Integrin-mediated axoglial interactions initiate myelination in the central nervous system

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All but the smallest-diameter axons in the central nervous system are myelinated, but the signals that initiate myelination are unknown. Our prior work has shown that integrin signaling forms part of the cell–cell interactions that ensure only those oligodendrocytes contacting axons survive. Here, therefore, we have asked whether integrins regulate the interactions that lead to myelination. Using homologous recombination to insert a single-copy transgene into the hypoxanthine phosphoribosyl transferase (hprt) locus, we find that mice expressing a dominant-negative β1 integrin in myelinating oligodendrocytes require a larger axon diameter to initiate timely myelination. Mice with a conditional deletion of focal adhesion kinase (a signaling molecule activated by integrins) exhibit a similar phenotype. Conversely, transgenic mice expressing dominant-negative β3 integrin in oligodendrocytes display no myelination abnormalities. We conclude that β1 integrin plays a key role in the axoglial interactions that sense axon size and initiate myelination, such that loss of integrin signaling leads to a delay in myelination of small-diameter axons.

Introduction

Myelination represents a spectacular cell–cell interaction in which axons are ensheathed by multiple layers of membrane from specialized glia—oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). The threshold axon diameter for myelination is a regulated process, with a minimum axon diameter for myelination of 1 μm in the PNS and of 0.2 μm in the CNS (Waxman and Bennett, 1972; Voyvodic, 1989). Moreover, the number of wraps is precisely related to the axon diameter such that the ratio of the diameter of the axon to that of the entire myelinated unit—the g-ratio—is constant (Friede, 1972). This points to the existence of signals on the axon surface that both initiate the myelination process and then determine exactly how many times the myelinating process wraps around the axon before the extrusion of cytoplasm (a process termed compaction) and the formation of the mature multilamellar sheath. In the PNS, a necessary and sufficient signal is type III neuregulin-1 (Nrg1), increased expression of which on the axon results in a thicker myelin sheath, whereas reduced axonal expression results in thinner myelin (Michailov et al., 2004; Taveggia et al., 2005). The overexpression of Nrg1 also causes the myelination of axons whose size is below the normal threshold (Taveggia et al., 2005), showing that Nrg1 is a signal that initiates myelination in addition to its role in regulating wrapping. In the CNS, however, the role and identity of any such initiation signals remains unknown, with the contribution of neuregulins at this and later stages of myelination being unclear. Although type III Nrg1−/− mice show reduced myelin sheath thickness in the corpus callosum (Taveggia et al., 2008), normal myelination in mice where the Nrg1 gene has been excised in the CNS (Brinkmann et al., 2008) shows that other signals must contribute to the precise relationship between axon and oligodendrocyte.

Cell adhesion molecules represent excellent candidates for these additional signals regulating myelination (Laurersen and ffrench-Constant, 2007). One of these is α6β1 integrin, a receptor expressed on oligodendrocytes for laminins expressed in...
axon tracts at the time of myelination that promotes oligodendrocyte survival by amplification of growth factor signaling (Colognato et al., 2002). This provides a mechanism for the target-dependent survival of oligodendrocytes, with those that fail to establish normal contact with axons during development undergoing programmed cell death. The importance of integrins in oligodendrocyte biology is further underscored by the finding that integrin-mediated signaling pathways are strongly represented in a genome-wide analysis of expression in differentiating oligodendrocytes (Cahoy et al., 2008). Here, therefore, we have asked whether integrins play a role in the regulation of myelination, either by contributing to the signals that initiate myelination or by regulating the thickness of the resulting myelin sheath.

Prior studies on the role of β1 integrin in CNS myelination in vivo have been contradictory. Constitutive disruption of the β1 integrin gene resulted in early lethality (Fassler and Meyer, 1995; Stephens et al., 1995), requiring the use of conditional ablation or dominant-negative strategies to examine function. Lee et al. (2006) reported that mice expressing a β1 integrin lacking the C-terminal cytoplasmic tail (β1ΔC) in oligodendrocytes displayed region-specific hypomyelination in optic nerve and spinal cord and no myelination abnormalities in the corpus callosum. In contrast, conditional inactivation of the β1 integrin gene in premyelinating oligodendrocytes showed that β1 integrin is not required for CNS axon ensheathment, myelination, or remyelination (Benninger et al., 2006). Nonetheless, the contribution made by integrin signaling to oligodendrocyte survival, evidenced by our earlier studies on the α6 knockout CNS, was confirmed by a transient reduction in oligodendrocyte numbers in the developing cerebellum (Benninger et al., 2006). However, it is possible that both these studies fail to reveal the specific function of β1 integrin in myelinating oligodendrocytes. In the knockout study, gene deletion occurs early, at the premyelinating stage of oligodendrocyte development, thus potentially enabling compensation by other integrins to occur by the stage of myelination. The ability of different integrin β subunits to compensate for one another has been previously shown in Drosophila embryogenesis (Martín-Bermudo et al., 1999). In the dominant-negative study, the construct used by Lee et al. (2006) contained a normal integrin extracellular domain that will bind laminin and compete with any other laminin receptors for available ligand in the myelinating tract. Here, therefore, we have used the reverse strategy—expression in oligodendrocytes of a well-characterized dominant-negative β1 integrin subunit containing only the intracellular domain that will not bind ligand and would thus be predicted to be more specific in targeting β1 integrin signaling (LaFlamme et al., 1994; Relvas et al., 2001). Furthermore, we have minimized the time available for compensatory mechanisms to develop by expressing the dominant-negative subunit only at the myelinating stage in oligodendrocytes using the myelin basic protein (MBP) promoter (Farhadi et al., 2003).

