

Neurite Fasciculation Mediated by Complexes of Axonin-1 and Ng Cell Adhesion Molecule

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Abstract. Neural cell adhesion molecules composed of immunoglobulin and fibronectin type III-like domains have been implicated in cell adhesion, neurite outgrowth, and fasciculation. Axonin-1 and Ng cell adhesion molecule (NgCAM), two molecules with predominantly axonal expression exhibit homophilic interactions across the extracellular space (axonin-1/axonin-1 and NgCAM/NgCAM) and a heterophilic interaction (axonin-1–NgCAM) that occurs exclusively in the plane of the same membrane (*cis*-interaction). Using domain deletion mutants we localized the NgCAM homophilic binding in the Ig domains 1-4 whereas heterophilic binding to axonin-1 was localized in the Ig domains 2-4 and the third FnIII domain. The NgCAM–NgCAM interaction could be established simultaneously with the axonin-1–NgCAM interaction. In contrast, the axonin-1–NgCAM interaction excluded

axonin-1/axonin-1 binding. These results and the examination of the coclustering of axonin-1 and NgCAM at cell contacts, suggest that intercellular contact is mediated by a symmetric axonin-1₂/NgCAM₂ tetramer, in which homophilic NgCAM binding across the extracellular space occurs simultaneously with a *cis*-heterophilic interaction of axonin-1 and NgCAM. The enhanced neurite fasciculation after overexpression of NgCAM by adenoviral vectors indicates that NgCAM is the limiting component for the formation of the axonin-1₂/NgCAM₂ complexes and, thus, neurite fasciculation in DRG neurons.

Key words: axonal fasciculation • axon guidance molecules • cell adhesion molecules • cell contact • cell recognition

THE precise wiring of the neuronal network is essential for the proper information-processing function of the nervous system. During the formation of the basic network neurons send out processes that reach their target regions in a highly stereotyped and precise manner and very few errors of navigation occur (Dodd and Jessell, 1988; Jessell, 1988; Hynes and Lander, 1992; Luo et al., 1993; Goodman and Shatz, 1993). The correct trajectory of growing fibers is determined by the growth cone, a specialized sensory motor organelle at the tip of the growing axon. Growth cones are capable of axon elongation in response to diffusible or surface-bound growth-promoting molecules and to make the correct decisions at pathway intersections and crossings (Tessier-Lavigne and Goodman, 1996; Cook et al., 1998). Axons growing out after the so-called pioneers tend to make use of the scaffold formed by earlier axons, by virtue of the growth-promoting mole-

cles displayed along their axonal shaft, giving rise to axon fascicles. Selective axonal fasciculation represents a simplifying strategy in the assembly of complex neuroanatomical structures (Van Vactor, 1998).

Two large families of neural cell adhesion molecules (CAMs)¹, the cell adhesion molecules of the Ig superfamily (IgSF) and the cadherins, represent local cues for axon guidance and specific fasciculation (Rathjen and Jessell, 1991). A large subfamily of IgSF CAMs, the immunoglobulin/fibronectin type-III (IgFnIII) class of molecules, is composed of Ig domains in conjunction with FnIII-like domains (Williams and Barclay, 1988; Chothia and Jones, 1997). One group of IgFnIII CAMs is attached to the cell membrane by a GPI-anchor and includes axonin-1/TAG-1 (Ruegg et al., 1989a; Stoeckli et al., 1989; Furley et al., 1990; Zuellig et al., 1992), F11 (contactin)/F3 (Bruemmen-

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1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; DRG, dorsal root ganglia; ECL, enhanced chemiluminescence; ECM, extracellular matrix; Fab, fragment with an antigen binding site; FnIII, fibronectin type-III; IgSF, immunoglobulin superfamily; IgFnIII, immunoglobulin/fibronectin type-III class of molecules; NBCS, newborn calf serum; pfu, plaque-forming unit.

dorf et al., 1989; Gennarini et al., 1989), and PANG/BIG1 (Connelly et al., 1994; Yoshihara et al., 1994, 1995). The molecules of this group are composed of six Ig domains in conjunction with four FnIII-like domains in their extracellular part. A second group includes transmembrane glycoproteins composed of six Ig domains and five FnIII-like domains, including NgCAM/L1 (Moos et al., 1988; Burgoon et al., 1991), neurofascin (Volkmer et al., 1992), and NrCAM (Grumet et al., 1991; Kayyem et al., 1992). The molecules of these subgroups are expressed predominantly on axons and are involved in neurite extension (Lemmon et al., 1989; Rathjen, 1991; Stoeckli et al., 1991; Lemmon et al., 1992), axonal pathfinding (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997; Stoeckli and Landmesser, 1998), and neurite fasciculation (Rathjen et al., 1987; Ruegg et al., 1989b; Rathjen and Jessell, 1991; Stoeckli and Landmesser, 1995). They exhibit a complex pattern of interactions: e.g., axonin-1 interacts with NgCAM (Kuhn et al., 1991), NrCAM (Suter et al., 1996), neurocan, and phosphacan/protein-tyrosine phosphatase β (Milev et al., 1996; Holland et al., 1998), whereas NgCAM binds to axonin-1, F11 (contactin; Bruemmendorf et al., 1993), DM-GRASP (DeBernardo and Chang, 1997), laminin (Grumet et al., 1993a) and the proteoglycans 3F8, neurocan, and phosphacan (Grumet et al., 1993b; Friedlander et al., 1994; Milev et al., 1994). Axonin-1 and NgCAM, in addition, exhibit homophilic interactions (Lemmon et al., 1989; Rader et al., 1993).

The role of axonin-1 and NgCAM in specific neurite fasciculation has been demonstrated by *in vitro* and *in vivo* studies (Ruegg et al., 1989a; Stoeckli et al., 1991; Stoeckli and Landmesser, 1995) and is further supported by the specific coclustering of these molecules at sites of contact between adjacent neurites *in vitro* (Honig and Kueter, 1995). A crucial role of NgCAM-mediated cell adhesion in the organization of the axonal cytoskeleton has been suggested by the fact that all vertebrate and invertebrate members of the NgCAM/L1 family contain two highly conserved amino acid sequences in their cytoplasmic domains, which were found to bind ankyrin, a protein that acts as an adaptor between membrane proteins and the actin spectrin-based cytoskeleton (Davis et al., 1993; Davis and Bennett, 1994). The intracellular association of ankyrin with neuroglian, the *Drosophila* homologue of NgCAM/L1, depends on the extracellular homophilic interaction (Dubreuil et al., 1996). Thus, members of the NgCAM/L1 family may be able to recruit cytoskeletal components to cell contact sites, and, thus, shape the connections between the cell surface and the cytoskeleton, depending on their pattern of extracellular interactions. We have recently observed that the progressing fasciculation in cultured sensory neurons is accompanied by the formation of heterodimeric and heterotetrameric complexes of NgCAM and axonin-1 (Kunz et al., 1996). Based on these results, we have speculated that heterooligomeric complexes composed of IgFnIII CAMs, such as NgCAM and axonin-1, may represent the functional units of cell recognition. It is, therefore, conceivable that the different functional roles of these molecules like those in axon growth-promotion, axon guidance, and axonal fasciculation, are determined by the composition of the CAM complexes at the sites of contact and the nature of the intracellular sig-

nals elicited by the specific complexes. The investigation of the structural and functional features of such complexes is therefore of pivotal importance for an understanding of CAM function at the molecular level.

In this study, we identified the NgCAM domains involved in the homophilic NgCAM/NgCAM interaction and the heterophilic axonin-1–NgCAM interaction. We investigated the binding pattern of axonin-1 and NgCAM in the areas of cell–cell contacts and found that homophilic NgCAM/NgCAM interaction can occur simultaneously with the heterophilic axonin-1–NgCAM binding, whereas the heterophilic axonin-1–NgCAM interaction and the homophilic axonin-1/axonin-1 binding are mutually exclusive. Based on these results, we suggest a model for a symmetric tetrameric axonin-1₂/NgCAM₂ complex that may represent the functional unit of cell adhesion and signal transduction of neurite fasciculation by axonin-1 and NgCAM.

Materials and Methods

Proteins and Antibodies

Axonin-1 was purified from chicken ocular vitreous fluid as described previously (Ruegg et al., 1989a). NgCAM was immunoaffinity purified from embryonic day 14 chicken brain membranes using the monoclonal antibody 12-I-14-E 311, provided by Fritz Rathjen (Rathjen et al., 1987). Laminin (mouse, isolated from Engelbreth-Holm-Swarm sarcoma) was purchased from GIBCO-BRL laboratories (Gaithersburg, MD). Polyclonal serum G67 against axonin-1 (Ruegg et al., 1989a) and G4 against NgCAM was raised in goat (Kuhn et al., 1991). IgG and Fab were obtained as previously specified (Stoeckli et al., 1991). Cy3-conjugated anti-mouse IgG and Texas red-conjugated anti-rabbit IgG was from Zymed Laboratories Inc. (South San Francisco, CA), FITC-conjugated anti-goat IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA), and anti-sheep/goat IgG secondary antibodies for enhanced chemiluminescence (ECL) detection were from Boehringer Mannheim (Mannheim, Germany).

Production of Soluble Axonin-1 Variants in Myeloma Cells

The production of the myeloma cell lines that constitutively express and secrete the fusions of axonin-1 (or fragments thereof) and the constant domain of the mouse Ig κ -light chain, axonin-1 Ig1234-C κ and Δ Ig1234-C κ is described by Rader et al. (1996). For the isolation of the axonin-1 variants, transfected myeloma cells were adapted to 2% FCS in DME and cultivated in roller bottles (Costar, Cambridge, MA). Supernatants were concentrated by ultrafiltration (Skanette; Skan AG, Basel, Switzerland) and loaded on an immunoaffinity column with rat anti-mouse C κ mAb 187.1 (American Type Culture Collection, Rockville, MD) coupled to Sepharose 4B. The column was washed with 40 column volumes of PBS and eluted with 50 mM diethylamine, 100 mM NaCl, pH 11.0. The eluate was immediately neutralized by the addition of 1 M Tris-HCl, pH 7.0, and dialysis against PBS. The integrity of the fusion proteins was tested by SDS-PAGE and Western blot analysis using anti-axonin-1 and anti-C κ antibodies for detection.

Construction of NgCAM Deletion Mutants

The domain deletion mutants of NgCAM were inserted downstream of the cytomegaloviral promoter into the eucaryotic expression vector pSCT (Buchstaller et al., 1996). Domain borders were defined according to the homology of the NgCAM domains to the Ig domains or the type-III domains of fibronectin (Burgoon et al., 1991). To construct the domain deletion mutants XhoI restriction sites were introduced at the domain borders by PCR cloning. In brief, the cDNA fragments 1Fc-Ig01B, Ig12F-10B, 1Fc-Ig12B, Ig23F-10B, 1Fc-Ig23B, Ig34F-10B, 1Fc-Ig34B, Ig45F-4B, 1Fc-

Ig45F, Ig56F-4B, 13F-Ig56B, Ig6Fn1F-4B, 13F-Ig6Fn1B, Fn12F-Fn45B, Ig6Fn1F-Fn12B, Fn23F-Fn45B, Ig6Fn1F-Fn23B, Fn34F-2B, Ig6Fn1F-Fn34B, Fn45F-2B, Ig6Fn1F-Fn45B, FN5TMF-2B were amplified using the polymerase chain reaction on the full-length NgCAM cDNA cloned into the pSCT vector (Buchstaller et al., 1996) as a template. PCR amplification was performed as described (Rader et al., 1993).

The following primer sequences were used: 1Fc: 5'CGTGAAGGCTCCGCCATGGCTCTGCCCATGGGCTCG3'; Ig01B: 5'CGGGGGCTCGAGGAAATCGTGTGC3'; Ig12F: 5'CCAACGTCATCC-TCCGAGAACACTCCG3'; Ig12B: 5'GGAGTGTCTCGAGGATGACGTTGGC3'; 13F: 5'CGAAGCTTCCGAGTGGCCGGAGAAGAAGG3'; Ig23F: 5'GAAGGAGCCCTCGAGTCCCG3'; Ig23B: 5'GCCACGCGGAGCTCGAGGGGCTCC3'; Ig34F: 5'CCACAGCGT-CACCCTCGAGGCCGCC3'; Ig34B: 5'GGGGCGGCTCGAGGG-TGACGC3'; Ig45F: 5'CCTGCGACTCCTCGAGTCCCGTCC3'; Ig45B: 5'GGACCGGAGCTCGAGGAGTCCAGG3'; 10B: 5'GAA-GGATCGATCGTCTCGAGAGC3'; Ig56F: 5'CGGCACTCTCGA-GGTCAGAGCC3'; Ig56B: 5'GGCCCTGACCTCGAGGAGCGCC3'; Ig6Fn1F: 5'CCGGCCCCCTCGAGGACCTCC3'; Ig6Fn1B: 5'CTT-GGAGTCTCGAGGGGGCC3'; Fn12F: 5'CCCCGGCTCTCG-AGCGCAACC3'; Fn12B: 5'CCGGTTGCGCTCGAGAGCCCG3'; Fn23F: 5'CGTTGGTTTACCTCGAGAATGTGGG3'; Fn23B: 5'CCCC-ACATTCTCGAGGTAACCAACCG3'; 4B: 5'GGAAGCTTACTTA-CCTATTGTCGACGACTCGCTCCCC3'; Fn34F: 5'GGCCCCCCTCGAGCCCATCCG3'; Fn34B: 5'GAAGCGATGGGCTCGA-GGGGGG3'; Fn45F: 5'CGGCCCGCCCTCGAGACGGTGGG3'; Fn45B: 5'GCTCCCCACCTCTCGAGGGCG3'; Fn5TMF: 5'CCGTG-GTGGCAGCCTCGAGG3'.

