

Neurofascin Induces Neurites by Heterophilic Interactions with Axonal NrCAM while NrCAM Requires F11 on the Axonal Surface to Extend Neurites

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Abstract. Neurofascin and NrCAM are two axon-associated transmembrane glycoproteins belonging to the L1 subgroup of the Ig superfamily. In this study, we have analyzed the interaction of both proteins using neurite outgrowth and binding assays. A neurofascin-Fc chimera was found to stimulate the outgrowth of tectal cells when immobilized on an inert surface but not as a soluble form using polylysine as substrate. Antibody blocking experiments demonstrate that neurite extension on immobilized neurofascin is mediated by NrCAM on the axonal surface. Under the reverse

experimental conditions where NrCAM induces neurite extension, F11, and not neurofascin, serves as axonal receptor. Binding studies using transfected COS7 cells and immunoprecipitations reveal a direct interaction between neurofascin and NrCAM. This binding activity was mapped to the Ig domains within neurofascin. The neurofascin-NrCAM binding can be modulated by alternative splicing of specific stretches within neurofascin. These studies indicate that heterophilic interactions between Ig-like proteins implicated in axonal extension underlie a regulation by the neuron.

THE molecular analysis of axonal growth and pathfinding during embryonic development using immunological as well as genetic approaches has led to the characterization of a large number of axon-associated glycoproteins that can be categorized according to their structure into the Ig superfamily (IgSF)¹ (Goodman, 1996; Brümmendorf and Rathjen, 1995; Cunningham, 1995). Neurofascin originally identified in the chick belongs to a subgroup of the IgSF including additional L1(NgCAM) and NrCAM in vertebrates (Grumet et al., 1991; Kayyem et al., 1992; Burgoon et al., 1991; Moos et al., 1988; Rathjen et al., 1987a; Volkmer et al., 1992) and neuroglian in invertebrates (Bieber et al., 1989). Characteristic features of these three vertebrate proteins are six Ig-like domains located in the NH₂-terminal half followed by four or five fibronectin type III (FNIII)-like repeats. They are transmembrane proteins with a cytoplasmic segment of ~110 amino acid residues that is highly conserved among these proteins and appears to interact with the membrane-cytoskeletal protein ankyrin (Davis et al., 1993; Davis and Bennett, 1994; Dubreuil et al., 1996).

An interesting feature of neurofascin is that it is expressed in several isoforms generated by alternative pre-mRNA splicing. For example, in the extracellular region of chick neurofascin, two alternatively expressed stretches of 17- and 12-amino acid residues are found between the

second and third Ig-like domain and between the Ig- and FNIII-like regions, respectively. Close to the NH₂ terminus, a stretch of six residues is expressed only in some isoforms of neurofascin. In addition to these short stretches, the complete third FNIII module is alternatively spliced. Some isoforms of neurofascin contain an unusual membrane-proximal 75-amino acid residue segment rich in proline, alanine, and threonine, termed PAT domain, which is extensively O-glycosylated (Volkmer et al., 1992; Davis et al., 1993).

Comparison of embryonic with adult brain by Northern blot analysis indicates that some isoforms of neurofascin are dominant in the adult brain (Volkmer et al., 1992; Moscoso and Sanes, 1995). At embryonic stages, neurofascin is like several other Ig-like proteins confined to layers bearing axons with a restricted and dynamic pattern of expression as revealed by immunocytochemistry (Rathjen et al., 1987a). For example, at very early stages of the developing spinal cord, neurofascin is localized primarily on axons of the primitive longitudinal pathway. Commissural axons weakly express neurofascin in the circumferential pathway but express neurofascin strongly on their surface when they cross the floor plate and join the contralateral longitudinal fascicle (Shiga and Oppenheim, 1991). This expression of neurofascin on a distal axon segment resembles that of L1 on rat commissural axons (Dodd et al., 1988). At early stages of the developing retina, neurofascin is exclusively expressed in the optic fiber layer when the ganglion cells send out their axons to the optic nerve, while in the embryonic tectum, neurofascin is concen-

1. *Abbreviations used in this paper:* FNIII, fibronectin type III; GPI, glycosylphosphatidylinositol; IgSF, Ig superfamily.

trated in the tectobulbar tract (Rathjen et al., 1987a; Kröger and Schwarz, 1990). At later stages of tectal development, neurofascin becomes confined to axons of retinononrecipient laminae and, at more advanced stages, weak labeling was observed throughout all the tectal laminae (Yamagata et al., 1995). The pattern and timing of localization of neurofascin at embryonic development suggest a function in neurite extension. Consistently, *in vitro* antibody perturbation experiments have shown that neurofascin is implicated in axonal fasciculation and extension on other axonal surfaces (Rathjen et al., 1987a).

In this report, we have extended our studies on the functional and molecular characterization of neurofascin, and we show that neurofascin serves as a permissive environment for neurite outgrowth when provided as immobilized substrate. We demonstrate that NrCAM functions as a cellular receptor for tectal neurons to adhere and to extend neurites on immobilized neurofascin. Immunoprecipitations and immunohistological studies suggest a direct association of neurofascin and NrCAM also in neural tissues *in vivo*. Mapping experiments using deletion mutant polypeptides of neurofascin reveal that the Ig-like domains are sufficient to bind NrCAM. Neurite extension on immobilized NrCAM is mediated by axonally expressed F11 and not by neurofascin, although neurofascin is expressed on growth cones.

Materials and Methods

Cloning Procedures and Purification of Neurofascin-Fc

Partial sequences of neurofascin cDNA clones NF82, NF180, NF192, NF527, and NF533 (Volkmer et al., 1992) containing different alternatively spliced sequences were cleaved by restriction digestion and cloned into pSG5 (Stratagene La Jolla, CA) to yield eukaryotic expression plasmids pNF4, pNF6, pNF15, pNF16, pNF17, pNF22, pNF23, and pNF24 with different sets of alternatively spliced sequences. The structural features of the expressed neurofascin isoforms are depicted in Fig. 7 A. Plasmid pNF17 was cleaved with *Sma*I or *Nru*I for the construction of deletion mutants pNF13 and pNF21 (see Fig. 6 G). The *Sma*I sites at position 277 (between the small alternatively spliced NH₂-terminal sequence and the first Ig-like domain) and position 2047 (between the sixth Ig-like domain and the first FNIII-like repeat) and a *Nru*I site at position 3508 (located 5' close to the sequence coding for the transmembrane domain) were used. Positions refer to the published cDNA sequence (Volkmer et al., 1992). All three cleavage sites generate blunt ends that are in frame to each other. pNF13 was generated by removal of the *Sma*I (277)–*Sma*I (2047) fragment, and pNF21 was generated by the removal of the *Sma*I (2047)–*Nru*I (3508) fragment from pNF17.

For the production of soluble recombinant neurofascin, the *Eco*RI–*Nru*I (position 3508) DNA fragment from pNF17 containing the neurofascin cDNA, except for the transmembrane region and the cytoplasmic tail, was introduced into a modified plasmid pIgG1 to generate pFcNF17, the fusion of neurofascin sequences to the human IgG1-Fc (see Fig. 1 B). The *Eco*RI site was derived from a linker sequence immediately 5' of the neurofascin cDNA (Volkmer et al., 1992). Before cloning, an oligonucleotide with appropriate cleavage sites and a splice donor sequence was introduced into IgG1 (Simmons, 1993). After transient transfection of pFcNF17 or pDIFc, the latter only expressing Fc sequences fused to the signal peptide of F11 (Brümmendorf, T., and U. Treubert, unpublished results), COS7 cells were cultivated for 2 d. Supernatants were then collected and soluble fusion neurofascin protein was purified by protein A affinity chromatography. The purity of fusion protein preparations was analyzed by SDS-PAGE as well as by Western blots.