Results
Generation of dominant-negative β1 integrin transgenic mice (dnβ1)
To examine the role of β1 integrin signaling in myelination in vivo, without perturbing the earlier stages of oligodendrocyte precursor migration and proliferation, we generated transgenic mice expressing a dominant-negative chimeric β1 integrin (dnβ1) under the control of the 9.5-Kb MBP promoter. This promoter drives specific expression in myelinating oligodendrocytes within the CNS (Farhadi et al., 2003), as we confirmed in lacZ reporter mice that showed expression of β-galactosidase only at the time of myelination in different white matter tracts (Fig. S1). The dominant-negative construct used contains the β1 intracellular domain and the extracellular and transmembrane domains were replaced by the nonsignaling α-subunit of the human interleukin-2 receptor (IL2Rα). This chimeric integrin has been shown to function in a dominant-negative manner in vitro (LaFlamme et al., 1994) and when expressed in oligodendrocyte precursors transplanted into demyelinated lesions (Relvas et al., 2001).

To overcome the usual limitations of random and multiple-copy insertion in standard transgenesis, we used the hypoxanthine phosphoribosyl transferase (hprt) targeting system (Farhadi et al., 2003) for the generation of the genetically modified embryonic stem (ES) cells expressing the dnβ1 integrin (Fig. 1 a). This technology uses homologous recombination to target a single copy of the desired transgene (in this case the dnβ1 integrin) driven by a specific promoter (in this case the MBP promoter) into the hprt locus on the X-chromosome. The ES cell line used lacks a complete hprt gene and the targeting vector “rescues” the hprt gene by replacing the lost sequences in addition to inserting the desired transgene and its promoter. As a result, ES cells with the correct insertion can easily be selected by virtue of their expression of hprt and the subsequent ability to grow in HAT medium. This process was extremely efficient, with 12 out of 12 clones selected having the correct insertion, as evidenced by PCR analysis performed as described in the Materials and methods (Fig. 1 b). We then generated transgenic mice from one of these ES cell lines to establish a dnβ1 transgenic line. These mice had no overt phenotype, and in particular showed no evidence of locomotor defects.

A second major advantage of using this technology is that single-copy insertion of the transgene into the same place in each line eliminates position and copy number effects, and makes comparison of multiple transgenic lines unnecessary. This has been confirmed by experiments where different mouse lines derived using this strategy from independently targeted ES cell clones expressing the same bcl-2 transgene exhibited comparable levels of expression (Bronson et al., 1996) and by our own previous studies showing that pairs of transgenic lines expressing different MBP regulatory sequences show similar levels of β-galactosidase expression within each pair (Farhadi et al., 2003). Hemizygous and wild-type littermate males of the single line were therefore used as mutant and control mice, respectively, throughout this study (Fig. 1 c).

Dnβ1 mice express the dominant-negative β1 integrin in white matter tracts
Expression of the dnβ1 integrin in the mutant was initially confirmed by RT-PCR. Primers hybridizing to the human IL2Rα gene, only present in the inserted dominant-negative construct, showed specific amplification in mutant mice (Fig. 2 a). To visualize the expression of the dnβ1 integrin protein in the myelinating tracts,
we used immunofluorescence labeling with an anti–human IL2Rα antibody on transverse sections of optic nerve, sagittal sections of cerebellum, and coronal sections of corpus callosum in wild-type and mutant mice (Fig. 2 b). The dnβ1 integrin was expressed in white matter tracts within these regions in mutant mice, whereas no expression was detected in the wild-type animals. These results confirm that the dnβ1 integrin is expressed in transgenic mice at the RNA and protein level in the predicted pattern within myelinating tracts.

Next, we tested if this expression was specific to the nervous system. We analyzed RNA from several tissues of the dnβ1 mice by RT-PCR (Fig. 2 c). Transgene expression was found to be restricted to cortex, cerebellum, brainstem, spinal cord, optic nerve, and sciatic nerve and absent from other tissues such as heart, liver, lungs, kidney, muscle, testis, spleen, and stomach. This confirms that, as we would predict from the known expression pattern of the MBP, the dnβ1 expression is restricted to the nervous system.

**Integrin signaling is inhibited in the dnβ1 mice**

To confirm the efficacy of our strategy, we asked to what extent the dnβ1 construct expressed in the transgenic mice inhibited the endogenous β1 integrin. First, we compared the expression levels of the endogenous and the transgenic β1 integrin. In vitro studies have demonstrated that approximately equal expression levels of the IL2R-β1integrin chimera to the endogenous integrin are sufficient to inhibit integrin-mediated cell spreading and migration, confirming that this level of transgene expression exerts a dominant-negative effect (LaFlamme et al., 1994). We therefore designed a semi-quantitative RT-PCR analysis using two distinct sets of primers, one specific for the endogenous β1 integrin and another for the dnβ1 integrin, taking advantage of the differences in their extracellular domains (Fig. 3 a).

To examine whether the levels of β1 integrin inhibition might vary in the different areas of the nervous system, we performed this RT-PCR analysis on several areas of the nervous system. We showed that in all CNS white matter tracts the dnβ1 integrin expression levels were equal to or greater than the levels of the mRNA for the endogenous integrin, with significantly higher levels seen in optic nerve (Fig. 3 b). The same expression pattern was observed in four pairs of mice analyzed, with the mean levels of the dnβ1 mRNA being 2.22 ± 0.37 in optic nerve (P = 0.033), 1.6 ± 0.37 in spinal cord, and 1.22 ± 0.17 in cerebellum, as compared with 0.92 ± 0.10 in cortex.

Second, to show directly that the dominant-negative strategy we used inhibits downstream signaling, as predicted from these expression studies, we examined activation of FAK by tyrosine phosphorylation. Activation of FAK via autophosphorylation, on tyrosine 397, occurs after integrin binding to ECM ligands (Schaller et al., 1994). We showed, by Western blot analysis for P-FAK (Y397), that β1 integrin signaling was significantly reduced (45 ± 9.3%, P = 0.018, n = 3 pairs of animals) in the optic nerve of P18 dnβ1 mice, as compared with wild-type mice of the same age (Fig. 3 c). Similar changes were seen in spinal cord, but these did not reach statistical significance.

