

# Dissection of Complex Molecular Interactions of Neurofascin with Axonin-1, F11, and Tenascin-R, Which Promote Attachment and Neurite Formation of Tectal Cells

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**Abstract.** Neurofascin is a member of the L1 subgroup of the Ig superfamily that promotes axon outgrowth by interactions with neuronal NgCAM-related cell adhesion molecule (NrCAM). We used a combination of cellular binding assays and neurite outgrowth experiments to investigate mechanisms that might modulate the interactions of neurofascin. In addition to NrCAM, we here demonstrate that neurofascin also binds to the extracellular matrix glycoprotein tenascin-R (TN-R) and to the Ig superfamily members axonin-1 and F11.

Isoforms of neurofascin that are generated by alternative splicing show different preferences in ligand binding. While interactions of neurofascin with F11 are only slightly modulated, binding to axonin-1 and TN-R is strongly regulated by alternatively spliced stretches located in the NH<sub>2</sub>-terminal half, and by the proline-alanine-threonine-rich segment.

In vitro neurite outgrowth and cell attachment assays on a neurofascin-Fc substrate reveal a shift of cellular receptor usage from NrCAM to axonin-1, F11, and at least one additional protein in the presence of TN-R, presumably due to competition of the neurofascin-NrCAM interaction. Thereby, F11 binds to TN-R of the neurofascin/TN-R complex, but not to neurofascin, whereas axonin-1 is not able to bind directly to the neurofascin/TN-R complex as shown by competition binding assays.

In conclusion, these investigations indicate that the molecular interactions of neurofascin are regulated at different levels, including alternative splicing and by the presence of interacting proteins.

**Key words:** neurofascin • cell adhesion • axonal outgrowth • Ig superfamily • tenascin-R

**A**XONAL growth and pathfinding by permissive cues in the local environment of the growth cone may involve the function of L1-like proteins that form a subgroup within the Ig superfamily comprising L1/neuron-glia cell adhesion molecule (NgCAM),<sup>1</sup> neurofascin, NgCAM-related cell adhesion molecule (NrCAM), and CHL1 in vertebrates (Cunningham, 1995; Hortsch, 1996; Brümmendorf et al., 1998). They are concentrated in axon tracts of the developing and adult nervous system in a partial overlapping distribution. The L1-like proteins share a common overall domain organization and related amino

acid sequences (Brümmendorf and Rathjen, 1996). They are composed of six NH<sub>2</sub>-terminal Ig-like domains followed by five fibronectin type III (FNIII)-like repeats, a transmembrane domain, and a highly conserved cytoplasmic domain that confers cytoskeletal associations via ankyrin (Davis and Bennett, 1994; Hassel et al., 1997; Davis et al., 1993; Brümmendorf and Rathjen, 1995). The importance of the L1-related proteins on the development of the nervous system is exemplified by the observation that mutations in the human L1 gene cause the related mental retardation diseases X-linked hydrocephalus, MASA syndrome (mental retardation, aphasia, shuffling gait, and adducted thumbs), and spastic paraplegia type I (Wong et al., 1995; Brümmendorf et al., 1998; Fransen et al., 1997; Kenrick et al., 1996). Consistently, the embryonic development of the corticospinal tract is compromised in L1-deficient mice, and depending on the genetic background, the lateral ventricles of the brain are enlarged (Dahme et al., 1997; Cohen et al., 1997).

Within the L1 subgroup, neurofascin is unique as it contains a proline-alanine-threonine-rich segment (termed PAT domain) between the fourth and fifth FNIII-like re-

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<sup>1</sup> *Abbreviations used in this paper:* ECM, extracellular matrix; FNIII, fibronectin type III; IgSF, Ig superfamily; NCAM, neural cell adhesion molecule; NgCAM, neuron-glia cell adhesion molecule; NrCAM, NgCAM-related cell adhesion molecule; PAT, proline-alanine-threonine-rich segment; TN-C, tenascin-C; TN-R, tenascin-R.

peats that may be the target of O-glycosylation (Volkmer et al., 1992). cDNA cloning and genomic analysis of neurofascin indicated that at least 50 different isoforms are generated by alternate usage of nine alternatively spliced exons spread over the complete gene (Hassel et al., 1997). There are three alternatively spliced sequences comprising complete domains, the third as well as the fifth FNIII-like repeat and the PAT domain, and four short stretches of 4–15 amino acid residues in length (see Fig. 2 D). They are located at the NH<sub>2</sub> terminus between the second and third Ig-like domain at the junction between the Ig- and the FNIII-like domains and in the cytoplasmic segment. Isoforms that contain either the third or the fifth FNIII-like repeat are selectively localized either on unmyelinated axons or on the nodes of Ranvier in the peripheral nervous system, respectively (Davis et al., 1996).

We extended antibody perturbation assays delineating a crucial role of neurofascin in axon fasciculation by studies of neurite outgrowth on recombinant neurofascin-Fc substrates, showing that the interaction with cellular NrCAM promotes neurite outgrowth of tectal cells (Volkmer et al., 1996; Rathjen et al., 1987a). This neurofascin–NrCAM interaction, however, appears not to be reciprocal for neurite outgrowth since tectal neurons use the F11 protein and not neurofascin to extend neurites on immobilized NrCAM, suggesting the ability of a neuron to regulate molecular interactions of Ig-like proteins (Volkmer et al., 1996). Analysis of the binding characteristics of neurofascin deletion mutants indicated that the Ig-like domains of neurofascin are sufficient for interaction with NrCAM. To some extent, interactions with NrCAM are modulated by using specific amino acid stretches located in the NH<sub>2</sub>-terminal half of the polypeptide, and by the PAT domain (Volkmer et al., 1996).

Up to now a variety of molecular interactions have been described for the L1-like proteins NgCAM, NrCAM, and for L1 itself (Brümmendorf and Rathjen, 1996). In addition to homophilic binding activities, NgCAM as well as NrCAM interact both with the glycosyl-phosphatidylinositol (GPI)-linked neural Ig superfamily (IgSF) members F11 and axonin-1 (Suter et al., 1995; Kuhn et al., 1991; Brümmendorf et al., 1993; Morales et al., 1993). The interaction between F11 and NrCAM induces neurite outgrowth of tectal cells (Volkmer et al., 1996; Morales et al., 1993) and retinal cells (Treubert and Brümmendorf, 1998), and the NrCAM/axonin-1 interaction contributes to the guidance of commissural axons to the floor plate of the spinal cord, and is implicated in neuron–glia interactions (Suter et al., 1995; Stoeckli et al., 1997; Stoeckli and Landmesser, 1995). NgCAM and F11 also provide linkages to the extracellular matrix (ECM) by interactions with laminin-1, phosphacan, and neurocan, and with tenascin-R (TN-R) and tenascin-C (TN-C; Milev et al., 1996; Grumet et al., 1993; Zisch et al., 1992; Nörenberg et al., 1995; Rathjen et al., 1991).

