Molecule

NCAM biosynthesis has been studied extensively in cultured fetal rat brain neurons [27, 28]. NCAM is synthesized in the endoplasmic reticulum as two polypeptides with 186 kD and 136 kD molecular weights that contain high-mannose cores and consist of four to five mannose residues. Twenty to thirty minutes after synthesis, the NCAM polypeptides are converted in the trans-Golgi compartment to more heterogeneous groups of larger molecules (187–201 kD and 137–157 kD) because of varying degrees of glycosylation [27]. NCAM polypeptides undergo rapid intracellular transport and are present at the cell surface within 35 min, where it becomes

phosphorylated. NCAM biosynthesis and polysialylation also have been studied in the AtT-20 cell line, in which polysialylation of newly synthesized NCAM was found to be a rapid process that occurs intracellularly in the late Golgi or post-Golgi compartment within 8–13 min after the beginning of translation, but before the molecule reaches the plasma membrane [29].

Biosynthesis of the NCAM-180 and NCAM-140 isoforms decreases with age, whereas biosynthesis of NCAM-120 and other high-molecular-weight NCAM polypeptides (250–350 kD) increases with age [27, 30, 31]. Neurons in culture can synthesize all three major NCAM isoforms, but the capacity for in vitro biosynthesis of NCAM-120 is low when compared with glial cell [32]. In chicks, NCAM turnover decreases in 3-day-old retinal ganglion cells, reflecting a decline of [^{35}S]-methionine-labeled NCAM of at least one order of magnitude in the fast component of axonal transport compared with the plasma membrane [33]. Cultured fetal rat neuronal cells express predominantly NCAM-180 and NCAM-140 isoforms [27]. In glial cell cultures, NCAM-140 and NCAM-120 are predominantly synthesized [34], whereas NCAM-180 was only occasionally observed in extracts of astrocytes [35]. Cultured skeletal muscle cells mainly produced the NCAM-140 polypeptide [34].

After synthesis, NCAM undergoes axonal transport. When studying the eyes of 20-day-old rats as the site of biosynthesis, NCAM was shown to be transported from retinal ganglion neurons along the optic nerve to nerve terminals in the lateral geniculate body [32]. Transmembrane NCAM isoforms, but not the GPI-linked isoform, have been demonstrated by [^{35}S]-methionine labeling to be axonally transported within 6 h in 3-day-old chick retinal ganglion cells within the fast component of axonal transport [33]. Experiments on organotypic cultures obtained from slices of postnatal hypothalamus demonstrated that NCAM reaches the cell surface of neurons and astrocytes via the constitutive pathway, independent from Ca^{2+} entry and enhanced neuronal activity [36].

NCAM biosynthesis is regulated by growth factors and intracellular calcium. In dibutyryl cyclic adenosine monophosphate (dBcAMP)-treated astrocytes, immunoblot analysis revealed unexpected NCAM-180 expression and an increase in NCAM-140 and NCAM-120 expression [37]. NCAM levels in myotube cultures were enhanced by nerve growth factor (NGF), insulin-like growth factor II, dBcAMP, the sodium ion channel agonist veratridine, and the calcium channel agonist nisoldipine [38].

Posttranslational Modifications: Glycosylation, Sulfation, Phosphorylation, and Palmitoylation

NCAM is co- and posttranslationally modified by *N*- and *O*-linked glycosylation, sulfation, Ser/Thr-phosphorylation, and palmitoylation. NCAM represents a complex glycoprotein, demonstrated by biosynthetic labeling with glucosamine, fucose, and mannosamine [28]. Two types of protein glycosylation occur: *N*-linked glycosylation

and *O*-linked glycosylation. *N*-linked glycosylation occurs when oligosaccharides are linked via *N*-acetylglucosamine to the amide group on asparagine. *O*-linked glycosylation occurs when oligosaccharides are linked via *N*-acetylglucosamine to the hydroxy group on the side chain of a serine or threonine residue or via galactose to the hydroxy group of a hydroxylisine residue.

N-Linked Glycosylation of NCAM

NCAM contains six potential sites for *N*-linked glycosylation, all of which are glycosylated [39]. Pulse-chase studies of NCAM biosynthesis in cultured fetal rat brain neurons demonstrated that the molecular weight of NCAM increases from 186 kD to 187–201 kD (NCAM-180) and from 136 kD to 137–157 kD (NCAM-140) owing the addition of carbohydrate moieties of various lengths [28]. Notably, newly synthesized NCAM molecules contain only four-to-five high mannose cores that later become converted to *N*-linked complex oligosaccharides, determined by treatment with endo-β-*N*-acetyl-glucosaminidase H that is known for specific degradation of high mannose cores.

O-Linked Glycosylation of NCAM

Among various NCAM isoforms, only the GPI-linked isoform from myotubes is known to carry *O*-linked carbohydrates of the common mucin type recognized by peanut lectin [40]. The presence of *O*-linked glycosylation correlated with the expression of MSD1 [40], which is known to be rich in serine and threonine residues [41]. One possible function of *O*-linked glycosylation may be the regulation of NCAM stability or NCAM turnover in the plasma membrane [40].

NCAM Polysialylation

NCAM is a major carrier of polysialic acid (PSA) that represents a linear homopolymer of α2-8-*N* acetylneuraminic acid [42]. The embryonic and neonatal forms of NCAM contain an unusually high proportion of PSA that decreases during development [42]. The large, negatively charged NCAM PSA chain can contain over 50 sialic acid residues and is suggested to be a spacer that reduces adhesion forces between cells and thus regulates cellular interactions. Two polysialyltransferases, ST8Sia II (STX) and ST8Sia IV (PST) are responsible for synthesizing PSA on NCAM [43] of which STX is predominant during embryonic development, whereas PST plays major role in the adult brain [44, 45]. Polysialylation occurs on fifth and sixth

N-glycosylation sites in the Ig5 of NCAM [46]. Recently, Mendiratta et al. has demonstrated that correct positioning of N-glycans on Ig5 depends on an interaction between Ig5 and the first fibronectin type III domain of NCAM [47].

The HNK-1/L2 Epitope on NCAM

The human natural killer-1 (HNK-1)/L2 epitope is a carbohydrate epitope recognized by two monoclonal antibodies: HNK-1 and L2. The epitope includes a terminal 3-sulphoglucuronyl residue [48]. Expression of the HNK-1/L2 epitope on NCAM is cell-specific, because only brain NCAM-180 and NCAM-140 express the epitope [49]. Moreover, the HNK-1/L2 epitope is not expressed by muscle NCAM isoforms [50] and is not involved in the regulation of NCAM-mediated adhesion [51]. Furthermore, HNK1 seems to play a role in neuron to glial cell adhesion [52].

NCAM Phosphorylation

NCAM contains up to 49 serine and threonine residues that can be phosphorylated. NCAM phosphorylation was demonstrated originally by means of [^{32}P]-labeled NCAM polypeptide phosphate incorporation [27, 53] and was demonstrated to be persistent during development [54]. NCAM was shown recently to become phosphorylated after neuronal differentiation of pheochromocytoma cells with NGF [55]. The phosphorylation was time dependent and peaked 30–60 min after NGF treatment [55]. Moreover, the phosphoamino acid analysis has shown that the only tyrosine residue in the cytoplasmic NCAM domain is phosphorylated [56]. Such phosphorylation has been suggested to stabilize cell–cell contacts via interactions with cytoskeletal components [56].

NCAM Sulfation

NCAM is a highly sulfated protein, accounting for 2–4% of total sulfated proteins in cultured rat neurons [27]. NCAM becomes sulfated upon incubation of brain tissue with $^{35}SO_4$ [53]. [^{35}S]-labeling can be removed by endoglycosidase F, indicating that sulfation occurs on Asn. Sulfation of NCAM-180 and NCAM-140 occurs during sorting through the trans-Golgi compartment and decreases during postnatal development [27]. Sulfate groups play important roles in conferring highly specific functions on glycoproteins taking part in the organization of the extracellular matrix of many cell types [57, 58].

NCAM Palmitoylation

Posttranslational NCAM modification includes fatty acid acylation [59]. Palmitoylation sites are located in the *N*-terminal part of the cytoplasmic NCAM domain, containing four cysteine residues of which the last three are palmitoylated [60]. NCAM palmitoylation possibly anchors the protein to the plasma membrane, thus organizing the remaining cytoplasmic part for interactions with other molecules important in NCAM-mediated signal transduction.

Cellular Distribution of NCAM

When synthesized in vitro on membrane-bound polysomes from whole rat brain, NCAM appears as three polypeptides, NCAM-180, NCAM-140, and NCAM-120 [30]. However, different types of brain cells express different sets of NCAM polypeptides. Biosynthetic labeling of different neuronal cells showed that retinal ganglionic cells synthesize small amounts of NCAM-120, though NCAM-180 and NCAM-140 predominate [32]. Other studies demonstrated that cultured brain astrocytes express mainly NCAM-140 and NCAM-120 [35, 52, 61]. NCAM also is expressed by cells in the peripheral nervous system, such as Schwann cells and satellite cells of sensory and sympathetic ganglia [62]. Minana et al. [63] have shown that during the proliferation and differentiation periods, cortical astrocytes in primary cultures express polysialylated NCAM, namely the NCAM-A and NCAM-B isoforms.

Biosynthesis rates and NCAM polypeptide turnover change during development, peak in early postnatal life, and decrease in aging organisms [64]. Linnemann and Bock [65] have demonstrated a two-fold increase in the amount of NCAM protein from embryonic day 17 to postnatal day 4, followed by a two-fold decrease at postnatal day 40. Generally, NCAM biosynthesis decreases 100-fold in neurons from embryonic day 17 to postnatal day 25, with a turnover decline of 350-fold during this period [54]. During development, the polypeptide pattern of NCAM at the cell surface changes from NCAM-140 at an early embryonic stage to polysialylated NCAM-180 and NCAM-140 at late embryonic and early postnatal stages [66–68]. In the adult brain, all three NCAM isoforms are present, though the level of their polysialylation is low [69–71].

NCAM Expression in Various Organs and Tissues During Development

NCAM also is present in non-neural tissues, such as muscle, kidney, sex organs, pancreas, and liver. NCAM was shown by immunofluorescence to be present at the onset of kidney development on cells of the uninduced metanephrogenic mesenchyme,

but it is gradually lost upon conversion of the cells into epithelia [72]. Recent research demonstrated that NCAM expression in adult kidney is restricted to rare interstitial cells with dendritic morphology that could represent renal progenitors [73]. Moreover, the NCAM-140 expression increases in the initial phases of interstitial fibrosis.

NCAM is involved in the development and formation of mouse gonads and ducts [74]. Of the three main NCAM isoforms, only transmembrane isoforms are expressed in gonads, rete, and ducts from young and adult mice. NCAM-140 also was found in the rete of both sexes and the adult uterus, whereas NCAM-180 was detected in the adult epididymis [74].

Immunoblot analysis demonstrated that all three major NCAM isoforms are expressed at all stages of heart development, with the NCAM-140 expression predominating; in aging heart, NCAM-140 and NCAM-120 expression increased [75]. Northern blot analysis was used to show that during postnatal development and aging, expression of 6.7 kb mRNA containing VASE was downregulated, whereas VASE was readily detectable in the 5.2 and 2.9 kb mRNA classes [75].

NCAM also is expressed by both embryonic and perinatal muscle, and its expression is regulated by the state of innervation during myogenesis [76]. In embryonic muscle, NCAM is uniformly present on the surface of myotubes and myoblasts. NCAM expression becomes downregulated as myelination proceeds and completely disappears from myotubes as they mature [76]. Myoblasts express predominantly the transmembrane NCAM-140 isoform, while myotubes express the GPI-linked NCAM-120 isoform [41]. During muscle development, NCAM-140 is replaced by NCAM-120 [76]. However, later studies have demonstrated that although NCAM levels decrease in rat skeletal muscle from perinatal to adult ages, its levels considerably increase in 24-month-old rat muscle [77]. In human muscle, only polysialylated NCAM-180 and NCAM-120, not polysialylated NCAM-120, were expressed during the early stages of myotube maturation [78]. In the latter study, the loss of polysialylated NCAM from maturing myotubes was correlated with the appearance of an adult myosin heavy chain phenotype. NCAM is expressed not only by adult skeletal and cardiac muscle, but also by nerves and muscles of the developing human large intestine when examined by immunohistochemical analysis at gestational ages of 8–20 weeks [79].

NCAM also is expressed in perisinusoidal stellate cells of the human liver [80] and in portal fibroblasts of regenerating liver after partial hepatectomy [81].

NCAM is widely expressed in endocrine tissues and endocrine tumors. NCAM presence has been demonstrated on the membranes of both adrenergic and nonadrenergic cultured adrenal medullary chromafinn cells and in islet of Langerhans endocrine cells [82, 83]. Of three isoforms, NCAM-140 is prevailing in endocrine cells [82, 84]. Immunohistochemical analysis of NCAM expression in neonatal, young, and adult rat islet cells and insulinomas demonstrated the presence of nonsialylated NCAM-180 and NCAM-140 [85]. In contrast, glucagonomas express highly glycosylated and polysialylated NCAM that appears as a broad 135–200 kDa band [85]. The NCAM biosynthesis has been also demonstrated on all neuroblastic tumors [86]. Furthermore, NCAM-120 was highly expressed in ganglioneuromas

and ganglioneuroblastomas, whereas NCAM-180 was predominantly expressed by neuroblastomas [86].

Conclusions

Finally, it can be concluded that the diversity of expressed NCAM isoforms is regulated on several levels including NCAM gene transcription, differential RNA splicing and posttranslational protein modifications, thereby modulating multiple aspects of NCAM functions during embryonic development and in the adult life of animals.

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Soluble NCAM

Thomas Secher

Introduction

The neural cell adhesion molecule (NCAM) belongs to the immunoglobulin (Ig) superfamily of cell adhesion molecules [1] and is a glycoprotein that is highly enriched in neural tissues. NCAM mediates structural integrity through cell–cell and cell–extracellular matrix interactions. However, NCAM also is a potent inducer of signal transduction and plays a pivotal role in the development and plasticity of the nervous system [2]. The extracellular part of NCAM is composed of five Ig-like homology modules (Ig1–Ig5) and two fibronectin type 3 (F3) modules [3]. NCAM is encoded from a single gene (*NCAM1*) containing at least 25 exons [4]. Several protein isoforms exist due to a variety of mRNA species produced by alternative splicing. The three major isoforms of NCAM in the nervous system are NCAM-180, NCAM-140, and NCAM-120 [5], named after their apparent molecular weights in kilo Daltons (kDa), when separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). NCAM-180 and NCAM-140 are transmembrane proteins that differ only in the length of their intracellular domains. NCAM-120 is tethered to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor and, thus, does not have an intracellular part [6]. Additional variability can be achieved by insertion of various other exons; for instance, variable domain alternatively spliced exon (VASE) can be included in the Ig4 module [7]. NCAM also can be modified at the posttranslational level, and several sites for *O*- and *N*-linked glycosylation and phosphorylation exist. The most prominent of these posttranslational modifications is the addition of polysialic acid (PSA) to the Ig5 module [8]. PSA-NCAM has reduced adhesive properties and is regarded as a plasticity-promoting form of the protein [2].

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In addition to the membrane-bound isoforms of NCAM, the existence of the protein in soluble form has long been known. Soluble NCAM has been demonstrated in human cerebrospinal fluid (CSF), serum, and amniotic fluid [9, 10], and in culture media of chick retinal cells and culture media from rat neurons and astrocytes [11, 12].

This review describes soluble NCAM and the various ways by which it is produced. The biological effects are discussed based on the available data from both in vitro and in vivo experiments. Finally, the dysregulation of soluble NCAM in human diseases is reviewed because levels of the protein are found to be altered under many pathological conditions.

Characterization of Soluble NCAM

Different species of soluble NCAM have been detected in rodent brain extracts, CSF, plasma, and media from cell cultures. Bands with molecular masses of 190, 135, and a double band of 110–115 kDa were demonstrated by Western blotting of rat CSF, plasma, and brain supernatant [13, 14]. Similar soluble NCAM species had been observed earlier following radioiodination and immunoisolation experiments in the same tissues [15, 16]. The two isoforms with higher molecular masses (190 and 135 kDa) comigrated with NCAM-140 and NCAM-180 in SDS-PAGE and behave like soluble molecules that cannot be sedimented by ultracentrifugation [13, 14] and thus these species might represent shedding of intact transmembrane NCAM-140 and NCAM-180. Accordingly, Western blotting revealed that these two species reacted with antibodies against the cytoplasmic domain of NCAM-140 and NCAM-180 [14]. The 110–115 kDa species does not have the cytoplasmic epitope and is produced in various ways (e.g., by enzymatic cleavage of the extracellular domain of membrane-bound NCAM; discussed below).

The fraction of soluble NCAM relative to total NCAM in rats was reported to increase with age from 0.7% on postnatal day 4 to 2.2% in adult rats [15]. Conversely, the concentration of soluble NCAM in CSF and plasma decreased during development in the same time period by 60–70% [15]. Similarly, serum levels of soluble NCAM were shown to decrease with age (19–87 years) in humans [17]. In CSF from human adults, the majority of soluble NCAM appears to be composed of a 100–120 kDa form [18–22], whereas in serum two forms of 100–120 kDa and 140–145 kDa are the most prominent [17, 18, 23].

Soluble NCAM can be polysialylated, and similar to membrane-bound NCAM polysialylation is developmentally regulated. In neonatal rat brain homogenates, soluble NCAM appears as a smear or diffuse bands on Western blots [13]. This smear disappears in adult animals or after enzymatic removal of PSA by endosialidase-N treatment [13]. In human infants, the amount of soluble PSA-NCAM in CSF decreased during the first year of development [24].

A 105–115 kDa isoform of soluble NCAM was purified and partially characterized from supernatant of human postmortem brain tissue; a fragment that also was

found in CSF [25]. Analysis of cleavage fragments produced by trypsin digestion of the purified NCAM isoform by mass-spectrometry revealed that the masses of eight cleavage fragments matched the theoretical masses of corresponding peptide sequences located within the extracellular domain of full-length NCAM [25]. Furthermore, antibodies recognizing the amino acid sequence specific to the secreted exon (SEC; discussed below), the cytoplasmic domain of NCAM-180, NCAM-140, VASE, and GPI-linked NCAM did not react with the purified soluble NCAM fragment [25]. Neuraminidase treatment led to a decrease in the size of the soluble NCAM fragment, indicating that it was partially polysialylated [25]. Taken together, this study indicated that this type of soluble NCAM is derived from the membrane-bound NCAM isoforms, possibly as a result of enzymatic cleavage of the extracellular domain.

The Source of Soluble NCAM

Soluble NCAM is released into the extracellular space by secretion, enzymatic processing of the extracellular domain of membrane-bound isoforms, and possibly by the release of detached NCAM-containing membrane fragments.

Secretion

Bock et al. [16] demonstrated that NCAM appeared in the media of neuronal cultures within 15–30 min after [S^{35}]methionine labeling. In contrast, accumulation of NCAM at the cell surface was not observed until 35 min after initiation of synthesis in an earlier study [26], suggesting that at least some of the soluble NCAM was directly secreted rather than enzymatically cleaved off ectodomain fragments.

Soluble NCAM can be produced by alternative splicing of the transcript from *NCAM1* (Fig. 1c). Gower et al. [27] showed that a secreted form of NCAM was generated in muscle and brain tissue as a result of alternative splicing. Thus, expression of the small SEC exon located between exon 12 and 13 introduces an in-frame stop codon, leading to a premature termination of the coding sequence and creating a truncated form of NCAM of 115 kDa consisting of the extracellular domain. Cells transfected with SEC NCAM cDNA were shown in vitro to accumulate the truncated form of NCAM in the cytosol and release the protein into the culture medium [27].

Enzymatic Cleavage of the Extracellular Domain

NCAM-120 is anchored to the cell membrane via a covalently bound GPI anchor. Phosphatidylinositol-specific phospholipase C was shown to release NCAM-120

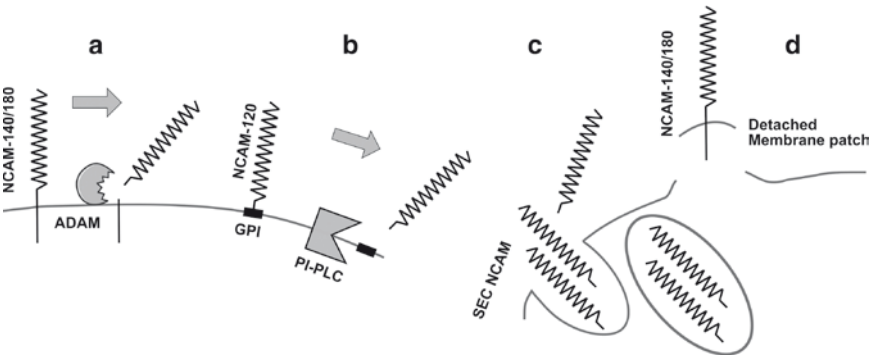

Fig. 1 The source of soluble NCAM. This figure summarizes the various ways soluble NCAM can be produced. (**a**) The extracellular domain of full-length NCAM can be enzymatically cleaved by metalloproteases, here illustrated by an a disintegrin and metalloprotease (ADAM). (**b**) NCAM-120 is fastened to the cell membrane via GPI anchor. The GPI anchor can be cleaved by a phosphatidylinositol-specific phospholipase C (PI-PLC). (**c**) Soluble NCAM can be produced by alternative splicing of the transcript from *NCAM1*. Expression of the small SEC exon introduces an in-frame stop codon. This leads to a premature termination of the coding sequence and creation a truncated form of NCAM, consisting of the extracellular domain. This NCAM isoform is secreted into the extracellular space. (**d**) Full-length NCAM can be associated with lipids and released into the extracellular space in a soluble form. Presumably, these lipids are minor membrane fragments that represent detached NCAM-containing membrane patches

from brain membranes and from the surface of neurons and astrocytes in culture (Fig. 1b) [6, 28].

The extracellular domain of membrane-bound NCAM can be enzymatically cleaved by metalloproteases (Fig. 1a). This was demonstrated in brain homogenates and cultures of primary neurons and various neuroblastoma cell lines [29–32]. Western blotting against NCAM in brain homogenate supernatants and cell culture media detected an NCAM fragment of 110–120 kDa. The presence of this NCAM fragment could be removed by pretreatment with the metalloprotease inhibitors GM6001, BB-3103, and TAPI-1, as well as the chelating agents EDTA and 1,10-phenanthroline, suggesting that the fragment is produced by proteolytic cleavage of membrane-attached NCAM by one or several extracellular proteases [29–32]. Serine and cysteine protease inhibitors were not able to block NCAM shedding, indicating that these proteolytic systems were not involved in NCAM cleavage [30–32]. Hinkle et al. [30] provided evidence suggesting that the proteases involved in NCAM cleavage was of the disintegrin and metalloprotease (ADAM) family and not the matrix metalloprotease (MMP) family. Thus, NCAM shedding was blocked in NCAM-expressing L-fibroblasts by the broad spectrum metalloprotease inhibitors GM6001 and TAPI-1, but not by a MMP-2/MMP-9 specific inhibitor. Furthermore, the active concentration of GM6001 (1 μM reduced shedding by ≈50%) suggested that the protease involved was of the ADAM family, because GM6001 inhibits MMPs with an IC50 in the nanomolar range [30]. ADAM 8, 10, and 17, but not ADAM 9, have been shown to cleave NCAM [30, 32].

Precisely how NCAM shedding is regulated remains to be determined. However, shedding can be modulated in various ways. Thus, the cleavage of NCAM could be stimulated by high concentrations of extracellular adenosine triphosphate (ATP) [31]. The tyrosine phosphatase inhibitor pervanadate also could stimulate shedding in an extracellular signal-regulated kinase 1/2-dependent manner [30]. Moreover, agents that disrupt lipid rafts or destabilize actin stimulated shedding [30, 32]. Finally, inhibition of calmodulin stimulated shedding, whereas inhibition of protein kinase C and phosphatidylinositol 3 kinase did not affect cleavage [30, 31]. Thus, ectodomain shedding of NCAM by ADAM proteases appears to be regulated by a complex system in which extracellular ATP stimulates cleavage, whereas tyrosine dephosphorylation and calmodulin inhibits the process. Furthermore, the cytoskeleton and lipid raft integrity seem to be important for the prevention of shedding.

Detached NCAM-Containing Membrane Fragments

Soluble NCAM can be associated with lipids. BT4C glioma cells transfected with NCAM-140 were shown to release both full-length NCAM-140 as well as a cleaved NCAM species of 110–115 kDa into the culture media [14]. The culture medium was analyzed by density-gradient ultracentrifugation, and the results showed that NCAM was present in both the high- and low-density fractions, indicating that some, but not all, soluble NCAM was associated with lipids [14]. These results suggest that full-length NCAM is released into the extracellular space in a soluble form and that a portion of this is attached to minor membrane fragments (Fig. 1d). The NCAM isoforms involved in the process are presumably NCAM-140 and NCAM-180 because they have a transmembrane domain. Several in vivo events may result in the detachment of membrane fragments containing transmembrane NCAM isoforms such as synaptic plasticity, cell death, and active cell movement where the extracellular domain adheres to the substrate.

Biological Effect of Soluble NCAM

In Vitro

Olsen et al. [14] demonstrated that soluble NCAM reduced the binding of NCAM-expressing BT4C glioma cells to immobilized NCAM, suggesting that soluble NCAM interferes with homophilic interactions between membrane-attached NCAM molecules and results in de-adhesion. Furthermore, inhibition of NCAM shedding by metalloprotease inhibitor treatment (GM6001 or BB-3103) in hippocampal neurons resulted in fewer and larger cell aggregates and enhanced adhesion of NCAM-expressing B35 neuroblastoma cells to culture wells coated

with fibronectin [29, 31]. These results provide support for the hypothesis that soluble NCAM interferes with NCAM-dependent cell adhesion.

Soluble NCAM can bind and modulate components of the extracellular matrix. Accordingly, soluble NCAM obtained from brain homogenate supernatants was shown to bind different types of collagen [33]. Also, the protein could modulate collagen fibrillogenesis and generate fibrils with a larger diameter [34].

Promotion of neurite outgrowth is one of the most studied cellular effects of homophilic NCAM interaction, and soluble NCAM has been shown to modulate NCAM-dependent neurite outgrowth. Thus, inhibition of NCAM shedding by a metalloprotease inhibitor (GM6001) or inactivation of the ADAM 17 metalloprotease in primary hippocampal neurons resulted in a decrease in neurite outgrowth in wells coated with purified NCAM or P2 [31, 32]. P2 is a peptide with an amino acid sequence derived from the Ig2 module of NCAM and is hypothesized to mimic NCAM homophilic interactions [35]. Inhibition of NCAM shedding by GM6001 treatment of NCAM-expressing B35 neuroblastoma cells resulted in reduced migration toward fibronectin in a haptotactic cell migration assay [29]. Schwann cell migration was promoted by a chimeric fusion protein composed of the extracellular part of NCAM attached to the Fc region of human IgG1. Enhanced Schwann cell migration was seen on poly-L-lysine-coated cover slips and from dorsal root ganglion explants on sciatic nerve substrate [36]. Taken together, these results suggest that de-adhesion promotes neurite outgrowth and cell migration. The de-adhesive effect is probably mediated by the interference of homophilic interactions, either because soluble NCAM is competing with existing NCAM–NCAM interactions or because NCAM membrane isoforms are enzymatically cleaved by metalloproteases.

In contrast to the aforementioned results, Hinkle et al. [30] reported that neurite outgrowth and branching in cortical neurons grown on a monolayer of NCAM-expressing L-fibroblasts was enhanced by GM6001 treatment. Furthermore, neurite outgrowth and branching were reduced in neurons isolated from NCAM-extracellular fragment (EC) transgenic mice that overexpress soluble NCAM (discussed below) [37] when compared with neurons from wildtype animals [30]. Thus, these results would suggest that de-adhesion inhibits NCAM-dependent neurite outgrowth and branching. Why this discrepancy exists is unclear. However, cell migration speed is a bell-shaped biphasic function of cell-substratum adhesiveness [38, 39], implying the existence of an optimum adhesiveness for cell migration speed. Thus, the effect of either increasing or decreasing adhesiveness on cell migration speed in a given situation will depend on which side of, and how far from, the optimum the particular biological system is. Similar biological rules theoretically apply to neurite outgrowth; the maximum speed of extending neurites is achieved at optimum cell-substratum adhesiveness. The studies described above could be interpreted in this context. A change in cell-substratum adhesiveness was accomplished by changing the concentration of soluble NCAM. The outcome of such experiments can swing either way, depending on which side of the optimum adhesiveness the particular test systems are located and how big a change in adhesiveness is produced. None of these parameters were known, and therefore it is very difficult to compare these results.

Another possible explanation to this discrepancy that should be considered is that different neuronal cell types were studied in these experiments (hippocampal vs. cortical primary neurons). Cell type is likely important for the levels of heterophilic receptors and growth and neurotrophic factors expressed as well as the presence of specific ADAM subtypes; factors that can influence the neuritogenic response. For instance, the expression of ADAM17 is more pronounced in the hippocampus than other brain regions whereas ADAM10 is more uniformly expressed throughout the brain [40]. Thus, the differential expression of these two ADAMs and the signaling pathways regulating their activity could account for the different results.

However, results from transgenic animals indicate that increased amounts of soluble NCAM, in general, have negative effects on synaptic connectivity and behavior in vivo (discussed below).

In Vivo

The biological effects of soluble NCAM have been studied in transgenic animals. A targeted mutation in *NCAM1* that introduces at stop codon in exon 13 (which codes for a part of the second F3 domain) was used to replace all of the membrane-associated forms of NCAM with a soluble, secreted form [41]. The mutation resulted in dominant embryonic lethality attributable to severe growth retardation and morphological defects occurring around embryonic day 8.5–9.5. In contrast, a null mutation in *NCAM1* or a deletion of the NCAM-180 isoform generated animals with no apparent phenotype that were both viable and fertile [42, 43]. However, a close examination of animals with these mutations revealed distinct cellular disturbances and changes in brain morphology and behavior [42–48]. Thus, the embryonic lethality in mice that only produce soluble NCAM is not due to the lack of the membrane-associated NCAM and abrogation of homophilic interactions mediated by membrane-attached NCAM isoforms. Therefore, the lethality might be attributable to the many possible heterophilic interactions mediated by soluble NCAM. NCAM makes heterophilic interactions with a number of targets, including other cell adhesion molecules, extracellular matrix components, growth factors, growth factor receptors, and more [2]. One can speculate that in the normal situation, membrane-bound NCAM can “absorb” or “buffer” the low amounts of soluble NCAM that are present in the brain, which was measured to be around 1–2% of the total amount of NCAM depending on the developmental stage [15]. In the mutant mice that only express soluble NCAM, this “absorption” cannot take place because membrane-bound NCAM forms are not present. The resulting higher availability of soluble NCAM might lead to undesirable heterophilic interactions or interactions with unknown partners to which soluble NCAM does not bind under normal conditions. Another possibility is that soluble NCAM forms aggregates. Krog et al. [13] demonstrated that purified soluble NCAM 110–115 kDa formed dimers when incubated at physiological concentration; increased amounts of soluble NCAM in vivo, therefore, could result in toxic deposits of NCAM aggregates.

To circumvent embryonic lethality, transgenic mice that overexpress soluble NCAM from the neuron-specific enolase (NSE) promoter were created[37]. This NSE promoter is expressed later in the development and attains maximal activation in adult animals [49]. These NCAM-EC mice have normal expression of NCAM-180 and NCAM-140, but as a result of the transgene, they have elevated levels of soluble NCAM [37]. The NCAM-EC mice are viable and fertile with normal gross sensory and motor functions. Unlike the other NCAM mutants, gross brain morphology is normal with no changes in the size of the olfactory bulbs or brain ventricles [37, 42, 46]. However, the NCAM-EC mice display a prominent decrease in the number of presynaptic terminals of a subpopulation of γ-aminobutyric acid interneurons in the frontal cortex and amygdala [37]. Also, a reduction in synaptophysin labeling and dendrite spine density was demonstrated in the cingulate cortex, suggesting that the animals exhibit a decrease in excitatory synapses [37]. These findings suggest that elevated levels of soluble NCAM perturb synaptic connectivity in vivo.

In addition, the effects of elevated levels of soluble NCAM on behavior have been studied. The NCAM-EC mice showed increased basal motor activity and increased behavioral activation in response to the psychogenic compounds amphetamine and MK801 [37]. Sensory gating was disturbed and could be normalized by treatment with the antipsychotic compound clozapine [37]. The NCAM-EC mice demonstrated impaired memory in contextual and cued fear conditioning [37]. Interestingly, the abnormal behaviors and cellular changes of the NCAM-EC mice have all been implicated in schizophrenia [50], and elevated levels of soluble NCAM are found in patients with schizophrenia (discussed below). Therefore, NCAM-EC mice could represent an animal model of aspects of this disease. The behavioral phenotype of the NCAM-EC mice has similarities to the other NCAM mutants that also display sensory gating deficits, hyperlocomotion, and impaired memory in context and cued fear conditioning [45, 46], suggesting that the behavioral changes are a consequence of disrupted NCAM function.

Soluble NCAM in Disease

Since the discovery of NCAM more than 30 years ago, researchers have been quantifying the soluble forms of NCAM in body fluids and postmortem brain tissue from an impressive list of human diseases, including schizophrenia, mood disorders, various neurodegenerative diseases and cancer types, neural tube defects, delirium tremens, and more (summarized in Table 1). The aim of this research has in many cases been to determine whether soluble NCAM can be utilized as a diagnostic marker for a particular disease.

Many early studies measured soluble NCAM by rocket immunoelectrophoresis or enzyme-linked immunosorbent assay (ELISA), thereby quantifying total NCAM immunoreactivity. However, following its introduction in the 1980s, Western blotting has been widely used for measuring soluble NCAM. The advantage of this method is that it can distinguish between NCAM isoforms with different molecular weights.

Table 1 Soluble NCAM in disease

Disease	Tissue/fluid	Conc.	Detection method	NCAM isoform	References
Schizophrenia					
	CSF	↑	WB	100–120	[19–21, 25]
	PFC, HIP	↑	WB	105–115	[53]
	CSF	↑		VASE	[54]
Mood disorder					
	CSF	↑	WB	120	[18]
	HIP	↑	WB	VASE	[57]
	HIP	↑	WB	SEC (115/108 ratio)	[58]
Neurodegenerative disorders					
Alzheimer's disease	Blood	↑	WB, ELISA	180–130, 130–100, Total	[17, 59]
Various disorders[a]	CSF	↑	WB, ELISA	110–115	[22]
Multiple sclerosis	CSF	↓	WB, ELISA	110–115, Total	[22, 60]
Cancer					
Small cell lung cancer	Blood	↑	ELISA	Total	[65–67]
	Blood	↑	WB, ELISA	PSA-NCAM (total, 110–130)	[23, 68]
Medulloblastoma	CSF	↑	ELISA	PSA-NCAM	[69]
Miscellaneous					
Delirium tremens	CSF	↑	Rocket	Total	[70]
Neural tube defect	Amniotic fluid	↑	Rocket	Total	[71, 72]
Normal pressure hydrocephalus	CSF	↓	ELISA	Total	[59]
Autism	Blood	↓	WB	Total	[73]

[a]Various neurodegenerative disorders were grouped together and compared to non-neurodegenerative diseases

The table provides a summary of concentration alterations of soluble NCAM in various human diseases. *Disease*: human disease studied. *Tissue/fluid*: compartment where the soluble NCAM concentration was found to be changed. *Concentration*: up or down arrow indicates if the soluble NCAM concentration was increased or decreased. *Detection method*: the method used to quantify soluble NCAM. In some instances, more than one method is indicated, because two or more studies exist that used different methods or two methods was used in the same study (e.g. ELISA was to quantify the amount of soluble NCAM and WB was used determine the NCAM isoform involved). *NCAM isoform*: soluble NCAM isoform studied. "Total" indicates that total NCAM immunoreactivity was measured, e.g. by ELISA or rocket immunoelectrophoresis. In some studies, soluble NCAM isoforms was grouped and quantified together, e.g. species with molecular weight between 180 kDa and 130 kDa. *References*: references to published studies. Abbreviations: CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; HIP, hippocampus; PFC, prefrontal cortex; Rocket, rocket immunoelectrophoresis; SEC, secreted exon; VASE, variable domain alternatively spliced exon; WB, Western blot

Thus, results from studies that have quantified a soluble NCAM fragment of a specific size using Western blotting can be difficult to compare with results from studies that have measured total NCAM immunoreactivity using ELISA or rocket immunoelectrophoresis.

Schizophrenia

The first studies of total NCAM immunoreactivity in serum from patients with schizophrenia were somewhat inconsistent. Lyons et al. [51] reported an increase in serum NCAM levels in patients with schizophrenia, and serum NCAM was found to comprise a 70-kDa fragment; no other isoforms could be detected by Western blotting. In contrast, other investigations reported decreased serum NCAM levels in patients as measured by ELISA [52]. However, many subsequent studies that quantified NCAM using Western blotting reported increased levels of a soluble fragment of 100–120 kDa in CSF samples [19–21, 25] and in the supernatant of postmortem brain extracts from the hippocampus and prefrontal cortex [53]. Soluble isoforms of NCAM with the VASE exon also have been reported to be increased in CSF of schizophrenic patients [54].

The increase in soluble NCAM levels in schizophrenia is likely related to the progression of the illness and its clinical state. In six pairs of monozygotic twins discordant for schizophrenia, only the affected twins were found to have increased soluble NCAM levels in the CSF [20]. Moreover, the CSF concentrations of soluble NCAM correlated with an increase in the size of the ventricles over a 2-year period and with the clinical score of positive symptoms [25, 54]. One study reported that CSF levels of soluble NCAM were decreased only in first-episode patients that had received antipsychotic treatment, whereas another study provided evidence showing that CSF levels were increased only in male chronic patients withdrawn from treatment [25, 55]. Other studies showed that antipsychotic treatment had no effect on soluble NCAM levels in the CSF [19, 21, 54]. Taken together, available evidence shows that the levels of soluble NCAM are increased in the CSF and specific brain regions in schizophrenia. This increase appears to be related to the progression of the illness and to a lesser degree to antipsychotic treatment.

Mood Disorders

Jorgensen et al. [56] measured total NCAM immunoreactivity in the CSF of patients with mood disorders. No differences were found between patients and controls, but the soluble NCAM concentration increased slightly when moods were normalized. In another study, Jorgensen [52] showed that total NCAM immunoreactivity in CSF of depressed patients was decreased by 15%. This decrease was partially normalized after recovery from the depressed state. A later study reported that the concentration of a soluble NCAM fragment of 120 kDa was increased in CSF of patients with unipolar and bipolar disorder type I [18]. However, an increase in soluble NCAM could not be demonstrated in postmortem hippocampal tissue from bipolar patients [53]. Soluble NCAM containing the region encoded by the VASE exon also was found to be increased in supernatants from hippocampal postmortem tissues [57], but not in CSF [54]. Specific antibodies against NCAM containing the region encoded by the SEC exon was reported to recognize two

bands of 115 kDa and 108 kDa on Western blots from hippocampal postmortem tissue [58]. Bipolar disorder patients were found to have an increased SEC NCAM 115/108 ratio compared with controls, suggesting that the expression of the 115 kDa NCAM isoform is altered in patients with bipolar disorder [58].

As with schizophrenia, soluble NCAM levels appear to be influenced by the progression of the disease and its clinical state. Poltorak et al. [18] showed that there was a negative correlation between soluble NCAM 120 kDa levels in the CSF and the Hamilton depression rating scale; however, no effect of medication could be demonstrated on the CSF levels of NCAM 120 kDa. Taken together, the aforementioned studies suggest that patients with mood disorders exhibit increased concentrations of soluble NCAM in the CSF and altered expression levels of NCAM isoforms containing the VASE and SEC exons in specific brain regions. Furthermore, these changes appear to be influenced by the progression of the illness.

Neurodegenerative Disorders

Soluble NCAM have been quantified in various neurodegenerative disorders. In Alzheimer's disease, total NCAM immunoreactivity was increased in blood plasma [59]. Similar results were reported in a later study, where various NCAM isoforms with sizes between 100 kDa and 180 kDa were detected by Western blotting in serum samples from Alzheimer's patients. Isoforms between 100–130 kDa and 130–180 kDa were clustered, and both the low and high molecular weight NCAM isoforms were increased in serum of Alzheimer's disease patients [17]. Moreover, the presence of the low molecular weight NCAM isoforms was associated with cognitive impairment [17]. One study reported no change in total NCAM immunoreactivity in CSF of Alzheimer's patients [59], whereas another study reported a tendency toward increased levels of the 110–115 kDa NCAM isoforms [22]. When patients with neurodegenerative disorders were grouped together and compared with patients with non-neurodegenerative disorders, CSF levels of the 110–115 kDa NCAM isoforms were found to be increased [22]. Taken together, soluble NCAM appears to be increased in serum and possibly in CSF in Alzheimer's patients.

CSF levels of total NCAM and the 110–115 kDa isoforms were decreased in multiple sclerosis patients [22, 60]. However, CSF levels of total NCAM increased dramatically in multiple sclerosis patients recovering from an acute phase attack [60].

No change in the amount of total NCAM and 110–115 kDa forms were detected in CSF or plasma of patients with amyotrophic lateral sclerosis [22, 61].

Cancer

NCAM is expressed in both neural and neuroendocrine tissues and tumors [62, 63]. Cell adhesion is an important determinant of organized growth and tissue integrity.

Therefore, the reduced adhesive properties of PSA-NCAM might favor metastatic spread and promote cancer [64]. In small cell lung cancer, both the amounts of total NCAM and PSA-NCAM immunoreactivity have been investigated in serum of patients, and the total amount of NCAM immunoreactivity was found to be increased in serum [23, 65–67]. Using PSA-NCAM specific antibodies, ELISA revealed that PSA-NCAM was increased in serum of patients with this type of cancer [68]. Furthermore, Takamatsu et al. [23] found that PSA-NCAM in serum was only present in patients and not in controls.

Soluble PSA-NCAM was only detected in patients with medulloblastoma and not in controls [69]. Moreover, there was good agreement between the clinical status of patients and the levels of PSA-NCAM in CSF. For this reason, PSA-NCAM levels in CSF were suggested to be a potential marker for medulloblastoma [69]. Taken together, NCAM and PSA-NCAM appear to be shed by certain types of tumors, increasing the levels in CSF and serum of cancer patients.

Others Disorders

Soluble NCAM has been investigated in several other disorders. Accordingly, Jorgensen et al. [70] studied the levels of total NCAM immunoreactivity in CSF of patient with delirium tremens (alcohol withdrawal syndrome) at admission to the hospital and after recovery. CSF levels of soluble NCAM were found to be increased dramatically (by approximately 100%) after recovery.

In addition, increased levels of total NCAM immunoreactivity were reported in amniotic fluids from pregnancies with fetal neural tube defects [71, 72]. Based on these results, the authors suggested that soluble NCAM could be utilized as a diagnostic marker for fetal neural tube defects because increased total NCAM immunoreactivity correctly predicted neural tube defects over other malformations and pregnancies with normal outcomes [71, 72].

Finally, total NCAM immunoreactivity was reported to be decreased in CSF of patients with normal pressure hydrocephalus [59] and decreased in serum of patients with autism [73].

Summary

Soluble NCAM exists in different isoforms, ranging in sizes from approximately 180 to 100 kDa depending on origin. The concentration of soluble NCAM in brain and CSF is developmentally regulated, and soluble NCAM can be polysialylated in a developmentally controlled manner. The protein can be produced in various ways, such as by alternative splicing of *NCAM1*, enzymatic processing of the extracellular domain at the cell membrane, and detachment of NCAM-containing membrane fragments. Soluble NCAM interferes with homophilic interactions between membrane-attached NCAM isoforms and reduces NCAM-mediated adhesion.

Soluble NCAM can modulate neurite outgrowth and branching in vitro, processes presumably governed by complex dose-response relationships. Overexpression of soluble NCAM in mice in vivo appears to perturb synaptic connectivity and results in abnormal behavior. The levels of soluble NCAM in brain tissue, CSF, and serum are altered in many human diseases, and in many cases, levels of soluble NCAM are related to the progression or clinical state of the disease.