The construction of Δ Ig1 was performed as follows: 1Fc-Ig01B was digested with XbaI/XhoI and Ig12F-10B with XhoI/RsrII. The two fragments were then cloned into the expression vector pSCTNgCAM (Buchstaller et al., 1996), previously digested with XbaI/RsrII. Using the same strategy, all the other NgCAM domain deletion mutants were generated: Δ Ig2: 1Fc-Ig12B digested with XbaI/XhoI, Ig23F-10B with XhoI/RsrII, cloned into pSCTNgCAM XbaI/RsrII; Δ Ig3: 1Fc-Ig23B digested with MluI/XhoI, Ig34F-10B with XhoI/SfiI, cloned into pSCTNgCAM MluI/SfiI; Δ Ig4: 1Fc-Ig34B digested with XbaI/XhoI, Ig45F-4B with XhoI/RsrII, cloned into pSCTNgCAM XbaI/RsrII; Δ Ig5: 1Fc-Ig45F digested with MluI/XhoI, Ig56F-4B with XhoI/HpaI, cloned into pSCTNgCAM MluI/HpaI; Δ Ig6: 13F-Ig56B digested with RsrII/XhoI, Ig6Fn1F-4B with XhoI/HpaI, cloned into pSCTNgCAM RsrII/HpaI; Δ Fn1: 13F-Ig6Fn1B digested with RsrII/XhoI, Fn12F-Fn45B with XhoI/SacI, cloned into pSCTNgCAM RsrII/SacI; Δ Fn2: Ig6Fn1F-Fn12B digested with HpaI/XhoI, Fn23F-Fn45B with XhoI/SacI, cloned into pSCTNgCAM HpaI/SacI; Δ Fn3: Ig6Fn1F-Fn23B digested with HpaI/XhoI, Fn34F-2B with XhoI/NarI, cloned into pSCTNgCAM HpaI/NarI; Δ Fn4: Ig6Fn1F-Fn34B digested with HpaI/XhoI, Fn45F-2B with XhoI/NarI, cloned into pSCTNgCAM HpaI/NarI; Δ Fn5: Ig6Fn1F-Fn45B digested with HpaI/XhoI, FN5TMF-2B with XhoI/NarI, cloned into pSCTNgCAM HpaI/NarI.

The double domain deletion mutants were constructed as follows: Δ Ig12: 1Fc-Ig01B digested with XbaI/XhoI, Ig23F-10B with XhoI/RsrII, cloned into pSCTNgCAM XbaI/RsrII; Δ Ig23: 1Fc-Ig12B digested with XbaI/XhoI, Ig34F-10B with XhoI/SfiI, cloned into pSCTNgCAM XbaI/SfiI; Δ Ig34: 1Fc-Ig23B digested with MluI/XhoI, Ig45F-4B with XhoI/RsrII, cloned into pSCTNgCAM MluI/RsrII; Δ Ig45: 1Fc-Ig34B digested with XbaI/XhoI, Ig56F-4B with XhoI/HpaI, cloned into pSCTNgCAM XbaI/HpaI. Δ Ig56: 1Fc-Ig45F digested with MluI/XhoI, Ig6Fn1F-4B with XhoI/HpaI, cloned into pSCTNgCAM MluI/HpaI; Δ Ig6Fn1: 13F-Ig56B digested with RsrII/XhoI, Fn12F-Fn45B with XhoI/SacI, cloned into pSCTNgCAM RsrII/SacI; Δ Fn12: 13F-Ig6Fn1B digested with RsrII/XhoI, Fn23F-Fn45B with XhoI/SacI, cloned into pSCTNgCAM RsrII/SacI; Δ Fn23: Ig6Fn1F-Fn12B digested with HpaI/XhoI, Fn34F-2B with XhoI/NarI, cloned into pSCTNgCAM HpaI/NarI; Δ Fn34: Ig6Fn1F-Fn23B digested with HpaI/XhoI, Fn45F-2B with XhoI/NarI, cloned into pSCTNgCAM HpaI/NarI; Δ Fn45: Ig6Fn1F-Fn34B digested with HpaI/XhoI, FN5TMF-2B with XhoI/NarI, cloned into pSCTNgCAM HpaI/NarI.

For the construction of the NgCAM mutant Δ Ig56Fn1 the fragment XbaI(565)-XhoI(1815), obtained from the XbaI/XhoI digest of Δ Ig5, and the fragment XhoI(2716)-SacI(3422), from a XhoI/SacI digest of Δ Fn1, was cloned into pSCTNgCAM XbaI/SacI. For the construct Δ Ig6Fn12, XbaI(565)-XhoI(2122), from a XbaI/XhoI digest of Δ Ig6, and XhoI(3040)-SacI(3422), from a XhoI/SacI digest of Δ Fn2, were cloned into pSCTNgCAM XbaI/SacI. For the cloning of Δ Ig56Fn12, XbaI(565)-XhoI(1815), obtained from XbaI/XhoI digest of Δ Ig5, and XhoI(3040)-SacI(3422), from a XhoI/SacI digest of Δ Fn2, were cloned into pSCTNgCAM XbaI/SacI.

All Deletion Mutants Were Verified by Double Strand DNA Sequencing

Based on the numbering of the amino acid sequence of NgCAM given by Buchstaller et al. (1996), the following segments were deleted in the mutants: Δ Ig1: Q35-A129; Δ Ig2: E130-L222; Δ Ig3: D223-V325; Δ Ig4: E326-V418; Δ Ig5: E419-L508; Δ Ig6: E509-R610; Δ Fn1: R610-P707; Δ Fn2: E708-P816; Δ Fn3: E817-S926; Δ Fn4: E927-L1024; Δ Fn5: Q1025-L1137; Δ Ig12: Q35-L222; Δ Ig23: E130-V325; Δ Ig34: D223-V418; Δ Ig45: E326-L508; Δ Ig56: E419-R619; Δ Ig6+Fn1: E509-P707; Δ Fn12: R610-P816; Δ Fn23: E708-S926; Δ Fn34: E817-L1024; Δ Fn45: E927-L1134; Δ Ig56Fn1: E419-P707; Δ Ig6Fn12: E509-P816; Δ Ig56Fn12: E419-P816.

The insertion of an XhoI site resulted in most of the cases in silent or conservative mutations of amino acids flanking the deletions. The following replacements were made: Δ Ig2: A129-L129, D223-E223; Δ Ig4: V325-L325; Δ Ig5: V418-L418; Δ Ig6: R610-E610; Δ Fn1: I609-L609; Δ Fn2: P707-L707; Δ Fn3: P816-L816; Δ Fn4: S926-L926, Q1025-E1025; Δ Fn5: V1137-E1137; Δ Ig12: D223-E223; Δ Ig23: A129-L129; Δ Ig45: V325-L325; Δ Ig56: V418-L418, R610-E610; Δ Fn12: I609-L609; Δ Fn23: P707-L707; Δ Fn34: P816-L816, Q1025-E1025; Δ Fn45: S926-L926, V1137-E1137; Δ Ig56Fn1 and Δ Ig56Fn12: V418-L418.

Construction of the Point Mutants NgCAM PM1-PM3 and the Deletion Mutant NgCAM Δ P871-V888

For the construction of NgCAM variants deficient for proteolytic processing, the following point mutations were introduced to the cleavage site: NgCAM PM1: R865A; NgCAM PM2: R865A and R864S; and NgCAM PM3: R865A, R864S, and R862A. R865, R864, and R862 correspond to the conserved Arg residues at the positions -1, -2, and -4 of the putative furin cleavage site described by Grumet and Sakurai (1996). The point mutations were introduced by PCR using the primers PM1, PM2, and PM3 that contained the necessary base exchanges.

The following primer sequences were used: PM1, 5'GTGGGGGAGCGCAGTCTGTCACAAGCCCCCCCC3'; PM2, 5'GTGGGGGAGCGCAGTAGTGCACAAGCCCCCCCC3'; PM3, 5'GTGGGGGAGGCGCAGTAGTGCACAAGCCCCCCCC3'; PMd, 5'GGGGAGCTCAGT-CGTGCACAAGCCCCCCCC3'.

The introduction of R865A resulted in a new ApaLI restriction site in position 3186 of pSCTNgCAM. The PCR fragments Fn12F-PM1, Fn12F-PM2, and Fn12F-PM3 were digested with KpnI/ApaLI. The PCR fragment PMd-Fn45B was digested with ApaLI/SacI. Fn12F-PM1, Fn12F-PM2, and Fn12F-PM3 KpnI/ApaLI and PMd-Fn45B ApaLI/SacI were cloned into pSCTNgCAM KpnI/SacI.

To construct the deletion mutant Δ P871-V888, a SacI restriction site was introduced at positions 870 and 889 of the amino acid sequence by PCR cloning, resulting in the exchanges D870E and A889L. The following primer sequences were used: LF, 5'GATTGGGGGAGCTCGGGGGGGGCTTG3' and LB, 5'CCCCCGAGCTCCTGACAGTGGGGGGGGAC3'.

In brief, the PCR fragments Fn12F-LB, digested with KpnI/SacI, and the fragment LF-Fn45B, digested with SacI/SacI were cloned into pSCTNgCAM KpnI/SacI. The NgCAM mutants were verified by double strand DNA sequencing.

Transient Transfection of COS7 Cells

Transient transfectants were obtained by electroporation according to Chu et al. (1987) and Rols et al. (1994). In brief, 10^6 COS7 cells were trypsinized and resuspended in 700 μ l of PBS and mixed with 100 μ l of PBS containing 10 μ g of vector DNA purified using the EndoFree plasmid purification kit (QuiaGen, Hilden, Germany). After a 10-min incubation on ice, the electroporation was carried out in a 1-cm cuvette by applying 960 μ F and 230 V (Bio-Rad Gene Pulser). After 10 min of incubation at 37°C, the cells were plated on 28 cm² cell culture dishes in DME supplemented with 10% FCS at 37°C in 5% CO₂. After 8 h the cells were washed twice with PBS and incubated for 16 h with 10 ml DME 10% FCS.

Immunoblotting

Transfected COS7 cells were washed twice with PBS and detached by treatment with 2 mM EDTA in PBS 2 d after transfection. For solubilization of membrane proteins the detached cells were lysed in 1% (wt/vol) Triton X-100, 1% (wt/vol) CHAPS, 0.1% (wt/vol) SDS, 5 mM EDTA, 1 mM PMSF, 1 mM iodacetamide, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5. Pro-

teins were precipitated (Wessel and Fluegge, 1984). After addition of sample buffer and boiling for 5 min, the samples were examined by gel electrophoresis and transferred to nitrocellulose. The nitrocellulose was blocked overnight with 1% (wt/vol) blocking reagent for chemiluminescence detection (Boehringer Mannheim) in TBS, then incubated for 2 h in 1% (wt/vol) blocking reagent containing the primary antibody (polyclonal serum G4 anti-NgCAM in a dilution of 1:5,000), and, subsequently, incubated for 1 h at room temperature in 0.5 μ g/ml anti-sheep/goat IgG Fab coupled to peroxidase in 0.5% (wt/vol) blocking reagent in TBS. After several washings in TBS and followed by one washing with 0.1% (wt/vol) Tween-20 in TBS, the chemiluminescence system from Boehringer Mannheim was used for detection. Exposure times for the autoradiographs was 10 s.

To assess the extent of proteolytic cleavage of the NgCAM mutants PM1, PM2, PM3, Δ P871-V888, and Δ Fn3 as well as wild-type NgCAM, quantitative Western blot analysis was performed, using the monoclonal anti-NgCAM antibody 12-I-14-E 311, that recognizes the 135-kD fragment of NgCAM, and enhanced chemiluminescence for detection. For the relative quantification of the NgCAM fragments of 135 and 200 kD, densitometric analysis with the Image QanNT software (version 4.1; Molecular Dynamics, Sunnyvale, CA) was performed. The extent of proteolytic cleavage was calculated as the ratio of cleaved NgCAM (represented by the 135-kD band) relative to total NgCAM (represented by the sum of the 135- and the 200-kD form): $I_{(135\text{ kD})}/(I_{(200\text{ kD})} + I_{(135\text{ kD})})$, where $I_{(200\text{ kD})}$ and $I_{(135\text{ kD})}$ represent the intensity of the bands of 135 and 200 kD, measured in densitometric units. The following percentages of cleavage were found: 61% (\pm 7.1) for wild-type NgCAM, 9.1 (\pm 2.8) for PM1, 8.7 (\pm 3.5) for PM2, and 2.6 (\pm 1.6) for PM3. No detectable cleavage was found for Δ P871-V888 and Δ Fn3.

Binding of Protein-conjugated Covaspheres to Transfected COS7 Cells and Immunofluorescence Staining

24 h after transfection, the cells were washed twice with PBS, detached by treatment with 2 mM EDTA in PBS, and resuspended in DME, 10% FCS. The cells were transferred to Lab-Tek glass slides coated with 250 μ g/ml poly-D-lysine (Nunc Inc., Naperville, IL), incubated at 37°C in 5% CO₂ for 24 h and then washed twice with DME, 5 mg/ml BSA. TRITC-Covaspheres were coupled to axonin-1, NgCAM, and the recombinant axonin-1-Ck fusion proteins Ig1234-Ck and Δ Ig1234-Ck as described elsewhere (Kuhn et al., 1991). The Covaspheres were sonicated for 2 min, diluted 1:1,000 in DME, 5 mg/ml BSA, and again sonicated for 3 min immediately before use. The cells were incubated with the prepared Covaspheres for 1 h at 37°C, washed twice with DME, 5 mg/ml BSA, and fixed with 2% formaldehyde for 1 h at 37°C. After washing twice with PBS, 1% FCS, the NgCAM expressing cells were visualized in direct immunofluorescence staining using a polyclonal anti-NgCAM serum (1:500) and FITC-labeled rabbit anti-goat IgG (1:100). The preparation was examined with a fluorescence microscope (Leitz DMR; Leica AG, Heerbrugg, Switzerland) using a green/red filter (Leica AG) for the simultaneous detection of FITC and TRITC fluorescence. For quantification, green fluorescing cells binding at least four Covaspheres were scored as binding cells (nonexpressing cells were found to bind 0-1 Covaspheres). In antibody blocking experiments the cells were preincubated with goat polyclonal anti-axonin-1 or anti-NgCAM Fab (250 μ g/ml) in DME, 10% FCS for 1 h. The cells were

washed three times with DMEM, 5 mg/ml BSA. Incubation with protein-conjugated Covaspheres and fixation were carried out as described above.

