

# Sodium channel $\beta 1$ and $\beta 3$ subunits associate with neurofascin through their extracellular immunoglobulin-like domain

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Sequence homology predicts that the extracellular domain of the sodium channel  $\beta 1$  subunit forms an immunoglobulin (Ig) fold and functions as a cell adhesion molecule. We show here that  $\beta 1$  subunits associate with neurofascin, a neuronal cell adhesion molecule that plays a key role in the assembly of nodes of Ranvier. The first Ig-like domain and second fibronectin type III-like domain of neurofascin mediate the interaction with the extracellular Ig-like domain of  $\beta 1$ , confirming the proposed function of

this domain as a cell adhesion molecule.  $\beta 1$  subunits localize to nodes of Ranvier with neurofascin in sciatic nerve axons, and  $\beta 1$  and neurofascin are associated as early as postnatal day 5, during the period that nodes of Ranvier are forming. This association of  $\beta 1$  subunit extracellular domains with neurofascin in developing axons may facilitate recruitment and concentration of sodium channel complexes at nodes of Ranvier.

## Introduction

Neuronal voltage-gated sodium channels are composed of three subunits: a large pore-forming  $\alpha$  subunit (260 kD) and two smaller auxiliary  $\beta$  subunits,  $\beta 1$  (36 kD) and  $\beta 2$  (33 kD) (Catterall, 1992; Isom et al., 1992, 1995). A third  $\beta$  subunit named  $\beta 3$ , which shares 57% identity with  $\beta 1$ , has been cloned recently (Curtis, R.A., D. Lawson, P. Ge, P.S. DiStefano, and I. Solis-Santiago. 2000. Cloning and localization of a novel  $\text{Na}^+$  channel  $\beta 3$  subunit. Society of Neuroscience Annual Meeting. 418.22 [Abstr.]; Morgan et al., 2000). The extracellular domains of all  $\beta$  subunits are predicted to form V-type Ig-like folds, and it is proposed that these domains have cell adhesion properties (Isom et al., 1995; Isom and Catterall, 1996). Clustering of sodium channels along myelinated axons at nodes of Ranvier is essential for efficient conduction of action potentials. This concentration of sodium channels at nodes of Ranvier is believed to involve interactions with glial cells, the extracellular matrix, and the cytoskeleton (Salzer, 1997). The 480- and 270-kD isoforms of the cytoskeletal spectrin-binding protein ankyrin<sub>C</sub> are highly concentrated at nodes (Kordeli et al.,

1995; Zhou et al., 1998), and have been copurified with sodium channels from rat brain membrane preparations (Srinivasan et al., 1988). This interaction may be mediated by the intracellular domains of  $\beta 1$  and  $\beta 2$ , which have been shown to recruit ankyrin (Malhotra et al., 2000). The neuronal extracellular matrix protein tenascin R, which localizes to nodes of Ranvier, has been shown to bind the  $\beta 2$  subunit of sodium channels (Srinivasan et al., 1998; Xiao et al., 1999) and the cell adhesion molecule (CAM)\* neurofascin (Volkmmer et al., 1998), suggesting a functional relationship among these molecular components. As the extracellular domains of sodium channel  $\beta$  subunits are homologous to CAMs belonging to the Ig superfamily, we predicted that they might interact with other neuronal CAMs in close proximity.

Neurofascin belongs to the L1 family of neuronal CAMs containing extracellular Ig- and fibronectin (FN) type III-like domains, as well as ankyrin binding activity in their cytoplasmic domains (see Fig. 1 A). FIGQY, a highly conserved sequence in L1 family intracellular domains, is required for ankyrin binding, and phosphorylation of the FIGQY tyrosine residue abolishes binding (Garver et al., 1997). Neurofascin and another L1 family member, NrCAM, become clustered along rat sciatic nerve axons early during postnatal

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\*Abbreviations used in this paper: CAM, cell adhesion molecule; FN, fibronectin; GPI, glycosylphosphatidylinositol; HA, hemagglutinin; RPTP $\beta$ , receptor protein tyrosine phosphatase  $\beta$ .

development, thus defining the sites for assembly of nodes of Ranvier (Davis et al., 1996). Ankyrin<sub>C</sub> and sodium channels are subsequently recruited to these sites as nodes mature (Lambert et al., 1997). Sodium channel  $\alpha$  and  $\beta$  subunits also assemble during this developmental period (Wollner et al., 1988). There are several splice variants of neurofascin, including a 155- and a 186-kD isoform (Davis et al., 1996). The 155-kD isoform contains six extracellular Ig domains followed by four FN domains, whereas the third FN domain is absent from the 186-kD isoform, and a mucin-like domain is inserted after FN domain 4. Only the 186-kD isoform, neurofascin 186, appears to be localized to nodes of Ranvier, whereas the 155-kD isoform is expressed only in unmyelinated fibers (Davis et al., 1996). Neurofascin 186 is also localized to Purkinje cell axon initial segments, whereas the 155-kD isoform is expressed in cell bodies and dendrites (Davis et al., 1996).

We show here that sodium channel  $\beta$ 1 subunits interact with neurofascin early in development and remain associated in adult rat brain. Only sodium channel  $\beta$ 1 and  $\beta$ 3 subunits are able to interact with neurofascin, and this interaction involves their extracellular domains.  $\beta$ 1 subunits localize to nodes of Ranvier in rat sciatic nerve with neurofascin. Together, these data suggest that the association of  $\beta$ 1 or  $\beta$ 3 subunit extracellular domains with neurofascin is involved in targeting sodium channels to nodes of Ranvier in developing axons and retaining channels at nodes in mature myelinated axons.

