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Cellular/Molecular

Neurofascin 140 Is an Embryonic Neuronal Neurofascin Isoform That Promotes the Assembly of the Node of Ranvier

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Rapid nerve conduction in myelinated nerves requires the clustering of voltage-gated sodium channels at nodes of Ranvier. The Neurofascin (Nfasc) gene has a unique role in node formation because it encodes glial and neuronal isoforms of neurofascin (Nfasc155 and Nfasc186, respectively) with key functions in assembling the nodal macromolecular complex. A third neurofascin, Nfasc140, has also been described; however, neither the cellular origin nor function of this isoform was known. Here we show that Nfasc140 is a neuronal protein strongly expressed during mouse embryonic development. Expression of Nfasc140 persists but declines during the initial stages of node formation, in contrast to Nfasc155 and Nfasc186, which increase. Nevertheless, Nfasc140, like Nfasc186, can cluster voltage-gated sodium channels (Nav) at the developing node of Ranvier and can restore electrophysiological function independently of Nfasc155 and Nfasc186. This suggests that Nfasc140 complements the function of Nfasc155 and Nfasc186 in initial stages of the assembly and stabilization of the nodal complex. Further, Nfasc140 is reexpressed in demyelinated white matter lesions of postmortem brain tissue from human subjects with multiple sclerosis. This expands the critical role of the Nfasc gene in the function of myelinated axons and reveals further redundancy in the mechanisms required for the formation of this crucial structure in the vertebrate nervous system.

Key words: axon initial segment; myelin; neurofascin; Nfasc140; node of Ranvier

Introduction

Myelinated nerves can conduct nerve impulses rapidly because voltage-gated sodium channels are concentrated at the nodes of Ranvier between successive myelinated segments. Nodes are the sites of action potential propagation due to the assembly of a macromolecular complex, including voltage-gated sodium channels. Components of the nodal complex include β1V-spectrin, ankyrinG, contactin (in the CNS), NrCAM and gliomedin (in the PNS), and a neuronal isoform of neurofascin, Nfasc186 (Davis et al., 1993; Berghs et al., 2000; Tait et al., 2000; Jenkins and Bennett, 2002; Yang et al., 2004; Eshel et al., 2005; Bekku and Oohashi, 2010). Flanking the nodes in both the CNS and PNS are the paranodal axoglial junctions where the terminal loops of the myelin sheath physically interact with the axon (Sherman and Brophy, 2005; Salzer et al., 2008). Axon–glial interaction at the paranode is mediated by an adhesion complex between the glial isoform of neurofascin, Nfasc155, and the axonal proteins caspr (also known as paranodin) and contactin (Einheber et al., 1997; Menegoz et al., 1997; Tait et al., 2000; Bhat et al., 2001; Boyle et al., 2001; Kazarinova-Noyes et al., 2001; Charles et al., 2002; Sherman et al., 2005).

The Neurofascin (Nfasc) gene is essential for the assembly of the nodal complex (Sherman et al., 2005). Nfasc has a unique role by encoding a glial isoform, neurofascin 155 (Nfasc155) and a neuronal isoform, neurofascin 186 (Nfasc186) (Davis and Bennett, 1993, 1994; Davis et al., 1993, 1996; Collinson et al., 1998; Tait et al., 2000), both of which have key roles in clustering sodium channels (Sherman et al., 2005; Zonta et al., 2008; Feinberg et al., 2010; Thaxton et al., 2011). A third isoform was also described in the original discovery of these proteins by Bennett and colleagues (Davis et al., 1993). This protein has an apparent size by SDS-PAGE of 140 kDa and was consequently named neurofascin 140 (Nfasc140). Nevertheless, little progress has been made in determining the function of Nfasc140.

