Thin myelin sheaths as the hallmark of remyelination persist over time and preserve axon function

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The presence of thin myelin sheaths in the adult CNS is recognized as a marker of remyelination, although the reason there is not a recovery from demyelination to normal myelin sheath thickness remains unknown. Remyelination is the default pathway after myelin loss in all mammalian species, in both naturally occurring and experimental disease. However, there remains uncertainty about whether these thin sheaths thicken with time and whether they remain viable for extended periods. We provide two lines of evidence here that thin myelin sheaths may persist indefinitely in long-lived animal models. In the first, we have followed thin myelin sheaths in a model of delayed myelination during a period of 13 years that we propose results in the same myelin sheath deficiencies as seen in remyelination; that is, thin myelin sheaths and short internodes. We show that the myelin sheaths remain thin and stable on many axons throughout this period with no detrimental effects on axons. In a second model system, in which there is widespread demyelination through this period with no detrimental effects on axons. In a second model system, in which there is widespread demyelination of the spinal cord and optic nerves, we also show that thinly remyelinated axons with short internodes persist for over the course of 2 years. These studies confirm the persistence and longevity of thin myelin sheaths and the importance of remyelination to the long-term health and function of the CNS.

Significance

The hallmark of remyelination in the CNS has been proposed to be the presence of thin myelin sheaths. This has been demonstrated in multiple experimental models and in multiple sclerosis. It is the only surrogate marker of remyelination, and therefore a crucial fingerprint of myelin repair. However, this has been challenged recently in separate studies, in which, by implication, the degree or presence of remyelinated axons has been underestimated. In this article, we provide evidence from two different models that thin myelin sheaths and short internodes persist almost indefinitely. Future attempts to promote myelin repair in models of multiple sclerosis will be crucially dependent on a definitive marker of repair. We propose that the thin myelin sheath remains the gold standard.

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sheaths to their normal thickness (31)? These are difficult questions to answer in MS, where it is impossible to determine how long axons have been remyelinated. In experimental rodent animal models, their short lifespan limits the length of time remyelinated axons can be followed. In an attempt to answer some of these questions, we studied two experimental models. In the first, for more than a decade, we followed the fate of thinly myelinated axons in the spinal cord of a canine mutant, resulting from a developmental delay in myelination, which we propose matches the changes seen in remyelinated fibers. Second, to test whether thinly remyelinated sheaths persist over time after demyelination, we turned to a model in which this was studied in animals over the course of 2 y after myelin repair.

Results
Genetic Disorder. The disorder presented here is a unique developmental disorder of myelination of the superficial tracts of the cervical and thoracic spinal cord caused by a mutation in the FNIP2 gene (32). At 2 wk of age, affected dogs have a severe tremor, resulting in difficulty in ambulation. We studied the thoracic spinal cord from a 2-wk-old affected dog and demonstrated a zone of hypomyelination of the superficial tracts of the lateral and ventral columns with almost a total lack of myelin (Fig. 1 C and D) compared with the same tracts in controls that were completely myelinated (Fig. 1 A and B). The dorsolateral column appeared most deficient in myelin. On higher magnification of this area, the myelin deficiency was more obvious despite the presence of many glial cells and intact axons (Fig. 1D). Importantly, however, some larger-diameter axons in this zone were myelinated normally (Fig. 1D, Inset). In contrast, in both of the affected 13-y-old dogs, these areas appeared completely myelinated, but the outstanding feature was the presence of many thinly myelinated axons of all axonal calibers in the lateral and ventral columns of the cervical and thoracic spinal cord (Fig. 2 and Fig. S1). These thinly myelinated axons were seen predominantly in the subpial zones of the lateral and ventral columns, the site of original myelination delay (Fig. 1). This was also seen at both levels of the cord in the second dog, although less obviously than in the first case (Fig. S1). There were no other abnormalities in any of the spinal cord sections evaluated. There was no evidence of demyelination or myelin debris that would suggest myelin was not stable. Myelinated fiber density appeared identical to controls, and although rare degenerating axons were seen, this was similar to controls. There was no evidence of abnormal axoplasm, as we have previously identified in 1-μm sections in another canine myelin mutant, in which a wide array of axonal changes was documented (33). In addition, no swollen axons or spheroids were present in either case in the cervical and thoracic spinal cord.