Confocal Laser Scanning Microscopy of Stably Transfected CV-1 Cells

CV-1 cells stably transfected with the full-length cDNA of axonin-1, NgCAM, or both (Buchstaller et al., 1996) were cultured in Lab-Tek glass slides coated with 250 μ g/ml poly-D-lysine in DME, supplemented with 2.5% FCS and 2.5% NBS, to a confluency of 20%. The cells were fixed in the culture medium by adding fixation solution to a final concentration of 2% formaldehyde and 0.1% glutaraldehyde for 1 h at 37°C. This fixation protocol was described to result in a complete immobilization of membrane proteins without permeabilization of the cells (Dubreuil et al., 1996). Axonin-1 and NgCAM were stained with goat anti-axonin-1 (1:500) and rabbit anti-NgCAM (1:1,000) serum in PBS, 10% FCS for 1 h at room temperature and rabbit anti-goat FITC and donkey anti-rabbit Texas red antibodies for detection. Confocal laser scanning microscopy was performed using a Multi Probe 2001 CLSM (Molecular Dynamics, Sunnyvale, CA) equipped with an argon/krypton laser and the single scanning mode. For the detection of FITC, the band-pass excitation filter 488DF10 was used in combination with a 510DLRP primary beam splitter and a long-pass barrier filter 510EPLP. For the detection of Texas red, the band-pass excitation filter 568DF10 was used in combination with a 488/568 dual dichronic filter as a primary beam splitter and a 590EFLP long-pass barrier filter. To obtain cell layers with comparable geometry, identical numbers of confocal sections were integrated for image generation and quantitative analysis of the distribution of axonin-1 and NgCAM. For quantification distribution coefficients were calculated. In cultures consisting of only one type of cells the distribution coefficient was calculated as: $q = 2 \times I_{\text{cell-contact}}/I_{\text{cell1}} + I_{\text{cell2}}$ and, in cases of contacts formed by different cell types (e.g., in a mixture of axonin-1 and NgCAM expressing cells), $q^* = I_{\text{cell-contact}}/I_{\text{expressing cells}}$ where the terms $I_{\text{cell-contact}}$ represents the fluorescence intensity per unit area at the cell contact, and the terms I_{cell1} , I_{cell2} , and $I_{\text{expressing cell}}$ represent the fluorescence intensity per unit area at the surface of the cells forming the contacts.

Cultivation of DRG Explants and Infection with Adenoviral Vectors for Gene Transfer

Dorsal root ganglia (DRG) were dissected from neonatal mice (ICR) and 2-3 ganglia were cultivated per well in Lab-Tek glass slides coated with 20 μ g/ml poly-D-lysine and 20 μ g/ml laminin in DME supplemented with 10% FCS, 1% N3 (Ruegg et al., 1989a; Stoeckli et al., 1991), and 40 ng/ml NGF. For infection the cultures were inoculated with 10⁷ pfu/well AdCMVax-1, AdCMVNgCAM, or AdCMVlacZ. For description of the adenoviral vectors see Giger et al. (1996) and Vogt et al. (1996). After 12 h the ganglia were washed three times with in DME supplemented with 10% FCS, 1% N3, and 40 ng/ml NGF and incubated for another 36 h. The explant cultures were fixed in the culture medium by adding fixation solution to a final concentration of 2% formaldehyde and 0.1% glutaraldehyde. For immunofluorescence staining the axonin-1-specific monoclonal antibody X7C11 or the NgCAM-specific monoclonal antibody 12-I-4E-311 (10 μ g/ml in PBS, 1% FCS for 1 h) and a Cy3-labeled donkey anti-mouse antibody (1:200, 30 min) were used. For β -galactosidase staining, the explants were fixed in 2% (wt/vol) formaldehyde for 10 minutes and

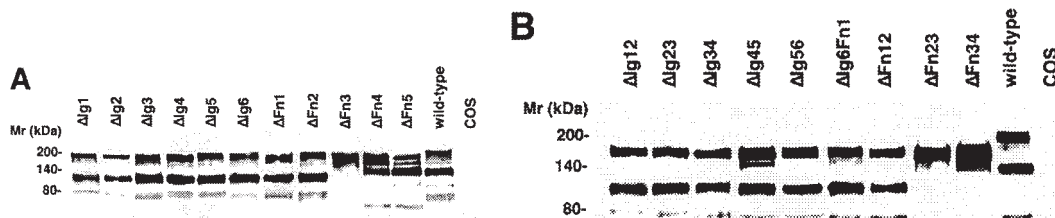


Figure 1. Analysis of the molecular masses of wild-type NgCAM and NgCAM domain deletion mutants transiently expressed in COS7 cells. Total protein of nontransfected COS cells (COS) and COS cells transfected with wild-type NgCAM (*wild-type*) and the NgCAM single (*A*) and double (*B*) domain deletion mutants was separated by SDS-PAGE, transferred to nitrocellulose and immunostained using a polyclonal anti-NgCAM antibody followed by enhanced chemiluminescence detection. Molecular masses are indicated.

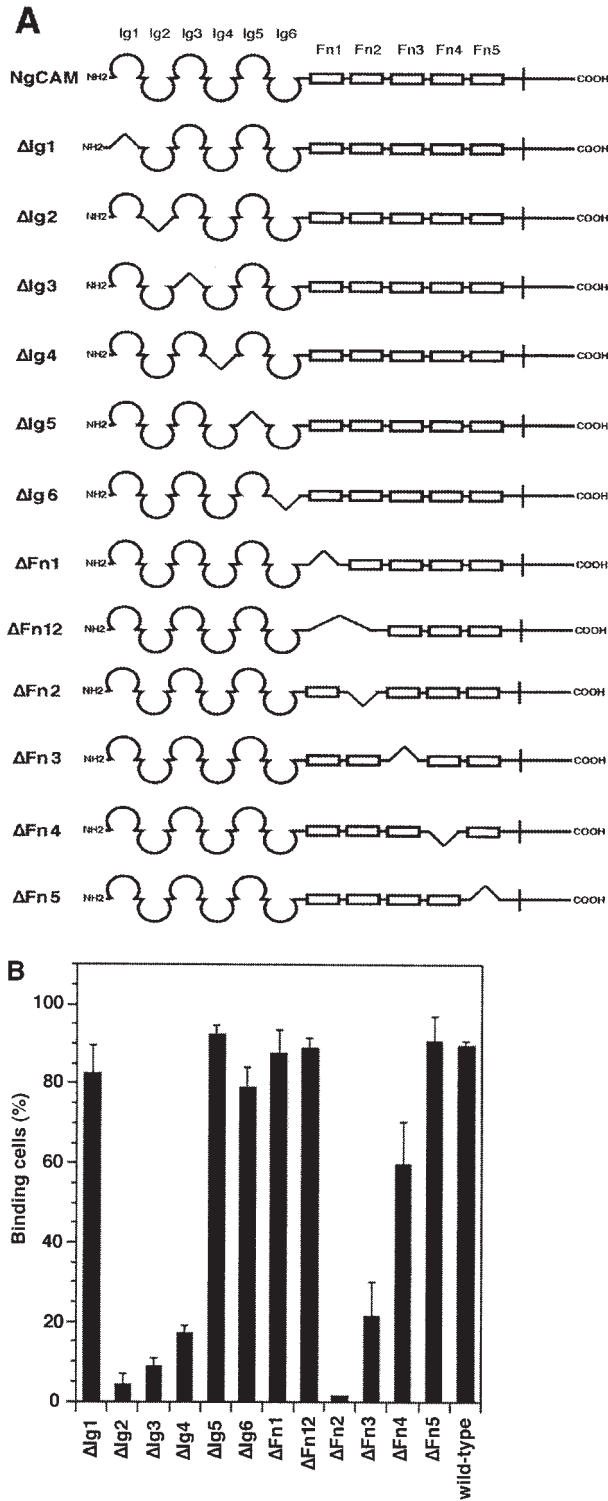


Figure 2. Axonin-1 binding to wild-type and domain deletion mutants of NgCAM. (A) Schematic representation of the NgCAM domain deletion mutants. NH₂-termini are on the left (NH₂) and COOH termini on the right (COOH). Ig domains are represented by half-circles and FnIII-like domains by rectangular boxes. The lines indicate the domain deletions. (B) Binding of axonin-conjugated TRITC Covaspheres to COS cells expressing wild-type NgCAM and the domain deletion mutants. NgCAM expressing cells were identified by indirect immunofluorescence staining using a polyclonal anti-NgCAM antibody and a FITC-labeled secondary antibody. For each mutant 100 green fluoresc-

incubated with staining solution: 5 mM K₃FeIII(CN)₆, 5 mM K₄FeII(CN)₆, 10 mM MgCl₂, and 5 mg/ml X-Gal in PBS at 37°C.

In antibody blocking experiments the explants were incubated with anti-axonin-1 or anti-NgCAM monoclonal antibodies (100 μg/ml IgG) during the whole duration of the experiment. After 48 h the explants were fixed and stained with Cy3-labeled donkey anti-mouse antibody.

For quantification of neurite fasciculation individual immunostained neurites were tracked from the periphery of the ganglion to a point at one-third of the total neurite length. Neurites that run into bundles consisting of >4 neurites formed one category and neurites found in bundles of <4 neurites the other (in each sample, 50 neurites were scored per ganglion and 6 ganglia were analyzed).

Results

The Ig Domains 2-4 and the Third FnIII Domain of NgCAM Are Essential for Axonin-1 Binding

Entire domains of NgCAM, as defined by their homology to the Ig domains or the type-III domains of fibronectin (Grumet et al., 1991; Buchstaller et al., 1996), were deleted. Two different sets of deletion mutants were generated, a complete set of single domain deletions and a complete set of double deletions of adjacent domains. This strategy was based on the observation that in many Ig-FnIII class molecules some of the folding units comprise two adjacent domains (Huber et al., 1994; Vaughn and Bjorkman, 1995). The NgCAM mutants were cloned into the NgCAM expression vector pSCTNgCAM. Transfection of COS7 cells by electroporation resulted in 30–40% of the cells expressing heterologous protein. The cells were solubilized 48 h after transfection and analyzed by immunoblotting using a polyclonal anti-NgCAM antibody. The molecular masses of the NgCAM domain deletion mutants were in agreement with the expected values (Fig. 1). In accordance with the localization of the natural proteolytic cleavage site of NgCAM in the third FnIII domain (Burgoon et al., 1991), proteolytic cleavage was absent in all mutants lacking the third FnIII domain. All mutants, except Δ Fn45, were expressed in similar quantities at the cell surface, as revealed by immunofluorescence analysis. 2 d after electroporation the cells were incubated with axonin-1-conjugated polystyrene microspheres (Covaspheres). After washing and fixation the NgCAM-expressing cells were identified by immunostaining. The deletion of each of the Ig domains Ig2-4 resulted in an almost complete loss of axonin-1 binding. Deletion of Fn2 and Fn3 resulted in strong reduction of axonin-1 binding, deletion of Fn4 in a much weaker, but still significant, reduction (Fig. 2 B). The axonin-1 binding capacities of the mutants with deleted Ig2, Ig3, Ig4, and Fn3, were in accordance with the data from the double deletion mutants lacking Ig1+2, Ig2+3, Ig3+4, Ig4+5, and the membrane proximal domain tandems Fn2+3 and Fn3+4 (data not shown). In contrast, the lack of axonin-1 binding by Δ Fn2 contradicts

ing cells were analyzed. Green fluorescing cells that bound four or more red fluorescing Covaspheres were scored as positive cells. Cells that did not express NgCAM were used as internal negative controls and were found to bind on the average 0.2 axonin-conjugated Covaspheres per cell. Each column corresponds to four independent experiments (\pm SD).

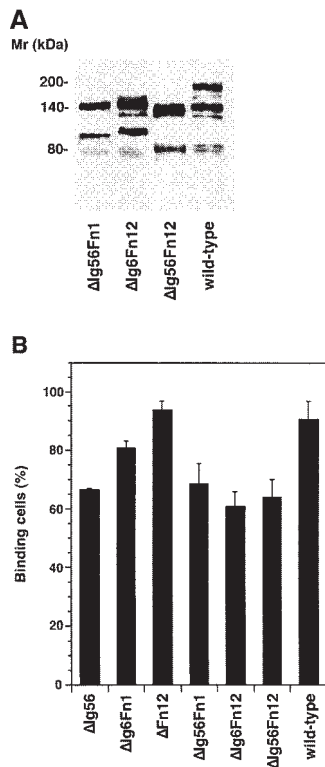


Figure 3. Axonin-1 binding to wild-type NgCAM and the domain deletion mutants Δ Ig56, Δ Ig6Fn1, Δ Fn12, Δ Ig56Fn1, Δ Ig6Fn12, and Δ Ig56Fn12. (A) Analysis of the molecular masses of the NgCAM domain deletion mutants Δ Ig56Fn1, Δ Ig6Fn12, and Δ Ig56Fn12 transiently expressed in COS7 cells. Western blot analysis was performed as described in Fig. 1, using a polyclonal anti-NgCAM antibody followed by enhanced chemiluminescence for detection. Molecular masses are indicated. (B) Binding of axonin-conjugated TRITC Covaspheres to COS cells expressing wild-type NgCAM and the domain deletion mutants Δ Ig56, Δ Ig6Fn1, Δ Fn12, Δ Ig56Fn1, Δ Ig6Fn12, and Δ Ig56Fn12. The detection of NgCAM expressing cells and the quantification was carried out as described in Fig. 2. Each column corresponds to three independent experiments (\pm SD).

the observation that axonin-1 binding is unaffected by the deletion of Fn1 and Fn2 in Δ Fn12 (Fig. 2 B). In consideration of the latter finding, a direct involvement of Fn2 in the binding to axonin-1 must be excluded. Interestingly, x-ray crystallographic analysis of the domain-tandem Fn1+2 of neuroglian, the homologue of NgCAM in *Drosophila melanogaster*, revealed an extended hydrophobic contact area between the two domains, which is conserved in the vertebrate isoforms (Huber et al., 1994). The deletion of Fn2 places Fn1 adjacent to the putative binding domain Fn3. It is conceivable that, in the absence of the cognate area on Fn2, the hydrophobic contact area of Fn1 prevents a correct folding of Fn3. Thus, we conclude that the Ig domains 2-4 and the third FnIII domain of NgCAM are essential for axonin-1 binding. The deletion of each of these domains resulted in a loss of binding. This indicates that Ig2-4 and Fn3 do not represent two alternative binding sites, but must engage cooperatively in a direct or indirect manner in order to establish a binding of axonin-1.