## Results and discussion

### Neurofascin interacts with $\beta$ 1 and $\beta$ 3 subunits in cotransfected tsA-201 cells

Neurofascin (Fig. 1 A) and sodium channels exist in close proximity at nodes of Ranvier (Davis et al., 1996). To determine if sodium channel subunits are involved in forming a complex with neurofascin 186, Na<sub>v</sub>1.2a  $\alpha$  subunits plus  $\beta$ 1,  $\beta$ 2, or  $\beta$ 3 subunits were expressed separately in tsA-201 cells with neurofascin 186. As no  $\beta$ 3 antibody was available,  $\beta$ 3 was fused to a c-myc epitope tag at its COOH terminus. Neurofascin 186 was fused to a hemagglutinin (HA) epitope (HA.11) tag at its NH<sub>2</sub> terminus for detection in transfected cells. 40 h after transfection, cells were lysed and their proteins immunoprecipitated using subunit-specific polyclonal antibodies anti-SP20, anti- $\beta$ 1CT, and anti- $\beta$ 2CT, and monoclonal antibody anti-myc, respectively. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed with monoclonal anti-HA.11 antibody, which detected coimmunoprecipitation of neurofascin 186 with  $\beta$ 1 and homologous  $\beta$ 3 subunits only (Fig. 1 B). Immunoblotting of cell lysates demonstrated robust expression of all subunits in cells cotransfected with neurofascin 186 and  $\alpha$  or  $\beta$ 2 subunits. Removal of the HA.11 tag from neurofascin 186 did not affect its interaction with  $\beta$ 1 (unpublished data).

$\beta$ 1 and  $\beta$ 3 mRNAs are both found in central and peripheral neurons, with overlapping as well as distinct patterns of expression (Curtis, R.A., D. Lawson, P. Ge, P.S. DiStefano, and I. Solis-Santiago. 2000. Cloning and localization of a

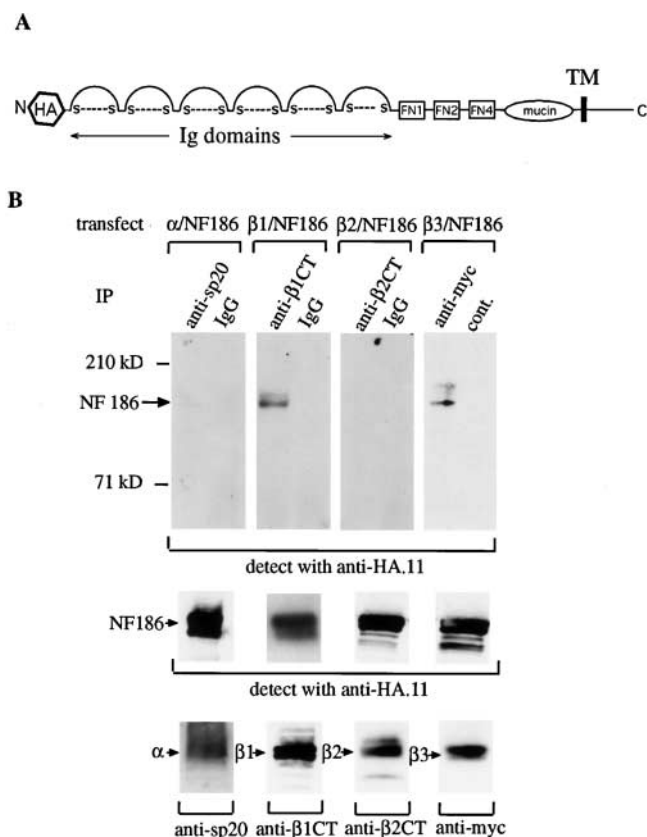


Figure 1. **Sodium channel  $\beta$ 1 and  $\beta$ 3 subunits coimmunoprecipitate with neurofascin in cotransfected tsA-201 cells.** (A) Representation of HA.11-tagged neurofascin 186. (B) TsA-201 cells were cotransfected with neurofascin 186 (NF186) and sodium channel subunits as indicated. Cell lysates were immunoprecipitated with the indicated antibodies and the blot was probed with monoclonal antibody anti-HA.11. Each cell lysate was probed with anti-HA.11 or specific antisodium channel antibodies as shown to confirm that all proteins were expressed (bottom).

novel Na<sup>+</sup> channel  $\beta$ 3 subunit. Society of Neuroscience Annual Meeting, 418.22 [Abstr.]; Morgan et al., 2000; unpublished data), suggesting that  $\beta$ 1 and  $\beta$ 3 play similar roles. This concept is supported by the ability of both subunits to associate with neurofascin, whereas  $\beta$ 2, which shares less homology with  $\beta$ 1 and  $\beta$ 3, does not interact with neurofascin.  $\beta$ 2 subunits share homology with contactin/F3/F11 (Isom et al., 1995), a glycosylphosphatidylinositol (GPI)-anchored CAM that interacts with NrCAM (Morales et al., 1993; Sakurai et al., 1997). It will be interesting to investigate whether  $\beta$ 2 subunits are able to interact with NrCAM at nodes of Ranvier, thus providing a further mechanism by which sodium channels are targeted to these specialized regions.