To date, the exact function of the different isoforms of soluble NCAM and what signaling pathways govern their production and how precisely they are regulated is not well understood. The role of soluble NCAM during development and plasticity of the nervous system is not well understood either. Is the release of soluble NCAM a biological mechanism that controls cell adhesion and regulates aspects of synapse formation and function? This will be important for future experiments to address and should help us understand what specific mechanisms are dysregulated in a number of human diseases like schizophrenia.

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Part V
NCAM, Synapses, Emotions, and Memory

Role of NCAM in Spine Dynamics and Synaptogenesis

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Introduction

Synapse formation and remodeling are one of the key mechanisms that regulate the development and function of synaptic networks. Recent evidence from in vivo imaging experiments has shown that synapse formation and elimination represent a major process contributing to the build-up and shape of brain networks [1–3]. Interestingly, this process remains active throughout life, although at a significantly lower level [4], it can be reactivated following injury or brain lesions [5], and it possibly underlies or is associated with cognitive functions and learning mechanisms [6]. Understanding how two partners establish and maintain a stable and functional synaptic connection between them has therefore become a major issue. The importance of this question is further strengthened by accumulating evidence suggesting that many of the cognitive or psychiatric disorders that affect human behavior, such as autism, mental retardation, or even schizophrenia, could involve alterations of the mechanisms regulating synapse formation in the brain [7].

At the molecular level, the number of proteins, enzymes, or messengers identified as contributing to the formation of a functional synapse has exploded over the last years. On the postsynaptic side, more than hundred molecules, including receptors, scaffold proteins, protein kinases, adhesion molecules, cytoskeletal components, or intracellular messengers, appear to participate one way or the other in the organization, function, or plasticity of the postsynaptic density. Similarly, the complexity and properties of the machinery responsible for transmitter release and the fusion of docked synaptic vesicles become progressively better and better elucidated. The challenge to establish a synaptic contact is to coordinate the interactions required between the two partners for the formation and differentiation of these specialized structures. Much recent evidence indicates that adhesion molecules

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play an important role in these mechanisms and analyses of their contribution to synaptogenesis have been the subject of several recent reviews [8–12]. Here, we focus on NCAM and discuss recent advances in our understanding of the mechanisms of excitatory synapse formation, as revealed by repetitive confocal imaging, and the role of NCAM in this process.

Adhesion Molecules and Synaptogenesis

Formation of a synaptic contact requires a complex coordinated interaction between pre- and postsynaptic structures leading to the differentiation of highly specialized and regulated structures [13]. At the presynaptic level, synaptic vesicles have to accumulate in the region of contact and become organized in a meshwork able to sustain vesicle fusion at high speed. This implicates various protein complexes, but also channels and signaling pathways. Postsynaptically, at excitatory synapses, a major step involves the formation of a protrusion, the differentiation into a dendritic spine and the expression of a postsynaptic density facing the release site. More than one hundred different proteins organized into complexes have been identified to contribute to these events [7]. In general, pre- and postsynaptic protein complexes are present in neurons and axons before the contact is made and they accumulate in mobile transport packets that are recruited to sites of contact [14, 15]. Once expressed at the synapse, these proteins still undergo a continuous turnover that may even be quite fast as demonstrated for example for glutamate receptors [16] or PSD-95 [17].

Several steps in the process of synapse formation can now be identified and most of these have been found in recent years to involve a contribution of adhesion molecules. A first step at excitatory synapses is to form a contact with an adequate partner on a dendritic spine. Early work in hippocampal neurons has suggested that this process could be initiated by the postsynaptic partner through the growth of a highly motile filopodium, which then stabilizes as a dendritic spine when it gets in contact with the right axon [18]. More recent 2-photon video time lapse analyzes have however shown that dendritic spines may also simply pop out from the dendrite within a few minutes without initial formation of a filopodium [19, 20]. It remains unclear in this case whether a presynaptic partner is already present to initiate the growth of the spine. However, as will be discussed later, the observation that newly formed spines usually lack postsynaptic densities until a few hours after appearance suggests that they might initially grow without identified partner [21, 22]. Expression of adhesion molecules on the growing structures could be important during this step for the matching of pre- and postsynaptic components. Molecules such as cadherins, nectins or neurofascin have been proposed to exhibit such recognition functions, although the evidence remains sparse for excitatory synapses [23]. A second important step is the differentiation of pre- and postsynaptic structures in order to make functional and morphologically mature synapses. This in particular implies the accumulation of release-competent vesicles and proper

receptors at the site of contact. Neurexin and neuroligins have been shown in this respect to be unique among synaptogenetic factors because each of them has the capacity to induce the differentiation of a complementary hemi-synapse on the corresponding partner [10]. Neuroligin expression can induce presynaptic differentiation of nearby axons, while neurexin stimulates the aggregation of postsynaptic components such as receptors or PSD proteins. While the example of neuroligins is certainly the most impressive, it is certainly not the only one and, as will be discussed later, the other adhesion molecules and in particular NCAM and PSA-NCAM might also contribute to bring specific molecular components to the synapse. Finally, a last important step might be the long-term stabilization of the newly formed synapse. Evidence from recent in vivo and in vitro work does indeed indicate that while some spines are definitely highly stables, there are others that are essentially transient [2]. It is very likely that this process could be directly related to activity or even also to synaptic plasticity. Molecules such as BDNF, but also ephrins, cadherins, PSD-95 or even NCAM have been shown to participate to forms of activity-dependent plasticity and could play a role in these aspects of synapse maturation [11, 24–26]. Together, these results point clearly to the notion that the same adhesion molecule may in fact contribute to various aspects of the mechanisms that are required to form a functional and mature synapse and that several adhesion molecules may very well have overlapping functions.

Role of NCAM and PSA-NCAM in Synaptic Function and Plasticity

An important step in our understanding of NCAM function in the brain has come from studies of transgenic mice deficient in NCAM, which showed deficits in learning and memory [27]. Consistent with this observation, numerous subsequent studies confirmed a role of NCAM and more specifically PSA-NCAM in mechanisms of synaptic plasticity such as long-term potentiation, an increase in synaptic strength considered as one of the physiological bases for information storage by a synaptic network. In the CA1 region of the hippocampus, interference with NCAM through specific antibodies or suppression of PSA from NCAM by the enzyme Endo-N or simply suppression of NCAM all resulted in a reduced or even abolished LTP [28–30]. This effect occurred without noticeable changes in the properties of synaptic transmission. Similar results were then further observed in conditional NCAM knockout mice [31], an experiment which allowed to exclude possibilities of developmental anomalies as a possible cause of the defect. Additionally, transgenic mice deficient in the sialyltransferase enzyme responsible for adding PSA to NCAM in adult mice (ST8SialV/PST-1) also showed impaired LTP and they were also selectively deficient in spatial learning tasks and contextual fear conditioning, which depend upon hippocampal plasticity [32]. Interestingly however, while NCAM knockout mice are impaired in cued fear conditioning experiments, a behavior, which depends on synaptic plasticity induced in the amygdala, ST8SialV/PST-1

deficient mice did not show any deficit, suggesting that different brain regions may differentially require PSA or NCAM for plasticity [33] A. Consistent with this interpretation, LTP experiments also suggest that the relative contribution of PSA-NCAM or NCAM to synaptic plasticity may vary as a function of the region analyzed. In the CA3 region of the hippocampus, where LTP properties clearly differ from those expressed in the CA1 region, but also in the dentate gyrus, NCAM seems to be more important than PSA-NCAM since LTP is present in ST8SialV/PST-1 deficient mice, but abolished in NCAM deficient mice [34]. Although it is therefore likely that NCAM and PSA-NCAM may play different roles at specific synapses, these results clearly point to the important regulatory function of PSA-NCAM and NCAM in the control of synaptic strength.

The mechanisms through which this might be achieved remain however unclear. Several hypotheses have been considered which mainly go along two main, non-exclusive lines of thinking. On the one hand, the ratio of PSA-NCAM to NCAM could modulate the adhesion properties between pre- and postsynaptic membrane and thus allow dynamic changes to take place [35]. In the case of LTP, it is now well accepted that a major contribution to the synaptic enhancement involves the expression of new AMPA receptors [16]. Associated to this, LTP in the CA1 region probably requires a remodeling of the synaptic structures, which includes a reorganization of the postsynaptic density and an enlargement of the postsynaptic spine head [36–39]. Variations in the level of expression of PSA-NCAM and changes in the ratio of PSA-NCAM to NCAM, as it occurs under conditions of increased activity [29, 40], could provide the structural plasticity required for these events. A second possibility however is that the ratio of PSA-NCAM to NCAM modifies homo- and heterophillic interactions and thereby affects NCAM signaling [41–43]. Evidence from a number of studies indicates that NCAM may indeed participate in a broad range of biological processes through activation or regulation of various molecular pathways [43–45]. These include growth factor receptors such as FGF receptors, which undergo dimerization and autophosphorylation upon NCAM homophillic binding [46, 47] [43, 48], but also neurotrophin receptors and in particular the BDNF TrkB or P75 receptors [49, 50], which play an important role for properties of synaptic plasticity [51], or direct interactions with either glutamate receptors, cytoskeletal proteins such as spectrin, or the Fyn/MAP kinase cascade [44] which are also critical for the remodeling of postsynaptic structures. Taken together, these results suggest that the ratio of PSA-NCAM to NCAM could represent an interesting system for setting the level of signaling in pathways involved in properties of synaptic plasticity, thus allowing to link activity with capacity for adaptation.

Role of PSA-NCAM in Synaptogenesis

In addition to their roles in regulation of the function and strength of synapses, several lines of evidence suggest that NCAM and PSA-NCAM could also participate in mechanisms of synapse formation. In cell cultures, NCAM accumulate rapidly, within minutes, at sites of contact formation in nascent synapses [52].

Because NCAM binds to spectrin coated trans-Golgi derived organelles, it was proposed that NCAM could work as a trap to allow accumulation of postsynaptic proteins and components necessary for the formation of the synaptic contact [53]. Consistent with this idea, the same authors recently showed that disruption of NCAM-spectrin complexes results in a decrease in the size of postsynaptic densities and a reduced targeting of proteins such as spectrin, NMDA receptors and CaMKIIa to the synapse [54]. They propose that this mechanism could be important not only for the formation of a nascent synapse, but also for activity-dependent plasticity, since, in NCAM deficient neurons, recruitment of CaMKIIa to the synapse by NMDA receptor activation is prevented [54]. Together, this very elegant work clearly emphasizes the important role of NCAM as a functional building block of the synapse. How is it then that NCAM knockout mice do not appear to show deficiencies in synapse number or even in synapse morphology? One possibility clearly is that some degree of overlap or compensation is made possible between the different adhesion molecules expressed at synapses. If neurons from NCAM knockout mice are cultivated together with neurons expressing NCAM and PSA-NCAM, they do show a reduced number of synapses and reduced excitatory activity, indicating preferential formation of synapses with NCAM expressing cells [55]. Similarly, removal of PSA from NCAM also abolished preferential formation of synapses on NCAM expressing cells, pointing to a role of PSA in this process [56]. Another study also indicates that an NCAM mimetic peptide promotes synapse formation [57]. Further analyzes showed that this synaptogenetic effect of NCAM probably involved an interaction between PSA-NCAM and a heparan sulfate proteoglycan and signaling through the FGF receptor [56]. Interference with this mechanism prevented both NCAM-driven synaptogenesis and activity-dependent structural remodeling of activated synapses as indicated by the absence of formation of perforated synapses upon induction of LTP [56, 57]. Together, these results are consistent with the idea that PSA-NCAM, by interfering with NCAM homophilic binding, plays a permissive role that promotes growth and reorganization of the postsynaptic structure such as is required both for the formation of a new contact and for activity-dependent plasticity. A further argument supporting a role for NCAM and more specifically PSA-NCAM in synaptogenesis is the very recent finding that elimination of PSA from NCAM results in the developing visual cortex in an early maturation of perisomatic GABAergic innervation by basket interneurons. In this example, PSA-NCAM also modulates synaptogenesis, but probably through a presynaptic expression, which prevents interneuron axons to form synapses until visual activity sets the stage for ocular dominance plasticity [58]. It is not unlikely therefore that NCAM and PSA-NCAM may have different functional and regulatory role at different synapses.

Dynamic Aspect of Spine Turnover and Synapse Formation

An important new aspect of the mechanisms of synaptic plasticity was revealed by experiments of repetitive confocal imaging examining spine remodeling in living mice. Studies by several groups provided evidence that dendritic spines undergo some

sort of turnover and that there exist a process of continuous growth and elimination of spines [1–4, 59]. Although there has been some debate about the magnitude of this turnover and the technological approach used to image living neurons in the cortex [60], current evidence indicates that spine turnover varies greatly during development, affecting as many as 10%–15% of spines per 24 h in very young animals [2, 3]. Later on, spines become progressively more and more stable with probably only a few percent of spines undergoing replacement in adult tissue [4]. Furthermore, this process appears to vary in different cortical regions and even show some cell-type specificity [6]. Finally, more and more data suggest that this spine turnover process is also affected by sensory activity and may again become very prominent in regions submitted to a lesion [5, 6].

To be able to better analyze the mechanisms regulating spine turnover, we recently developed an in vitro approach based on the use of hippocampal slices cultures. Through repetitive imaging of the same cells over several days (Fig. 1), we found that dendritic spines showed a high level of turnover, affecting about 20% of all spines over a 24 h period in 15 days old cultures, but only about 10% after 25 days in vitro [21]. These values, which are quite close to those obtained in very young animals, suggest that spine turnover retains similar properties in vitro and in vivo and particularly its developmental dependency. Another interesting aspect was that this rate of spine turnover is actually underestimated by the use of long observation intervals. It turns out that in 15 DIV slice cultures, the basal rate of protrusion formation reaches values in the order of 2% of all spines per hour, which represents hundreds of new protrusions per day and per neuron. The reason why spine density remains nevertheless stable is that most of these new protrusions do in fact disappear fairly quickly and only a small proportion of them become stable spine synapses. Interestingly, while filopodia were proposed in previous studies to be precursors of spine synapses [18], we found that they only exceptionally lead to the formation of stable spine synapses (Fig. 1d), a result consistent with other in vivo data [3]. Protrusions are thus generated at a high rate, but only a fraction of them become finally stable spines (Fig. 1e). We also found that this required a process of maturation that lasted about 24 h. During this period, new spines usually grew in size (Fig. 1f) and started to express a PSD (Figs. 1g, h). It took however about 5 h in our in vitro experiments to start detecting PSDs on newly formed spines (Fig. 1i), a result that is consistent with results obtained through EM reconstruction of newly formed spines [22]. Interestingly, expression of this PSD was activity-dependent in hippocampal slice cultures, since blockade of AMPA and NMDA receptors prevented its expression and reduced the probability of the spine to be stabilized.

exceptionally lead to the formation of a stable spine (*black columns*). (**e**) Stability of newly formed spines (*black columns*) and of their transformation into stubby spines (*grey columns*). (**f**) Progressive enlargement of newly formed spines (bar: 0.5 µm). (**g**) Illustration of a newly formed spine (age < 5 h; *arrow head*, *middle panel*), which do not express PSD-95-DsRed2 (*arrow head*, *right panel*; bar: 0.5 µm). (**h**) Electron microscopic illustration of a spine devoid of PSD and presynaptic partner (bar: 0.5 µm). (**i**) Time course of Psd-95-DsRed2 expression in newly formed spines

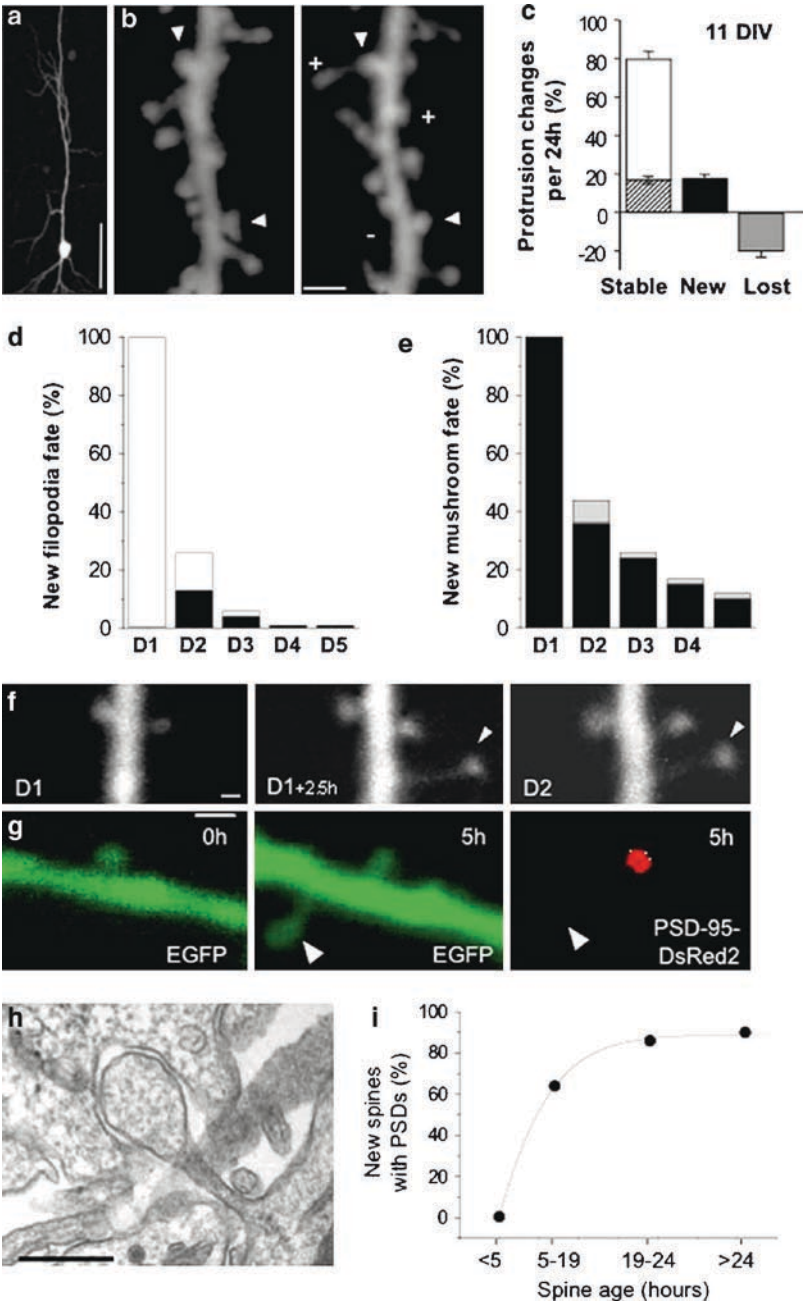

Fig. 1 Spine dynamics in hippocampal organotypic slice cultures. (**a**) EGFP transfected CA1 pyramidal neuron (bar: 100 μm). (**b**) Repetitive imaging of a dendritic segment at 24 h interval reveals the occurrence of new and lost protrusions (bar: 1 μm). (**c**) Summary of the proportion of stable spines (*open column*), which include spines exhibiting changes in morphology (*dashed column*), of newly formed (*black column*) and disappearing (*gray column*) spines. (**d**) Stability over 5 days of newly formed filopodia. Note that most of them disappear within 1–2 days and only

Taken together, these experiment suggest a model in which development of synaptic networks proceed through an extensive, non specific growth of dendritic protrusions, followed by the stabilization of a small number of spines. This stabilization process involves the expression of a PSD through mechanisms that appear to be driven by synaptic activity [21].

Regulation of Spine Stability and Function by PSA-NCAM/NCAM Ratio

The capacity to now examine on a longer time scale the behavior of specific synapses makes it possible to investigate new aspects of the role of adhesion molecules and in particular how they may regulate some of the initial or activity-dependent steps of synaptogenesis. In the case of NCAM and PSA-NCAM, preliminary experiment in this laboratory (Mendez et al., unpublished) suggest that upon removal of PSA from NCAM by the enzyme Endo-N, the rate of spine formation is reduced and more importantly the stability of spines is decreased. At the presynaptic level, other studies also suggest that the lack of NCAM function leads to a reduction of the stability of synaptic contacts [61]. These observations would actually be consistent with the different results reported above and in particular with the hypothesis that one important function of NCAM might be to help build the architecture of the postsynaptic density, probably by interacting with molecules such as spectrin and indirectly with constituents of the PSD such as PSD-95 or CaMKII [54]. One might also hypothesize that NCAM molecules through homophillic cis- or trans-interactions tend to form clusters that could give some rigidity to the synapse. It is interesting in this respect that electron microscopic analyzes of high pressure quickly frozen hippocampal synapses revealed the presence of a protein network within the synaptic cleft that shows the periodic arrangement expected from a zipper organization of NCAM or cadherin molecules [62]. However, in order to remodel or enlarge the PSD, as it happens during the initial formation of the PSD in a new synapse or following activity-induced plasticity, one might assume that less rigid interactions and additional signaling promoting turnover of PSD components would be important. These changes could be promoted by increasing the ratio of PSA-NCAM to NCAM, a change that can be mediated by neuronal and synaptic activity [29, 40]. The increase in PSA-NCAM could at the same time disrupt or affect the zipper organization of NCAM and, through interactions with heparan sulfate proteoglycans and FGF receptors or even other neurotrophin receptors, activate the signaling pathways regulating receptor and other PSD protein dynamics [43, 57]. An increased ratio of PSA-NCAM to NCAM could represent therefore an important regulator or trigger of plasticity. Accordingly, suppression of PSA from NCAM by the enzyme Endo-N would be expected to affect several important properties of excitatory synapses: this should interfere with the process of synapse formation by limiting the initial growth of the PSD in newly formed spines and thus reduce the probability for new protrusions to become stabilized, accounting therefore for the decreased

number of synapses detected on young NCAM knockout neurons [56]; the same mechanism would however also account for the role of PSA-NCAM in LTP, since the remodeling of the postsynaptic density and namely the expression of new receptors and other PSD constituents such as PSD-95 or CaMKII could also require to loosen the zipper organization of adhesion molecules at the synapse. Elimination of PSA from NCAM has indeed been shown to prevent the formation of perforated synapses upon application of high frequency stimulation [56]. Finally, the ratio of PSA-NCAM to NCAM could also have direct consequences on the long-term stability of excitatory synapses, if, as suggested by several results, stability correlates with size [63] and if size changes are mediated or associated to expression of properties of plasticity [24, 39, 64]. The interest of this general hypothesis about the role of PSA-NCAM and NCAM is that it makes predictions that should now become testable with the new developments carried out in confocal imaging. For example the dynamic of synaptic proteins at the PSD should depend upon the ratio of PSA-NCAM to NCAM or the stabilization of new spines by activity should also require expression of PSA-NCAM. Additionally, the development of new molecular tools such as peptides mimicking specific regions of these adhesion molecules could represent powerful new approaches to identify specific interactions involving adhesion molecules and their role in synapse formation and plasticity [65]. Evidence from recent results indeed indicates the great potential interest of these new tools with regard to therapeutic applications [57, 66].

Conclusion

The work reviewed here clearly points to an important function of NCAM and PSA-NCAM at excitatory synapses. While the evidence that these molecules regulate synapse formation mechanisms remains as yet scarce, solid data now firmly established that they play an important role in synaptic plasticity. As proposed here, these two aspects might actually reflect the same function of NCAM and PSA-NCAM at synapses, i.e., to regulate the capacity of the PSD to undergo remodeling and therefore set the stage for the formation and stabilization of the synapse. This hypothesis is already supported by interesting recent data, but it is very likely that some new developments in several methodological approaches will make it possible to test this idea more thoroughly.

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NCAM in Long-Term Potentiation and Learning

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Abbreviations

Ab	Antibody
AD	Alzheimer's Disease
BDNF	Brain-derived neurotrophic factor
CAM	cell adhesion molecule
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
EM	Electron microscopy
Endo-N	Endoneuraminidase N
EPSP	Excitatory postsynaptic potential
FGFR	Fibroblast growth factor receptor
GluR2/3	Glutamate receptor 2/3
Ig	Immunoglobulin
KO	Knock-out
LTP	Long-term potentiation
NCAM	Neural cell adhesion molecule
PKC	Protein kinase C
PSA	Polysialic acid
PSD	Postsynaptic density
TTX	Tetrodotoxin

Introduction

Neural cell adhesion molecule (NCAM) is a cell adhesion molecule of the immunoglobulin (Ig) superfamily and the first NCAM to be discovered and characterized. Neural cell adhesion molecule was first reported in 1974 as "D2,"

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a glycoprotein enriched in synaptic membrane fractions, by Jørgensen and Bock. Independent work by others subsequently described the "N-CAM" and "BSP-2" proteins, which were later realized to be identical to D2 and shown to mediate cell–cell adhesion [1–5]. Neural cell adhesion molecule has since been studied intensely and reported to mediate cell adhesion and signaling through homophilic (NCAM–NCAM) binding as well as heterophilic interactions with a number of membrane protein binding partners including L1, FGF-receptors, TAG1/axonin-1, and GDNF/GFRα1; extracellular matrix components including fibronectin and heparin sulfate glycans; and intracellular binding partners including spectrin, focal adhesion kinase $p125^{FAK}$, and fyn (reviewed in [6–8]).

Neural cell adhesion molecule is the carrier of an unusual carbohydrate called polysialic acid (PSA), which consists of large sialic acid homopolymers. PSA-NCAM is widely expressed in the embryonic brain, whereas expression becomes much more restricted postnatally. PSA-NCAM has been regarded as a "plasticity-promoter," possibly acting permissively by decreasing adhesion mediated by homophilic NCAM binding as well as by other adhesion molecules, thereby facilitating neurite outgrowth and synaptic remodeling (For review, see [9–12]). In the adult rodent, PSA-NCAM expression is striking in the mossy fiber system and is also expressed in the CA1 stratum radiatum on pyramidal dendrites in vivo and in slice cultures [13, 14].

Due to its expression pattern and possible involvement in synaptic plasticity, PSA-NCAM has been studied in relation to long-term potentiation (LTP), a widely studied form of synaptic plasticity. LTP was first described in 1966 by Terje Lømo and colleagues who described a long-lasting increase in synaptic efficacy after high-frequency stimulation of the perforant pathway (see [15]). The phenomenon immediately caught attention as a synaptic strengthening mechanism and possibly a cellular substrate of memory. Enormous efforts have since been done to dissect the molecular mechanisms in LTP. Several reviews have discussed these mechanisms in detail [16–19]. At the CA3-CA1 synapse, there is consensus that the NMDA-receptor plays a crucial role in LTP induction, while downstream effectors are still subject to debate. The NMDA-receptor activation requires both glutamate binding and membrane depolarization. The NMDA-receptor may therefore mediate Ca^{2+}-influx when a given pyramidal neuron is activated to the level where significant membrane depolarization occurs such as when several afferents fire simultaneously. Thus, the NMDA-receptor activation occurs only when the postsynaptic cell is depolarized to a sufficient level and only at synapses that actually sense glutamate release from a presynaptic terminal. Due to the properties, the NMDA-receptor has been termed "coincidence detector." Downstream of the NMDA-receptor activation, it is widely believed that postsynaptic recruitment of AMPA-receptors from vesicular pools and/or perisynaptic membrane plays an important role in synaptic strengthening in LTP. Different stimuli induce synaptic potentiation of varying duration, which may correspond to several mechanistically distinct phases or LTP forms. The induction of LTP at the CA3–CA1 synapse requires synaptic glutamate release in a manner and magnitude sufficient to induce Ca^{2+}-influx through NMDA-receptors. Other sources of Ca^{2+}-influx or -release from intracellular stores may modulate induction. Suggested effector mechanisms in LTP maintenance include posttranslational

modifications such as phosphorylation of AMPA-receptors by protein kinases CaMKII and PKC; local synthesis of new proteins near dendritic spines; and somatic gene expression. In addition to the CA3–CA1 synapse, LTP has been described in several other brain areas and it is realized that the molecular mechanisms underlying LTP induction and maintenance differ.

A major part of the fascination of LTP is the possible relation to learning and memory. Thus, LTP may represent a mechanism for synaptic enhancement in learning. This has not been unequivocally demonstrated although numerous studies have pursued the relation between learning and LTP. A major obstacle is the difficulty in identifying and monitoring the actual synapses being modified in a complex in vivo learning paradigm. However, various indirect approaches have correlated LTP and learning including by showing the importance of the NMDA-receptor in hippocampal LTP as well as in spatial learning. Likewise, the involvement of NCAM in LTP and animal behavioral models have been studied in parallel in an attempt to dissect the putative role of NCAM in activity-dependent synaptic strengthening and remodeling in various model systems and its relevance to learning and memory.

NCAM in Learning and LTP

Theoretically, cell adhesion molecules (CAMs) appear well suited as mediators of long-lasting synaptic changes in learning. Ultimately, long-lasting changes in synaptic efficacy are paralleled by structural synaptic change. Cell adhesion molecules may mediate such changes through their adhesive properties. Moreover, most CAMs, including NCAM, are coupled to the cellular signaling machinery and could transmit shorter lasting activity changes into long-lasting structural synapse modifications.

Direct experimental support for a role of NCAM in LTP was first obtained by infusion of antibody (Ab) in the attempt to block normal NCAM function. Thus, infusion of NCAM Ab into the CA1 stratum radiatum in acute hippocampal slices reduced the potentiation induced by high-frequency stimulation. Similar effects were found using three different polyclonal NCAM antibodies [20, 21]. The reduced potentiation of the field excitatory postsynaptic potential (EPSP) was observable immediately following high-frequency stimulation. A similar effect on LTP was demonstrated after bath application of agents believed to inhibit L1-NCAM binding, namely (1) synthetic carbohydrates mimicking a carbohydrate expressed by L1 and thought to be recognized by NCAMs Ig4 module and (2) synthetic peptides mimicking the stretch in the fourth Ig-domain of NCAM, which supposedly is involved in L1 binding [20, 22].

An involvement of NCAM in learning was first demonstrated by intracerebroventricular infusion of NCAM antibodies in the rat [9, 23]. In these studies, NCAM antibodies impaired consolidation of a passive avoidance response when administered in a specific time period occurring 6–8 h post-training. This time-specific memory impairment by NCAM antibodies was also demonstrated in pas-

sive avoidance learning in the chick [24] and in odor discrimination in rats [25]. In agreement with this, NCAM was synthesized among other glycoproteins in a glycoprotein-dependent phase, occurring 5–8 h after training in a passive avoidance paradigm in the chick [26]. Thus, it appears that NCAM is required in this specific phase of memory consolidation. Additionally, injection of NCAM antisense oligonucleotides twice before training impaired memory consolidation of a passive avoidance response in the chick [27] and continuous intraventricular infusion of NCAM antibodies impaired spatial learning in the Morris water-maze in rats providing further support for a role of NCAM in learning [28]. Injection of large molecules, such as antibodies, directly into the brain may result in unspecific effects causing memory impairment by an unphysiological clustering of NCAM and other proteins on neuronal membranes. Thus, it cannot be ruled out that the memory impairments observed in the described studies could be due to such confounding factors and not to a direct NCAM-mediated effect. However, much evidence, as outlined above, indicates that the memory impairment caused by NCAM antibodies is time specific supporting the theory that the impairments are not due to confounding factors, but are indeed caused by the specific binding of NCAM antibodies to NCAM and the cellular processes that follow. In addition, the described studies have used different controls, i.e., preimmune serum, in order to show that the effects seen with NCAM antibodies were specific to these antibodies and importantly, in most studies it has been demonstrated that the injected animals did not show any overt behavior, indicating that the impairment was not due to nonspecific effects. Thus, all in all it seems that NCAM antibodies impair memory consolidation probably by disrupting NCAM homophilic binding and thereby disrupting cell adhesion and stabilization of synaptic connections necessary for consolidation of memory.

Recently, the involvement of NCAM in learning and memory has also been investigated via synthetic peptides mimicking or interfering with NCAM binding [29–33]. The first of these peptides discovered was the C3 peptide, which is a synthetic peptide binding to the first N-terminal Ig-like module of NCAM and has been demonstrated to impair long-term memory in a passive avoidance task [31], in an approach avoidance task [32] and of contextual fear conditioning [30] when injected intracerebroventricularly immediately prior to training or 5–6 h posttraining. The latter time period corresponds to the specific time window sensitive to NCAM antibodies as described above. C3 also impaired spatial memory in the Morris water-maze when injected 24 h before training [33]. Thus, the C3 peptide seems to disrupt NCAM functions necessary for acquisition and consolidation of different forms of hippocampus-dependent memory in two distinct time windows. By binding to the first Ig module of NCAM, C3 probably disrupts NCAM homophilic binding, resulting in reduced cell adhesion and/or interferes with NCAM-mediated intracellular signaling as demonstrated in vitro [29]. This reduced cell adhesion may reduce stability in synapses during learning and prevent new synapse formation necessary for memory consolidation, resulting in the amnesia demonstrated. Another hypothesis may be that C3 interferes with the structural remodeling occurring in the synapse during consolidation by preventing internalization of NCAM after training [31].

Other synthetic peptides derived from NCAM, the FGL and P2 peptides, have in contrast to the C3 peptide recently demonstrated procognitive effects in vivo [34, 35]. The FGL peptide corresponds to a 15 amino acid sequence in the second F3 loop of NCAM and this sequence is presumably involved in the binding of NCAM to the fibroblast growth factor receptor 1 (FGFR1) [36]. It has been demonstrated that the FGL peptide enhanced spatial memory in the Morris water-maze and fear conditioning when administered intracerebroventricularly immediately post-training and this effect lasted up to 2 and 4 weeks, respectively [34]. Additionally, FGL improved social recognition and expressed both as short- and long-term social memory, when administered either 1 h or 73 h pretraining [37], indicating that the FGL peptide may induce long-lasting changes in the cells involved in acquisition or consolidation, probably by activating FGFR1 and initiating intracellular signaling. Interestingly, the FGL peptide has recently shown potential for treatment of Alzheimer's disease (AD), as it both prevented and rescued AD-like pathological signs and cognitive impairment induced by $A\beta_{25-35}$ [38].

The P2 peptide is derived from the second Ig module and this sequence is involved in cis-homophilic NCAM binding [39]. P2 showed procognitive effects both in the T-maze, representing spatial working memory, and in the social recognition task with a 2 h intertrial interval, representing short-term social memory, when administered 1 h pretraining and 1 h or 24 h pretraining, respectively, whereas no effects were observed in the Morris water-maze [35]. P2 probably mediates these procognitive effects by disrupting cis-homophilic NCAM binding and thereby reduce cell adhesion and shift the function of NCAM to mediating intracellular signaling possibly via the FGFR [35].

Thus, the three synthetic peptides mimicking NCAM binding all affected learning and memory; however, C3 induced amnesia, whereas FGL and P2 showed procognitive effects. FGL differs from C3 and P2 by binding directly to FGFR1, whereas C3 and P2 both are involved in NCAM homophilic binding and are therefore supposed to disrupt homophilic NCAM binding and thereby reduce cell adhesion and/or interfere with NCAM-mediated intracellular signaling. Even though both the C3 and P2 peptides are sequences supposed to be involved in NCAM homophilic binding, they mediate opposite effects with regard to cognitive processes as mentioned above. This is surprising as these peptides would be expected to mediate similar effects. The FGL peptide enhanced both short- and long-term memory in contrast to the P2 peptide, which only enhanced short-term memory. The differences in the procognitive effects mediated by the FGL and P2 peptides are most likely due to the fact that FGL binds FGFR directly presumably acting as an NCAM mimetic, whereas P2 binds to NCAM and therefore disrupts homophilic NCAM binding, meaning that their modes of action are different.

As reviewed above, a number of experimental studies using NCAM antibodies and synthetic peptides mimicking or interfering with NCAM binding have supported a role for NCAM in LTP and learning. Neural cell adhesion molecule antibodies impaired learning and LTP and the three peptides either impaired or enhanced memory consolidation. Neural cell adhesion molecule acts as a mediator of cell adhesion and intracellular signaling via FGFR and probably thereby regulates synaptic remodeling and morphological changes necessary for learning and memory consolidation.

NCAM Expression and Localization in Synaptic Plasticity

Several studies have shown that the expression of NCAM changes during memory consolidation and LTP. A decrease in NCAM expression has been demonstrated in the hippocampus 3–4 h following passive avoidance training, probably due to internalization [31]. Further, a reduced NCAM expression has been shown 12 h following fear conditioning [40]. In contrast, an increase in the expression of NCAM has been demonstrated in the hippocampus 24 h following both fear conditioning and training in the Morris water-maze [33, 40]. These findings indicate that NCAM expression is regulated in phases during memory consolidation, where NCAM may be downregulated at first in order to allow destabilization and remodeling of synaptic connections. After that, an upregulation of NCAM may mediate stabilization of newly formed synaptic connections.

Parallel studies have explored NCAM expression after LTP induction. In 1994, an overall increase in extracellular NCAM protein associated with LTP in vivo was reported [41]. The increased NCAM was observed 90 min after induction of LTP in the dentate gyrus. In addition, increased hippocampal immunoreactivity corresponding to an extracellular NCAM fragment has been observed in vitro in hippocampal slice cultures after a brief NMDA application and in vivo after kainate administration [42]. These changes were suggested to result from proteolysis of NCAM molecules resulting in the extracellular release of NCAM fragments and possibly facilitating synaptic remodeling by cleavage of existing adhesive contacts. Interestingly, mice expressing lacZ under control of the NCAM promoter were reported to respond to treatment with the ampakine, CX547 (80 mg/kg i.p.) with increased hippocampal expression of the reporter βgal as well as NCAM mRNA 8 h later indicating that NCAM expression may be regulated by AMPA-receptor activation [43].

One NCAM form is of particular interest in relation to synaptic plasticity, namely, NCAM-180, which has been localized to the postsynaptic density (PSD). Immunogold electron microscopy has been applied to study postsynaptic NCAM-180 expression in dentate synapses and its regulation by perforant path high-frequency stimulation in vivo. 24 h following LTP induction a marked increase in the fraction of NCAM-180 immuno-positive spine synapses in the outer molecular layer was observed [44] and shown to be NMDA-R-dependent. Concomitantly, the fraction of AMPA-R immuno-positive synapses increased in accordance with theories on recruitment of AMPA-Rs to silent synapses following LTP induction. Later the same group reported that the synaptic NCAM-180 distribution changed [45]. In unstimulated rats, NCAM-180 is predominantly localized in the center of postsynaptic densities (PSDs). 24 h following LTP induction, an increased fraction of NCAM-180 molecules was localized at the edges of postsynaptic densities. Interestingly, similar changes were observed for the NMDA-receptor subunit NR2A, whereas more of the AMPA-receptor subunits, GluR2/3 immunogold particles were localized more centrally and the fraction of GluR2/3 positive synapses decreased. It is possible that changes in synaptic NCAM expression and localization in

response to LTP induction in turn contribute to the regulation of Glu-R redistribution by mediating recruitment and stabilization of organelles at synapses [46]. In addition, adhesion changes resulting from synaptic NCAM relocalization from central to more peripheral zones may be permissive for synaptic remodeling and thereby NCAM may serve to translate transient patterns of neuronal activity into long-term morphological change.

Taken together, the findings from the diverse in vitro and in vivo studies of NCAM expression indicate that indeed expression of NCAM protein may change in an activity-dependent manner. Initial studies have detected changes in overall expression of NCAM forms, which is now pursued in more detail studying expression and activity-dependent relocalization of specific NCAM forms in synaptic regions.

PSA-NCAM in Synaptic Plasticity

A particular form of NCAM, PSA-NCAM, carrying large sialic acid homopolymers, is believed to facilitate neurite outgrowth and synaptic remodeling, possibly by decreasing cell adhesion mediated by CAMs and by permitting NCAM-mediated activation of FGF-receptors. PSA-NCAM expression shows pronounced developmental regulation and expression in the adult brain is restricted, but it is found in areas involved in learning such as the hippocampus, the amygdala, the piriform cortex, and the neocortex (for review, see [12]). In the hippocampus, a high PSA-NCAM expression is observed in the entire mossy fiber system. In addition, PSA-NCAM expression has been demonstrated in the CA1 region.

The expression of PSA-NCAM in the hippocampus has been investigated in relation to learning in vivo and it has been demonstrated that the expression of PSA-NCAM changes during learning. Thus, an increase in hippocampal PSA-NCAM was detected following training in a passive avoidance task [47, 48], in the water-maze [33, 49] and after contextual but not auditory fear conditioning, auditory fear conditioning being more amygdala-dependent than contextual fear conditioning [50, 51]. This increase in polysialylation seemed to occur in the time window 10–24 h following training and probably reflects that the adhesive properties of NCAM are reduced by polysialylation of NCAM in this phase of memory consolidation allowing structural remodeling of synaptic connections.

Further evidence for a role of PSA-NCAM in learning in vivo has been supported by studies demonstrating that enzymatic removal of PSA by Endo-neuramidase (Endo-N) generated deficits in spatial learning in the Morris water-maze in wildtype mice [13, 33]. In addition, mice deficient in PSA-NCAM due to lack of polysialyltransferase-1 (ST8SialV/PST-1), an enzyme, which is responsible for adding PSA to NCAM, exhibited impaired spatial learning in the Morris water-maze as well as impaired contextual fear conditioning [52]. These studies provide further support for PSA-NCAM playing a role in these types of hippocampus-dependent learning. Recently, the effect of exogenously added PSA-NCAM has also been investigated in learning. Interestingly, intrahippocampal

injections of PSA-NCAM impaired fear conditioning when injected into wildtype mice, whereas injections of PSA-NCAM, but not of NCAM, partly restored an impaired fear conditioning in NCAM-deficient mice [51], indicating that exogenously added PSA-NCAM disrupts learning when naturally occurring NCAM is present, but promotes learning when NCAM is not present.

Removal of PSA by Endo-N treatment has also been shown to markedly inhibit LTP in CA1 in acute slices [13] and in organotypic slice cultures [14]. In addition, an impaired CA1 LTP was demonstrated in mice lacking ST8SiaIV/PST-1 [53]. Moreover, Endo-N treatment has been demonstrated to inhibit the increased fraction of perforated spine synapses otherwise observed in the CA1 region of hippocampal slice cultures after LTP [54]. In this study, putative activated synapses were identified by means of a Ca^{2+} precipitation technique [55, 56]). By electron microscopy (EM), an increased fraction of synapses containing an organelle Ca^{2+}-precipitate showed a perforated PSD after LTP when compared to nonstimulated controls. No difference was observed after Endo-N or heparinase treatment. In addition to influencing cell adhesion and adhesion-induced signaling, PSA has been reported to downregulate NMDA-R function in primary hippocampal cultures, presumably by decreasing open probability of NR2B subunit containing NMDA-Rs [57].

In hippocampal slice cultures, the re-expression of PSA-NCAM subsequent to Endo-N treatment and wash out has been shown to depend on neuronal activity. Thus, PSA re-expression under these circumstances could be inhibited by 1 μM tetrodotoxin (TTX) or low Ca^{2+} and conversely promoted by the presence of 10 μM Bicuculline. Activity-dependent mobilization of PSA-NCAM has also been reported in cultured neurons and cell lines [58, 59]. These observations suggest that PSA-NCAM may mediate adhesive changes in an activity-dependent manner and support a role for PSA-NCAM in regulating morphological synaptic change in LTP and learning.

In conclusion, the role of PSA-NCAM in learning and LTP has been investigated using enzymatic removal of PSA and genetically modified mice deficient in PSA-NCAM. It is clear from these studies that PSA-NCAM is involved in learning and LTP possibly by shifting the function of NCAM from primarily mediating cell adhesion to favor intracellular signaling and synaptic remodeling and thereby allowing morphological changes necessary for learning and memory consolidation.

Synaptic Plasticity in NCAM-Deficient Mice

The characterization of NCAM knock-out (KO) mice has shed additional light on the role of NCAM in synaptic plasticity. Neural cell adhesion molecule-deficient mice had an obvious phenotype, as the olfactory bulb was markedly reduced in size, but otherwise they appeared healthy and fertile [60]. Later more subtle changes have been reported including a change in mossy fiber fasciculation [61]. Despite morphological abnormalities including in the intrahippocampal circuitry, NCAM-deficient mice have been most valuable in the attempts to understand the role of NCAM in synaptic plasticity.