Binding of NrCAM-conjugated Microspheres to Neurofascin-expressing Cells

Red fluorescing Covaspheres (Duke Scientific Corp., Palo Alto, CA) with a 0.5- μ m diam were covalently coupled with affinity-purified NrCAM as

detailed elsewhere (Kuhn et al., 1991; Brümmendorf et al., 1993). COS7 cells were transfected with neurofascin encoding plasmids by the DEAE-dextran method and grown overnight (Brümmendorf et al., 1993). At day 1 after transfection, the cells were trypsinized, washed, and replated on collagen-treated 8-well chamberslides (Nunc Roskilde, Denmark). At day 2 after transfection, the supernatant was removed, and 150 μ l DME/10% FCS was added containing 1 μ l of fluorescent microspheres per well. After a 1.5-h incubation, cells were washed, fixed, and stained for neurofascin expression by indirect immunofluorescence using an mAb to neurofascin that does not interfere with binding. In blocking experiments, antibodies were added to a final concentration of 200 μ g/ml for polyclonal Fab fragments or 20 μ g/ml for mAbs. Bound beads were analyzed in the fluorescence microscope and binding was quantified using a confocal microscope (MRC1000; Bio Rad Laboratories, Hercules, CA). For the quantification of Covasphere binding, confocal images were analyzed with the Comos software (Bio Rad Laboratories). Single neurofascin-expressing cells were identified in the FITC channel, and Covasphere binding was quantified in the Texas red channel. The shape of cells loaded with beads was delineated with a mouse cursor. The defined area was analyzed in the Texas red channel with regard to the average pixel intensity. This provided a reliable measurement for the number of beads bound to a single cell. The mean pixel intensity of several measurements was expressed as the percentage of the mean control level.

Neurite Outgrowth Assay

Culture dishes (Petriperm; Bachhofer, Reutlingen, Germany) were coated with 100 μ l of affinity-purified neurofascin-Fc, Fc alone, or NrCAM at concentrations of 100 to 250 μ g/ml in the coating solution that was spread over 1 cm² delineated by a silicon fitting at 4°C overnight. An efficient coating of NrCAM was only obtained by incubating the culture dishes for 48 h at 4°C. The different neurofascin-Fc and NrCAM charges varied slightly in their biological activities, and an optimum concentration was titrated for each preparation. NgCAM was coated on nitrocellulose-containing culture dishes as detailed elsewhere (Lemmon et al., 1989; Brümmendorf et al., 1993). After removal of the supernatant, the spot was washed with PBS and blocked with DME/10% FCS for 30 min. Single cells were obtained by dissociation of chick tecta in a trypsin/EDTA solution and were resuspended in DME/10% FCS for plating on the immobilized neurofascin-Fc fusion protein, NrCAM, the Fc fragment without neurofascin sequences, or NgCAM (30,000 cells per well). In blocking experiments, Fab fragments of polyclonal antibodies were added at 200 μ g/ml and monoclonal IgG to neurofascin or NrCAM were used at 20 μ g/ml. Cultures were grown for 16 or 24 h at 37°C followed by staining by indirect immunofluorescence using mAb A2B5. Neurite outgrowth was quantified with an image analysis system as described previously (MEASURE-MOUSE; AI Tektron; Meerbusch, Germany; Brümmendorf et al., 1993) or with genias imaging software (image works; Teltow, Germany). The measure mouse system analyzes the longest neurite of a neuron, while the genias software measures the total length of neurites of a single neuron including the cell body. Neurite lengths obtained by the genias imaging software are therefore longer when compared with the measure mouse system. Parallel measurements of the same cultures by each system show a shift to higher mean neurite lengths in the genias system in comparison with the measure mouse system. Other differences in the length distribution of different samples were not observed.

Neurite outgrowth of tectal cells (6,000 cells per well) in the presence of soluble neurofascin-Fc, or soluble NgCAM, or soluble NrCAM was on polylysine-pretreated (100 μ g/ml) culture dishes. DME/FCS was used to block polylysine-coated culture dishes. Both proteins were added at the time of cell plating at concentrations of 20 μ g/ml in DME/FCS and cultures were grown for 48 h. Neurite outgrowth was analyzed using the genias software.

Antibodies and Purification of NrCAM, NgCAM, F11, and Neurofascin from Brain

The purification of F11, NgCAM(G4), NrCAM(Bravo), neurofascin, and NCAM from detergent extracts (1% Triton X-100) of plasma membrane preparations of adult chicken brain, and the specificity of the corresponding monoclonal and polyclonal antibodies and their Fab fragments have been detailed elsewhere (Rathjen et al., 1987a,b; Wolff et al., 1987; Brümmendorf et al., 1989; Morales et al., 1993; de la Rosa, 1990). NrCAM purified from detergent extracts appeared biologically active in contrast with that obtained from urea extracts of adult brains which were used in a pre-

vious communication and shown not to induce neurites (Morales et al., 1993). mAb W1B10 was purchased from Sigma Chemical Co., (St. Louis, MO), and hybridomas producing JG22 were obtained from the Developmental Studies Hybridoma Bank (Johns Hopkins University School of Medicine, Baltimore, MD)

Protein Analytical Procedures

Concentrations of protein solutions were determined according to Peterson (1977). SDS-PAGE (Laemmli, 1970) was performed with 7% acrylamide under reducing or nonreducing conditions followed by silver staining (Ansorge, 1985). Western blots of neurofascin fusion proteins or mutant polypeptides were analyzed using polyclonal antibodies to neurofascin followed by labeling with alkaline phosphatase-conjugated secondary antibodies and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium staining.

Immunoprecipitations and Immunohistological Studies

For the immunohistological localization of neurofascin and NrCAM, formaldehyde-fixed cryostat sections of embryonic retinae and tecta were incubated with primary and FITC-conjugated secondary antibodies as described elsewhere (Rathjen et al., 1987a). Immunoprecipitations of detergent extracts (1% octylglucoside in PBS supplemented with protease inhibitors) of chick retinae of embryonic day 12 were performed with mAbs to NrCAM or neurofascin. Immunocomplexes were precipitated with an mAb (HB58) directed to the mouse κ light chain conjugated to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) followed by several washing steps using a buffer containing 150 mM NaCl, 1% Triton X-100, 0.5% desoxycholate, 0.1% SDS, 50 mM Tris/Cl, pH 7.4, and protease inhibitors. Immunoprecipitates were analyzed in 7% SDS-PAGE followed by Western blotting and staining with mAbs to neurofascin, NrCAM, or NgCAM as primary antibodies and peroxidase-conjugated anti-mouse polyclonal antibodies as secondary antibodies. Binding of antibodies was visualized by chemiluminescence using the ECL system (Amersham Intl., Little Chalfont, UK).

Results

Induction of Neurite Extension by a Neurofascin-Fc Chimera

Previous in vitro antibody perturbation experiments have shown that neurofascin is implicated in axonal fasciculation and neurite extension over axonal surfaces (Rathjen et al., 1987a). However, a direct demonstration of neurite elongation using different neural cell types on immunoaffinity-purified neurofascin immobilized on an inert surface failed in contrast with other axonal IgSF members such as L1, axonin-1, or F11. One reason for this failure might be, as previously reported, that neurofascin is very sensitive to proteolytical degradation during purification leading to the inactivation of its biological activity. Alternatively, the immunoaffinity isolate might contain several alternatively