Given the complementary roles of glial Nfasc155 and neuronal Nfasc186 in the assembly of nodal complexes, we wished to determine whether Nfasc140 might have a related function. First, we determined that Nfasc140 is a neuronal protein. We then investigated whether Nfasc140 was reexpressed in demyelinated axons in multiple sclerosis (MS) lesions. To address its function, we used a transgenic rescue approach, as described previously.
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Thy1.2 with a FLAG tag at its C terminus under the control of the
predicted sizes for Nfasc186 or Nfasc140 mRNA were 411 bp and 732 bp
cedures) Act 1986, and to the University of Edinburgh Ethical Review
nal cord ventral funiculus nerves, and cerebellar sections was as described
ogy, and behavioral analysis.

rescues the normal localization of NrCAM also supports the view
in the assembly of the node of Ranvier. The fact that transgenic
acin family with a complementary role to Nfasc155 and Nfasc186
Zonta et al., 2001

Materials and Methods
Isolation of Nfasc140 cDNA and detection of Nfasc186, Nfasc155, and
Nfasc140 isoforms by RT-PCR. RNA was extracted from mouse hind
brain at E15 and subjected to RT-PCR as described previously (Tait et al.,
2000) using 5'-AGGCCAGGCAGCCGGCCAC-3' and 5'-CCAT
CTATCCCCGTGGCTGA-3' forward and reverse primers, respectively, which
span the full coding regions of all neurofascins. The gel-purified product
of 3.2 kb was sequenced. To detect Nfasc186, Nfasc155, and
Nfasc140 mRNAs in P21 hind brain or optic nerve, RNA primers span-
ning the second and fourth FNIII repeats common to all three isoforms
were used (5'-CGCTACATTGTCAAGTGGCG-3' and 5'AAGCCGT
AAGGGGACACGGG-3' forward and reverse primers, respectively). The predicted sizes for Nfasc186 or Nfasc140 mRNA were 411 bp and 732 bp
for Nfasc155.

Animals. All animal work conformed to UK legislation (Scientific Pro-
cedures) Act 1986, and to the University of Edinburgh Ethical Review
Committee policy. Thy1Nfasc186 transgenic mice expressing full-length
Nfasc186 with a FLAG tag at its C terminus under the control of the
Thy1.2 promoter have been described and are here abbreviated as T186
(Zonta et al., 2011). The Nfasc140 cDNA with a FLAG tag at its C termi-
nus was cloned into the XhoI site of the pTSC21k vector (Lüthi et al.,
1997), released by digestion with NotI and transgenic mice expressing
Nfasc140 under the control of the Thy1.2 promoter (T140) were similarly
generated by pronuclear injection. The tamoxifen-inducible Thy1CreERT2
and PLPCreERT2 lines and the floxed Neurofascin line (Nfasc3r0/H11002/H11032)
have been described (Leone et al., 2003; Zonta et al., 2011). Induction of recombi-
nation using tamoxifen has been described (Zonta et al., 2011). All lines
were back-crossed to at least F6 to a C57BL/6 background. To express transgenic Nfasc140 and Nfasc186 on a neurofascin-null background,
the T140 and T186 lines were interbred with Nfasc140-/- and mice to generate T140/Nfasc140-/- and T186/Nfasc186-/- mice.

Antibodies, microscopy, Western blots, yeast two-hybrid, electrophysiol-
ogy, and behavioral analysis. Immunostaining of teased quadriceps, spi-
nal cord ventral funiculus nerves, and cerebellar sections was as described
previously (Sherman et al., 2005; Zonta et al., 2011). Most antibodies
used to stain these preparations have been described (Zonta et al., 2008,
2011) with the addition of anti-GFAP (GA-5, dilution 1:100, Sigma) and
anti-IBA1 (dilution 1:1000, Wako). Samples were mounted in Vectashield (Vector Laboratories). For confocal microscopy, we used a Leica TCS-LS confocal microscope and 63× objective, numerical aper-
ture 1.4, and Leica proprietary software. Conventional fluorescence mi-
croscopy was performed using an Olympus BX60 microscope and 40×
objective lens, numerical aperture 0.75, and images were captured using a
Hamamatsu Orca-ER camera and Improvision Openlab software. All
figures were prepared using Adobe Photoshop version 7.0 and were not
subjected to any subsequent image processing. Western blotting was
performed as described previously (Sherman et al., 2005). Yeast two-
hybrid interactions were tested exactly as previously described (Sherman
et al., 2001; Gunn-Moore et al., 2006). Electrophysiological measure-
ments of nerve conduction velocity were performed on acutely isolated
quadriceps nerves and performance on rotarod as described previously
(Court et al., 2004; Sherman et al., 2012). All quantitative measurements
were from an equal number of males and females.