First, it was demonstrated that NCAM-deficient mice exhibited impaired spatial learning in the Morris water-maze [62]. Later, it was shown that mice deficient in NCAM also had deficits in contextual and cued fear conditioning [51, 63] and this impairment could be partially restored by intrahippocampal injections of PSA-NCAM, but not of NCAM, when injected before training as mentioned previously.

In organotypic slice cultures from NCAM-deficient mice, a decaying LTP was observed, which differed from LTP in WT slice cultures [64]. It should be mentioned that in another NCAM-deficient mouse strain, LTP appeared normal [43]. It is not known whether this apparent discrepancy is due to strain differences or differences in the electrophysiological approach. Later, mice in which NCAM was inactivated predominantly postnatally in the hippocampus by means of CRE-loxP recombination using the CaMKII promoter [65] were generated. LTP was reduced in CA1 in acute slices from these mice. The latter mice did not show the same morphological abnormalities regarding the olfactory bulb and the mossy fiber system as the constitutive NCAM KOs supporting a specific role of NCAM in LTP in the Schaffer collateral – CA1 synapses. In contrast, in slices from the conditional hippocampal NCAM-deficient mice, no change in mossy fiber – CA3 LTP was observed indicating that structural abnormalities and/or abnormal mossy fiber development may be responsible for the deficient LTP observed in this region in constitutive NCAM KOs.

Several studies have reported rescue of the LTP impairment in NCAM-deficient mice. Thus, the impaired LTP in CA1 has been restored by infusion of PSA-NCAM in hippocampal slices [51]. LTP impairment could also be rescued by raising extracellular Ca^{2+} in mutants with reduced hippocampal NCAM expression [65], suggesting an involvement of NCAM in regulation of Ca^{2+}-dependent signal transduction in LTP in CA1. Interestingly, brain-derived neurotrophic factor (BDNF) was reported to restore LTP in slices from NCAM KO mice [66], indicating that NCAM may be important for BDNF responsiveness of CA1 neurons in synaptic plasticity.

Thus, an involvement of NCAM in learning and LTP has been supported by the analysis of mice deficient in NCAM as outlined above. Neural cell adhesion molecule-deficient mice showed impaired hippocampus-dependent learning and decaying LTP in the hippocampus, and soluble PSA-NCAM, in contrast to NCAM, could at least partially rescue these deficits.

NCAM in Synaptogenesis

As several studies have supported an involvement of NCAM in LTP and learning based on Ab studies, enzymatic removal of PSA and KO mice, a key question is to understand the actual role of NCAM in synaptic plasticity. One approach to address this question has been to culture primary hippocampal neurons from both wildtype and NCAM-deficient mice together and to investigate the possible differences in synaptogenesis in vitro. In such cocultures, an increased synapse density was observed on neurites from NCAM-expressing neurons when compared to neurites

from NCAM-deficient neurons as estimated by synaptophysin immunoreactivity and FM4-64 imaging [67]. All NCAM forms (120, 140 and 180) had this effect to a roughly similar magnitude when transfected into NCAM-deficient neurons. Thus, new synapses may preferentially form on neurons expressing NCAM at least in vitro. The increased synapse formation on NCAM-expressing neurites appears to be dependent on synaptically activated NMDA-receptors, as the response was blocked by AP5 but not TTX or NBQX.

Interestingly, the increased synaptogenesis observed on NCAM-expressing neurons was inhibited or abolished by removing PSA from NCAM by Endo-N treatment or by NCAM-PSA-Fc or NCAM-Fc constructs. Likewise, the synaptogenic response to NCAM expression was inhibited by heparinase treatment and by the pharmacological FGF-receptor blocker PD173074. These observations support a synaptogenic role of PSA-NCAM in vitro dependent on NCAM interaction with heparin sulfates involving FGF-receptor activation similar to what has been reported for the neurite outgrowth response to NCAM expression on contacting cells. In summary, the possible role of NCAM and PSA in activity-dependent synaptogenesis in vitro has been supported in studies of primary NCAM-expressing neurons from WT mice cocultured with neurons from NCAM-deficient mice. This type of experimental approach will be valuable in the attempts to understand NCAMs role in synaptic plasticity at the molecular level.

Concluding Remarks

In conclusion, considerable evidence has now been accumulated that NCAM is involved in synaptic plasticity in hippocampal-dependent learning and LTP. Impaired LTP and learning have been reported following blockade of normal NCAM function using Ab or PSA-removal. Moreover, NCAM-deficient mice or mice lacking enzymes necessary for expression of PSA-NCAM show impaired LTP and learning. Synaptic NCAM expression has been shown to change in association with LTP and conversely NCAM appear to be necessary for synaptic changes observed in association with LTP in slices and to facilitate synaptogenesis in cell culture. Based on these findings, NCAM and its PSA moiety appear capable of mediating and permitting adhesive changes in an activity-dependent manner and thereby to play an important role in synaptic remodeling in LTP and learning. The exact mechanisms for NCAM's role in cognitive processes, however, remain to be clarified. A better understanding of NCAM and its PSA moiety in pathological conditions characterized by cognitive impairment would be most valuable in the search for new treatment options for these conditions.

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Role of NCAM in Emotion and Learning

Lisa Conboy, Reto Bisaz, Kamila Markram, and Carmen Sandi

Introduction

In this review, we will discuss the role of the neural cell adhesion molecule [1] in emotion and learning. Classically, the behavioral role attributed to NCAM has been in learning, memory, and neural plasticity [2, 3]. However, increasing evidence presented over the past few years is also unraveling a role of NCAM on emotional behavior. After a brief introduction about the NCAM molecule, we will start reviewing its role in emotion, particularly in unlearned emotional responses, such as anxiety and aggression. In the second part of this review, we will address the role of NCAM in learning and memory processes to finally propose a role for this molecule at the interface between emotion and learning.

General Features of NCAM in the Central Nervous System: Molecular Structure and Function

NCAM is a member of the immunoglobulin superfamily of cell adhesion molecules. It is characterized by the presence of immunoglobulin homology domains (Ig-domains) in its extracellular part. NCAM is encoded by a single gene on chromosome 9 in mice [4] and 11 in humans [5] and undergoes differential splicing of the messenger RNA [6, 7]. The three main splice variants of NCAM are named according to their approximate molecular weights: NCAM180, NCAM140, and NCAM120. Within the central nervous system, NCAM180 appears to be the isoform enriched at postsynaptic sites, while NCAM140 is expressed both in neurons (pre and postsynaptically) and glia, and NCAM120 predominantly in glia [8, 9].

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Post-translational attachment of long chains of the polysaccharide polysialic acid (PSA) to NCAM (PSA-NCAM) allows NCAM an additional mechanism to control synaptic functioning. In the adult brain, PSA-NCAM is mainly present in regions capable of undergoing some kind of structural plasticity [10], such as the hypothalamo-neurohypophyseal system [11], the olfactory bulb [12], the piriform and entorhinal cortices [13], the amygdala [14] hippocampus [13], and prefrontal cortex [14]. PSA is proposed to decrease NCAM-mediated membrane–membrane adhesion in vitro [15, 16], presumably due to its very large hydrated volume or negative charge or both [9, 17, 18].

NCAM is highly expressed at synaptic junctions. Neuronal activity regulates the functioning of synapses with a potential to either enhance or depress synaptic strength. During development, selective expression of cell adhesion molecules is proposed to regulate embryogenesis by dictating patterns of cell differentiation followed either by stabilization or selective elimination of synapses, as a mechanism of finetuning cellular connections [19]. Moreover, during development, neurite outgrowth is associated with the cell being in a de-adhesive state. When the neurite reaches and innervates the correct area, adhesiveness is increased so that the cell becomes locked in position. This modification in adhesion is controlled by homophilic cell–cell binding via cell adhesion molecules such as NCAM [20]. In addition to development, learning, and memory, LTP, aging, stress, and neuro-regeneration are all events that can also stimulate synaptic reorganization. Cumulative evidence indicates a key role for NCAM in the neural remodeling accompanying all these events.

NCAM and Emotion

Altered emotional behaviors have mainly been studied in adult male mice expressing a null mutation in the NCAM gene. Initial work demonstrated that NCAM-KO mice show enhanced anxiety in an emergence test, as indicated by their increased latencies to leave from a small box and explore an open environment [21]. An increased anxiety-like behavior of NCAM-deficient mice was also found in the light/dark avoidance test [22]. This effect was then shown to be gender and genetic background-independent and could be influenced by application of a low dose of the 5-HT_{1A} serotonin receptor agonists, either buspirone or 8-hydroxy-2-(di-n-propylamino)-tertraline (8-OH-DPAT), which at the same dose showed no anxiolytic effect in wild-type controls [22]. This suggested a functional alteration of the serotonergic system in these mice likely to be involved in their altered anxiety-like responses. Although the authors found little evidence for the existence of regional differences in serotonin receptor expression, they did find a slight reduction in the level of the 5-HT_{1A} receptor expression in the hippocampus and the amygdala of NCAM-KO mice.

In support of an altered serotonergic system is also the finding that NCAM-KO mice show enhanced aggression toward an unfamiliar intruder male, which was

accompanied by a greater post-intruder hormonal stress response [23]. Analysis of c-fos mRNA levels to monitor neuronal activation after the intruder stress revealed greater neuronal activity when compared with wild type controls in limbic areas, including the amygdala, a brain region known to be involved in modulating emotional responses.

Exploratory behavior is a basic adaptive behavioral response in rodents. When an animal is presented with a new environment, it is normally motivated to explore. Perturbations in this response are indicative of alterations in emotionality. NCAM-KO mice were found to show enhanced exploratory activity in response to challenging and novel environments, such as the light/dark avoidance test [22] and the elevated plus maze [24], which has been proposed to be related to their enhanced amygdala activation and/or the greater stress response (see above). On its turn, this enhanced exploratory behavior of NCAM-deficient mice could also explain the contradictory anxiolytic behavior, which was reported in the elevated plus maze [24].

In order to further investigate the role of specific NCAM isoforms in emotional behaviors, the effect of manipulating the levels of the NCAM180 isoform was analyzed in the presence or absence of endogenous NCAM in mice. While transgenic overexpression of NCAM180 was without apparent behavioral and morphological effects, its expression in NCAM-deficient mice rescued many of the effects induced by NCAM ablation; i.e., it counteracted the following effects observed in NCAM-KO: (1) the enhanced intermale aggression displayed in the intruder test [25]; (2) the increased anxiety-like behavior in the light/dark avoidance test; and (3) partially, the hyperactivity displayed in the elevated plus maze [24]. Transgenic induction of NCAM180 in NCAM-KO mice also prevented the hypersensitivity of NCAM-KO mice to the anxiolytic effects of buspirone, which suggests the involvement of NCAM180 on the development and/or maintenance of the functionality of the serotonergic system, which might represent an important link between NCAM and the regulation of emotional behaviors. Together, these studies indicate that deletion of the NCAM gene is associated with abnormal emotional responding and that the NCAM180 isoform plays a pivotal role in emotional behavior.

Research to elucidate the role of NCAM in learning and memory has been greatly aided by the development of mimetic peptides with the ability to bind and modulate the activity of NCAM. In addition to the above described findings in genetically mutant mice, there is upcoming evidence from pharmacological studies that further supports a role for NCAM in emotional behaviors. Peptides which interact either with NCAM homophilic binding (P2 peptide; a 12-amino-acid peptide derived from the second immunoglobulin-like (Ig) module of NCAM and able to interact with cis-homophilic) have been shown to alter emotional behaviors. For example, administration of the P2 peptide intracerebroventricularly was shown to reduce anxiety, associated with performance in a learning task (i.e. the T-maze), and exploratory activity in an open field test [26]. Another peptide, C3d, binds to the NCAM IgI module and is able to trigger intracellular signaling cascades in vitro, which are similar to those activated by homophilic NCAM binding [27]. Intracerebroventricular injection of this peptide had no acute effect on exploratory

behavior in an open field test, but dramatically reduced exploratory activity when C3d injected rats were re-exposed to the same arena 3-h and 24-h post-injection. This effect was not a consequence of sensorimotor impairments as peptide-treated rats performed without any significant differences on a rotarod and when explorative motility was accessed in an activity cage [28].

Emotional behavior is to a large extent associated with the development of psychiatric disorders. A few recent studies have shown some evidence to implicate NCAM in mood disorders. Thus, an increase in the CSF levels of the soluble 100–120kDa NCAM fragments has been found in patients with bipolar mood disorder type I and recurrent unipolar major depression [29, 30]. This increased presence of 120-kDa NCAM fragment in the CSF was postulated to derive form enhanced proteolytic cleavage of most of the extracellular region of transmembrane NCAM isoforms [31]. The complex nature of the extracellular region of the NCAM molecule has been also analyzed by creating transgenic mice that overexpress a soluble extracellular fragment of NCAM from the neuron-specific enolase promoter which leads to expression of this transgene in late neuronal differentiation (maximally in the neocortex and hippocampus) in adult mice [32]. Although these mice have normal sensory and motor functions, as compared to wild-type controls they display enhanced basal activity in the open field and in response to amphetamine and MK-801. However, and despite presenting a decreased number of synaptic terminals of subpopulations of GABAergic interneurons in the amygdala, these mice showed no alterations in anxiety-levels when tested in the zero maze [32].

Further evidence for the importance of NCAM and its polysialylated form PSA-NCAM in depression comes also from animal studies, which show that prolonged exposure of rodents to aversive stimuli (i.e. chronic stress protocols are widely used to model depression-like symptoms in rodents) lead to a reduction of NCAM mRNA and protein levels mainly in the hippocampus and the prefrontal cortex [33–43]. In contrast, PSA-NCAM expression was upregulated in the hippocampus, but downregulated in the amygdala of rats, exposed to 3 weeks of chronic stress [44]. Interestingly, recent studies in NCAM-KO (either constitutive or conditional, in which the cre-recombinase is regulated under the control of the αCaMKII promoter) mice have shown that these mice show enhanced depressive-like symptoms such as higher immobility time in the tail suspension test (Bisaz et al. unpublished observations) and a lower preference for sucrose solution [45]. In addition, chronic antidepressant treatments of rats and mice, with either the selective serotonin reuptake inhibitor fluoxetine or the tricyclic antidepressant imipramine, have shown to increase PSA-NCAM expression in the hippocampus and the prefrontal cortex [14, 46, 47]. These findings strongly support the hypothesis of a critical link between stress-related mood disorders and altered NCAM and PSA-NCAM expression [45].

In conclusion, the revised work indicates an important association between NCAM and emotion, particularly in domains such as anxiety, intermale aggression and depression. (See Table 1 for a comprehensive summary.) We will now review the literature that indicates a link between NCAM and learning and memory processes.

Table 1 Role of NCAM and PSA-NCAM in emotion

Model	Effect	Location	References
Anxiety			
NCAM$^{-/-}$ mice	Enhanced anxiety in an open field emergence test		[21]
NCAM$^{-/-}$ mice	Enhanced anxiety in Light/Dark avoidance test		[22, 24]
	Reduced anxiety in Elevated plus maze due to increased locomotion		[22, 24]
NCAM$^{-/-\ 180+}$ mice	Normal anxiety in Light/dark avoidance test and Elevated plus maze		[24]
ST8SiaIV/PST$^{-/-}$ mice	Reduced Anxiety in the Elevate Plus maze		[81]
Startle response and prepulse inhibition			
ST8SiaII/STX$^{-/-}$ mice	No effect in Startle response and Prepulse inhibition		[84]
NCAM$^{-/-}$ mice	No effect in Prepulse inhibition and facilitation		[109]
NCAM$^{-/-}$ mice	Reduced sensitivity to acoustic, but enhanced sensitivity to tactile stimuli		[59]
	Reduced footshock sensitization in startle response		[59]
Cleavage of PSA-NCAM by endoN	No effect in Startle response, 72-h post-enzyme injection	Bilateral in dorsal HC	[95]
Aggression			
NCAM$^{-/-}$ mice	Enhanced intermale aggression in an intruder aggression test		[23, 24]
NCAM$^{-/-\ 180+}$ mice	Normal intermale aggression in an intruder aggression test		[24]
Exploration and locomotion			
NCAM$^{-/-}$ mice	No effect on exploration and locomotion in open field test		[21]
NCAM$^{-/-}$ mice	Enhanced exploratory behavior in Light/dark avoidance test and Elevated plus maze		[22, 24]
C3d injected rats	No effect in exploratory behavior in the open field, 15-min post-injection Reduced exploration when retested 24 h later	Ventricle	[28]
C3d injected rats	No effect in mobility in the activity cage, 24-h post-injection	Ventricle	[28]
C3d injected rats	No effect in exploratory behavior in the open field test, 48-h post-injection	Ventricle	[67]
FGL injected rats	No effect in exploratory behavior in open field test, 24-h post-injection	Ventricle	[70]

(continued)

Table 1 (continued)

Model	Effect	Location	References
ST8SiaII/STX$^{-/-}$ mice	Enhanced exploratory behavior in the open field and enhanced rearing		[84]
FGL injected rats	No effect on anxiety and locomotion in the open field test, when injected for two consecutive days before test	Ventricle	[70]
P2 injected rats	Reduced exploratory behavior in the open field test, 1-h post-injection	Ventricle	[26]
Cleavage of PSA-NCAM by endoN	No effect in exploratory behavior in the open field test, 48-h post-enzyme injection	Bilateral in dorsal HC	[95]
ST8SiaIV/PST$^{-/-}$ mice	No effect in exploratory behavior in the open field test		[81]
Depression			
Soluble NCAM in CSF of patients with mood disorders	Enhanced levels of soluble NCAM120 fragment in CSF of patients with bipolar and major depression		[29]
NCAM$^{-/-}$ mice	Reduced immobility in the Forced swim test due to enhanced swimming activity		[24]
NCAM$^{-/-180+}$ mice	Enhancement of immobility (but below wt) and reduction of swimming activity (but above wt) in Forced swim test		[24]
NCAM$^{-/-}$ mice	Enhanced immobility in the tail suspension test and reduced sucrose preference in the sucrose preference test		[44]
FGL injected NCAM$^{-/-}$ mice	Reduction of depressive-like behaviors (in tail suspension test and sucrose preference test) by FGL		[44]
Aged conditional NCAM$^{-/-}$ mice	Enhanced depressive-like behavior in the tail suspension test		Bisaz et al. unpublished data
Stress			
NCAM$^{-/-}$ mice	Enhanced CORT levels after intruder (social) stress		[23]
CORT administration	Increase total NCAM expression after acute CORT administration	Forebrain chicken	[97]
	Increased total NCAM expression 8-h and 24-h post- injection	Frontal cortex	[40]
	Reduced total NCAM expression after 21-day chronic treatment	Frontal cortex, hypothalamus	[40]
	Reduced PSA-NCAM levels after 21-day chronic treatment	Piriform cortex, dentate gyrus	[32, 41]

Acute stress	Reduced NCAM180 protein levels immediately after stress	Hippocampus	[99]
	Reduction in all NCAM isoforms immediately after stress	Prefrontal cortex	[99]
	No effect in NCAM isoform expression immediately after stress	Amygdala, cerebellum	[99]
Chronic stress	Reduced totalNCAM protein expression after 21-day chronic restrain stress	Hippocampus	[37]
	Increased PSA-NCAM expression after 21-day chronic restrain stress	Hippocampus	[37]
	Increased PSA-NCAM after 21-day chronic restrain stress	Dentate gyrus	[38]
	No effect on PSA-NCAM after 42-day chronic restrain stress	Dentate gyrus	[38]
	Reduced total NCAM mRNA after 21-day chronic restrain stress	Hippocampus	[33]
	No effect on total NCAM mRNA after 21-day chronic restrain stress	Prefrontal cortex, frontal cortex, striatum and thalamus	[33]
	Reduced NCAM140 after 21-day chronic restrain stress	Hippocampus	[34]
	Reduced NCAM140 in high reactive rats after 21 days of chronic psychosocial stress	Hippocampus	[35]
	Reduced PSA-NCAM after 21-day chronic restrain stress	Central medial and medial amygdala	[43]
	Reduced totalNCAM mRNA after 21-day chronic restrain stress	Hippocampus	[39]
	Reduced NCAM180 in early aged rats after 28-day unpredictable chronic stress during adulthood	Hippocampus	[36]
	No effect in PSA-NCAM levels in early aged rats after 28 days unpredictable chronic stress during adulthood	Hippocampus	[36]

NCAM in Learning: Functional Studies

Learning can be defined as a process by which new information is acquired, whereas memory is the process by which this information is retained. The process of transferring learned information into memory is known as consolidation and is intimately linked to the functioning of the hippocampus [48, 49]. Cognitive tests for rodents have been developed to capitalize on normal behavioral responses often using fear, hunger or innate curiosity to motivate and strengthen learning. For example, in the watermaze, animals are forced to learn a novel spatial map in order to escape from water, whereas in avoidance and fear conditioning paradigms electrical shocks are used to initiate associative memory formation. In particular, a key role and requirement of NCAM function during learning has been particularly demonstrated using hippocampus-dependent tasks, including avoidance conditioning [50] and spatial learning [51]. In addition, the role of NCAM in "emotional learning" is more typically studied with fear conditioning paradigms. Classical contextual and auditory fear conditioning are often utilized to this end. These tasks involve the induction of learned fear (generally manifested as a freezing response) to an initially neutral stimulus (respective, either a new context or a tone) that is associated with a naturally aversive stimulus (normally a footshock). Recall of the fear response can be tested by re-exposing the animal to either the context or tone and evaluating its conditioned fear (freezing) responses. Acquisition and consolidation of either fear conditioning modality relies on the basolateral complex of the amygdala [52–58], while the hippocampus is also required in the case of contextual fear conditioning [59].

The role of NCAM in learning has been investigated by a variety of approaches, including the examination of particular behaviors in genetically mutant (transgenic or knockout) mice, after application of "blocking" NCAM-related antibodies or peptides that affect (either mimicking or impairing) NCAM functioning. Constitutive NCAM-KO are perturbed in the Morris water maze when compared to wild-type controls [21]. As mentioned in the introduction, NCAM expression is critical during post-natal development and, therefore, one of the problems with the constitutive NCAM-KO mice is that their altered learning and memory might be related to the elimination of NCAM during such period (and its consequent developmental effects), but unrelated to the absence of NCAM in the adult brain. To overcome this problem, a conditional knockout mouse was generated where expression of NCAM was controlled by a Cre-recombinase system using a forebrain specific promoter. In this conditional knockout mouse, NCAM expression is reduced starting at around P22 onwards and, therefore, its deficit occurs after the major neurodevelopmental events have already taken place [60]. This conditional KO of NCAM also leads to a spatial memory deficit in adulthood, demonstrating that post-natal NCAM expression is required for learning and memory. Evidence that NCAM mediated processes contribute to fear memories also stem from work performed in constitutive NCAM-KO mice. In these mice, both auditory and contextual fear memories are impaired [24], suggesting that NCAM-mediated consolidation processes might also be implicated in brain regions other than the

hippocampus, because auditory fear conditioning relies on the amygdala, but does not implicate the hippocampus.

The role of NCAM in non-associative learning was also tested by examining habituation (a decrease in behavioral responding to a stimulus) and sensitization (increased responding to an aversive stimulus) processes in NCAM-KO mice. While acoustic and tactile responses were altered in NCAM-KO mice (as evaluated by their startle responses), their ability to habituate to these stimuli was the same as wild type mice, demonstrating habituation learning is intact. In contrast, NCAM-KO mice exhibited impaired footshock sensitisation learning when compared with the wild type controls [61]. Footshock sensitization is a form of contextual conditioning, during which the context becomes a fear inducing stimulus leading to an increase in startle response to a tone [62].

In addition to the work reviewed above in genetically mutant mice, the critical role played by cell adhesion molecules in learning-related synaptic plasticity has been further demonstrated using blocking antibodies and peptides that bind to NCAM. Through these pharmacological approaches, the temporal implication of NCAM molecules during memory consolidation could also be explored. For example, studies where antibodies against NCAM were infused by intracerebroventricular injection in rats trained in the passive avoidance task found NCAM antibodies to induce memory impairment only when they where administered between 6 and 8 h post-training, but they were ineffective if given at the time of training or at any other time point up to 10 h following training [63]. In agreement with these observations, NCAM-specific antibodies were also found to impair passive avoidance learning in chicks when administered to a brain region critical for that learning (the intermediate medial hyperstriatum ventrale) at around 6–8 h post-training time [1, 64, 65]. The requirement of NCAM in long-term memory formation was similarly demonstrated through the use of oligonucleotides directed to NCAM [66].

In vitro evidence demonstrated that the C3d peptide could disrupt NCAM mediated cell adhesion and modulate neuritogenesis and synaptogenesis. When given to rats following training in the passive avoidance task, C3d prevented memory consolidation but only when the peptide was administered within a restricted time window either 20 min before training or at 6–8 h post-training [67]. Moreover, the C3d peptide also impaired both acquisition and recall of the Morris water maze [68] and the consolidation of contextual fear memories when administered 5.5 post-training [69].

After homophilic binding, NCAM promotes neurite outgrowth through mechanisms involving its interaction with the fibroblast growth factor receptor (FGFR1) [25, 70, 71]. The region of NCAM that binds FGFR1 is found in the second FnIII module of NCAM. A 15-aa peptide mimicking this region, termed the FGL peptide, has been shown to bind to and activate FGFR1 and to stimulate neurite outgrowth [25]. In vitro, the FGL peptide promotes synaptogenesis, synaptic outgrowth and pre-synaptic functioning [25, 72], as opposed to the C3d peptide that it similarly induces synaptogenesis and synaptic outgrowth, but impairs presynaptic functioning [27]. In vivo, FGL effects also contrast to those induced by C3d peptide (for details, see above). FGL strongly enhances spatial memory as shown in experiments, in which it was administered in rats immediately after the first and second day of

spatial training in the water maze [72]. This peptide was ineffective if given 2 days prior to training indicating that it does not perturb learning of new information. Moreover, post-training FGL treatment also improved performance in a subsequently given reversal learning challenge, suggesting that FGL is beneficial in promoting behavioral flexibility [72].

The FGL peptide has also significantly enhanced our understanding of the role of NCAM in the prototypic emotional learning task, fear conditioning. Intracereboventricular infusion of FGL just after fear conditioning improved contextual memory performance when tested 24 h, and 7 and 28 days later. However, auditory fear memories were only enhanced when tested 28 days later, but not earlier, suggesting different consolidation mechanisms for conditioned fear to tones, which might become apparent only after longer time periods. FGL did not affect emotional responses per se, having no affect on open field behavior when administered for 2 days prior to testing. Therefore, similarly to its effect in spatial learning, FGL was shown to also enhance emotional learning.

NCAM in Learning: Correlative Studies

Functional studies reviewed above suggest a role for NCAM in memory formation. However, they do not allow one to address whether the functional consequences derived from interference with NCAM (expression or function) are due to a primary effect on already existing molecules or, instead, on learning-induced NCAM regulation. This is a critical issue as the former possibility might imply a non-specific effect of treatments on normal circuit functioning, whereas the latter would highlight NCAM as a key player in memory-associated circuit remodeling [68]. We will review here those studies that have addressed a potential regulation of NCAM expression by learning experiences.

The 6–8 h post-training period was highlighted by interventive (antibody injection) studies as critical for the involvement of NCAM in memory consolidation, Interestingly, in chicks, NCAM was found to be enriched in synaptic active zones in a memory-relevant region (the lobus parolfactorius) 5–6 h after a one-trial passive avoidance learning experience [73]. In rats, spatial training in the water maze was found to induce an increase in synaptosomal, but not total, expression level of hippocampal NCAM140 24 h post-training [68]. In the zebrafish, using an active avoidance paradigm (fish learn to cross a hurdle to avoid mild electric shocks when presented with a conditioned light signal) NCAM mRNA levels were increased in the optic tectum (a region important for avoidance learning) 3 h following avoidance conditioning, indicating that some learning-dependent changes in NCAM expression are transcriptionally mediated [74].

In addition to this temporal increase in NCAM expression observed several hours after training, there is also evidence that NCAM expression is decreased for a restricted period on the first phases of memory consolidation. Work in simple invertebrate organisms such as the sea snail *Aplysia* has allowed the study

of neurobiological mechanisms linked to different types of learning, notably habituation and sensitisation [75]. Sensitisation in *Aplysia* is induced by presenting a noxious stimulus to the tail of the snail whereupon it withdraws its siphon and gill. If this is followed by stimulation of the siphon, it will withdraw both the siphon and gill in a more sensitized manner [76]. The neuronal circuit involved in long-term sensitisation has been identified [77]. Elements of this circuit (a siphon sensory neuron synapsing onto a gill motor neuron) can be isolated and cultured in vitro and synaptic facilitation can be induced artificially by presenting a puff of 5-TH directly to the cultured sensory neuron [78]. Interestingly, *Aplysia* expresses a homologue of mammalian NCAM known as apCAM. Induction of long-term sensitisation in cultured sensory neurons of *Aplysia* is accompanied by the growth of new synaptic connections and requires downregulation of apCAM [79].

Remarkably, in rodents NCAM is also selectively degraded 2–6 h post-training and this is necessary for passive avoidance memory consolidation [67]. Moreover, in this study the authors demonstrated that the C3d peptide, which impairs memory formation, seemed to prevent the temporal reduction in NCAM that occurs 2–6 h following learning in the passive avoidance paradigm indicating, as had been demonstrated in *Aplysia*, that a temporal reduction in NCAM expression is required for effective learning and memory.

PSA-NCAM in Learning

Polysialylation of NCAM is a potent modulator of NCAM functioning that significantly impacts on the role of NCAM in learning. Substantial evidence indicates that this posttranslational modification mechanism plays a key role in activity-dependent synaptic plasticity [2, 80] and memory formation. Most of the original work on this topic focused in the hippocampus. The requirement of PSA-NCAM for spatial learning has been indicated by different approaches. Removal of PSA from NCAM by endo-neuraminidase NE (endo-N), an enzyme which specifically cleaves α-2, 8-linked PSA, impedes the acquisition and retention of spatial memory [81, 82]. Mice expressing a null mutation in the polysialyltransferase (PST) gene, an enzyme critical for the postnatal polysialylation of NCAM, are impaired in spatial learning [83]. Conversely, a synthetic PSA-mimetic peptide administered in the mouse hippocampal CA3 region 5 h after massed training in the water maze was shown to significantly improve recall up to 4 weeks after training [84].

The role of PSA-NCAM in fear conditioning has received much attention in the past few years. Mice deficient in the PST gene were shown to display a very mild deficit in contextual fear conditioning. By contrast, auditory fear conditioning was normal in these PST-KO mice [83]. Strikingly, contextual fear conditioning was also impaired by application of PSA or PSA-NCAM 6 h, but not 2 h, following training [85]. Adult mice lacking the prenatally important St8SiaII/STX polysialyltransferase exhibit impaired memories (but not acquisition) in fear learning-related paradigms, auditory and contextual fear conditioning [86], suggesting an involvement

of PSA-NCAM in memory processes related to fear conditioning. However, since PSA-NCAM expression levels in the amygdala of adult St8SiaII/STX knockout mice are normal, the possibility exist that their alterations in emotional learning are due to developmentally caused alterations in amygdala function.

Given the differential disruption of auditory fear conditioning in PST as compared to SXT-KO mice it initially seemed, at least in adulthood, PSA-NCAM may be more important in regulating hippocampal learning when compared with amygdala-dependent learning. To resolve this issue, our group examined the role of PSA-NCAM in the amygdala during fear learning and found that auditory fear conditioning under conditions that employed a high intensity shock (1 mA) enhances the amygdaloid expression of PSA-NCAM 12 h post-training [87]. However, fear learning appeared not to require the induction of PSA-NCAM since endo-N cleavage failed to prevent either fear learning or its consolidation. However, removal of PSA-NCAM in the amygdala enhanced memory extinction, suggesting that PSA-NCAM modulations during emotional learning may be important in determining the intensity of the memory trace.

On the other hand, there is extensive evidence showing that PSA-NCAM levels in the hippocampus are modified by learning experiences. Notably, selective enhancement of PSA-NCAM-positive cells in the rat hippocampal dentate gyrus (in particular in a population of cells located at the dentate infragranular zone) has been found following initiation of learning in numerous behavioral tasks. For example, a temporal modification of PSA-NCAM levels is known to occur 10–12 h after passive avoidance [88–92], water maze [93, 94], olfactory learning [95, 96] and contextual fear conditioning [97, 98]. This up-regulation in PSA-NCAM can be sustained, being in some cases also evident at 24 h post-training [68, 94]. Moreover, repetitive training in the water maze induces PSA-NCAM upregulation following each training session while animals are still improving their performance levels [93] suggesting that enhanced expression of PSA-NCAM in the hippocampus is a molecular signature of plasticity-related to hippocampal learning. Despite PSA-NCAM being a marker for immature neurons, the spatial learning-dependent increase in PSA-NCAM does not result from increased neurogenesis or progenitor cells survival [95], indicating some other function for this selective modification in PSA-NCAM. Together, these studies suggest that selected enhancement of hippocampal PSA-NCAM can facilitate memory formation, whereas the role of amygdaloid PSA-NCAM in memory function deserves further studies.

NCAM and PSA-NCAM: Sensitive Indices of "Emotional Learning"

In this review about the role of NCAM and PSA-NCAM in learning, we have detailed many instances were both NCAM and PSA-NCAM are important for "emotional learning" particularly in the context of auditory and contextual

fear conditioning. We should also note that in our view the concept of "emotional" learning goes beyond fear learning tasks, since in our view virtually all animal learning models involve an emotional motivation too (for example, escaping from water stress in the water maze task). Work from our lab has found that NCAM [99] and PSA-NCAM [98] expression in the hippocampus are regulated by emotional learning depending on the intensity of the emotional experience. Contextual fear conditioning was found to induce time- and shock-intensity dependent alterations in the expression of hippocampal NCAM and PSA-NCAM. The intensity of the training experience can be modulated by altering the shock received by the animal, applying 0.2, 0.4 or 1 mA, which corresponds to low, medium and high intensity shocks, respectively. Previously, we showed that the intensity of the shock has a positive correlation with both the extent and duration of conditioned fear and post-training corticosterone levels [100]. Training rats at a moderate intensity (0.4 mA) led to a significant enhancement of hippocampal PSA-NCAM 12 h post-training [98], similarly to changes found after passive avoidance conditioning and spatial learning [68, 88]. By contrast, 24 h hours post-training only animals trained at 1 mA showed a significant enhancement of NCAM expression and, interestingly, this group also exhibited the greatest retention of the task and highest post-training corticosterone induction.

We have recently examined the regional specificity of contextual fear conditioning on hippocampal PSA-NCAM expression. We found [97] differential expression of hippocampal PSA-NCAM in the ventral and dorsal hippocampus that corresponds to a different functional involvement of these discrete regions in learning tasks [51]. Context exposure alone led to a significant increase in PSA-NCAM in the ventral and dorsal hippocampal dentate gyrus 24 h post-training [97]. However, following training in the contextual fear conditioning task (i.e., when context was paired with a shock), PSA-NCAM expression was only enhanced in the dorsal hippocampus. Moreover, infusion on Endo-N to the dorsal, but not the ventral, hippocampus impaired retention of the contextual memory [97]. More recently, we have demonstrated that prevention of a very rapid reduction in PSA-NCAM in the ventral hippocampus of rats exposed to the radial arm water maze is linked to a facilitation of memory retrieval (Conboy et al. unpublished observations).

Exposing rats during 30 min to a traumatic experience (i.e., predator stress, more specifically cat exposure) immediately following massed training in the radial arm water maze can impair recall of the platform location and induce a correlative reduction in hippocampal NCAM180 expression [101]. In contrast, the novel anti-depressant treatment agomelatine can prevent this stress-induced deficit in memory retrieval. Our recent findings show that in parallel to this behavioral effects, agomelatine facilitates the synaptic insertion of NCAM within 30 min of cessation of the learning task (Conboy et al. unpublished observations).

For a more comprehensive list of the role of NCAM and PSA-NCAM in learning and memory see Table 2.

Table 2 NCAM and PSA-NCAM in learning and memory

Model	Effect	Location	References
Passive avoidance			
NCAM expression	Increased expression in synaptic active zones 5–6-h post-training	Lobus parolfactorius (chick striatum)	[73]
NCAM expression	Decreased due to enhanced NCAM180 internalization 3–4-h post-training. Could be prevented by C3 infusion	Dentate gyrus	[67]
Anti-NCAM infusion	Impaired when infused 5–6-h and tested 48-h post-training	Ventricle	[63]
Polyclonal anti-NCAM (1 and 2) infusion	Impaired when injected 6–8-h post-training and tested 24-h post-training No effect at any other time point	Intermediate medial hyperstriatum ventrale (IMHV) of chick	[64]
Polyclonal anti-NCAM R1 and R2 infusion	Impaired when injected 5.5-h post-training and tested 24-h post-training	IMHV (chick)	[65]
Polyclonal anti-NCAM R1 infusion	Impaired when injected 5.5-h post-training and tested 48 h later No effect when tested 24 h later	Ventricle	[1]
Anti-NCAM	Impaired the facilitatory effect of CORT on learning	Forebrain (chick)	[99]
C3 infusion	Impaired when infused during training and 6–8 post-training and tested 48 h later, but not 24-h post-training. Prevents NCAM180 internalization 3–4 post-training in dentate gyrus	Ventricle	[67]
C3 infusion	Impaired when infused pre-training and tested 48 post-training	Ventricle	[28]
NCAM antisense oligonucleotides infusion	Impaired when injected twice: both 12 h before training and immediately afterwards and tested 3-h and 24-h post-training	IMHV (chick)	[66]
PSA-NCAM expression	Increase 12-h and 24-h post-training	Hippocampus	[102]
PSA-NCAM expression	Increase 12–24-h post-training, can be abolished with amnesia inducing agents	Hippocampus	[110]
PSA-NCAM expression	Increase 10–12 h post-training	Dentate gyrus	[88]
PSA-NCAM expression	Increase 10–12 h post-training	Dentate gyrus (granular cells)	[93]

PSA-NCAM expression	Increase 10–12 h, persisting up to 24–48-h post-training	Layer II of the enthorinal, perirhinal and piriform cortex	[90]
PSA-NCAM expression	Increase 12-h post-training at subtriangular septal zone in GABAergic interneurons	Septal nuclei and septohippocampal pathway	[91]
St8SiaII/STX$^{-/-}$ mice	Impaired		[86]
Active avoidance			
NCAM expression	Increased 3-h post-training	Optic tectum (zebrafish)	[74]
Auditory fear conditioning			
Constitutive NCAM$^{-/-}$ mice	Impaired tone memory		[85]
Constitutive NCAM$^{-/-}$ mice infused with Fc	Impaired when injected pre-training (4 h) and tested 24 h and 72 h later	Dorsal hippocampus	[85]
NCAM$^{-/-180-}$ mice	Impaired tone memory		[24]
NCAM$^{-/-180+}$ mice	Impaired tone memory		[24]
FGL infusion	No effect 24-h and 7-day post-training, but: Enhanced 28-day post-training	Ventricle (post-training)	[72]
NCAM-Fc infusion (C57Bl/6J mice)	No effect when injected pre-training (4 h) or post-training (2 or 6 h) and tested 24 h and 72 h later	Dorsal hippocampus	[85]
NCAM-Fc infusion (Constitutive NCAM$^{-/-}$ mice)	No effect when injected pre-training (4 h) and tested 24 h and 72 h later	Dorsal hippocampus	[85]
PSA-NCAM expression	No effect	Dentate gyrus	[97]
Cleavage of PSA-NCAM by endoN	No effect	Dorsal dentate gyrus	[97]
PSA-NCAM expression	Increase in BLA and CE after 1 mA, not 0.4 mA, 24-h post-training	Amygdala	[87]
Cleavage of PSA-NCAM by endoN	No effect	Amygdala	[87]
pr 2 infusion	No effect 24-h and 28-day post-training	Amygdala	[87]
PSA-NCAM-Fc infusion (C57Bl/6J mice)	No effect when injected pre-training (4 h) or post-training (2 or 6 h) and tested 24 h and 72 h later	Dorsal hippocampus	[85]

(continued)

Table 2 (continued)

Model	Effect	Location	References
PSA infusion (C57Bl/6J mice)	No effect when injected pre-training (4 h) or post-training (2 or 6 h) and tested 24 h and 72 h later	Dorsal hippocampus	[85]
PSA-NCAM-Fc infusion (Constitutive NCAM$^{-/-}$ mice)	Enhanced when injected pre-training (4 h) and tested 24 h and 72 h later (when compared to NCAM$^{-/-}$ injected with Fc)	Dorsal hippocampus	[85]
St8SiaII/STX$^{-/-}$ mice	Impaired		[86]
St8Sia-IV/PST$^{-/-}$ mice	No effect		[83]
St8Sia-IV/PST$^{-/-}$ mice	No effect		[85]
Auditory fear extinction			
Cleavage of PSA-NCAM by endoN	Enhanced extinction memory 24-h post-training	Amygdala	[87]
Contextual fear conditioning			
NCAM expression	Decrease 12 h post-training after 0.2, 0.4 and 1 mA Increase 24-h post-training after 1 mA	Hippocampus	[98]
C3 infusion	Impaired when injected 5.5-h post-training and tested 2–3 and 7 days later. (0.4 mA) No effect when injected 2 days pre-training	Ventricle	[69]
FGL infusion	Enhanced contextual fear 24-h, 7-day and 28-day post-training	Ventricle (post-training)	[72]
NCAM-Fc infusion (C57Bl/6J mice)	No effect when injected pre-training (4 h) or post-training (2 or 6 h) and tested 24 h and 72 h later	Dorsal hippocampus	[85]
Constitutive NCAM$^{-/-}$	Impaired context memory		[85]
NCAM$^{-/-180-}$ mice	Impaired context memory		[24]
NCAM$^{-/-180+}$ mice	Impaired context memory		[24]
Constitutive NCAM$^{-/-}$ mice infused with Fc	Impaired when injected pre-training (4 h) and tested 24 h and 72 h later (when compared to NCAM$^{+/+}$)	Dorsal hippocampus	[85]
NCAM-Fc infusion (Constitutive NCAM$^{-/-}$ mice)	Impaired when injected pre-training (4 h) and tested 24 h and 72 h later (when compared to NCAM$^{-/-}$ injected with Fc)	Dorsal hippocampus	[85]

PSA-NCAM expression	No effect 12 and 24 h post-training after 0.2 and 0.4 mA Decrease 12 and 24 h post-training after 1 mA	Hippocampus	[98]
PSA-NCAM expression	Increase 12-h post-training after moderate shock intensities (0.4 mA) Decrease 12-h post-training after traumatic shock intensity (1 mA)	Dentate gyrus	[109]
PSA-NCAM expression	Increase 24 h post-training in dorsal, but not ventral hippocampus (both after 0.4 and 1 mA shock)	Dentate gyrus	[97]
Cleavage of PSA-NCAM by endoN	Impaired	Dorsal hippocampus	[97]
PSA-NCAM-Fc infusion (C57Bl/6J mice)	Impaired when injected pre-training (4 h) or 6 h post-training and tested 24 h and 72 h later No effect when injected 2-h post-training and tested 24 h and 72 h later	Dorsal hippocampus	[85]
PSA infusion (C57Bl/6J mice)	Impaired when injected pre-training (4 h) and tested 24 h and 72 h later No effect when injected 2-h or 6-h post-training and tested 24 h and 72 h later	Dorsal hippocampus	[85]
PSA-NCAM-Fc infusion (Constitutive $NCAM^{-/-}$ mice)	Enhanced when injected pre-training (4 h) and tested 24 h and 72 h later (when compared to $NCAM^{-/-}$ injected with Fc)	Dorsal hippocampus	[85]
PSA-NCAM expression	No effect at any time point (30 min, 24 h) and intensity (0.4, 1 mA)	Amygdala	[87]
Cleavage of PSA-NCAM by endoN	No effect	Amygdala	[87]
St8Sia-II/$STX^{-/-}$ mice	Impaired		[86]
St8Sia-IV/$PST^{-/-}$ mice	Impaired (mildly)		[85]
Spatial learning			
NCAM expression	No effect immediately post-training	Hippocampus, thalamus, striatum, PFC, frontal cortex	[111]
NCAM expression	Increase 24-h post-training	Hippocampus	[112]
C3 infusion (pre-training infusions)	No effect when averaged over all days Impaired on second trial on day one	Ventricle	[28]

(continued)

Table 2 (continued)

Model	Effect	Location	References
C3d infusion	Impaired in learning Impaired in probe test	Ventricle	[112]
FGL infusion after 1st and 2nd training session	Enhanced on 2nd training session in first trial Enhanced 24-h, 7-day and 14-day post-training No effect in probe trials Enhanced reversal learning	Ventricle	[72]
Conditional hippocampus NCAM$^{-/-}$ mice	Impaired (day 3)	Hippocampus	[60]
Constitutive NCAM$^{-/-}$ mice	Impaired during acquisition Impaired in probe trial		[21]
NCAM$^{-/-180-}$ mice	Impaired acquisition Impaired probe trial memory Increased floating		[24]
NCAM$^{-/-180+}$ mice	No effect		[24]
Polyclonal anti-NCAM	Impaired	Ventricle	[113]
PSA-NCAM expression	Increase 10–12-h post-training	Dentate gyrus (granular cells)	[93]
PSA-NCAM expression	Increase 12-h post-training at subtriangular septal zone in GABAergic interneurons	Septal nuclei and septohippocampal pathway	[91]
PSA-NCAM expression	Increase 10–12-h post-training	Enthorinal cortex	[92]
PSA-NCAM expression	Increase 12-h post-training	Dentate gyrus	[109]
PSA-NCAM expression	Increase 12-h post-training Negative correlation between PSA-NCAM expression and performance in WM	Dentate gyrus	[114]
PSA-NCAM expression	Increase 12-h post-training, no effect on neurogenesis	Dentate gyrus	[95]
PSA-NCAM expression	Increase 24-h post-training in synaptosomes	Hippocampus	[112]
Cleavage of PSA-NCAM by endoN	Impaired on learning day 2, impaired in probe test	Dorsal hippocampus	[81]
Cleavage of PSA-NCAM by endoN	Impaired learning	Ventricle	[112]
pr2 infusion	Enhanced learning	Hippocampus	[115]

St8Sia-II/STX$^{-/-}$ mice	No effect		[86]
St8Sia-IV/PST$^{-/-}$ mice	Impaired learning, impaired reversal learning		[83]
Water maze followed by passive avoidance			
PSA-NCAM expression	Increase 10–12-h post-training	Dentate gyrus (granular cells)	[93]
PSA-NCAM expression	Increase 12-h post-training	Dentate gyrus	[94]
Olfactory discriminative learning and memory			
NCAM expression	No effect at any time point of measurement (3rd, 5th day of learning, 3-day post-training) Positive correlation between learning performance and NCAM expression	HippocampusPiriform cortex	[116]
Constitutive NCAM$^{-/-}$ mice	Impaired odor discrimination No effect short-term memory No effect odor detection thresholds		[117] [112]
Polyclonal anti-NCAM	Impaired when injected 5.5-h post-training and tested 48 h later	Ventricle	[113]
PSA-NCAM expression	Increase 12-h post-training	Dentate gyrus	[96]
PSA-NCAM expression	Increase 24-h post-training in the hippocampus, but not piriform cortex	Hippocampus Piriform cortex	[116]

Studies were conducted on rats or mice unless specified otherwise
Compounds infused: C3 or C3d, synthetic peptide ligand of NCAM with an affinity for the IgI domain and the capability of inhibiting NCAM-mediated neurite outgrowth in vitro
FGL, synthetic 15 amino acid peptide corresponding to the binding site of NCAM for the fibroblast growth factor receptor 1 (FGFR1)
pr2, synthetic peptide mimicking PSA
NCAM-Fc or PSA-NCAM-Fc, recombinantly produced proteins in which the extracellular portion of NCAM is fused with a human Fc fragment of IgGs. Fc alone has no effect when infused into mouse brains

Mechanisms Related to NCAM Actions on Learning

Humans retain the ability to form new memories, in the absence of dementia, throughout their whole life, which indicates that the implicated brain structures must retain the potential to continuously restructure their synapses. Adult learning and memory has been proposed to imply, to a certain extent, a replay of neurodevelopmental events and as such utilize the same plasticity-related molecules, including NCAM [102, 103]. Numerous studies have shown modifications of hippocampal synaptic morphology as a result of learning and memory [104–107]. As the selective expression of cell adhesion molecules during neurodevelopment is important in determining synaptic structuring [19], analogous cell adhesion molecule modulations presumably regulate synaptic restructuring during memory formation.