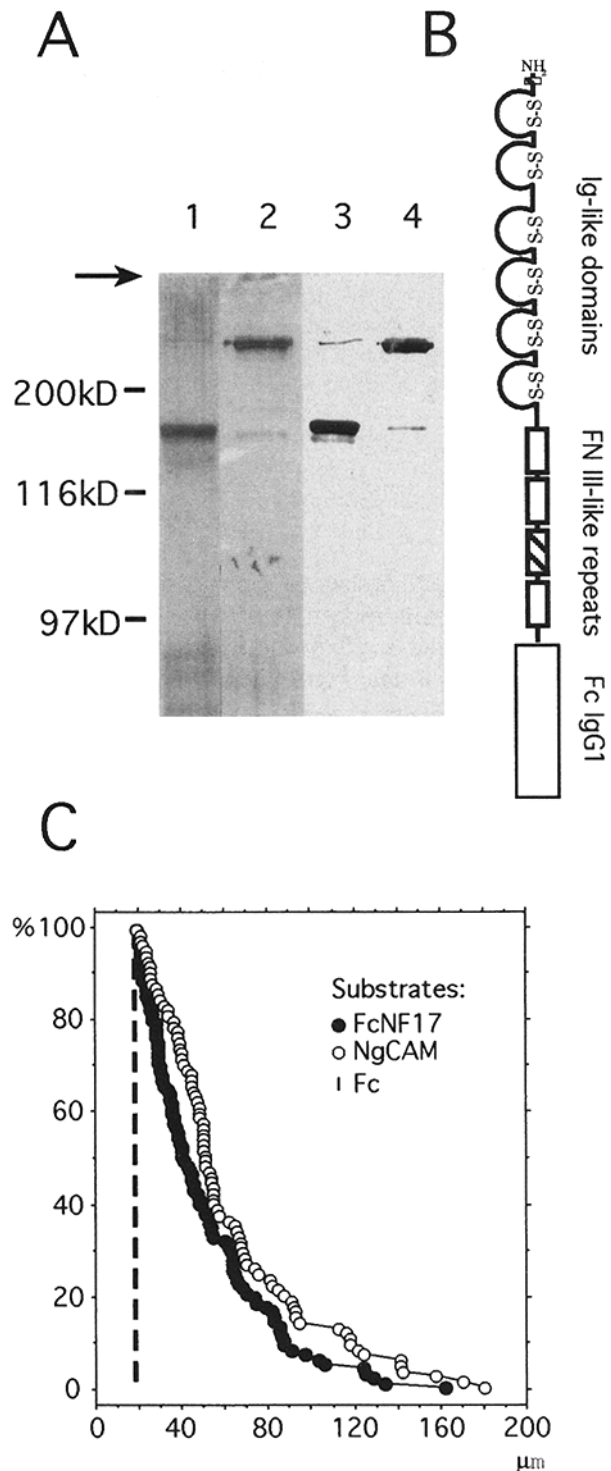


Figure 1. A neurofascin-Fc chimera induces neurites. (A) Analysis of the neurofascin-Fc fusion protein in SDS-PAGE and Western blotting. The neurofascin-Fc fusion protein (FcNF17) was purified from COS7 cell supernatants, and 0.5 μ g was resolved in SDS-PAGE followed by silver staining under reducing (lanes 1 and 3) or nonreducing conditions (lanes 2 and 4) followed by silver staining (lanes 1 and 2) or transferred to nitrocellulose and stained by polyclonal antibodies to neurofascin (lanes 3 and 4). Molecular mass markers are indicated at left. The arrow indicates the upper border of the separating gel. (B) Schematic model of neurofascin-Fc chimera (FcNF17). Loops indicate Ig-like domains; FNIII-like repeats are shown as small rectangles. The Fc portion is represented by a large rectangle below the FNIII-like

domains. Alternatively spliced regions are indicated as hatched symbols. (C) Neurite extension of tectal cells on immobilized neurofascin-Fc (FcNF17), the Fc fragment without neurofascin sequences, or NgCAM. Single cells were prepared from chick tecta of embryonic day 6 and cultivated for 16 h. Neurite lengths were measured with the measure mouse system, and the percentages of neurons (vertical axis) with neurites longer than or equal to 20 μ m (horizontal axis) are plotted as introduced by Chang et al. (1987). For each experimental condition, 80–120 neurites were measured. The broken line indicates that tectal cells do not extend neurites on the immobilized Fc protein lacking neurofascin sequences.

spliced isoforms with distinct or contrasting functions with respect to neurite extension.

To analyze a direct neurite outgrowth-promoting activity of neurofascin, we have therefore generated a neurofascin-Fc chimera composed of the extracellular portion of neurofascin and the Fc portion of human IgG1 (FcNF17; Fig. 1 *B*). Individual alternatively spliced sequences were added or omitted to generate a polypeptide that is closely related in its overall domain organization to outgrowth-promoting L1. Neurofascin-Fc was then purified from COS7 cell supernatants by protein A affinity chromatography. The isolate consisted of a single band at 165 kD in SDS-PAGE under reducing conditions (Fig. 1 *A*, lane 1), which differed from the expected size (139.8 kD), suggesting that the neurofascin-Fc generated in COS7 cells is glycosylated to a similar extent as brain neurofascin. The 165-kD component is also recognized by antibodies generated against brain neurofascin in Western blots (Fig. 1 *A*, lane 3). Under nonreducing running conditions, a band of ~300 kD appeared that was also recognized by antibodies to neurofascin, indicating that neurofascin-Fc is synthesized as a dimer linked by disulfide bridges within the Fc portion (Fig. 1 *A*, lanes 2 and 4). Neurons from chick tecta of embryonic day 6 were cultured for 16 h on the neurofascin-Fc chimera immobilized on a culture dish. The lengths of neurites were then measured with an image analysis system and compared with those of neurites on immobilized NgCAM. The quantitative analysis shows that, under identical assay conditions, the recombinant neurofascin fusion protein is as active as NgCAM in stimulating neurites (Figs. 1 *C* and 2 *A*). The immobilized Fc portion without neurofascin sequences was not found to promote neurite outgrowth (Fig. 1 *C*). This indicates that neurite induction is due to the neurofascin portion of the neurofascin-Fc chimera. Furthermore, attachment of neural cells and the neurite outgrowth-promoting activity of neurofascin-Fc are completely inhibited by Fab fragments of polyclonal antibodies to neurofascin (Fig. 2 *B*), providing additional evidence for the specific outgrowth-promoting activity of the neurofascin portion of the neurofascin-Fc chimera.

Axonal NrCAM Is Required to Induce Neurites on Immobilized Neurofascin

To identify an axonal receptor required for neurite extension on neurofascin-Fc, antibodies to several axon-associated proteins including several axonal Ig-like proteins (NCAM, NgCAM, NrCAM, F11, gicerin, and HT7) were added to tectal cultures. In the presence of Fab fragments of polyclonal antibodies to NrCAM, tectal cells were found not to adhere and extend neurites on immobilized neurofascin-Fc (Fig. 2 *C*). Antibodies to other axon-associated proteins were found not to effect neurite extension on neurofascin-Fc (not shown). The same polyclonal antibodies to NrCAM or neurofascin did not block neurite outgrowth on an NgCAM substratum (not shown; Morales et al., 1993). Moreover, one out of six mAbs to NrCAM (antiNr5) was found to completely inhibit outgrowth on neurofascin, indicating further specificity for the NrCAM-neurofascin interaction (Fig. 2, *D-F*; data not shown). Although neurofascin contains an RGD peptide sequence in the third FNIII-like domain, which is also conserved in

mammalian neurofascin (Volkmer et al., 1992; Davis et al., 1993), mAbs to β 1 integrins (W1B10 and JG22) did not effect neurite extension (not shown).

The blocking experiments using mAbs to NrCAM also suggest that different regions within NrCAM might be important for the F11-NrCAM and neurofascin-NrCAM binding. While mAb antiNr5 blocks neurite outgrowth on both neurofascin (Fig. 2 *D*) and F11 (see Morales et al., 1993), mAb antiNr1 and antiNr8 do not interfere with elongation on neurofascin but block neurite extension on F11 (Fig. 2, *E* and *F*).

