Axonal Neuregulin-1 Regulates Myelin Sheath Thickness

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In the nervous system of vertebrates, myelination is essential for rapid and accurate impulse conduction. Myelin thickness depends on axon fiber size. We use mutant and transgenic mouse lines to show that axonal Neuregulin-1 (Nrg1) signals information about axon size to Schwann cells. Reduced Nrg1 expression causes hypomyelination and reduced nerve conduction velocity. Neuronal overexpression of Nrg1 induces hypermyelination and demonstrates that Nrg1 type III is the responsible isoform. We suggest a model by which myelin-forming Schwann cells integrate axonal Nrg1 signals as a biochemical measure of axon size.

Myelination requires axonal signals. Millisecond precision is an important aspect of nervous system function (1). Myelin sheath thickness, which is one determinant of nerve conduction velocity (2), must thus be carefully controlled in development. Nearly 100 years ago, it was observed that the axonal ensheathment is related to axon size (3). Myelin thickness is proportional to the diameter of the axon and the internodal length, with only minor species-specific and nerve-specific differences in this ratio (4–8). Axon caliber also appears to be a critical determinant of myelination for small axons (9). Thus, Schwann cells detect the diameter of the axonal segment that they engulf. This correlation is established during initial development: Peripheral axons with a secondarily reduced diameter have unchanged myelin thickness (10).

We have hypothesized that interactions between axonal ligands and glial receptors, such as neuregulins (Nrg) and ErbB receptor tyrosine kinases (11–14), can integrate cell surface signals and provide an indirect biochemical measure of the axon caliber. The Nrg1 gene encodes more than 15 transmembrane and secreted protein isoforms, generated by alternative promoter usage and mRNA splicing. Nrg1 subtypes I through III share the epidermal growth factor–like signaling domain and are defined by different amino-termini (15). The N-terminal cystein-rich domain (CRD), as found in the sensory and motor neuron-derived factor (SMDF), defines Nrg1 type III, which in embryonic development is responsible for survival of Schwann cell precursors (16–21). No such function has been demonstrated for Nrg1 type I (Heregulin (HRG) or acetylcholine receptor–inducing activity (ARIA)) or Nrg1 type II (glial growth factor).

Neuregulin-1 and ErbB receptors in mature peripheral nerves. Expression of Nrg1 and the ErbB2 and ErbB3 genes is maintained in the sciatic nerve of adult mice. Nrg1 was immunodetectable (22) in axons (Fig. 1A) and detected by in situ hybridization in the dorsal root ganglia (DRG) and in spinal cord motoneurons (23, 24). Similarly, ErbB2 was immunostained in mature Schwann cells and was associated with myelin (Fig. 1B). Moreover, by using polymerase chain reaction (RT-PCR) analysis in mature axons, we found that Nrg1 is selectively expressed in sciatic nerves of wild-type mice (25). In addition, we used Western blot analysis to confirm expression of Nrg1 in total protein lysates from sciatic nerves of wt animals and mice in which reduced gene dosage. (Top) Reduced amount of ErbB2 is obvious in Nrg1+/ErbB2 double heterozygotes (N1+/−E2+/−) and in ErbB2 single heterozygotes (E2+/−), but not in Nrg1 heterozygous (N1+/−) mice (age P2). A similar reduction is shown for Nrg1 in sciatic nerves of N1+/−E2+/− and N1−/− (but not in E2+/−), analyzed at age P10 (middle) and in the adult (bottom). Probing blots for β-tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a loading control.
double heterozygotes and controls. Staining of teased-fiber preparations with a Nrg1 type III–specific antibody revealed that the axonal ligand was uniformly distributed along the internode (Fig. 1, C and D).

If Schwann cells obtained information concerning axon size from the steady-state amount of (axon-to-glia) Nrg1 signaling, an experimental reduction in either ligand or receptor number should be perceived by Schwann cells as an apparent “decrease” in axonal caliber. The anticipated reduced amount of myelination would be reflected in an increase in the g ratio of the fiber (the numerical ratio between the diameter of the axon proper and the outer diameter of the myelinated fiber). To test this hypothesis, we generated mice with reduced Nrg1, ErbB2, and/or ErbB3 gene dosages, obtained by gene targeting (22), and produced compound heterozygotes. Whereas the null mutants of these genes die before myelination (15, 16), all compound heterozygotes were fully viable and exhibited normal motor behavior. Peripheral nerves were myelinated and populated with a normal density of Schwann cells (24). Sciatic nerves analyzed at different ages (postnatal day 2 (P2), P10, and 6 months) showed steady-state amounts of Nrg1 and glial ErbB2 reduced to half of wild-type amounts (Fig. 1E).