In contrast to many extracellular interactions that have been described for L1/NgCAM and NrCAM, for neurofascin only the neurite outgrowth–promoting interaction with NrCAM has been characterized so far (Volkmer et al., 1996). To further characterize the molecular function of neurofascin, we were therefore interested in defining new binding partners of neurofascin using a transfection-based

binding assay. We observed that the IgSF members F11 and axonin-1, and the ECM glycoprotein TN-R specifically interacted with neurofascin. We then asked at which levels the molecular interactions of neurofascin can be regulated that finally might contribute to axonal extension. The analysis of the binding of different neurofascin isoforms to axonin-1, F11, and TN-R indicates that alternative splicing is one regulatory element to modulate the activities of neurofascin. Cell adhesion and neurite outgrowth assays then demonstrated that binding of TN-R to neurofascin induces a shift in receptor usage of tectal cells to adhere and extend neurites, suggesting that the molecular composition of these interacting proteins appears to be important for neurite outgrowth of tectal cells.

## Materials and Methods

### Cloning Procedures and Purification of Neurofascin-Fc

Construction of neurofascin isoforms NF4, 6, 15, 16, 17, 22, 23, and 24 as well as the deletion mutants NF13 (without Ig-like domains) and NF21 (without FNIII-like repeats) for expression in COS7 cells are detailed in Volkmer et al. (1996). For construction of neurofascin expression plasmids pNF32→pNF38, either pNF6, pNF22, or pNF23 were digested by restriction endonuclease SmaI which cleaves within the first Ig-like domain as well as within the first FNIII-like repeat, removing all Ig-like domains. This region was then replaced by the Ig-like domains of pNF6, pNF15, pNF23, or pNF24 to obtain permutations of the three small alternatively spliced exons in the context of either a 3rd FNIII<sup>+</sup>/PAT<sup>-</sup> or a 3rd FNIII<sup>-</sup>/PAT<sup>+</sup> construct (for a scheme of the isoforms see Fig. 2 D). An expression plasmid coding for FcNF15 was generated by fusing the extracellular part of NF15 to the Fc portion of human IgG1 (Simmons, 1993). Generation and isolation of the neurofascin-Fc fusion protein (FcNF15) and of the Fc portion of human IgG1 (DiFc) alone by Protein A affinity chromatography was done essentially as described previously (Volkmer et al., 1996).

### Immunoaffinity Purification of F11, Axonin-1, NgCAM, NrCAM, Neural Cell Adhesion Molecule (NCAM), Neurofascin, TN-R and TN-C, and Antibodies

F11, axonin-1, NgCAM, NrCAM, NCAM, neurofascin, TN-R, or TN-C were purified from adult chicken brains by immunoaffinity chromatography as described previously (Volkmer et al., 1996). Different TN-R charges reveal a certain degree of variability to increase the relative cell adherence to immobilized neurofascin-Fc. Isolation of monoclonal antibodies, generation of Fab fragments of polyclonal antibodies to the proteins listed above, and generation of bacterial fusion proteins containing TN-R domains and polyclonal antibodies to these domains are detailed elsewhere (Nörenberg et al., 1995; Nörenberg et al., 1992; Brümmendorf et al., 1989; Wolff et al., 1987; Rathjen et al., 1987a; Rathjen et al., 1987b). An mAb to axonin-1, kindly provided by P. Sonderegger (University of Zürich), was used to purify axonin-1 from phosphatidylinositol-specific phospholipase C extracts of chicken brains (Rader et al., 1996). Polyclonal antibodies to axonin-1 were generated by immunizing rabbits at 2-wk intervals in Freund's adjuvans.

### Microsphere Binding Assay and Transfection of COS7 Cells

Immunoaffinity-purified F11, axonin-1, NgCAM, NCAM, TN-R, or TN-C were conjugated to red fluorescent microspheres 0.5 μm in diameter according to the manufacturer's protocol (Bioscience Resource Project, Palo Alto, CA), and residual binding sites were blocked by BSA. COS7 cells were transiently transfected with neurofascin-encoding plasmids as detailed previously (Volkmer et al., 1996). At day 1 after transfection, cells were transferred to collagen-coated 8-well chamber slides (Nunc, Roskilde, Denmark), grown overnight, and then incubated at 37°C with 150 μl DMEM/10%FCS containing 1 μl of microsphere suspension. In antibody inhibition experiments, Fab fragments of polyclonal antibodies to neurofascin, TN-R, F11, or axonin-1 were added at a concentration of 200

$\mu\text{g/ml}$ . After 1 h of incubation, cells were washed, fixed in 4% formaldehyde/PBS, and stained for neurofascin expression by indirect immunofluorescence using an antibody to neurofascin that does not interfere with binding. For the quantification of microsphere binding and of neurofascin expression on single cells, fluorescence levels were measured with a confocal microscope (MRC1024; Bio-Rad Laboratories, Hercules, CA). Images were analyzed for red fluorescence, indicating microspheres binding to the cell and green fluorescence for neurofascin staining. Digital images were analyzed using the public domain program NIH IMAGE (developed at the United States National Institutes of Health and available on the internet at <http://rsb.info.nih.gov/nih-image/>). The procedure in general conforms with previously published methods (Volkmer et al., 1996). Individual neurofascin-expressing cells were outlined using a mouse cursor, and the mean fluorescence intensity of each cell was determined as a measure for the level of neurofascin expression. Microsphere binding was quantified by calculating the mean fluorescence intensity in the red channel within the same outlined area of individual cells. Microsphere binding to an individual cell was normalized with respect to the level of neurofascin expression of the same cell, and background values measured over single cells that did not bind microspheres were subtracted. About 20 cells per experiment were measured. For competition assays, neurofascin-expressing COS7 cells were preincubated for 15 min with soluble TN-C or TN-R at a concentration of 150  $\mu\text{g/ml}$  before adding bioclean beads coated with NrCAM, F11, or axonin-1.

### **Analysis of Cell Attachment and Neurite Outgrowth of Tectal Cells**

For analyzing neurite extension, 100  $\mu\text{l}$  of FcNF15, collagen, BSA, or the Fc portion of human IgG1 (DiFe) were immobilized at a concentration of 10  $\mu\text{g/ml}$  or of F11 at a concentration of 100  $\mu\text{g/ml}$  per  $\text{cm}^2$  culture dish (Petriperm; Bachhofer GmbH, Reutlingen, Germany). Residual binding sites were blocked by washing and incubating with DMEM/10% FCS. 10,000 chick embryonic day 6 tectal cells per  $\text{cm}^2$  delineated by a silicon fitting were plated in a volume of 100  $\mu\text{l}$  DMEM/10%FCS and incubated for 22 h. TN-R or TN-C was applied at a final concentration of 10 or 50  $\mu\text{g/ml}$  during the cultivation period. The different concentrations used gave qualitatively the same results; however, the percentage of adhering cells increased in the presence of the higher TN-R concentration (Fig. 3, A and C). Seven independent experiments were performed, two of these with 50  $\mu\text{g/ml}$  of TN-R. In cases of preincubation with TN-R, immobilized neurofascin-Fc was incubated with TN-R at a concentration of 10  $\mu\text{g/ml}$  after blocking for 1 h. Unbound TN-R was removed by extensive washing with DMEM/FCS before adding cells. Soluble axonin-1 or F11 was used at 20  $\mu\text{g/ml}$ . Fab fragments of polyclonal antibodies to the different cell surface proteins were added at a final concentration of 100  $\mu\text{g/ml}$ , and monoclonal antibodies were added at a final concentration of 10  $\mu\text{g/ml}$  of culture medium. After a 22-h cultivation, cells were fixed and stained by indirect immunofluorescence using mAb A2B5 followed by Cy3-conjugated rabbit anti-mouse as secondary antibody (Dianova, Hamburg, Germany). The cultures were analyzed using the GENIAS imaging software to measure the number of attached cells and the length of neurites (Image works, Teltow, Germany).