At the conceptual level, the reviewed evidence led us to propose the following role for NCAM during memory consolidation. During the early consolidation period, 2–6 h following training NCAM expression seems to be downregulated [67]. A transient increase in spine number has been found in the hippocampus in the early hours after a training experience [104–106]. In *Aplysia*, it has been shown that the growth of new synaptic connections requires endocytosis and degradation of the NCAM homologue apCAM [79]. It is therefore conceivable that the temporal reduction in NCAM found in rodents may similarly enable synaptic loosening to facilitate synaptic growth. During the later periods of consolidation, robust learning and memory has been associated with an enhancement of synaptosomal NCAM expression [68, 98]. In chicks, avoidance training induces the localisation of NCAM in the synaptic active zone of the lobus parolfactorius 5–6 h after a one-trial passive avoidance learning experience [73], indicating that NCAM may localize to newly formed synapses. Moreover, contextual fear conditioning leading to a strong memory [100] correlates with an enhancement of synaptically localized NCAM [98]. Correlative work in vitro has suggested that increased concentrations of NCAM can selectively increase synapse formation. For example, transfection of NCAM deficient neurons with any of the three NCAM molecules leads to the formation of synapses preferentially between NCAM-NCAM containing neurons [108].

Learning and memory also require temporal modulations in PSA-NCAM. Learning induced synaptic modifications occurring in the hippocampus are transient [106] indicating that a period of synaptic pruning or selection also contributes to memory consolidation. As PSA attachment to NCAM reduces NCAM mediated cell adhesion, activity dependent upregulation in PSA-NCAM during the later memory consolidation period (12–24 h) [68, 88] may enable synaptic loosening which facilitates selection and pruning of hippocampal connections.

In conclusion, we have presented evidence that NCAM and PSA-NCAM regulate both emotion and learning and memory processes, and we have presented a model suggesting that the functioning of these molecules might be related to the modulation of learning induced by emotional aspects. This evidence supports the use of recently developed NCAM-related compounds, such as the FGL peptide, for the treatment of devastating neurological disorders of cognitive dysfunction like Alzheimer's disease.

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Part VI
NCAM in Disease

NCAM in Neuropsychiatric and Neurodegenerative Disorders

Leann H. Brennaman and Patricia F. Maness

Introduction

The neural cell adhesion molecule, NCAM, is a key mediator of axon/dendrite growth and branching and synaptic strength and plasticity and is important in cognitive function. Through alternative splicing, three major isoforms of NCAM are generated: NCAM120, NCAM140, and NCAM180. NCAM120 is found predominantly in glia and is anchored to the cell membrane through a GPI linkage (Fig. 1). NCAM140 participates in several signaling pathways to activate axonal and dendritic growth and branching, while NCAM180 interacts with cytoskeletal components and may have a more prominent role in synaptic stability and memory formation (reviewed in [1, 2]). NCAM is modified by the addition of α-2,8-polysialic acid (PSA). PSA-NCAM is a highly glycosylated form of NCAM, that is required for synaptic plasticity, and is increased during learning and memory formation (reviewed in [2, 3]). Mice deficient for the polysialyltransferase PST display reduced spatial and reversal learning, which are dependent on prefrontal cortical (PFC) and hippocampal function [4]. Similarly, mice lacking all isoforms of NCAM have disrupted long-term potentiation and depression (LTP and LTD, respectively) and have impaired spatial learning and sensorimotor gating [5, 6]. NCAM has a prominent role in normal cognitive functions of the hippocampus and PFC and is dysregulated in a variety of neuropsychiatric and neurodegenerative disorders (Table 1).

This review will focus on the evidence for dysregulation of NCAM in neuropsychiatric and neurodegenerative disorders, specifically schizophrenia, mood disorders

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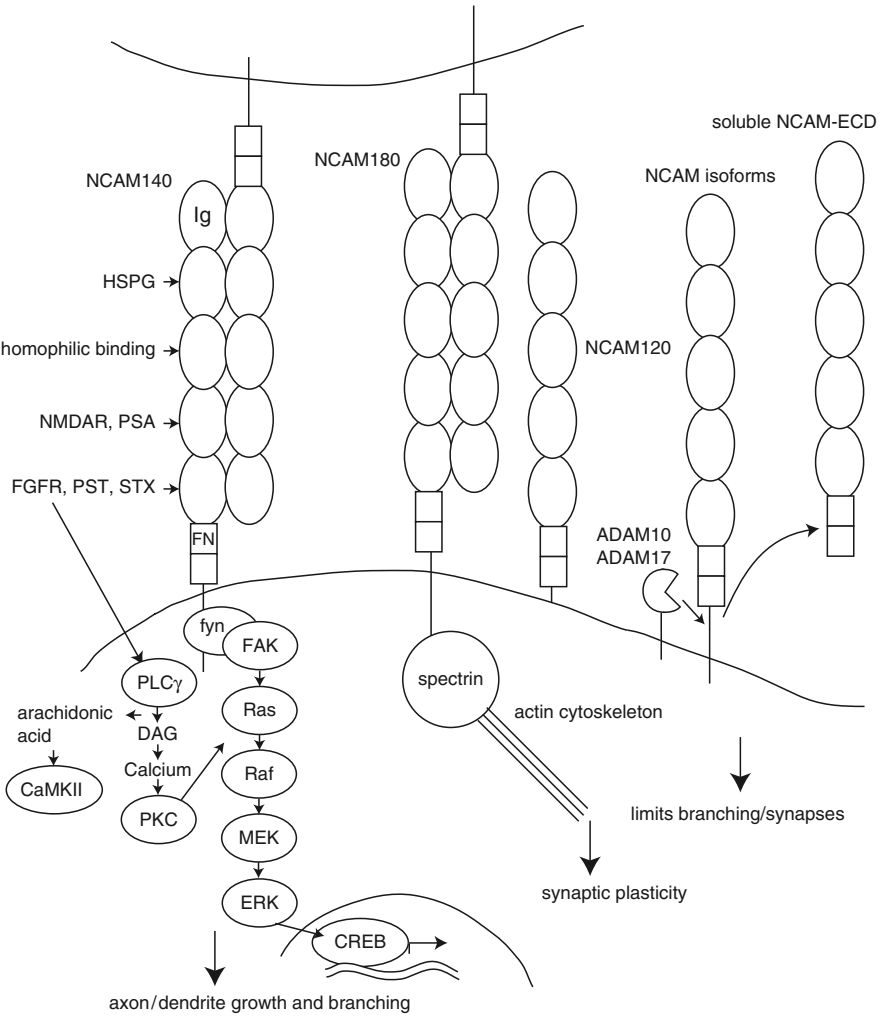

Fig. 1 NCAM signaling and neuronal functions. NCAM has three major isoforms: NCAM140, NCAM180, and NCAM120 (GPI-linked on glia) (reviewed in [1, 2]). The extracellular domain of NCAM participates in homophilic and heterophilic protein–protein interactions. The second Ig domain of NCAM binds heparin sulfate proteoglycans (HSPG), while the fourth and fifth Ig domains bind to the NMDA receptor and the fibroblast growth factor (FGF) receptor, respectively. The fifth Ig domain also binds the polysialyltransferases PST and STX, which add PSA to the fourth Ig domain. The third Ig domain of NCAM is critical for NCAM-NCAM homophilic binding. NCAM140 has critical roles in axon and dendrite growth and branching, which are mediated by intracellular signaling. Upon binding of NCAM140 on opposing cell membranes, the src family kinase fyn is activated that binds and activates FAK, leading to activation of ERK MAP kinase and CREB-mediated transcription. NCAM140 homophilic binding can also activate FGF receptor signaling pathways that lead to the activation CaMKII and protein kinase C (PKC). In contrast, NCAM180 is important in synaptic stabilization and plasticity. Upon homophilic binding, NCAM180 interacts with spectrin, linking it to the actin cytoskeleton. NCAM isoforms are also subject to proteolytic cleavage by ADAM10 and/or ADAM17 to release the soluble extracellular domain (NCAM-ECD), which serves to limit axonal and dendritic branching and the number of synapses

Table 1 Summary of neuropsychiatric/neurodegenerative disorder characteristics and NCAM modulations

Disorder	Age of onset	Neurotransmitter(s)	Affected Brain Region(s)	NCAM modulation
Schizophrenia [7–18]	Late adolescence, early adulthood	Dopamine GABA	Hippocampus Prefrontal cortex Ventricular enlargement	SNPs in NCAM, PST, STX [22–29] ↓PSA-NCAM [30] ↑NCAM deavage [31–37]
Bipolar disorder [55–57]	Late adolescence, early adulthood	Mania – dopamine, GABA, depression – serotonin, norepinephrine	Hippocampus Prefrontal cortex Ventricular enlargement	SNPs in NCAM [58–59] ↑Secreted isoforms [16,60–62]
Depression [63–66, 80–82]	Can occur at any age	Serotonin, norepinephrine	Hippocampus Frontal cortex Hypothalamus Amygdala Striatum	↑Soluble NCAM [75,80–82] ↓PSA- NCAM [83–85] ↑PSANCAM [90–94] ↓NCAM signaling [49,53–54] ↓NCAM14O [90–94]
Anxiety Disorders [104–109]	Median age 11 years	Serotonin, norepinephrine	Amygdala Prefrontal cortex	↓NCAM [90–94] ↑PSA-NCAM [90–94] ↓NCAM signaling [49,53–54]
Alzheimer's disease [112–113]	Early adulthood (rare familial), Late adulthood	Acetylcholine	Hippocampus Frontal cortex General brain atrophy	↓NCAM [116] ↑PSA-NCAM [118–120] ↑NCAM cleavage [121–122] ↓HNK-1 glycosylation [126] ↓NCAM signaling [112–114]

(bipolar disorder and major depressive disorder), anxiety disorders, and Alzheimer's disease (AD). Each section is divided into a brief introduction to the major symptoms, brain regions, and neurotransmitter systems implicated, followed by the evidence for NCAM in each disorder including expression, genetic association, and animal-based studies. A more complete understanding of how alterations in NCAM may disrupt normal connectivity leading to cognitive dysfunction may aid in our ability to further comprehend the complexities of various neuropsychiatric and neurodegenerative disorders.

Schizophrenia

Schizophrenia is a devastating disease that affects 1% of the world's population [7]. Onset generally occurs during late adolescence to early adulthood (Table 1). Several genes have been proposed as vulnerability loci, but environmental risk factors also exist. Psychosis is the most common clinical feature of schizophrenia, but cognitive dysfunction (memory, executive control/information processing, attention), particularly associated with the dorsolateral prefrontal cortex (DLPFC), may be more disabling and can predict long-term outcome [8–11].

For over 30 years, the dopamine hypothesis has dominated the field, as amphetamines and dopamine induce schizophrenia-like symptoms in normal patients, and amphetamines exacerbate symptoms in individuals with the disease [12, 13]. Moreover, the dopamine D_2 receptor is increased in the schizophrenic brain, but antipsychotics can also increase receptor levels. Currently prevailing is the neurodevelopmental hypothesis of schizophrenia, which implicates altered neuronal development in disrupted connectivity and cognitive dysfunction [14, 15]. At the neuronal level, abnormalities in both excitatory (pyramidal) and GABAergic inhibitory neurons have been identified in individuals with schizophrenia [8, 9, 12, 16–18]. There are decreases in the glutamate transporter (VGlut1), glutamate uptake sites, the NR1 subunit of the NMDA receptor, AMPA receptors, and the presynaptic marker synaptophysin in the PFC and hippocampus. Levels of the GABA synthetic enzymes, GAD65 and GAD67, and the GABA transporter, GAT1, have been found to be reduced in the PFC of individuals with schizophrenia. There are also increased compensatory levels of the $GABA_A$ receptor. More importantly, many reports have indicated that populations of GABAergic interneurons are selectively affected in schizophrenia, which may be attributed to their later time course of development as compared to pyramidal neurons (reviewed in [8, 9, 12, 17–19]). Expression of the calcium binding proteins, parvalbumin (PV) and calbindin, are decreased in schizophrenia. Calbindin is expressed in Martinotti, neurogliaform, and Cajal-Retzius cells, which target the dendrites of pyramidal cells [8, 20, 21]. Parvalbumin is expressed in wide arbor basket cells and chandelier cells, which target the cell body (soma) or axon initial segment (AIS) of pyramidal neurons, respectively, suggesting that affecting multiple types of GABAergic interneurons contributes to inhibitory dysfunction in schizophrenia.

NCAM ranked fourth in a meta-analysis of schizophrenia susceptibility loci [22], and single nucleotide polymorphisms (SNPs) are associated with cognitive impairment [23]. Most of the SNPs identified in NCAM are located in intronic regions, and may regulate splicing or expression of NCAM. However, SNP rs58447 represents a mutation in exon 12, encoding the second half of the first fibronectin type III domain. Although this is a synonymous mutation, it is interesting that this domain is the binding site for the polysialyltransferases, PST, and STX, which adds PSA to the fifth Ig domain of NCAM [24, 25]. Accordingly, SNPs in STX and PST have been reported as susceptible loci for schizophrenia [26–29], and PSA-NCAM levels are decreased in the hippocampus of individuals with schizophrenia [30].

Increases in the levels of a soluble fragment consisting of most of the extracellular domain of NCAM in the CSF and affected brain regions (PFC, hippocampus) of individuals with schizophrenia have been correlated with disease severity and duration [31–37]. Soluble NCAM fragments (105–115 kDa) do not arise from alternative splicing as they were not recognized by antibodies raised against the secreted isoform (NCAM-SEC) [36]. Instead, they result from metalloprotease-mediated cleavage of NCAM by ADAM (a disintegrin and metalloprotease) type proteases [38–40] (Fig. 1). In vitro studies have demonstrated that broad spectrum inhibitors of metalloproteases block NCAM shedding, but increase NCAM-dependent neurite growth and branching from cortical neurons [38], whereas they decrease outgrowth from hippocampal neurons [39, 40]. Thus, NCAM shedding may provoke cell-type specific responses, possibly due to differing expression patterns of ADAM proteases [41].

Two ADAM family proteases have been implicated as NCAM convertases, ADAM10 and ADAM17/TACE. Human ADAM10 is found on chromosome 15q22 [42], a region that was ranked 17th in the genomic meta-analysis of schizophrenia [22]. Interestingly, human ADAM17/TACE is located on chromosome 2p25 [43], a region close to the top-ranked 2p12-q22.1 region. While ADAM17/TACE lies outside the affected region, suggesting that mutations in the coding region are unlikely to be linked to schizophrenia, the possibility exists that mutations in promoter or enhancer regions could affect ADAM17/TACE levels in the disease. Support for these two ADAMs as NCAM sheddases has predominantly come from in vitro studies. Transfection of ADAM10 increased NCAM shedding in fibroblast cultures, while ADAM17/TACE transfection had no effect [38]. Moreover, brain lysates from wild-type mice and mice homozygous for a deletion in the zinc binding region of the ADAM17/TACE metalloprotease domain showed no differences in NCAM expression or cleavage fragments. In cortical neurons, transfection with a dominant negative ADAM10 construct resulted in increased NCAM-dependent neurite growth and branching, and ADAM10 coimmunoprecipitates with NCAM in brain homogenates throughout postnatal development (L.H. Brennaman and P.F. Maness, unpublished observations). In contrast, NCAM shedding from ADAM17/TACE-null fibroblasts is not evident, but can be elicited by reintroducing ADAM17/TACE expression [40]. Lack of ADAM17/TACE activity also results in decreased NCAM-dependent neurite outgrowth from hippocampal neurons. The apparent discrepancy between the two results may lie in cell-type specific expression of the ADAMs. ADAM10 is widely expressed throughout the brain, whereas ADAM17/TACE has been shown to be more selectively expressed in the hippocampus [41]. Both ADAM10 and ADAM17/TACE could cooperate to cleave NCAM, similar to alpha secretase processing of amyloid precursor protein [44]. However, neither ADAM10 nor ADAM17/TACE has been shown to directly cleave NCAM in vitro or to be a physiological convertase.

Studies in mouse models have furthered our understanding of the potential roles of NCAM in schizophrenia. Ventricular enlargement is the most reliable morphological feature in the schizophrenic brain [45], and is often accompanied by cognitive impairments. Mice lacking only NCAM180 display increased lateral ventricles, and also have deficits in learning and prepulse inhibition of acoustic startle (PPI, a

test used to measure sensory gating) [46]. In contrast, mice lacking all isoforms of NCAM have abnormalities in the hippocampus and olfactory bulb, two regions affected in the disease, without ventricular enlargement [6, 47–50]. Furthermore, addition of NCAM antibodies to hippocampal slice cultures results in reduced long-term potentiation (LTP) [51, 52], a measure of synaptic plasticity.

A mouse model of the increased NCAM cleavage (NCAM-EC) seen in schizophrenia displays decreased emotional memory (contextual and cued fear conditioning) and PPI, which is attenuated by the atypical antipsychotic drug clozapine, but not the dopamine D_2 drug haloperidol [53]. NCAM-EC mice also show increased hyperactivity and enhanced sensitivity to amphetamines, as well as stereotypy. While no differences were observed in the lateral ventricles, NCAM-EC mice display decreased levels of PV, GAD65/67, GAT-1 and synaptophysin in the cingulate cortex and amygdala, but not hippocampus. Apical dendritic spines from pyramidal neurons are reduced in density, suggesting that pyramidal neuron development may also be affected. Recently, development of a subpopulation of PV^+ basket cells was shown to be stunted at postnatal day 20 (P20), which corresponds to adolescence in mice [54]. The basket cells show impaired branching of axonal and dendritic processes and decreased numbers of synapses onto pyramidal neuron soma. Moreover, cultured cortical neurons from NCAM-EC mice display dramatically reduced NCAM-dependent neurite outgrowth and branching [38]. This can be attributed to a dominant inhibitory mechanism, as soluble NCAM-EC added directly to the culture media also blocks NCAM-dependent growth and branching [54]. Future testing to determine if deficits exist in working memory in NCAM-EC mice may implicate altered NCAM proteolytic cleavage in the cognitive dysfunction observed as a part of the schizophrenia endophenotype. Thus, these mice may provide a test model for novel treatment options, as blocking NCAM cleavage could restore the altered development of GABAergic interneurons in the PFC and possibly the learning dysfunctions.

Mood Disorders: Bipolar Disorder

Bipolar disorder affects ~3%–5% of the world's population and is the sixth most common cause of disability in the United States [55, 56] (Table 1). It is a chronic disease characterized by recurring episodes of mania alternating or occurring with depression. Bipolar disorder has an early age of onset (~20 years old) and the majority of individuals also suffer from a comorbid condition that can often trigger manic episodes such as substance abuse, anxiety, or attention-deficit hyperactivity disorder. Causes for bipolar disorder include familial inheritance and environment. Bipolar disorder and schizophrenia share some common susceptibility genes [55, 57] and neuroanatomical characteristics, such as ventricular enlargement, decreased temporal lobe volume, and disrupted synaptic plasticity.

NCAM has been postulated as a candidate susceptibility gene for bipolar disorder. SNPs from different NCAM regions were associated with a risk for bipolar disorder in Japanese [58] and North American [59] subpopulations. In the North American

subpopulation, there was a significant association between alternative splicing of the mini exon b, also associated with the alternatively spliced secreted isoform (NCAM-SEC) [59]. Levels of NCAM-SEC protein (108–115 kDa) are also increased in the hippocampus of individuals with bipolar disorder [16]. Other soluble NCAM proteins (120 kDa) are elevated in the hippocampus, prefrontal cortex, and CSF of affected patients compared to normal controls [16, 60, 61], although this did not correlate with drug treatment status. However, in one patient, soluble CSF NCAM levels decreased by ~40% following electroshock therapy. These soluble NCAM forms do not appear to be derived from the cleavage of full-length NCAM isoforms (120, 140, 180) in a sequence that would be upstream of the GPI and SEC splice sites to produce 105–115 kDa [36, 38–40]. Nor are they likely to be derived from NCAM-SEC as this isoform does not appear in CSF. Thus, the increased levels of the 120 kDa soluble NCAM in bipolar disorder are likely from release of GPI-linked NCAM120 from the plasma membrane [60]. Moreover, a soluble form of the NCAM-VASE isoform (140 kDa) associated with decreased neurite outgrowth and plasticity [62] is increased in hippocampus and prefrontal cortex of some affected individuals [61]. The elevated levels of soluble NCAM suggest that subcellular or synaptic functions of NCAM are compromised, perhaps contributing to the pathology of the disorder.

Mood Disorders: Depression

Major depressive disorder, or depression, is defined by the American Psychiatric Association as “a despairing mood and the loss of interest or pleasure in nearly all activities previously considered pleasurable [63].” It is accompanied by at least four other symptoms including: appetite or weight change; sleep change; psychomotor activity change; feelings of worthlessness or guilt; difficulty thinking, concentrating, or making decisions; or recurrent thoughts of suicide. One prevailing hypothesis is that deficits in norepinephrine and serotonin (5-HT) are a major cause of depression, and may be associated with hippocampal atrophy, neuronal loss and dendritic reorganization, resulting in decreased synaptic connectivity [64–66] (Table 1). Most antidepressants increase the levels of these two neurotransmitters, and increase neuronal connectivity. However, antidepressant therapy may only be effective in 60%–75% of patients and take weeks to months to have an effect [65].

Recently, changes in neurotrophins such as brain derived neurotrophic factor (BDNF) have been implicated in the structural reorganization observed in depression. BDNF promotes growth and arborization of neurons [67–70] and is required for adult plasticity. BDNF increases neurotransmitter release from presynaptic terminals, increases synaptogenesis, and promotes stable induction of LTP (reviewed in [71, 72]). BDNF can signal through two receptors, p75 neurotrophin receptor and TrkB, to induce activation of a MAP kinase signaling pathway. Expression of BDNF and TrkB is decreased in the hippocampus and serum of depressed individuals, a response that is exacerbated by stress and reversed by antidepressants (reviewed in

[65, 66, 72, 73]). Importantly, the decrease in BDNF expression correlates with the severity of symptoms. BDNF may also modulate serotonin levels. Intracerebral infusion of BDNF increased 5-HT synthesis and induced sprouting of 5-HT axons in rats [74–77]. Moreover, loss of one copy of the BDNF gene in mice results in serotonin deficits and renders the animals insensitive to antidepressants [78, 79]. Therefore, modulating BDNF levels may have direct implications on circulating serotonin. However, neither BDNF nor serotonin can explain all of the symptoms associated with depression. Thus, the prevailing hypothesis is the network hypothesis of depression, which postulates that hypofunction of brain regions involved in positively motivated behaviors, such as executive control, attention, cognition, and effort or reward-motivated behavior (hippocampus, PFC) coordinated with hyperfunction of regions involved in stress responses (amygdala, striatum, paraventricular nucleus of the hypothalamus), underlie the symptoms of depression [66, 80–82]. During depression, neural activity of the hippocampus and frontal cortex is decreased, while the activity of the stress response regions is increased. Structural remodeling including decreased synapses, dendritic atrophy and decreased neurogenesis are observed in the hippocampus and frontal cortex as a result of stress and/or depression, while these conditions increase arborization in the amygdala. Importantly, excitatory connections from the cortex to interneurons of the amygdala can inhibit output, suggesting that decreases in cortical activity or connectivity could result in the hyperactivity in the amygdala seen in depression. Several animal models have been used to substantiate the network hypothesis: forced swim test, chronic restraint stress, immune system activation, intracerebral administration of galanin (an inhibitor of neurotransmitter release), and depletion of monoamines (dopamine, 5-HT, etc.) with reserpine [82]. All models have shown that stress and learned helplessness reduce signal transduction and excitatory output in the cortex and hippocampus. Moreover, serotonergic modulation directly affects positive behavior, suggesting that defects in hippocampal or cortical activity/connectivity cause the symptoms of depression.

NCAM has been implicated in serotonergic and neurotrophic function in the PFC [83, 84]. Normally, the PFC has high levels of PSA-NCAM, as it is expressed in the regions of synaptic plasticity. PSA-NCAM-positive neurons (excitatory and inhibitory) express the serotonin receptor (5-HT-3), and serotonin increases PSA-NCAM levels. Depletion of serotonin in PFC decreases PSA-NCAM and results in dendritic remodeling. The antidepressant fluoxetine increases levels of serotonin and PSA-NCAM, and enhances neuronal connectivity in chronic treatment of adult rats [83–85]. However, NCAM-null mutant mice display increased sensitivity to 5-HT_{1A} serotonin receptor agonists without changes in serotonin or receptor levels. Instead, it is thought that the lack of NCAM affects pathways involving K^+ channels and G proteins that lie downstream of 5-HT_{1A} receptors [49]. Moreover, NCAM-null mice have increased levels of stress-induced corticosteroids and show vulnerability to develop depression, as evidenced by increased intermale aggression [86], anxiety [49], decreased LTP and plasticity [87, 88], and decreased learning and memory [87, 89]. Chronic restraint stress or early postnatal stress as models of depression associated with increased corticosteroids have further implicated NCAM, as mRNA

and levels of the NCAM140 isoform are significantly reduced in the hippocampus and PFC, while levels of PSA-NCAM are increased [90–94]. Intriguingly, these deficits correlate to decreased levels of BDNF [95], and treatments such as exercise [96] or antidepressants (rolipram or fluoxetine) increase both proteins [83, 97, 98]. PSA-NCAM may bind to BDNF resulting in TrkB phosphorylation, and has been described as a sensitivity switch for BDNF levels [99–102]. Thus, the increased levels of PSA-NCAM may be a compensatory mechanism to respond to the lower levels of BDNF in depressed hippocampus and PFC. In CSF, there is an increase in levels of soluble NCAM, particularly the 120 kDa form [60, 103]. Soluble NCAM could disrupt homophilic or heterophilic interactions required for synaptic plasticity. The decrease in NCAM expression or function could result in synapse instability and reduced axonal/dendritic outgrowth. Thus, depression may be a consequence of the inability of the brain to adapt to synaptic and structural changes, and may be alleviated by antidepressant therapy.

Anxiety Disorders

Anxiety encompasses a range of disorders, including: specific phobia, separation anxiety, social phobia, obsessive-compulsive disorder, panic disorder, post-traumatic stress disorder, and generalized anxiety disorder [104–106]. Together they account for a 29% lifetime prevalence and are the most common form of mental illness. Anxious individuals have selective attention toward an anticipated threat and interpret nonthreatening or mildly aversive expressions, comments or events as negative, resulting in generalized distress and worry. Anxiety disorders are highly comorbid with each other and with depression. Often, anxiety disorders precede depression, and early treatment could prevent depression onset. However, only less than half of the individuals with anxiety disorder receive treatment.

Circuitry between the amygdala and PFC is dysfunctional in anxiety disorders [107–109]. The amygdala is critical site for acquisition and expression of fear, while excitatory input from the PFC downregulates amygdalar output by targeting GABAergic interneurons to inhibit fear response. As described above for depression, chronic stress exacerbates anxiety through structural changes in the PFC. Animal models of fear conditioning and anxiety (light/dark avoidance, anxiety in open field or in memory testing) have also been used to examine the circuitry and proteins involved in anxiety disorders. Fear conditioning [110] is elicited when a conditioned or contextual stimulus (a tone or specific location) precedes a negative stimulus (foot shock). When the tone or location is subsequently presented, the animal demonstrates fear responses such as freezing and startle.

NCAM has been implicated in anxiety disorders primarily through the use of these behavioral models. Chronic stress exacerbates anxiety and facilitates fear conditioning. Chronic stress decreases NCAM expression and increases PSA-NCAM levels [90–94]. NCAM-null mutant mice display increased anxiety in light/dark avoidance testing and increased sensitivity to anxiolytic 5-HT_{1A} receptor agonists [49].

Mice that overexpress the soluble NCAM-EC fragment display decreased excitatory and inhibitory synapses in the PFC and amygdala [53], as a result of decreased growth/branching of the axonal and dendritic arbors [54], suggesting impaired function of both PFC and amygdala due to decreased NCAM interactions. Intriguingly, NCAM-EC transgenic mice are significantly impaired in contextual and cued fear conditioning, and show increased locomotor activity in open field [53]. Administration of the P2 peptide to WT mice, a 12 amino acid peptide derived from the second Ig-like domain of NCAM and thought to mimic homophilic interactions, decreases certain forms of anxiety (T-maze testing) [111]. Thus, modulating NCAM homophilic and heterophilic interactions could result in subsequent plastic changes in the PFC and amygdala to inhibit anxiety responses.

Alzheimer's Disease

AD is the most common neurodegenerative disease, affecting more than 20 million people worldwide [112, 113]. AD is predominantly a disease of the aging population (Table 1). Most cases of AD are sporadic, and familial inheritance accounts for only 1% of total AD cases. AD features include: neuronal death, synapse loss, reduced brain volume, disruption of the cholinergic system, and cognitive dysfunction. The hallmark features of AD are neurofibrillary plaques and tangles in the brain, which are composed of amyloid β (Aβ) and Tau proteins, respectively (reviewed in [112, 113]. Total Aβ levels, and not necessarily plaque formation, are important for the progression of AD and resulting cognitive dysfunction [112, 114]. Many familial cases of AD are attributed to mutations in presenilin-1 (PS1), the proteolytic component of the γ-secretase complex that increases the production of Aβ (reviewed in [112, 113]).

NCAM expression was not different when comparing gross neuroanatomy of Alzheimer's and control samples [115]. However, upon closer examination of individual cortical regions, there was a significant decrease in the numbers of NCAM-positive neurons in the frontal cortex, but not occipital cortex or hippocampus [116]. There are conflicting reports on NCAM expression in plaques and tangles, suggesting that it may be altered in only a subset of individuals with AD [115, 117]. Treatment with cholinesterase inhibitors, which improve cholinergic function, increases PSA-NCAM in the hippocampus [118]. This increase has been correlated with increased neurogenesis in the hippocampus of AD patients and may indicate disease severity [119, 120]. Soluble forms of NCAM are increased in the cerebrospinal fluid of AD patients and also correlate with age, neurodegeneration, and degree of cognitive impairment [121, 122]. There is evidence that surviving neurons attempt to compensate for the loss of neurons and synapses by reorganizing or sprouting axons and dendrites [120]. NCAM and PSA-NCAM are required for axon growth, and recent evidence has shown that NCAM cleavage to generate soluble fragments inhibits growth and branching of axons and dendrites, both in vitro and in vivo [38, 54]. Thus, the increased PSA-NCAM may be required for axon growth as surviving neurons try to reform lost connections in the brain, while the increased levels of soluble NCAM are likely inhibiting growth and synaptogenesis.

The dysregulated production of Aβ and Tau associated with AD also affects NCAM expression and function. Total loss of the γ-secretase component PS1 decreases NCAM expression, while mice expressing the familial human PS1 mutant show increased levels of NCAM in the frontal and piriform cortices and hippocampus [123]. Aβ has also been shown to directly affect NCAM function. HNK-1 is a glycosylation moiety on NCAM that has been implicated in synaptic transmission [124, 125]. Aβ generates reactive oxygen species (ROS) that oxidize glucuronyl transferase, the enzyme that synthesizes HNK-1. HNK-1 is decreased by ROS without affecting NCAM levels, suggesting that synaptic function of NCAM may be compromised by the increased Aβ levels in AD brain [126].

NCAM may also serve neuroprotective roles. NCAM can functionally interact with the fibroblast growth factor receptor (FGFR) to activate signaling through protein kinase C (PKC) and phosphatidyl inositol-3 kinase (PI-3 kinase), leading to the phosphorylation of GSK3β at an inhibitory site [114]. GSK3β is upregulated during AD by Aβ and leads to increased Tau filaments, neuronal death, axon/dendrite retraction and impaired cognition. Tau is a microtubule-associated protein, which becomes hyperphosphorylated by dysregulated GSK3β activity (the Tau kinase) in AD and may be required for its neurotoxic effects (reviewed in [112, 113]). Lack of NCAM signaling leading to inhibition of GSK3β would allow the progression of tangle pathology.

While NCAM may be implicated in the progression of AD through its function or lack of activity, peptide derivatives of NCAM are currently in clinical trials as a potential treatment for AD. An ideal AD drug would reduce Aβ and/or Tau levels, foster neuronal connectivity, stop neuron loss, and improve cognition and memory. The FGL peptide is a short NCAM peptide of the FGFR binding site from Glu_{681}-Ala_{695}, which seemingly meets these criteria. It has been shown to bind FGFR and activate PI-3 kinase and PKC signaling to inhibit GSK3β, promoting neuronal survival, differentiation, and synaptogenesis [127–129]. In rats, Aβ injection impairs cognition, increases neuronal death, degeneration and Tau phosphorylation, and induces brain atrophy, all hallmarks of AD. Early FGL peptide administration following Aβ injection (1 week later) decreased Aβ and Tau phosphorylation, prevented neuronal loss, and improved cognitive function [114]. FGL may also have "curative potential" as treatment after plaques had formed and significant cognitive impairment was present, resulting in enhanced learning and memory, and prevented or overcame memory loss due to disrupted cholinergic system function. Thus, regulating NCAM signaling in the brain may provide a novel treatment possibility for AD.

Future Directions

Schizophrenia, bipolar disorder, depression, anxiety disorders and AD present very different clinical symptoms, but share cognitive dysfunction at their core. The implication of NCAM in all of these diseases suggests that treatments to increase or mimic the function of NCAM, including increased PSA expression and decreased NCAM cleavage may improve neuronal connectivity and cognition. The effects of NCAM

cleavage are manifest around adolescence as PSA levels are decreasing, allowing soluble NCAM-EC to bind and possibly inhibit the signaling of full length NCAM downregulating and synapse formation [54]. NCAM-EC also prevents neurons from correcting the synaptic deficiency by inhibiting growth and branching [38, 54]. Targeted inhibition of NCAM cleavage in the brain could help alleviate these deficiencies. However, general treatment with currently available metalloprotease inhibitors is not possible as these compounds produce various nonneuronal side effects such as rheumatoid arthritis, tendonitis, fibroplasias, or exacerbate pulmonary disease or cancer (reviewed in [130]). Treatment with PSA may aid in improving plasticity, as addition of PSA may alleviate the learning and memory deficits caused by its absence [88, 101, 131–133] and allow for axonal growth and synaptogenesis [134–137]. Though one protein cannot be solely responsible for all aspects of these diseases, NCAM treatments may help alleviate a subset of symptoms in conjunction with other therapies and give hope to patients for the possibility of a brighter tomorrow.

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Neural Cell Adhesion Molecule in Cancer: Expression and Mechanisms

Silvia Zecchini and Ugo Cavallaro

Introduction

Tumorigenesis is a multistep process that often culminates with the invasion of cancer cells from the primary site into the surrounding tissue, their entry into the vascular network, and their metastatic dissemination to distant organs. The mechanisms underlying these events are only partially understood. However, it has become increasingly clear that changes in the adhesive properties of cancer cells, in terms of both cell–cell adhesion and adhesion to the extracellular matrix, are involved in tumor progression. Indeed, alterations in the levels and/or function of adhesion molecules, namely the membrane proteins that mediate intercellular and cell–matrix adhesion, have been associated with cancer invasion and metastasis. This is best exemplified by the loss of E-cadherin, the prototypic member of the cadherin superfamily of calcium-dependent cell adhesion molecules, which is a hallmark of many epithelial tumors when they acquire an invasive phenotype. Both in vitro and in vivo approaches have conclusively demonstrated that the downregulation of E-cadherin is not just an epiphenomenon of tumor progression, but it actually contributes to cancer invasion [1].

Recently, various lines of evidence have supported the notion that besides regulating cell adhesive properties, adhesion molecules are also involved in the modulation and transmission of intracellular signals [1]. Nevertheless, the contribution of deregulated signaling from adhesion molecules to cancer progression has remained elusive and is the focus of intense research in the field of molecular oncology.

This review will focus on the alterations of NCAM expression in cancer and on the possible role of NCAM-mediated signaling in tumor development.

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NCAM in Human Cancer: An Overview

As described in other chapters of this book, NCAM was first identified and characterized in the central nervous system. However, subsequent observations in various human cancers as well as in several experimental models have led to the hypothesis that NCAM plays also an important role in tumor progression. Indeed, the correlation between deregulated NCAM expression and poor prognosis has been found in different cancer types. These observations are summarized in Table 1. In addition, during tumor development NCAM often undergoes an isoform switch from the adult, GPI-linked 120-kDa isoform to the embryonic, transmembrane isoforms of 140 kDa or 180 kDa [1]. Post-translational modifications also seem to affect NCAM function in cancer cells. In particular, polysialylation, a modification that is mostly restricted to NCAM [2], has a profound impact on the role of NCAM in tumorigenesis. Nevertheless, the effect of NCAM polysialylation is still controversial: in certain tumor cell lines, polysialylated NCAM reduces tumor growth due to a decreased cell–cell and cell–matrix adhesions [3]. In contrast, the aggressiveness of neuroblastoma and of certain neuroendocrine cancers, correlates with the extensive polysialylation of NCAM [4, 5].

Table 1 Changes in NCAM expression in cancer and correlation with tumor progression

Tumor type	NCAM expression in tumor	References
Neuroblastoma	• ⇑ Upregulated NCAM expression correlates with cancer progression • NCAM inhibits cancer cells adhesion to endothelium PSA-NCAM enhances tumor invasion	[4, 8, 10–15, 68]
Glioma	• ⇓ Downregulated NCAM expression correlates with poor prognosis	[6, 7]
Myeloma	• ⇑ Upregulated NCAM expression correlates with poor prognosis	[16–19]
Acute myeloid leukemia	• ⇑ Upregulated NCAM expression correlates with poor prognosis	[21–25]
Colon cancers	• ⇓ Downregulated expression of the NCAM180 isoform correlates with tumor progression • Soluble NCAM is a marker of aggressive cancers	[26, 27]
Pancreatic cancer	• NCAM expression correlates with survival • PSA-NCAM induces neural invasion	[28–30]
Thyroid cancer	• ⇓ Downregulated NCAM expression in tumors • PSA-NCAM is highly expressed in medullary thyroid carcinoma	[8, 32–35, 69, 70]
Small cell lung cancer	• ⇑ Upregulated PSA-NCAM expression correlates with tumor progression and shorter survival	[9, 38]

Brain Tumors

The alterations in NCAM expression in human brain tumor suggest that this adhesion molecule can either counteract or promote cancer malignancy, depending on the tumor type. Early-stage astrocytic tumors express all three NCAM isoforms, whereas the expression of the adhesion molecule decreases with the progression toward malignant glioma: in advanced glioma, the loss of NCAM correlates with a more aggressive tumor phenotype and with poor prognosis [6]. In vitro studies have revealed that homophilic NCAM interactions repress the proliferation of astrocytes stimulated with fibroblast growth factor (FGF) [7]. Overall, these findings point to a tumor-suppressive function of NCAM in cancers of glial origin.

Neuroblastoma and tumors of neuroendocrine origin, instead, exhibit high expression of NCAM that is frequently associated with cancer progression [4, 8–10]. Hence, in these tumor types, the role of NCAM would be consistent with that of a tumor-promoting factor. Since a high degree of NCAM polysialylation is often observed in neuroblastoma and neuroendocrine tumors [4, 8, 9], and since polysialylation induces NCAM-dependent invasion [2], it is conceivable that such a post-translational modification determines the role of NCAM in cancer cells.