The axonin-1 binding properties of Δ Ig5, Δ Ig6, and Δ Fn12 show clearly that the domains Ig5, Ig6, Fn1, and Fn2, which link the NgCAM domains Ig2-4 and Fn3, are not essential for axonin-1 binding. However, a marked reduction of axonin-1 binding was observed with the mutant Δ Ig56 (Fig. 3 B). Because the deletions of Ig5 and Ig6 individually do not reduce axonin-1 binding, the possibility of an involvement of Ig5 or Ig6 in the axonin-1–NgCAM interaction must be excluded (Fig. 2 B). The effect of the deletion of the domain tandem Ig5+Ig6 cannot be ascribed to a reduced spacing between Ig2-4 and Fn3, because no

comparable effect was observed with the deletion mutants Δ Ig6Fn1 and Δ Fn12. The mutants Δ Ig56Fn1, Δ Ig6Fn12, which lack three domains, and Δ Ig56Fn12, which lacks all four domains between Ig2-4 and Fn3 exhibit axonin-1 binding comparable to Δ Ig56 (Fig. 3 B). This indicates that the domains Ig5-Fn2 do not participate directly in axonin-1 binding but rather provide the conformational flexibility required for an optimal arrangement of Ig2-4 and Fn3 with respect to the corresponding binding areas on axonin-1.

Proteolytic Cleavage of NgCAM Is Not Required for Axonin-1 Binding

In neural tissue, NgCAM is proteolytically processed, resulting in a NH₂-terminal fragment of 135 kD and a transmembrane fragment of 80 kD (Grumet et al., 1984). The cleavage site is localized within the third FnIII-type domain, between the residues Q866 and A867 (Burgoon et al., 1991). NgCAM from COS7 cells exhibits a similar, although less pronounced, proteolytic processing (Buchstaller et al., 1996). The function of the proteolytic cleavage of NgCAM is currently unclear. As the deletion of Fn3 resulted in an uncleaved form of NgCAM that exhibited a strongly reduced binding of axonin-1, a possible effect of

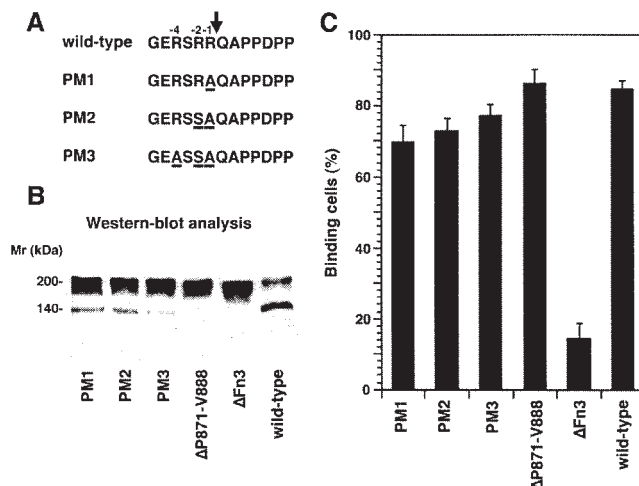


Figure 4. Binding of axonin-1 to the NgCAM mutants NgCAM PM1-3, NgCAM Δ P871-V888, and wild-type NgCAM. (A) Schematic representation of the point mutations introduced into positions R865, R864, and R862 of NgCAM PM1, NgCAM PM2, and NgCAM PM3. Amino acid exchanges are underlined and the site of cleavage marked by an arrow. The relative positions of the residues with respect to the cleavage site are indicated. (B) Western blot analysis. The NgCAM mutants were transiently expressed in COS7 cells, total protein separated by SDS-PAGE, transferred to nitrocellulose and immunostained using a monoclonal anti-NgCAM antibody against the 135-kD fragment followed by enhanced chemiluminescence for detection. Molecular masses are indicated. (C) Binding of axonin-conjugated TRITC Covaspheres to COS cells expressing wild-type NgCAM and the NgCAM mutants NgCAM PM1-3, NgCAM Δ P871-V888. The identification of NgCAM expressing cells and the quantification of the binding of axonin-1 Covaspheres was carried out as described in Fig. 2. Each column corresponds to three independent experiments (\pm SD).

the proteolytic processing of NgCAM on the interaction with axonin-1 was investigated.

The cleavage site that is localized within the third FnIII domain is preceded by conserved Arg residues at positions -1 (R865), -2 (R864), and -4 (R862). This pattern of conserved residues resembles the recognition sequences for furin-type proteases (Burgoon et al., 1991; Hosaka et al., 1991; Grumet and Sakurai, 1996). To generate NgCAM variants deficient in proteolytic cleavage we introduced the following point mutations: NgCAM PM1, R865; NgCAM PM2, R865A and R864S; and NgCAM PM3, R865A, R864S, and R862A (Fig. 4 A). In another construct, the segment P871-V888 that is localized COOH terminally to the cleavage site was deleted. Sequence alignments revealed that this Pro-rich sequence occurs only in NgCAM but not in its putative mammalian homologue L1, which is found primarily in the uncleaved 200-kD form (Grumet et al., 1991). Based on comparisons of the sequences of NgCAM and L1 (Grumet et al., 1991) and considering the recently published hypothetical outline structure of Fn3 of mammalian L1 (Bateman et al., 1996), we expected the deletion of the sequence P871-V888 to have an effect on proteolytic cleavage without extensive changes in the tertiary structure of Fn3.

The NgCAM mutants were expressed in COS7 cells as described above. Proteolytic cleavage of these NgCAM variants was assessed by quantitative Western blot analysis using a monoclonal anti-NgCAM antibody that recognizes only the 135-kD fragment for detection. The extent of proteolytic processing is reflected by the relative intensities of the 135- and the 200-kD band, that represents the uncleaved form of NgCAM (Fig. 4 B). Compared with wild-type NgCAM that was cleaved to ~60%, densitometric analysis revealed a sixfold reduction of proteolytic cleavage for PM1, carrying the point mutation R865A in position -1. No further reduction was observed with the additional point mutation R864S in position -2. In contrast, a strong additional reduction in cleavage, to ~25-fold compared with wild-type, was observed after the additional exchange R862A in position -4. Cleavage was entirely absent from NgCAM Δ P871-V888 (for details see Materials and Methods). To investigate the possible effects of the reduced cleavage of these NgCAM variants on their interaction with axonin-1, binding studies using axonin-1-conjugated Covaspheres were performed. Surprisingly, none of the NgCAM mutants exhibited a significant reduction in axonin-1 binding when compared with the wild-type (Fig. 4 C). These data clearly demonstrate that proteolytic cleavage of NgCAM is not required for the binding to axonin-1. Thus, the strongly reduced binding of Δ Fn3 cannot be ascribed to the lack of proteolytic processing of this mutant but must be caused by the actual deletion of the domain.

Ig2-4 and Fn3 of NgCAM Interact with the Four NH₂-terminal Domains of Axonin-1

Because the four NH₂-terminal Ig domains of axonin-1 were previously found to form a domain conglomerate that is necessary and sufficient for NgCAM binding (Rader et al., 1996), we also tested the binding of the domain deletion mutants of NgCAM with two truncated forms of

axonin-1, axonin-1 Ig1234-C κ , and axonin-1 Δ Ig1234-C κ . The soluble axonin-1 fragments were produced as fusion proteins with the mouse C κ domain, as described by Rader et al. (1996), coupled to fluorescent microspheres, and analyzed for their NgCAM binding as described for wild-type axonin-1 (Fig. 5). The binding pattern of axonin-1 Ig1234-C κ to the NgCAM mutants (Fig. 5 C) was very similar to that of wild type axonin-1 (Fig. 2). In contrast, the complementary axonin-1 fragment axonin-1 Δ Ig1234-C κ did not show any binding to the NgCAM mutants (Fig. 5 C). This result provides experimental evidence that the binding of axonin-1 to both, Ig2-4 and Fn3 of NgCAM is mediated by the four NH₂-terminal Ig domains of axonin-1. The loss of binding of axonin-1 Ig1234-C κ to NgCAM by

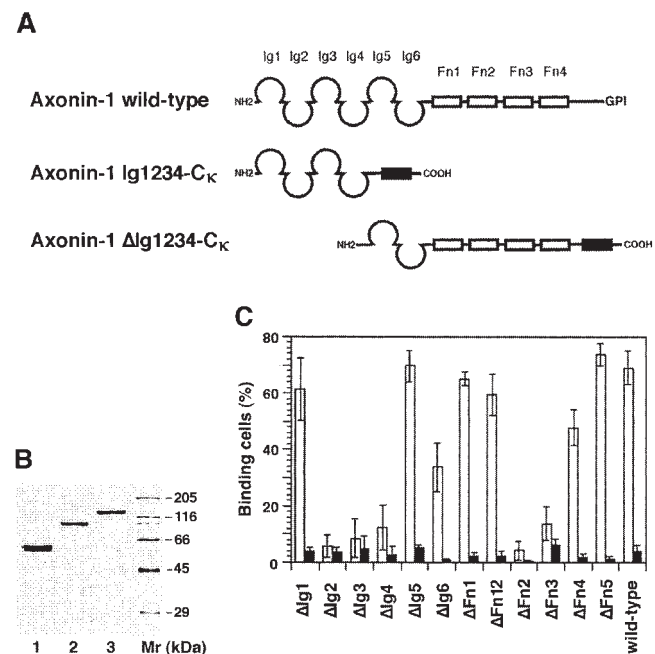


Figure 5. Binding of axonin-1 Ig1234-C κ and axonin-1 Δ Ig1234-C κ to wild-type NgCAM and the two series of domain deletion mutants. (A) Schematic representation of the soluble axonin-1-fragments fused to mouse Ig C κ domain (filled bars). As in Fig. 2, Ig domains are represented by half-circles and FnIII-like domains by empty bars. The molecules were expressed in stably transfected myeloma cells and purified from the supernatants by immunoaffinity-chromatography using an anti-mouse Ig C κ monoclonal antibody. (B) Purified proteins were subjected to SDS-PAGE and visualized by silver staining. Lane 1, axonin-1 Ig1234-C κ ; lane 2, axonin-1 Δ Ig1234-C κ ; lane 3, native axonin-1 purified from the vitreous fluid of E14 chicken embryos; lane Mr represents the molecular mass standard. (C) The soluble fusion proteins axonin-1 Ig1234-C κ and axonin-1 Δ Ig1234-C κ were coupled to TRITC-conjugated Covaspheres and tested for binding to the NgCAM mutants expressed on COS cells. The experiment was carried out as described in Fig. 2. Again, green fluorescing cells that bound four or more red fluorescing Covaspheres were scored as positive cells. Binding of Ig1234-C κ is represented by the empty bars, Δ Ig1234-C κ by the filled bars. Cells that did not express NgCAM were used as internal negative controls and were found to bind on the average 0.1 Covaspheres per cell for Ig1234-C κ and 0.5 Covaspheres per cell in case of Δ Ig1234-C κ . Each column corresponds to four independent experiments (\pm SD).

deletion of one of the domains Ig2-4 and Fn3 indicates that the domain conglomerate formed by the four NH₂-terminal Ig domains of axonin-1 must interact simultaneously with Ig2-4 and Fn3 of NgCAM.

The Four NH₂-terminal Ig Domains of NgCAM Are Involved in NgCAM Homophilic Binding

For the identification of the NgCAM domains involved in homophilic binding we investigated the binding of NgCAM-conjugated microspheres to both series of domain deletion mutants of NgCAM (Fig. 6). The data derived from both sets of NgCAM deletion mutants show clearly that deletions of the four NH₂-terminal Ig domains of NgCAM resulted in a strong reduction of the homophilic binding. In addition, a significant reduction of the homophilic binding was observed with Δ Fn2. In contrast, no reduction of binding was found with the mutant lacking the domain tandem Fn1+Fn2, Δ Fn12. This result resembles the situation regarding the binding of axonin-1 described above. The reduced homophilic binding of Δ Fn2 could reflect an impaired folding of the NgCAM mutant due to the deletion of Fn2 rather than an involvement of Fn2 in the homophilic interaction.

To address the question whether proteolytic cleavage of NgCAM in Fn3 has an influence on the homophilic interaction we tested the binding of NgCAM-conjugated Covaspheres to the NgCAM mutants NgCAM PM1-3 and NgCAM Δ P871-V888. All NgCAM mutants, including Δ Fn3 exhibited homophilic binding comparable to the wild-type (Fig. 6 B). As the axonin-1–NgCAM interaction, NgCAM homophilic binding was not affected in mutants that were not processed.