NrCAM Induces Neurite Extension by Interactions with Axon-associated F11

In a previous communication, we reported that NrCAM immunopurified from urea extracts of adult brain did not induce neurite outgrowth if immobilized on an inert surface (Morales et al., 1993). Modification of the extraction protocol by using detergent instead of urea, and prolongation of the immobilization procedure up to 48 h, led to a biological active NrCAM preparation that stimulated neurite outgrowth of tectal cells. The length distribution after 24 h is comparable to that of neurofascin-Fc (Fig. 3 *A*). This induction can be completely blocked by antibodies to NrCAM (Fig. 3 *B*). Surprisingly, polyclonal antibodies to neurofascin were not found to block outgrowth on NrCAM (Fig. 3 *B*), although axons and growth cones extending on an NrCAM substratum express neurofascin on their surface (not shown). This finding indicates that, under these experimental conditions, NrCAM and neurofascin do not interact to induce neurite extension. To identify axonal receptors responsible for neurite outgrowth on immobilized NrCAM, *in vitro* blocking experiments with antibodies to other cell surface proteins were performed (see above). Of these, Fab fragments of polyclonal antibodies to F11 almost completely block extension, revealing that predominantly the glycosylphosphatidylinositol (GPI)-anchored F11 protein functions as a receptor for tectal cells to adhere and extend neurites on immobilized NrCAM (Fig. 3 *B*).

In contrast with immobilized neurofascin-Fc or NrCAM, tectal neurons cultivated on a polylysine substratum were not found to respond to neurofascin-Fc or NrCAM if added in soluble form. The percentage of cells with neurites was identical with or without soluble neurofascin-Fc or NrCAM (Fig. 4, *A* and *B*). In the presence of soluble NgCAM, however, the percentage of cells with neurites increases on a polylysine substratum (Fig. 4 *C*), which is in agreement with studies on a soluble L1-Fc chimera (Doherty et al., 1995). Taken together, these data provide evidence that neurofascin and NrCAM, in an immobilized but not soluble form, are able to promote neurite extension.

Ig-like Domains of Neurofascin Are Sufficient for NrCAM Binding

To demonstrate a direct interaction between neurofascin and NrCAM and to analyze the interaction of both proteins at the submolecular level, the ability of NrCAM-coupled fluorescent microspheres to bind to neurofascin expressed on the surface of COS7 cells after cDNA transfection was tested. An isoform of neurofascin corresponding to the

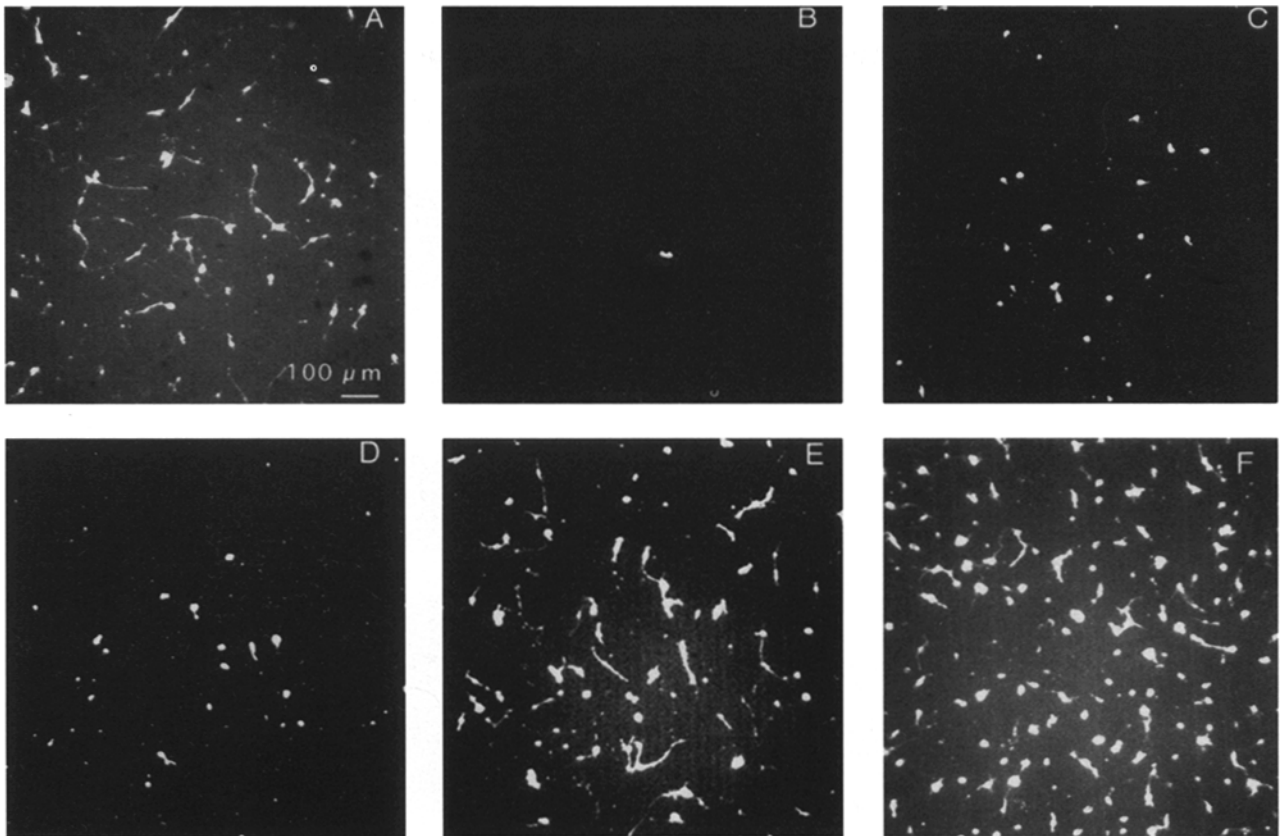


Figure 2. Neurite extension of tectal cells on immobilized neurofascin-Fc chimera in the presence of antibodies to neurofascin and NrCAM. Neurite extension after 16 h on immobilized neurofascin-Fc (FcNF17) is shown in the absence of antibodies (*A*), or in the presence of Fab fragments of polyclonal antibodies to neurofascin (*B*), Fab fragments of polyclonal antibodies to NrCAM (*C*), mAb antiNr5 directed to NrCAM (*D*), mAb antiNr8 directed to NrCAM (*E*), or mAb antiNr1 directed to NrCAM (*F*). For visualization, cultures were indirectly stained by mAb A2B5 and Cy-3-conjugated secondary antibodies and analyzed in an inverted fluorescence microscope. Fab fragments of polyclonal antibodies to neurofascin (*B*) or to NrCAM (*C*) and a mAb to NrCAM (*D*) completely blocked neurite extension on neurofascin-Fc. The same antibodies did not inhibit extension on NgCAM (Brümmendorf et al., 1993; Morales et al., 1993). Bar, 100 μ m.

fusion protein FcNF17 was linked to the neurofascin transmembrane and cytoplasmic region to generate NF17. Confluent COS7 cell cultures including a subpopulation of 10–20% of cells that express neurofascin (NF17) were incubated with NrCAM-conjugated beads, and neurofascin-expressing cells were identified by antibodies to neurofascin. Binding of NrCAM beads was confined to neurofascin-expressing cells as shown by double fluorescence analysis (Fig. 5, *A–C*). Mock-transfected or nontransfected COS7 cells did not bind NrCAM microspheres (Fig. 5; not shown). This binding could be almost completely blocked by Fab fragments of polyclonal antibodies to NrCAM or to neurofascin (Fig. 5 *D*). Moreover, one mAb to neurofascin (antiNF6) and one mAb to NrCAM (antiNr5) were found to reduce binding significantly (Fig. 5 *D*), providing additional specificity of the observed neurofascin–NrCAM interaction. Both mAbs were also found to inhibit neurite extension on neurofascin-Fc.