A peculiar property of NCAM in neuroblastoma cells is the inhibition of tumor cell adhesion to the endothelium [11]. Indeed, the acquisition of chemoresistance by neuroblastoma cells is accompanied by the downregulation of NCAM expression, thus enabling the transendothelial migration of cancer cells and their dissemination to distant organs [12]. Taken together, these observations suggest that the regulation of NCAM expression during the progression of neuroblastoma is highly dynamic, with a marked increase during the invasive phase and a reduction concomitant to tumor intravasation and metastatic dissemination.

Finally, the serum and the cerebrospinal fluid of patients affected by brain tumors frequently contain high levels of soluble NCAM, likely deriving from the shedding of the extracellular portion [13, 14]. Hence, soluble NCAM represents a suitable biomarker for these tumor types. In addition, since the serum level of soluble NCAM in glioma patients decreases significantly upon surgical removal of the tumor [15], this protein could be used as a marker for monitoring the response to therapy.

Myeloma

NCAM (often referred to as CD56 in hematopoietic lineages and malignancies) is one of the most reliable markers of multiple myeloma (MM), where it is highly expressed in 94% of the cases, while normal plasma cells, that give rise to MM, show no expression of NCAM [16]. In this context, NCAM has proven very useful to distinguish MM from other B-cell neoplasms [17, 18]. In addition to these biomarker properties, NCAM is likely to play a biological role in MM, based on the observations that (1) NCAM expression correlates inversely with bone marrow infiltration and with the number of circulating tumor cells [19], and (2) higher levels

of NCAM are associated with lytic bone lesions. The latter correlation is probably dependent on the NCAM-mediated homophilic interactions between MM cells and osteoblasts in bone marrow lesions [17]. Nevertheless, the lack of CD56 is associated with extramedullary involvement and poorer prognosis [20], raising the intriguing hypothesis that NCAM-mediated adhesion to osteoblasts prevents the dissemination and limits the aggressiveness of MM cells.

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a lympho-hematopoietic malignancy characterized by heterogeneous genetic, morphological and immunophenotypic patterns. Such heterogeneity is often an obstacle to a more reliable prognostic assessment and an appropriate therapeutic approach. Among the few antigens that have proven useful for the prognosis in AML patients, NCAM is certainly one of the most prominent. Indeed, NCAM is found in a subset of AML cases, ranging from 15 to 25%, where its expression is associated to unfavorable prognosis [21–23]. Moreover, NCAM has been associated to reduced remission and therapeutic response, even among patients carrying the chromosomal translocation t(8;21)(q22;q22), generally considered at lower risk of relapse [23].

The possibility that NCAM contributes functionally to the course of AML has not been thoroughly investigated yet. A recent report implicated NCAM in severe leukostasis, where NCAM would induce the adhesion of leukemic blasts to vascular endothelium and/or the clumping of blasts in small vessels, eventually leading to tissue infiltration and multiorgan failure [21]. NCAM also inhibits apoptosis in AML cells via the induction of the nuclear factor (NF)-kB pathway [24], which might contribute to the higher relapse rate associated with NCAM expression in AML patients.

Finally, with regard to the regulation of NCAM expression in leukemic blasts, recent findings pointed to RUNX1, a transcription factor encoded by one of the most frequently mutated genes in AML [25]. Indeed, RUNX1 is specifically overexpressed in NCAM-positive AML cases, and the forced expression of RUNX1 in AML cells induced NCAM transcription [24]. Interestingly, the same study identified novel RUNX1 isoforms that exert a dominant-negative activity on NCAM expression and, accordingly, are expressed at higher level in NCAM-negative AML.

Gastrointestinal Cancers

The expression pattern of NCAM in colon cancer is consistent with a tumor-suppressive function. Normal colon epithelial cells express the NCAM180 isoform [26], while no information is available on the other NCAM isoforms. The development

of colon carcinoma, however, is frequently accompanied by a decrease in NCAM180 expression in tumor cells, which correlates with the clinical aggressiveness of the disease [26]. Such a decrease is due to an alternative splicing mechanism that causes a truncation of NCAM mRNA between exons 12 and 13 [27]. Since this splicing should result in the truncation of NCAM's ectodomain between the two FNIII repeats, it would imply the production of a soluble form of NCAM as a marker of aggressive colon cancer. This hypothesis deserves further investigation, as it may provide a novel biomarker useful in the prognostic evaluation of colon cancer patients.

The expression of NCAM has been also assessed in pancreatic carcinoma, with results that failed to provide a clear picture. While NCAM is consistently undetectable in normal exocrine pancreas [28, 29], its expression is induced in a significant fraction of pancreatic carcinomas. In these patients, the level of NCAM correlated with the survival rate [29]. Another study, however, reported that the expression of NCAM in pancreatic cancer lesions is restricted to the inflammatory tissue surrounding the neoplastic foci, rather than the cancer cells themselves [30]. Kameda et al. [28] reported an intriguing correlation of NCAM expression with neural invasion, a peculiar feature of pancreatic cancer which often accounts for its recurrence. Nevertheless, NCAM appeared not to serve as an adhesion molecule between tumor and neural cells, but rather to promote cancer cell invasion thanks to its polysialylation [28].

In this context, it is interesting that a synergism between activated K-Ras and p53 induced pancreatic cell invasion by disrupting a complex containing NCAM, N-cadherin and keratinocyte growth factor receptor (KGFR), a member of the FGFR family [31]. Hence, the crosstalk between NCAM, N-cadherin and KGFR in pancreatic ductal cells would be required for the maintenance of the tissue architecture.

Thyroid Cancer

Follicular epithelial cells of normal thyroid express NCAM at the cell surface [32, 33]. The neoplastic transformation of thyrocytes, however, is accompanied by the downregulation of NCAM, a phenomenon observed in most thyroid tumor types, including follicular anaplastic and papillary carcinoma [33–35]. An exception is represented by medullary thyroid carcinoma that exhibits a strong expression of polysialylated NCAM, thus contributing to differential diagnosis [8].

Whether the loss of NCAM is functionally involved in thyroid cancer development remains unresolved. It is conceivable that, by analogy to other adhesion molecules, the disruption of NCAM-mediated cell adhesion increases the invasive capacity of malignant thyroid cells. However, the knockdown of NCAM in a cell line derived from papillary thyroid carcinoma resulted in the reduction of cell migration and invasion [35].

Small Cell Lung Cancer

Small cell lung cancer (SCLC) is the most aggressive tumor among pulmonary neoplasias, as the median survival time from diagnosis is only one month. Both surgery and radiotherapy are frequently unsuccessful, while chemotherapy shows some degree of response. SCLC cells exhibit a neuroendocrine phenotype and express a wide range of neuronal markers [36]. In this context, the expression of NCAM is a reliable indicator of neuroendocrine differentiation and has long been exploited as a SCLC biomarker [9, 37]. Among NCAM-positive SCLC cases, the polysialylation of NCAM correlates with higher grade and shorter survival [9, 38]. While there is no information on the potential role of NCAM in the progression of SCLC, the widespread expression of NCAM in this cancer type has led many researchers to exploit NCAM as a target for both imaging and therapeutic purposes. Indeed, radioactively labeled antibodies against NCAM have shown a good biodistribution in SCLC-bearing mice [39, 40] as well as in patients [41]. Furthermore, the successful delivery of toxins into SCLC cells by conjugation with anti-NCAM antibodies [13, 42–45] indicated that NCAM is an attractive therapeutic target for the treatment of this neoplastic disease.

How Does NCAM Modulate Tumor Development?

NCAM and Tumor–Microenvironment Interactions: The Rip1Tag2 Model

The only mouse model of spontaneous tumorigenesis used to assess the functional contribution of NCAM to cancer development is the Rip1Tag transgenic mouse. In these mice, the expression of the SV40 Large T antigen is driven by the rat insulin promoter, resulting in the neoplastic transformation of beta cells within the pancreatic islets of Langerhans [46]. Such a model recapitulates some of the properties of human beta-cell cancer, also known as insulinoma, including the expression of NCAM in tumor cells [47, 48]. The inactivation of the *Ncam* gene in Rip1Tag2 mice has pointed to a key role for this adhesion molecule in governing the interplay between tumor cells and their microenvironment. In particular, the ablation of NCAM induced tissue disaggregation of the primary tumor [49] and the formation of metastases in the local draining lymph nodes of the pancreas [50], thus suggesting that NCAM controls tissue architecture and prevents metastatic dissemination in that tumor type. In an attempt to identify the molecular mechanisms underlying this function of NCAM, we have discovered a signaling complex on the surface of beta cells, where NCAM associates with the fibroblast growth factor receptor (FGFR). By virtue of this interaction, NCAM was able to trigger FGFR-mediated signaling that, in turn, modulated the activation of beta1 integrin and, therefore, cell adhesion to the extracellular matrix [49]. Furthermore, the loss

of NCAM caused tumor-associated lymphangiogenesis, thus providing cancer cells with a route for their dissemination to the regional lymph nodes [51]. It is conceivable that lymphangiogenesis represents a secondary event due to the increase in interstitial fluid pressure which follows the tissue disaggregation caused by the loss of NCAM [49].

The phenotype of metastatic lesions within the lymph nodes of NCAM-deficient Rip1Tag2 tumors was frequently benign in nature [49], thus implying that metastatic dissemination is not restricted to cancer cells that have acquired a fully malignant phenotype. Indeed, based on the phenotypic characteristics of NCAM-negative Rip1Tag2 mice, we propose a model that would account for the metastatic spread of benign cancer cells. According to this model, depicted in Fig. 1, the loss of integrin-mediated matrix adhesion generates clusters of neoplastic cells that are detached from the tumor mass and "float" in the lymphatic

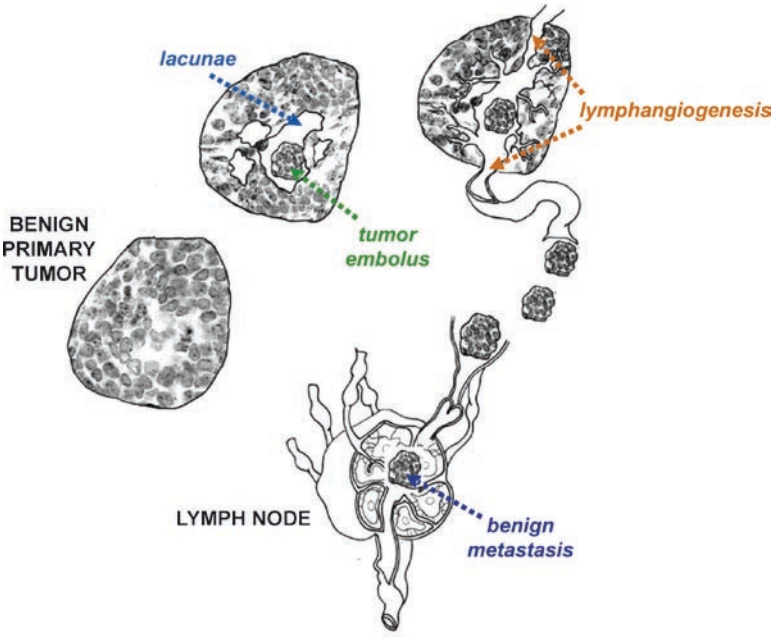

Fig. 1 A model for passive metastasis**.** The picture illustrates the series of events that may lead to the passive dissemination of tumor cells in the absence of an overt malignant phenotype (see text for more details). The loss of integrin-mediated adhesion (e.g., following the down-regulation of NCAM in the Rip1Tag2 mouse model), causes tissue disaggregation, with the detachment of tumor cell clusters (emboli) and the appearance of hemorrhagic lacunae. The consequent fluid leakage and the increase in interstitial fluid pressure stimulate lymphangiogenesis, required for the drainage of excess fluid. Tumor emboli are then passively "washed out" together with the drained fluid, entering the lymphatic vessels and reaching the regional lymph nodes. Here, they get trapped and give raise to the metastatic lesion. The entire process does not implicate "active" invasion by the tumor cells, thus accounting for the dissemination of benign tumor cells

lacunae that are formed upon tissue disaggregation. The increase in interstitial fluid pressure and the subsequent lymphangiogenesis (required to drain the excess fluid) would then force these tumor cell clumps into the lymphatic circulation and eventually into the lymph node tissue, where they would get trapped and form the metastatic lesions. Since these events are consistent with a passive washout of tumor cells from the primary site (together with the drained fluid) and do not require any invasive activity by the cancer cells, we have termed this process as "passive" metastasis [52]. It is noteworthy that an analogous mechanism of passive dissemination of benign tumor cell clusters has been reported in xenotransplantation model of breast carcinoma [53]. The formation of benign metastasis might also account for the favorable clinical course observed in a subset of cancer patients (e.g., with breast cancer), in spite of the presence of lymph node metastases [1].

A recent study on the Rip1Tag2 model uncovered another important function of NCAM in tumor–microenvironment interactions. The tumors of NCAM-deficient mice exhibited a dramatic increase in blood vessel leakage, suggesting an impaired stabilization of the tumor vasculature. Indeed, it was found that NCAM promotes the recruitment of pericytes to the vascular wall, thus stabilizing the cancer-associated blood vessels. This, in turn, counteracted the dissemination of tumor cells, contributing to the role of NCAM in limiting metastasis [54]. This model, that was confirmed in a fibrosarcoma xenotransplantation system [54], highlighted a novel mechanism underlying metastatic dissemination, namely the perturbation of endothelium–pericyte interaction as a way to destabilize tumor vessels and facilitate the intravasation of cancer cells.

NCAM and Tumor Angiogenesis

A number of studies have reported the expression of NCAM in blood-vessel endothelial cells from different tissues [55–59]. Despite the biological role of vascular NCAM has not been deeply investigated yet, some recent reports highlighted its relevance in tumor-associated angiogenesis. First, Bussolati et al. [60] have shown that NCAM is specifically overexpressed in tumor-derived endothelial cells isolated from renal carcinoma, as compared to normal endothelial cells. These findings provided the rationale for the successful use of endothelial NCAM as a target for imaging tumor angiogenesis in preclinical models [61]. Furthermore, the expression of NCAM was shown to be highly dynamic during the endothelial cell remodeling that accompanies the formation of capillary-like structures. In particular, NCAM was undetectable in resting cells, but it was readily induced upon stimulation of in vitro angiogenesis, and decreased again once the morphogenetic process was completed. Notably, NCAM is functionally involved in the angiogenic potential of tumor-derived endothelial cells, a property that implicates, at least to some extent, its interplay with FGFR [62].

The NCAM–FGFR Crosstalk

By analogy to other adhesion molecules, the ability of NCAM to modulate intracellular signaling is likely one of its most prominent activities in the context of cancer progression. While many studies have delineated the signaling cascades controlled by NCAM in neural cells (for reviews, see other chapters in this book), only limited information is available in non-neural cell types. Nevertheless, one of the most intriguing activities of NCAM in this context is its crosstalk with the FGFR signaling machinery. Despite the NCAM–FGFR functional interaction was initially reported in neurons [63], the first demonstration of a physical association between the two proteins was obtained in neoplastic beta cells from the Rip1Tag2 mice [49]. Subsequently, our group has also detected the NCAM–FGFR crosstalk in various other cell types ([64]; SZ and UC, unpublished observations), while the group of Berezin and Bock has demonstrated the direct binding of NCAM to FGFR [65]. Notably, NCAM appears to play a dual role on FGFR activity: on one hand it acts as an agonist of FGFR inducing MAPK activation, neurite-like protrusions and integrin-mediated adhesion [49] (Fig. 2); on the other hand, NCAM appears to

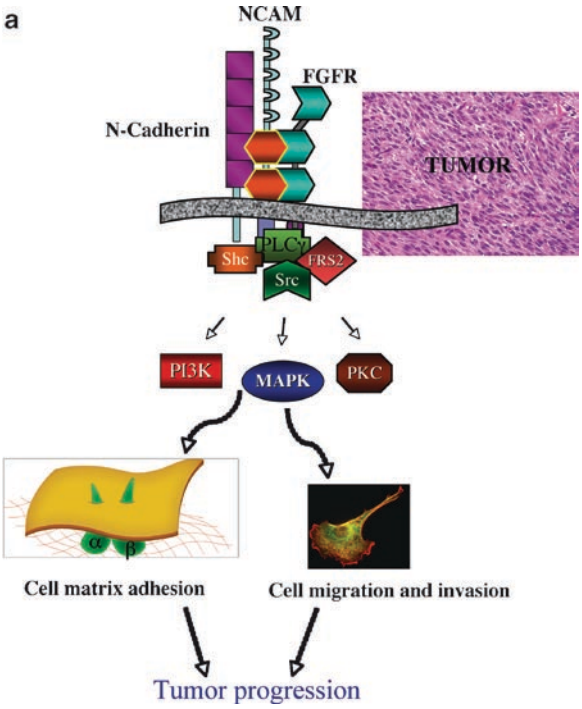

Fig. 2 The NCAM–FGFR signaling complex**.** The physical interaction of NCAM with FGFR in tumor cells (**a**) and in neurons

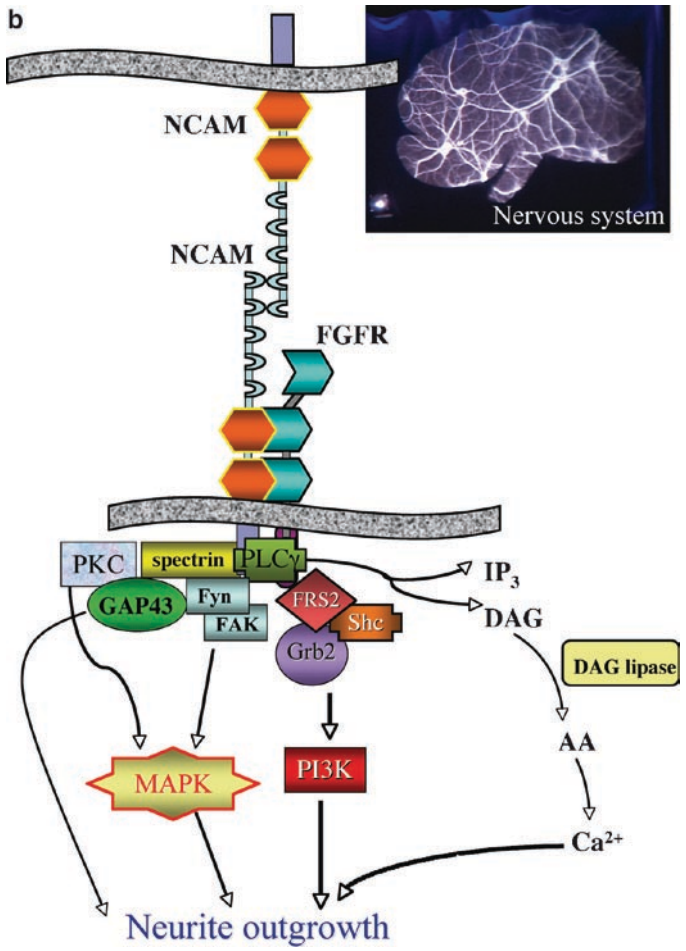

Fig. 2 (continued) (**b**) triggers a variety of signaling cascades that ultimately modulate a broad spectrum of cellular functions, including cell–matrix adhesion, cell migration and neurite outgrowth. The biological role of N-cadherin, that associates with NCAM and FGFR [31, 49] is not completely understood. N-cadherin associated with the NCAM–FGFR complex has been proposed to regulate cancer cell migration and invasion in an adhesion-dependent manner [31]. See the text for more details. PLCγ, phospholipase C-γ; FRS2, FGF receptor-substrate 2; PI3K, phosphatiylinositol 3-kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; α and β, integrin subunits, DAG, diacylglycerol; AA, arachidonic acid; FAK, focal adhesion kinase

exert a negative regulation on the cellular response evoked by the classical FGFR ligands, the FGFs. In particular, FGF-induced cell proliferation is inhibited by the expression of NCAM or even by a small peptide that mimics the direct binding of NCAM to FGFR [64]. Such a mechanism could account for the reported inhibition of NCAM on FGF-induced growth of astrocytoma cells [7] and could contribute to the inhibitory function of NCAM in some tumor types (see above).

Given that aberrant FGFR signaling has been implicated in many tumor types (reviewed in [66]), and that, as outlined above, NCAM deregulation is a hallmark of many neoplasias, it is tempting to speculate that a tightly regulated crosstalk between NCAM and FGFR is required to counteract certain steps of tumor progression. Further studies will be needed to address this issue in a systematic manner in order to elucidate whether the NCAM–FGFR interaction represents a suitable target for novel therapeutic approaches to be tested in appropriate preclinical models.

Perspectives

Various lines of evidence point to NCAM as an important player in the development of several cancer types. The expression patterns of NCAM in human tumors are consistent with both a negative and a positive effect on cancer development, depending on the tumor type. This is in agreement with functional studies performed on several cancer-derived cell lines, where NCAM affects a broad spectrum of cellular processes. Changes in the intercellular adhesive properties of cancer cells as a consequence of altered NCAM expression certainly play a role in tumorigenesis. However, the ability of NCAM to modulate intracellular signaling is emerging as an important factor in dictating its effect on cancer development.

Together with the use of NCAM as a tumor antigen suitable for antibody-based targeting strategies [67], the identification of NCAM-dependent signaling in tumor cells should provide the rationale for novel therapeutic strategies against specific cancer types.

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Part VII
Pharmacology of NCAM: NCAM Mimetics

NCAM Mimetic Peptides: An Update

Vladimir Berezin and Elisabeth Bock

Introduction

Recent advances in the characterization of genomes of different species have led to the cloning of a large number of new cell adhesion molecules (CAMs) [1]. These molecules play major roles in virtually every physiological and pathological process in multicellular organisms. Studies of specific aspects of CAM functions have, until recently, been hindered by the availability of reliable pharmacological tools. CAMs are, in most cases, involved in multiple, low affinity interactions with themselves and with other cell surface receptors, and the majority of CAMs does not possess any intrinsic enzymatic activity. Currently available pharmacological tools for the study of the roles of CAMs in physiological and pathological processes are antibodies recognizing specific epitopes of CAMs, recombinantly produced functional modules of CAMs, and mimetic peptides targeting CAMs at different binding sites (for review, see [2]). Recent progress in our understanding of a structural basis of CAM-mediated cell adhesion and signaling has allowed the structure-based design of CAM mimetic peptides. A first review summarizing current knowledge about peptides mimicking various functional aspects of the neural cell adhesion molecule (NCAM) was published in 2004 [3]. The purpose of the present review is to give an update on NCAM mimetic peptides as regards their structure, identification, in vitro and in vivo functional properties. NCAM mimetic peptides fall in three distinct groups, (1) NCAM-binding peptides with artificial sequences, see Table 1, (2) peptides, whose sequences contain one or several NCAM homophilic binding sites (i.e., sites involved in NCAM binding to itself), see Table 2, and (3) peptides, whose sequences contain one or several NCAM heterophilic binding sites (i.e., sites involved in NCAM interactions with other proteins), see Table 3.

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Table 1 NCAM-binding peptides with artificial sequences

Peptide name/sequence	Mode of identification	Sequence identity/ localization	Binding to receptor/module	Effects in vitro	Effects in vivo	Reference
C3/ASKKPKRNIKA	Screening of combinatorial library	Artificial	NCAM/Ig1	Induction of neurite outgrowth by increasing cytoplasmic calcium, activation of ERK1/2, Akt/ PKB and CREB and induction of c-Fos.	Protection against embryotoxic effect of teratogen.	[17, 19–31, 35]
				Protection against apoptosis.	Impairment of short- and long-term memory.	
				Inhibition of proliferation, NCAM –mediated adhesion and presynaptic function.	Protection against chronic pain.	
NBP10/AKKMWKKTW	Screening of combinatorial library	Artificial	NCAM/Ig1	Induction of neurite outgrowth by increasing cytoplasmic calcium.	N.D.	[32]
				Inhibition of NCAM-mediated adhesion.		

ENFIN2/AFYRTIQWTME	Screening of combinatorial library	Artificial	NCAM/FN3,1-2	Induction of neurite outgrowth by activation of heterotrimeric G-protein pathway.	N.D.	[33]
ENFIN11/ ARWSKGFDQWM	Screening of combinatorial library	Artificial	NCAM/FN3,1-2	Induction of neurite outgrowth by activation of a heterotrimeric G-protein pathway.	N.D.	[33]

Table 2 Synthetic peptides that contain one or several NCAM homophilic binding sites

Peptide name/sequence	Mode of identification	Sequence identity/ localization	Binding to receptor/module	Effects in vitro	Effects in vivo	Reference
P2/GRILARGEINFK	Structural analysis	NCAM/Ig2	NCAM/Ig1	Induction of neurite outgrowth by increasing cytoplasmic calcium, activation of FGFR, Src-family kinases, Akt/PKB and ERK1/2.	Enhancement of short-term memory.	[35–40]
				Protection against apoptosis.	Protection against the amnestic effect of scopolamine.	
				Inhibition of NCAM –mediated adhesion.	Facilitation of recovery following traumatic brain injury.	
P1-B/GEISVGESKFFL	Structural analysis	NCAM/Ig1	NCAM/Ig2	Inhibition of NCAM-mediated adhesion.	N.D.	[4, 37]
P-3-G/SIHLKVFAK	Structural analysis	NCAM/Ig3	NCAM/Ig2	Inhibition of NCAM-mediated neurite outgrowth by monomer.	N.D.	[4]
P-3-DE/ KHIFSDDSSELTI-RNVDKNDE	Structural analysis	NCAM/Ig3	NCAM/Ig1	Inhibition of NCAM-mediated neurite outgrowth by a monomer.	N.D.	[4]

Table 3 Synthetic NCAM-derived peptides targeting heterophilic NCAM interaction partners

Peptide name/sequence	Mode of identification	Sequence identity/ localization	Binding to receptor/ module	Effects in vitro	Effects in vivo	Reference
FGL/EVYVVAENQQGKSKA	Structural analysis	NCAM/ FN3,2	FGFR/Ig2-Ig3	Induction of neurite outgrowth by binding and activation of FGFR followed by activation of ERK1/2 and Akt/PKB.	Enhancement of spatial and social memories.	[42–51]
				Protection against apoptosis.	Promotion of postnatal sensorimotor development.	
				Promotion of synapse formation and enhancement of presynaptic function.	Protection of hippocampal neurons against ischemic insult.	
				Attenuation of inflammatory impact.	Reduction of neuropathological signs and cognitive impairment in AD model.	
					Attenuation of age-related changes in LTP and inflammatory signs and prevention of stress-induced dementia.	
FRM/SIDQVEPYSSTAQ	Sequence homology analysis	NCAM/ FN3,1	N.D.	Induction of neurite outgrowth in the absence of FGF and NCAM-NCAM interaction.	N.D.	[53, 54]

(continued)

Table 3 (continued)

Peptide name/sequence	Mode of identification	Sequence identity/ localization	Binding to receptor/ module	Effects in vitro	Effects in vivo	Reference
				Promotion of cell survival.		
DekaCAM/AALNGKGL	Sequence homology analysis	NCAM/ FN3,1	FGFR/Ig2-Ig3	Induction of neurite outgrowth by binding and activation of FGFR.	N.D.	[55]
BCL/NLIKQDDGGSPIRHY	Structural analysis and molecular modeling	NCAM/ FN3,2	FGFR/Ig2-Ig3	Induction of neurite outgrowth by binding and activation of FGFR.	N.D.	[56]
EncaminA/ SIDRVEPYSSTAQVQFD		NCAM/ FN3,1	FGFR/Ig2-Ig3	Induction of neurite outgrowth.	N.D.	[57]
EncaminC/ KAEWKSLGEEAWHSK	Structural analysis and molecular modeling	NCAM/ FN3,1	FGFR/Ig2-Ig3	Induction of neurite outgrowth.	N.D.	[57]
				Protection against apoptosis.		
				Enhancement of presynaptic function.		
EncaminE/TIMGLKPETRYAVR	Structural analysis and molecular modeling	NCAM/ FN3,1	FGFR/Ig2-Ig3	Induction of neurite outgrowth.	N.D.	[57]
				Protection against apoptosis.		
				Enhancement of presynaptic function.		
HBP/KGRDVILKKDVRFI	Structural analysis	NCAM/Ig2	Heparin/ HSPG	Induction of neurite outgrowth through HSPG.	N.D.	[60]

Ectodomain Structure of NCAM and Natural Extracellular Interaction Partners

NCAM belongs to the immunoglobulin (Ig) superfamily. Alternative splicing and posttranslational modifications lead to the generation of a number of distinct NCAM forms, all arising from a single *NCAM1* gene. The extracellular part of NCAM (NCAM ectodomain) consists of five *N*-terminal Ig modules followed by two fibronectin type III (FN3) modules. The NCAM ectodomain interacts with itself through both homophilic *cis* and *trans*-interactions. The crystal structure of the *N*-terminal triple Ig module (Ig1-2-3) of NCAM solved by X-ray crystallography suggest that NCAM molecules form *cis*-dimers on the cell membrane, which, in turn, mediate *trans*-interactions between cells via formation of two types of "zippers," flat or dense zippers [4, 5].

In addition to homophilic binding, the NCAM ectodomain may interact with other CAMs, such as the CAMs L1 and TAG-1 [6, 7], ATP [8], the fibroblast growth factor receptors (FGFRs) [9], glial cell line-derived neurotrophic factor (GDNF) and its cognate receptor, GDNF family receptor α (GFRα) [10], heparin and chondroitin sulfate proteoglycans (HSPG and CSPG, respectively) [11–13], collagens I-IV and IX [14], rabies virus [15], and the cellular prion protein [16].

Artificial NCAM-Binding Peptides

A number of NCAM-binding peptides have been identified employing an approach, in which peptides from combinatorial libraries attached to polystyrene beads are incubated with a biotin-labeled target molecule (e.g., whole NCAM or one or more NCAM-derived modules), and the beads binding the target are identified by a streptavidin-based staining reaction, isolated and microsequenced [17] (Table 1).

C3

The C3 peptide was the first peptide mimetic of NCAM identified by screening a combinatorial peptide library of undecapeptides for binding to the first Ig module of NCAM [17]. C3 has, by NMR and SPR analysis, been shown to interact with the first Ig module of NCAM. The C3 binding site has been mapped and shown to be different from the binding site of the NCAM Ig2 module. The latter is involved in NCAM homophilic *cis*-interactions [18]. Pharmacological effects of C3 have been extensively studied both in vitro and in vivo.

C3 has been shown to induce neurite outgrowth in primary cultures of neurons by increasing the cytoplasmic calcium concentrations, activation of the extracellular regulated kinase ERK1/2, protein kinase B (PKB) and cAMP response element

binding protein (CREB), and by induction of c-Fos [19–21]. C3 also promotes survival of cerebellar granule neurons and dopaminergic neurons. Interestingly, whereas Fyn, FGFR, ERK1/2 kinase, protein kinase A (PKA) and C (PKC) are required for both the neuritogenic response and survival response, PLC and calcium calmodulin-dependent kinase II are only necessary for the C3-induced neurite outgrowth response, but dispensable for C3 mediated neuroprotection [22, 23]. Cyclic guanosine monophosphate (cGMP) is also involved in C3 induced neurite outgrowth from hippocampal neurons and survival of dopaminergic neurons [24]. Thus, in in vitro systems, C3 mimics NCAM functions such as induction of neurite outgrowth and neuroprotection. However, C3 is also capable of inhibiting NCAM functions by interfering with NCAM-mediated cell adhesion [17], a property that probably explains the inhibitory effect of C3 on presynaptic function in cultures of hippocampal neurons [25]. C3 has also been shown to inhibit proliferation of PC12 cells [17]. In neuroblastoma cells, C3 has been shown to prevent the effect of polysialic acid (PSA) removal from the cell surface on proliferation and activation of ERK [26].

In vivo, C3 has considerable effects. Intra-amniotic administration of C3 in rats treated with the teratogen pyrimethamine has been shown to rescue the deficit in brain weight, reverse the decrease in thickness of the cortical plate, and significantly reduce the number of malformed fetuses [27]. Thus, C3 demonstrates neuroprotective effects both in vitro and in vivo. In accordance with the inhibitory effect of C3 on neurotransmitter release in hippocampal cultures, treatment with C3 inhibits learning and memory. The C3-mediated memory impairment has been observed in the approach avoidance and passive avoidance responses, contextual fear conditioning and water maze tests [28–30]. It has been suggested that C3 might be a potential drug candidate to reduce the cognitive impact induced by exposure to intensive stress experiences [30]. In addition, C3 has recently been demonstrated to have analgesic properties; it partially reduces the chronic pain induced by a chronic constrictive injury [31].

NBP10

An NCAM binding peptide 10 (NBP10) was identified by screening a combinatorial library of synthetic peptides with NCAM purified from postnatal day 10 rat brains [32]. Like C3, NBP10 binds to the first Ig1 module of NCAM. Unlike C3, the binding site in the Ig1 module of NCAM for NBP10 peptide and the binding site for the Ig2 module partially overlap, indicating that NBP10 can compete with the formation of NCAM *cis*-dimers made through the reciprocal interaction between two NCAM Ig modules. Like C3, NBP10 displays a dual function. As antagonist, it inhibits NCAM mediated adhesion. On the other hand, NBP10 exhibits an agonistic effect, inducing neurite outgrowth by activating signal transduction pathways similar to those activated by NCAM itself [32].

ENFIN2 and ENFIN11

The ENFIN2 and ENFIN11 peptides have been identified by screening a combinatorial library of synthetic undecapeptides with recombinantly produced FN3 modules 1 and 2 of NCAM [33]. ENFIN2 and 11 bind to the combined two FN3 modules of NCAM with apparent K_D values of 84 nM and 3,4 μM, respectively. Both peptides induce neurite outgrowth in an NCAM-dependent manner, since knock-down of NCAM abolishes the peptide-induced neuritogenic response in vitro. Unlike C3 and NBP10, the ENFIN peptides do not inhibit NCAM mediated adhesion. Also, unlike C3, the effect of the ENFIN peptides on neurite outgrowth is not dependent on the activation of FGFR. Moreover, neither of two peptides stimulate neurite outgrowth through signaling mediated by Fyn or related kinases of the Src family. However, the peptide effect on neurite outgrowth requires a pertussis toxin (PTX) sensitive signaling pathway. Thus, the ENFIN peptides probably employ a restricted number of signaling pathways activated through NCAM [33].

Synthetic NCAM-Derived Peptides Targeting NCAM

Structural studies of NCAM homophilic interactions have made it possible to point out sequential motifs, which are involved in NCAM intermodular interactions, thereby opening the possibilities to design peptides targeting particular NCAM binding sites, thus mimicking specific NCAM functions (Table 2).

P2

P2 is a peptide corresponding to a 12 amino acid long sequence in the second Ig module of NCAM, which represents the natural binding partner of the first Ig module of NCAM involved in the reciprocal interaction with NCAM Ig2 and the formation of NCAM dimers in *cis* [4, 18, 34, 35]. In vitro, the P2 peptide is a very strong inducer of neurite outgrowth through increased cytoplasmic calcium concentrations, activation of FGFR, PLCγ, Src family kinases and ERK1/2 [35, 36]. P2 is a "competitive antagonist" of NCAM because, under certain conditions, it inhibits NCAM-mediated adhesion. However, if NCAM is not involved in *trans*-homophilic interactions, P2 acts as an agonist binding to NCAM, thereby inducing the neuritogenic response [37]. P2 also has been shown to enhance the survival rate of cerebellar neurons, although not of mesencephalic dopaminergic neurons [38].

In vivo, P2 has been shown to enhance short term memory, inhibit decreased anxiety behavior during learning, and protect against the amnesic effects of scopolamine [39]. The P2 peptide appears rapidly in blood and cerebrospinal fluid (CSF) after subcutaneous administration in rat [40]. P2 administered subcutaneously has been shown to facilitate recovery of motor and cognitive functions and counteract neuropathological signs following traumatic brain injury [40].

P1-B

The P1-B peptide is a conserved sequence fragment of NCAM derived from the Ig1 module. The peptide represents the Ig1–Ig2 contact area involved in the *cis* dimeric formation of NCAM [4]. P1-B has, by NMR titration analysis, been shown to bind to the second Ig module of NCAM, and to induce chemical shifts of a number of amino acid residues representing the P2 sequence. The chemical shift describes the dependence of nuclear magnetic energy levels on the electronic environment in a molecule, thus indicating that the P1-B peptide binds Ig2 at the site that includes the P2 motif. Like P2, the P1-B peptide acts as conventional antagonist, agonist, and reverse agonist of NCAM at low, intermediate and high peptide concentrations, respectively [37]. interfering with the NCAM homophilic binding in *cis* [4].

P-3-G

The P-3-G peptide corresponds to a Ser281-Lys289 fragment of mouse and rat NCAM, and covers the *C*-terminal part of the G β strand of the Ig3 module. It represents the Ig2-to-Ig3 contact, which, according to the crystal structure of the triple Ig1-3 module of NCAM, is engaged in a flat zipper formation following NCAM homophilic *trans*-interactions [4]. A monomeric form of this peptide has been shown to inhibit NCAM-mediated neurite outgrowth from PC12E2 cells [4].

P-3-DE

The P-3-DE peptide corresponds to Lys244-Glu264 of rat NCAM, and covers the D and E β strands and the EF loop of the third Ig module. It represents the Ig1-to-Ig3 contact, which, according to the crystal structure of the triple Ig1-3 module of NCAM, is engaged in a dense zipper formation following NCAM homophilic *trans*-interactions. P-3-DE has been shown to interfere with NCAM-induced neurite outgrowth [4].

Synthetic NCAM-Derived Peptides Targeting Heterophilic Ligands of NCAM

It is well established that NCAM can be involved in a variety of heterophilic interactions with growth factors, growth factor receptors and various components of the extracellular matrix [41]. Structural studies and sequence homology analysis of NCAM heterophilic interactions have resulted in identification of a number of sequential motifs, which are involved in NCAM interactions with FGFR and HSPGs (Table 3).

FGL

The FGL peptide represents a conserved motif encompassing the F and G β strands and the interconnecting loop in the second FN3 module of NCAM. The peptide has been shown by NMR and SPR analyses to bind to FGFR [42]. It has been shown to bind not only FGFR1, but also FGFR2, splice variant IIIc [43]. FGL is the most studied NCAM mimetic peptide. Interaction with FGFR1 results in receptor phosphorylation. In vitro, FGL has been demonstrated to induce neurite outgrowth in primary rat dopaminergic neurons, hippocampal neurons and CGNs. It also has been shown to increase neuronal survival and reduce DNA fragmentation when apoptosis is induced. These in vitro effects of FGL are dependent on the activation of FGFR, MAP and ERK kinase MEK and phosphatidylinositide-3-kinase (PI3K) [44]. FGL also has been shown to protect neurons in the dissociated rat hippocampal cultures and cultures of hippocampal slices following oxygen/glucose deprivation [45]. FGL has been demonstrated to promote synapse formation and enhance presynaptic function in hippocampal cultures by facilitating transmitter release in an FGFR activation-dependent manner [46]. Finally, FGL has recently been shown to attenuate interleukin-1β production and enhance interleukin-4 release in mixed glial cell cultures, indicating that FGL might have an anti-inflammatory potential [47].

In vivo, FGL has been shown to induce a long-lasting improvement of memory in contextual fear conditioning and water maze tests [46]. Enhanced spatial learning after FGL treatment has been shown to be accompanied by rapid phosphorylation of FGFR in the hippocampus and by specific changes in dendrites and dendritic spines of CA1 pyramidal neurons (Knafo S, personal communication). A single intracisternal injection of FGL has been shown to significantly protect hippocampal CA1 neurons from death in a transient global ischemia model in the gerbil [45]. In a rat model of β amyloid $(A\beta)_{25–35}$ peptide-induced neurotoxicity, FGL has been demonstrated to strongly reduce all investigated signs of Aβ-induced neuropathology and cognitive impairment. FGL both prevents and ameliorates neurotoxic effects of Aβ. Interestingly, the beneficial effect of FGL is seen both after intranasal and subcutaneous administration of the peptide [48]. In newborn rats, the FGL peptide, when administrated intranasally, accelerates early postnatal development of coordination skills. In adult animals, subcutaneous administration of FGL results in a prolonged retention of social memory [49]. In aged rats, subcutaneous treatment with FGL has recently been shown to attenuate the impairment in long-term potentiation and the age-related changes in CD200, and the markers of activated microglia, CD86 and ICAM [47], and to induce significant structural alterations in synapses and dendritic spines [50]. FGL treatment of aged rats during 4 weeks of continuous stress followed by intermittent stress once a week during the subsequent 6 months has been shown to prevent stress-induced cognitive impairment [51]. FGL also has been demonstrated to rapidly reach the blood and CSF after both intranasal and subcutaneous administration in the rat. It remains detectable in

these fluids for up to 5 h [49]. Thus, the FGL peptide has a distinctive pharmacological profile targeting neurodegeneration and impaired cognition. Recently, preclinical studies in rats, dogs and monkeys have demonstrated exposure in plasma and cerebrospinal fluid after parenteral or intranasal administration of FGL, with no systemic toxicity. Intranasal administration of FGL has been shown to be well tolerated in healthy human male volunteers, with no safety concerns and a dose-related pharmacokinetic profile. Further studies are currently being planned to evaluate the effects of FGL in patients with Alzheimer's disease [52].

FRM

Apart from FGL, a putative FGFR binding and activation motif, FRM, corresponding to Ser504 – Gln516 of human NCAM contained within the first FN3 module has been suggested to contribute to NCAM activation of FGFR. The synthetic FRM peptide has been demonstrated to induce tyrosine phosphorylation of FGFR and FRS-2, stimulate neurite outgrowth and promote neuronal survival. However, at low concentrations, it inhibits both NCAM and FGF2-induced neurite outgrowth responses. FRM has also been demonstrated to inhibit FGF2-induced proliferation of 3T3 fibroblasts. It has been suggested that FRM, in conjunction with FGL, forms an NCAM FGFR activation site [53, 54].

DekaCAM

Sequence alignment of the first NCAM FN3 module and various FGFs resulted in the identification of a six amino acid motif, ALNGKG, localized in the *C*-terminal sequence of the first FN3 module of NCAM encompassing parts of the F and G β strands and the short connecting loop, and in the β10-β11 region of FGF10. The motif has been termed decaCAM [55]. The decaCAM peptide has been shown to bind both FGFR1 splice variant IIIc, and FGFR2 splice variant IIIb. The binding of decaCAM to FGFR results in receptor phosphorylation and induction of neurite outgrowth in CGN cultures. Thus, decaCAM represents one of the multiple NCAM binding sites for the FGFR [55].

BCL

The BCL peptide is a conserved motif encompassing the B and C β strands and the interconnecting loop in the second FN3 module of NCAM. It binds and activates FGFR and induces neurite outgrowth in an FGFR activation-dependent manner.

A glutamine residue in the fifth position of the peptide is critical for its neuritogenic activity. The peptide appears to act as a partial agonist of FGFR inhibiting FGF2-induced receptor activation. Together with FGL, FRM and DekaCAM, BCL has therefore been suggested to contribute to the multiple FGFR binding sites in NCAM [56].

Encamin Peptides

NCAM probably interacts with FGFR through multiple binding sites, four of which represented by motifs FGL, decaCAM, FRM and BCL, respectively, have been discussed above. In a recent study [57], peptides encompassing all six strand-loop-strand regions in the first FN3 module of NCAM, termed encamins, were synthesized and tested for their ability to bind and activate FGFR. Three encamins, EncaminA, C and E, all localized to the *C*-terminal part of the module, have been shown to bind and activate FGFR and induce neurite outgrowth. EncaminC and E promote neuronal survival and enhance presynaptic function. The sequence of EncaminA is actually an elongated (from the C-terminus by four amino acids) form of the FRM peptide (Table 3). Thus, the interaction between NCAM and FGFR probably involves multiple binding sites at an interface formed by the two FN3 modules of NCAM and FGFR. There are at least six distinct regions in the NCAM molecule involved in the binding, four sites in the first (dekaCAM, FRM/EncaminA, EncaminC and encaminE) and two sites in the second (FGL and BCL) FN3 modules. It has, however, to be determined whether these six sites interact with FGFR simultaneously or alternatively.