### Neurofascin and $\beta$ 1 localize to nodes of Ranvier and associate in rat brain

Sodium channel  $\alpha$  subunits have been shown to localize to nodes of Ranvier (Ellisman and Levinson, 1982; Vabnick et al., 1996; Rasband et al., 1999), but concentration of  $\beta$ 1 subunits at nodes has not been confirmed. The localization of both neurofascin and  $\beta$ 1 subunits in sciatic nerve was de-

terminated by probing tissue from adult rats with polyclonal anti-FN domain antibody and anti- $\beta 1$ CT, respectively. Neurofascin has previously been localized to nodes of Ranvier in adult rats (Davis et al., 1996), and we confirmed these data for the sciatic nerve (Fig. 2 A) while demonstrating that  $\beta 1$  subunits also localize to highly concentrated sites that appear identical to those stained by anti-FN antibodies (Fig. 2 B). This result shows that both  $\beta 1$  subunits and neurofascin are highly concentrated at nodes of Ranvier.

In developing nodes of Ranvier,  $\beta 1$  subunits are localized in the sciatic nerve at postnatal days 3 and 10, during the process of myelination and maturation of the nodes (Fig. 2, E and F). Neurofascin is also localized at developing nodes of Ranvier at these ages (unpublished data; Lambert et al., 1997). Therefore, these two molecules are appropriately positioned to interact with each other as sodium channels are clustered and immobilized in developing nodes of Ranvier.

Neurofascin and NrCAM cluster at nodes of Ranvier in rat sciatic nerve between postnatal days 2 and 5, followed by the recruitment of ankyrin<sub>G</sub> and sodium channels to these sites (Lambert et al., 1997). To examine whether  $\beta 1$  subunits form a complex with neurofascin from early postnatal development through to adulthood, both P5 and adult rat brain lysates were immunoprecipitated with polyclonal  $\beta 1$

antibody, anti- $\beta 1$ CT, or control rabbit IgG. After SDS-PAGE of immunoprecipitated proteins and immunoblotting, polyclonal antimucin domain antibody detected the 186-kD neurofascin isoform coimmunoprecipitating with  $\beta 1$  subunits in both P5 and adult brain lysates (Fig. 2 C). The antimucin domain antibody was raised against the mucin domain present only in the neurofascin isoform detected at nodes. P5 and adult rat brain lysates were probed with anti- $\beta 1$ CT to confirm that  $\beta 1$  is expressed during early postnatal development (Fig. 2 D).

These results show that  $\beta 1$  subunits interact with neurofascin in developing rat brain, and we propose that this association is involved in targeting sodium channels to specialized regions of the neuron such as nodes of Ranvier and axon initial segments. Previous reports suggest that mature nodes of Ranvier contain sodium channels, neurofascin, and NrCAM, bound in a complex by ankyrin<sub>G</sub> (Davis et al., 1996; Volkmer et al., 1996; Zhou et al., 1998) that is able to interact with several transmembrane molecules. Ankyrin<sub>G</sub> is likely to be present in our immunoprecipitates as well, but the experiments presented below show that sodium channels and neurofascin interact through their extracellular domains, so it is unlikely that ankyrin<sub>G</sub> is required for formation of the complex. Our data show that sodium channel  $\beta 1$