**The relationship between axon diameter and myelin thickness is normal in dnβ1 mice**

To examine myelination in the dnβ1 mice, we first compared white matter tracts in several CNS regions of wild-type and dnβ1 mice by immunohistochemistry using an anti-MBP antibody. We observed no differences in these studies, with the white matter tracts visualized in sections of corpus callosum and optic nerve showing identical thickness and labeling intensity (Fig. S2).

To assess myelination in more detail, we performed an ultrastructural electron microscopy (EM) analysis on optic nerve, spinal cord, and cerebellum at P28, an age when myelination is largely complete (Fig. 4). We found that the relationship between axon diameter and myelin thickness (g-ratio) was not significantly different between dnβ1 mutants and their wild-type
The axon diameter required to initiate myelination is increased in the dnβ1 mice. We reasoned that an analysis of development might reveal transient abnormalities of myelination not seen in the fully developed myelinated tracts. We therefore examined optic nerve at P17, a time at which myelination is not yet complete. As in the later stages, no significant differences in the thickness of those myelin sheaths that had formed and appeared fully compacted by this age were detected in the optic nerve (P = 0.65), with average g-ratios for control and mutant mice of 0.79 ± 0.007 and 0.80 ± 0.016, respectively (Fig. 5, a and b). Interestingly, however, we noted a higher frequency of dysmyelinated axons in mutant optic nerve, corpus callosum, or spinal cord was observed. We conclude that, for myelinated axons, the myelin morphology and the ratio of myelin thickness/axon diameter in the dnβ1 mutants are the same as in wild-type mice at P28.

Figure 2. Dnβ1 mice express the dominant-negative β1 integrin in white matter tracts. (a) RT-PCR analysis from cortex of 3-mo-old wild-type (Wt) and transgenic mice (dnβ1) reveals RNA expression of β1 integrin in both Wt and transgenic mice but dnβ1 integrin (IL2Rα) only in the mutant. (b) Anti–human IL2Rα immunolabeling of optic nerve transverse sections (bar: 33 μm), cerebellar sagittal sections (bar: 33 μm), and corpus callosum coronal sections (bar: 66 μm) from P18 wild-type and dnβ1 mice shows that the dominant-negative integrin protein is expressed in the myelinating tracts of mutant mice. (c) Dnβ1 expression is nervous system specific as confirmed by an RT-PCR analysis on various tissues from P22 mutant mice. IL2Rα primers were used for detection of the dnβ1 expression (top). Actin primers were used as control (bottom).

counterparts in P28 optic nerve, cerebellum, and spinal cord, with average g-ratios for control and mutant optic nerves of 0.79 ± 0.013 and 0.77 ± 0.020 (P = 0.57); for control and mutant cerebella of 0.80 ± 0.014 and 0.82 ± 0.019 (P = 0.43); for control and mutant spinal cords of 0.80 ± 0.001 and 0.79 ± 0.004 (P = 0.36), respectively. Additionally, no evidence of a higher frequency of dysmyelinated axons in mutant optic nerve, corpus callosum, or spinal cord was observed. We conclude that, for myelinated axons, the myelin morphology and the ratio of myelin thickness/axon diameter in the dnβ1 mutants are the same as in wild-type mice at P28.
this age that wild-type oligodendrocytes were myelinating smaller axons to a larger extent than their dnβ1 counterparts. This became apparent in the g-ratio analysis by a reduced population of myelinated axons with small diameters in dnβ1 optic nerves as compared with wild-type nerves (Fig. 5 c). These very small axons must therefore have been unmyelinated in the dnβ1 nerve. We therefore hypothesized that oligodendrocytes with inhibited β1 integrin signaling would require a higher axon diameter to initiate myelination. To address this question, we compared the relationship between axon diameter and the percentage of axons myelinated for wild-type and dnβ1 mice. We measured the axon diameter of more than 400 axons from at least three random nonoverlapping photos and scored them as myelinated or unmyelinated. The axon diameters were then subdivided into 0.1-µm intervals and plotted against the percentage of axons that were myelinated within each interval (Fig. 5 d). As some variability was noted between litters, littermate controls were examined in each case. The percentage of myelinated axons across all axon diameter intervals for the dnβ1 mutant optic nerves (62.08 ± 10.55%, n = 2,494 from 3 animals) was lower (P = 0.023) than for their wild-type littermates (66.30 ± 10.09%, n = 2,506 from 3 animals). This phenotype was most evident in small axons of ≤0.6 µm diameter (P = 0.0085), whereas a similar percentage of myelination was seen in larger axons of >0.6 µm of diameter. In particular, mutant axons of a diameter within the 0.3–0.4-µm range showed the most significant reduction in myelination as compared with wild-type controls (P = 0.023). These results therefore show that the threshold diameter required to initiate myelination is increased in the dnβ1 optic nerve.

To confirm that this effect was transient and only seen during myelinating stages of development, we next repeated the analysis on the older P28 optic nerves. Here no difference was seen between wild-type and dnβ1 littermate animals (Fig. S3), showing that the effect of loss of integrin signaling is only on the initiation of myelination and results in a delay, rather than in a long-lasting perturbation, in the formation of normal myelin sheaths. This correction resulted from restoration of the ability of the mutant oligodendrocytes to myelinate smaller-diameter axons with age, rather than an overall increase in axon diameter, as evidenced by the higher percentage of axons with a diameter less than 0.5 µm that are myelinated at P28. Thus, at P17 the percentage of axons myelinated of diameters 0.2–0.3, 0.3–0.4 and 0.4–0.5 µm were 1.2, 12.7, and 37.2%, whereas at P28 the percentages were 6.1, 51.4, and 83.6%, respectively.