HBP

NCAM has the ability to bind the heparin sulfate proteoglycans (HSPG), thereby mediating cell adhesion [58]. An NCAM heparin-binding site has been shown to be localized in the second Ig module [12, 59]. A peptide encompassing the heparin binding site (HBP), termed the HBP, has been shown to promote neurite outgrowth in CGNs [60]. Thus, HBP can mimic NCAM functions through binding to HSPG.

Conclusions

During the last decade, a number of NCAM mimetics have been identified employing several approaches such as screening of combinatorial libraries of peptides, X-ray crystallography and NMR titration analyses of binding sites, and molecular modeling.

Some mimetics target various regions/modules of NCAM interfering with homophilic NCAM interaction and acting as antagonists and/or agonists. Other peptides target heterophilic NCAM ligands such as FGFR and HSPG. The NCAM-derived, FGFR-targeting peptides mimic NCAM functions entirely through the activation of FGFR, whereas some NCAM-targeting peptides also involve activation of FGFR-independent signaling pathways. A number of NCAM mimetics display neuritogenic, neuroprotective and synaptic plasticity-modulating properties in vitro and in vivo, making them attractive pharmacological tools and compounds for development of drugs, for the treatment of neurodegenerative disorders characterized by memory impairment.

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Synthetic NCAM-Derived Ligands of the Fibroblast Growth Factor Receptor

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Introduction

The neural cell adhesion molecule (NCAM) belongs to the immunoglobulin (Ig) superfamily of cell adhesion molecules and was first described in rat and human synaptosomal fractions more than three decades ago by Jørgensen and Bock [1]. Subsequently, NCAM has been demonstrated to be expressed in the brain in three major isoforms, NCAM-120, NCAM-140, and NCAM-180, named according to their apparent molecular weights.

Originally, NCAM was characterized as a glue that "stuck" cells together via homophilic (NCAM–NCAM) interactions between NCAM molecules expressed on opposing cells. However, extensive research over the past several years has challenged this view and revealed that NCAM is much more than a simple glue. NCAM is now considered as a signal transduction receptor with the ability to trigger intracellular signaling cascades and regulate cytoskeletal dynamics. NCAM exerts these functions partly through subplasmalemmal interactions with intracellular molecules such as spectrin [2–4], nonreceptor tyrosine kinase $p59^{fyn}$ [5], and growth-associated protein-43 (GAP-43) [6]. In addition, NCAM engages in extracellular interactions with a number of molecules, including cell adhesion molecules L1 and axonin-1 [7, 8], glial cell line-derived neurotrophic factor (GDNF) and its receptor GFRα [9], and, most notably, the fibroblast growth factor receptor (FGFR) [10] thereby regulating downstream signaling and cellular functions of these molecules. NCAM has the potential to regulate multiple processes in the nervous system and

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has profound effects on diverse cellular responses, such as proliferation of neuronal progenitor cells [11], neuronal survival [12, 13], migration [14], differentiation [15], synaptic plasticity [16–18], and memory formation. [19–22]. The involvement of NCAM in such diverse aspects of neuronal function makes this molecule and its interaction partners potential targets for pharmacological regulation in a number of diseases in which normal neuronal functions are disturbed. However, considering the complexity of NCAM interactions and downstream signaling and the resulting complexity of cellular responses, specifically mimicking or inhibiting a specific NCAM interaction may be necessary to modulate a specific NCAM-induced response. For example, the adhesive properties of NCAM may be important in some cellular events, but interactions with FGFR may be important for other events. Thus, to fully exploit NCAM as a pharmacological target, identification of important NCAM interactions and downstream signals for specific neuronal functions is required.

Although peptide fragments have occasionally been used previously to investigate NCAM function [e.g. see 23, 24], the use of peptides for NCAM research has truly accelerated over recent years [25]. At first, synthetic NCAM-binding peptides were identified by combinatorial chemistry [26, 27], but as the atomic structure of NCAM was more fully characterized, an alternative approach was developed, namely structure-based peptide design, to identify peptide fragments that would bind to NCAM or mimic NCAM functions [28]. With this approach, it became possible not only to identify peptides that would bind to NCAM, but also to synthesize peptides that could mimic NCAM interactions with other molecules. Accordingly, structure-based peptide design has greatly improved the investigation, understanding, and modulation of specific NCAM-mediated events. This review focuses on the development of a specific set of peptides, NCAM-derived FGFR agonists, that have been developed based on the structural analysis of NCAM and its interactions with FGFR and are hypothesized to mimic NCAM-FGFR interactions.

NCAM Interactions with FGFR

Early evidence suggesting that NCAM may regulate cellular events via a heterophilic interaction with a receptor tyrosine kinase came from a study showing that NCAM-induced differentiation of PC-12 cells is inhibited by the application of a protein tyrosine kinase inhibitor [29]. Subsequently, the kinase was identified as FGFR by a number of experiments demonstrating that NCAM-induced differentiation was inhibited by antibodies directed against FGFR [30, 31] and by expression of a dominant-negative, kinase-deleted FGFR [32]. Deeper insight into how signaling by the different NCAM isoforms depends on FGFR signal transduction was provided by Niethammer and colleagues, who demonstrated that pharmacological inhibition of FGFR inhibited NCAM-180 signal transduction, whereas NCAM-140 signal transduction was less affected, and NCAM-120 signaling was completely unaffected [33]. Thus, of the three major NCAM isoforms, NCAM-180-mediated signaling is most dependent on FGFR.

Structural Basis for the Interaction Between NCAM and FGFR

Although the aforementioned studies highlight the involvement of FGFR in NCAM-mediated signaling, they do not provide evidence for a direct interaction between NCAM and FGFR. All three major isoforms of NCAM have a similar extracellular domain structure consisting of five membrane-distal Ig modules (Ig1-5) and two membrane-proximal fibronectin type III (FN3) modules (FN3,1-2). This extracellular part of NCAM provides the structural basis for homophilic and many heterophilic interactions at the cell surface. The first suggestion of a direct interaction between NCAM and FGFR came from a study demonstrating that a construct corresponding to the two NCAM FN3 modules engaged in heterophilic interactions with an unknown interaction partner, suggested to be FGFR, on the cell surface [34]. Furthermore, although not tested experimentally, another group suggested that several cell adhesion molecules, including NCAM, may interact with FGFR via a putative FGFR activation motif (FRM, previously termed as the CAM homology domain [CHD] binding motif). FRM has been identified in NCAM, as well as in other CAMs, and is speculated to bind to the CHD motif in FGFR [35].

Four major transmembrane isoforms of FGFR tyrosine kinases have been identified in the brain (termed FGFR1-4) that have a prototypic extracellular domain structure consisting of three Ig domains (Ig1-3) with a long linker region between Ig1 and Ig2 [36]. The CHD is localized in the second β-strand in FGFR Ig2 [37]. In NCAM, the FRM motif is localized to the FN3,1 module, and NCAM was suggested to interact directly via this motif with the CHD in FGFR [35]. However, side chains of histidine and valine in the FGFR CHD sequence were shown later by crystallography not to be available for ligand interactions [37]. Moreover, co-immunoprecipitation studies showed that NCAM continues to co-immunoprecipitate with FGFR1 without the CHD motif [38], suggesting that this sequence in FGFR is not essential for NCAM binding to FGFR.

In 2003, a direct interaction between NCAM and FGFR was demonstrated [10]. Both NCAM FN3,1 and FN3,2 were shown by surface plasmon resonance (SPR) to be involved in binding to an FGFR1 Ig2–Ig3 construct. Further SPR analysis of direct NCAM interactions with FGFR, demonstrated that a construct of the two FN3 modules also interact with an Ig2–Ig3 construct from FGFR2 [39], suggesting that NCAM interacts with several FGFR isoforms via the FN3 modules. The FN3 module is composed of seven antiparallel β-strands (termed A-G) arranged in a sandwich of two β-sheets [40] (see Fig. 1). More detailed studies of the NCAM-FGFR1 interaction with the NCAM FN3,2 module and its interaction with the FGFR1 Ig2–Ig3 construct by nuclear magnetic resonance (NMR) titration analysis revealed that the FGFR binding site in the NCAM FN3,2 module involves a sequence corresponding to part of the F and G β-strands and the connecting loop [10]. Thus, evidence so far suggests that the NCAM FN3 modules engage in FGFR interactions via an interaction with the FGFR Ig2-Ig3 domains. Notably, these two FGFR Ig domains also are the site of interaction with FGF ligands and heparin, but evidence about whether the exact binding sites of NCAM and FGF overlap is not

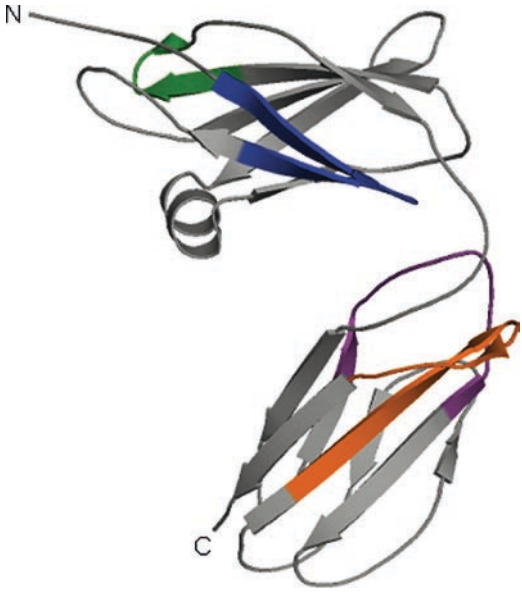

Fig. 1 *Peptide location in backbone models of the first and second NCAM FN3 modules.* The model was developed based on the experimental structure obtained by X-ray crystallography (Human NCAM FN3,1 and FN3,2: [69]). FN3,1 is likely at an 80–120° angle to FN3,2 and may rotate around the axis relative to FN3,2. Each fibronectin module contains six strand-loop-strand regions. The colored areas demarcate the strand-loop-strand motifs from which the peptides have been synthesized: dekaCAM (*green*), FRM (*blue*), FGL (*orange*), and BCL (*magenta*)

yet available. Interestingly, deletion of the acidic box that is localized in the linker region between FGFR Ig1 and Ig2 abolished NCAM co-immunoprecipitation with FGFR, indicating that this region of FGFR may modulate NCAM-FGFR interactions [38].

On the basis of the available structural information on NCAM-FGFR interactions, a number of peptides (Fig. 1) have been generated from sequences in the two NCAM FN3 modules and have been used as pharmacological tools to study the possible involvement of FGFR in a variety of NCAM-mediated cellular events.

FGL, a Synthetic FGFR-Ligand Derived from the Second NCAM FN3 Module

The 15 amino acid-long FG loop (FGL) peptide was the first peptide to be synthesized based on structural analysis of the interaction of the NCAM FN3,2 module with FGFR. NMR experiments by Kiselyov and colleagues [10] suggested that, within the second NCAM FN3 module, a sequence corresponding to the F and G

β-strands and the connecting loop interacts specifically with FGFR Ig2-Ig3. On the basis of these findings, investigations commenced to determine whether a synthetic peptide spanning these amino acid residues also would bind to FGFR. The peptide (termed FGL because it spans the F and G strands and the interconnecting loop; Fig. 1) was first produced in a dendrimeric version in which four peptide monomers are linked together via their C-terminal to a three-lysine backbone. This peptide composition allows one dendrimer to bind several FGFRs, whereby dimerization/oligomerization and subsequent activation of FGFR could occur. The dendrimeric FGL peptide was shown by SPR analysis to bind to a recombinant protein comprising the FGFR1 Ig2-Ig3 domains. Furthermore, subsequent studies showed that the binding induced full-length FGFR1 activation [10]. Interestingly, a monomeric version of FGL also induced FGFR1 activation, although with lower potency than the dendrimeric version [10], suggesting that the monomeric version also is capable of inducing dimerization and autophosphorylation of FGFR.

FGL-Induced Intracellular Signaling

Autophosphorylation of tyrosine residues in the FGFR intracellular domain results in the activation of a number of different intracellular signaling pathways [41]. One of the most well-documented signal transduction molecules linked to FGFR signaling is the FGF receptor substrate 2 (FRS2) [41, 42]. FRS2 is constitutively bound to FGFR, but its activity likely depends on Tyr653 and Tyr654 phosphorylation in the activation loop of the FGFR kinase domain [43]. Activation of FRS2 that is bound to FGFR may induce activation of a number of signaling cascades, including the mitogen-activated protein (MAP) kinase and phosphoinositide-3 (PI3) kinase pathways [44, 45]. Accordingly, FGL has been demonstrated in cerebellar neurons to induce FGFR-dependent activation of the MAP kinases, extracellular regulated kinase 1/2 (Erk1/2), as well as transient activation of Akt, a serine/threonine kinase acting downstream of PI3 kinase [46].

A putative target for activated Akt is the glycogen synthase kinase 3β (GSK3β) [47, 48] and Akt-induced GSK3β phosphorylation at serine 9 has been demonstrated to inhibit GSK3β activity. FGL has been shown to inhibit GSK3β activity in the cingulate cortex and since FGL activates Akt, FGL has been hypothesized to exert its effect on GSK3β via this pathway [49]. Activated GSK3β phosphorylates the microtubule-associated Tau protein, and Tau protein hyperphosphorylation and subsequent microtubule destabilization have been implicated in Alzheimer's disease [50]. In this regard, it is interesting that FGL not only inhibits GSK3β activity, but also prevents Tau hyperphosphorylation in vivo, suggesting that FGL may be a potential therapeutic agent for Alzheimer's disease [49].

Finally, FGL has been demonstrated to modulate caspase-3 activity, a protease that is best known for its involvement in apoptosis. Surprisingly, caspase-3 activity in cerebellar granule neurons is upregulated by FGL [46]. Exactly how this increased caspase-3 activity is induced by FGL remains unknown.

Cellular Responses to FGL In Vitro

The cellular response to FGL has been addressed in a number of different experimental conditions. One of the well-documented cellular effects of NCAM-induced FGFR activation is the induction of neuronal differentiation in various types of neurons and assuming that FGL mimics the interaction of NCAM with FGFR, neuronal differentiation induced by FGL is a reasonable expectation. In accordance with this assumption, FGL in various versions (monomeric, dimeric, dendrimeric) has been demonstrated to induce differentiation of dopaminergic, hippocampal, and cerebellar granule neurons [10, 46]. This stimulation of neuronal differentiation was shown to be FGFR-dependent as antibodies directed against FGFR, pharmacological inhibition of FGFR, and transfection with a dominant negative FGFR inhibited FGL-induced differentiation [10, 46].

Both NCAM and FGFR have been shown to have survival-promoting effects on neurons [13, 51], and the interaction between the two molecules could be important for this induction of survival [13]. In support of this hypothesis, FGL has been shown to promote survival of dopaminergic, hippocampal, and cerebellar granule neurons [46]. In cultures of dopaminergic neurons, FGL rescues dopaminergic neurons induced to undergo apoptosis by treatment with the neurotoxin 6-hydroxydopamine [46]. Hippocampal neurons induced to undergo cell death by application of the highly toxic β-amyloid peptide or by oxygen-glucose deprivation are likewise partially rescued by FGL [46, 52] and in oxygen-glucose deprived hippocampal slices, the survival-promoting effect of FGL is abolished by concomitant pharmacological inhibition of FGFR [52]. In cerebellar granule neurons induced to undergo cell death by deprivation of potassium, FGL acts as a neuroprotectant and in this cell type, the neuroprotective effect of FGL was correlated with its ability to prevent DNA fragmentation [46].

Interestingly, as mentioned earlier, FGL treatment was reported to induce caspase-3 activity in these cerebellar granule neurons induced to undergo cell death. Caspase-3 activity normally is considered as a pro-apoptotic signal; therefore, increased activity of this caspase induced by FGL does not intuitively suggest a neuroprotective role of FGL. However, cell fate appears to be regulated largely by a balance between pro- and anti-survival factors [53, 54]. FGL also induces activation of Erk1/2 and Akt, both of which have been suggested to be pro-survival molecules [55]. In addition, activity of the upstream activators of Erk1/2 and Akt, MAP/Erk kinase (MEK) and PI3 kinase, respectively, is necessary for the FGL-induced survival-promoting effect, suggesting that FGL-induced survival is mediated by MAP kinase and PI3 kinase pathways. Accordingly, the FGL-induced neuroprotective effect possibly depends on a shift in the balance between pro- and anti-apoptotic pathways. The activity of MAP kinase and PI3 kinase pathways promotes survival and overcome caspase-3-induced apoptotic signaling.

Postsynaptically expressed NCAM favors synapse formation, and neurons from NCAM null mutant mice develop fewer synapses compared with wildtype neurons [17]. Furthermore, inhibition of FGFR signaling inhibits NCAM-dependent

synapse formation [17], suggesting that NCAM-induced synapse formation is dependent on NCAM-induced FGFR activation. In accordance with this observation, treatment of hippocampal neurons with FGL has been shown to result in an increased number of synapses [22]. Furthermore, several studies have implicated NCAM in long-term potentiation (LTP) [16, 56, 57], suggesting that NCAM also modulates the signal strength at a given synapse. One important feature of LTP is an increase in the probability of presynaptic release and a failure of presynaptic transmission at the neuromuscular junction has been observed in NCAM null mutant mice and NCAM-180 null mutant mice when the neuron is challenged with continued high stimulation [18, 58]. Interestingly, treatment of hippocampal neurons with FGL increases vesicle release from the presynaptic terminal, and this increase is inhibited by pharmacological inhibition of FGFR [22], suggesting that the modulation of synaptic plasticity by NCAM depends on FGFR activation and is mimicked by the FGL peptide.

Effects of FGL In Vivo

Synaptic plasticity is hypothesized to underlie learning and memory, and accumulating evidence suggests that NCAM is involved in several types of memory consolidation processes encompassing a broad spectrum of brain structures. In particular, NCAM homophilic binding and complex regulation of NCAM and polysialylated NCAM (PSA-NCAM) expression in specific time windows appear to be important for normal consolidation of different types of learning paradigms [20, 59–61]. More recently, FGFR also has been shown to be important for NCAM-mediated modulation of memory processes. Thus, FGL pretreatment in healthy rats before a social recognition memory test robustly prolonged short-term social memory from 1 h in untreated rats to 73 h in FGL-treated rats, suggesting that FGL has memory-enhancing effects [62]. In addition, FGL administration in β-amyloid peptide-treated rats reversed social memory deficits induced by β-amyloid [49]. However, whether this reversal was attributable to FGL's neuroprotective effect or to a specific memory-enhancing effect has yet to be determined.

Posttraining administration of FGL also has been demonstrated to have a long-term memory-enhancing effect in two hippocampus-dependent tests, spatial learning and context-related fear conditioning, as well as in a hippocampus-independent test, auditory cued fear conditioning [22], suggesting that FGL modulates memory processes that depend on different brain areas and most likely also different consolidation processes. With regard to hippocampus-dependent tasks, subcutaneous injections of FGL to aged rats recently has been demonstrated to induce structural changes in synapse and dendritic spine structures in the dentate gyrus, suggesting that structural modifications of synaptic contacts could underlie hippocampus-dependent learning [63]. Finally, FGL administration to newborn rats accelerated the maturation of motor coordination skills [62], but the cellular functions that are

influenced by FGL to obtain this acceleration are unknown, although the previously mentioned effect on synaptic changes could be involved.

In addition to the demonstration of an in vitro effect of FGL on neuronal survival, a neuroprotective role of FGL has been confirmed in two different in vivo models. Global brain ischemia, which ultimately results in neuronal death, can be induced by occlusion of the carotid arteries. Using this approach in the gerbil, pretreatment with FGL has been shown to result in increased survival of neurons in the CA1 area of the hippocampus 24 h after the induction of ischemia [52]. Neuronal cell death also is observed during the progression of Alzheimer's disease, and Alzheimer's-related cell death can be mimicked by injection of β-amyloid peptide into the brains of rodents [49]. Rats injected with β-amyloid peptide and treated with FGL either before or after β-amyloid peptide injection showed significantly decreased neuronal cell death, suggesting that FGL may be able to both prevent and reverse the neuropathological changes induced by β-amyloid peptide injection [49]. Increased activity of GSK3β is associated with the induction of apoptosis [64] and since FGL induced GSK3β phosphorylation at serine 9, thereby inhibiting activity of this kinase, it is possible that FGL may induce some of its neuroprotective effects by inhibition of this kinase, together with activation of the MAP kinase and PI3 kinase pathways. However, Klementiev and colleagues furthermore showed that FGL treatment decreases glial activation and it can not be excluded that the effect of FGL on the microglia response may also be responsible, at least in part, for the neuroprotective effect of FGL.

Altogether, evidence so far has suggested that the NCAM-derived peptide FGL acts as an FGFR ligand and stimulates neuronal differentiation, survival, and synaptic changes in vitro via activation of FGFR and FGFR-induced downstream signaling. In this regard, FGL can be considered as a mimetic of NCAM-mediated FGFR activation and many of its downstream cellular effects. In vivo, the FGL peptide enhances memory and prevents neuropathology in a rodent model of Alzheimer's disease, suggesting that FGL may be a therapeutic candidate for Alzheimer's disease. Interestingly, FGL administered subcutaneously or intranasally to rats has been detected in cerebrospinal fluid, suggesting that the peptide may penetrate the blood–brain barrier [62] and in humans, single intranasal doses of FGL are well tolerated and FGL is measurable in plasma for 4 h [65].

BCL, a Synthetic FGFR Ligand Derived from the Second NCAM FN3 Module

The FGL peptide is derived from a strand-loop-strand region in the FN3,2 module and as this module contains six such loop structures, it has been of interest to test the involvement of other strand-loop-strand FN3,2 regions in binding of NCAM to FGFR. Jacobsen et al. [66] therefore investigated whether additional loop regions in the NCAM FN3,2 module are able to bind and activate FGFR. Five peptides, ABL, BCL, CDL, DEL, and EFL, each encompassing the sequence of a specific

strand-loop-strand FN3,2 region of NCAM, were synthesized as dendrimers. Structurally, the BCL and FGL sequences are believed to be positioned at the interface between FN3,1 and FN3,2, whereas ABL, CDL, and EFL interface the NCAM transmembrane domain. The DEL loop region, in contrast, appears to protrude somewhat in the middle of the module (Fig. 1). When the five peptides derived from the five strand-loop-strand regions were tested for binding to a recombinant protein comprising the FGFR1 Ig2-3 domains, SPR analyzes showed that the BCL peptide was the only peptide that convincingly bound to the FGFR1 construct. Both the FGL and BCL sequences are located at the interface between FN3,1 and FN3,2 in the three-dimensional NCAM structure, suggesting that NCAM interacts with FGFR at this interface.

Further analysis of the bioactivity of BCL showed that BCL dose-dependently stimulated FGFR1 phosphorylation. Interestingly, FGF2-induced FGFR1 phosphorylation was inhibited by BCL at high concentrations, indicating that BCL might be a FGFR inverse agonist. Furthermore, BCL, similarly to FGL, induced FGFR-dependent neurite outgrowth in cerebellar granule neurons. However, some differences appear to exist in the kinetics of FGL and BCL-induced cellular events. Although BCL and FGL appear to have similar apparent dissociation constants [10, 66], BCL stimulates differentiation at high concentrations, whereas FGL stimulates differentiation only at considerably lower concentrations [46]. In contrast to FGL, BCL did not stimulate survival of cerebellar granule neurons [66]. In summary, although these two peptides both bind and activate FGFR, they induce somewhat different neuronal responses.

FRM, a Synthetic FGFR Ligand Derived from the First NCAM FN3 Module

When a direct interaction between NCAM and FGFR was first demonstrated [10], optimal binding was obtained when a protein construct containing both FN3,1 and FN3,2 was used, suggesting that both modules are involved in FGFR binding. Furthermore, the FRM motif, which was suggested to be a putative binding site for FGFR, is located in the FN3,1 module. A recent study by Anderson and colleagues [67] has further elucidated this possibility and provided support for an important role of FN3,1 in NCAM-FGFR interactions. The authors showed that antibodies directed against FN3,1 inhibited NCAM-induced neurite outgrowth in cerebellar granule neurons (a response that is known to be FGFR-dependent), suggesting that the steric availability of FN3,1 is necessary for NCAM-induced differentiation. Following this initial experiment, a peptide corresponding to the 13 amino acid-long FRM sequence in NCAM (Fig. 1) was synthesized and tested. Interestingly, this sequence, similarly to both FGL and BCL, encompasses a strand-loop-strand region in the fibronectin module. More specifically, the sequence maps to a site comprising part of the A β-strand, the A-B turn, and half of the B β-strand in the NCAM FN3,1 module [35, 67] and the FRM loop region recently has been shown

by crystallography to be localized to the interface between FN3,1 and FN3,2 [68, 69]. Although no direct interaction was demonstrated between the FRM peptide and FGFR, the peptide was shown to induce FGFR phosphorylation and activation of the downstream signaling molecule FRS2.

Furthermore, results from a number of cellular assays suggest that this peptide interacts with FGFR and stimulates FGFR-mediated cellular responses. Thus, high concentrations of a monomeric version of the FRM peptide stimulated neurite outgrowth in cerebellar granule neurons at levels comparable to the NCAM-induced response, suggesting that FRM mimics this NCAM- and FGFR-dependent cellular response. At low concentrations, however, the monomeric version of FRM specifically inhibited NCAM-induced neurite outgrowth. Furthermore, this inhibition was shown to apply to FGF2-induced differentiation, suggesting that the monomeric FRM peptide, similarly to BCL, can function as an FGFR inverse agonist. The neuritogenic activity of FRM was shown to be FGFR-dependent as FRM did not stimulate neurite outgrowth of cerebellar neurons from transgenic mice expressing a dominant-negative FGF receptor. Notably, a tetrameric form of FRM is 125-times more potent than the monomeric form of FRM with regard to its neuritogenic activity and cyclization also promoted FRM activity 50-fold, suggesting that stereo FRM structures are more effective than monomeric FRM structures in activating FGFR. Similarly to what was found for the FGL peptide, FRM at high concentrations was ineffective in stimulating neurite outgrowth, indicating that FRM and FGL stimulate neurite outgrowth with a bell shaped dose-response curve, similarly to FGF2 [70] and other growth factors.

Further studies demonstrated that FRM stimulated survival of fibroblasts, premyelinating oligodendrocytes, and cerebellar neurons equivalent to the effects of FGF2 and insulin. The neuroprotective effect of FRM was dependent on FGFR activation, demonstrated by the finding that neither FGF2 nor FRM could prevent cell death in FGFR-negative L6 cells, whereas FRM induced survival of FGFR1-transfected L6 cells. Interestingly, the peptide was shown to have no effect on cell proliferation [67], another FGFR-mediated event. This suggests that FRM induces only a specific subset of FGFR-mediated cellular responses, a conclusion that could have important implications for future development of FRM as a putative therapeutic agent.

DekaCAM, a Synthetic NCAM and FGF10-Derived FGFR Ligand

In a recent study, Li and colleagues [71] investigated whether the NCAM FN3,1 module and a number of FGFs share any homologous sequence motifs, hypothesizing that such shared sequences could be a common motif for FGF and NCAM binding to FGFR. Sequence alignment of the FN3,1 module and various FGFs resulted in the identification of a six-amino acid motif, ALNGKG, localized to the C-terminal sequence of the FN3,1 module encompassing parts of the F and G β-strands and the short connecting loop [68] (Fig. 1) that is identical to a six-amino

acid motif in the FGF10 β10-β11 region. On the basis of this finding, a peptide termed dekaCAM, encompassing the ALNGKG motif, was synthesized and shown by SPR analysis to bind to Ig2-Ig protein constructs of both FGFR1-IIIc and FGFR2-IIIb with an apparent K_D of about 0.1 μM, which is somewhat lower than that for FGL and BCL. Further analysis demonstrated that treatment with dekaCAM resulted in dose-dependent FGFR phosphorylation in FGFR1-transfected TREX cells and dose-dependent neurite outgrowth in cerebellar granule neurons. Interestingly, dekaCAM corresponds to a loop region of FN3,1 that interfaces the Ig5 module [68] rather than the FN3,1 and FN3,2 modules. However, further studies are needed to evaluate which aspects of NCAM and FGF10 function can be mimicked by dekaCAM.

Mechanism of FGFR Activation by the Peptides

A current model for NCAM-induced FGFR activation suggests that only NCAM in clusters activates FGFR. In cells without contact with other cells or the extracellular matrix, NCAM is hypothesized to exist in *cis* dimers, but these are scattered over the cell membrane, suggesting that contact is fairly unlikely between two NCAM *cis* dimers that are both bound to an FGFR (approximately one FGFR exists for every 1,000 NCAMs, and thus only a few NCAM dimers will bind to FGFR). In contrast, when cells form contact points, NCAM will engage in clusters of *trans* homophilic interactions, bringing into contact many *cis* dimers and the probability of NCAM-mediated FGFR dimerization thereby increases (for further explanation see [72]). Accordingly, a current view is that NCAM may only activate FGFR at contact points where NCAM is engaged in *trans* homophilic interactions. Importantly, these *trans* homophilic interactions are likely to be modulated by the attachment of PSA to the fifth NCAM Ig module, and a current hypothesis is that PSA attachment favors loose *trans* homophilic interactions that in turn favor NCAM-FGFR interactions.

Although the exact binding mechanisms of the peptides to FGFR have not yet been elucidated experimentally, at least two theories could explain how peptides interact with and activate FGFR. For the dendrimeric version of FGL, activation of FGFR may be induced by a clustering of FGFR molecules bound to each of the four peptide "arms" of the dendrimer. Thus, each peptide arm "catches" an FGFR molecule. Several FGFRs, therefore, would be placed in close proximity to one another, thereby enabling transphosphorylation of the FGFR intracellular tyrosine kinase domains. Although more speculative, a similar mechanism may apply to the monomeric version of the peptide. Because all of the peptides are derived from strand-loop-strand regions of the FN3 module, it is possible that on either side of the loop, a β-strand sequence may interact with FGFR. Accordingly, a monomeric peptide version of the loop region will contain two binding sites for FGFR, and each of these binding sites may interact with an FGFR molecule, thereby bringing two FGFRs into close proximity, thus resulting in dimerization and activation. The possibility that two FGFR molecules interact with one monomeric peptide is substantially

lower than the possibility of two FGFR molecules interacting with four peptide chains in the dendrimeric version, which to some extent explains the lower potency of monomeric peptides. Interestingly, cyclization of a peptide by adding a cysteine at either end appears to increase monomer potency. For example, cyclization of the FRM peptide resulted in a strikingly increased potency of the monomeric peptide [67], suggesting that the formation of a loop-like structure is important for optimal FGFR interaction and activation. However, because even very short versions of FGL and FRM appear to have bioactivity, peptide-mediated clustering of FGFR may not be the entire explanation for peptide-induced FGFR activation.

Ligands of the epidermal growth factor receptor (EGFR) family have been proposed to activate EGFRs via complex extracellular and intracellular conformational changes [73]. This so-called "rotational-twist" model proposes that, in the inactive form, EGFR exists in an autoinhibitory conformation where interactions between extracellular modules prevent the receptor from obtaining its active conformation. When a ligand binds to the receptor, it induces a large conformational change in the extracellular part of the receptor that, in turn, induces a rotation of the transmembrane domain with subsequent reorientation of the intracellular tyrosine kinase domains to form an active kinase conformation. Thus, in this model, the activation of the tyrosine kinase receptor by ligands depends largely on ligand-induced conformational changes in the receptor. An explanation for the ability of the monomeric version of peptides to induce FGFR activation likewise could be that the interaction between the peptide and FGFR induces conformational changes in FGFR, whereby dimerization of FGFR and activation of the tyrosine kinase domain are induced. The acidic box in the Ig1-2 linker region, as well as the Ig1 domain of FGFR, apparently exhibits an auto-inhibitory function on FGFR activation [74–76]. Accordingly, an FGFR3 construct, including the Ig1 domain, has been demonstrated to bind FGF1 or heparin with lower affinity than a construct excluding the Ig1 domain [74]. Furthermore, structural analysis has shown that the FGFR1 Ig1 domain binds to Ig2 at a site overlapping with both the FGF and heparin binding sites, as well as the contact site for Ig2–Ig2 interactions, suggesting that Ig1 regulates both FGFR1-ligand binding and spontaneous FGFR1 dimerization [75]. Thus, NCAM-derived FGFR agonists, upon binding to FGFR1, possibly induce conformational changes that result in relief of autoinhibition by Ig1 and subsequent FGFR spontaneous dimerization and activation.

Different NCAM-Derived FGFR Ligands Induce Differential Responses

Regardless of the mode of binding, the NCAM-derived FGFR agonists FGL, FRM, BCL, and dekaCAM all appear to stimulate FGFR phosphorylation, downstream signaling, and cellular responses. Importantly, although many similarities in the cellular response to different NCAM-derived FGFR agonists exist, some significant differences also are apparent. For example, BCL, in contrast to FGL and FRM, does

not modulate survival of neurons, but rather stimulates differentiation at high concentrations, which does not appear to be true for FGL or FRM. Although these differences need to be evaluated further before any conclusions can be made, it could be speculated that different peptide ligands of FGFR may induce differential responses. One explanation may be that different peptides preferentially bind different FGFR isoforms. However, the differences also may be attributable to different binding kinetics of the peptides to FGFR. In FGFR signaling, activation of different signaling cascades depends on phosphorylation of different tyrosines in the FGFR kinase domain. A recent study that employed chemical quench and mass spectrometry showed that at least five of the seven tyrosine phosphorylation sites in FGFR are both sequentially and temporally phosphorylated [43]. After exposing the kinase domain of FGFR to ATP for various times, a clear pattern was observed in which tyrosine 653 (Y653) in the activation loop was first phosphorylated, followed by Y583, Y463, Y585, and Y654, the second tyrosine of the activation loop [72]. Therefore, the biological outcome of FGFR activation likely depends on the kinetics of the binding. Strong ligand binding, lasting for longer periods of time, induces phosphorylation of all tyrosines and thereby activates several downstream molecules, in contrast to weak binding that induces phosphorylation of only a subset of tyrosines and, therefore, activates fewer downstream molecules. In this regard, it is notable that Carafoli and colleagues [69] recently demonstrated that the hinge region between the two NCAM FN3 modules may allow considerable flexibility, resulting in different orientations of the two FN3 modules with each other. The two loop regions comprising the FRM and FGL sequences were shown in one crystal of tandem FN3 modules to be positioned in close proximity to one another, particularly due to a bend conformation, suggesting that these two NCAM loops likely interact with FGFR simultaneously. However, mutation of one single amino acid residue in FN3,2 opened the closed conformation and resulted in a twist of the FN3,1 module relative to the FN3,2 module, suggesting that the hinged region allows for considerable flexibility. Interestingly, this latter conformation positioned the FGL and FRM loops further apart, making it less likely that these two loops interact with FGFR simultaneously. Thus, in this conformation, only one of the loops may interact with FGFR. Conversely, the FGL or FRM loops may be positioned such that they interact with FGFR in concert with yet another loop from the opposing FN3 module. The hinge region is subject to alternative splicing, and Carafoli and colleagues suggested that different NCAM splice variants and/or subjecting NCAM to different biomechanical forces (e.g. different *trans*-homophilic interactions) may yield different FN3,1-FN3,2 conformations. This, in turn, could modulate the interaction of NCAM with FGFR, i.e., different loops will be available for NCAM interaction with FGFR, perhaps ultimately resulting in different cellular responses. Thus, the difference observed with the different peptides may simply reflect the fact that NCAM interactions with FGFR differs depending on the NCAM splice variant and the biomechanical forces that NCAM is subjected to.

Although the NCAM-derived FGFR ligands all induce cellular responses that also have been attributed to NCAM, the peptides may induce effects that do not reflect the physiological functions of NCAM because NCAM in similar circumstances may not

induce FGFR dimerization. For example, various FGFs have higher affinity for FGFR than NCAM, and in situations where FGF is upregulated, NCAM may not interact with FGFR, although peptides might. Furthermore, when NCAM is engaged in tight homophilic interactions in the absence of PSA attachment to NCAM, or when NCAM is scattered over the cell membrane, NCAM unlikely induces FGFR dimerization and activation [72]. However, these differences between the actions of the peptides and physiological NCAM may have an advantage with regard to developing NCAM mimetics as therapeutic agents. For example, PSA attachment to NCAM is speculated to be a prerequisite for NCAM-FGFR interactions. A number of studies have demonstrated that modulation of PSA expression on NCAM is involved in several types of neuronal plasticity, including those involved in learning and memory [20, 21, 77], thus suggesting a link between PSA, NCAM-FGFR interactions, and neuronal plasticity. In cases where the PSA expression pattern on NCAM does not favor an NCAM-FGFR interaction, the peptides still may mimic an NCAM-FGFR interaction and modulate (at least some) aspects of neuronal plasticity.

Conclusions and Future Directions

Both the first and second NCAM FN3 modules have been demonstrated by means of SPR and biological activity analyses to be important for NCAM activation of FGFR. Four sequence motifs involved in NCAM interactions with FGFR have been identified. Two (FRM and dekaCAM) are localized to the first NCAM FN3 module, and two (FGL and BCL) are localized to the second NCAM FN3 module. It would be interesting in future studies to investigate whether other loop regions in the NCAM FN3 modules are involved in NCAM-FGFR interactions and determine how (or if) the various sites activate the receptor in concert. In addition, future research should address the question about whether the diversity of binding sites reflects differences in the ability of different sites to induce different signaling cascades with subsequently different cellular responses.

NCAM-derived FGFR ligands may be considered as valuable new pharmacological tools for investigating the role of NCAM-FGFR interactions in NCAM function and modulating NCAM-mediated events via FGFR. Furthermore, considering the involvement of FGFR in a number of pathological conditions and its role in repair processes following neuronal degeneration NCAM-derived FGFR ligands may have significant therapeutic potential.

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Dendritic Spine and Synapse Morphological Alterations Induced by a Neural Cell Adhesion Molecule Mimetic

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Introduction

The neural cell adhesion molecule (NCAM) is a membrane-associated glycoprotein expressed on the surface of neurons and glial cells and is also a member of the immunoglobin (Ig) superfamily [1, 2]. It promotes synaptogenesis, enhances presynaptic function, and facilitates synaptic plasticity in relation to learning and memory consolidation [3, 4]. This short review will focus on evidence of the role of NCAMs in synaptic plasticity and learning, and the ability of an NCAM mimetic to influence morphological changes in dendritic spines and synapses.

NCAM Involvement in Synapse Formation

Persuasive evidence for the involvement of NCAM in synapse formation comes from the work of Dityatev et al. [5] which examined the mechanism of synaptogenic activity of NCAM and focused on the role of polysialic acid (PSA), which is a carbohydrate preferentially associated with NCAM. They demonstrated that enzymatic removal of PSA with endoneuraminidase-N (endo-N) abolished preferential formation of synapses on NCAM-expressing cells in heterogenotypic cocultures of wild-type and NCAM-deficient hippocampal neurons. It was shown that enzymatic removal of heparan sulphates from cultured neurons and a mutation in the heparin-binding domain of NCAM reduced synaptogenic activity of neuronally expressed PSA-NCAM. This led to the suggestion that neuronal PSA-NCAM in complex with heparan sulphate proteoglycans promotes synaptogenesis and

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therefore activity-dependent remodelling of synapses. NCAMs also have a role in the determination of synaptic form because differential expression of neural cell adhesion molecules leads to morphologically different excitatory glutamatergic synapses in the "trisynaptic circuit" in the adult rodent [2].

NCAM Involvement in Memory

It has been more difficult to visualise the direct involvement of NCAM in memory formation process at synaptic level, but immunocytochemical labelling in the electron microscope has been used to demonstrate NCAM involvement in memory formation processes. Skibo et al. [6] showed that an increased immunogold labelling of neural cell adhesion molecule isoforms occurred in synaptic active zones of the chick striatum 5–6 h after one-trial passive avoidance training, suggesting that its impact was more at the edges of the active zone. Moreover, in one of the best models of synaptic plasticity, hippocampal long-term potentiation (LTP) where the mechanisms may underlie or be similar to processes involved in memory formation [7], there is an increase in the proportion of hippocampal spine synapses expressing the neural cell adhesion molecule NCAM180 following LTP [18]. More evidence of the importance of NCAM in synaptic plasticity comes from the use of genetically modified mice, deficient in NCAM, which are impaired in LTP induced by theta-burst stimulation, suggesting that LTP in the dentate gyrus depends on the NCAM glycoprotein [9].

What Is the Mode of Action of NCAM at the Cellular/Synaptic Level?

A clue as to the mode of action of NCAM comes from the finding that it mediates cell–cell adhesion because the extracellular domain of NCAM engages in homophilic interactions (NCAM binding to NCAM) and in heterophilic interactions between NCAM and proteins such as the fibroblast growth factor receptor (FGFR), and the neuronal cell adhesion molecule L1 [10, 11]. Of particular note is the finding that an NCAM-derived FGF-receptor agonist, the FGL-peptide, induces neurite outgrowth and neuronal survival in primary rat neurons [12]. When the FGL-peptide is administered intranasally to newborn rats, it accelerates early postnatal development of coordination skills and in adult animals s.c. administration of FGL results in prolonged retention of social memory. It appears that FGL rapidly penetrates into the blood and cerebrospinal fluid after both intranasal and s.c. administration and remains detectable in the fluids for up to 5 h [13]. The FGL-peptide also reduces neuropathological signs and cognitive impairment induced by $A\beta_{25\text{-}35}$ [14].

How Does NCAM Influence Synaptic and Dendritic Morphology?

Aged Rats

Until recently, little was known about how exactly NCAM mediates its effect on the synaptic and neural structure, and indeed whether it exerts an effect on the existing structures or via synthesis of synaptic and dendritic structures de novo. One approach to this issue has been to examine the effect of the remarkable FGL peptide on synaptic and dendritic structure in the hippocampus of rats using transmission electron microscopy and three-dimensional (3-D) reconstruction of serial ultrathin sections. Rats were used at an age when it is known that they suffer from cognitive deficits, and deficits in measured physiological parameters such as the ability to maintain LTP in perforant path granule cell synapses in the hippocampus LTP as a result of tetanic stimulation [15–17]. Aged rats (22 months old) were injected subcutaneously (8 mg kg^{-1}) at 2-day intervals until 19 days after the experiment start. Animals were perfused with fixative, brains removed and coronal sections cut at 50 μm. Full details of the protocols are given in Popov et al. [18]. It was noted that hippocampal volume was not significantly affected by treatment with FGL, hence any morphometric measures of synaptic or dendritic parameters would not be affected by gross hippocampal volume changes. Tissue from the dentate gyrus was embedded, and ultra-thin sections viewed in a JEOL 1010 electron microscope.

Analyses were made of synaptic and dendritic parameters following three-dimensional (3-D) reconstruction of images from a series of serial ultrathin sections. Examples of these reconstructions are shown in Fig. 1: the dendritic branches (Fig. 1A) have two major spine types, mushroom and thin spines, on which the majority of synapses occur (~90%), whilst synapses on stubby spines and synapses directly on dendritic shafts together comprise ~10% of the total. A mushroom spine is shown in Fig. 1Ba. These large spines are contacted by synapses and a complex perforated postsynaptic density (PSD) at the spine head is shown in Fig. 1Bb. There were no significant differences in synapse or spine density following treatment with FGL-peptide.

Spine Volume, and Percentage Distribution of Synapse on Spine Types

There were also no significant differences in the volume of thin and mushroom spines measured before and after FGL treatment. However, there was a marked difference in the distribution of synapses on the different spine types as shown in Table 1 which summarises the morphometric parameters for spines and synapses following FGL treatment of the aged rats. There is a significant increase in the percentages of mushroom spine synapses and a concomitant decrease in thin spine synapse, with small changes in stubby spines and synapses directly on shafts.