To analyze the NrCAM-binding region within neurofascin, deletion constructs of neurofascin encoding cDNA (pNF17) were generated and expressed on the surface of COS7 cells by transfection. The truncated protein NF13 lacked the Ig-like domains, while mutant polypeptide

NF21 was composed of the Ig-like domains only (Fig. 6 *G*). Although both mutant polypeptides were found to be expressed on the surface of COS7 cells at wild-type levels as judged by immunofluorescence analysis, only mutant polypeptide containing the Ig-like domains was found to bind NrCAM beads (Fig. 6, *E* and *F*), suggesting that the Ig-like domains are sufficient for binding to NrCAM. This binding could be blocked by polyclonal antibodies to neurofascin and NrCAM as well as mAb antiNr5 (Fig. 6 *H*).

Using similar procedures as outlined above for the analysis of the neurofascin–NrCAM interaction, we found no evidence for a homophilic binding activity of soluble neurofascin-Fc to surface-expressed neurofascin (not shown), as had been shown for other axonal members of the IgSF such as NCAM, L1, NrCAM, or axonin-1. Similarly, beads conjugated with other proteins of the IgSF such as NCAM were not found to bind to neurofascin-expressing COS7 cells, indicating further specificity of the neurofascin–NrCAM interaction.

cDNA cloning revealed that neurofascin contains at least six alternatively spliced sequences that yield an unknown number of isoforms. There are two alternatively spliced sequences including the third FNIII repeat, a pro-

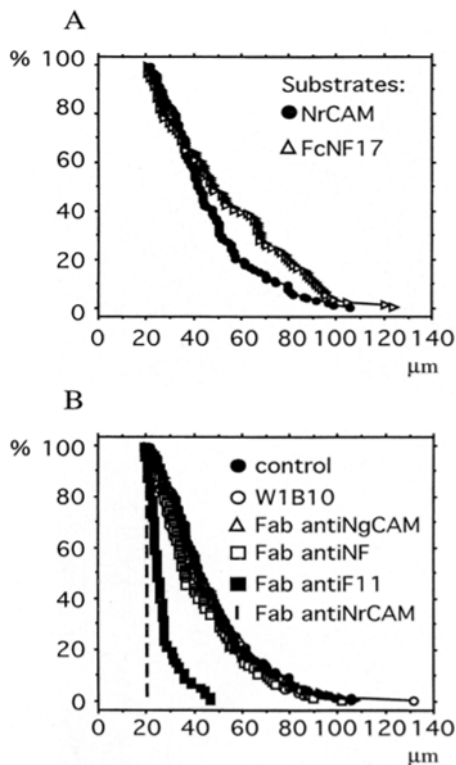


Figure 3. Neurite extension on immobilized NrCAM is inhibited by antibodies to F11. NrCAM was purified by immunoaffinity chromatography from detergent extracts of adult brains and immobilized on culture dishes. Tectal cells were grown for 24 h on neurofascin-Fc (FcNF17) or NrCAM (A). In B neurite lengths were analyzed on immobilized NrCAM (control) or NrCAM in the presence of Fab fragments of polyclonal antibodies to NrCAM, neurofascin, F11, or NgCAM or in the presence of mAb W1B10. Neurite outgrowth on NrCAM in the presence of Fab fragments of polyclonal antibodies to NrCAM resulted in a complete inhibition of neurite outgrowth as shown by a broken line in B. The lengths of 80–120 neurites were measured by the measure mouse system for each experimental condition. Data were compiled from two independent experiments.

line, alanine, and threonine segment, designated PAT domain, and four alternatively spliced short stretches of 4–17-amino acid residues. One of these short segments is found close to the NH₂ terminus, the second is between the second and third Ig-like domains, the third is between the Ig and FNIII domains, and the fourth is composed of 4-amino acid residues in the cytoplasmic domain. To analyze the influence of these alternative spliced stretches on NrCAM binding, several isoforms of neurofascin were expressed on the surface of COS7 cells (Fig. 7 A). In general, all isoforms were found to bind NrCAM to a similar extent in relation to standard NF17; however, the removal of two short stretches in the NH₂-terminal region in NF16 decreased NrCAM binding, while inclusion of the PAT domain (NF17 vs NF22) led to an increase of NrCAM binding (Fig. 7 B).

Interaction of Neurofascin and NrCAM in Embryonic Tissue

To demonstrate a direct interaction between neurofascin

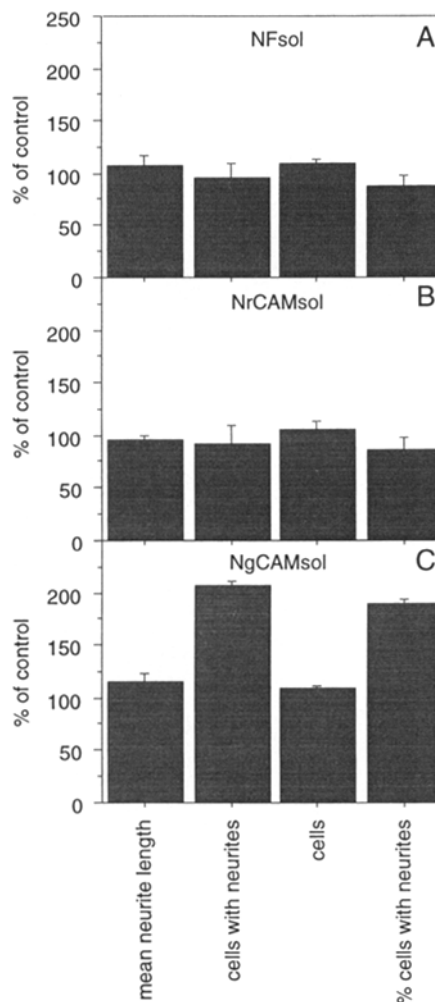


Figure 4. Neurite extension on a polylysine substratum in the presence of soluble neurofascin-Fc, NgCAM, or NrCAM. Tectal cells were grown on a polylysine substratum for 48 h in the presence of 20 μg/ml soluble neurofascin-Fc (A), soluble immunoaffinity-purified NrCAM (B), or soluble immunoaffinity-purified NgCAM (C). The mean neurite length, cells with neurites, total cells, and the relative proportion of cells with neurites were calculated as the percentage of the control cultures without soluble neurofascin or NgCAM using the genias software. Data were compiled from two independent experiments. Error bars represent SEM. In the presence of soluble NgCAM, the percentage of cells with neurites increased in contrast with soluble neurofascin or NrCAM.

and NrCAM in embryonic tissue, neurofascin was immunoprecipitated from detergent extracts of embryonic day 12 retinae with an mAb to neurofascin. Coprecipitating proteins were then analyzed in Western blots using mAbs to NgCAM or NrCAM. While NgCAM was found not to coprecipitate with neurofascin, NrCAM is clearly detectable (Fig. 8 A). The same result was obtained in the reverse experiment when antibodies to NrCAM were used to immunoprecipitate NrCAM, followed by the analysis of precipitates in Western blots with antibodies to neurofascin or NgCAM (not shown).