As an alternative method to demonstrate the increased threshold diameter required to initiate myelination in the dnβ1 mice, we compared the frequency distribution of the diameters of all myelinated and total axons (irrespective of myelination status) between wild-type and dnβ1 mutants in P17 optic nerve (Fig. 5 e). As predicted from the observations above, both the mean myelinated and unmyelinated axon diameters were significantly larger (P < 0.0001) in mutant optic nerves (0.86 ± 0.015 µm for myelinated axons, 0.38 ± 0.004 µm for unmyelinated axons) than in their wild-type littermate controls (0.75 ± 0.012 µm and 0.35 ± 0.004 µm, respectively). By contrast, the mean diameter of all axons was not significantly different, with an average axon diameter of 0.47 ± 0.006 µm and 0.48 ± 0.007 µm for wild-type and mutant, respectively (P = 0.21). This excludes the possibility that changes in the distribution of axon diameters might contribute to the effects on myelination we observe.

A greater threshold for myelination is also seen in FAK-deficient mice

If the increased threshold for myelination we observed in the dnβ1 mice reflects an inhibition of integrin signaling, then we
would predict that other transgenic mice having perturbations of downstream signaling molecules should show a similar phenotype. To test this we examined P16–P21 optic nerves from mice in which FAK had been deleted in myelinating glial cells by crossing mice homozygous for a floxed FAK allele with a Cnp-Cre line (Grove et al., 2007). As discussed above, FAK is a key part of the integrin signaling pathway, binding to the integrin cytoplasmic domain and being activated by phosphorylation after integrin ligand binding, and loss of FAK activity in oligodendrocytes has very recently been shown to result in hypomyelination in the developing mouse optic nerve (Forrest et al., 2009). Similarly to the dnβ1 mice, we found that an increased threshold diameter was required for myelination at this age, as evidenced by a reduced percentage of myelinated axons in knockout ($57.37 \pm 10.38\%$, $n = 2,186$ from 4 animals) compared with wild-type ($64.93 \pm 10.70\%$, $n = 2,560$ from 4 animals) optic nerves of P16–P21 animals ($P = 0.0087$; Fig. 6), whereas there was no difference in the percentage of myelinated axons in adult (P60) nerves (Fig. S3).

**Dnβ1 oligodendrocytes myelinate a reduced number of internodes in myelinating co-cultures**

There are two general mechanisms by which the increase in axon diameter required for myelination in the P17 optic nerve might be explained. First, an intrinsic abnormality of the oligodendrocyte such that it cannot respond appropriately to the axoglial signal proportional to axon diameter that is required to initiate myelination. As a result, the smallest axons no longer generate a signal within the oligodendrocyte of sufficient strength to trigger myelination. Second, a reduction in oligodendrocyte numbers such that there are no longer sufficient cells to myelinate all available axons. In this last situation, it would be expected that those axons myelinated last during development, i.e., the smallest-diameter axons (Remahl and Hildebrand, 1982), would be myelinated late or not at all during development in the mutant mouse. The former mechanism represents an intrinsic defect in the oligodendrocyte, whereas the latter is an extrinsic effect secondary to a reduction in cell numbers.

To discern which mechanism is responsible for our observations, we performed co-culture experiments in which oligodendrocytes are provided in considerable excess (thus eliminating any extrinsic effects of cell number variation) and examined myelination by oligodendrocytes derived from wild-type or dnβ1 mutant mice. These oligodendrocytes were cultured in the presence of wild-type dorsal root ganglion (DRG) neurons and their myelination efficiency was evaluated as described elsewhere (Wang et al., 2007) by determining the percentage of oligodendrocytes forming myelin. Although not the normal target of CNS oligodendrocytes, axons formed by DRG neurones grown in culture have a range of diameters of less than 2 µm (Fig. S4 a), similar to those in the optic nerve studied here, and are myelinated by oligodendrocytes added to these cultures. They therefore provide an appropriate test of the intrinsic myelogenic capacity of the oligodendrocyte. We found that dnβ1 oligodendrocytes myelinated DRG axons significantly less efficiently...
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wild-type: 45.8 ± 0.9 µm; dnβ1: 44.2 ± 0.8 µm, n = 5) or in the number of primary processes generated by each oligodendrocyte (wild-type: 4.12 ± 0.10, n = 74 from 4 experiments; dnβ1: 4.25 ± 0.12, n = 79 from 4 experiments). We conclude, therefore, that the increased axon diameter required for myelination reflects an intrinsic abnormality of axoglial signaling within the dnβ1 oligodendrocytes.

Dnβ3 mice show no evidence of altered axon diameter threshold for myelination

A concern with the use of the dominant-negative expression strategy is that the phenotype might reflect nonspecific effects of transgene expression on the cell surface. To control for this, we generated a second line of mice expressing a dominant-negative
subunit for β3 integrin (Fig. S5). β3 integrin is expressed on oligodendrocyte precursors but is not present at the myelinating stages (Milner et al., 1997). The construct used is identical to that for the dnβ1 mice except for the substitution of a β3 cytoplasmic domain for the β1 sequences. The same hprt targeting method was used to generate the dnβ3 transgenic mice, thus creating single-copy insertion into exactly the same locus as with the dnβ1 mice. This ensures comparable expression of the two coding sequences, as confirmed by immunofluorescence analysis showing the distribution and expression pattern of the dnβ3 protein to be the same as that previously seen for dnβ1 protein (Fig. S5). Hemizygous and wild-type male littermate mice were generated and compared with the dnβ1 mice. Dnβ3 mice revealed no defect in CNS myelination at P17 with no significant differences seen in the percentage of axons myelinated across all axonal intervals (dnβ3: 58.73 ± 10.54, n = 1,397 from 3 animals; wild type: 54.07 ± 10.41, n = 1,416 from 3 animals; P = 0.24) and no evidence for a lack of myelination in the smaller-diameter axons seen as for the dnβ1 mice (Fig. 8). Equally, no effect of the expression of dnβ3 on the myelination efficiency, the number of internodes, and the number of processes formed by each individual oligodendrocyte was detected in the myelinating co-culture assay. Dnβ3 oligodendrocytes generated 47 ± 1 internodes of average length 43.6 ± 0.6 µm and 4.44 ± 0.10 primary processes (n = 98 from 4 experiments), numbers not significantly different from wild-type cells (Fig. 7). We conclude that the phenotype we observed in the dnβ1 mice is not a nonspecific effect of transgene expression, but rather a specific effect of perturbing β1 integrin signaling.