MS postmortem tissues. All postmortem tissues were obtained from the
UK Multiple Sclerosis Society Tissue Bank at Imperial College. The tis-
uess were all collected via a prospective donor scheme following fully
informed consent (08/MRE09/31). The demographic data and clinical
and neuropathological features of the secondary progressive MS cases and
controls are shown in Table 1. The clinical diagnosis of MS was
confirmed based on the patient history and a detailed neurophysiological
analysis, as described previously (Reynolds et al., 2011). MS white matter
lesions were classified according to the UK MS Society Tissue Bank’s
published criteria (Reynolds et al., 2011) and tissue blocks chosen con-
taining chronic active lesions. The presence of chronic active lesions was
confirmed in serial sections as described previously (Mahad et al., 2009).
Briefly, snap frozen tissue blocks and cryostat sections, cut to a thickness
of 15 μm, were kept at ~80°C for storage. Sections were air dried for an
hour before being fixed in a cold solution of 4% PFA. Antigen retrieval
using 10 mM EDTA, pH 8, was performed, and a blocking solution of 1%
normal goat serum was used. Primary antibodies HLA-DF, DQ, DR
(dilution 1:200, Dako), PLP (dilution 1:500, AbD Serotec), and total
neurofilament (dilution 1:1000, Thermo Scientific) were applied at room
temperature for 90 min. Biotinylated secondary antibodies were added
for 30 min and visualized using DAB. Sections were immersed in a
hematoxylin solution for 1 min followed by mounting. The borders of the
lesions were scored on the snap frozen tissue block with a scalpel blade,
which allowed separation of the lesion and normal-appearing white mat-
er when cryosectioning at 50 μm, and 50–150 mg tissue isolated for
subsequent Western blotting.

Results
Nfasc140 is an embryonic neurofascin isoform in neurons
Western blotting of lysates from mouse hindbrains at different
ages showed that a neurofascin band at 140 kDa was expressed in the
embryonic mouse hind brain, whereas Nfasc186, although
also present, became more abundant at the onset of myelination
when Nfasc140 was downregulated (Fig. 1A). The identity of the
band that has an apparent size larger than Nfasc155 between
Nfasc140 and Nfasc186 at E15 is unknown. Nfasc155 expression
is associated with the initiation of myelination (Collinson et al.,
1998), and this was reflected in its increased abundance at P8 (Fig.
1A). Because the band with a mobility of 140 kDa was very abund-
ant at E15, we purified hindbrain RNA at E15 and performed
RT-PCR to isolate full-length cDNA clones of Nfasc140. These
cloned were sequenced, and the deduced protein domain struc-
ture was compared with the two well-characterized neurofascin
isoforms (Fig. 1B). This confirmed that Nfasc140 lacked the
mucin and FNIII5 domains characteristic of neuronal Nfasc186, and
the FNIII3 domain unique to Nfasc155 (Fig. 1C).

Table 1. Details of MS and control subjects used in this study*

| Case no. | Sex | Age (yr) | PMD (h) | Disease duration (yr) | Cause of death | Course |
|----------|-----|----------|---------|-----------------------|----------------|--------|
| C032     | M   | 88       | 22      | Prostate cancer       | —              | SP      |
| C036     | M   | 68       | 30      | Fibrosing alveolitis  | —              | SP      |
| C037     | M   | 84       | 5       | Bladder cancer        | —              | SP      |
| C045     | M   | 77       | 22      | Pneumonia             | —              | SP      |
| M200     | F   | 43       | 20      | Urinary tract infection| SP             | SP      |
| M203     | F   | 53       | 17      | Multiple sclerosis    | SP             | SP      |
| M207     | F   | 46       | 10      | Pneumonia             | SP             | SP      |
| M230     | F   | 42       | 31      | Multiple sclerosis    | SP             | SP      |
| M256     | F   | 53       | 21      | Pneumonia             | SP             | SP      |
| M252    | M   | 47       | 10      | Pneumonia             | SP             | SP      |

*PMD, Postmortem delay; SP, secondary progressive MS.