### **Immunoprecipitations**

Immunoprecipitations of detergent extracts (0.5% octylglucoside and 1% Triton X-100 in PBS supplemented with protease inhibitors) of chick retinae of embryonic day 11/12 were performed with mAbs to neurofascin, axonin-1, or F11 as detailed (Volkmer et al., 1996). Immunocomplexes were precipitated with an mAb (HB58) directed to mouse  $\kappa$  light chain conjugated to CNBr-activated Sepharose (Pharmacia Biotech Sverige, Uppsala, Sweden) followed by several washing steps using the solubilization buffer. Immunoprecipitates were analyzed in 8% SDS-PAGE followed by Western blotting with mAbs to neurofascin, NgCAM, axonin-1, F11, or NCAM as primary antibodies, and alkaline phosphatase-conjugated anti-mouse polyclonal antibodies as secondary antibodies.

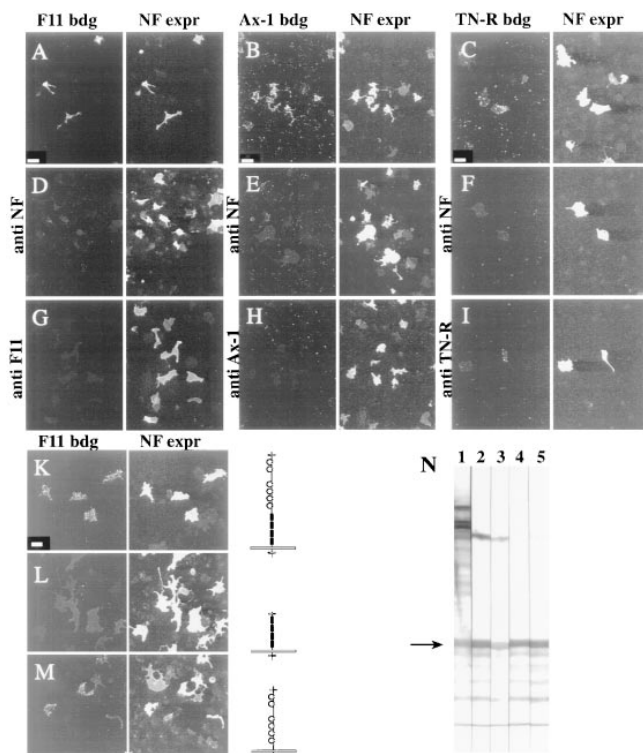
## **Results**

### **F11, Axonin-1, and TN-R Bind to Neurofascin**

An interesting feature of several axonal members of the L1- and F11-subgroups of the IgSF is their complex bind-

ing pattern with other surface-associated proteins or ECM glycoproteins (Brümmendorf and Rathjen, 1996). As neurofascin-mediated neurite extension and fasciculation might be modulated by distinct molecular interactions, we are interested in defining novel binding partners of this protein. As a first step we therefore analyzed whether neurofascin also binds to the axon-associated IgSF members NCAM, NgCAM, F11, axonin-1, or the ECM glycoproteins TN-C or TN-R. To this end, binding of protein-coated fluorescent microspheres to COS7 cells that express neurofascin on their surface was examined. Since neurofascin is generated in several isoforms by alternative splicing (Hassel et al., 1997) in the initial screen for novel binding partners, a neurofascin isoform (NF22, see Fig. 2 D) was used for transfection that shows the strongest binding activity to NrCAM beads (Volkmer et al., 1996). While incubation of microspheres coated with TN-C, NgCAM or NCAM did not lead to detectable binding to neurofascin-expressing COS7 cells (data not shown), beads conjugated with F11 (Fig. 1, A, D, and G), axonin-1 (Fig. 1, B, E, and H), or TN-R (Fig. 1, C, F, and I) were found to bind to neurofascin-expressing COS7 cells. Binding to untransfected cells within the same culture or to mock-transfected cells was not observed (data not shown). To provide additional data on the specificity, microspheres were incubated with transfected COS7 cells in the presence of Fab fragments of polyclonal antibodies to the proteins under investigation. While anti-neurofascin antibodies blocked binding of F11-, axonin-1-, or TN-R-coated microspheres to neurofascin-expressing COS7 cells (Fig. 1, D-F), Fab fragments to F11, axonin-1, or TN-R specifically blocked the interaction between neurofascin and the respective protein (Fig. 1, G-I). Incubating F11-coated beads with COS7 cells expressing neurofascin deletion mutants reveals that F11 specifically interacts with the Ig-like domains (Fig. 1 M), but not with the FNIII-like repeats of neurofascin (Fig. 1 L). TN-R or axonin-1-coated beads did not bind to either deletion mutant, suggesting that binding of these proteins to neurofascin is more complex, and that both proteins most likely require an intact neurofascin polypeptide to bind (data not shown).

To provide further independent evidence on these molecular interactions of neurofascin, and to study direct complex formation of neurofascin with F11, and of neurofascin with axonin-1 in neural tissues, neurofascin was immunoprecipitated from detergent extracts of embryonic day 11/12 chick retinae using an mAb to neurofascin. Immunoprecipitates were then analyzed in Western blots using mAbs to different axonal IgSF members. While NgCAM or NCAM were found not to coprecipitate with neurofascin, the F11 polypeptide and axonin-1 are clearly detectable in these blots, suggesting a direct interaction within the tissue (Fig. 1 N). The same result was obtained in the reverse experiment when antibodies were used to precipitate F11 or axonin-1, followed by an analysis of these precipitates in Western blots with antibodies to neurofascin (data not shown). A coprecipitation analysis of TN-R could not be performed since the solubilization of TN-R requires use of urea in the extraction buffer (Rathjen et al., 1991), which most likely destroys the conformation and therefore dissociates these protein complexes (data not shown).