To further study this association of neurofascin and NrCAM in neural tissue, the localization of both proteins in

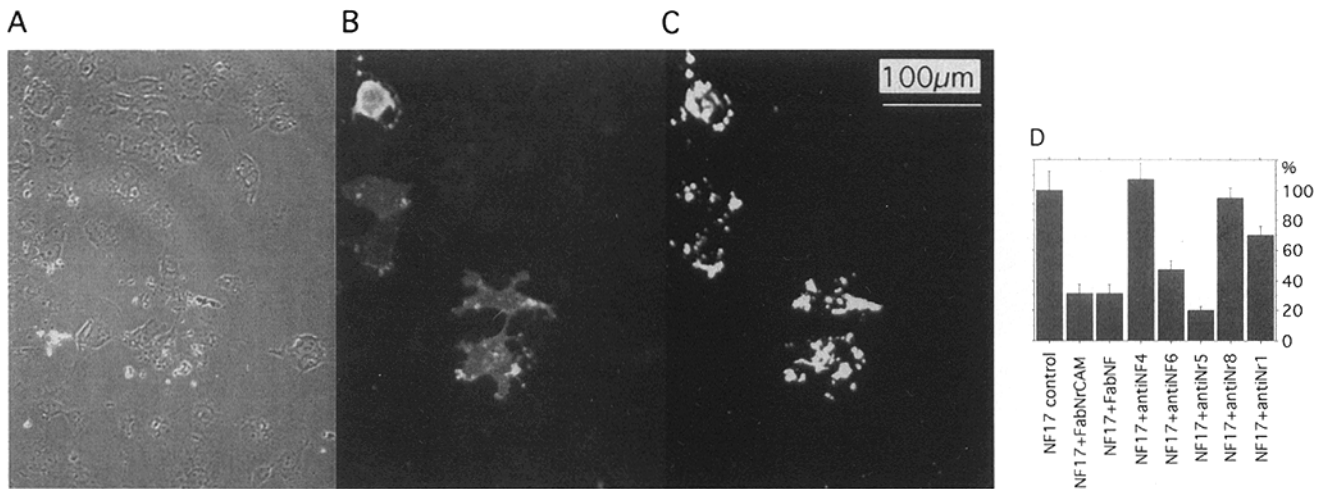


Figure 5. Binding of NrCAM to neurofascin expressed on the surface of COS7 cells. Neurofascin encoding cDNA (pNF17) was transiently transfected into COS7 cells. (A) Phase contrast of the same field as in B and C. (B) Cells expressing neurofascin were identified after microspheres bound to cells by indirect fluorescein immunofluorescence using an mAb to neurofascin. (C) Binding of NrCAM-conjugated red fluorescing microspheres to cells. Cells expressing neurofascin bind NrCAM-conjugated microspheres. (D) Fab fragments of polyclonal antibodies or mAbs to NrCAM or neurofascin block binding of NrCAM-conjugated microspheres to neurofascin-expressing COS7 cells. mAb antiNF6 also interferes with neurite extension on FcNF17 (data not shown). Binding was quantified in a confocal microscope equipped with the appropriate software. Bar graphs are expressed as the percentage of binding without antibodies (control). Error bars indicate the SEM. Bar, 100 μ m.

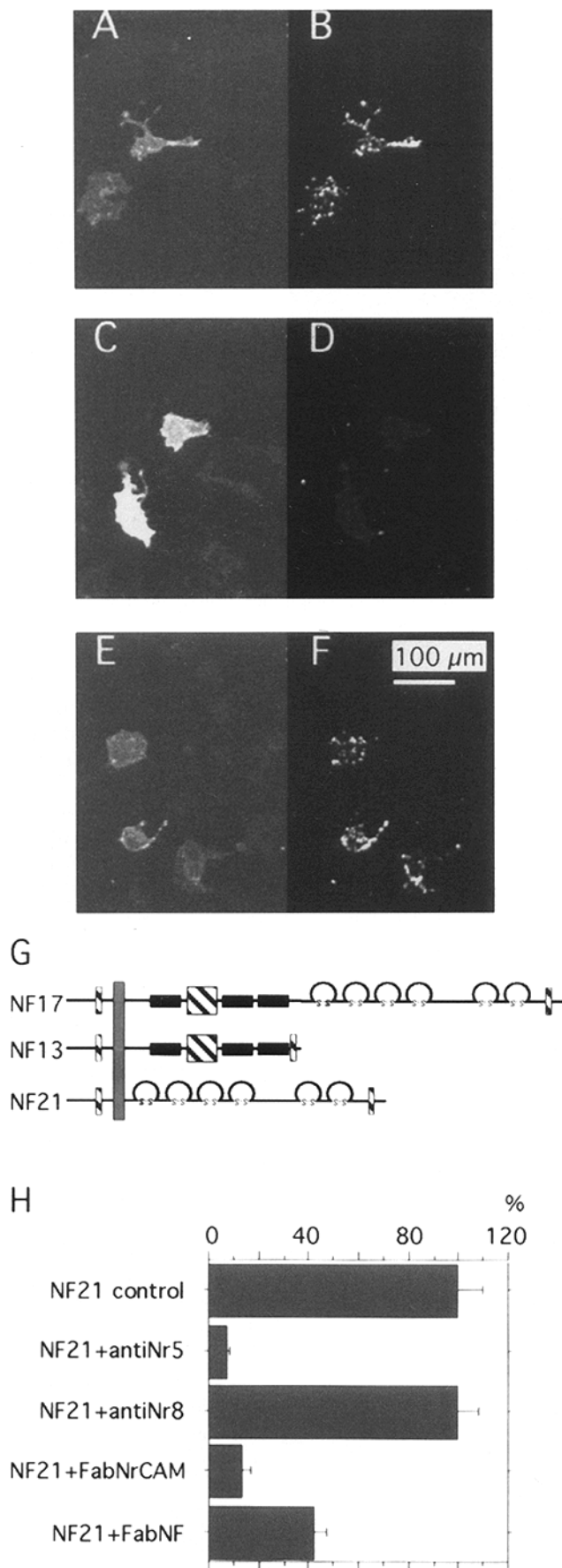
the embryonic visual system was analyzed by immunohistochemistry. At early stages of the developing retina, NrCAM and neurofascin colocalized exclusively in the developing optic fiber layer that contained the axons of the retinal ganglion cells. Within this layer, both proteins are concentrated in regions facing the vitreous body (Fig. 8, B and C). At more advanced stages, both proteins are also found in the inner plexiform layer of the retina and very weakly in the outer plexiform layer (Fig. 8, D and E). In the tectum, NrCAM and neurofascin appear to be expressed on tectobulbar axons that grow in fascicles at embryonic day 5 within the most superficial layer, termed marginal zone (Fig. 8, F and G). At embryonic day 10, both proteins are weakly expressed in the stratum opticum and the stratum album centrale (Fig. 8, H and I). Taken together, these studies on the localization and the immunoprecipitations also suggest an interaction between neurofascin and NrCAM in embryonic neural tissues.

Discussion

Several members of the IgSF with Ig- and FNIII-like domains coexist on many growth cones during embryonic development and participate in axonal extension and pathfinding. An interesting feature of these proteins is their complex binding pattern. To understand the function of the individual axonal Ig-like proteins, it is important to characterize the interacting counterreceptors implicated in neurite extension. In this communication, we have analyzed by *in vitro* experiments the cellular interactions and functions of neurofascin and NrCAM. Our studies show that both proteins are able to induce neurite extension, if immobilized on an inert surface. NrCAM serves as an axonal receptor of E6 tectal cells on a neurofascin substrate,

while F11 is required on the axonal surface to extend neurites on immobilized NrCAM. This was demonstrated by antibody perturbation assays of neurite outgrowth on immobilized recombinant fusion proteins of neurofascin and on immunoaffinity-purified NrCAM. Binding of NrCAM-coupled microspheres to COS7 cells transfected with neurofascin implied that the direct interaction of neurofascin and NrCAM accounts for the neurite outgrowth on an immobilized neurofascin-Fc substrate. An NrCAM binding site was located within the Ig-like domains of neurofascin. Our previous investigations have demonstrated a direct interaction between F11 and NrCAM and revealed that NrCAM serves as axonal receptor for immobilized F11 (Morales et al., 1993). Since neurite extension appears to be a complex process of which contact formation is only one parameter, our results do not exclude the possibility that other molecular interactions occur between the neuron and immobilized neurofascin or NrCAM that are required for axonal extension. *In vitro* antibody blocking experiments, however, exclude the participation of β 1 integrins, NCAM, NgCAM, HT7, and gicerin and axonin-1 in neurite extension on neurofascin or NrCAM.