Discussion

We show that oligodendrocytes expressing a single copy of a dominant-negative β1 integrin transgene myelinate small-diameter axons in optic nerve less efficiently than wild-type or control (dominant-negative β3) oligodendrocytes. This presents as a transient failure to myelinate small-diameter axons during development, demonstrated by a shift to the right of the dose–response curve created by plotting axon diameter versus the percentage myelinated as schematized in Fig. 9. A similar phenotype was seen when the downstream signaling molecule FAK was deleted in myelinating glial cells. In mice expressing the dominant-negative β1 integrin transgene, we also observed that the thickness of the myelin sheath in those axons that do become myelinated is normal, as is the length of the myelin internodes as measured in myelinating co-cultures. We conclude, therefore, that inhibition of integrin signaling delays the initiation of myelination but has no effect on the signals required for the formation of the sheath once the axoglial interactions that lead to myelination have been established. It follows, therefore, that integrins contribute to the axoglial interactions that initiate myelination.

Given the evidence from myelinating co-culture experiments revealing an intrinsic defect in oligodendrocyte myelination, two broad mechanisms can be put forward to explain our observations—perturbation of the axoglial signaling that initiates myelination or a failure of oligodendrocyte differentiation (Fig. 10). The first would be based on the observation that in the PNS an axonal signal proportional to the diameter and above a certain threshold is required to initiate myelination by the contacting glial cell (Taveggia et al., 2005; Nave and Salzer, 2006). If there is a reduction in the glial response to the axonal signal due to perturbation of integrins, then the prediction would be that the signal on the small axons will not be detected as above the required level and a reduced percentage of myelination of these small-diameter axons will be observed. In contrast, the large axons could either provide a signal that is in excess of the threshold level or express an additional signaling molecule that initiates myelination without any requirement for integrin function, and thus would not be affected by integrin perturbation. In the second mechanism, small-diameter axons could be preferentially affected by the reduction in β1 integrin signaling as a result of a compromised ability of the oligodendrocyte to change their morphology and generate multiple processes for myelination. It has been shown that toad oligodendrocytes myelinating small axons extend more processes than those myelinating large axons (Stensaas and Stensaas, 1968; Hildebrand et al., 1993).
reorganization would therefore be expected to reveal a phenotype in those tracts where more processes are required, i.e., those with small-diameter axons. In agreement with this, knockout mice for the regulator of the actin cytoskeleton WAVE1 exhibited a reduced number of myelinated axons in the optic nerve. Additionally, oligodendrocytes in rat white matter areas constituted of small axons possess more branches than those in areas with both large and small axons (Matthews and Duncan, 1971). A reduction in β1 integrin signaling that compromises process outgrowth via the established role of this receptor in cytoskeletal reorganization would therefore be expected to reveal a phenotype in those tracts where more processes are required, i.e., those with small-diameter axons. In agreement with this, knockout mice for the regulator of the actin cytoskeleton WAVE1 exhibited a reduced number of myelinated axons in the optic nerve.

Figure 7. Dnβ1 oligodendrocytes myelinate a reduced number of internodes in myelinating co-cultures. (a) Representative pictures are shown for wild-type, dnβ1, and dnβ3 MBP+ oligodendrocytes myelinating neurites within DRG neuron cultures at low (top panels) and high (bottom panels) magnification. Bars: top, 133 μm; bottom, 50 μm. (b) (left) Neurosphere-derived dnβ1 oligodendrocytes myelinate DRG neurons less efficiently than their wild-type or dnβ3 counterparts. Error bars represent SD (**, P < 0.01). The internodal number (middle) and length (right) were quantified for each oligodendrocyte (10 oligodendrocytes per experiment, n = 5). Dnβ1 oligodendrocytes showed a decrease in the internodal number per oligodendrocyte (*, P < 0.05), whereas the internodal length was unchanged for the three genotypes.

Figure 8. Dnβ3 mice show no evidence of altered axon diameter threshold for myelination. (Left) Representative electron micrographs of optic nerve sections of P17 wild-type and dnβ3 transgenic mice show no differences. Bar: 1 μm. (Right) Representative graph from one litter shows the percentage of myelinated axons with respect to axon diameter at 0.1-μm intervals for wild-type and dnβ3 mice. A polynomial trendline was adjusted to the data. The axon diameter threshold for myelination is unaffected in dnβ3 transgenic mice.
and corpus callosum, with WAVE1 knockout oligodendrocytes showing a reduced number of processes in cell culture (Kim et al., 2006).

Three observations argue against the latter mechanism—perturbation of cytoskeletal function as the cause of the phenotype in the dnβ1 mice. First, although the number of internodes myelinated by each dnβ1 oligodendrocyte in the myelinating cocultures is reduced, the length of internodes is unaltered, with the majority of internodes myelinated by wild-type, dnβ1, and dnβ3 oligodendrocytes distributed between 27 and 40 µm as recently reported for cortical oligodendrocytes in vivo (Murrie et al., 2007; Fig. S4 c). A compromised ability of the oligodendrocyte to extend processes would be expected both to reduce the number of internodes and to shorten internodal length, as this latter measurement must reflect process extension along the axon once contact has been established. Second, the similar g-ratios seen in the P17 optic nerve of wild-type and dnβ1 mice show that the cytoskeletal reorganization required for myelin sheath compaction is not affected by inhibition of integrin signaling. Third, the number of primary processes is not significantly different between wild-type, dnβ1, and dnβ3 oligodendrocytes in premyelinating co-cultures. Dnβ1 oligodendrocytes are, therefore, able to extend processes normally, as evidenced by their number of primary processes and internodal length, but then myelinate less efficiently with fewer internodes.