**Figure 1.** (A–I) Binding of TN-R, axonin-1, and F11 by neurofascin isoform NF22 expressed on the surface of transfected COS7 cells. Double fluorescence images obtained using a confocal microscope are shown. The right half of each micrograph of A–I reveals neurofascin expression (*NF expr.*) by indirect immunofluorescence in the FITC channel, while the left half depicts the binding of F11– (A, D, and G), of axonin-1– (B, E, and H) or of TN-R– (C, F, and I) conjugated microspheres in the same microscopic field as detected in the Texas Red channel. Each microscopic field also contains unstained cells that do not express neurofascin, and concomitantly do not bind beads. Beads were incubated in the absence (A–C) or presence of Fab fragments of polyclonal antibodies to neurofascin (D–F), to F11 (G), to axonin-1 (H), or to TN-R (I), which is indicated at the left of each panel. (A) Bar, 100  $\mu\text{m}$ . (K–M) F11-coated beads bind to the Ig-like domains of neurofascin. COS7 cells transfected with plasmid NF17 (K) expressing both Ig- and FNIII-like domains of neurofascin, deletion mutant NF21 lacking the Ig-like domains (L), or deletion mutant NF13 lacking the FNIII-like repeats (M) were incubated with F11-coated beads. (K) Bar, 100  $\mu\text{m}$ . NF17 and the deletion constructs NF21 and NF13 are shown schematically at the right of each panel. (N) Neurofascin and F11 or neurofascin and axonin-1 coprecipitate in immunocomplexes. Retinae from embryonic day 11/12 were solubilized in detergent containing buffer, and were subjected to immunoprecipitation using an mAb to neurofascin. Immunocomplexes were separated by SDS-PAGE and transferred to nitrocellulose, followed by staining with antibodies to neurofascin (lane 1), F11 (lane 2), axonin-1 (lane 3), NCAM (lane 4), or NgCAM (lane 5). Binding of antibodies was visualized by alkaline-conjugated secondary antibody. The arrow indicates the heavy chain of the mAb used to immunoprecipitate neurofascin.

In summary, the data obtained demonstrate that in addition to NrCAM (Volkmer et al., 1996) the GPI-anchored IgSF members F11 and axonin-1 and the ECM glycoprotein TN-R are novel binding partners of neurofascin if neurofascin is expressed on the cell surface.

### Differential Binding of TN-R, Axonin-1, and F11 to Isoforms of Neurofascin

Different neurofascin isoforms are generated by alternative splicing during embryonic development and, as shown previously, different combinations of alternatively spliced stretches of neurofascin modulate the binding to NrCAM to some extent (Hassel et al., 1997; Volkmer et al., 1996). To analyze whether distinct neurofascin isoforms bind differentially to TN-R, to axonin-1, or to F11, 15 selected neurofascin isoforms were expressed in COS7 cells (Fig. 2 D), and binding of microspheres was quantified and normalized with respect to neurofascin expression on the cell surface using a confocal microscope as described previously (Volkmer et al., 1996). In contrast to F11, which bound to all tested neurofascin isoforms with different efficiency (Fig. 2 A), significant TN-R binding was only observed to neurofascin isoforms NF15, NF24, NF22, NF34, NF36, NF38, and NF35 (Fig. 2 B). Removing the NH<sub>2</sub>-terminal miniexon and/or the miniexon between the Ig- and FNIII-like repeats resulted in a considerable reduction of TN-R binding, while inclusion of the PAT domain increased TN-R binding. Furthermore, addition of the miniexon between the second and third Ig-like domain slightly decreased binding in several isoforms (NF35 vs. NF36 or NF38 vs. NF37).

Similar to TN-R binding, a crucial influence of the alternatively spliced short stretches and of the PAT domain of neurofascin was observed on axonin-1 binding. For example, removing the miniexon at the junction between the Ig-like and FNIII-like repeats (NF15 vs. NF17) strongly reduced axonin-1 binding; however, binding could be restored if the third FNIII-like repeat was replaced by the PAT domain (NF17 vs. NF22).

Taken together, these binding data on neurofascin isoforms indicate that alternative splicing of the neurofascin gene may be one mechanism to modulate the preference of neurofascin for one of its ligands, and emphasizes a pivotal role of the miniexons within the NH<sub>2</sub>-terminal half as well as the PAT domain of neurofascin in the regulation of binding. In comparison to F11, interactions of neurofascin with axonin-1 or TN-R are more strongly regulated by these alternatively spliced stretches.

### TN-R Induces a Shift in Receptor Usage of Tectal Cells to Adhere and Extend Neurites on a Neurofascin/TN-R Complex

In addition to alternative splicing, the activities of neurofascin may be regulated by the presence of specific binding proteins. Therefore, to analyze the function of the interaction between neurofascin and axonin-1, between neurofascin and F11, and between neurofascin and TN-R, we used in vitro neurite outgrowth and cell attachment assays in which tectal cells were cultivated on neurofascin-Fc immobilized on culture dishes in the presence of blocking antibodies or of purified soluble proteins. Antibodies to F11, axonin-1, or TN-R were not found to affect neurite extension on immobilized neurofascin-Fc, which is known to be mediated by neuritic NrCAM (see Fig. 4 B; Volkmer et al., 1996), suggesting that F11, axonin-1, or TN-R are not important for neurite extension in this experimental system. We therefore incubated tectal cells on immobilized neuro-