**Note:** The Nfasc140 cDNA was expected to be smaller than
Nfasc155 (140 kDa) because the mucin and FNIII5 domains
are absent. The observed molecular weight of the band
was 140 kDa, which may have been due to the presence
of a mucin exon. The results from RT-PCR, however,
suggest that Nfasc140 does not contain the mucin or
FNIII5 domains.
F I G U R E 1.  Nfasc140 is a neuronal isoform of neurofascin. A, Developmental Western blot of hind brain lysates on two separate gels using a pan anti-neurofascin antibody (NFC) that recognizes the C terminus of the cytoplasmic domain shows abundant but declining expression of Nfasc140 relative to Nfasc186 in the embryo up until birth (E13–P1), which declines further postnatally at the onset of myelination (P4–P12) when glial Nfasc155 becomes prominent. Actin was used to ensure equal loading in each group. B, The domain structure of the three neurofascin isoforms. Sequencing of the Nfasc140 RT-PCR product generated from E15 RNA showed that it lacked the mucin and FNIII domains characteristic of neuronal Nfasc186 or the FNIII domain unique to Nfasc155. Ig. Immunoglobulin-like domain; FNIII, fibronectin Type III domain; TM, transmembrane domain. C, Western blots of hind brain lysates at P45 confirmed that Nfasc140 lacked the FNIII and mucin/FNIII domains found in Nfasc155 and Nfasc186, respectively. As described previously (Tait et al., 2000), the pan anti-neurofascin antibody (NFC) was raised against a region at the extreme COOH terminus of the intracellular domain common to Nfasc186, Nfasc155, and Nfasc140 (CGNESSEATSPVNAAIYSLA), the second (NFF3) to the third FNIII domain specific to Nfasc155 (CLWVSOKROQASFGDPGRPR), and the third (MNF2) to a sequence within the mucin domain specific to Nfasc186 (CTESTPPTTGKIIHETAPDEQS). Note the presence of Nfasc155 and Nfasc186 bands that lack the C terminus of the full-length proteins. Actin was the loading control. D, Neuron- and glia-specific Cre-mediated inactivation of the Nfasc gene shows that Nfasc140 is a neuronal protein. Mice heterozygous for a floxed allele of Nfasc and heterozygous null for Nfasc and either expressing the transgene encoding a tamoxifen-inducible neuronal Cre (Thy1CreERT2/Nfascfl/H11002) or glial Cre (PLPCreERT2/Nfascfl/H11002) were treated with tamoxifen at P30, and hind brain lysates were analyzed by Western blot 2.5 months later using the pan anti-neurofascin antibody (NFC). Controls (Nfascfl/H11002) lacked Cre. Actin was the loading control. E, Neurofascins are not expressed in astrocytes or microglia. Double-label immunofluorescence using the pan anti-neurofascin antibody (NFC) (green) with either anti-GFAP for astrocytes (red) or anti-IβA1 for microglia (red) reveals neurofascins at axonal nodes and paranodes (arrows) but not in either astrocytes or microglia. Nfasc186 is not expressed in oligodendrocytes. Double-label immunofluorescence using the Nfasc186-specific antibody (MNF2) (green) and the oligodendrocyte-specific antibody APC shows labeling of axonal nodes (arrow) but no Nfasc186 in oligodendrocytes (red). Scale bar, 5 μm. F, Neither Nfasc140 nor Nfasc186 is expressed in non-neuronal cells. RT-PCR was used to discriminate between mRNAs encoding either Nfasc186 and Nfasc140 (411 bp) or Nfasc155 (732 bp) in total hind brain (HB) RNA or in optic nerve (ON) RNA.