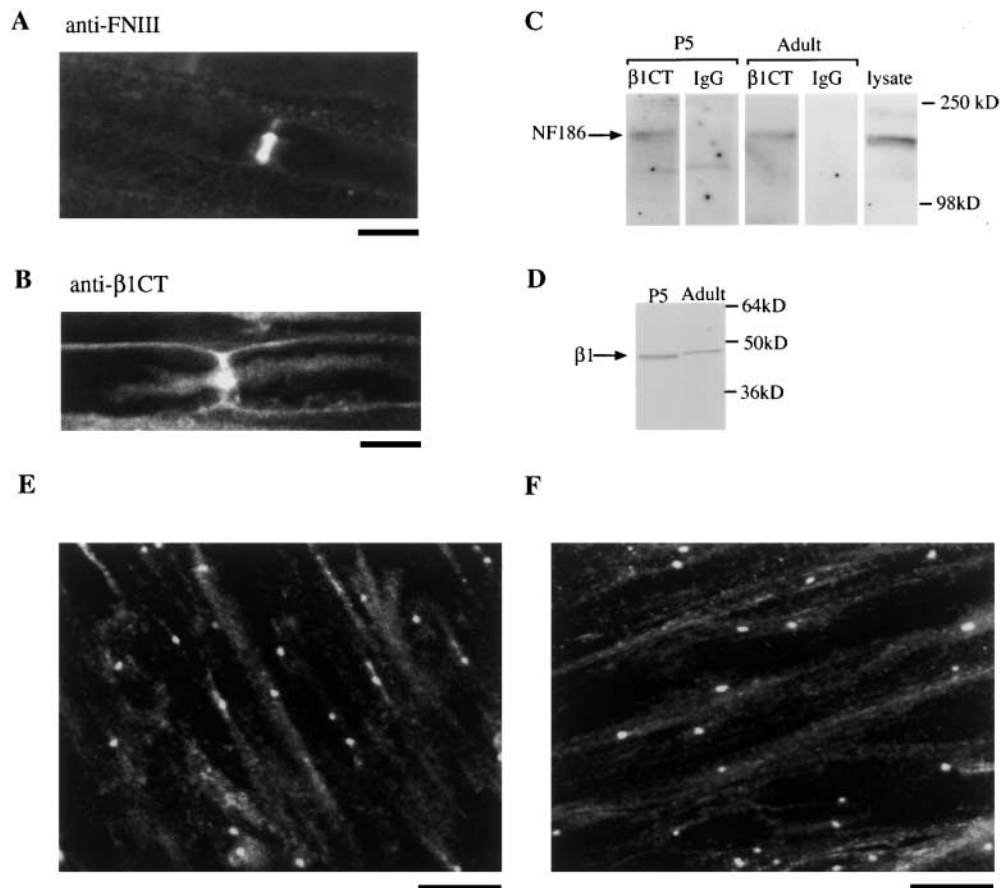


Figure 2. **Neurofascin and  $\beta 1$  localize to nodes of Ranvier and associate in rat brain.** Rat sciatic nerve was labeled with neurofascin antibody anti-FNIII (A) or anti- $\beta 1$ CT (B). Expression of both  $\beta 1$  and neurofascin is concentrated at nodes of Ranvier. (C) P5 or adult rat brain lysates were immunoprecipitated with the indicated antibodies. Blots were probed with neurofascin antimucin antibody. (D) Expression of  $\beta 1$  in both P5 and adult rat brain lysates. (E and F) Teased sciatic nerve stained with anti- $\beta 1$  extra from 3-d-old (E) and 10-d-old (F) rats, demonstrating the localization of  $\beta 1$  at the nodes of Ranvier in development. NF, neurofascin. Bars: 2  $\mu$ m (A and B) and 10  $\mu$ m (E and F).

subunits can interact directly with neurofascin in transfected cells and are associated with neurofascin in postnatal and adult rat brain, indicating that this interaction may be involved in both forming the nascent node of Ranvier and stabilizing the mature node.

### Neurofascin and $\beta 1$ interact in cis in transfected tsA-201 cells

Both neurofascin and NrCAM form trans-interactions with molecules on adjacent cells (Volkmer et al., 1996). To investigate whether  $\beta 1$  interacts with neurofascin in cis or in trans, tsA-201 cells were transfected together or separately with neurofascin 186 and  $\beta 1$ . 20 h after transfection cells were removed from culture dishes by treatment with EDTA, and the separately transfected  $\beta 1$ -expressing cells were mixed thoroughly with neurofascin-expressing cells and cultured for a further 24 h to 80% confluency. Cells were lysed and their proteins were immunoprecipitated with anti- $\beta 1$ CT. SDS-PAGE and immunoblotting with monoclonal antibody anti-HA.11 detected interaction of  $\beta 1$  and neurofascin in cis (Fig. 3 A), but were unable to detect any neurofascin 186 coimmunoprecipitating with  $\beta 1$  subunits in trans following separate transfection (Fig. 3 B). Immunoblotting of cell lysates showed that both  $\beta 1$  and neurofascin 186 were well expressed (Fig. 3 B). As a control, transfected cells were fixed and stained with anti- $\beta 1$ CT (Fig. 3 C) or anti-HA.11 antibody to tagged neurofascin (Fig. 3 D). Both proteins localized to the plasma membrane, which would allow trans-heterophilic interactions to occur between them. Thus, our results in tsA-201 cells demonstrate that interaction of  $\beta 1$  and neurofascin only occurs in cis within the same cell membrane in this experimental system. This type of interaction would be important in the formation of sodium channel clusters and targeting to nodes of Ranvier in the axonal membrane. However, it is possible that weaker trans-interactions occur between  $\beta 1$  and neurofascin expressed on opposing cells such as the axon and perinodal astrocyte or Schwann cell microvilli. Such trans-interactions may be easily disrupted during cell lysis, and therefore are not detected in our immunoprecipitation experiments.

### The $\beta 1$ extracellular domain is sufficient for association with neurofascin

The intracellular domain of neurofascin 186 interacts with ankyrin<sub>G</sub> (Zhang et al., 1998), and it has recently been suggested that  $\beta 1$  and  $\beta 2$  subunits may also interact with ankyrin (Malhotra et al., 2000). That study shows that ankyrin is concentrated at points of cell contact in aggregates formed by cells expressing full-length  $\beta 1$  and  $\beta 2$  subunits, but that this concentration is not detected when cells are transfected with  $\beta$  subunits lacking their intracellular domains. This observation suggests that  $\beta 1$  and  $\beta 2$  intracellular domains are able to interact directly or indirectly with ankyrin. An isoform of ankyrin is expressed in the HEK293 cell line from which tsA-201 cells are derived (Zhang et al., 1998), but we were unable to detect either ankyrin<sub>B</sub> or ankyrin<sub>G</sub> expression in tsA-201 cells (unpublished data). To investigate whether the presence of an ankyrin isoform in tsA-201 cells mediates the interaction between  $\beta 1$  and neu-

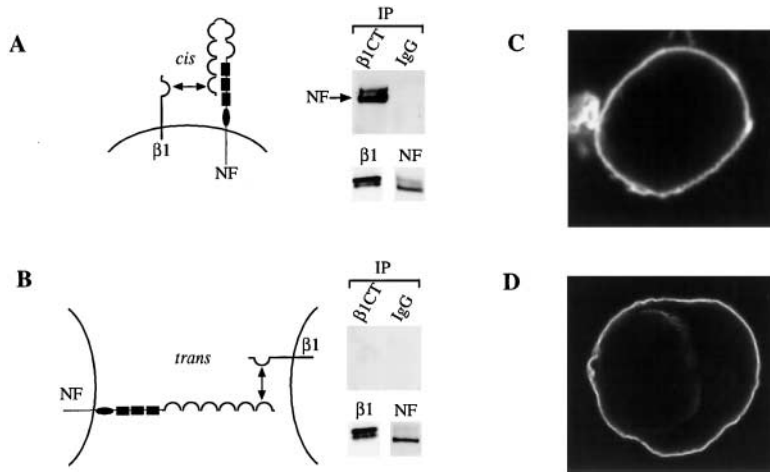
rofascin 186, the intracellular domain of neurofascin 186 was deleted from E1129, thus removing the ankyrin binding site. It has been demonstrated that this mutant is unable to associate with ankyrin<sub>G</sub> in transfected cell lines (Zhang et al., 1998). TsA-201 cells transfected with this mutant (NF186 $\Delta$ ic) and  $\beta 1$  were lysed, and cell lysates were immunoprecipitated with polyclonal antibody  $\beta 1$ CT or control IgG. After resolution of proteins by SDS-PAGE and transfer to nitrocellulose, probing blots with monoclonal antibody anti-HA.11 showed that NF186 $\Delta$ ic is able to interact with  $\beta 1$  (Fig. 4 A). These results indicate that ankyrin is not necessary for the formation of this complex.