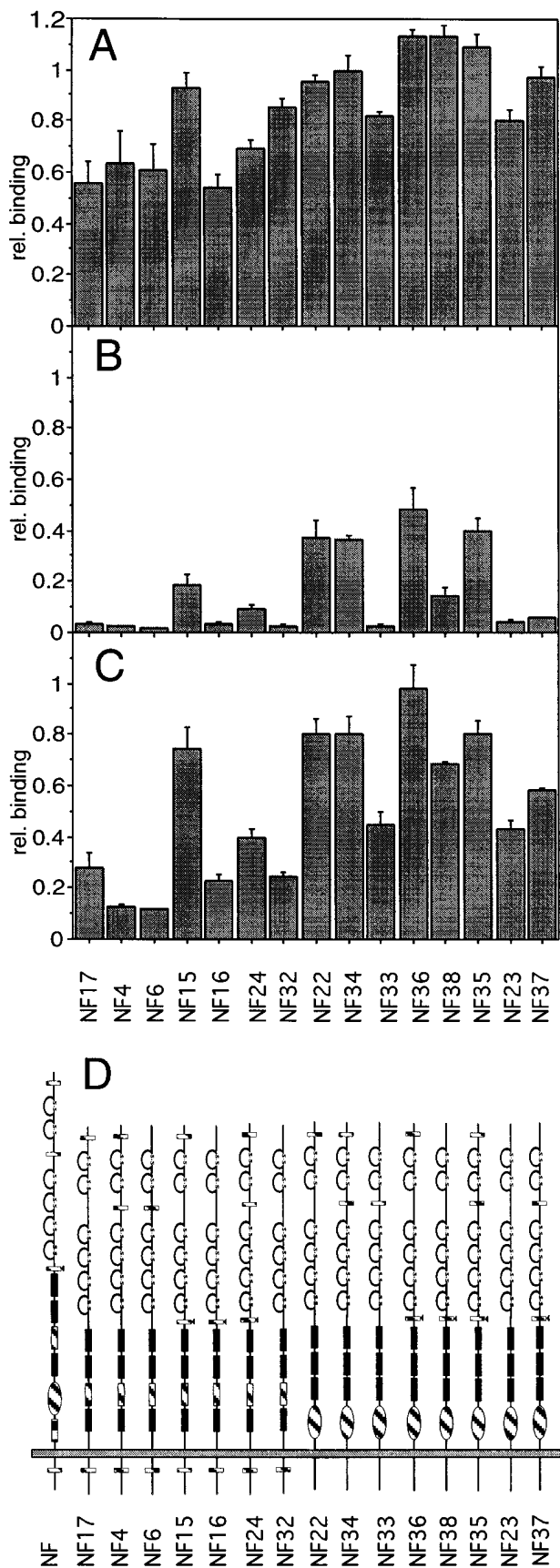


Figure 2. Binding of F11-, axonin-1-, or TN-R-conjugated microspheres to different isoforms of neurofascin. After transfection of neurofascin plasmids into COS7 cells and incubation with

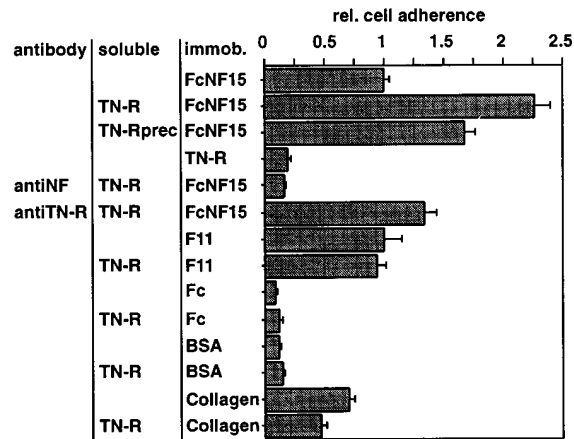
fascin-Fc in the presence or absence of soluble F11, axonin-1, or TN-R to study their influence on neurite outgrowth and long-term cell attachment. A neurofascin isoform was chosen that binds to TN-R and to the other ligands, and is composed of the extracellular region of NF15 fused to the Fc portion of human IgG<sub>1</sub>. It therefore contains the short exons at the NH<sub>2</sub> terminus, and between the Ig- and FNIII-like domains; however, it lacks the PAT segment of neurofascin (see Fig. 2 D for a schematic representation of NF15). Among the different combinations that we analyzed in these assays, the interaction between neurofascin and TN-R appeared to be of particular importance since it was found to modulate the behavior of tectal cells to neurofascin as described below.

In contrast to axonin-1 or F11, application of soluble TN-R to the immobilized neurofascin-Fc protein resulted in considerable stimulation of neural long-term cell attachment (Fig. 3, A and B), whereas analysis of the length distribution of neurites performed in the presence of soluble TN-R remained unchanged (Fig. 4 E; see below). Increased neural attachment was also observed after preincubating the neurofascin-Fc substrate with TN-R, followed by removal of unbound TN-R by washing before adding tectal cells, whereas immobilized TN-R alone does not allow long-term cell attachment (Fig. 3 A) and neurite extension of tectal cells of embryonic day 6 (Fig. 4 E; Rathjen et al., 1991; Nörenberg et al., 1995). Attachment of tectal cells to the neurofascin-Fc/TN-R complex could be blocked by polyclonal antibodies to TN-R to levels using neurofascin-Fc alone, while antibodies to neurofascin inhibited attachment completely (Fig. 3 A). TN-R did not enhance neural attachment on the Fc portion without neurofascin sequences, collagen, or F11 substrates (Fig. 3 A). Application of soluble TN-C, which does not bind to a neurofascin substrate, did not result in an enhancement of cell attachment (data not shown), emphasizing the specificity of the interaction between neurofascin and TN-R (see Fig. 6 B). These findings indicate that the increase of attached cells was due to the presence of TN-R and to an interaction between soluble TN-R with immobilized neurofascin-Fc.

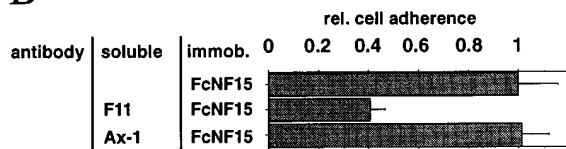
In accordance with previous results, anti-NrCAM antibodies completely abolished cell attachment and neurite outgrowth on a pure neurofascin-Fc substrate (Fig. 4 B; Volkmer et al., 1996). In the presence of soluble TN-R, however, cell attachment (Fig. 3 C) as well as neurite outgrowth (Fig. 4 D) on neurofascin-Fc remained unaffected by Fab fragments of polyclonal antibodies to NrCAM, suggesting that NrCAM does not function as a cellular re-

fluorescent beads coated with F11 (A), TN-R (B), or axonin-1 (C), the binding of beads on individual cells was quantified and normalized with respect to the expression level of neurofascin as described in Materials and Methods. Bars indicate SEM. (D) Overview of neurofascin isoforms encoded by individual plasmids to transfect COS7 cells. Alternatively expressed segments are hatched. Ig-like domains are indicated by loops, while FNIII-like repeats are shown as rectangles. All identified neurofascin sequences (Hassel et al., 1997) are summarized at the left as NF and isoforms tested in the assay are indicated by NF and a number. The left schematic version containing all spliced segments has not been detected in brain tissue (Hassel et al., 1997).