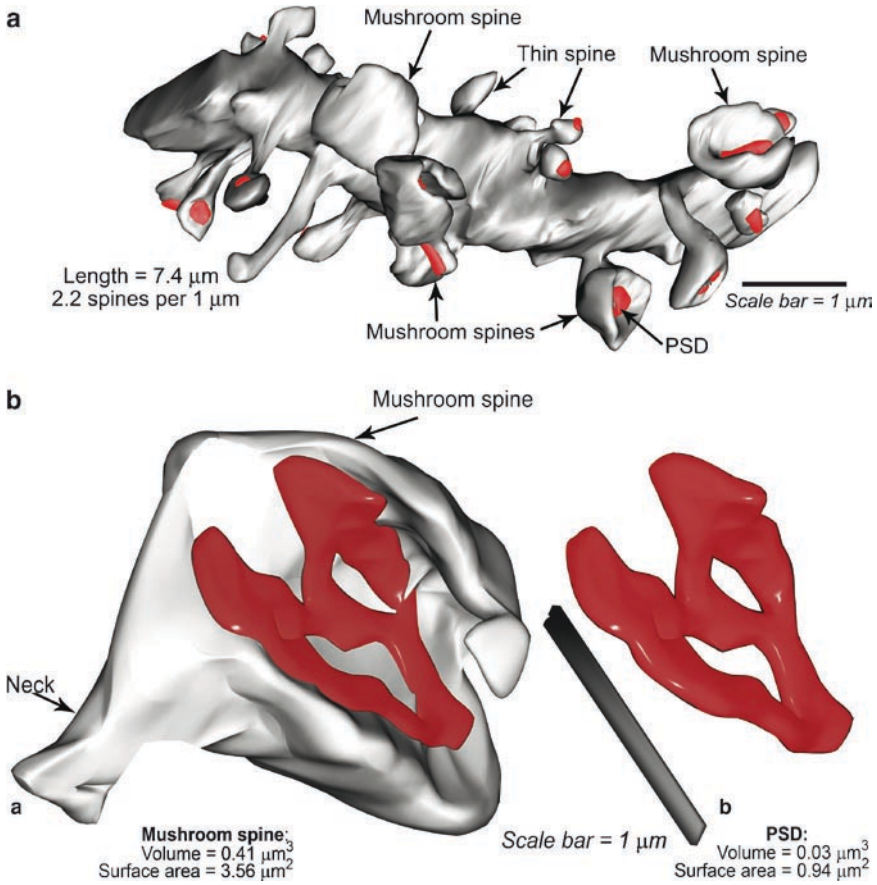

Fig. 1 (a) Three-dimensional (3-D) reconstruction of images from a series of ~100 serial ultrathin (60 µm) sections taken in the middle molecular layer of the dentate of the aged rat hippocampus. There are two major spine types, mushroom and thin spines, protruding from the dendrite and it is on these spines that the majority of synapses occur (~90%), while synapses on stubby spines and synapses directly on dendritic shafts together comprise ~10% of the total. The PSD-post-synaptic densities on thin and mushroom spines are red in colour. (b) A mushroom spine is shown in 3-D (ba). This large spine is contacted by a synapse with a complex perforated post-synaptic density (PSD) in red at the spine head, as shown in (bb). There are no significant differences in synapse or spine density following treatment with FGL-peptide; however, the proportion of mush-room spines increases and that of thin spines decrease after FGL treatment, while spine head curvature also changes (see Table 1)

Curvature Changes

Spine head curvature is taken as an ultra structural estimation of functional activity in dendritic spines or synapses [19–21]. Curvature was measured in the FGL-peptide study using a novel method to determine curvature in three-dimensions.

Table 1 Summary of three key measures of morphometric parameters for spines and synapses in dentate gyrus of aged rats: either control, or after treatment with the peptide FGL which is an NCAM-derived FGF-receptor agonist. (n = 5 animals per group)

	FGL	Control	Significance (p)
Percentage of synapses on spine or shaft[a]			
Mushroom spine	15	10	<0.001
Thin spine	75	83	<0.0001
Stubby	4	6	<0.003
Shaft	6	3	<0.035
Curvature of apposition zone between presynaptic bouton and mushroom spines[b]	41.47	20.91	$<10^{-5}$
Curvature of PSDs on mushroom spines	19.15	14.45	<0.004
Curvature of apposition zone between presynaptic bouton and thin spines	–4.86	–7.81	<0.02
Curvature of PSDs on thin spines	1.57	1.02	n.s.
Volume of multivesicular bodies (μm^3)[c]	0.02	0.01	<0.01
Surface area of multivesicular bodies (μm^2)	0.30	0.19	<0.001

[a]Percentage of synapses on spines or shaft

[b]Curvature changes measured using a 3-D approach (see Popov et al. [8]) – a negative value represents a convex shape and a positive value represents a concave shape

[c]Effect of FGL on volume and surface area of multivesicular bodies (MVB)/endosomes. Significance obtained via ANOVA followed by Tukeys Unequal N Honest Significant Differences tests; (n.s. not significant)

This was because 2-D estimators from sections of ~60 nm are likely to give only a snapshot of curvature at the synaptic junction which is actually ~350 nm in diameter. Nonetheless, the nomenclature of earlier studies was employed: a synapse whose presynaptic element protrudes into the postsynaptic element is referred to as a concave synapse, and a synapse whose postsynaptic element protrudes into the presynaptic element is classified as convex. Curvature measurements are given in Table 1 and the details of the methodology are provided in Popov et al. [18]. For synapses on mushroom spines following FGL treatment spines, there is a significant decrease in concavity of both the PSD and the apposition zone whilst for thin spines the PSDs exhibit little curvature, but the apposition zone increases in convexity after FGL treatment

Volume and Surface Area of Endososmes/Multivesicular Bodies

Endosomes play a key role in sorting membrane proteins for degradation or recycling [22]. They are believed to be major sorting stations in the endocytotic process, sending proteins and lipids to multiple destinations including the cell surface, Golgi complex, and lysosomes [23, 24], which have described protein trafficking to and from the postsynaptic membrane as a key mechanism underlying various forms of synaptic plasticity. AMPAR sorting occurs in early endosomes and is regulated by

synaptic activity and activation of AMPA and NMDA receptors [25]. Following treatment with FGL-peptide (Fig. 2a–l), MVBs were notably more frequent in dendrites (and spines) of the dentate gyrus and coated vesicles were also more notable in the vicinity of the synaptic membranes (Fig. 3a–c). When the MVBs are quantified (Table 1), their volume and surface area was significantly greater in FGL treated animals.

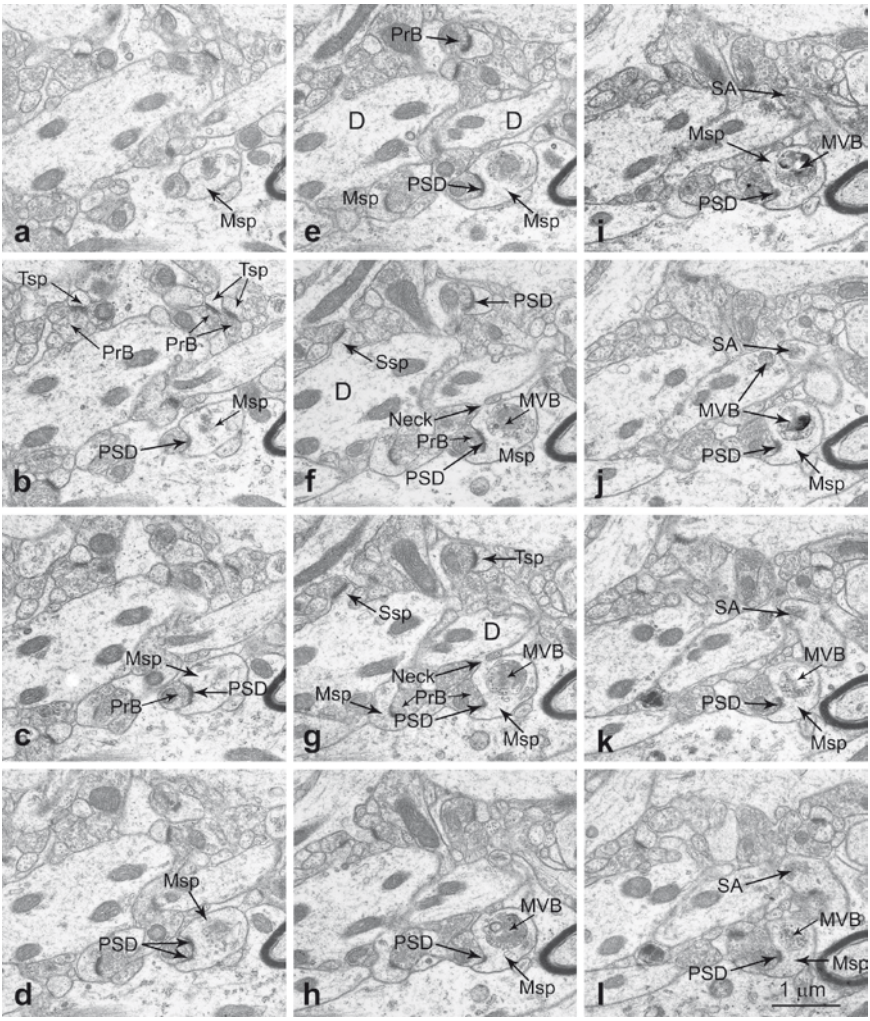

Fig. 2 Following treatment with FGL-peptide (**a–l**), MVBs (multivesicular bodies) are notably more frequent in dendrites (and spines) of the dentate gyrus as can be seen in (**f–k**), of the series in the figure. Abbreviations of other items indicated by arrows: Msp, mushroom spine; Tsp, thin spine; Ssp, stubby spine; PrB, pre-synaptic bouton; PSD, post synaptic density; SA, spine apparatus; MVB, multivesicular bodi; D, dendrite; Neck, spine neck(stalk). When the MVBs are quantified (Table 1), their volume and surface area is significantly greater in FGL treated animals

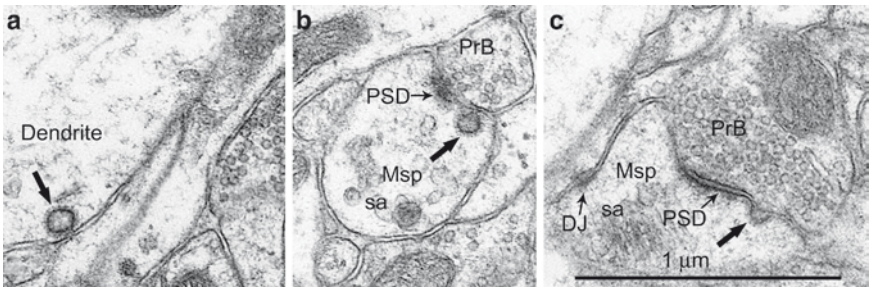

Fig. 3 Coated vesicles (*arrows*) are more notable in the vicinity of the synaptic membranes (**a**–**c**) after treatment with the NCAM mimetic, FGL-peptide. The three micrographs (**a**–**c**) show coated vesicles in different stages of budding from the PSD; in (**a**), a complete coated vesicle is visible, whereas in (**b**) the vesicle is more attached to the PSD but in (**c**) the vesicle is just beginning to bud off from the PSD. Abbreviations: Msp, mushroom spine; PSD, post-synaptic density; PrB, pre-synaptic bouton; sa, spine apparatus; DJ, dense junction

Does FGL Exert a Similar Effect on Younger Animals?

Studies similar to those described for aged rats have not yet been performed on younger rats. However, an interesting approach has been to examine the effect of FGL-peptide on dendritic structure in rat hippocampus following treatment with amyloid β-25-35 [26]. This is known to generate neuropathological signs related to those of early stages of Alzheimer's disease (AD) and results in the deposition of endogenously produced amyloid protein [14] whilst quantitative immunohistochemistry showed a variety of increasing of neuropathology including increases in amyloid immunoreactivity, tau phosphorylation, microglial activation, and astrocytosis. Moreover, neuronal cell death and brain atrophy increased in response to $A\beta_{25\text{-}35}$ together with impaired short-term memory as determined by a social recognition test. However, the peptide termed FGL peptide alleviated the $A\beta_{25\text{-}35}$-induced neuropathology and cognitive impairment [14]. When Golgi impregnated CA1 pyramidal neurons (Fig. 4) were examined following similar $A\beta_{25\text{-}35}$ treatment of rats, preliminary data showed that there are no differences in dendritic diameters between B-amyloid treated and control/FGL groups. However, in Aβ injected rats spine densities were significantly lower ~ 14% than in untreated rats ($p < 0.05$). Moreover, the morphology of dendritic spines differed between groups. Treatment with FGL-peptide alleviates this reduction and so would potentially help maintain cognitive function [26].

The Mechanism of NCAM Action at the Synaptic Level?

On the basis of the very marked effects of an NCAM in synaptic plasticity and development as reviewed above [2, 8, 12], combined with evidence of the influence of the NCAM mimetic – FGL peptide, on synaptic and dendritic structure in aged rats [18], and its apparent effect on dendritic spines in younger animals [26], in

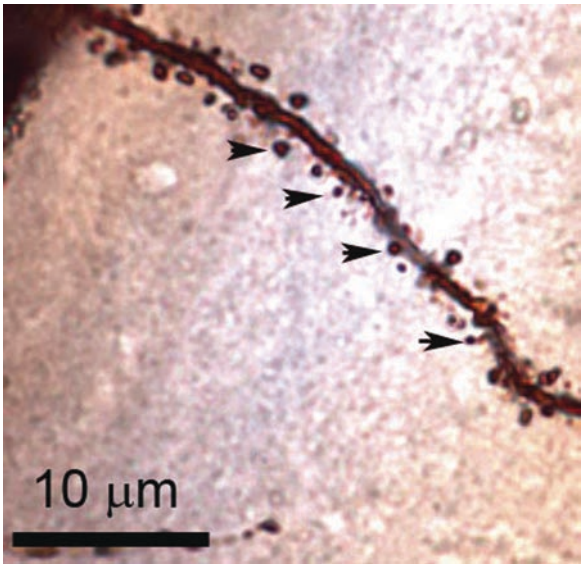

Fig. 4 Golgi impregnated CA1 pyramidal neuron material from rat hippocampus with a dendritic segment showing here. Four of the many twig like spines extending from the dendrite stem are indicated by four arrows. Preliminary evidence suggests that spine density is reduced by β-amyloid treatment of rats but that the NCAM mimetic FGL-peptide, alleviates this reduction

alleviating the neuropathlogical effects of β-amyloid, it is clear that NCAMs have a great influence on morphology. Although there do not appear to changes in synapse or spine density following FGL-peptide use, at least in older rats, there are changes in both structure of spines and their PSDs, most significantly alterations occur in the proportion of the large mushroom spines in the middle molecular layer of the dentate with a concomitant decrease in the smaller sized thin spine population. This would suggest that FGL treatment stimulates transition of thin spines into mushroom spines as may occur during learning or physiological plasticity [27, 28]. Since most mushroom-shaped spines have perforated synapses [29], which have more AMPA and NMDA receptors than those on the smaller thin spines [30, 31], the increased proportion of mushroom-shaped spines suggests an increase in the efficacy of synapses generating action potentials in the soma/axon [32]. As we have argued [18], this presumed increase in postsynaptic efficacy in FGL-treated rats may be amplified by the enhanced presynaptic function reported previously [3], and therefore contribute to the cognitive benefits of FGL treatment in young adult rats.

Multivesicular Bodies and Clathrin Coated Pits

One of the most interesting findings from an ultra structural analyses of the effects of the FGL-peptide on dendrites and spines, is an increase in multivesicular bodies (MVBs) following FGL treatment and an increase in clathrin-coated pits on the

postsynaptic membrane of mushroom spines (see Fig. 3) – these play a key role in endocytosis [33] and we have previously observed an increase in the number of MVBs in thorny excrescences in CA3 following water maze training and a decrease following stress [34]. Since FGL enhances spatial learning and synaptic plasticity [3], it seems reasonable to believe that the altered receptor function may play a role in its mode of action. In this connection, the role of endosomes in endocytotic processes and receptor recycling would seem to be likely [22]. Coated pits in mushroom spines after FGL treatment indicate high levels of endocytotic processes on postsynaptic membranes and a result of such endocytosis would be a decrease in volume and surface area after FGL treatment.

In summary, NCAMs are intimately involved in synaptic plasticity and development in a variety of situations and model systems, and would appear to exert considerable influences on neural morphology. Convincing evidence that this is so is provided by ultrastructural studies following treatment of rats with an NCAM mimetic (FGL-peptide), in which synapses and spines were studied in 3-D at the electron microscope level. This shows that increases occur in the ratio of mushroom to thin spines (mushroom spines increase in stimulated physiological plasticity), numbers of MVBs, and also the frequency of appearance of coated pits. Three-Dimensional analysis also show a significant decrease in both PSD and apposition zone curvature of mushroom spines following FGL treatment, whilst for thin spines, convexity of the apposition zone increases. These data indicate that the NCAM mimetic used induces large changes in the fine structure of synapses and dendritic spines in hippocampus of aged rats, complementing behavioural data showing its effect on cognitive processes.

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Part VIII
Orthologs and Paralogs of NCAM

Fasciclin II: The NCAM Ortholog in *Drosophila melanogaster*

Lars V. Kristiansen and Michael Hortsch

Introduction

Fundamental principles of nervous system development, including the molecular mechanisms that regulate neuronal differentiation and axon guidance, have been largely conserved throughout evolution [1]. This is illustrated by the presence of homologous genes governing these processes in the genomes of a wide range of species, from invertebrate to humans. One of these conserved gene families encodes NCAM-type neural cell adhesion molecules (CAMs) (Fig. 1). Contrary to vertebrates, the genome of the fruit fly *Drosophila melanogaster* and that of other invertebrate species contains only one single NCAM-type gene, which in *Drosophila* is named as *fasciclin II* (*fasII*) [2]. Similar observations have been made for other conserved gene families, including other neural CAMs. E.g., whereas the *Drosophila* genome contains only one gene for the L1-type CAM, named *neuroglian (nrg)* [3, 4], vertebrate genomes encode up to four paralogous L1-type genes, L1-CAM, CH-L1, Neurofascin and NrCAM [5]. This suggests that large scale gene duplication events have resulted in the generation of multiple paralogous genes in the chordate lineage after it split from the arthropod phylum some 600–900 million years ago [6, 7]. The structural and functional conservation of NCAM-type genes in different phyla is the evidence that an ancestral NCAM-like protogene existed early during the evolution of metazoa. This indicates an important role for these neural CAMs in the development of simple, as well as more complex nervous systems. For NCAM and other important genes that have been conserved through evolution, gene duplication has allowed for the divergence of gene expression and function between the paralogous genes, including the adaptation to novel functions and interactions. At the same time, ancestral functions are either shared by these paralogous gene products or have been maintained by at least one family member [8].

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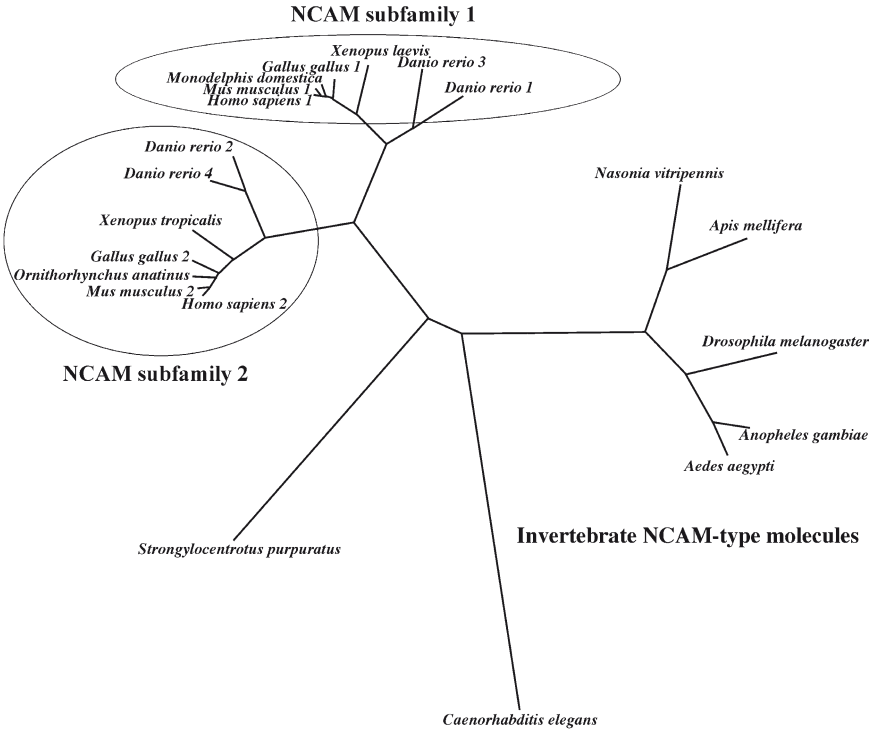

Fig. 1 Phylogenetic tree of NCAM-type genes in different species. With the exception of the *C. elegans* sax-3 protein, all other 20 polypeptides, which were selected for this phylogenetic analysis, conform to the 5 Ig plus 2 FNIII consensus protein domain structure of NCAM-type proteins. Polypeptide sequences, which cover four and a half of the Ig and both FNIII protein domains, were used for a protein sequence comparison. The multiple sequence alignment was performed using the on-line version of the MAFFT program (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). An unrooted phylogenetic tree was constructed using the Promlk and the Drawtree subroutines of the Phylip v3.65 program package [107]. The genomes of the included invertebrate species [*Caenorhabditis elegans* (nematode; NP_741748), *Nasonia vitripennis* (jewel wasp; XP_001606611), *Anopheles gambiae* (malaria mosquito; XP_312112), *Aedes aegypti* (yellow fever mosquito; XP_001659793), *Drosophila melanogaster* (fruit fly; P34082), *Apis mellifera* (honey bee; XP_393339), and *Strongylocentrotus purpuratus* (purple sea urchin; XP_001177682)] all contain only one NCAM-type gene. In contrast, most vertebrate species, such *Gallus gallus* (chicken; XP_001234122 and XP_425540), *Mus musculus* (mouse; NP_001074914 and NP_035084), and *Homo sapiens* (human; NP_000606 and NP_004531), have 2 distinct NCAM-type genes, with the *Danio rerio* (zebrafish; NP_571277, NP_571905, NP571906 and NP_001070212) genome encoding a total of 4 separate NCAM-type polypeptides. As only partial genomic sequences are currently available for *Xenopus tropicalis* (Western clawed frog; Xentr4|393939), *Xenopus laevis* (African clawed frog; NP_001081296), *Ornithorhynchus anatinus* (duck-billed platypus; XP_001511913), and *Monodelphis domestica* (gray short-tailed opossum; XP_001381275), it appears likely that these genomes also contain a second NCAM-type gene

Similar to vertebrate NCAMs, Fasciclin II belongs to the immunoglobulin (Ig) superfamily and is expressed as several protein isoforms with a characteristic pattern of five Ig-like and two fibronectin type III protein domains [9] (Fig. 2). Although NCAM and Fasciclin II share an amino acid identity of only 26% across

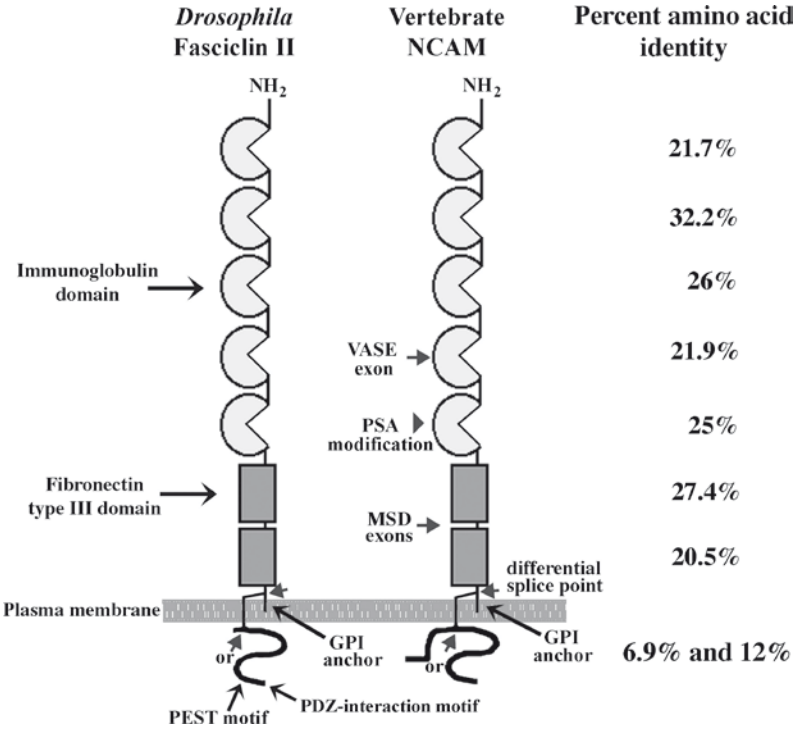

Fig. 2 Structural comparison of *Drosophila* Fasciclin II and vertebrate NCAM. Alternative splice points for the VASE and MSD mini exons and the major protein isoforms are indicated by arrows. The arrowhead marks the site at which vertebrate NCAMs are optionally modified by polysialic acid (PSA). The sequence identity scores were obtained using the EMBOSS alignment tool from the EMBL-EBI website (www.ebi.ac.uk/emboss/align). The amino acid sequences for human $NCAM^{140}$ (P13591), $NCAM^{180}$ (EAW67201), and *Drosophila* Fasciclin II^{PEST+} (P34082) including information about their domain organization were obtained from the Expert Protein Analysis System (expasy) from the Swiss Institute of Bioinformatics. For the intracellular domains, the highest amino acid identity score was obtained for $NCAM^{140}$ and the PEST+ isoform of Fasciclin II (12%). The cytoplasmic domain of $NCAM^{180}$ shares only 6.9% identity with the PEST+ domain of Fasciclin II

all seven extracellular protein domains and a much lower level of conservation between their intracellular sequences, functional and comparative studies indicate that these molecules should be considered true orthologs [2]. The *Drosophila fasciclin I* and *fasciclin III* genes also encode adhesive proteins, which are expressed in the fly's nervous system. However, these polypeptides exhibit no sequence or functional similarities with NCAM-type proteins.

Alternative Splicing and Posttranslational Modifications

Similar to vertebrate NCAM-type proteins, differential splicing of the *fasciclin II* transcript gives rise to three major protein isoforms; one with a glycosyl-phosphatidylinositol (GPI) membrane anchor and two transmembrane isoforms (Fig. 2). However, the

cytoplasmic domains of the two Fasciclin II transmembrane isoforms display very little similarity to those of the vertebrate 140 and 180 kDa NCAM isoforms (Fig. 2). The cytoplasmic domain of the larger transmembrane Fasciclin II isoform includes an amino acid motif that is rich in proline, glutamic acid, serine, and threonine residues (PEST) [10]. The biological significance of this PEST sequence in Fasciclin II is currently unknown. In other proteins, this amino acid motif represents a signal for the rapid proteolytic degradation of the polypeptide [11]. Therefore, the presence of the PEST motif might provide a mechanism for the regulation of Fasciclin II protein levels. Whereas, vertebrate NCAM genes produce a multitude of alternatively spliced transcripts that are developmentally regulated (Fig. 2), the transcription and alternative splicing of the *Drosophila fasciclin II* gene is not significantly altered during development. Furthermore, none of the Fasciclin II protein isoforms is modified by polysialic acid (PSA), which in vertebrates constitutes an important, developmentally-regulated post-translational modification of NCAM proteins [12, 13]. In *Drosophila*, PSA is only expressed during early embryonic development and is linked to proteins other than Fasciclin II [14]. Interestingly, a selective posttranslational modification of the Fasciclin II protein in the fly's nervous system involves a different type of carbohydrate moiety that is specifically recognized by anti-horseradish-peroxidase antibodies (HRP epitope) [15–17]. In contrast, Fasciclin II protein that is expressed by non-neuronal cells does not contain this modification [16, 18].

Fasciclin II Expression

NCAM expression levels are the highest in the nervous system, in which it is almost ubiquitously expressed by most neurons [19, 20]. However, NCAM is also found in many other tissues, including liver and muscles, as well as in glial cells [19, 21–23]. Levels of PSA-modified NCAM are at their peak during the early development and decrease markedly in the adult nervous system [24–26]. Compared to NCAM, Fasciclin II expression in the *Drosophila* nervous system is rather restricted. Fasciclin II protein is first expressed during oogenesis, where it is involved in establishing tissue polarity in inner polar cells of epithelium-derived border cells through its selective localization to basolateral junctions [27, 28]. During later stages of embryogenesis, Fasciclin II protein is dynamically expressed on a subset of neuronal tracts, including the MP1 fascicle and other longitudinal nervous system pathways. Subsequently, Fasciclin II expression expands to include axons and growth cones of other axonal tracts, such as embryonic PNS motor neurons [2, 29–31]. Several Fasciclin II-positive ganglia in the embryonic nervous system continue to express Fasicilin II protein throughout the larval development and remain Fasciclin II-immunopositive in the fly's adult central and peripheral nervous system. Interestingly, in the insect *Manduca sexta,* only the transmembrane isoforms of Fasciclin II are expressed by neurons. In contrast, the GPI-linked Fasciclin II isoform is exclusively associated with non-neuronal cell types, especially with glial

cells, in which it provides an adhesion and axon-guidance substrate for growth cones of extending axons [32, 33]. Hence, in addition to its expression by neuronal cells, the expression of Fasciclin II by glia cells indicates its importance for non-neuronal cell differentiation.

Cell Adhesion Mechanism

Like its vertebrate NCAM ortholog, Fasciclin II is an adhesion-competent membrane protein that mediates neuronal migration and axonal guidance by a *trans*-homophilic binding mechanism [2, 9, 29, 34]. Despite the low degree of amino acid sequence identity between vertebrate NCAM and *Drosophila* Fasciclin II (Fig. 2), overall structural features are remarkably well conserved. This suggests that Fasciclin II's homophilic binding mechanism most likely is governed by similar molecular interactions as those reported for NCAM [35]. A conservation of adhesive specificity and molecular mechanisms has been described for the *Drosophila* Neuroglian and human L1-CAM proteins. Although the specificity of their homophilic interaction has drifted apart, *Drosophila* and vertebrate L1-type proteins still exhibit a weak, but measurable "homophilic" interaction across phylogenetic boundaries. [36].

Proneural Functions

During early neurogenesis, Fasciclin II is expressed by several clusters of differentiating neuroblasts in the *Drosophila* embryo [37, 38]. The functional role of Fasciclin II at this initial stage of neuronal development is unclear, but likely involves the induction of downstream proneural genes, including the *achaete* (*ac*) and *atonal* (*ato*) genes [39]. The *ac* and *ato* gene products regulate the developmental programs that lead to sensory organ precursor cell formation in the fly [40]. Genetic analyses have demonstrated that loss-of-function, as well as gain-of-function mutations in the *fasII* gene induce alterations in proneural cluster formation of the eye-antennal imaginal disc, which leads to compromised adult sensory organ formation [39]. In addition, loss-of-function mutations in the *fasII* gene, results in alterations of the adult nervous system in the wing [41]. The mechanism, by which Fasciclin II regulates proneural gene expression during imaginal disc development and whether it involves an adhesion-dependent signaling event, is currently unknown. However, genetic evidence suggests that the activation of the Abelson (*abl*) tyrosine kinase is a crucial step in this process [39]. This non-receptor tyrosine kinase is highly active during CNS development and also plays an essential role during midline crossing of axons [42–44]. Whether PSA-NCAM has a similar morphogenetic function during neurogenesis in vertebrates remains unclear. However, its high expression levels during early stages of neuronal development suggest such a possibility.

Axon Growth and Guidance

During initial elongation of pioneer axons, a select group of Fasciclin II-immunopositive neuroblasts are located in an uninterrupted longitudinal pattern along the midline of the embryo [31]. These neuroblasts, have been termed as "fibre tract founder clusters" and serve as physical guidance cues for pioneer axon growth cones. The interaction between extending growth cones and these founder cluster cells represents the first example of Fasciclin II's role during axonal pathfinding. [31]. At later stages, after initial Fasciclin II-positive axonal pathways have been established, subsequent Fasciclin II-positive axons use this pioneer axon framework for their own growth and axonal pathway choices, which also involve homophilic Fasciclin II-mediated adhesion. The principles that govern these early Fasciclin II functions also appear to apply to the development of adult nervous system structures.[45]. The importance of Fasciclin II during axonal pathfinding has been demonstrated in vivo by gene knock-out and overexpression studies. These genetic manipulations of Fasciclin II expression cause severe axonal fasciculation phenotypes in the *Drosophila* embryo [2, 10]. This suggests that selective expression of Fasciclin II by a group of neuronal axons and grown cones is required for the fasciculation of specific axonal tracts [2, 29]. In addition, expression of Fasciclin II by certain glial cells provides additional axonal pathfinding cues during this process [33, 46]. Besides homophilic binding, Fasciclin II-mediated axonal pathfinding involves the activation of various cellular signaling processes, including the induction of mitogen-activated protein kinase (MAPK) activity and the regulation of intracellular Ca^{2+} levels [47, 48]. MAPK, which functions upstream of the extracellular signal-regulated protein kinases (ERK), leads to an increase in ERK-mediated Fos and c/EBP activation [49]. Fasciclin II functions during these processes are closely linked and co-regulated with other mechanisms for axon outgrowth, including semaphorin-mediated repulsive clues [50].

Several lines of evidence suggest that similar principles govern axon growth and guidance in the invertebrate and vertebrate nervous systems [51]. E.g., interference with NCAM function during axon outgrowth in the retina has been shown to cause altered fasciculation [52, 53]. In addition, animals lacking NCAM during nervous system development have changes of axon guidance and fasciculation in the olfactory bulb and the mossy fiber system in the hippocampus [20, 54]. Consistent with the functional importance of these areas for activity induced plasticity, NCAM knock-out animals have reduced learning capacity [55, 56]. Similar to Fasciclin II, NCAM mediated axon guidance involves activation of various signaling pathways including MAPK activation, which acts to modify downstream gene transcription in the neuron [47, 57, 58].

Activation of EGFR and FGFR

In addition to Fasciclin II, the L1-type neural CAM Neuroglian also plays an important role during axonal outgrowth, guidance, and fasciculation in the embryonic and postembryonic *Drosophila* nervous system [59]. In the adult ocellar sensory system

(OSS), which is located on the dorsal surface of the *Drosophila* head (Fig. 3c), both Fasciclin II and Neuroglian are involved in establishing two distinct axonal pathways. These pathways originate from different types of sensory neurons; the bristle mechanoreceptors (BM) and ocellar pioneer (OP) neurons. Whereas, axons from OP neurons directly project towards the brain, axons from BM neurons initially fasciculate and grow attached to the head epithelium, before detaching and entering the CNS at a more anterior point [60]. Both of the two Neuroglian isoforms, the non-neuronal Nrg^{167} and the neuronal Nrg^{180} isoforms, are differentially expressed in the OSS; Nrg^{167} in the epithelium and the Nrg^{180} protein exclusively expressed by both OP and BM neurons (Fig. 3a, b) [61, 62]. In contrast, Fasciclin II is only expressed by OP axons, but not by BM axons and in other regions of the OSS (Fig. 3d). Therefore, in the OSS, only OP neurons co-express both Fasciclin II and Nrg^{180}.

The use of a temperature-sensitive *nrg* mutant allele allows the conditional expression of Neuroglian protein at distinct developmental stages. Thereby, the early embryonic lethal phase, which is associated with a loss of Neuroglian, can be bypassed and the requirement for Neuroglian during development of adult structures can be studied[63]. Using this technique, Garcia-Alonso et al. studied the

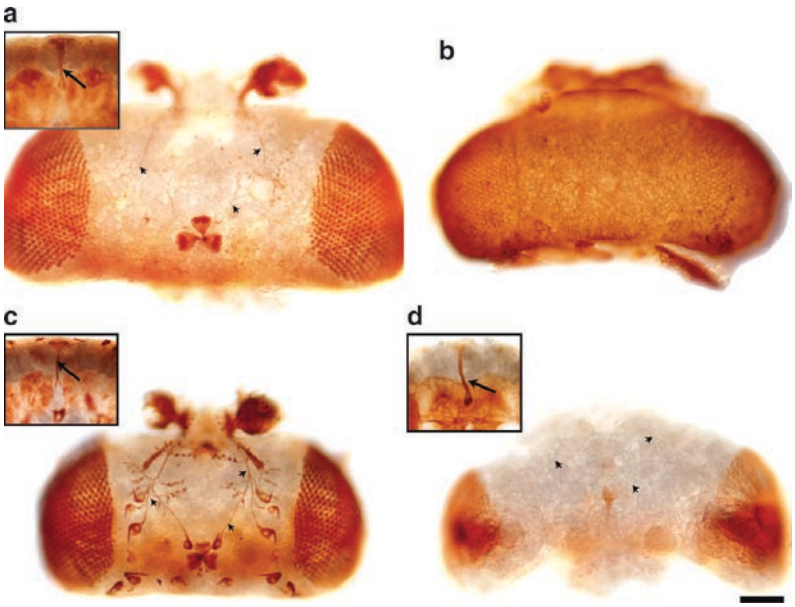

Fig. 3 Immunohistochemical detection of Neuroglian, Fasciclin II and the neuronal marker Futsch in the ocellar sensory system (OSS) of *Drosophila melanogaster*. A: Nrg^{180} (BP104 [62]); B: Nrg^{167} + Nrg^{180} (1B7 [3]); C: Futsch (22C10 [108]); D: Fasciclin II (1D4 [10]). Large images (**a**–**d**) are dorsal views of the OSS. Inserts (**a**–**d**) are coronal sections corresponding to the anterior-posterior level of the ocellar pioneer (OP) fascicle. Arrowheads indicate the pattern of BM axons in the OSS (not detected in **d**). The *arrows* in inserts **a**, **c**, and **d** indicate the immuno-positive OP axon fascicle. The scale bar represents 100 μm in the large images and 50 μm in the inserts

importance of Neuroglian expression during OSS development and demonstrated an essential requirement for Neuroglian during BM and OP axonal fasciculation and pathfinding [64]. In addition, this study showed that pathfinding of both BM and OP axons involves the Neuroglian-dependent activation of *Drosophila* epidermal growth factor (DER) and fibroblastic growth factor receptors (Heartless, *Htl*) [64]. This is consistent with results reported by Forni et al., who used a cell-culture based system to demonstrate that both Fasciclin II and Neuroglian promote neurite outgrowth from *Drosophila* primary neurons by specifically activating Htl signaling [65]. In vertebrates, FGF receptor activation by NCAM and L1-CAM has been thoroughly studied and, in a similar way, is believed to be central for promoting axon growth and guidance [35, 47, 66, 67].

Re-introduction of Nrg^{180} into the OSS by UAS/GAL4-mediated transgene expression [68] rescues both the BM and the OP phenotype that is associated with the *nrg* loss-of-function condition [61]. In contrast, the ectopic expression of Fasciclin II in this *nrg*-mutant background rescues only the OP pathfinding phenotype (where endogenous Fasciclin II is normally co-expressed with Neuroglian), but exacerbates the BM phenotype (where FasII is not normally expressed). Furthermore, a genetic reduction of the Fasciclin II gene dosage to 50% in a *nrg*-mutant background causes a significant increase in the penetrance of the OP phenotype [61]. These genetic experiments strongly suggest a functional redundancy between Neuroglian and Fasciclin II during guidance of OP axons, which normally co-express the two proteins. This supports the notion that the *Drosophila* orthologs of L1-CAM and NCAM serve both specific, as well as overlapping functions during nervous system development. These findings in the *Drosophila* model system might provide novel insight into molecular mechanisms, which are also at play during nervous system development in higher organisms, including humans [61].

Synaptic Functions

A number of studies in vertebrate model systems point to a role of NCAM-type proteins in synaptic plasticity [13, 24, 69–71]. Through its role in establishing long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus and other areas, NCAM is postulated to be of central importance for several aspects of learning and memory. Similarly, results obtained in *Drosophila* indicate that Fasciclin II is not only involved in the modification of existing synapses, especially during activity-induced synaptic re-modulation [72, 73]. In concert with other adhesion molecules, it also participates in the de-novo formation of synapses during nervous system development [74, 75]. Due to its easy accessibility, well-described morphology, and stereotyped pattern of connections, the *Drosophila* embryonic/larval glutamatergic neuro-muscular junction (NMJ) has widely been used as a model system for studying synaptogenesis in vivo [76–78].

The process of synaptogenesis involves the formation of dendritic filopodia from the target muscle cell, called myopodia, which establishes synaptic active zones. This is followed by a flow of retrograde information from the postsynaptic compartment to the growth cone, which connects and stabilizes immature synaptic contacts [79, 80]. Dynamic Fasciclin II expression at both the developing pre- and postsynaptic entities is essential for the stabilization, growth and pruning of larval NMJs [81–83]. Interestingly, in *fasII*-mutant animals, NMJs form normally during the initial phases of synaptogenesis. However, these newly established synaptic boutons subsequently retract during larval development, eventually resulting in lethality. In addition, alterations in the level of synaptic Fasciclin II expression have a profound impact on the final size of the NMJ and result in either a reduction or significant enlargement of the synapse [82]. Based on these observations, it is not surprising that the levels of NMJ-expressed Fasciclin II protein are controlled by synaptic activity, which involves a cAMP response element binding protein (CREB)-dependent signaling mechanism [73, 84, 85]. Prior to NMJ formation, all muscles express low levels of Fasciclin II protein [86]. Later, during synapse formation, Fasciclin II becomes concentrated at developing pre- and postsynaptic sites. Besides stabilizing synaptic contacts, the level of Fasciclin II protein exhibited by individual muscle fibers also has a profound impact on target selection and patterning of synapses [86]. Interestingly, Fasciclin II expression and the subsequent establishment of synaptic contacts is sensitive to various environmental factors, such as population density during larval development and larval locomotor activity [85, 87]. In addition, several genetic studies have demonstrated that in vivo synaptic target selection and stabilization is a complex process that involves a precisely balanced expression of several close-range repulsive and attractant molecular cues [88, 89]. In addition to Fasciclin II, Netrins, Semaphorins and other Ig-CAMs are involved in this process. More recent studies have also linked the *sidestep* (*side*), *highwire* (*hiw*), and *wishful thinking* (*wit*) gene products to NJM synapse maturation [90–92].

Similar to the embryonic NMJ, initial contacts of adult central cholinergic synapses and peripheral glutamatergic NMJs appear to be independent of Fasciclin II expression. However, the subsequent proliferation and growth of these synapses involves Fasciclin II [93]. As previously shown for embryonic NMJs [84], the development of these adult synapses is not only influenced by the level of Fascicilin II expression, but is also regulated through synaptic neuronal activity [94].

The role of Fasciclin II in NMJ development not only depends on its homophilic adhesion, but also involves interactions with cytoskeletal elements and activation of intracellular signaling pathways [95]. The precise localization and clustering of Fasciclin II protein at developing synaptic contacts is regulated through interactions with the Discs-Large (DLG) protein, a member of the postsynaptic density-95 protein (PSD95) membrane-associated guanylate kinase (MAGUK) family [96]. The association with the DLG protein and the subsequent synaptic clustering of Fasciclin II molecules is mediated by a specific interaction between the COOH-terminus of Fasciclin II and the PDZ domains located in DLG [81, 96]. Interactions with the fly homolog of the amyloid precursor protein (APPL), with *Drosophila*

integrins, and with the dX11/Mint protein have also been shown to influence Fasciclin II patterning [97, 98]. Interestingly, the binding between Fasciclin II and DLG is reduced by the activity-induced phosphorylation of DLG by the calcium-calmodulin protein kinase II (CaMKII), which causes the dissociation of DLG from the postsynaptic density, in turn causing de-clustering and removal of Fasciclin II from the synapse [99, 100]. Through this mechanism, Fasciclin II localization and synaptic clustering are inversely linked to synaptic activity and plasticity. In addition, retrograde modulation of presynaptic terminals through CaMKII activation at the postsynaptic side was recently shown to depend on Fasciclin II and DLG [101]. In both *fasII* and *dlg* knock-out animals, this presynaptic modulation is abolished, but can be rescued by reintroducing these molecules. Interestingly, the involvement of similar signaling pathways has been well established in vertebrate animal models during the induction of memory and learning. This indicates that the molecular mechanisms that govern synaptogenesis have been conserved during evolution [102–106].