Coexpression of Axonin-1 and NgCAM in the Same Membrane Does Not Affect the Homophilic Binding Capacity of NgCAM, but Reduces Homophilic Binding of Axonin-1

The results of the domain mapping studies described above indicate an overlap of the domains of NgCAM involved in axonin-1 binding, Ig2-4 and Fn3, and the domains involved in the homophilic NgCAM interaction, Ig1-4. Likewise, localization of the binding sites on axonin-1 had revealed that the axonin-1 domains Ig1-4, which mediate NgCAM binding, overlap with those identified recently to be involved in axonin-1 homophilic binding, Ig1 and Fn4 (Kunz, B., R. Lierheimer, C. Rader, U. Ziegler, L. Vogt, M. Spirig, and P. Sonderegger, manuscript submitted for publication). The participation of some domains of both axonin-1 and NgCAM in homophilic and heterophilic interaction rises the possibility of a competition between the axonin-1–NgCAM binding and the homophilic interactions of each of the two molecules. To address this question, we investigated the effect of a coexpression of axonin-1 with NgCAM on the homophilic interactions of axonin-1 and NgCAM. For this purpose, the expression constructs pSCTaxonin-1 (Rader et al., 1993) and pSCT-NgCAM were introduced individually or together into COS7 cells by electroporation, resulting in the expression of either axonin-1 or NgCAM alone or the coexpression of axonin-1 and NgCAM. 48 h after transfection the cells were preincubated with either control Fab (prepared from

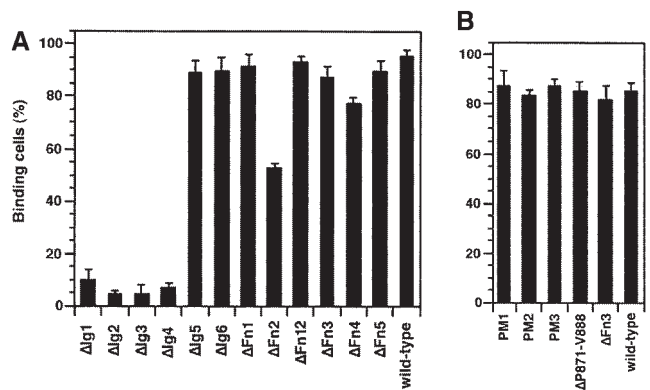


Figure 6. Binding of NgCAM-microspheres to wild-type NgCAM, the domain deletion mutants, and the NgCAM variants deficient in proteolytic processing. Binding of NgCAM-conjugated TRITC Covaspheres to COS cells expressing wild-type NgCAM and the domain deletion mutants (A) and the mutants NgCAM PM1-3 and NgCAM Δ P871-V888 (B). NgCAM expressing cells were identified as described in Fig. 2. For each mutant 100 green fluorescing cells were analyzed. Green fluorescing cells that bound four or more red fluorescing Covaspheres were scored as positive cells. Cells that did not express NgCAM were used as internal negative controls and were found to bind on the average 0.1 NgCAM-conjugated Covaspheres per cell. Each column corresponds to three independent experiments (\pm SD).

preimmune serum), anti-axonin-1, or anti-NgCAM Fab, washed extensively, and subsequently incubated with microspheres conjugated to NgCAM or axonin-1. The expression of axonin-1 and NgCAM was detected by immunofluorescence staining using goat anti-axonin-1 and rabbit anti-NgCAM antibodies and the corresponding secondary antibodies conjugated to FITC or Texas red, respectively. The coexpressing cells were clearly identified by their yellow staining using a narrow band-pass double filter for analysis. For quantification, we selected cells with comparable intensity of the individual immunofluorescence staining of axonin-1 and NgCAM. The results of this study are partially presented in Fig. 7. A summary of all results is represented by Table I and a quantitative analysis is shown in Fig. 8.

The binding of NgCAM microspheres to cells coexpressing axonin-1 and NgCAM was similar to the binding observed with cells expressing NgCAM alone (Fig. 7). This indicates that the capability of NgCAM to undergo homophilic binding is not altered by the coexpression of axonin-1 in the same cell. However, a change in the accessibility of axonin-1 in the axonin-1–NgCAM coexpressing cells was observed by antibody perturbation experiments. Binding of axonin-1- and NgCAM-microspheres to cells coexpressing NgCAM and axonin-1 was completely abolished after masking of cellular NgCAM by preincubation with anti-NgCAM Fab (Fig. 7 h, data not shown). No effect was observed with anti-axonin-1 Fab (Fig. 7 i, data not shown). This indicates that axonin-1- and NgCAM-microspheres bind to coexpressing cells exclusively via cellular NgCAM. In contrast, cellular axonin-1 seems to be inaccessible for an interaction with axonin-1 or NgCAM when coexpressed with NgCAM. These observations suggest

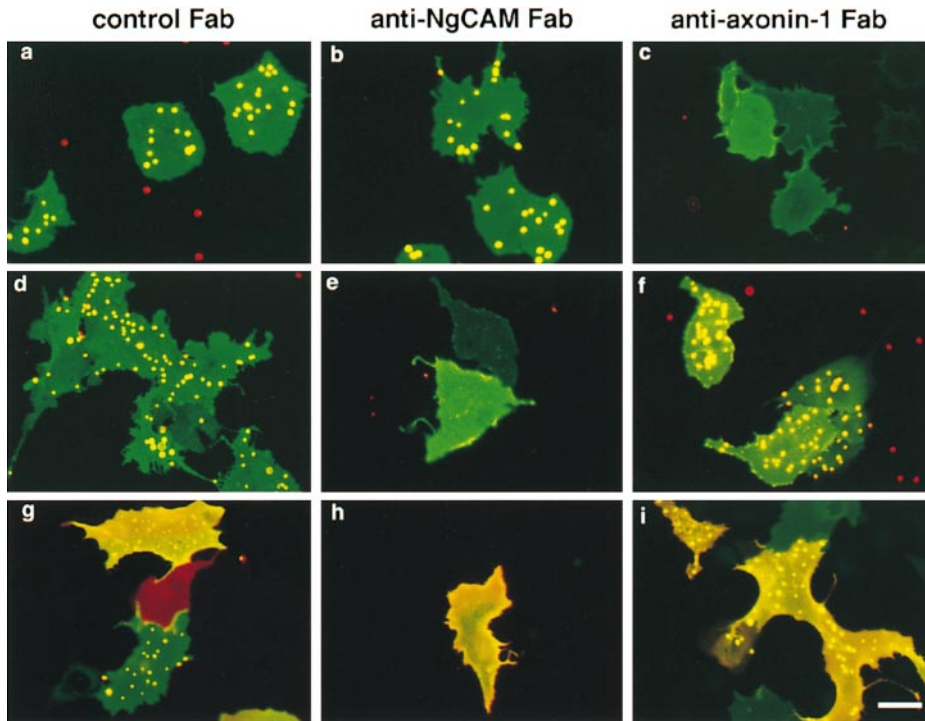


Figure 7. Binding of NgCAM-microspheres to cells expressing axonin-1, NgCAM or coexpressing axonin-1 and NgCAM. COS cells were transfected with axonin-1 (*a–c*), NgCAM (*d–f*) and cotransfected with axonin-1 and NgCAM (*g–i*). The cells were preincubated with anti-axonin-1, anti-NgCAM, and control Fab as indicated. Binding of TRITC-conjugated NgCAM Covaspheres was assessed. Axonin-1 expressing cells in *a–c* were identified by indirect immunofluorescence staining using a polyclonal anti-axonin-1 antibody and a FITC-labeled secondary antibody. An identical staining protocol was applied for the detection of NgCAM in *d–f*, using a polyclonal anti-NgCAM antibody. Axonin-1–NgCAM coexpressing cells were detected in *g* and *h* by the application of goat polyclonal anti-NgCAM antibody (green fluorescence due to the FITC labeled secondary antibody) combined with rabbit anti-axonin-1 antibody (red fluorescence due to the Texas red-labeled secondary antibody). In *i*, axonin-1 was detected by a goat polyclonal antibody, combined with the FITC-labeled secondary antibody and NgCAM by a rabbit anti-NgCAM antibody using the Texas red-labeled secondary antibody. Axonin-1–NgCAM coexpressing cells appear as a Texas red/FITC double filter device was used. TRITC-conjugated NgCAM Covaspheres can be easily recognized on coexpressing cells. Bar, 20 μ m.

the Texas red-labeled secondary antibody). In *i*, axonin-1 was detected by a goat polyclonal antibody, combined with the FITC-labeled secondary antibody and NgCAM by a rabbit anti-NgCAM antibody using the Texas red-labeled secondary antibody. Axonin-1–NgCAM coexpressing cells appear as a Texas red/FITC double filter device was used. TRITC-conjugated NgCAM Covaspheres can be easily recognized on coexpressing cells. Bar, 20 μ m.

that the heterophilic axonin-1–NgCAM interaction, which is thought to take place in the plane of the membrane (Stoeckli et al., 1996; Buchstaller et al., 1996), excludes homophilic axonin-1 binding but does not interfere with the homophilic NgCAM interaction.

Coclustering of Axonin-1 and NgCAM at Cell Contacts Is Mediated by NgCAM Homophilic Binding Across the Extracellular Space and an Interaction of Axonin-1 with NgCAM in the Plane of the Same Membrane

To investigate the molecular interactions underlying the formation of complexes of axonin-1 and NgCAM at cell contacts we used CV-1 cells stably transfected with full-length cDNAs of axonin-1 or NgCAM alone or stably cotransfected with both cDNAs. Cultures of single transfectants, double transfectants and mixtures of single transfectants were kept for 24 h to allow the formation of extended cell contacts. Subsequently, the cells were fixed using 2% (wt/vol) formaldehyde and 0.1% (wt/vol) glutaraldehyde to obtain complete immobilization of membrane proteins without permeabilization of the cells (Dubreuil et al., 1996). Axonin-1 and NgCAM were localized by immunofluorescence staining using goat anti-axonin-1 and rabbit anti-NgCAM antibodies and the corresponding secondary antibodies conjugated to FITC or Texas red, respectively. The distribution of axonin-1 and NgCAM was quantitatively analyzed by confocal laser scanning microscopy. To obtain cell layers of comparable thickness we in-

tegrated identical numbers of confocal sections for image generation and quantitative analysis (Figs. 9 and 10). The accumulation of axonin-1 and NgCAM in the different situations is reflected by the distribution coefficient that represents the ratio of protein detected at the cell contact versus protein at the surface of the adjacent cells (Fig. 10). These distribution coefficients represent underestimations of the actual amount of protein accumulated at the cell contact region due to a limited accessibility of the molecules localized in closely apposed membranes (for details see Materials and Methods).

Accumulation of NgCAM was observed at contacts formed by cells expressing NgCAM alone (Fig. 9 *a*) or NgCAM in combination with axonin-1 (Fig. 9 *c*). Accumulation of axonin-1 at cell contacts was found to be critically dependent on the presence of NgCAM in the same cell. Strong axonin-1 accumulation was detected at cell contacts formed by cells coexpressing axonin-1 and NgCAM (Fig. 9 *d*), but only a slight accumulation was found at contacts formed by axonin-1 single transfectant cells (Fig. 9 *b*). No accumulation of axonin-1 and NgCAM was found at mixed contacts formed by axonin-1 and NgCAM single transfectants (Fig. 9, *e* and *f*). This indicates the absence of a heterophilic interaction between axonin-1 and NgCAM, when the two molecules are present in different cells (*trans*-interaction). Based on these findings and the results of the preceding section, we propose that the coclustering of NgCAM and axonin-1 at the cell contact area of axonin-1–NgCAM coexpressing cells is driven by a *trans*-homophilic NgCAM binding. The accumulation of axonin-1 at the same sites results from an interaction of axonin-1 with

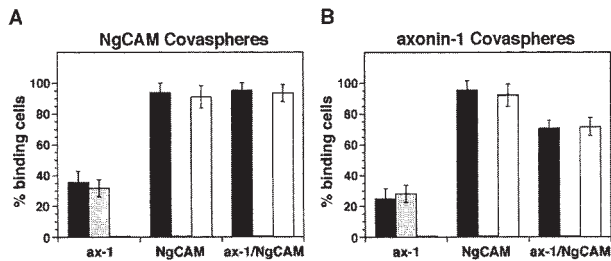


Figure 8. Binding of NgCAM and axonin-1-microspheres to cells that express axonin-1, NgCAM or coexpressed axonin-1 and NgCAM: Quantitative analysis of the binding of NgCAM (A) and axonin-1 (B) Covaspheres to COS cells expressing axonin-1 (*ax-1*), NgCAM (NgCAM) and coexpressing axonin-1 and NgCAM (*ax-1/NgCAM*). Cells were preincubated with control- (filled bars), anti-NgCAM- (shaded bars), and anti-axonin-1- (empty bars) Fab. In case of the single axonin-1 or NgCAM transfections, green fluorescing cells that bound four or more red fluorescing Covaspheres were scored as positive cells. In axonin-1–NgCAM cotransfection cells of comparable fluorescence for axonin-1 and NgCAM staining were selected by examination with the corresponding single-pass filters. Again, cells that bound four or more red fluorescing Covaspheres were scored as positive. Cells that did not express NgCAM or axonin-1 were used as internal negative controls and were found to bind on the average 0.1 axonin-1 or NgCAM Covaspheres. Each column corresponds to four independent experiments (\pm SD). The capability of NgCAM to bind axonin-1 microspheres is only partially reduced in axonin-1–NgCAM coexpressing cells. This may be due to a stoichiometric excess of NgCAM versus axonin-1 in the coexpressing cells we selected for quantification. Combined analysis using immunofluorescence microscopy and quantitative immunoblotting, performed on axonin-1–NgCAM coexpressing cells indeed revealed a comparably weaker immunostaining of NgCAM on cells with similar amounts of axonin-1 and NgCAM protein (data not shown).

NgCAM in the plane of the membrane that occurs simultaneously with the *trans*-homophilic NgCAM binding.

Overexpression of NgCAM Enhances Neurite Fasciculation in DRG Explants

The data presented above indicate that in the fasciculation

Table I. Binding of Axonin-1 and NgCAM to Cells That Express Axonin-1, NgCAM, or Coexpress Axonin-1 or NgCAM

Expression on COS cells	Preincubation with Fab			Binding of microspheres	
	Control	Anti-NgCAM	Anti-axonin-1	NgCAM	Axonin-1
Axonin-1	X			+	+
Axonin-1		X		+	+
Axonin-1			X	–	–
NgCAM	X			+	+
NgCAM		X		–	–
NgCAM			X	+	+
Axonin1/NgCAM	X			+	+*
Axonin1/NgCAM		X		–	–
Axonin1/NgCAM			X	+	+*

COS cells were transfected with axonin-1 (*Axonin-1*), NgCAM (*NgCAM*), and cotransfected with axonin-1 and NgCAM (*Axonin-1/NgCAM*). The cells were preincubated with control, anti-axonin-1, and anti-NgCAM Fab as indicated (X). Binding of TRITC-conjugated Covaspheres conjugated to axonin-1 and NgCAM was assessed as described in Fig. 2. Binding comparable to controls is represented by +, no detectable binding by –, and partially reduced binding by +*, according to the quantitative analysis represented in Fig. 8.