Finally, to exclude the possibility that Nfasc140 is expressed in neural cells other than neurons, we used primers that span the second and fourth FNIII repeats that are present in Nfasc186, Nfasc155, and Nfasc140 in RT-PCR of RNA purified from whole-mouse hind brain or optic nerve (Fig. 1B). Postnatal optic nerve was chosen because it contains a variety of neural cells, including oligodendrocytes, astrocytes, and microglia, but no neuronal cell bodies, and therefore no neuronal mRNA. The primers were chosen to discriminate between isoforms where the second and fourth FNIII repeats are contiguous (Nfasc186 and Nfasc140) from mRNA where these two domains are separated by FNIII13 (Nfasc155) (Fig. 1B). The predicted size of the products derived from Nfasc186 and Nfasc140 mRNA is identical (411 bp) and smaller than that from Nfasc155 (732 bp). Both are readily detectable in hind brain. However, only the product derived from Nfasc155 was obtained from optic nerve (Fig. 1F).

Hence, Nfasc140 is a neuronal neurofascin isoform that is expressed somewhat earlier than its neuronal counterpart, Nfasc186. However, although it persists, Nfasc140 is downregulated as myelination proceeds, in contrast to Nfasc186. This suggests that Nfasc140 has a role in the earliest stages of sodium channel clustering, whereas Nfasc186 has the primary role in neurons during the period of most active myelination.
Nfasc140 is reexpressed in demyelinated MS lesions

We have previously shown that demyelination in MS lesions leads to disruption of the nodal and paranodal domains (Howell et al., 2006). Because Nfasc140 is normally expressed strongly at the very earliest stages of myelination, we asked whether this neurofascin isoform might be reexpressed in chronic active lesions where demyelination was occurring. Lesions were identified as described in Materials and Methods (Fig. 2A). As expected, Nfasc140 is either not detectable or present at very low levels by Western blot in mature non-neurological control white matter (Fig. 2B, C). In contrast, chronic active lesions in four different MS patients reexpress a smaller form of neurofascin, with a mobility in SDS-PAGE identical to Nfasc140 (Fig. 2B). Although the levels of Nfasc186 were highly variable (data not shown), expression of the paranodal glial neurofascin, Nfasc155, persisted, indicating the presence of oligodendrocytes in the lesions (Fig. 2B). The variations in the relative amounts of Nfasc140 and Nfasc155 (the latter being a marker of oligodendrocytes) presumably reflect the fact that the MS patients were of varying age and disease duration and the fact that the samples were not from anatomically identical places. Comparison of normal-appearing white matter with adjacent chronic active lesions in two different subjects confirmed that this lower band was much more abundant in the lesions (Fig. 2C). Domain-specific antibodies supported the
view that this lower band was indeed Nfasc140 (Fig. 2D) and not a variant of Nfasc155 as has been detected in the brains of MS patients (Pomicter et al., 2013).

Nfasc140 is targeted to the node of Ranvier and axon initial segment

To study the function of Nfasc140, it was necessary to use a transgenic approach because this neurofascin isoform does not possess any unique domains not also found in Nfasc186 and Nfasc155 that would permit either the generation of isoform-specific antibodies, as is the case for Nfasc155 and Nfasc186, or an RNAi knockdown approach (Tait et al., 2000). To investigate the expression of the transgenically expressed protein, we attached a C-terminal FLAG-tag.

We generated transgenic lines expressing Nfasc140 with a C-terminal FLAG-tag, and one founder (T140) was selected in which FLAG-tagged Nfasc140 was robustly and ubiquitously expressed in neurons in both the CNS and PNS in similar fashion to the T186 transgenic line expressing the FLAG-tagged Nfasc186 (T186) we have described previously (Zonta et al., 2011). The level of expression of transgenically expressed Nfasc140 and Nfasc186 in the CNS was comparable, and the sizes of the proteins from Western blots were as predicted (Fig. 3A).