$\beta$  subunit extracellular domains were previously proposed to mediate adhesion through interaction with other CAMs (Isom and Catterall, 1996). As the  $\beta 2$  subunit does not interact with neurofascin 186, a chimera between  $\beta 1$  and  $\beta 2$  was used to locate the site of interaction of  $\beta 1$  with neurofascin 186. TsA-201 cells were cotransfected with neurofascin 186 and a  $\beta 1\beta 2\beta 2$  chimera consisting of  $\beta 2$  intracellular and transmembrane domains fused to the  $\beta 1$  extracellular domain. Cells coexpressing neurofascin 186 with  $\beta 1\beta 2\beta 2$  were lysed and immunoprecipitated with anti- $\beta 2$ CT. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Blots probed with the anti-HA.11 antibody showed neurofascin 186 coimmunoprecipitating with  $\beta 1\beta 2\beta 2$  (Fig. 4 B). This result suggests that the extracellular domain of  $\beta 1$  is sufficient for the interaction with neurofascin 186. To confirm this observation, tsA-201 cells were cotransfected with neurofascin 186 and a GPI-linked  $\beta 1$  extracellular domain protein,  $\beta 1_{ec}$ -GPI. This fusion protein consists of the  $\beta 1$  extracellular domain, immediately followed by the GPI anchor recognition sequence from human placental alkaline phosphatase (McCormick et al., 1999). The majority of this recognition sequence is cleaved from the protein upon attachment of the GPI moiety. Cells coexpressing  $\beta 1_{ec}$ -GPI and neurofascin 186 were lysed and immunoprecipitated using polyclonal antibody anti- $\beta 1_{extra}$  raised against the extracellular domain of  $\beta 1$ . After SDS-PAGE and immunoblotting,  $\beta 1_{ec}$ -GPI was found to associate with neurofascin 186 (Fig. 4 C), confirming that the  $\beta 1$  extracellular domain alone is sufficient for this interaction. Heterologous expression of  $\beta 1$  or  $\beta 2$  subunits in *Drosophila* S2 cells induces aggregation, which is proposed to occur through homophilic interactions between the extracellular domains of the  $\beta$  subunits (Malhotra et al., 2000). Our data demonstrate that  $\beta 1$  subunit extracellular domains can also associate heterophilically with another CAM.

### Determination of the neurofascin binding site

The extracellular region of neurofascin 186 consists of multiple domains. To investigate which extracellular domains of neurofascin were able to interact with  $\beta 1$ , the Ig domains Ig1–6, the FN domains FN1, 2, and 4, and the mucin-like domain were expressed separately, fused at the NH<sub>2</sub> terminus to the HA.11 tag, and at the COOH terminus to the GPI anchor sequence from human placental alkaline phosphatase. These constructs were coexpressed with sodium channel  $\alpha$  and  $\beta 1$  subunits in tsA-201 cells. After immunoprecipitation with anti-SP20, only Ig1–GPI and FN2–GPI were able to associate with  $\alpha/\beta 1$  complexes in tsA-201 cells, suggesting that