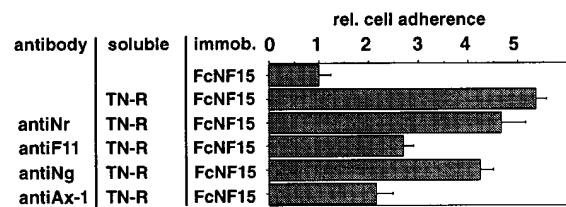
**A**



**B**

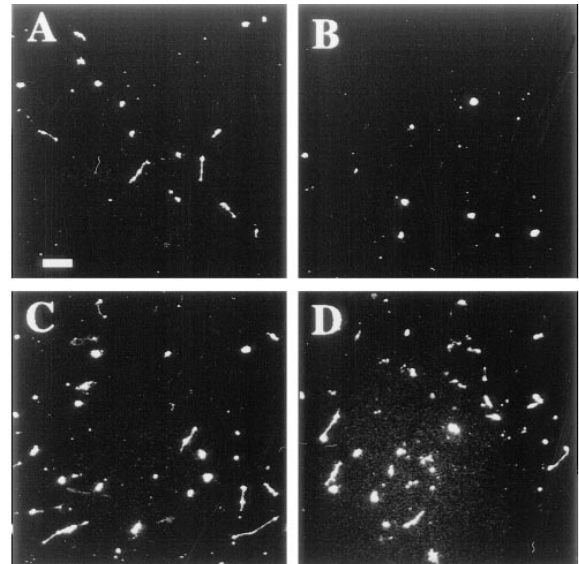


**C**

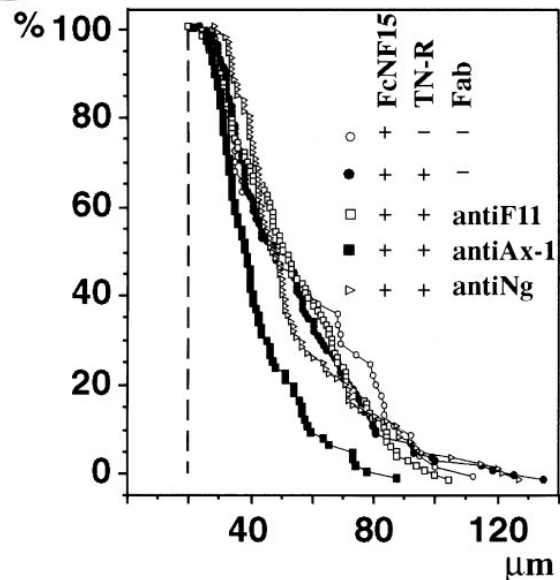


**Figure 3.** (A–C) The adherence of tectal cells to a neurofascin-Fc substrate is enhanced in the presence of TN-R. Neurofascin fusion protein with the Fc portion of human IgG1 (*FcNF15*), the Fc portion without neurofascin sequences (*Fc*), F11, BSA, or collagen were immobilized on petriperm dishes, and dissociated tectal cells were incubated in the presence or absence of TN-R (A and C) or in the presence of F11 or axonin-1 (B). The immobilized proteins, the protein in solution, or the applied antibody is given at the left of each panel. (A) 10  $\mu\text{g/ml}$  or (C) 50  $\mu\text{g/ml}$  of TN-R was used to monitor the effects of the different antibodies. The concentration of soluble F11 or axonin-1 was 20  $\mu\text{g/ml}$  in B. Long-term attachment (after 22 h) of cells was quantified with the GENIAS imaging software, and the adherence of tectal cells to neurofascin (*FcNF15*) was calculated to 1. Error bars indicate SEM. TN-R was either provided in the supernatant of a neurofascin substrate during the whole culture period (*FcNF15* + TN-R) or washed out after a 1 h incubation period before adding the cells (*FcNF15* + TN-Rprec). Cells were also incubated in the presence of Fab fragments of polyclonal antibodies specific for neurofascin (*antiNF*), NrCAM (*antiNr*), F11 (*antiF11*), NgCAM (*antiNg*), axonin-1 (*antiAx-1*), or TN-R (*antiTN-R*) at a concentration of 100  $\mu\text{g/ml}$ .

ceptor for cell attachment and neurite extension on the neurofascin-Fc/TN-R complex, in contrast to the pure neurofascin-Fc substrate. Fab fragments of polyclonal antibodies to NgCAM had a slight effect, whereas Fab fragments to F11 and axonin-1 both interfered strongly with cell attachment (Fig. 3 C). Neurite extension was partially



**E**



**Figure 4.** Receptor switch from NrCAM to axonin-1 in the presence of TN-R. (A–D) Representative micrographs of cultures of tectal cells on a neurofascin-Fc substrate in the absence (A and B) or in the presence of TN-R (C and D). Anti-NrCAM antibodies block neurite outgrowth on a neurofascin substrate in the absence (B) but not in the presence of TN-R (D). Bar, 100  $\mu\text{m}$ . (E) Quantification of neurite extension on neurofascin-Fc (*FcNF15*) in the presence or absence of TN-R, and Fab fragments of polyclonal antibodies to F11, axonin-1, or NgCAM. The broken line indicates that tectal cells do not extend neurites on immobilized TN-R. The lengths of individual neurites were measured with the GENIAS imaging software, and are plotted as introduced by Chang et al. (1987). The percentage of neurons (vertical axes) with neurites longer than or equal to 20  $\mu\text{m}$  (horizontal axis) is shown. For each experimental condition, 80–120 neurites were measured. Neurite length of tectal cells is not affected in the presence of TN-R. Only Fab fragments of polyclonal antibodies to axonin-1 and not to NgCAM or F11 reduce neurite length on a combined neurofascin/TN-R substrate (*P* values of the *t* test: TN-R vs. TN-R+anti-F11, 0.98; TN-R vs. TN-R+anti-NgCAM, 0.81; TN-R vs. TN-R+anti-Ax-1; 0.0002).

inhibited by Fab fragments to axonin-1, while antibodies to NgCAM or F11 did not inhibit neurite outgrowth (Fig. 4 E) on the neurofascin-Fc/TN-R complex. These observations suggest that under these experimental conditions, F11, axonin-1, and probably an unknown component (however not NrCAM) are important for the attachment of neural cells to the neurofascin-Fc/TN-R complex, while neurite extension appears to be dependent on axonin-1 and at least one unknown surface protein. In comparison to a pure neurofascin substratum, formation of a neurofascin/TN-R complex therefore results in a shift of receptor usage of tectal cells to adhere and extend neurites.

### Competition Among F11, Axonin-1, NrCAM, and TN-R to Bind to Neurofascin

One explanation for the finding that NrCAM is replaced as receptor for cell adhesion and neurite extension on the neurofascin-Fc/TN-R complex might be that TN-R sterically blocks the NrCAM binding region within neurofascin, but still allows binding of axonin-1 and F11 expressed by tectal cells to neurofascin or to the neurofascin/TN-R complex. To mediate the attachment of tectal cells, axonin-1 may directly interact with neurofascin and not with TN-R of the complex since no direct binding between axonin-1 and TN-R has been detected so far in different binding assays (Nörenberg et al., 1995 and data not shown). F11 may either bind to TN-R as shown in a previous communication (Brümmendorf et al., 1993; Rathjen et al., 1991; Nörenberg et al., 1995) or to neurofascin as shown above. To address these questions we made use of the observation that some neurofascin isoforms bind TN-R, and others do not. COS7 cells were transfected with the TN-R-binding isoform NF22 and the nonbinding isoform NF17 (see Fig. 2 B), followed by an incubation with F11-, axonin-1-, or NrCAM-coated microspheres in the presence or absence of soluble TN-R or of soluble TN-C as a control (Fig. 5).