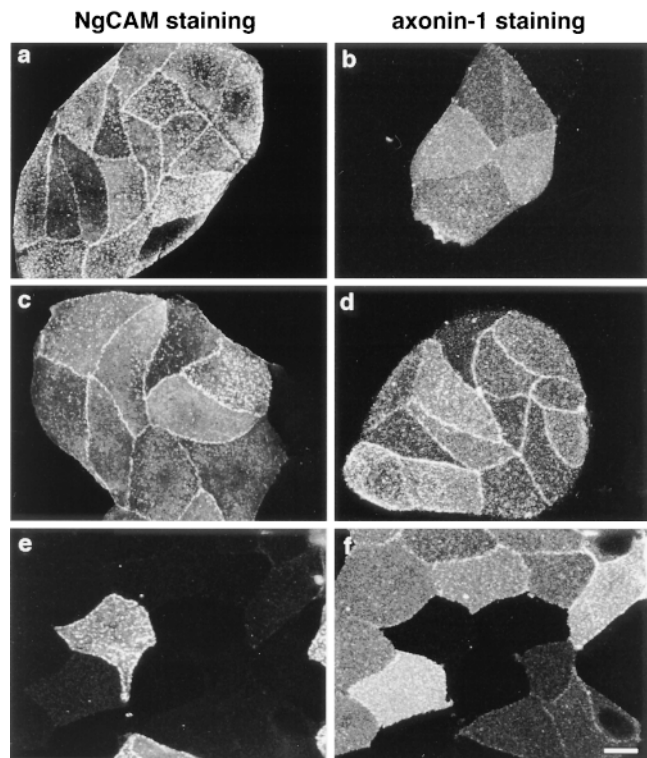


Figure 9. Localization of axonin-1 and NgCAM at cell contacts of stably transfected CV-1 cells: CV-1 cells were stably transfected with NgCAM (a) and axonin-1 (b) or stably cotransfected with axonin-1 and NgCAM (c and d). e and f represent mixtures of single axonin-1 and NgCAM transfectants. Axonin-1 and NgCAM were stained using a rabbit polyclonal anti-axonin-1 antibody (*axonin-1 staining*) combined with a Texas red-labeled secondary antibody and a goat anti-NgCAM antibody (*NgCAM staining*) combined with a FITC-labeled secondary antibody. Images were generated by confocal laser-scanning microscopy. To examine cell layers of equal thickness, equal numbers of confocal sections were integrated for image generation. Bar, 20 μ m.

of neurites bearing axonin-1 and NgCAM it is a homophilic NgCAM interaction that mediates the contacts between adjacent neurite membranes. The *cis*-axonin-1–NgCAM interactions that occur simultaneously in both membranes would result in the formation of a symmetric tetrameric axonin-1–NgCAM complex. Complexes of the expected size and composition were indeed detected after chemical cross-linking on strongly fasciculated but not on unfasciculated neuronal cultures (Kunz et al., 1996). This distinctive interactive pattern of axonin-1 and NgCAM in fascicles formed by neurons coexpressing the two molecules introduced the possibility that changes in the stoichiometric ratio of the two molecules might modulate neurite fasciculation. To test this hypothesis we changed the stoichiometric ratios of axonin-1 and NgCAM in DRG explants using adenoviral gene transfer vectors of axonin-1 (AdCMVax-1) and NgCAM (AdCMVNgCAM). Mouse DRG explants were used because they develop long neurites that extend away from the ganglion in a radial orientation and are highly susceptible to adenoviral infection. Cross-species studies demonstrated that the mouse homologues of axonin-1 and NgCAM, TAG-1, and L1, can

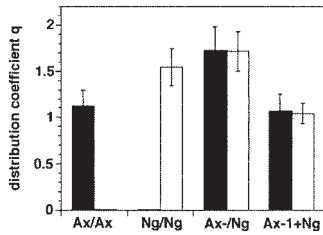


Figure 10. Quantitative analysis of the distribution of axonin-1 and NgCAM in stably transfected CV-1 cells: The distributions of axonin-1 (filled bars) and NgCAM (empty bars) were quantified by analysis of the fluorescence intensity of the axonin-1 and NgCAM immuno-

fluorescence staining in axonin-1 single transfectants (Ax), NgCAM single transfectants (Ng), axonin-1–NgCAM cotransfectants (Ax-/Ng) and mixtures of axonin-1 and NgCAM single transfectants (Ax-1+Ng). The combinations of antibodies used correspond to those in Fig. 9. To obtain cell layers with comparable geometry, identical numbers of confocal sections were integrated for quantitative analysis of the distribution of axonin-1 and NgCAM. For quantification distribution coefficients were calculated. In case of cultures consisting of only one type of cell the distribution coefficient was calculated as: $q = 2 \times I_{\text{cell-contact}} / (I_{\text{cell1}} + I_{\text{cell2}})$ and in cases of contacts formed by different cell types (e.g., axonin-1 and NgCAM expressing cells) $q^* = I_{\text{cell-contact}} / I_{\text{expressing cell}}$. The term $I_{\text{cell-contact}}$ represents the fluorescence intensity per area at the cell contact and the terms I_{cell1} , I_{cell2} , and $I_{\text{expressing cell}}$ represent the fluorescence intensity per area at the surface of the cells forming the contacts. Bars represent means \pm SD, $n = 30$ for NgCAM and $n = 24$ for axonin-1 single transfectants, $n = 24$ for axonin-1–NgCAM double transfectants, and $n = 24$ and for the mixtures of axonin-1 and NgCAM single transfectants.

undergo functional interactions with their chicken counterparts (Lemmon et al., 1989; Felsenfeld et al., 1994). Therefore, heterologous expression of axonin-1 and NgCAM in mouse DRG neurons corresponds functionally to an overexpression of TAG-1/axonin-1 and L1/NgCAM.

Adenoviral gene transfer was initiated after 12 h in culture by infection of the DRG explants with AdCMVax-1, AdCMVNgCAM, and AdCMVlacZ. At the time of infection, the ganglia had already initiated neurite outgrowth. 48 h after infection the DRG explants were fixed by 2% formaldehyde and 0.1% glutaraldehyde. Axonin-1 and NgCAM that appeared on the extracellular face of the axonal membrane were visualized by immunofluorescence staining using specific monoclonal antibodies that did not cross-react with TAG-1 and L1. Strong heterologous expression of axonin-1 and NgCAM was detected on ~80% of the neurites. β -galactosidase staining of ganglia infected with AdCMVlacZ revealed a similar rate of infection. The morphological integrity of the explants infected with the various viral constructs in comparison to uninfected explants was examined microscopically using Nomarski optics. No significant morphological alterations due to viral cytotoxicity were detected. After 60 h in culture most neurites stained homogeneously for axonin-1 and NgCAM from the growth cone at the leading tip to the neurite shaft proximal to the ganglion. In contrast, at an earlier time point, after only 20 h in culture, a predominant staining of the growth cone tip and the neurite shaft distally from the ganglion was detected. This is in good accordance with previous studies indicating that newly synthesized axonin-1 and NgCAM are inserted into the axonal membrane of the growth cone (Vogt et al., 1996).

Overexpression of axonin-1 resulted in a slightly re-

duced neurite fasciculation (Fig. 11, a, b, e, and f) compared with control explants treated with AdCMVlacZ (not shown) or without virus (Fig. 11, i–m). In contrast, overexpression of NgCAM resulted in strongly enhanced fasciculation (Fig. 11, c, d, g, and h). Quantitative analysis of the effects of axonin-1 and NgCAM overexpression on neurite fasciculation are summarized in Fig. 11 m (for details see Materials and methods). The effect of NgCAM overexpression on neurite fasciculation was blocked specifically by the addition of monoclonal anti-NgCAM IgG (Fig. 12, c and f) but not by control IgG (Fig. 12, b and e). The enhanced fasciculation observed with neurites overexpressing NgCAM can therefore be ascribed specifically to the enhanced NgCAM expression in the neuronal membrane. The observed effects of axonin-1 and NgCAM overexpression on neurite fasciculation are in accordance with the observation that NgCAM but not axonin-1 can efficiently mediate contact across the extracellular space in the context of heterooligomeric axonin-1–NgCAM complexes.

Discussion

The Ig Domains 2-4 and the Third FnIII Domain of NgCAM Are Essential for the Binding to Axonin-1 in the Plane of the Membrane

A direct binding between axonin-1 and NgCAM was originally demonstrated using the purified proteins coupled to the surface of microspheres (Kuhn et al., 1991). Further studies, including this paper, revealed that axonin-1 and NgCAM cannot interact when present on the surface of different cells (Buchstaller et al., 1996). In contrast, antibody-induced cocapping and chemical cross-linking indicated a specific and direct binding of the two molecules in the plane of the same membrane (*cis*-interaction; Buchstaller et al., 1996). In the Covaspheres-binding assays performed in this study, membrane-bound NgCAM can engage in a heterophilic binding with axonin-1 that is presented in the Covaspheres-conjugated form. This indicates that at least one fraction of the axonin-1 molecules on Covaspheres is bound in an orientation appropriate for binding cell surface exposed NgCAM, whereas membrane-anchored axonin-1 is presented only on one orientation, that allows *cis*- but not *trans*-interaction. We suggest that the fraction of axonin-1 on Covaspheres that binds to cellular NgCAM is oriented in a way that it approaches NgCAM in the same relative orientation as axonin-1 present in the same membrane. The binding of axonin-1-conjugated Covaspheres corresponds therefore most probably to a *cis*-axonin-1–NgCAM interaction and does not represent an actual *trans*-binding.

Binding studies revealed that the Ig domains 2-4 (Ig2-4) and the third FnIII-type domain (Fn3) are necessary for axonin-1 binding. Both Ig2-4 and Fn3 were required for axonin-1 binding as demonstrated by the loss of axonin-1 binding after deletion of any one of the domains. These results indicate that Ig2-4 and Fn3 may contribute cooperatively to axonin-1 binding and do not represent alternative axonin-1 binding sites. The question arises whether Ig2-4 and Fn3 undergo a direct interaction with axonin-1 in a cooperative manner or whether the deletion of Fn3 simply

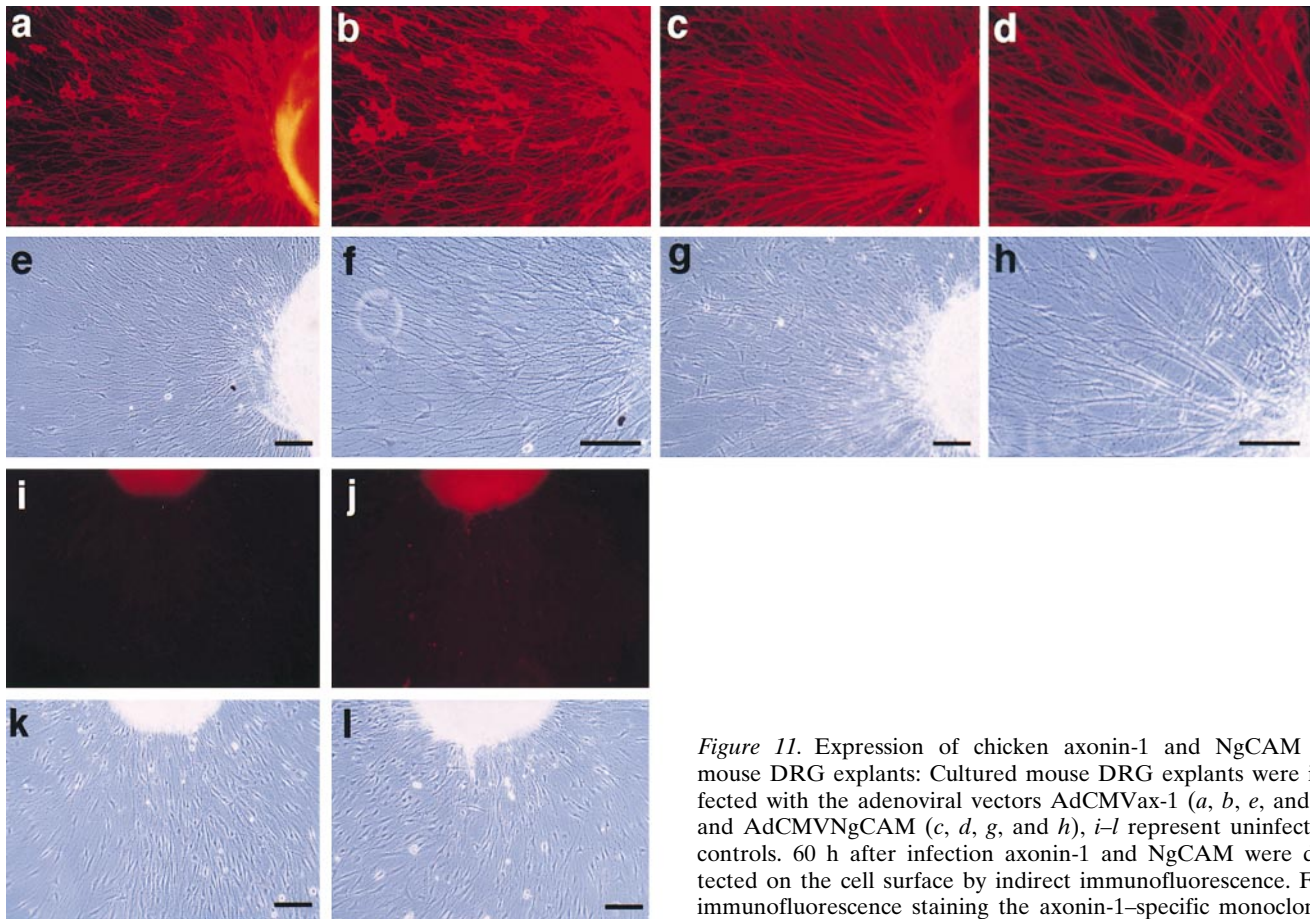
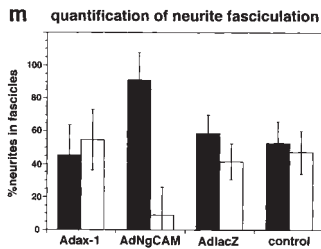


Figure 11. Expression of chicken axonin-1 and NgCAM in mouse DRG explants: Cultured mouse DRG explants were infected with the adenoviral vectors AdCMVax-1 (*a, b, e, and f*) and AdCMVNgCAM (*c, d, g, and h*), *i-l* represent uninfected controls. 60 h after infection axonin-1 and NgCAM were detected on the cell surface by indirect immunofluorescence. For immunofluorescence staining the axonin-1-specific monoclonal antibody X7C11 and the NgCAM-specific monoclonal antibody 12-I-4E-311, and a Cy3-labeled donkey anti-mouse secondary antibody were used. Axonin-1 staining: *a, b, and i* (fluorescence optics); *e, f, and k* phase optics); NgCAM staining: *c, d, and j* (fluorescence optics; *g, h, and l* phase optics). Quantitative analysis of neurite fasciculation is represented by *m*. In brief, for quantification individual neurites that stained positively for the heterologously expressed protein were tracked from the periphery of the ganglion to a point at one-third of the total neurite length. Neurites that coalesced into bundles consisting of more than 4 neurites were scored as the stronger fasciculated category (*filled bars*) and neurites found in bundles of less than 4 neurites belonged to the other category (*empty bars*). 50 neurites were scored per ganglion, *n* = 6 (6 ganglia were analyzed per sample). Bars represent means \pm SD. Bars, 100 μ m.



results in a changed conformation of Ig2-4 that is incompatible with the binding of axonin-1. An extensive conformational change in the NH₂-terminal Ig domains of NgCAM due to the deletion of Fn3 appears rather unlikely in the light of the absence of any effect on the homophilic NgCAM binding that is mediated by the Ig domains 1-4. However, since the third FnIII domain of NgCAM is subject to proteolytic cleavage (Burgoon et al., 1991) we addressed the possibility that the absence of proteolytic cleavage in Δ Fn3 rather than the actual lack of the domain may be responsible for the loss of axonin-1 binding.