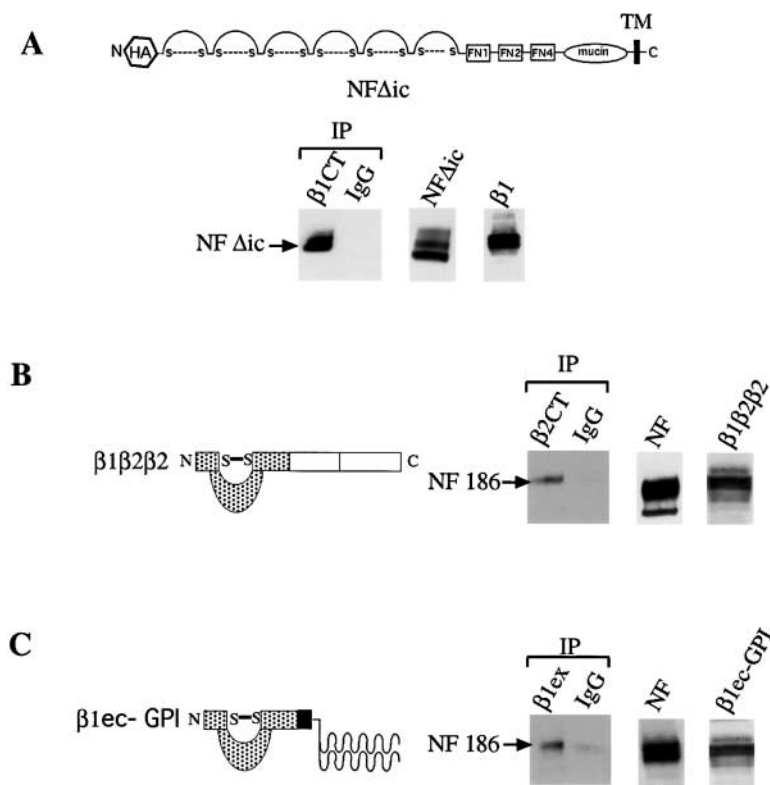


**Figure 3.  $\beta 1$  and neurofascin associate in cis but not in trans.** (A) TsA-201 cells cotransfected with  $\beta 1$  and neurofascin (NF) were lysed and immunoprecipitated (IP) as described previously. (B) TsA-201 cells were transfected separately and cocultured. Lysed cells were immunoprecipitated with the indicated antibodies and the blot was probed with anti-HA.11. The bottom panel shows cell lysates probed with anti- $\beta 1$ CT or anti-HA.11 to demonstrate that proteins were well expressed. (C) Cells transfected with  $\beta 1$  were stained with anti- $\beta 1$ CT, and (D) cells transfected with HA-tagged neurofascin 186 were stained with anti-HA.11.

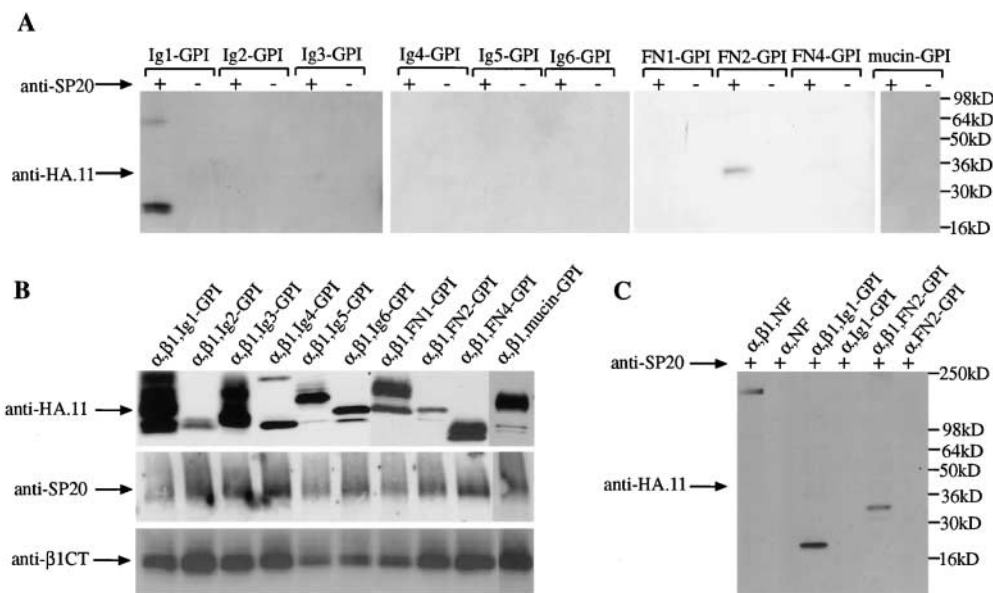
the neurofascin binding site is assembled from amino acids in both of these domains (Fig. 5 A). It was necessary to express  $\beta 1$  complexed with  $\alpha$  subunits in these experiments, as the GPI constructs were expressed at high levels and all of them bound nonspecifically to  $\beta 1$  in the absence of  $\alpha$  subunits. When  $\alpha$  subunits were expressed alone with Ig1–GPI or FN2–GPI no interaction was observed, confirming that this association is  $\beta 1$  dependent (Fig. 5 C). Both the 155- and 186-kD isoforms of neurofascin contain Ig1 and FN2, so  $\beta 1$  should interact with both isoforms. However,  $\beta 1$  is localized to nodes of Ranvier in sciatic nerve and is most likely to interact with neurofascin 186, which also concentrates at nodes. The interaction of Ig1 with  $\beta 1$  in cis suggests that the neurofascin molecule folds back on itself to make this domain accessible to  $\beta 1$ , as drawn in Fig. 3 A; thus, binding of

amino acids on Ig1 and FN2 with  $\beta 1$  could then occur. It is also possible that the FN2 domain of neurofascin could interact with  $\beta 1$  subunits in a cis configuration, whereas Ig1 interacts with  $\beta 1$  subunits in trans on an opposing membrane. Development of new methods to measure lower-affinity trans-interactions of  $\beta 1$  will be required to test this idea. Similar interactions are made by axonin 1/TAG-1-like glycoproteins, a family of neural CAMs containing six Ig domains and four FN domains. Homophilic trans-interactions occur between Ig domains 2 and 3 of axonin 1 (Freigang et al., 2000), whereas the FN domains of TAX-1, the human homologue of axonin 1, are thought to form cis-homophilic interactions (Tsiotra et al., 1996).

These results demonstrate that the extracellular domain of  $\beta 1$  functions as a CAM by adhering to neurofascin. Neuro-



**Figure 4. The extracellular domain of  $\beta 1$  interacts with neurofascin.** (A) TsA-201 cells cotransfected with NF186 $\Delta$ ic and  $\beta 1$  were lysed and probed with the indicated antibodies. Blots were probed with anti-HA.11. Cell lysates were also probed with anti-HA.11 and anti- $\beta 1$ CT to show that both proteins were expressed. (B) Cells cotransfected with neurofascin and  $\beta 1\beta 2\beta 2$  were lysed and immunoprecipitated (IP) with the indicated antibody. Blots were probed with anti-HA.11 and anti- $\beta 2$ CT to show expression of both proteins. (C) Cells cotransfected with  $\beta 1_{ec}$ -GPI and neurofascin were lysed and immunoprecipitated with antibodies as indicated. Cell lysates were probed with anti-HA.11 or anti- $\beta 1$ extra to show both proteins were expressed.