Incubation of NrCAM-coated beads with NF22-expressing COS7 cells led to a significant reduction of NrCAM bead binding in the presence of TN-R (Fig. 5 B). In accordance, NrCAM bead binding to TN-R nonbinder NF17 (Fig. 5 A) remained unaffected, indicating that binding of TN-R to neurofascin is required for the competition of the neurofascin-NrCAM interaction, and that NrCAM does not bind to the neurofascin-TN-R-complex (Fig. 5 B). TN-C instead of TN-R that binds neither NrCAM nor neurofascin did not compete NrCAM bead binding, indicating further specificity (Fig. 5, A and B). Binding of F11-coated beads to NF17 is markedly reduced in the presence of soluble TN-R or TN-C (both are known to bind F11 [Zisch et al., 1992; Nörenberg et al., 1995]), indicating that both compete with neurofascin for the binding site on F11 (Fig. 5 D). In contrast, if the TN-R-binding neurofascin isoform NF22 was expressed, F11 bead binding to neurofascin-expressing cells remained unaffected in the presence of soluble TN-R, but not TN-C (Fig. 5 E). Binding of axonin-1-coated beads to NF22 (TN-R binder) was completely blocked by soluble TN-R (Fig. 5 C). Competition binding experiments using NF17 and axonin-1-conjugated beads could not be performed due to its low binding activity to axonin-1.

In summary, these binding assays indicate that TN-R in-

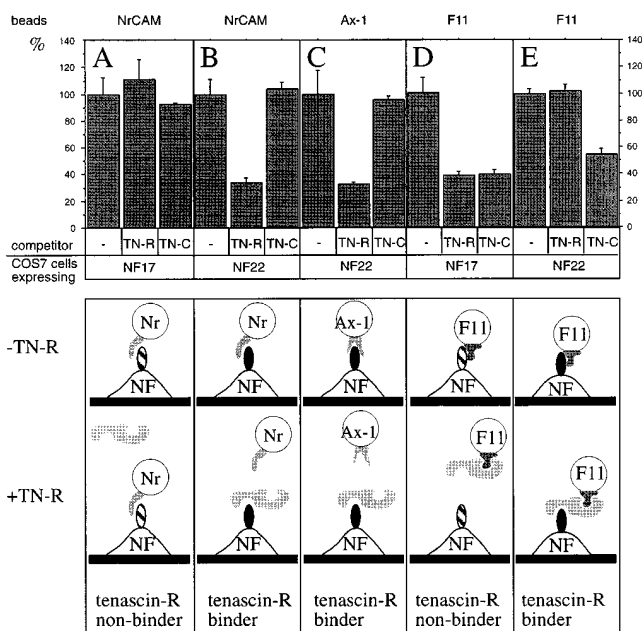


Figure 5. Soluble TN-R and TN-C differentially compete for ligand binding to neurofascin. Microspheres coated with NrCAM (A and B), axonin-1 (C), or F11 (D and E; indicated on the top of the panel) are incubated with COS7 cells expressing neurofascin variants NF17 or NF22 (see bottom row). Competitor indicates TN-R or TN-C in solution at a concentration of 150  $\mu$ g/ml. The interpretation of the results is illustrated schematically in the lower half of the figure. Circles indicate fluorescent beads conjugated with NrCAM, axonin-1, or F11. NF22 is shown as a filled and NF17 as a hatched ellipse. The indication that F11 binds to TN-R while NrCAM and axonin-1 do not is based on previous studies (Nörenberg et al., 1995). Bars on the columns indicate SEM.

terferes with neurofascin-NrCAM and neurofascin-axonin-1 interactions if an isoform of neurofascin is tested that is able to bind to TN-R. Furthermore, axonin-1 does not bind to the neurofascin/TN-R complex while F11 binds to oligomeric TN-R (Nörenberg et al., 1995) within the neurofascin/TN-R complex. In the latter case, oligomeric TN-R might link F11-conjugated beads to neurofascin-expressing COS7 cells by generating a bridge. In contrast, in the case of a neurofascin TN-R nonbinder, a decreased neurofascin-F11 interaction is observed. Cell attachment to neurofascin/TN-R complex mediated by F11 might therefore include binding of F11 expressed on tectal cells to TN-R while axonin-1-mediated cell attachment and neurite outgrowth does not include binding of axonin-1 to neurofascin/TN-R. This interpretation suggests an involvement of an additional cell surface receptor for TN-R for neurite extension on the neurofascin-TN-R complex.

### Analysis of the Neurofascin-TN-R Binding by Domain-specific Antibodies to TN-R

Since the neurofascin-TN-R interaction appeared to be of particular interest, and since cell attachment and neurite extension is only partially blocked by antibodies to F11 or axonin-1, we further analyzed binding of TN-R to neurofascin and cell attachment to the neurofascin-TN-R complex. Also, to address the question whether additional cel-





(Pesheva et al., 1993; Friedlander et al., 1994; Peles et al., 1995; Sakurai et al., 1997; Peles et al., 1997; Kunz et al., 1996; Rader et al., 1996; Suter et al., 1995; Buchstaller et al., 1996; Kuhn et al., 1991; Morales et al., 1993; Brümmendorf et al., 1993; Schumacher et al., 1997; Nörenberg et al., 1995), it appears that neurofascin is a component of a complex network of molecular interactions, and that it probably acts in the form of complexes in the developing nervous system to regulate axonal growth. This view is also supported by the partial overlapping distribution of these proteins in several vertebrate fiber tracts, including the visual system (Brümmendorf and Rathjen, 1995). Although the membrane topology of the neurofascin interactions has not been investigated in this study, neurofascin might also recruit F11 or axonin-1 within the same plasma membrane to form receptor complexes, as it has been found for NgCAM that cooperates with axonin-1 on DRG neurons to extend neurites, or with F11 (Stoeckli et al., 1996; Kunz et al., 1996; Brümmendorf et al., 1993; Buchstaller et al., 1996). Similarly, NrCAM forms complexes with F11 (Sakurai et al., 1997; Morales et al., 1993), which is implicated in neurite outgrowth induced by the receptor tyrosine phosphatase  $\beta/\zeta$  (Peles et al., 1995).

To gain further insight into the function of the individual isoforms of neurofascin, we have analyzed 15 distinct isoforms to bind to TN-R, axonin-1, and F11. These studies show that the different isoforms of neurofascin reveal a differential binding to TN-R and axonin-1, indicating that one function of the extensive alternative splicing of the neurofascin gene is to modulate ligand recognition. The interaction of F11 with different neurofascin isoforms roughly resembles the binding behavior of NrCAM to neurofascin, which has been detailed in a previous communication (Volkmer et al., 1996). The Ig-like domains of neurofascin are sufficient to bind both proteins. In contrast, TN-R and axonin-1 binding requires additional regions, and is regulated more tightly by alternative splicing, which is also reflected by the observation that certain isoforms do not display any binding to TN-R-coated microspheres. The generation of different isoforms might therefore provide a fine tuning of the binding activities of neurofascin. Several investigations have addressed the function of alternative splicing in extracellular regions of other IgSF members. For example, an alternatively spliced exon termed VASE introduces 10 additional amino acid residues within the fourth Ig-like domain (Small and Akeson, 1990; Small et al., 1988; Santoni et al., 1989), resulting in a modulation of neurite outgrowth promotion (Liu et al., 1993; Doherty et al., 1992) and the signaling activity of NCAM (Saffell et al., 1997; Brittis et al., 1996). In the FGF receptor 1, the alternate usage of two exons encoding two different COOH-terminal halves of the third Ig-like domain modulate the affinity for one of its ligands, bFGF (Werner et al., 1992). Furthermore, alternatively spliced exons coding for the Ig-like domains of vascular cell adhesion molecule-1 generate either one or two binding sites for an integrin receptor, resulting in different adhesive properties of these isoforms (Vonderheide and Springer, 1992). The interaction between neurofascin and axonin-1, and between neurofascin and TN-R is enhanced either by introduction of the PAT domain or by the short stretches at the NH<sub>2</sub> terminus, and between the Ig-like and the