Proteolytic Processing of NgCAM Is Not Required for Binding to Axonin-1 and NgCAM Homophilic Binding

The strong and specific reduction in cleavage after amino acid exchanges at positions -1 (R865A) and -4 (R862A)

from the cleavage site is in good accordance with previous studies (Hosaka et al., 1991; Jiang et al., 1993) and makes furin family proteases likely candidates for proteolytic processing of NgCAM in COS7 cells. The absence of proteolytic processing after deletion of the sequence P871-V888 that is localized COOH terminally from the cleavage site, was also not unexpected. According to sequence alignments, this Pro-rich sequence occurs specifically in NgCAM and is absent from mammalian L1, which occurs mainly in the uncleaved 200-kD form (Grumet et al., 1991). Considering the recently published outline structure of Fn3 from mammalian L1 (Bateman et al., 1996) this sequence may represent an NgCAM-specific extension of the polypeptide chain that makes the cleavage site more accessible for proteolytic enzymes. Despite their pronounced effect on proteolytic cleavage, none of these mutations had a significant effect on the heterophilic binding to axonin-1 or the homophilic interaction. These re-

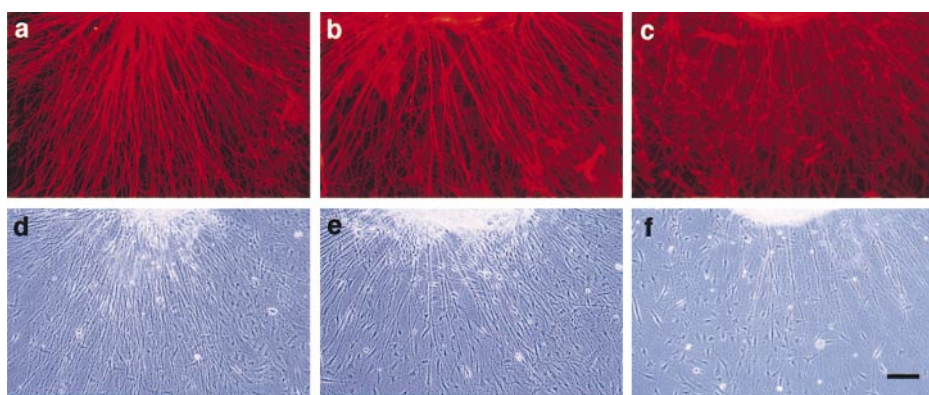


Figure 12. Perturbation of neurite fasciculation with species-specific anti-NgCAM antibodies. Cultured mouse DRG explants were infected with the adenoviral vector AdCMV-NgCAM. The explants were incubated in the presence of control mouse IgG (*a* and *d*), monoclonal anti-axonin-1 IgG X7C11 (*b* and *e*) and the monoclonal mouse anti-NgCAM IgG 12-I-4E-311 (*c* and *f*). NgCAM was detected on the cell surface by indirect immunofluorescence as described in Fig. 11; *a*–*c* represent fluorescence optics and *d*–*f* the corresponding phase optics. Bar, 100 μ m.

sults clearly demonstrate that proteolytic processing of NgCAM is not required for binding to axonin-1 or NgCAM. It is conceivable, that after cleavage, the third FnIII domain may retain enough tertiary structure to maintain its overall conformation and the association between the two fragments of 135 and 80 kD. However, a possible functional relevance of the proteolytic processing of NgCAM cannot be excluded. Proteolytic cleavage may affect other functionally important interactions of NgCAM. Alternatively, it may exert more subtle influence on the conformational dynamics of the molecule that could be relevant for example in signal transduction.

In the context of our investigation, the reduced binding of axonin-1 to the NgCAM mutant Δ Fn3 cannot be ascribed to the lack of proteolytic processing but may rather reflect the loss of an actual binding domain. Thus, a direct interaction of axonin-1 with both, Ig2-4 and Fn3 of NgCAM appears likely.

Implications for the Conformations of Axonin-1 and NgCAM within the Postulated *Cis*-Axonin-1–NgCAM Complex

Domain deletion studies had revealed that the first four NH₂-terminal Ig domains of axonin-1 are necessary and sufficient for NgCAM binding (Rader et al., 1996). In this study, evidence was provided that the four NH₂-terminal Ig domains of axonin-1 form a conglomerate that maintains its native structure only when all domains are present. Our results indicate that the binding sites for Ig2-4 and Fn3 of NgCAM reside also on this domain conglomerate. A possible simultaneous interaction of the NH₂-terminal domain conglomerate of axonin-1 with Ig2-4 and Fn3 of NgCAM may bend the NgCAM molecule around the Ig1-4 conglomerate of axonin-1. This conformational change of NgCAM due to the binding of the Ig1-4 conglomerate of axonin-1 within a *cis*-axonin-1–NgCAM complex may require a flexible hinge between Ig2-4 and Fn3. Some evidence for this is derived from the reduced axonin-1 binding of the mutant Δ Ig56 that cannot be due to a simple space effect, because no reduction was observed after deletion of the domain tandems Ig6+Fn1 and Fn1+Fn2 (Fig. 2 B). The binding properties of Δ Ig56 suggests a requirement for conformational flexibility in order

to fit Ig2-4 and Fn3 to the domain conglomerate of axonin-1. The comparably strong axonin-1 binding of the NgCAM mutants Δ Ig56Fn1, Δ Ig6Fn12, and Δ Ig56Fn12 may therefore reflect a close proximity of Ig2-4 and Fn3 in the axonin-1–NgCAM complex.

Based on these results we suggest the following molecular model: to establish a concomitant binding of Ig2-4 and Fn3 of NgCAM with the NH₂-terminal Ig1-4 domain conglomerate of axonin-1, with both molecules being anchored to the same membrane by their COOH-terminal end, a backfolded structure of axonin-1 would be required. Interestingly, negative staining electron microscopy had revealed that monomeric axonin-1 has a backfolded, horseshoe-shaped domain-arrangement (Fig. 13 A; Rader et al., 1996). Thus, monomeric axonin-1 can readily fit its NH₂-terminal domain conglomerate into a hypothetical binding pocket of NgCAM when both molecules are bound to the same membrane (Fig. 13 B). Although a direct interaction between Fn3 of NgCAM and the Ig1-4 domain conglomerate appears likely, we cannot exclude the possibility that axonin-1 interacts with NgCAM exclusively via a binding site localized in Ig2-4 of NgCAM. In the latter case a partial unfolding of axonin-1 from its horseshoe like conformation in the monomeric state would be required (Fig. 13 C). However, compared with the situation in a *cis*-axonin-1–NgCAM complex, a considerable conformational change of the axonin-1 molecule would be required to establish a contact with NgCAM residing in the membrane of an opposed cell.

Homophilic Interaction of NgCAM but Not Axonin-1 Can Occur Simultaneously with the Heterophilic Axonin-1–NgCAM Binding

The domain deletion studies also revealed that the four NH₂-terminal Ig domains of NgCAM are essential for its homophilic interaction. Thus, in the segment of the Ig domains 2-4, binding sites for both the homophilic binding as well as the NgCAM/axonin-1 binding are located. Domain localization studies of the homophilic axonin-1 interaction revealed an involvement of Ig1 and Fn4 of axonin-1 and, interestingly, a heterotypic binding modus (Ig1 of one axonin-1 molecule binds to Fn4 of the other molecule and vice versa) and prompted the postulation of an extended con-

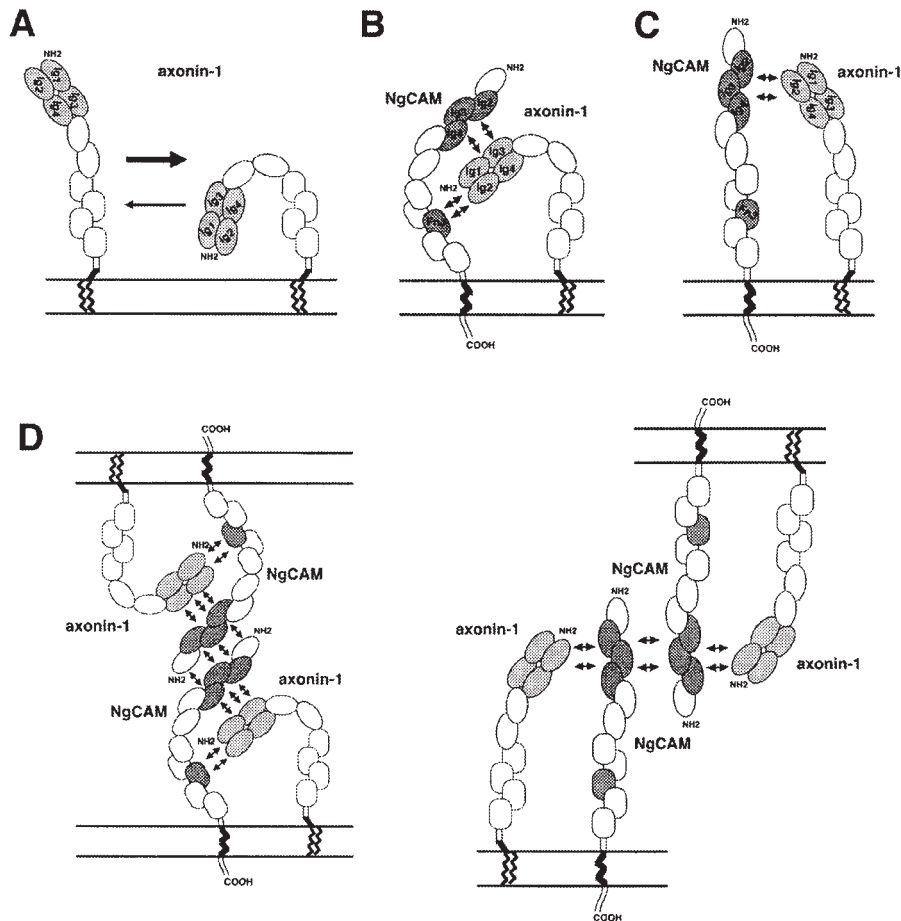


Figure 13. Molecular models of complexes formed by axonin-1 and NgCAM. (A) Previous studies revealed that a major proportion of monomeric axonin-1 has a backfolded, horseshoe-like conformation in which the six Ig domains fold back towards the membrane. The four NH₂-terminal Ig domains (light gray) are shown in close physical association. This conglomerate bears the NgCAM binding site (after Rader et al., 1996). (B) In one model for a *cis*-axonin-1–NgCAM complex the domain conglomerate formed by the four NH₂-terminal Ig domains of axonin-1 interacts directly with the Ig domains 2–4 and the third FnIII-like domain of NgCAM, Fn3 (dark gray). In this model, a partially backfolded conformation of axonin-1 would be required. NgCAM is thought to bend around the Ig1–4 conglomerate of axonin-1 to allow a simultaneous interaction of Ig2–4 and Fn3 of NgCAM with Ig1–4 of axonin-1. (C) An alternative model for the *cis*-axonin-1–NgCAM complex in which the axonin-1 domains Ig1–4 interact only with Ig2–4 of NgCAM. The absence of a direct interaction between Fn3 of NgCAM and axonin-1 Ig1–4 suggests a partially unfolded conformation for axonin-1 in this model of the complex. (D) Models for the presumptive axonin-1–NgCAM tetramer that is formed at cell contacts. Intercellular binding is mediated by NgCAM

homophilic interaction that can occur simultaneously with the *cis*-binding between axonin-1 and NgCAM. The *cis* interaction between axonin-1 and NgCAM may involve a direct interaction of the axonin-1 domains Ig1–4 with Ig2–4 and Fn3 of NgCAM (B, left model) or only a binding between axonin-1 and Ig2–4 of NgCAM (C, right model).

formation of axonin-1 in the homodimeric complex (Kunz, B., R. Lierheimer, C. Rader, U. Ziegler, L. Vogt, M. Spirig, and P. Sonderegger, manuscript submitted for publication). In analogy to NgCAM, axonin-1 exhibited domains that contributed to both the homophilic binding as well as the heterophilic axonin-1–NgCAM binding. The locations of the binding sites and the different conformational requirements for axonin-1 in the heterophilic versus homophilic complex indicate the possibility of competition between the heterophilic axonin-1–NgCAM binding and the homophilic interactions of axonin-1 and NgCAM, respectively.