In this review, we present a short summary of our current knowledge about the *Drosophila* NCAM ortholog Fasciclin II and its diverse functions during the development of neural and other tissues in the fruit fly. Vertebrate NCAMs and Fasciclin II share many structural and functional similarities, but also display several important differences, which likely represent newer evolutionary adaptations that have developed over time. Similar to NCAM in the vertebrate nervous system, Fasciclin II expression in *Drosophila* is important for early neuronal development, axonal growth and pathfinding, and during synapse formation and remodeling. Despite almost 1 billion years of separate evolution between arthropod and chordate species, a significant number of molecular features, such as homophilic adhesion and activation of receptor tyrosine kinases have been conserved between Fasciclin II and NCAM.

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The Neural Cell Adhesion Molecule NCAM2/ OCAM/RNCAM, a Close Relative to NCAM

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List of Abbreviations

ATP	Adenosine 5′-triphosphate
CAM	Cell adhesion molecule
CHL1	Close homolog of L1
CNS	Central nervous system
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FGFR	Fibroblast growth factor receptor
Fn3	Fibronectin type 3
GPI	Glycosylphosphatidylinositol
Ig	Immunoglobulin
MAG	Myelin-associated glycoprotein
MCAM	Melanoma cell adhesion molecule
M/T-cell	Mitral and/or tufted cell
NCAM	Neural cell adhesion molecule
NQO1	NADPH: Quinone oxidoreductase1
OB	Olfactory bulb
OCAM	Olfactory cell adhesion molecule
OE	Olfactory epithelium
OR	Odorant receptor
OSN	Olfactory sensory neuron
PSA	Polysialic acid
RNCAM	Rb-8 neural cell adhesion molecule
RPTP	Receptor protein tyrosine phosphatase

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SH3 Src-homology 3
STAT Signal transducer and activator of transcription
VSN Vomeronasal sensory neuron

Cell Adhesion Molecules in the Nervous System

The development, maintenance, and dynamics of the nervous system depend on processes such as the migration and differentiation of neural precursor cells [1] and the formation of neuronal connections through outgrowth, guidance, and target recognition of neurites. Furthermore, cell numbers are controlled through regulated cell survival and apoptosis, and neural connections are strengthened by the fasciculation of neurites, and the formation, maturation, and plasticity of synapses [2].

Many of the aforementioned processes are modulated or regulated by cell adhesion molecules (CAMs). These proteins mediate attachment of cells to neighboring cells through homophilic *trans*-interactions (a CAM binding to a similar CAM on an opposing cell surface) or to components of the extracellular matrix (ECM) through heterophilic interactions. Furthermore, CAMs can form, through their extracellular domains, *cis*-interactions with molecules located in the same plasma membrane and through their cytoplasmic tails interact with cytosolic proteins, thereby modulating intracellular signal transduction cascades and the organization of the cytoskeleton [3].

In addition to their importance for healthy nervous system function, CAMs expressed in the nervous system often are important elements of processes occurring in the diseased nervous system, including neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease [4–7].

The first neural cell adhesion molecule to be identified was appropriately termed "neural cell adhesion molecule" (NCAM) [8, 9]. The sequence of NCAM demonstrates a high identity to that of the neural cell adhesion molecule 2 (NCAM2) (Fig. 1), and both proteins are abundant in the central nervous system (CNS), suggesting that they also may share functional similarities. However, whereas the expression of NCAM is characterized by a relatively uniform expression pattern in the developing CNS, NCAM2 has been demonstrated to exhibit a less uniform expression pattern with high expression in specific regions of the olfactory system [10, 11]. In contrast to NCAM, NCAM2 has only been the focus of a few studies, and consequently, the properties and functions of NCAM2 are largely uncharacterized. This review summarizes some of the available information about NCAM2, with an emphasis on similarities and differences between NCAM and NCAM2.

The Identification of NCAM2

The first NCAM2-related study was published in 1985, where the monoclonal antibody R4B12 directed against rabbit olfactory bulb (OB) tissue was found to recognize an antigen expressed on axons of an olfactory sensory neuron subgroup [12].

Later, the same antibody was shown to recognize two isoforms of a membrane-localized protein, with molecular weights of 90 kDa, and 115 kDa, respectively, of which the smaller isoform was attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor [13]. Another monoclonal antibody, Rb-8, obtained by immunization with neonatal rat piriform cortex tissue was found to recognize a 125 kDa membrane-integral protein [14, 15]. However, the protein corresponding to the antigens of R4B12 and Rb8 was not identified until 1997, when NCAM2 was independently cloned by three laboratories.

The protein encoded by the human gene was named NCAM2 (due to the high sequence homology with NCAM) [16], whereas the proteins encoded by the genes cloned from rat, mouse, and rabbit were named as OCAM (olfactory cell adhesion molecule; a name referring to the specific expression pattern of the protein) [17] and RNCAM (Rb-8-neural cell adhesion molecule; a name referring to the previously described Rb-8 antigen) [10, 15]. Currently, the official names for the genes encoding the NCAM2 protein is *NCAM2* in human and *Ncam2* in mouse, and consequently, we refer to the protein as NCAM2 throughout this review.

Isoforms and Protein Structure of NCAM2

NCAM2 belongs to the immunoglobulin (Ig) superfamily and has the same overall structure as NCAM (Fig. 1). Thus, the extracellular part of NCAM2 consists of five Ig-homology modules (presumably of the C2 type) followed by two fibronectin type 3 (Fn3)-homology modules. Alternative splicing of the transcript from *NCAM2* results in the production of either a glycosylphosphatidylinositol (GPI)-anchored isoform of the protein or a transmembrane isoform that, in addition to the extracellular domain, contains a 20–25 amino acid-long transmembrane region followed by a 106–119 amino acid-long cytoplasmic region [10, 17]. The two isoforms of NCAM2 are comparable with two of the main isoforms of NCAM: NCAM-120 (which is GPI-anchored) and NCAM-140 (which is transmembrane), respectively. However, *NCAM2* does not contain the region corresponding to exon 18 of the gene encoding NCAM, *NCAM1*, and consequently, there is no isoform of NCAM2 resembling the large transmembrane isoform of NCAM, NCAM-180 [10, 15]. Furthermore, in contrast to *NCAM1*, *NCAM2* does not appear to be alternatively spliced in the region encoding the extracellular domain of the protein.

The overall amino acid sequence identity between human NCAM and NCAM2 is around 44.5% [10, 16, 17]. As indicated in Fig. 1, the sequence identity between NCAM and NCAM2 is highest in the Ig1, Ig2, and Ig5 modules and in the cytoplasmic region (~45–55% identity), whereas the sequence identities between the Ig3, Ig4, and the two Fn3 modules are notably lower (~36–40% identity). The fact that the two proteins, domain-for-domain, exhibit a high degree of identity suggests that the genes encoding NCAM and NCAM2 are paralogs.

NCAM and NCAM2 contain a number of regions with consensus sequences for various types of posttranslational modifications, some of which are conserved

Region	Protein	% Identity	Sequences
Ig1-module	NCAM NCAM2	49.5	QVDIVPSQGEISVGESKFFLCQVAGDAKDKDISWFSPNGEKLTPNQQRISVVWNDDSSSTLTIYNANIDDAGIYKCVVTGEDGSESEATVNVKIFQK QVTISLSKVELSVGESKFFTCTAIGEPE--SIDWYNPQGEKIISTQR--VVVQKEGVRSRLTIYNANIEDAGIYRCQATDAKGQTQEATVVLEIYQK ** * *: *:******** * . *:.: .*.*:.*:***: ..*: ** ::. * ********:*****:* .*. .*. .**** ::*:**
Ig2-module	NCAM NCAM2	55.3	Heparin N-glyc# LMFKNAPTPQEFREGEDAVIVCDVVSSLPPTIIWKHKGRDVILKKDVRFIVLSNNYLQIRGIKKTDEGTYRCEGRILARGEINFKDIQVIVNVP LTFREVVSPQEFKQGEDAEVVCRVSSSPAPAVSWLYHNEEVTTISDNRFAMLANNNLQILNINKSDEGIYRCEGRVEARGEIDFRDIIVIVNVP * *::. :****::**** :** * ** .*:: * ::..:* .* ** :*:** *** .*:*:*** ******: *****:*:** ******
Ig3-module	NCAM NCAM2	39.6	N-glyc PTIQARQNIVNATANLGQSVTLVCDAEGFPEPTMSWTKDGEQIEQEEDDEKYIFSDDSSQLTIKKVDKNDEAEYICIAENKAGEQDATIHLKVFAK PAISMPQKSFNATAERGEEMTFSCRASGSPEPAISWFRNGKLI---EENEKYILKGSNTELTVRNIINSDGGPYVCRATNKAGEDEKQAFLQVFVQ *:*. *: .****: *:.:*: * *.****::** ::*: * *::****:....::**:::: :.* . *:* * *****:: .*:**.:
Ig4-module	NCAM NCAM2	37	N-glyc N-glyc* PKITYVENQTAMELEEQVTLTCEASGDPIPSITWRTSTRNISSEEKASWTRPEKQETLDGHMVVRSHARVSSLTLKSIQYTDAGEYICTASNTIGQDSQSMYLEVQYA PHIIQLKNETTYE-NGQVTLVCDAEGEPIPEITWKRAV--------DGFTFTEGDKSLDGRIEVKGQHGSSSLHIKDVKLSDSGRYDCEAASRIGGHQKSMYLDIEYA *:* ::*:*: * : ****.*:*.*:***.***: :. .:* .* :::***:: *:.: *** :*.:: :*:*.* * *:. ** ..:****:::**
Ig5-module	NCAM NCAM2	45.3	N-glyc# N-glyc N-glyc N-glyc PK-LQGPVAVYTWEGNQVNITCEVFAYPSATISWFRDGQLLPSSNYSNIKIYNTPSASYLEVTPDSENDFGNYNCTAVNRIGQESLEFILVQADT PKFISNQTIYYSWEGNPINISCDVKSNPPASIHWRRDKLVLPAKNTTNLKTYSTGRKMILEIAPTSDNDFGRYNCTATNHIGTRFQEYILALADV ** :.. . *:**** :**:*:* : *.*:* * ** :**:.* :*:* *.* **::**:****.*****.*:** . *:**. **.
Fn3I-module	NCAM NCAM2	37.9	N-glyc# PSSP-SIDQVEPYSSTAQVQFDEPEATGGVPILKYKAEWRAVGEEVWHSKWYDAKEASMEGIVTIVGLKPETTYAVRLAALNGKGLGEISAASEFKTQPVRKE PSSPYGVKIIELSQTTAKVSFNKPDSHGGVPIHHYQVDVKEVASEIWKI----VRSHGVQTMVVLNNLEPNTTYEIRVAAVNGKGQGDYSKIEIFQTLPVR-E **** .:. :* .:**:*.*::*:: ***** :*:.: : *..*:*: .:. .:: :*.: .*:*:*** :*:**:**** *: * . *:* *** *
Fn3II-module	NCAM NCAM2	35.9	WA ATP *FGFR* PSAPKLEGQMGEDGNSIKVNLIKQDDGGSPIRHYLVRYRALSSEWK-PEIRLPSGSDHVMLKSLDWNAEYEYYVWAENQQGKSKAAHFVFRTSAQPTAIPANG PSPPSIHGQ-PSSGKSFKLSITKQDDGGAPILEYIVKYRSKDKEDQWLEKKVQGNKDHIILEHLQWTMGYEVQITAANRLGYSEPTVYEFSMPPKPNII--KD **.*.:.** ..*:*:*:.: ******:** .*:*:**: ..* : * :: ...**::*: *:*. *** :.* *: * *:.: : * ..:*. * :.
TM region	NCAM NCAM2	37.5	SPTSGLSTG AIVGILIVIFVLLLVVVDITCYF TLFNGLGLG AVIGLGVAALLLILVVTDVSCFF : .**. * *::*: :. ::*:***.*::*:*
Cytoplasmic Region	NCAM NCAM2	53.0	P P P # # # PDZ LNKCGLFMCIAVNLCGK-AGPGAKGKDMEEGKAAFSKDESKEPIVEVRTEEERTPNH-DGGKHTEPNETTPLTEPEKGPVEAKPECQETETKPAPAEVKTVPNDATQTKENESKA IRQCGLLMCITRRMCGKKSGSSGKSKELEEGKAAYLKDGSKEPIVEMRTEDERVTNHEDGSPVNEPNETTPLTEPEKLPL--KEEDGKEALNPETIEIK-VSNDIIQSKEDDSKA :.:***:***: .:*** :*...*.*::****** ** *******:***:**..** **. .************ *: * * : :* . *:* *.** *:**::***

Fig. 1 Alignment of NCAM and NCAM2 Sequences. Alignment and subsequent investigations of sequences of human NCAM [Ensembl Peptide ID ENSP00000318472] and human NCAM2 [Ensembl Peptide ID ENSP00000383392] were made using the T-Coffee multiple sequence alignment program (http://www.ebi.ac.uk/t-coffee/) and additional programs as indicated in the legend to Fig. 2. Column 1 indicates which part of the proteins the given sequences represent. Column 2 indicates from which protein the respective sequences are derived. Column 3 indicates the amino acid identity between the aligned sequences. N-glycosylation sites conserved between NCAM and NCAM2 are indicated with bold "N-glyc" above a bold "N"-residue; non-conserved NCAM and NCAM2 *N*-glycosylation sites are indicated by a bold "N-glyc*" and a bold "N-glyc#", respectively. The heparin binding site on NCAM is indicated by a bold, highlighted "Heparin" above bold, highlighted residues. The walker A motif and amino acids demonstrated to be involved in ATP binding in NCAM are indicated by "ATP" above bold, highlighted residues. The walker A motif in NCAM2 is indicated by a bold, highlighted "WA" above bold, highlighted residues. The binding site for FGFR on NCAM is indicated by a bold, italic "FGFR" above bold, italic residues. The PDZ-like binding motif of NCAM is indicated by a bold "PDZ" above bold residues. Conserved palmitoylation sites are indicated by a bold "P" above a bold residue. Conserved phosphorylation sites are indicated by a bold "#" above bold residues

between the two proteins (see in the following section). Furthermore, NCAM2 intracellularly contains a potential internalization signal and potential SH2 and SH3 domains (Fig. 2). However, despite the high overall sequence homology between NCAM and NCAM2 in the intracellular region, none of these regions are conserved between the two proteins. Whether these regions have any functional significance remains to be determined.

The cytoplasmic part of NCAM2 also has been reported to contain a so-called PEST sequence, with regions rich in proline (P), glutamic acid (E), serine (S), and threonine (T) [17] that have been proposed to make proteins susceptible to rapid proteolytic degradation [18]. Using web-based tools for the identification of PEST sequences and calculations of so-called PEST scores (see legend to Fig. 1), the cytoplasmic region of NCAM2 can be shown to contain a PEST sequence with a relatively high PEST score of 19. Interestingly, the majority of the identified PEST sequence is highly conserved between NCAM and NCAM2, and an analysis of human NCAM-140 revealed that this molecule also contains a PEST sequence (with a PEST score of 20). However, NCAM is not known to be a molecule that is rapidly degraded, so the presence of a PEST sequence in NCAM2 should not necessarily indicate that NCAM2 is a protein with a short half-life. Nevertheless, the region in question is almost completely conserved between NCAM and NCAM2, so it is highly likely to be important for a function shared by the two proteins.

In NCAM-140 from rat, the cytoplasmic region stretching from amino acid 839 to 843 (encoding the sequence TEVKT) has been reported to be important for NCAM-mediated neurite outgrowth [19]. This sequence is only partially conserved between NCAM molecules from different species, and between NCAM and NCAM2. Furthermore, the amino acid residues within this region that are important for neurite outgrowth have not yet been determined, and consequently, the corresponding region in NCAM2 that might be important for the function of the protein is not clear.

Extracellular Posttranslational Modifications of NCAM2

Glycosylation

NCAM2 contains eight potential sites for *N*-linked glycosylation that are conserved within all of the vertebrate NCAM2 sequences presented in Fig. 2. For comparison, NCAM contains six sites for *N*-linked glycosylation; of these, five are conserved between NCAM and NCAM2. Only the third (counting from the N-terminal) *N*-linked glycosylation site of NCAM, located in the Ig4 module, is not conserved between the two proteins (Fig. 1). All five glycosylation sites that are conserved between NCAM and NCAM2 are known to be glycosylated in NCAM [20] and, therefore, are likely also to be glycosylated in NCAM2.

Two of the glycosylation sites conserved between NCAM and NCAM2 (the two most membrane-proximal glycosylation sites in the Ig5 module) are able, in

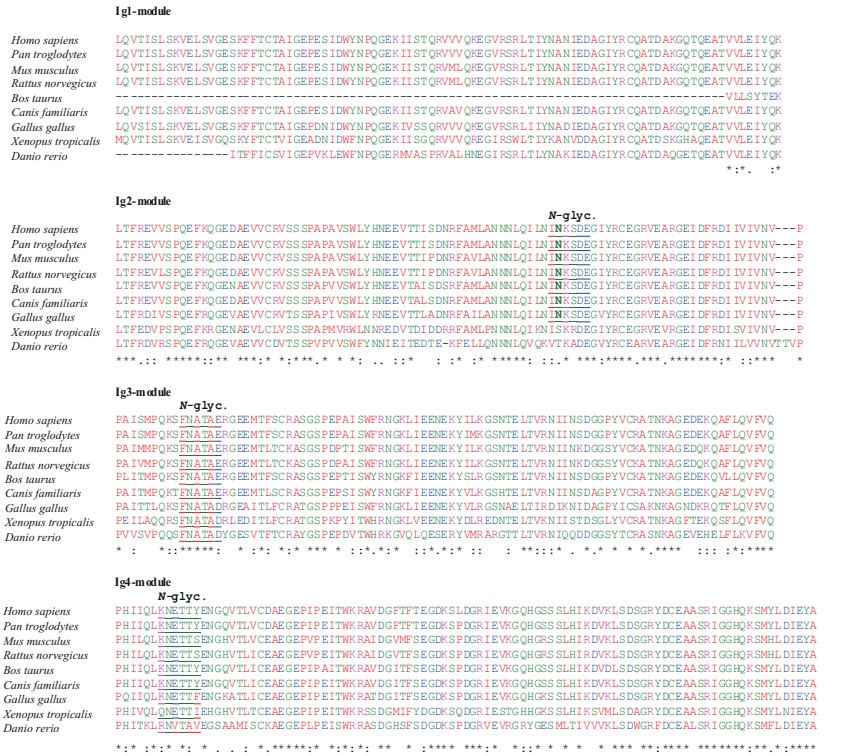

Fig. 2 Alignment of NCAM2 Sequences. Alignments of NCAM2 sequences from selected vertebrate species were made using the T-Coffee multiple sequence alignment program (http://www.ebi.ac.uk/t-coffee/) and include human (Ensembl Peptide ID ENSP00000383392), chimpanzee (Ensembl Peptide ID ENSPTRP00000023782), mouse (Ensembl Peptide ID ENSMUSP00000063468), rat (Ensembl Peptide ID ENSRNOP00000002895), cow (Ensembl Peptide ID ENSBTAP00000034315), dog (Ensembl Peptide ID ENSCAFP00000012401), chicken (Ensembl Peptide ID ENSGALP00000025332), frog (Ensembl Peptide ID ENSXETP00000015377), and zebrafish (Ensembl Peptide ID ENSDARP00000091452). Amino acid residues were colored by the alignment program according to the following physiochemical criteria. Red: nonpolar residues (A, F, I, L, M, P, V, W; excluding C, G). Blue: acidic residues (D, E). Magenta: basic residues (K, R). Green: remaining residues (C, G, H, N, Q, S, T, Y). "*" indicates that the residues are identical in all aligned sequences. ":" indicates that conserved substitutions exist in the aligned sequences (corresponding to the grouping of the amino acids in the abovementioned color scheme). "." indicates semi-conserved substitutions in the aligned sequences. Potential *N*-glycosylation sites (indicated with bold "N-glyc" above a bold "N" residue) were identified using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/; [75]). Potential walker A motifs (P-loops) (indicated by bold "WA" above bold sequences) were identified from the consensus pattern [AG]-x(4)-G-K-[ST] (PROSITE documentation PDOC00017; http://www.expasy.ch/prosite/). Transmembrane regions (surrounded by a rectangular box) were identified using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). For investigations of the intracellular regions, potential palmitoylation sites (indicated by a bold "P" above bold residues) were identified using CSS-Palm (http://bioinformatics.lcd-ustc.org/css_palm/index.php; [76]). Potential sorting and internalization signals (indicated by a bold "I" above underlined sequences), SH2 domains (indicated with bold "SH2" above underlined sequences), and SH3 domains (indicated with bold "SH3" above underlined sequences) were identified using the ELM server (http://elm.eu.org/; [77]).

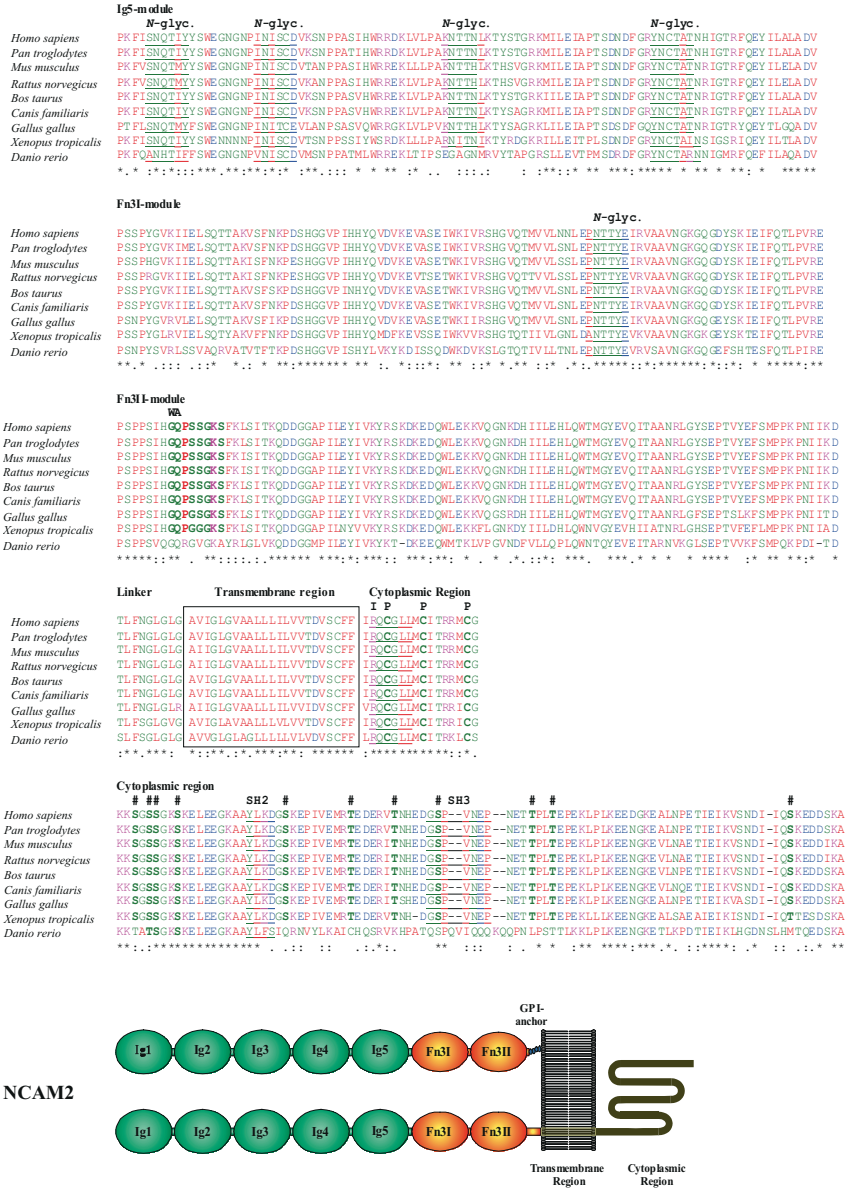

Fig. 2 (continued) Potential phosphorylation sites (indicated by a bold "#" above bold residues) were identified using the NetPhosK 1.0 Server (http://www.cbs.dtu.dk/services/NetPhosK/; [75]). Sites of interest are only indicated if they are conserved in at least seven of the nine aligned sequences

NCAM, to be glycosylated with polysialic acid (PSA; long, negatively charged homopolymers of α2-8-*N* acetylneuraminic acid) but can also be glycosylated with more traditional carbohydrates bearing the so-called human natural killer-1 (HNK-1)

epitope [21, 22]. NCAM is one of very few vertebrate proteins glycosylated with PSA, and on NCAM, this glycosylation is believed to interfere with some of the extracellular NCAM protein interactions, thereby reducing NCAM-mediated adhesion. The degree of polysialylation on NCAM is particularly high during embryonic development, and PSA-NCAM is sometimes referred to as embryonic NCAM, [23] but is also prominent in regions of the brain that require a high degree of synaptic plasticity [24].

In NCAM2, some of the glycosylations contain the HNK-1 epitope [17]. Furthermore, NCAM2 is potentially heavily glycosylated in the Ig5 module, which contains as many as four of the eight potential glycosylation sites, suggesting that regulated changes in the degree of glycosylation within the Ig5 module of NCAM2 are a way of modulating the function of NCAM2.

The HNK-1 epitope (also referred to as L2 or CD57) [25, 26] is also found in a number of other neural CAMs, including L1 [25], close homolog of L1 (CHL1) [27, 28], myelin-associated glycoprotein (MAG) [29], melanoma cell adhesion molecule (MCAM) [30], NCAM [21, 22, 31], contactin, P0, and F3 [32]. One of the functions suggested of glycosylations with HNK-1 epitopes in the nervous system is to facilitate the migration of neural crest cells [33]. If the HNK-1 epitope indeed is modulating the migration of cells or neurites, then the glycosylations on NCAM2 might play a role in the suggested importance of the protein for axon guidance and target recognition in, for example, the olfactory system (see in the following section).

Intracellular Posttranslational Modifications of NCAM2

No studies have investigated intracellular posttranslational modifications of NCAM2 and their potential importance for the distribution and function of the protein. Nevertheless, it is possible, based on various studies of NCAM, to identify intracellular regions or amino acids in NCAM2 that may be of central importance for the function of the molecule.

Acetylation

NCAM2 contains three potential palmitoylation sites that all are conserved between NCAM2 and NCAM (Fig. 1). In NCAM, all three cysteine residues are palmitoylated [34], suggesting that NCAM2 also exists in a palmitoylated form. Abrogation of the palmitoylations of NCAM results in a ~80% decrease in the amount of NCAM-140 localized in lipid rafts, and the palmitoylations are important for NCAM-mediated neurite outgrowth and for activation of the focal adhesion kinase (FAK) and extracellular signal–regulated kinase 1 and 2 (ERK1/2) downstream of the Ras-MAP-kinase pathway [35]. These observations suggest that

palmitoylation of the transmembrane isoform of NCAM2 might be important for the subcellular distribution and function of the protein and for its effects on intracellular signal transduction.

Phosphorylation

Trans-homophilic NCAM interactions lead in astrocytes to activation of the transcription factor NF-κB. Essential for this process is the phosphorylation of one to three threonine residues in the middle part of the NCAM cytoplasmic region (T788, T794, and T797 in NCAM-140) [36]. Two of the indicated phosphorylation sites, including the supposedly most essential residue, T794, are conserved between NCAM and NCAM2 (Fig. 1), suggesting that they may be relevant for NCAM2-mediated signal transduction. The expression of NF-κB in the nervous system is activated and repressed by a large number of molecules, and the protein has been shown to be involved in the regulation of neuroprotection, synaptic plasticity, and learning and memory formation (reviewed in [37]). However, whether NCAM2 can modulate any of these NF-κB-regulated processes remains to be determined.

In a recent study of NCAM, Polo-Parada *et al*. [38] identified a postsynaptic density-95, disks large, zonula occludens-1 (PDZ)-like binding motif (KENESKA) with a putative serine phosphorylation site in the C-terminal end of NCAM (Fig. 1). The authors show that this region is important for neurotransmission, supposedly through a pathway involving myosin light chain kinase and myosin II. Moreover, phosphorylation of the serine residue within the region is essential for this process. The consensus sequence for the PDZ-like binding motif (-X-[S/T]-X-[V/A]) is conserved between NCAM and NCAM2 in some species. However, in NCAM2 derived from mouse and rat, for example, the essential serine residue is not found. Thus, the reported function of the C-terminal end of the cytoplasmic region may not be conserved between NCAM and NCAM2.

Expression of NCAM2

During human embryonic development, NCAM2 is expressed in several tissues, including brain, lung, liver, and kidney [16]. In the adult human, *NCAM2* transcripts have been identified by Northern blots in many regions of the brain (including amygdala, caudate nucleus, corpus callosum, hippocampus, substantia nigra, subthalamic nucleus, and thalamus) and in several other tissues, including heart, small intestine, colon, and testis [16]. Surprisingly, the expression pattern of NCAM2 is reported to be somewhat different in mouse than in human, being confined almost exclusively to neuronal tissues where it predominantly is found in the olfactory sensory system [10, 17]. In mouse, the protein is detectable from embryonic day 13. The expression level peaks around postnatal day 21, but is maintained

in the adult animal [17], suggesting that the protein is necessary both during development and throughout adulthood.

NCAM2 demonstrates a specific expression pattern within the olfactory sensory system, in which it is expressed by olfactory sensory neurons (OSNs) in specific zones of the olfactory epithelium (see below), on the dendrites of mitral/tufted (M/T) cells in the accessory OB [39, 40], and by vomeronasal (VSNs) in the vomeronasal organ expressing heterotrimeric G-proteins of the $G_{\alpha i2}$-type [10, 17]. Furthermore, the expression level of the transmembrane isoform of NCAM2 in mouse brain is generally higher than that of the GPI-anchored isoform [17].

Homophilic NCAM2 Interactions

Cell aggregation assays have demonstrated that NCAM2 forms homophilic *trans*-interactions [17], but the molecular details of the interaction have not been investigated. In contrast, the homophilic interactions mediated by NCAM have been the topic of numerous studies, and the protein has been proposed to form homophilic *cis*-dimers as well as homophilic *trans*-interactions (reviewed in [41]). The homophilic *cis*- and *trans*-interactions of NCAM were demonstrated to involve, on the basis of protein structures obtained by X-ray crystallography, the Ig1, Ig2 and Ig3 modules of NCAM. However, additional modules also may be involved. Most noticeably, the Ig1 and Ig2 modules form a cross-like structure through reciprocal Ig1–Ig2 interactions between modules from different NCAM molecules [42, 43]. As mentioned above , the Ig1 and Ig2 modules are among the most conserved regions between NCAM and NCAM2 (Fig. 1). However, the homophilic interaction sites identified in NCAM are not well-conserved between NCAM and NCAM2, suggesting that the molecular basis for homophilic interactions may vary between the two proteins.

Heterophilic Binding Partners of NCAM2

In one study, the extracellular part of NCAM2 was found not to form *trans*-interactions with NCAM [17]. However, no further studies of heterophilic NCAM2 interactions have been published, and no heterophilic extracellular or intracellular ligands for NCAM2 have been identified.

Conversely, NCAM has been demonstrated to have numerous binding partners, both intra- and extracellularly (reviewed in [44]). The high sequence identity between NCAM and NCAM2 indicates that the proteins might share binding partners. Unfortunately, only a few heterophilic binding sites on NCAM have been mapped, despite the extensive number of known NCAM binding partners. The most well-described heterophilic binding sites of NCAM include the binding sites for heparin, the fibroblast growth factor receptor (FGFR), and adenosine 5′-triphosphate

(ATP) [45–47]. These binding sites are marked in Fig. 1, which also indicates that none of these binding sites are well conserved between NCAM and NCAM2.

NCAM exhibits low extracellular ATPase activity, and the interaction between ATP and NCAM has been mapped to a region close to a so-called Walker A motif (or P-loop) located in the Fn3II module of NCAM [47, 48]. The binding of ATP to NCAM interferes with the binding of FGFR to NCAM, and thereby with NCAM-mediated signaling through FGFR [47]. Furthermore, the binding of ATP to NCAM promotes extracellular proteolysis of NCAM by matrix metalloproteases [49]. NCAM2 also contains a Walker A motif in the Fn3II module. However, the motif is located within a different loop of the module, and it is not known whether ATP binds to NCAM2.

The cytoplasmic region of NCAM-140 has been reported to interact with a number of proteins, including phospholipase Cγ, acidic leucine-rich nuclear phosphoprotein 32 family member A (LANP; a phosphatase inhibitor), syndapin (an SH3-domain-containing protein involved in vesicle trafficking), the receptor protein tyrosine phosphatase RPTPα, the serine/threonine protein phosphatases PP1 and PP2A, the actin-binding protein α-actinin, and α- and β-tubulin [50–52]. The exact binding sites on NCAM for these proteins have not been identified, and consequently, it is not immediately apparent whether some of the same proteins also might interact with NCAM2.

Functions

NCAM2 in the Olfactory System

During neuronal development, guidance of axons to their respective targets is based on the ability of proteins located on axons and growth cones to recognize guidance signals in the form of soluble molecules or cell surface proteins. Proteins involved in the guidance and growth of axons include CAMs [53], and in the olfactory sensory system NCAM2 appears to play a central role [54, 55].

In the main olfactory system, axons from OSNs connect the olfactory epithelium (OE) with the main OB and in the accessory olfactory system axons from VSNs connect the vomeronasal epithelium with the accessory OB. The individual OSNs each express a single type of odorant receptor (OR), and OSNs expressing a specific OR are confined to specific regions of the OE [56–60]. The OE and main OB are divided into four zones, and NCAM2 has been found to be one of the few proteins demonstrating a distinct expression pattern in the olfactory system [13, 14], being expressed in zones Z2, Z3, and Z4, but not in zone Z1 [10, 11]. Furthermore, axons of NCAM2-positive sensory neurons in the olfactory and vomeronasal epithelium form synapses with NCAM2-negative M/T-cells, whereas, axons of NCAM2-negative sensory neurons form synapses with NCAM2-positive M/T-cells [39, 40]. NCAM2 is the only vertebrate CAM shown to exhibit an expression pattern

correlating with OR expression zones [61, 62], and to date, this has only been demonstrated for few other proteins. These include the enzyme NADPH:quinone oxidoreductase (NQO1), a member of the medium-chain acyl-CoA synthetase family O-MACS, and members of the "regulators of G-protein signaling" family, RGS9, and RGSZ1. [61–64].

The unique expression pattern of NCAM2 suggests that the protein plays an important role in the development of the olfactory sensory system, and NCAM2-positive neurons cultured *in vitro* have been demonstrated to grow larger axonal arbors and are more fasciculated than explants of NCAM2-negative neurons[55].

Unexpectedly, NCAM2 knockout mice do not demonstrate alterations in the distribution of OSNs in the olfactory epithelium, although the compartmental organization of OSN axons and target cell dendrites within neuropils (glomeruli) of the OB is disturbed [61]. Thus, further studies are required to establish the importance of NCAM2 for the development and function of the olfactory sensory system.

Interestingly, odor treatment of rabbit olfactory epithelium has been shown to cause a reduction in NCAM2 immunoreactivity in regions of the main OB, when identified by the R4B12 antibody using an immunohistochemical technique, whereas, no reduction could be demonstrated by Western blotting of OB membrane lysates using the same antibody. These observations suggest that odor treatment of the olfactory epithelium subsequently leads to a conformational change of NCAM2, or promotes interactions between NCAM2 and other molecules, thereby preventing an identification of NCAM2 in regions of the OB by R4B12 using immunohistochemical techniques. [12]

In addition to the potential participation of NCAM2 in the development of the olfactory sensory system, NCAM2 has been suggested to contribute to the establishment and maintenance of dendritic bundles in the granular retrosplenial cortex, a region involved in spatial learning and memory [65–68].

NCAM2 in Down Syndrome, Autism, and Cancer

The function of NCAM2 might be involved in a number of neurological diseases, including Down syndrome and autism [16, 69]. In humans, *NCAM2* is located on chromosome 21. Trisomy 21 (the presence of three copies of chromosome 21 in somatic cells) is the cause of Down syndrome, and consequently, a potentially excessive expression of *NCAM2* has been suggested to be one of the factors contributing to the symptoms of this disease [16]. However, a mouse model of Down syndrome that involves trisomy of a distal part of chromosome 16 (mimicking trisomy 21 in humans), does not affect the number of *Ncam2* genes, suggesting that the expression levels of NCAM2 may be unrelated, or at least not pivotal, to the development of Down syndrome [70].

NCAM2 is one of the genes suspected to be involved in the development of autism, a neurological disease estimated to be >90% hereditary [69]. However, the relationship between *NCAM2* and autism has not been studied in detail.

Finally, NCAM2 may be a marker of certain types of cancer, including human prostate cancer [71, 72]. The transcription from *NCAM2* was recently demonstrated to be under the control of STAT5, an oncogenic transcription factor dysregulated in numerous cancers [73]. However, it has not been demonstrated whether increased activity of STAT5 leads to increased levels of NCAM2.

Concluding Remarks

The first reports describing NCAM and NCAM2 were published more than 20 years ago [8, 9, 14, 15]. Since then, these proteins have been demonstrated to be more than mere mediators of cell adhesion. Whereas NCAM still is the subject of many investigations, surprisingly few studies have focused on NCAM2. As described earlier, the high sequence homology between NCAM and NCAM2 suggests that the two proteins have a number of properties in common. However, distinct differences also exist between the two proteins, with regard to both their expression patterns and their primary structures.

Studies of NCAM2 have predominantly focused on the expression of the protein in the olfactory sensory system [11, 17, 54, 55]. However, some of the early studies of NCAM2 demonstrated that the protein is expressed in many regions of the human CNS as well as in other tissues [16], suggesting that NCAM2 is not only a protein involved in the development and maintenance of the olfactory sensory system. Furthermore, as mentioned earlier, NCAM2 may be a marker or a modulator of certain types of cancer and neurological diseases, suggesting that more attention ought to be given to the function and expression of the protein.

A better understanding of the mechanisms underlying the functions of NCAM2 can be obtained by investigating the homo- and heterophilic interactions mediated by the protein. Much of the knowledge of the homo- and heterophilic interactions of NCAM derives from studies of recombinant NCAM constructs from which protein structures have been obtained by nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography [42, 43, 47, 74]. Nevertheless, different models for the homophilic *cis*/*trans*-interactions mediated by NCAM still exist (reviewed in [41]). Knowledge of the molecular interactions mediated by NCAM2 might help to clarify the molecular mechanisms of homophilic interactions mediated by NCAM, suggesting that more attention should be given to the protein structure of NCAM2. The structure of the Fn3I module of NCAM2 recently was solved by NMR spectroscopy (PDB code 2doc). However, the potential protein interactions mediated by this module have not been investigated.

This review raises many fundamental questions regarding the function of NCAM2 that remain to be answered. NCAM2 appears to play a role in the normal nervous system as well as in certain diseases, and more research will elucidate the function of the protein. Furthermore, knowledge about the structure and function of NCAM2 will contribute to the understanding of neural CAMs in general, and can,

therefore, be important for the understanding of neurodegenerative diseases and other diseases of the nervous system.

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Part IX
Conclusion

Honoring Dr. Elisabeth Bock

Vladimir Berezin

I first met Elisabeth in October 1980 when I came to the Protein Laboratory and spent 9 months working under her supervision as a guest researcher. It was an exciting time where I was introduced to the most powerful tools of protein purification and identification at the time and also to NCAM-related research. I returned to the laboratory in 1993, and since then Elisabeth and I have been working in close collaboration on determining the structure and function of the NCAM molecule.

Elisabeth did not plan to become a neuroscientist. In high school, she studied Latin and Greek and read Plato and Cicero in their original languages. She seriously considered the study of linguistics, but employment opportunities seemed dim. She therefore chose the path of her father and became a medical doctor. While studying medicine, she was attracted to psychiatry and neurology, and already as a student she worked for periods in various psychiatric hospitals. After receiving her medical degree, she obtained a research position at the Psychiatric Department at the University State Hospital in Copenhagen. Her project was the study of proteins in cerebrospinal fluid, and she immediately realized that she had found her true calling. She employed refined techniques of quantitative immunoelectrophoresis, which at that time represented a major step forward in protein chemistry. She soon applied this technology to the study of brain proteins, and was the first to identify two important brain proteins, NCAM, originally named D2 [1] and synaptophysin, also called C3/synaptin [2–4]. Simultaneously, she identified the function of the first described neuron-specific protein, the 14-3-2 protein, which she discovered was a γ-enolase (today termed neuron-specific enolase [2]). In 1974, Elisabeth was employed by the Protein Laboratory at the Medical Faculty of Copenhagen University, and she has worked there ever since, becoming Professor in Cell Biology in 1987. She was the first woman to receive a full professorship at the Medical Faculty of Copenhagen University. During subsequent years, Elisabeth has

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received several prizes and honors including a prestigious honorary doctorate from Bergen University in Norway.

Her scientific efforts have resulted in more than 400 articles focused mainly on determining the structure and function of NCAM, including structural determination of NCAM fragments by means of X-ray crystallography and nuclear magnetic resonance analysis [5–8]. Moreover, signal transduction mediated via NCAM has been studied in detail, leading to a precise characterization of the activated pathways and the relationship between these pathways and various functions [9–11]. As part of the characterization of NCAM, a series of binding sites involved in homophilic interaction and heterophilic binding to various counter-receptors have been identified. Most untraditionally, these sites have been prepared as peptides; in many cases, functional peptides mimicking NCAM function have been prepared. The peptide mimetics include the C3 peptide [12], the P2 peptide [7], the FGL peptide [13, 14], and many others. Some of these peptides have turned out to have highly intriguing and beneficial effects on learning and memory in normal animals [15], and in various animal models of neurological diseases such as Alzheimer's disease [16] and traumatic brain injury [17].In addition to her scientific work, Elisabeth has also undertaken a long series of administrative tasks. At the University of Copenhagen, she chaired the committee for the establishment of an elitarian Master's study in Human Biology, which today is highly respected by students, employers, and official authorities. For this, she received the Harald of the Year, a prestigious prize given by Copenhagen University to professors that have made a unique contribution within teaching and education.

Elisabeth has also headed a committee that has planned the strategy of Copenhagen University within the field of biotechnology and in this connection she proposed the establishment of a biotechnological center, the BioCenter. A governmental appropriation of approximately 150 mio € for the BioCenter building was elicited by two reports written by Elisabeth for the Copenhagen University and the Ministry of Science, Technology and Development. The Center was inaugurated in 2007 and is now fully functioning and already highly successful.

Elisabeth has held a series of honorary offices and positions of trust, including membership on the Danish Medical Research Council and on boards of various international societies, including the International Society for Neurochemistry (ISN) and the European Society for Neurochemistry (ESN). In ISN, she acted first as secretary and subsequently as president during the 1990s. In ESN, she was treasurer and head of the Program Committee for the biannual meeting in St. Petersburg in 1998. This international work has given Elisabeth an extensive professional network, resulting in her participation as partner and coordinator of a long series of European Union Consortia. Recently, she was the coordinator of the successful PROMEMORIA Consortium that had seventeen partners, three of which were small- to medium-sized enterprises. The Consortium focused on the role of cell recognition processes and dysfunctional plasticity, learning and memory. In this connection, the Consortium characterized the many peptide mimetics developed by Elisabeth in close collaboration with me. These inventions have led to a series of patent applications and to the establishment of ENKAM Pharmaceuticals A/S.

Elisabeth's first article was published in 1970 and she has ever since been highly productive. In her daily professional life, Elisabeth is a very energetic colleague and supervisor. She seeks to create a positive environment for the many students and guest researchers at the Protein Laboratory, and she designs projects for the students aimed at yielding publishable and relevant results giving them a solid foundation for a career in science. She stresses the role of scientific integrity and advises her students to focus on important problems. She does not take no for an answer, and she continually challenges colleagues and students to perform optimally.

Privately Elisabeth is very entertaining with a lot of humor and an impressive knowledge about literature, art, history, and politics. She has three children and four grandchildren with Nils Axelsen, to whom she has been happily married for 40 years. For relaxation, she and Nils spend their vacations on exhausting hiking, preferably in the Alps or the mountains of Corsica.

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