The observation that the NrCAM-neurofascin interaction is not reciprocal, in contrast with the F11-NrCAM interaction (both can serve as substrate or axonal receptor for each other), raises the question of why tectal neurons use F11 and not neurofascin as receptor to extend on NrCAM although these proteins are expressed on their surface. These findings suggest the ability of a neuron to regulate the molecular interactions of Ig-like proteins. Regulation of the activity of Ig-like proteins by a neuron might be obtained through the existence of multiple states, perhaps generated by changes in the conformation of their extracellular regions. It is also conceivable that the association of an Ig-like protein with another protein within the



plane of the plasma membrane may result in a steric hindrance for a specific *trans* interaction (Sonderegger and Rathjen, 1992). Furthermore, neurofascin in tectal cells growing on NrCAM might not be linked to a signaling system that allows neurite extension, whereas F11 is. Regulation of the activity induced by different conformational states, or through the association with other surface components or by coupling to different signaling systems, would greatly expand the number of guidance cues provided by Ig-like proteins in the developing brain.

The extensive use of alternative splicing as it has been described for neurofascin (Volkmer et al., 1992) and NrCAM (Grumet et al., 1991; Kayyem et al., 1992) might be another possibility to change the binding activities of neurofascin or NrCAM. In this study, we found that the various, putative isoforms of chick neurofascin are able to bind to NrCAM. Addition or omission of individual alternatively spliced sequences did not result in the loss of neurofascin–NrCAM binding, indicating that they do not contain NrCAM binding sites or a decisive influence on the binding behavior. However, certain combinations of alternatively spliced sequences may at least exert some modulatory effects. Removal of short stretches close to the NH₂ terminus of neurofascin results in a decrease of NrCAM binding. In contrast, the inclusion of a proline-, alanine-, and threonine-rich segment, termed PAT domain, close to the plasma membrane–spanning domain, enhanced the binding of NrCAM to neurofascin. This highly O-glycosylated structure might induce a longer stiff structure within neurofascin and could extend the amino-terminal Ig-like domains well above the axonal glycocalyx, as it has been proposed for the low density lipoprotein receptor (Jentoft, 1990) and NCAM (Walsh and Doherty, 1991). It is currently unknown if this enhanced NrCAM–neurofascin binding modulates neurite outgrowth. Since the PAT domain is primarily expressed in forms of neurofascin at late developmental stages and in the adult nervous system, it is conceivable that glycosylation of this region might slow down neurite extension and stabilize cellular contacts. In contrast, the expression of polysialic acid, a specific carbohydrate moiety on NCAM, leads to a decrease of NCAM-mediated cell contacts, and its removal decreases NCAM-dependent neurite outgrowth (Rutishauser, 1993; Doherty and Walsh, 1994; Tang et al., 1992; 1994).

Other examples of heterophilic interactions of axonal Ig-like proteins include F11–NgCAM (Brümmendorf et al., 1993), F11–NrCAM (Morales et al., 1993), axonin-1–NrCAM (Stoekli and Landmesser, 1995; Suter et al., 1995), axo-

Figure 6. Binding of NrCAM microspheres to deletion mutants of neurofascin. Confocal images of neurofascin-expressing COS7 cells in the fluorescein channel (A, C, E) and of cells bearing NrCAM Covaspheres of the same microscopic fields (B, D, and F). (A and B) COS7 cells expressing NF17. (C and D) NF13. (E and F) NF21. (G) Structure of the full-length isoform of neurofascin NF17 and the deduced deletion mutants NF13 and NF21. Alternatively spliced sequences are shown as hatched symbols. (H) Quantification of NrCAM Covasphere binding to deletion mutant NF21 in the absence (control) or presence of mAbs to NrCAM or polyclonal Fab fragments to NrCAM or neurofascin. 21–30 individual cells were measured per sample. Bar, 100 μ m.

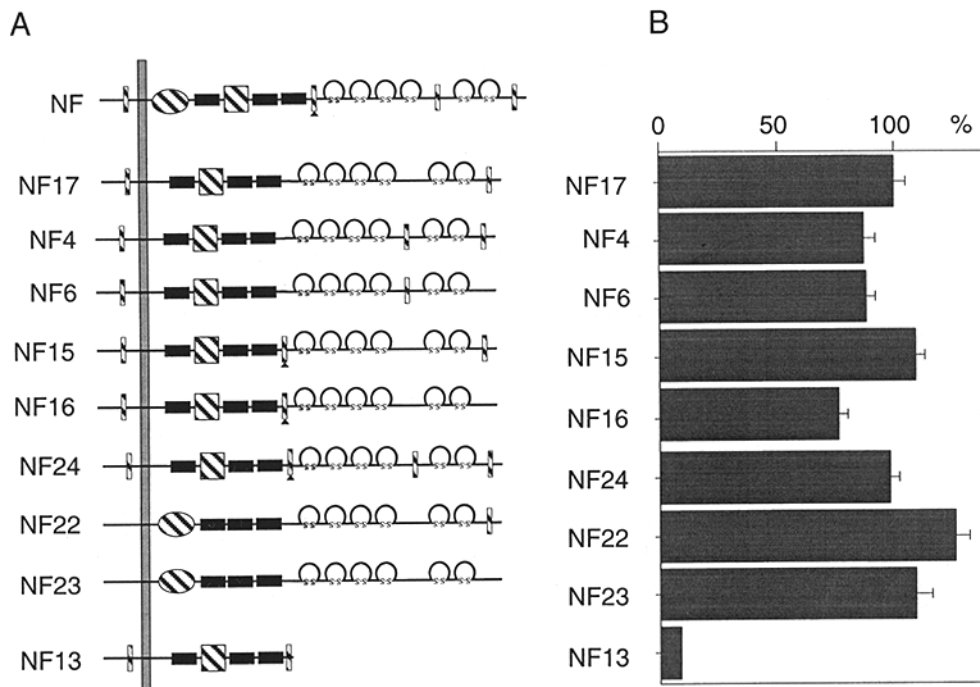


Figure 7. Binding of NrCAM microspheres to different isoforms of neurofascin. (A) Schematic model of neurofascin including all alternatively spliced regions in several neurofascin isoforms. All described neurofascin sequences from different cDNA clones are summarized at the top, as NF and isoforms generated by cloning are indicated by NF and a number. (B) Quantification of NrCAM Covasphere binding to different isoforms of neurofascin. As different plasmids were transfected for each sample, bead binding of individual cells was first related to the neurofascin expression, as determined by the measurement of the fluorescence intensity in the fluorescein channel, and then related to mean control levels. NF13 serves as negative control.

control, as it does not bind NrCAM beads (see also Fig. 6, C and D). 41–44 cells were compiled from two independent experiments for each sample. *p* values of a *t* test: NF15 vs NF16, *p* < 0.0001; NF15 vs NF17, *p* = 0.1973; NF17 vs NF22, *p* = 0.001.

nin-1–NgCAM (Kuhn et al., 1991; Felsenfeld et al., 1994; Rader et al., 1996), NgCAM–DMGRASP (DeBernardo and Chang, 1996), and NgCAM and NrCAM to so far uncharacterized components on L-cells (Mauro et al., 1992; Burgoon et al., 1995). The F11 protein has also been shown to bind to the receptor tyrosine phosphatase β/ζ (Peles et al., 1995), and the extracellular matrix proteins tenascin-C (Zisch et al., 1992) and tenascin-R (Rathjen et al., 1991; Brümmendorf et al., 1993). This latter interaction is also implicated in neurite extension, in that TN-R enhances F11-mediated neurite extension (Nörenberg et al., 1995) or that axon-associated F11 mediates a repulsive activity of TN-R (Pesheva et al., 1993). The multitude of binding partners seen in binding assays suggests a broad binding specificity for these Ig-like proteins. A simple explanation of the multitude of binding activities may be that the primary function of the NH₂-terminal Ig-like region is to establish an intimate contact between axons or between axons and the extracellular matrix, allowing other regions, including their FNIII domains, to operate. In all cases where binding regions have been localized in these proteins using mutant polypeptides, the NH₂-terminal Ig-like domains have been found to be sufficient for binding. In F11 and axonin-1, the smallest units active in binding were the first four Ig-like domains, and, on the basis of domain-specific antibodies, it was shown that specific Ig-like domains of F11 were more important for a specific type of binding than others (Brümmendorf et al., 1993; Morales et al., 1993; Rader et al., 1996). It is therefore conceivable that the first Ig domains function as a unit and might be in close physical association, as has been suggested for axonin-1 (Rader et al., 1996).