Because Nfasc140 is a neuronal neurofascin isoform, we speculated that, like Nfasc186, it might also be targeted to the node of Ranvier (Tait et al., 2000). Immunofluorescence labeling showed that FLAG-tagged Nfasc140 was indeed concentrated at nodes both in the PNS and the CNS in comparable fashion with Nfasc186 in wild-type (WT) mice (Fig. 3B). We then asked whether Nfasc140 was, like Nfasc186, also targeted to AISs where Nfasc186 is known to play a crucial role in stabilizing the complex of proteins, including voltage-gated sodium channels in the adult cerebellum (Zonta et al., 2011). Nfasc140 was targeted to the AIS in Nfasc186−/− mice (Fig. 3C). Further, because the absence of neuronal neurofascin during development causes the concomitant loss of the cell adhesion molecule NrCAM (Zonta et al., 2011), we asked whether Nfasc140 was able, like Nfasc186, to rescue...
Neuronal Neurofascin Nfasc140 Clusters Nodal Nav

Figure 4. Introduction of the FLAG tag at the C terminus of Nfasc186 or Nfasc140 prevents interaction with ankyrin G. A. The 109 amino acid cytoplasmic tail common to Nfasc186, Nfasc140, and Nfasc155 (NfascIC) containing AnkBD (Zhang et al., 1998) was used as bait in a yeast two-hybrid interaction with empty vector (pAS2-1) or clones encoding regions of ezrin, ankyrin, and syntenin-1 that had previously been identified as interactors (Davis and Bennett, 1994; Koroll et al., 2001; Gunn-Moore et al., 2006). B. The degree of interaction was scored (−, +, ++, +++). NfascICFlag was no longer capable of interacting with ankyrin G.

NrCAM expression at the AIS in neurofascin-null mice, which it did (Fig. 3D) (Zonta et al., 2011).

It has been proposed that the ankyrin binding site (AnkBD) in the C-terminal domain of the neurofascins assists in the recruitment of ankyrin G to the node of Ranvier (Davis and Bennett, 1994; Lambert et al., 1997). Therefore, we wished to test whether the AnkBD domain was still functional when a FLAG tag was present at the C terminus. We used the yeast two-hybrid technique to test the interaction of the intracellular domain common to Nfasc186, Nfasc140, and Nfasc155, with or without the presence of the FLAG epitope (Fig. 4A). The ankyrin G clone comprised amino acids 464–759, previously isolated using NfascIC as bait (unpublished data), and the controls were clones encoding regions of ezrin and syntenin-1 that had previously been shown to be interactors with the neurofascin C terminus (Koroll et al., 2001; Gunn-Moore et al., 2006).

The prediction was that the FLAG-tag would not affect interaction with the ezrin-binding domain because this lies close to the transmembrane segment, although because syntenin-1 interacts with the PDZ-binding motif at the extreme C terminus of the neurofascins, interaction with syntenin-1 would be abolished (Koroll et al., 2001; Gunn-Moore et al., 2006). As predicted, interaction of the C-terminal domain of tagged neurofascin with ezrin was unaffected, whereas syntenin-1 no longer bound when the FLAG tag was at the C terminus (Fig. 4B). However, and perhaps surprisingly, the FLAG-tag also inhibited interaction of ankyrin with the AnkBD in this assay (Fig. 4B). Unfortunately, it was not possible to confirm this interaction by coimmunoprecipitation because the presence of the FLAG tag blocked the activity of the best antibody for the purpose, namely, the NFC antibody. Hence, this must remain a tentative conclusion. Nevertheless, it was interesting that the presence of the FLAG does not prevent targeting of both Nfasc140 and Nfasc186 to the AIS and nodes, which suggests that a direct interaction between neuronal neurofascins and ankyrin G may not be essential for node assembly.