**Figure 5. Determination of the neurofascin binding site.** (A) TsA-201 cells were cotransfected with  $\alpha$  and  $\beta$  subunits and GPI-tagged constructs Ig1-GPI, Ig2-GPI, Ig3-GPI, Ig4-GPI, Ig5-GPI, Ig6-GPI, FN1-GPI, FN2-GPI, FN4-GPI, and mucin-GPI, respectively. Cell lysates were immunoprecipitated with anti-SP20 and blots were probed with anti-HA.11 antibody. (B) Lysates were probed with anti-SP20, anti- $\beta$ 1CT, and anti-HA.11 to demonstrate that all proteins were expressed. (C) TsA-201 cells were cotransfected with (lane 1)  $\alpha$ ,  $\beta$ 1, and neurofascin 186; (lane 2)  $\alpha$  and neurofascin 186; (lane 3)  $\alpha$ ,  $\beta$ 1, and Ig1-GPI; (lane 4)  $\alpha$  and Ig1-GPI; (lane 5)  $\alpha$ ,  $\beta$ 1, and FN2-GPI; and (lane 6)  $\alpha$  and FN2-GPI. Cell lysates were immunoprecipitated with anti-SP20 and blots were probed with anti-HA.11 antibody.

fascin and NrCAM clusters appear along sciatic nerve axons at postnatal day 2, followed by recruitment of ankyrin<sub>G</sub> and sodium channels (Lambert et al., 1997). Sodium channel recruitment appears to be dependent on ankyrin<sub>G</sub>, as sodium channels are no longer present at axon initial segments in the granule cells of ankyrin<sub>G</sub>-null mice, and Purkinje cells show reduced ability to fire action potentials (Zhou et al., 1998). Therefore, neurofascin and NrCAM may initially recruit ankyrin<sub>G</sub>, and sodium channels may subsequently be targeted to these sites by the interactions between the extracellular domain of  $\beta$ 1 and neurofascin, and the intracellular domains of  $\beta$ 1 and  $\beta$ 2 with ankyrin<sub>G</sub>. We show that  $\beta$ 1 subunits interact with neurofascin in developing rat brain, and propose that this association is involved in targeting sodium channels to specialized regions of the neuron such as nodes of Ranvier and axon initial segments.

Neurofascin and NrCAM are only able to associate with ankyrin<sub>G</sub> when the conserved tyrosine residue within the FIGQY sequence is dephosphorylated (Garver et al., 1997). We reported recently that sodium channels associate with receptor protein tyrosine phosphatase  $\beta$  (RPTP $\beta$ ) in developing rat brain (Ratcliffe et al., 2000). This interaction is mediated through the extracellular domain of the  $\alpha$  subunit, and the intracellular domains of both the  $\alpha$  and  $\beta$ 1 subunit; it is observed in neonatal tissue, but is absent at postnatal day 16. RPTP $\beta$  is expressed predominantly in glia, but RPTP $\beta$  mRNAs have also been detected in neurons (Snyder et al., 1996). The close localization of neurofascin and NrCAM to RPTP $\beta$  suggests that they could be substrates, with dephosphorylation occurring early in postnatal development, thus allowing interactions with ankyrin<sub>G</sub> to occur. As expression of RPTP $\beta$  is restricted to the brain (Levy et

al., 1993), a related tyrosine phosphatase may perform a similar function in the peripheral nervous system.

## Materials and methods

### Antibodies and DNA constructs

Polyclonal antibodies anti- $\beta$ 1CT and anti- $\beta$ 2CT raised against the COOH termini of  $\beta$ 1 and  $\beta$ 2, respectively, and sodium channel  $\alpha$  subunit antibody anti-SP20 were prepared as described previously (Ratcliffe et al., 2000). Polyclonal antimucin domain antibody raised against the mucin domain of neurofascin 186, and anti-FN domain antibody raised against all four FN domains of neurofascin, were gifts from Dr. Vann Bennett (Duke University, Durham, NC) (Davis et al., 1996). Polyclonal antibody anti- $\beta$ 1extra was a gift from Dr. Lori Isom (University of Michigan, Ann Arbor, MI) (Xiao et al., 1999). Monoclonal antibody anti-HA.11 was purchased from Covance Research Products, Inc., and monoclonal anti-myc antibody was purchased from Invitrogen.