Reduced neuregulin-1/erbB signaling limits myelination. The degree of peripheral myelination was also altered. We determined the thickness of sciatic nerve axons and their myelin sheaths for wild-type mice and Nrg1*ErbB2 compound heterozygotes with the use of light microscopy and digital imaging (22) of 0.5-μm semithin cross sections (Fig. 2A). The g ratios were calculated from no fewer than 100 myelinated axons per mouse and genotype (n = 6). In wild-type mice, the average g ratio was 0.68 (±0.032) at P10 and 0.66 (±0.013) at 6 months of age, in agreement with published data (5). In contrast, the myelin of compound heterozygotes (Nrg1+/− ×ErbB2+/−) was significantly thinner (P < 0.001), both at age P10 (g = 0.81) and at 6 months (g = 0.79) (fig. S1). The internodal length of sciatic nerve axons was 0.61 ± 0.10 mm in wild-type and 0.64 ± 0.15 in double heterozygous mice (fig. S2), respectively, and the axonal size distribution was also unchanged (Fig. 2, B and C).

All compound heterozygotes were behaviorally normal, but nerve conduction velocity (NCV) was reduced. With the use of the tail nerve of double heterozygotes (n = 4), we measured a motor NCV of only 26 ± 1.34 m/s (22), in contrast to 34 ± 0.65 m/s in wild-type mice (fig. S3). Current amplitudes were not reduced, and muscle compound action potentials exhibited a normal profile (24), bearing no spontaneous electromyogram signals that would be indicative of muscle denervation (25).

To determine whether the axonal ligand or the glial receptor was the limiting parameter of myelin thickness, we also analyzed adult mice heterozygous for only the Nrg1 or the ErbB2 null allele. Reducing Nrg1 gene dosage was sufficient to cause the same phenotype as observed in double heterozygotes and even in Nrg1*ErbB2*/ErbB3 triple heterozygotes (Fig. 3A and fig. S4). No decrease in myelin thickness was observed in the ErbB2+/− mice. Thus, under normal conditions, axonal Nrg1 expression appears to serve as a rate-limiting factor for myelination, whereas Schwann cells possess saturating amounts of ErbB receptors. For Nrg1−/− mice, the reduction in myelin thickness was more pronounced for small than for large caliber axons (Fig. 3B) but the axonal size distribution did not differ (Fig. 3C).

Neuregulin-1 overexpression increases myelin thickness. It is possible that the reduction in myelin thickness in Nrg1+/− mice could be caused by a minor delay in Schwann cell maturation. Moreover, because some Schwann cells express Nrg1 (26), its reduction could contribute to hypomyelination. Both hypotheses were tested by cell-specific expression of elevated Nrg1 amounts in axons, beginning in postnatal development. Nrg1 overexpression should mimic an enlarged axonal size from the perspective of the Schwann cells. We generated transgenic mice (22) expressing different Nrg1 isoforms under control of the murine Thy 1.2 promoter, which is active in postnatal motoneurons and DRG neurons (27), i.e., when most Schwann cells have reached the promyelination stage (28). Transgene constructs (Fig. 4A) were derived from murine cDNAs encoding either Nrg1 type I (as present in ARIA/HRG) or Nrg1 type III with an SMDF/CRD domain [we chose the β1a isoforms for each (11)]. Microinjection of fertilized mouse oocytes resulted in five transgenic founders, three of which developed mild tremors. Because these mice proved difficult to breed, we included the founders in the analyses at 5 months of age.

We obtained sciatic nerves from three individual Nrg1 type III transgenic founders for biochemical and microscopic analyses. In all transgenics, the overexpression of Nrg1 type III was evident by Western blot analysis when compared with wild-type littermates (Fig. 4B). Morphologically, the peripheral myelin of the adult transgenic mice was thicker than that of controls (Fig. 4C), with some fibers having g ratios below 0.4 (lowest was 0.28), a level never observed in wild-type mice. Although the Thy-1.2 promoter is expressed in a mosaic fashion in transgenic mice (27), fiber g ratios were significantly lower in Nrg1 type I transgenics (g ratio of 0.53 ± 0.014) than in nontransgenic littermates (g ratio of 0.65 ± 0.014; P < 0.001) (fig. S5). Sometimes, normally myelinated axons were in direct proximity to hypermyelinated axons (Fig. 5A), suggesting that
these differences reflect mosaic Thy1.2-Nrg1 type III transgene expression. The ultrastructure (22) and periodicity of the hypermyelinated fibers was normal (Fig. 5B), demonstrating that the increase in myelin thickness was the result of an increase in the number of wrappings. Longitudinal sections revealed that hypermyelination was uniform along the axon and morphologically unrelated to tomacula (24).