Fig. 3 shows the distribution of g ratio measurements within each image, by subject and ventral column (VC) or lateral column (LC) locations. The number of axons measured at each position ranged from 249 to 505, and mean g ratio ranged from 0.62 to 0.84, with the variability having an SD of 0.07–0.10 (Table S1). We tested the difference between mean affected and WT g ratio adjusting for VC or LC location, using random effects for the subject and 1 μm image factors, to account for potentially higher correlation of measurements for axons within the same subject and/or the same 1-μm image (Table S2). g ratios measured in affected subjects were, on average, 0.126 (SE = 0.027; P < 0.0001) higher than in WT subjects. There was no difference in the g ratio between VC and LC locations of the cord.

To determine whether these abnormally thin myelin sheaths also had short internodes, a hallmark of remyelination, tissue from the VC of the cervical and thoracic cord of the male dog was embedded for longitudinal sectioning. These sections enabled visualization of myelinated axons from the subpial surface to those in the more medial gray matter interface. Thinned myelinated short internodes were only seen in the subpial area and not more medially, as would be expected from the transverse sections (Fig. 2). In addition, some axons had adjacent, normal-thickness/thin myelin paranodes (Fig. 2J). This confirmed that this is a primary glial defect and not an abnormality of axons. Not all thinly myelinated internodes were short, however, and on occasion, they were more than 100 μm. It was also noted that nodal gaps were greater than normal at many nodes of the thinly myelinated axons (Fig. 4). In the more medial, normally myelinated areas, nodes of Ranvier with normal nodal gaps were seen on large-, medium-, and small-caliber axons, but it was impossible to measure internode length on these axons because of variation in fiber plane. However, those internodes that could be followed over some length were always longer than the thinly myelinated internodes seen in the subpial zone.

Fig. 1. Myelin deficiency in the subpial zone of the ventral and lateral tracts of the spinal cord in the mutant. Hemisections of the thoracic spinal cord from a 2-wk-old control dog (A and B) and age-matched affected dog (C and D). In the control, the spinal cord appears completely myelinated, and a higher-power image of the dorsolateral column (arrows) in B confirms this. In contrast, in the affected dog, there is a clear, pale subpial zone (C) that on higher power shows a marked myelin deficiency compared with the medial white matter (D). However, occasional large-diameter axons (arrow) are myelinated in this area (D, Inset). [Scale bars: 0.2 mm (A and C), 20 μm (B and D), and 10 μm (D, Inset).]
We have previously shown that irradiation of food at more than 30 Kgray results in a widespread and severe demyelinating and remyelinating disorder in cats (1). Here we studied this phenomenon in the spinal cord of three cats that were examined over the course of 16 mo to 2 y after neurologic recovery, and found thinly myelinated axons interspersed between mature myelin sheaths (Fig. 5A). These appeared to be stable lesions, as there was no myelin vacuolation and no foamy, lipid-filled macrophages, which are seen during and shortly after demyelination and remyelination. Longitudinal sections of the spinal cord showed

Fig. 2. In the aged mutant, hypomyelination persists in the affected tracts, and internodes can be short. In the control dog, the spinal cord is normally myelinated (A), and this is confirmed on higher-power examination of subpial areas in the LCs (B) and VCs (C). The g ratio of myelinated axons in these areas was measured at two sites and the ratio noted A. In the first affected dog, the cord appears completely myelinated (D), yet on higher power, there are many thin myelin sheaths of all axon diameters in both the LC (E) and VC (F) in the subpial zone. This was confirmed by the g ratio measurements from these sites (D). High power of areas (*) in the control (G) and affected dog (H) show that these areas have a mix of normally myelinated (thick myelin sheaths) and hypomyelinated axons, as reflected by the g ratio range. (J) A short internode with a thin myelin sheath is seen between thicker internodes. (L) In the ventral column, a 7.6-μm-diameter axon has two short internodes, as marked by arrows. The node of Ranvier on the right has a paranode with a normal myelin sheath adjacent to the thin, short internode (g ratio, 0.83) (A). Arrows mark nodes of Ranvier. Internode lengths are detailed. [Scale bars: 0.5 mm (A and D), and 20 μm (B, C, E–J).]
Widened nodal gaps seen on affected fibers. Although normal nodes
Measurements of g ratios in the dorsolateral columns and VCs confirms
myelination, similar to what occurs in the Weimaraner