Nfasc140 rescues the nodal complex

We then asked whether Nfasc140 FLAG-tagged at its C terminus could reconstitute the nodal complex in the axons of Nfasc−/− mice in vivo in the absence of an intact axoglial adhesion complex. We have used this transgenic rescue approach previously to show that neuronal Nfasc186 and glial Nfasc155 have complementary functions in node assembly (Sherman et al., 2005; Zonta et al., 2008). We compared Nfasc140 with Nfasc186 because we knew the latter could rescue the nodal components on a neurofascin-null background (Sherman et al., 2005; Zonta et al., 2008). Expression of transgenic Nfasc140 on a neurofascin-null background was fully able to rescue the nodal complex in both the PNS and CNS (Fig. 5). Thus, sodium channels ankyrin G and βIV-spectrin were all targeted to CNS and PNS nodes by Nfasc140 similar to Nfasc186 (Fig. 5). Furthermore, the fact that we assessed targeting at P60 suggests that these nodal components are quite stable at the node in the presence of Nfasc140. NrCAM and gliomedin are PNS-specific nodal components, whereas brevican is CNS-specific, and all were targeted appropriately (Fig. 5).

The fact that the transgenic mice were null for Nfasc155 meant that they could not have had intact paranodal axoglial junctions (Sherman et al., 2005; Zonta et al., 2008). This was reflected in the redistribution of Kv1.1 channels to a paranodal location from their normal juxtaparanodal domain (Fig. 5). However, this did not affect the ability of Nfasc140 (or Nfasc186) to recruit nodal proteins to the complex.

Amelioration of nerve conduction velocity by Nfasc140 rescue of the PNS node

Nerve conduction velocity measurements on quadriceps nerves showed that expression of Nfasc140 at PNS nodes restored nerve conduction velocities to the same degree as Nfasc186 (Fig. 6A) (41.2 ± 1.6 m/s for WT, 24.1 ± 2.3 for T140/Nfasc−/−, and 23.0 ± 1.4 for T186/Nfasc−/−, values are mean ± SEM; n = 6 mice for each condition; p < 0.0001, one-way ANOVA test followed by Tukey’s Multiple Comparison Test) (Fig. 6A). Interestingly, it has been previously shown that, when axoglial junctions are disrupted by the loss of Caspr, conduction velocities in the PNS are 59% of WT (Bhat et al., 2001). This agrees remarkably well with the values for nerves in which nodes were rescued by Nfasc140 (59%) or Nfasc186 (56%), where in both cases paranodal axoglial junctions were deranged due to the absence of Nfasc155 (Fig. 5) (Sherman et al., 2005). Although still not at WT values, these conduction velocities are a considerable improvement on the 81% reduction in the speed of conduction in the peripheral nerves of neurofascin-null mice, which die at P7 (Sherman et al., 2005). Consistent with their electrophysiological deficits, neurofascin-null mice rescued with Nfasc140 or Nfasc186 performed poorly on the rotator motor behavioral test (Fig. 6B). Nevertheless, it was noteworthy that, even though the “rescued” mice had significant gait problems, they were otherwise healthy and survived up to 9 months of age after which they were killed.

Discussion

Our data provide further evidence for the essential role played by the Neurofascin gene in the assembly of the node of Ranvier, a key axonal domain in myelinated nerves. Hence, three isoforms of the same protein expressed in two distinct cell types cooperate to achieve the goal of clustering a macromolecular axonal complex, namely, voltage-gated sodium channels and its associated pro-
proteins. This represents an unusual example of functionally cooperative, cell-type-specific alternative splicing of a single gene in cell biology. In the case of the neurofascins, this cooperativity results in the assembly of an essential structure in the development of the vertebrate nervous system.

Further, when considering the function of the rescued mice, we have previously found that neurofascin-null mice rescued with Nfasc186 survive into adulthood (Zonta et al., 2011). We also found that the same was true for mice rescued with the Nfasc140 transgene. This is quite surprising given the fact that both types of mice lack intact paranodal axoglial junctions. This may be due to the fact that the heterologous promoter used to drive expression of the Nfasc transgenes, namely, Thy1.2, remains active throughout adult life. In contrast, the endogenous neuronal Nfasc promoter is probably downregulated in adulthood due to the fact that Nfasc186, at least, is a very stable protein, which turns over very slowly at the node of Ranvier (Zonta et al., 2011).