Sodium channel subunit mammalian expression plasmids pCDM8 $\alpha$  (encoding Na<sub>v</sub>1.2a), pCDM8 $\beta$ 1, pCDM8 $\beta$ 2, and chimera pCDM8 $\beta$ 1 $\beta$ 2 $\beta$ 2 have been described previously (Auld et al., 1990; Ratcliffe et al., 2000).  $\beta$ 3 cDNA was amplified and subcloned into pcDNA3.1myc-his (Invitrogen) for expression of tagged  $\beta$ 3.  $\beta$ 1<sub>ec</sub>-GPI was subcloned from pSP64T (McCormick et al., 1999) into pCDM8. cDNA encoding neurofascin 186 tagged at the NH<sub>2</sub> terminus with HA.11 was provided by Dr. Vann Bennett. This cDNA was subcloned into the EcoRI site of pcDNA3.1 (Invitrogen). pcDNA3.1NF186 $\Delta$ ic was constructed by introducing a stop codon after residue E1129. Neurofascin 186 GPI-tagged constructs were made as follows. A ClaI restriction site was introduced into pcDNA3.1NF186 through a silent change of the codon for S8 from AGC to TCG. The ClaI-EcoRV fragment of pcDNA3.1NF186, which includes all but the first eight amino acids of neurofascin 186 and the HA.11 tag, was removed and replaced with a PCR product encompassing the mucin domain, amino acids P897–A1084. The GPI anchor recognition sequence from human placental alkaline phosphatase (McCormick et al., 1999) was then cloned in frame into the EcoRV-XhoI sites of this construct to produce pcDNA3.1mucin-GPI. For construction of remaining GPI-tagged domain expression vectors, the ClaI-EcoRV fragment of pcDNA3.1mucin-GPI was replaced with PCR products coding for Ig1 (P16–Q11), Ig2 (V112–T212), Ig3 (R213–P313), Ig4 (Y314–P406), Ig5 (R407–T498), Ig6 (R499–L586), FN1 (A587–P703), FN2 (E704–L801), and FN4 (P802–A905).

## Coimmunoprecipitation experiments

Membrane fractions were prepared from P5 and adult rat brains as described previously (Nishiwaki et al., 1998). For immunoprecipitation reactions, 500  $\mu\text{g}$  of total protein was added to 20  $\mu\text{g}$  of anti- $\beta 1$ CT or control IgG antibody, and incubated for 2 h at 4°C. Protein A-agarose was added, and the incubation was continued overnight. The complex bound to the agarose beads, was washed extensively, and bound proteins were heat eluted in SDS loading buffer at 100°C for 10 min. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose, and neurofascin 186 was detected using polyclonal antimucin domain antibody. For coimmunoprecipitation assays performed using tsA-201 cell lysates, 50  $\mu\text{g}$  of an equimolar ratio of expression plasmids was transfected into cells in DMEM F12 supplemented with 10% FBS, 100 U/ml penicillin, and streptomycin plated on 150-mm dishes using the calcium phosphate method. At 40 h after transfection, cell monolayers were washed in PBS, lysed in 1% Nonidet P-40, 0.25% sodium deoxycholate, 15 mM NaCl, 1 mM EGTA, 50 mM Tris-Cl, pH 7.4, 1 mM PMSF, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 10  $\mu\text{g}/\text{ml}$  aprotinin, and incubated at 4°C for 30 min. Lysates were centrifuged and the supernatant was used in immunoprecipitation experiments essentially as described above. HA-tagged neurofascin 186 was detected with monoclonal antibody anti-HA.11 (Covance Research Products, Inc.).

## Immunocytochemistry

Transfected tsA-201 cells were plated on poly-L-lysine-coated glass coverslips for 24 h and fixed in 4% paraformaldehyde. Coverslips were blocked in 5% avidin, followed by 5% biotin, and then 10% milk solution in TBS. Primary antibody was added at a 1:100 (anti- $\beta 1$ extra) or 1:1,000 dilution (anti-HA.11) in TBS containing 10% milk and 0.025% Triton X-100, and coverslips were incubated overnight at 4°C. Coverslips were then incubated with a 1:300 dilution of biotin-conjugated secondary antibody for 1 h at 37°C, followed by a 1-h incubation with fluorescein-conjugated avidin (Vector Laboratories). Coverslips were washed, dried, and mounted on glass slides in Vectashield (Vector Laboratories), and visualized under a confocal laser scanning microscope (model MRC 600; Bio-Rad Laboratories).

For staining of intact sciatic nerve, adult rats were anesthetized with nembutal and intracardially perfused with a solution of 4% paraformaldehyde in PB (0.1 M sodium phosphate, pH 7.4). The sciatic nerves were removed, postfixed for 2 h, and sunk in successive solutions of 10 and 30% (wt/vol) sucrose in PB at 4°C over a period of 72 h. Sciatic nerves were embedded in OCT compound, and 20  $\mu\text{m}$  sections were cut and thaw mounted onto Superfrost Plus slides (Fisher Scientific). Sciatic sections were fixed in 4% paraformaldehyde, rinsed, and then blocked using 5% normal goat serum and 5% nonfat milk in 0.1 M TBS for 1 h. The sections were then incubated in anti- $\beta 1$ extra antibody (diluted 1:15) overnight at room temperature, rinsed in TBS for 30 min, incubated in biotinylated goat anti-rabbit IgG (diluted 1:300; Vector Laboratories), rinsed in TBS for 30 min, and finally incubated in avidin D-fluorescein (diluted 1:300; Vector Laboratories) for 1 h. All antibodies were diluted in TBS containing 5% milk, 5% normal goat serum, and 0.05% Triton X-100. The slides were then rinsed in TBS for 5 min, in TB for 20 min, and in distilled water for 2 min, coverslipped with Vectashield, sealed with nail polish, and viewed using an MRC 600 confocal microscope. Control sections were incubated in normal rabbit serum, or the primary antibody was omitted. In both instances, no specific staining was observed.

For staining of teased sciatic nerves, fresh sciatic nerve removed from 3- and 10-d-old rats was rinsed in PB, treated with collagenase (3.5 mg/ml of PB) for 15 min, rinsed in PB, teased apart on a slide coated with cell tak, rinsed with PB, fixed in 4% paraformaldehyde, rinsed, and then blocked using 10% normal goat serum in TBS for 1 h. The sections were then incubated in anti- $\beta 1$ extra (diluted 1:15) overnight at room temperature, and processed for immunocytochemistry.

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