Given that neurologic function was restored in the affected dogs by 4–6 mo of age, and this is correlated with the myelination of the spinal cord superficial tracts (32), it can be concluded that the thin myelin sheaths restored conduction. It has been known for some time that endogenous remyelination of CNS axons restores secure conduction from previous conduction block of axons demyelinated by lysolecithin injection (11). Despite the neurologic normality and likely normal conduction in the thinly myelinated axons, we saw numerous cases of nodal gap widening on the thinly myelinated axons that may also result in conduction abnormalities. Nodal gap widening is common and a nonspecific finding in many neurologic disorders (35). Despite these findings of thin myelin sheaths and nodal widening, they appear not to have any negative effect on neurologic function.

Thus, although we propose that the thin myelin sheaths that persist in this mutant are the same as those seen after remyelination of the adult CNS, the question remains, as with remyelination: Why do these myelin sheaths remain thin? Franklin and Hinks (36) proposed that thin myelin sheaths in remyelination result from Ols unsheathing mature axons of mature diameter and length. In contrast in normal development, early myelin internodes adapt to changes in axon caliber and length by becoming thicker in diameter, and longer. We propose that the Franklin and Hinks hypothesis applies in the developmental delay in myelination in these mutants. Myelin sheath thickness in the peripheral nervous system is controlled by neuregulin III signaling (37), whereas in the CNS, ERK1/ERK2 signaling, as found in both loss- and gain-of-function experiments, decreases or increases myelin sheath thickness, respectively (38, 39). The model proposed by Franklin and Hinks (36) to explain why remyelinated sheaths are thin could equally apply to the late-stage developmental myelination documented here. As oligodendrocytes are myelinating (or remyelinating) axons that are close to their maximum diameter and length, there is no need to undergo remodeling that is required in the early developing CNS. That is, the original association of the OL process with an axon is when the axon reaches a critical diameter, although it is still to undergo increases in its diameter and length. Indeed, Franklin and Hinks (36) suggest that remyelination in the adult CNS resulting from oligodendrocytes derived from adult progenitor cells represents “delayed” myelination, similar to what occurs in the Weimaraner mutant. The similarities between myelination and repair have led to the supposition that remyelination is simply a recapitulation of development, and although there are differences, these may be redundant (40). This has been confirmed recently in an investigation of the key transcription factor, myelin regulatory
factor in experimental remyelination after lysolecithin-induced demyelination of the mouse corpus callosum, and in MS lesions (41). Myelin regulatory factor expression, which was essential for remyelination in both, broadly recapitulated the OL expression seen during development (41).

Although we propose that the thin myelin sheaths result from delayed myelination of the subpial axons, it could be suggested that the causative mutation in FNIP2 has a negative effect on myelination in addition to the proposed effect on migration and differentiation of OLs and their progenitors (32). However, we would argue against this, as some OLs that are found early (2 wk) in the subpial zone are able to myelinate axons normally (Fig. 1D), leading to the presence of scattered, thickly myelinated axons with a normal g ratio at 13 y, interspersed with the late-mytelinated thin sheaths (Fig. 1H). There is no published evidence to indicate there is heterogeneity of OLs in the subpial zone of the spinal cord that could result in a difference in myelinating capacity of a subset of OLs in mutations of FNIP2.