The conclusion that oligodendrocyte integrins form part of the recognition complex for axonal signals that determine whether or not myelination occurs is important, as the identity of such axonal signals in the CNS is unknown. In the PNS, the level of axonal type III Nrg1 has been shown to play a crucial role in determining whether an axon is myelinated and in the regulation of myelin thickness (Michailov et al., 2004; Taveggia et al., 2005). We have shown that laminin-binding integrins amplify neuregulin signaling as part of the mechanisms that regulate target-dependent survival of newly formed oligodendrocytes in the CNS (Colognato et al., 2002). Our results here would therefore be consistent with a model in which axonal neuregulins in the CNS, recognized by oligodendrocyte ErbB receptors, generate a signal to initiate myelination that is further amplified by integrins. In the case of small-diameter axons, this amplification is essential for triggering myelination, whereas large-diameter axons generate sufficient signal without integrin amplification. However, the role of neuregulins in CNS myelination has not been resolved. Exogenous addition of Nrg1 promotes myelination in co-cultures of oligodendrocytes and DRG neurons (Wang et al., 2007) and myelination of type III Nrg1-deficient DRG neurons is significantly reduced in co-culture (Taveggia et al., 2008). Hypomyelination is seen in transgenic mice expressing a dominant-negative ErbB receptor in oligodendrocytes, and in mice haploinsufficient for type III Nrg1 (Roy et al., 2007; Taveggia et al., 2008). However, in contrast to the PNS, increased expression of this axonal signal does not promote myelination of very small, and normally unmyelinated, axons in co-cultures of superior cervical ganglion neurons and oligodendrocytes (Taveggia et al., 2008). Moreover, conditional mutants with ablation of Nrg1, ErbB3, or ErbB4 exhibit no cortical myelination abnormalities (Brinkmann et al., 2008), a result that rather compellingly argues against a necessary role for Nrg1 signaling in CNS myelination. Additional neuregulin isoforms may be important in the CNS but equally, and in contrast to the PNS, other axonal signals may be required for regulating myelination. These may also be amplified by integrins, as seen for a number of different growth factors in other cell types. The hypothesis that such multi-component signaling complexes initiate and regulate myelination by oligodendrocytes provides a mechanism to explain the “catch-up” by the mutant oligodendrocytes in the older animals, as compensatory interactions within the complex will facilitate restoration of normal axoglial interactions.

Interestingly, both in monkeys and humans the area of the CNS most prone to remyelination failure is the optic nerve (Lachapelle et al., 2005). A model in which amplification of a myelination signal is required for small- but not large-diameter axon myelination could explain both the vulnerability of the optic nerve and the regional selectivity of the phenotype seen in the dnβ1 mouse, as the optic nerve contains entirely small-diameter axons. Similar regional differences in myelination are observed in other mutants that perturb integrin signaling, such as the Fyn knockout and the laminin-2–deficient (dy/dy) mouse. In these mice myelination defects are seen in optic nerve but not in spinal cord, where the density of larger axons is higher. Another possibility to explain regional heterogeneity, intrinsic differences in the oligodendrocytes arising from different regions of the developing CNS, seems less likely given that ablation of one such population is followed by replacement from a different region (Kessaris et al., 2006). In addition, transplantation of oligodendrocytes from optic nerve into spinal cord reveals that the transplanted cells can myelinate the full range of axon diameters in their new environment, even though these diameters are much greater than those present in their original location (Panarraga et al., 1998).

Although the two previous studies examining the role of β1 integrins in regulating CNS myelination appear contradictory (Benninger et al., 2006; Lee et al., 2006), the current study allows their reconciliation. Here we have concluded that inhibition of integrins perturbs axoglial signaling and delays myelination of
extracellular domain will form heterodimers with integrin-α subunits and thus compete for ligand with other oligodendroglial laminin receptors. One of these is dystroglycan (Colognato et al., 2007), for which the main binding site on laminin overlaps with that of integrins at the laminin G domains LG1-3 (Talts et al., 1999; Wizemann et al., 2003). We have shown that dystroglycan is a significant laminin receptor for CNS myelination in vitro, and transgenic studies reveal an essential role in PNS small-diameter axons, but has no effect on the subsequent formation of the myelin sheath. This conclusion is consistent with our previous report on the absence of g-ratio disturbances in the oligodendrocyte-specific conditional β1 knockout (Benninger et al., 2006), where the analysis focused on myelin sheath thickness in older animals. Hypomyelination after expression of dnβ1 integrin (Lee et al., 2006) can be explained by a requirement for dystroglycan in sheath formation. As noted in the introduction, the β1ΔC integrin extracellular domain will form heterodimers with integrin-α subunits and thus compete for ligand with other oligodendroglial laminin receptors. One of these is dystroglycan (Colognato et al., 2007), for which the main binding site on laminin overlaps with that of integrins at the laminin G domains LG1-3 (Talts et al., 1999; Wizemann et al., 2003). We have shown that dystroglycan is a significant laminin receptor for CNS myelination in vitro, and transgenic studies reveal an essential role in PNS small-diameter axons, but has no effect on the subsequent formation of the myelin sheath. This conclusion is consistent with our previous report on the absence of g-ratio disturbances in the oligodendrocyte-specific conditional β1 knockout (Benninger et al., 2006), where the analysis focused on myelin sheath thickness in older animals. Hypomyelination after expression of dnβ1 integrin (Lee et al., 2006) can be explained by a requirement for dystroglycan in sheath formation. As noted in the introduction, the β1ΔC integrin extracellular domain will form heterodimers with integrin-α subunits and thus compete for ligand with other oligodendroglial laminin receptors. One of these is dystroglycan (Colognato et al., 2007), for which the main binding site on laminin overlaps with that of integrins at the laminin G domains LG1-3 (Talts et al., 1999; Wizemann et al., 2003). We have shown that dystroglycan is a significant laminin receptor for CNS myelination in vitro, and transgenic studies reveal an essential role in PNS
myelination (Saito et al., 2003; Occhi et al., 2005; Colognato et al., 2007). We propose, therefore, that the study of Lee et al. (2006) expressing B1aC reveals a requirement for dystroglycan in later stages of myelin sheath formation in addition to the role of integrins in the earlier stages of initiation. Our present and previous studies, by contrast, specifically target integrin signaling and would thus not reveal any later role of other laminin receptors.