In this study we found that coexpression of axonin-1 and NgCAM in the same cell, and, thus, the formation of heterodimeric axonin-1–NgCAM complexes, reduced the accessibility of axonin-1 for interactions with axonin-1 and NgCAM presented on the surface of microspheres. The inaccessibility of axonin-1 for *trans*-homophilic interaction in this situation indicates that the axonin-1–NgCAM *cis*-interaction excludes the axonin-1 homophilic binding. Considering the domain arrangement of axonin-1 and Ng-

CAM within the *cis*-axonin-1–NgCAM complex as proposed in the model of Fig. 13 B, it is conceivable that the heterodimeric complex stabilizes the bent conformation of axonin-1 and prevents its transition into the extended conformation required for homophilic binding. In contrast, the capability of NgCAM to undergo homophilic binding was not altered by the complexation with axonin-1, suggesting that the *cis*-heterophilic axonin-1–NgCAM interaction does not require a major conformational change of NgCAM. A *cis*-interaction of axonin-1 with NgCAM that is further involved in a *trans*-homophilic binding may have important physiological functions. Two independent studies demonstrated a requirement for axonin-1 for neurite outgrowth on NgCAM (Stoeckli et al., 1996; Buchstaller et al., 1996). In this situation growth cone-substratum contact is thought to be mediated by homophilic NgCAM interaction (Lemmon et al., 1989). Axonin-1 interacting in *cis* with NgCAM of the growth cone may act as an essential costimulatory factor for neurite growth. A ternary axonin-1–NgCAM₂ complex could represent a minimal functional unit in neurite outgrowth on NgCAM substratum.

Homophilic NgCAM Binding Across the Extracellular Space and Axonin-1–NgCAM Binding in the Plane of the Membrane May Result in the Formation of Axonin-1₂/NgCAM₂ Tetramers at Cell Contacts

Complexes of axonin-1 and NgCAM were implicated in neuronal cell recognition processes serving distinct developmental functions such as axonal pathfinding *in vivo* (Stoeckli and Landmesser, 1995) and *in vitro* (Stoeckli et al., 1997), substratum recognition by growth cones (Stoeckli et al., 1996), and neurite fasciculation (Ruegg et al., 1989b; Stoeckli et al., 1991; Honig and Kueter, 1995; Kunz et al., 1996). To investigate the detailed interactions of axonin-1 and NgCAM at cell contacts we used CV-1 cells transfected with axonin-1 or NgCAM alone as well as cotransfectants expressing both molecules. The main result of these studies was a strong discrepancy in the accumulation of NgCAM and axonin-1 at homophilic cell–cell contact sites. Whereas contacts between cells expressing NgCAM exhibited a pronounced accumulation of NgCAM, only a minor accumulation of axonin-1 was observed when axonin-1–expressing cells were in contact. The accumulation of NgCAM at contacts between NgCAM transfectants reflects the well described *trans*-homophilic binding capacity of NgCAM (Lemmon et al., 1989; Kuhn et al., 1991; Buchstaller et al., 1996). The weak accumulation of axonin-1 at contacts between axonin-1 single transfected cells was not unexpected despite the clear demonstration of the axonin-1/TAG-1 homophilic interaction in various systems (Rader et al., 1993; Felsenfeld et al., 1994; Kunz, B., R. Lierheimer, C. Rader, U. Ziegler, L. Vogt, M. Spirig, and P. Sonderegger, manuscript submitted for publication). When expressed alone, the favored state of axonin-1 may be the backfolded structure (Rader et al., 1996). Evidence from domain deletion studies suggests that the horseshoe form of axonin-1 is stabilized by an intramolecular interaction of the Ig1-4 domain conglomerate with Fn4, the most membrane proximal domain. In axonin-1 single transfected cells, a major proportion of the axonin-1 molecules may therefore adopt this conformation and the likelihood of the extended state, which would favor the formation of a homophilic interaction and the accumulation of axonin-1 at cell contact regions, may be relatively low. Strong accumulation of axonin-1 at cell contacts was only observed when NgCAM was expressed in the same cell. Because the *cis*-axonin-1–NgCAM binding makes axonin-1 inaccessible for *trans*-interactions with axonin-1, the accumulation of axonin-1 at the cell contact regions is unlikely to be driven by a homophilic axonin-1 interaction. We suggest that the coclustering of axonin-1 and NgCAM at the cell contacts is driven by a NgCAM *trans*-homophilic binding, whereas the accumulation of axonin-1 is due to its *cis*-heterophilic binding with NgCAM that occurs simultaneously and results in the formation of a symmetric tetrameric axonin-1–NgCAM complex with a composition of axonin-1₂/NgCAM₂ (Fig. 13 D). Chemical cross-linking studies of axonin-1, performed previously on cultured DRG neurons, revealed the aggregation of axonin-1 and NgCAM into a complex with a molecular mass of ~500 kD during the formation of cell–cell contacts (Kunz et al., 1996; Fig. 9). Based on its size and composition this complex most

probably represents the symmetric tetrameric axonin-1₂/NgCAM₂ complex suggested by the results of this study (Fig. 13 D).

Overexpression of NgCAM but Not Axonin-1 Results in Enhanced Neurite Fasciculation

Several independent pieces of evidence implicate axonin-1 and NgCAM as key players in neurite fasciculation. Immunocytochemical analysis revealed coclustering of axonin-1 and NgCAM at sites of close contact between neurites (Honig and Kueter, 1995). Antibody perturbation experiments as well as chemical cross-linking studies indicated that the formation of neurite fascicles *in vitro* involves axonin-1 and NgCAM (Rathjen et al., 1987; Ruegg et al., 1989b; Stoeckli et al., 1991; Kunz et al., 1996). Further, an interaction between axonin-1 and NgCAM was demonstrated to be of crucial importance for the fasciculation of commissural axons *in vivo* (Stoeckli and Landmesser, 1995). We have previously found a progressive accumulation of heterotetrameric complexes of axonin-1 and NgCAM during the formation of neurite fascicles in cultures of DRG neurons (Kunz et al., 1996). It is an intriguing possibility that heterooligomeric complexes of axonin-1 and NgCAM, such as the tetrameric axonin-1₂/NgCAM₂ complex, represent functional units in neurite fasciculation mediated by axonin-1 and NgCAM. Size and composition of such axonin-1–NgCAM complexes formed at cell contacts would be determined by the stoichiometric ratios of the two molecules in the cells involved. This would open the possibility to regulate axonal fasciculation by changing the relative expression levels of axonin-1 and NgCAM. To test this hypothesis we overexpressed axonin-1 and NgCAM in DRG explants using adenoviral vectors for gene transfer and found that overexpression of axonin-1 resulted in a slight but statistically not significant defasciculation whereas NgCAM overexpression caused strongly enhanced fasciculation. The effects of axonin-1 and NgCAM overexpression on neurite fasciculation correlate very well with the observations in the CV-1 cell model system. NgCAM mediates the contact between adjacent neurite membranes across the extracellular space by homophilic interaction. In contrast, axonin-1 is a *cis*-interacting component of the heterotetrameric cell contact complex that does not directly engage in an interaction across the extracellular space.

According to these findings one would expect that the extent of neurite fasciculation strongly depends on the level of NgCAM expression. Indeed, strong defasciculation was observed upon down-regulation of axonal L1 in mouse DRG neurons dependent on the pattern of electrical activity (Itoh et al., 1995, 1997). These findings are in line with our results of enhanced neurite fasciculation due to NgCAM overexpression and indicate that NgCAM is the limiting component for neurite fasciculation in DRG neurons.

The role of axonin-1 in the heterotetrameric cell contact complex is currently not clear. Yet, results of the studies of the intracellular signaling associated with axonin-1, NgCAM, and complexes thereof suggest a role of axonin-1 in the modulation of intracellular phosphorylation events. Studies on the signal transduction of axonin-1 and Ng-

CAM revealed that the formation of higher molecular axonin-1–NgCAM complexes during neurite fasciculation was accompanied by a reduction of axonin-1-associated fyn tyrosine kinase activity and an increased NgCAM phosphorylation by a casein kinase II related kinase (Kunz et al., 1996; Wong et al., 1996). The effects of the two distinct protein kinases associated with axonin-1 and NgCAM on the cytoskeleton may contribute to the observed effects of axonin-1 or NgCAM overexpression on neurite fasciculation. The high expression of fyn in developing fiber tracts during the phase of axon outgrowth and a comparably low abundance in consolidated fascicles (Bare et al., 1993) suggests that fyn activity may contribute to a dynamic state of the cytoskeleton in transient cell contacts. In contrast, phosphorylation of specific cytoskeletal proteins by casein kinase II could result in a stabilization of the axonal cytoskeleton. Phosphorylation of microtubule-associated proteins such as MAP1B by casein kinase II was reported to be essential for the consolidation of stable neurite structures (Matus, 1991; Brugg et al., 1993; Ulloa et al., 1993). Therefore, an excess of free axonin-1 could destabilize contacts between adjacent neurite membranes due to the predominance of fyn over casein kinase II. Overexpression of NgCAM would drive more axonin-1 into axonin-1–NgCAM complexes resulting in reduced activity of fyn and predominance of casein kinase II. This could result in an enhanced stability of the neuronal cytoskeleton at the cell contact sites and lead to a stronger association of NgCAM with cytoskeletal components.

Similar mechanisms may underlie the strong defasciculation that is observed after application of soluble axonin-1 to neurite bundles *in vitro* and *in vivo* (Stoeckli et al., 1991; Stoeckli and Landmesser, 1995). Soluble axonin-1 may interfere with the complexation of cellular axonin-1 by NgCAM in a competitive manner. This would result in an enhanced concentration of monomeric axonin-1 in the neuronal cell membrane. The enhanced fyn activity that may accompany the excess of monomeric axonin-1 (Kunz et al., 1996) may alter the phosphorylation pattern of cytoskeletal proteins and result in a destabilization of cell contacts. Thus, differential fasciculation that represents a crucial prerequisite for functional target innervation during development and regeneration could be regulated not only by changes in the relative expression of CAMs like axonin-1 and NgCAM but also by variations in the local concentrations of soluble axonin-1 during development.

Axonin-1 and NgCAM Are Implicated in Axonal Fasciculation In Vivo

The findings on the interactions of axonin-1 and NgCAM, suggest an important role of the two molecules in axonal growth and fasciculation. During neural development axonin-1 and NgCAM are coexpressed predominantly in outgrowing fiber tracts (Thiery et al., 1985; Daniloff et al., 1986; Shiga et al., 1990, 1991; Shiga and Oppenheim, 1991; Halfter et al., 1994; Wolfer et al., 1994; Moscoso and Sanes, 1995). A strong coexpression of axonin-1 and NgCAM is found on DRG axons during their entry into the dorsal horn of the spinal cord (Shiga et al., 1997). Antibody perturbation experiments suggest that these CAMs mediate fasciculation among the DRG axons in the pro-

spective dorsal funiculus that is thought to be crucial for the correct pathfinding of these axons *in vivo*, probably by preventing their entry into the mantle layer of the spinal cord (Shiga et al., 1997). Specific fasciculation mediated by axonin-1 and NgCAM was also demonstrated for the commissural axons of the spinal cord. Several IgFnIII molecules are expressed in the commissural system of the chicken embryo when commissural growth cones are making pathfinding decisions. NgCAM and axonin-1 are both expressed on commissural axons (Shiga et al., 1990; Shiga and Oppenheim, 1991; Stoeckli and Landmesser, 1995) and NrCAM/Bravo is expressed in the floor plate cells (Krushel et al., 1993; Denburg et al., 1995). The perturbation of the *in vivo* guidance of chick commissural axons with antibodies demonstrated that the heterophilic interaction between axonin-1 and NgCAM and the homophilic binding of NgCAM are involved in the bundling or fasciculation of these axons whereas heterophilic interactions between axonin-1 and NrCAM appear to be involved in pathfinding (Stoeckli and Landmesser, 1995). An important role for axonin-1 and NgCAM in the organization of neuronal projections is further suggested by the analysis of their expression during the development of the retinotectal system. Both are present in the area where retinal ganglion cells are generated and the expression of axonin-1 and NgCAM along the optic nerve, chiasm, optic tract, and in the superficial layers of the optic tectum follows the chronotropic pattern of axon formation. The temporal correlation of the coexpression of these proteins with the onset and the duration of fiber growth makes it highly probable, that these proteins play an important role in the formation of the fiber pathway (Rager et al., 1996; Morino et al., 1996).

The *in vivo* coexpression suggests that heterooligomeric complexes of axonin-1 and NgCAM that are formed at cell contacts may provide signals for axonal growth and fasciculation during the development of fiber tracts. In early stages of fiber tract development, axons may grow along each other in a way that the membranes of earlier axons may provide the substratum for the growth cones of later ones. This situation may be mimicked *in vitro* by neurite outgrowth on axonin-1 and NgCAM substratum. Several lines of evidence suggest that complexes formed by axonin-1 and NgCAM mediate the signals for neurite elongation elicited by axonin-1 or NgCAM substratum (Kuhn et al., 1991; Stoeckli et al., 1996; Buchstaller et al., 1996). As mentioned above, neurite outgrowth of DRG neurons on NgCAM substratum for example is thought to involve a heterooligomeric complex, formed by the interaction of a *cis*-axonin-1–NgCAM heterodimer of the growth cone membrane with substratum NgCAM (Stoeckli et al., 1996; Buchstaller et al., 1996). Similar complexes formed at growth cone/neurite contacts may provide the signals for the specific axonal elongation on preexisting fibers *in vivo*. The composition of such complexes may be determined by the relative expression of axonin-1 and NgCAM in the axons that form the contact. Contacts between axons that show coexpression of axonin-1 and NgCAM, like the DRG projections of the dorsal horn, the commissural axons of the spinal cord, and the axons of the retinotectal projection mentioned above may result in the formation of a symmetric axonin-1–NgCAM heterooligomer. This as-

sumption is supported by the detection of a presumptive axonin-1/NgCAM tetramer in strongly fasciculated DRG neurons in vitro (Kunz et al., 1996) and the interactive pattern of axonin-1 and NgCAM at cell contacts described in this study. In later stages of fiber tract development, distinct complexes of axonin-1 and NgCAM, that elicit different intracellular signals, may be involved in the consolidation of the fascicle structure.

We expect that the axonin-1–NgCAM complexes involved in axonal growth along preexisting fibers in early stages of fiber tract development are functionally different from those that are involved in the consolidation of the fascicle structure in later phases. Axonin-1–NgCAM complexes of distinct compositions, that elicit different intracellular signals, may be generated by developmental changes in the relative expression of axonin-1 and NgCAM. This assumption is supported by in vivo studies that reveal marked changes in the expression of axonin-1 and NgCAM during fiber tract development (Dodd and Jessell, 1988; Dodd et al., 1988; Stoeckli and Landmesser, 1995). The aggregation of axonin-1 and NgCAM could be further influenced by the expression of other binding partners for the two molecules, that may change their signaling properties or alter the equilibrium of complex formation.

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