FNIII-like domains. These elements may modulate the flexibility of the neurofascin polypeptide either by providing an extended O-glycosylated segment in the case of the PAT domain, or a hinge-like structure. As these alternatively spliced segments within neurofascin can complement each other with respect to the enhancement of binding to axonin-1 or TN-R, there appear to be independent regions in the extracellular part of neurofascin that affect binding. Interestingly, expression of the exons that encode these stretches are developmentally regulated. While exons 3 and 17 (segments at the NH<sub>2</sub> terminus and between the Ig- and FNIII-like regions, respectively) are found early in development, exon 27 (PAT domain) appears late in the development of the chicken brain (Volkmer et al., 1992; Hassel et al., 1997). The 17 amino acid residue-long segment between Ig-like domains two and three that is expressed late in development and that slightly abolishes binding to axonin-1 or TN-R in several neurofascin isoforms might increase the distance between these neighboring domains, and therefore might decouple intrachain interactions. This interpretation is supported by a key residue analysis of the related human L1, indicating that Ig-like domains two and three are linked by amino acid residues that are not involved in  $\beta$ -sheet formation of the preceding or following Ig-like domain (Bateman et al., 1996). This structural feature may influence the orientation of neighboring domain faces, and can also be expected to be shared by neurofascin due to its relationship to L1. The modulation of binding might also affect the localization of neurofascin isoforms into specialized structures. For example, a neurofascin form that contains the fifth FNIII-like repeat is localized at the nodes of Ranvier, whereas an isoform encompassing the FNIII-like domains 1–4 was found on unmyelinated axons (Davis et al., 1996).

Our previous investigations have shown that neurite extension of tectal cells mediated by a pure neurofascin substratum requires NrCAM on the axonal surface (Volkmer et al., 1996). In the present study neurite outgrowth and cell attachment assays in the presence of specific antibodies show that the usage of NrCAM as a cellular receptor for substrate-bound neurofascin is replaced if TN-R is bound to neurofascin. Instead, F11, axonin-1, and at least one additional cellular receptor are involved in axon extension and cell attachment on the combined neurofascin–TN-R substrate. Such a shift in receptor usage might be important within the retina, where neurofascin and TN-R colocalize in the inner and outer plexiform layer, but not in the optic fiber layer at specific developmental stages (Rathjen et al., 1991; Volkmer et al., 1996). It is therefore conceivable that neurofascin–NrCAM interactions are of importance on ganglion cell axons in the optic fiber layer, while the presence of TN-R reduces a neurofascin–NrCAM interaction on the neurites of the interneurons in the plexiform layers of the retina. One explanation that NrCAM is replaced as a receptor might be that TN-R sterically blocks the NrCAM-binding region within neurofascin. Previous investigations have shown that F11 binds directly to TN-R, while axonin-1 and NrCAM are not able to bind to TN-R (Brümmendorf et al., 1993; Pesheva et al., 1993; Xiao et al., 1996; Nörenberg et al., 1995). Our competition-binding experiments also suggest an interaction of cellular F11 with TN-R of the TN-R–neurofascin complex,

but not with neurofascin itself. First, TN-R strongly competes with neurofascin (NF17) to bind F11, making it unlikely that F11 directly binds to neurofascin in the presence of TN-R. Second, although Fab fragments of polyclonal antibodies to F11 block adherence to the combined neurofascin–TN-R substrate, they neither affect cell attachment nor neurite outgrowth on the pure neurofascin substrate, indicating that in the absence of TN-R a putative neurofascin–F11 interaction has no functional consequences in this experimental system (Volkmer et al., 1996). Third, the competition binding assays further suggest an independent binding of F11 and neurofascin (NF22) to TN-R, as F11-coated microspheres may be linked to neurofascin (NF22)-expressing COS cells by a TN-R bridge. Mapping studies indicate that F11 binds to the FNIII-like repeats 2–3 of TN-R (Nörenberg et al., 1995), while neurofascin presumably binds to a broader overlapping region.

In contrast, axonin-1, which is implicated in neurite extension and cell attachment on the neurofascin–TN-R complex on the basis of the antibody perturbation experiments (Figs. 3 and 4), does not directly bind to the neurofascin–TN-R complex as it has been shown by the competition-binding assay (Fig. 5). Therefore, axonin-1 may interact with other cellular surface proteins within the same plasma membrane to mediate neurite outgrowth on the neurofascin–TN-R complex. For example, the homophilic interaction between a substrate of purified Ng-CAM and cellular NgCAM has been shown to require a *cis* interaction of cellular axonin-1 with NgCAM within the same plasma membrane of DRG neurons (Buchstaller et al., 1996; Kuhn et al., 1991). The participation of NgCAM in a *cis* complex with axonin-1 can be excluded on the combined neurofascin–TN-R substrate since polyclonal antibodies directed to NgCAM did not interfere with neurite outgrowth or cell attachment (see Fig. 4). On the basis of the experiments using domain-specific antibodies to TN-R, it is conceivable that additional so far unknown receptor protein(s) on tectal cells in association with axonin-1 and/or F11 probably interact with the FNIII-like domains 6–8 and the fibrinogen segment within TN-R.

In conclusion, this and our previous study suggest that neuronal cells are able to regulate the molecular activities of the L1-like protein neurofascin at different levels. First, the neurofascin–NrCAM interaction is not reciprocal, in contrast with the F11–NrCAM interaction (Volkmer et al., 1996). Second, the presence or absence of specific interacting proteins such as TN-R in the local environment of an extending axon might be a possibility to influence receptor usage as shown for the neurofascin–NrCAM interaction. Alternative splicing of the pre-mRNA might be a third possibility in that it modulates the preferences for specific ligands (e.g., TN-R binder or TN-R nonbinder). Regulation of the binding activities would greatly expand the number of guidance cues provided by neurofascin in the developing brain.

We thank Dr. P. Sonderegger (University of Zürich) for providing monoclonal antibodies to axonin-1, and F. Kirsch, H. Drechsler, and M. Henning for expert technical assistance. Critical reading of the manuscript by Dr. T. Brümmendorf and M. Koroll is acknowledged.

These studies were supported by Fritz-Thyssen-Stiftung grant Az1995–2035, by SFB515, and by EU Biotechnology Programme BIO4-CT96–0450 to F.G. Rathjen.

Received for publication 23 March 1998 and in revised form 13 July 1998.

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