The identification of the signals regulating the initiation of myelination may lead to the development of strategies to promote effective remyelination by oligodendrocytes within MS lesions arrested at the premyelinating stage and therefore unable to contribute to repair (Chang et al., 2002). Further studies examining the partners and ligands of the integrins expressed on oligodendrocytes represent promising new approaches toward understanding this goal.

Materials and methods

Cloning and dnβ1/dnβ3 ES cell generation

For the generation of the genetically modified embryonic stem (ES) cells expressing the dnβ1/dnβ3 integrin we used the hypoxanthine phosphoribosyl transferase (hprt) targeting system (Farhadi et al., 2003). This system relies on the use of an hprt-deficient ES cell line (Bronson et al., 1996; Farhadi et al., 2003) and on the cloning of the desired sequences onto a vector containing the deleted hprt sequences as well as the hprt homologous arms required for recombination.

The dominant-negative sequences were initially cloned into a Gateway entry vector containing the 9.5-kb Bmp promoter (pENTR1A9.5). The I2R2β1 integrin sequence was available (Relvas et al., 2001) cloned as an Xhol fragment into Xhol of pLUXN vector (Clontech Laboratories, Inc.). We subsequently subcloned I2R2β1 as an Xhol-NotI fragment from pLUXN vector into pENTR1A9.5 linearized with Sall-Clal. The I2R2β3 integrin sequence was also available cloned as an Xhol fragment into Xhol of pLUXN vector (Clontech Laboratories, Inc.). We subsequently subcloned I2R2β3 as an Xhol-HindIII-NotI fragment from pLUXN vector into EcoRV-HindIII fragment of pLUXN vector. The I2R2β3 was then excised as an Xhol-ClaI fragment from pLUXN vector and cloned into pENTR1A9.5 linearized with Sall-Clal. The clones were all confirmed by an extensive series of restriction digests and the final clones in pENTR1A9.5 were also confirmed by automatic sequencing using the primers pE9.5bs 5′-aagccccgtttgtaagccc-3′, pE9.5acs 5′-gatggctgtccatagctgtaa-3′, and pE9.5mrbs 5′-tcctcggagggatcc-3′. Transgenes cloned into pENTR1A9.5 vector were subsequently transferred to the hprt targeting destination vector, in vitro, using the Gateway technology (Invitrogen). Both transgenes were docked, in single copy and fixed orientation, in the 5′-flanking sequence of the hprt locus on the X chromosome. BpES5 ES cells (Denarier et al., 2005), bearing the same hprt deletion as the original BK4 ES cells (Bronson et al., 1996), were electroporated with 40 µl of linearized DNA and selected on HAT-supplemented medium as described previously (Farhadi et al., 2003). The HAT+ ES cell clones were screened by PCR using the primers E12R2β3lf 5′-caggtgcttcatcagtga-3′ and pE9.5acs.

Generation of dominant-negative β1/β3 integrin transgenic mice

The genetically altered agouti ES cells were subsequently injected into C57BL/6-derived blastocysts that were then transplanted into the uteri of recipient females. Resulting chimeric mice were bred with C57BL/6 females, and the F1 female agouti offspring were backcrossed with C57BL/6 females. Genotyping was performed by PCR with a set of primers for the wild-type (Phprt mts 5′-ggagggatagctgaagttg-3′ and Pfrhpt mts 5′-ctcggagggatagctgaagttg-3′) and a set of primers for the transgenic (ES12R2β1lf and pE9.5acs) alleles. When further confirmation of sex determination was required, especially at young ages, PCR for the sex-determining region Y (Sry) gene was additionally performed. Mutant homozygous females and hemizygous males of the two dominant-negative lines were viable and showed no obvious behavioral abnormalities.

FAK knockout mice

The conditional inactivation of FAK in myelinating cells by crossing transgenic mice with lac sites inserted into the FAK gene (McLean et al., 2004; Charlesworth et al., 2006) with transgenic mice expressing cre recombinase under the regulation of the CNP promoter (a gift of Dr Klaus Nave, Gottingen, Germany) has been described elsewhere (Grove et al., 2007).

Mylinating co-cultures

Dorsal root ganglia (DRGs) were dissected from E14–E16 rats and digested for 45 min at 37°C with 1.2 U/ml pepsin (Worthington), 0.24 mg/ml cysteine (Sigma-Aldrich), and 40 µg/ml Dnase I (Sigma-Aldrich). The dissociated cells were plated onto 22-mm coverslips precoated with poly-l-lysine (10 µg/ml; Sigma-Aldrich) followed by matrigel (1:20 dilution; BD Biosciences) at a density of 5 × 10^4 cells/ml. The DRGs were grown for two weeks in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and nerve growth factor (NGF, 100 ng/ml; AbD Serox). The cultures were pulsed three times, for 2 d at each time, with fluorodeoxyuridine (10 µM; Sigma-Aldrich) to remove contaminating fibroblasts and glial cells. Neuronspheres from wild-type or mutant litters were added to each coverslip with purified DRGs. The medium used for the co-cultures was 50:50 DMEM/Neurobasal medium (Invitrogen) supplemented with Sato and B27 (Invitrogen), NGF (100 ng/ml), N-acetyl cysteine (5 µg/ml, Sigma-Aldrich), and α-tubulin (10 ng/ml). Cocultures were maintained for 3 wk with medium and supplements changed every 3–4 d. After 3 wk, the extent of myelination was quantified by determining the slope of the best-fit line defining the relationship between the percentage of oligodendrocytes (defined by the expression of MBP as detected by immunohistochemistry) with myelin sheaths and the density of the underlying neurite network (Wang et al., 2007). The number and length of internodes was traced and measured using ImageJ software. The average number of primary processes (defined as those originating directly from the cell body) was quantified in MBP+ oligodendrocytes at the initial stages of myelination, before internode formation, using OpenLab image analysis software (Improvision).