Neuregulin-1 type III and myelin growth. The growth-promoting effect on Schwann cell myelin appeared specific for the Nrg1 type III isoform, because transgenic founder mice overexpressing Nrg1 type I exhibited normal g ratios of sciatic nerve fibers (Fig. 4C). Nevertheless, the latter revealed differences in CNS myelin (29), suggesting a functional Nrg1 type I transgene expression also in these lines. Adult heterozygous mice with reduced Nrg1 type III gene dosage (21) also possessed abnormally thin myelin, with g ratios of 0.77 ± 0.014 (Fig. 4C and Fig. S5), similar to those in mice heterozygous for the Nrg1 null allele. Because the type III–specific knock-out mouse leaves the expression of all other Nrg1 isoforms intact, it is apparent that Nrg1 type I and type II are both unable to functionally compensate for the loss of type III. Although we cannot formally rule out the participation of additional axonal signals and growth factors (30), Nrg1 type III emerges as a critical regulator of Schwann cell myelin thickness.

Hypermyelination (reduced g ratio) could be mimicked by pathological shrinkage of the axon caliber without altering myelin (31). We therefore determined the size distribution of axons in comparable cross sections prepared from adult sciatic nerve (Fig. 5C). This morphometric analysis failed to detect meaningful size differences between Nrg1 transgenic mice and age-matched controls (Mann-Whitney U test, P = 0.917).

It was also important to rule out the possibilities that Nrg1 overexpression had changed the absolute number of axons and/or Schwann cells and that hypermyelination was merely the result of a reduced axon:glia ratio. Histology and immunohistochemistry of comparable nerve cross sections revealed no difference in the number of Schwann cell nuclei (240 ± 8 for wild type and 237 ± 23 for Nrg1 type III transgenic; n = 3) or the number of sciatic nerve axons (3957 ± 19 for wild type and 3883 ± 138 for Nrg1 type III transgenic; n = 3) (figs. S6 and S7).

Lastly, we calculated the g ratios of sciatic nerve fibers from Nrg1-overexpressing mice, plotted as a function of their respective axonal diameters (Fig. 5D). This revealed that hypermyelination was more pronounced for smaller axons than for larger axons. A possible explanation is that a linear increase of axon diameter requires an exponential increase of myelin membrane synthesis (if g ratios are maintained constant). Thus, axon size–dependent myelination may well require an exponential increase of neuronal Nrg1 gene expression as a function of the overall axonal dimension. This regulation is likely not provided for a transgene with the Thy-1.2 promoter.
Schwann cells differentiation depends on the phosphatidyl-inositol-3-phosphate (PI3) kinase and Akt pathway downstream of activated ErbB2/3 receptors (36, 37). In our working model, phosphorylated lipids generated by PI3 kinase at the adaxonal membrane diffuse through myelin membranes to reach the Schwann cell soma. Alternatively, signaling intermediates could travel to the perinuclear cytoplasm through Schmitt-Lantermann incisures or radial gap junctions (38).

An important future goal is to understand how neurons regulate Nrg1 expression as a function of their own axonal dimension. Also other axonal proteins, including sodium and potassium channels (1), require axon size- and length-dependent gene regulation. It is intriguing that the Nrg1 C-terminal domain can be proteolytically cleaved to become a signaling molecule itself (39). Whether this cleavage contributes to a back-propagation of axonal size information remains to be determined.

References and Notes
22. Materials and methods are available as supporting material on Science Online.
24. G. V. Michaliov et al., unpublished data.
29. B. G. Brinkmann et al., unpublished data.
33. J. Salzer, personal communication.
40. We thank E. Nickisch and A. Fahrnenholz for excellent technical help, J. Archelos (Graz) for providing us with P0 antibodies, S. S. Scherer (Pennsylvania) for advice on teased-fiber preparations, J. Salzer (New York) for helpful comments on the manuscript, and members of the Nave lab for discussion. This work was supported by the Deutsche Forschungsgemeinschaft (SFB523), the Hertie Institute of Multiple Sclerosis Research, and by grants of the European Union (to K.-A.N.). M.W.S. was supported in part by the Deutsche Forschungsgemeinschaft.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1095862/DC1
Materials and Methods
Figs. S1 to S7.
References and Notes
21 January 2004; accepted 8 March 2004.
Published online 25 March 2004, 10.1126/science.1095862.