In a recent publication, Doherty and colleagues showed

that a soluble L1–Fc chimera is effective in stimulating neurite outgrowth from primary neurons. This finding, as pointed out by the authors, allows the separation of the signaling activity of L1 from the substrate function of L1 (Doherty et al., 1995). Neurite extension on immobilized L1 was found to be mediated primarily by a homophilic interaction of L1 (Lemmon et al., 1989; Williams et al., 1992). In our study, soluble neurofascin or NrCAM was not found to modulate neurite growth, in contrast with soluble NgCAM, the species equivalent of mammalian L1. This suggests that a dissociation of signaling from the substrate function is not possible in neurite extension mediated by heterophilic interactions, in contrast with primarily homophilic-mediated interactions such as L1 or NCAM (Doherty and Walsh, 1994). Furthermore, it is conceivable that the reciprocal F11–NrCAM and the non-reciprocal neurofascin–NrCAM interaction with respect to neurite extension might not result in a convergent signaling pathway. F11, which is in contrast with neurofascin, L1, and NrCAM, linked to the outer leaflet of the plasma membrane via GPI. How F11 transduces signals into the interior of a growth cone during neurite extension on NrCAM has not yet been established. There is, however, evidence that F11 is linked to an intracellular signaling system. Analysis of immunoprecipitations of nonionic detergent extracts followed by *in vitro* kinase assays revealed complexes of F11 with the nonreceptor tyrosine kinase Fyn (Zisch et al., 1995; Cervello et al., 1996). These immunocomplexes also contained an additional, yet uncharacterized, 75/80-kD component that may serve as a transmembrane “linker” protein, and that might bind to myristylated Fyn and to the GPI anchor of the cell surface–exposed F11. Other candidates required for a signaling function of

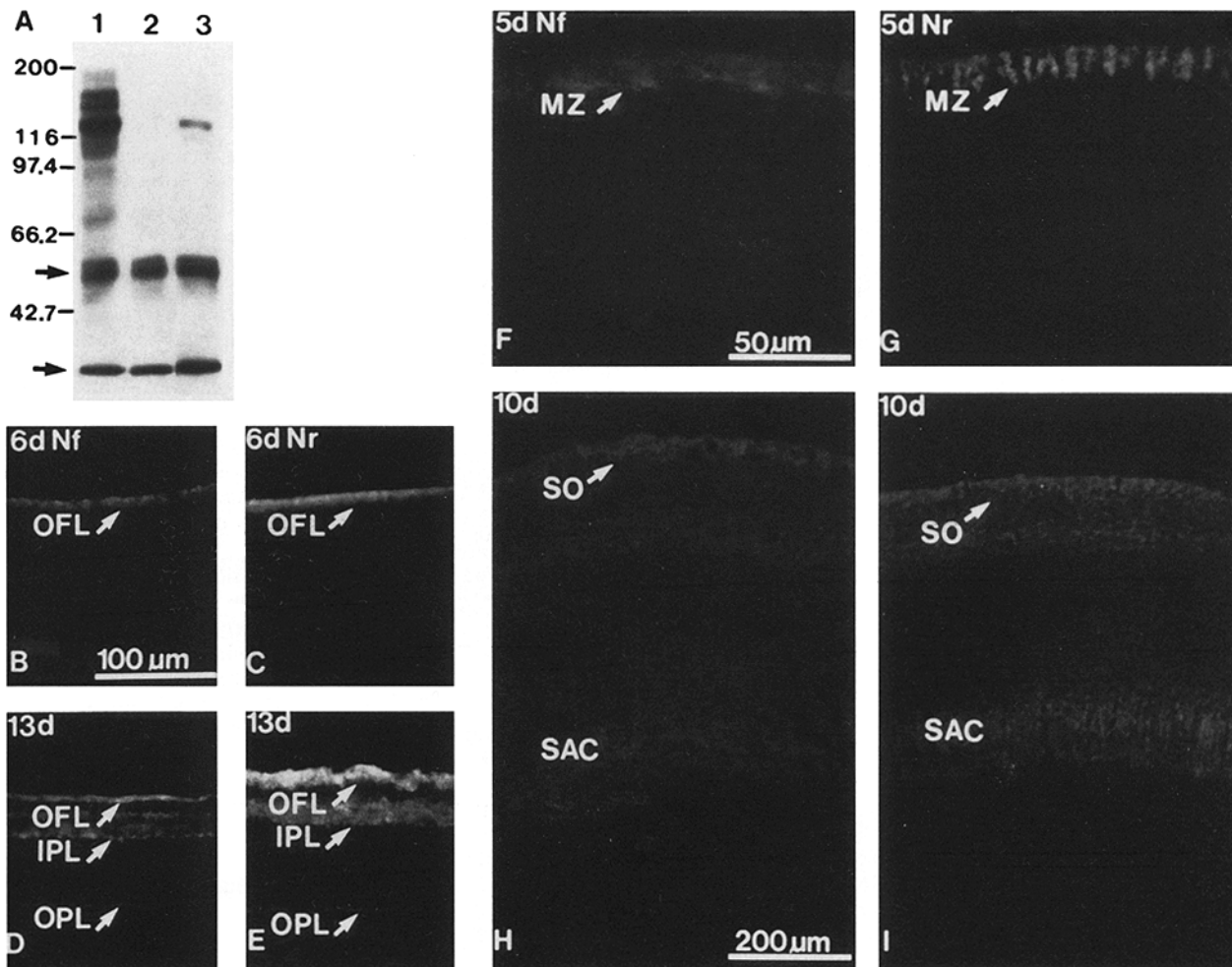


Figure 8. Neurofascin and NrCAM coprecipitate in immunocomplexes and colocalize in the embryonic visual system of the chick. (A) Retinæ from embryonic day 12 were solubilized in octylglucoside and precipitated with an mAb to neurofascin. Immunocomplexes were separated by SDS-PAGE and transferred to nitrocellulose followed by staining with antibodies to neurofascin (lane 1), NgCAM (lane 2), or NrCAM (lane 3). The molecular mass markers are indicated at left, and the arrows indicate the heavy and the light chain of the mAb used to precipitate neurofascin. Localization of neurofascin and NrCAM in two stages of the embryonic retinæ (B–E) or tectum (F–I) by indirect immunofluorescence using mAbs to neurofascin or NrCAM. The embryonic age is indicated at the left of each micrograph. OFL, optic fiber layer; OPL, outer plexiform layer; IPL, inner plexiform layer; MZ, marginal zone; SAC, stratum album centrale; SO, stratum opticum. Bars: (B) 100 µm; (F) 50 µm; (H) 200 µm.

F11 might be the transmembrane protein NgCAM (Brümmendorf et al., 1993) or the carbonic anhydrase domain of the receptor tyrosine phosphatase β/ζ (Peles et al., 1995). However, antibodies to NgCAM do not block neurite extension on NrCAM, and the receptor protein tyrosine phosphatase β/ζ is primarily found on glial cells (Engel et al., 1996).

The results described here indicate that the Ig/FNIII-like axonal proteins undergo multiple heterophilic interactions to regulate axonal extension. Several of the conclusions drawn from the neurite outgrowth and mapping experiments may extend to other members of this group of proteins and may allow us to dissect binding activities from other signals generated by these proteins, which are essential for neurite extension *in vitro* and *in vivo*.

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