RT-PCR

Total RNA was extracted from mouse tissues using the RNeasy mini kit (QIAGEN), according to the manufacturer’s instructions, and subjected to one-step RT-PCR (QIAGEN). The following primers were used for the extracellular β1 integrin domain: B1ecf 5′-ggagacagtctcgcttggaa-3′, B1ecf 5′-gtgccacgtctgtgggca-3′; and for the intracellular domain: IL2Rα 5′-cctggaggaaggtc-3′, IL2RaR 5′-cagggagggagatcc-3′. Data were normalized to β-actin levels (5′-agcctgctgctcagtc-3′ and 5′-ctcagctgctggtgga-3′). ImageJ software was used to quantify mRNA expression levels.

Western blot

Dissected tissues from dnβ1 and wildtype mice were homogenized in 1% SDS and subjected to SDS-PAGE using appropriate percentage of acrylamide minigels. They were then transferred onto 0.45-µm nitrocellulose membranes (HybondC; GE Healthcare) and blocked with 5% nonfat dry milk and 0.1% Tween 20 Tris-buffered saline (TBS) for 1 h at room temperature (RT). The primary antibody diluted in blocking solution was incubated for 2 h at RT or overnight (ON) at 4°C. The blots were then washed three times in TBS 0.1% Tween (TBS-T) and incubated for 1 h at RT with a horseshad peroxidase-labeled secondary antibody (GE Healthcare). After washing again in TBS-T the immunoreactive proteins were revealed by chemiluminescence (ECL; GE Healthcare) according to the manufacturer’s instructions. Digitalized images were obtained and bands quantified using ImageJ software.

Immunofluorescence and image acquisition

When staining for MBP on tissue sections, a postfixation step prior to staining was performed with 95% ethanol and 5% acetic acid for 30 min at −20°C, followed by three rinses in PBS. Sections were then blocked for 1 h in PBS containing 10% normal goat serum (NGS) and 0.1% Triton X-100 at RT, after which the primary antibody incubation was performed either ON at 4°C or for 2 h at RT. The following primary antibodies were used for immunofluorescence or Western blot: rat anti-MBP (AbD Serotec), mouse anti-nerfilament goat anti-Sigma-Aldrich, rabbit polyclonal anti-FAK (Y397; Invitrogen), rabbit polyclonal anti-FAK (Invitrogen), and monoclonal mouse anti-human CD25/interleukin-2 receptor (Dako). After washing in PBS, the sections were incubated for 1 h with the secondary antibody (FITC or Alexa 588 and TRITC or Alexa 488 labeled) and Hoechst. After further washes in PBS, slides were mounted in fluorescent mounting (SouthernBiotech). All fluorescence images were acquired at RT with a fluorescence microscope (Axioskop; Carl Zeiss, Inc.), fitted with 10x eyepiece magnification, 20x (0.5 NA) and 40x (0.75 NA) objectives, and a digital camera (model C4742-95; Hamamatsu) using OpenLab image analysis software (Improvision).
Electron microscopy

Hemizygous and wild-type males were used as mutant and control mice, respectively. Anaesthetized mice were perfused intracardially with freshly prepared 4% glutaraldehyde containing 2 mmol/l CaCl₂ in 0.1 M phosphate buffer (pH) at RT. Dissected tissues were incubated in the same solution ON. After rinsing in 0.1 M PB, samples were post-fixed in 1% osmium tetroxide ON and dehydrated in a grade series of ethanol. The tissues were embedded in TAAB resin, and sections for light and electron microscopy were prepared using a microtome (Ultratcut UCT; Leica). Semi-thin sections were stained with methylene blue. Ultra-thin sections were stained with saturated uranyl acetate in 50% ethanol and lead citrate and examined in a transmission electron microscope (model CM100; Philips).

Morphometry

Digitalized images were obtained from corresponding levels of the various mouse tissues. The g-ratio was calculated by the ratio between the area of the axon and the area of the same axon including myelin using OpenLab image analysis software (Improvision). A minimum of 100 randomly chosen axons, from at least 5 nonoverlapping images per animal, were analyzed for calculating the g-ratio. A minimum of 400 axons from at least 3 nonoverlapping images per animal were analyzed to calculate the percentage of myelination per axon diameter.

Statistical analysis

The data show the mean ± SEM, unless otherwise indicated. Statistical significance for the expression of the dn integrin by RTPCR and changes in pFAK by Western blot was determined by using paired t test; for evaluation of myelination efficiency in co-culture by repeated measures ANOVA with Tukey’s multiple comparison posttest; and for percentage of myelination per axon diameter by one-way ANOVA with Tukey’s multiple comparison posttest. n represents the number of axons examined for each control and mutant littermate, with at least three independent litters of transgenic mice analyzed unless otherwise indicated or, in the case of the co-cultures, the number of independent experiments performed. Statistical analysis was performed using GraphPad Prism software.

Online supplemental material

Figure S1 shows the expression pattern in the brain and optic nerve of the 9.5-Kb MBP promoter used for the dominant-negative integrins. This promoter is expressed only at the time of myelination in different white matter tracts. Figure S2 shows that the morphology of corpus callosum and optic nerve in the dn11 mice is normal, as assessed by immunohistochemistry. Figure S3 shows that the threshold axon diameter for myelination is normal in adult dn1 and FAK KO mice. Figure S4 provides further quantification of the myelinating cocultures, showing the distribution of axon diameters and that oligodendrocyte differentiation and internodal length are similar in cultures using cells from wt, dn11, and dn3 mice. Figure S5 illustrates the steps taken to generate and characterize the dn3 transgenic line used as a control in our study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200807010/DC1.

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