The Neurofascin gene encodes six Ig repeats, five FNIII repeats, and a mucin domain (Davis et al., 1996) (Fig. 1). All three major neurofascin isoforms have the six Ig repeats; however, Nfasc186 has FNIII-1, 2, 4, and 5 repeats and the mucin domain, whereas Nfasc155 lacks the mucin domain and has the FNIII-1, 2, 3, and 4 repeats. Therefore, the mucin and FNIII-5 domains are unique to neuronal Nfasc186, whereas the FNIII-3 domain is unique to glial Nfasc155. In contrast, Nfasc140 has the FNIII-1, 2, and 4 repeats found in Nfasc186 but lacks FNIII-5 and the mucin domain. The fact that Nfasc140 lacks these domains shows that they are neither required for targeting of Nfasc140 to the node nor are they necessary for sodium channel clustering. It remains to be determined what the function of these domains might be. Other work has shown that Ig5–6 are also dispensable for Nfasc186 targeting to the node (Thaxton et al., 2010), although the ectodomain of Nfasc186 is necessary for targeting to the PNS node.

![Figure 5](image1.png)

**Figure 5.** Nfasc140 rescues the PNS and CNS nodal complexes as efficiently as Nfasc186 in Nfasc−/− mice. Immunostaining of teased quadriceps (PNS) and spinal cord ventral funiculus fibers (CNS) at P60 shows that both FLAG-tagged Nfasc140 and Nfasc186 can rescue the nodal components, voltage-gated sodium channels (Nav), ankyrinG, and βIV-spectrin when expressed on a neurofascin-null background. Additionally, the PNS-specific nodal proteins NrCAM and gliomedin were also rescued, as was the CNS-specific nodal protein brevican. Rescue of the node was not accompanied by reformation of the axoglial junction; and, as a consequence, juxtaparanodal Kv1.1 channels were mislocalized compared with WT. Scale bars, 5 µm.

![Figure 6](image2.png)

**Figure 6.** Nfasc140 restores limited nodal function in 2-month-old Nfasc−/− mice. A, Conduction velocities were measured in quadriceps nerves from mice at P60. Although significantly less than WT mice, values from mice expressing Nfasc140 were not significantly different from those expressing Nfasc186. B, T140/Nfasc−/− and T186/Nfasc−/− P60 animals were significantly worse than WT mice on the rotarod. And the deficit comparing the two different transgenic animals was not significantly different. Data are mean ± SEM; n = 6 mice for electrophysiology and n = 4 for rotarod. ***p < 0.0001 (one-way ANOVA test followed by Tukey’s Multiple Comparison Test).
(Dzhashiashvili et al., 2007). Further, although there is evidence that ankyrinG promotes sodium channel clustering at the PNS node (Dzhashiashvili et al., 2007), our data show that interaction of ank- 
rinG with either Nfas186 or Nfas140 is not necessary for either targeting or maintenance of sodium channels at the node in both the CNS and PNS.

Nfas140 appears to have a similar structure and developmental pattern of expression to NF166, previously described in chick (Pruss et al., 2006). The early expression of Nfas140 during development suggests that Nfas140 complements the function of Nfas155 and Nfas186 in the initial stages of the assembly and stabilization of the nodal complex. Further, Nfas140 is reex- pressed in demyelinated regions of postmortem brain tissue from human subjects with MS. Our previous studies demonstrated that Nfas186 expression was disrupted in MS white matter lesions and became diffusely distributed along demyelinated axons in a manner similar to sodium channels (Howell et al., 2006). The demonstration that Nfas140, which is normally absent from adult axons, becomes reexpressed in demyelinated lesions sug-
gests a return to a premyelinating state, perhaps in preparation for remyelination to occur. This is in keeping with the reduced expression of Nfas155 in the same demyelinated lesions, indic-
ating the presence of some oligodendrocytes or residual myelin.

In conclusion, this work expands the critical role of the Nfas gene in the function of myelinated axons and reveals further redundancy in the mechanisms required for the formation of this crucial structure in the vertebrate nervous system.

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