The persistence of thin, remyelinated sheaths after demyelination was also clearly seen in the FIDID model. In all three cases in which tissue was collected over the course of 1–2 y after neurologic recovery, scattered thin myelin sheaths were seen in the spinal cord VCs and LCs, with these fibers being interspersed between mature myelin sheaths. This pattern of fiber mixing is seen during acute disease; that is, scattered myelinated axons (with mature myelin sheaths) escape demyelination and persist adjacent to demyelinated and remyelinated axons. In the three recovered cats recorded here, however, there was no evidence of active myelin breakdown, demyelinated axons, or macrophages; that is, these were nonactive areas of myelin damage and repair. The persistence of thin myelinated sheaths and short internodes confirms that these abnormalities had remained for more than 2 y. In one cat, this was even more dramatic in the optic nerves, where over 95% of axons had thin myelin sheaths with only occasional residual mature sheaths, primarily on large axons. The more extensive remyelination seen in the optic nerves reflects the global demyelination in the nerve compared with the spinal cord.

Are there alternative explanations for the finding of thinly myelinated axons with short internodes in the mature CNS? The concept that myelin sheaths remain static throughout life has been challenged by recent studies on oligodendrocyte dynamics in the normal adult CNS, which showed continuous division of OPCs in the brain and shortening of internode lengths in some tracts (42). This was not related to ongoing myelination of nonmyelinated axons; instead, it was suggested that there is continuous myelin remodeling that results in shortened internode lengths. Could this be the reason for this finding in the two dogs examined here? This would seem to be unlikely, however, as the only areas with thin myelin/short internodes were in the superficial tracts of the LCs and VCs. Myelinated axons medial to these zones had normal myelin sheath thickness and no evidence of shortened internodes as far as could be determined in longitudinal sections.

Despite the fact that many axons in the spinal cord were only ensheathed by thin myelin sheaths, there was no evidence that this compromised axon health or resulted in degeneration (29). The interactions between the axon and its myelin sheath/oligodendrocyte that are essential for axon survival are well recognized (43–46). Although the primary source of support for the axon has been shown to the metabolic coupling between the oligodendrocyte and axon, involving importing glucose and lactate, the myelin sheath itself is also likely to be involved. In Plp1 knockout mice, there is late-stage axonal degeneration (47), whereas mutation of the myelin gene Cnp1 likewise results in axon degeneration (48). In 19-mo-old rumpshaker mice which have a mutation in the Plp1 gene, thin myelin sheaths persist, yet the genetically deficient myelin results in a late-stage axonopathy (49). Here, however, we show...
that biochemically normal, yet thin, myelin sheaths appear to support the long-term integrity of axons as the myelin fiber density was qualitatively normal (Fig. 2).

The tissue fixation precluded immunolabeling axons for amyloid precursor protein or for demonstrating any increase in neurofilament phosphorylation (50), both of which may have been used to indicate axonal disturbance. However, from observations of axonal pathology in 1-μm sections from another canine myelin mutant (33), we believe we would have identified most potential changes in the axoplasm in the present model and certainly noted if axons were swollen. Absence of any such abnormalities leads us to conclude that thin myelin sheaths can support axon health and function for over the course of 13 y, and potentially indefinitely. Hence, the need to develop remyelinating strategies that restore myelin to its normal thickness to protect axons and sustain function (31) is not required.

**Materials and Methods**

All animals were handled and treated according to the guidelines and recommendations of the Research Animal Resources Center and the Animal Care and Use Committee at the University of Wisconsin–Madison. The genetic model studied here is a disorder in the Weimaraner breed of dog that has a unique developmental deficiency in myelination of the superficial tracts of the cervical and thoracic spinal cord (32, 51). Affected dogs develop a severe tremor and ataxia by 12–14 d of age, which gradually diminish and are lost in most cases by 3–4 mo. The disorder is inherited as an autosomal recessive trait, and the mutation is in the folliculin-interacting protein 2 (FNIP2) (32). We have proposed that the mutation results in a failure of migration or differentiation of a subpopulation of oligodendrocytes (32).

In the present study, we followed two homozygous affected littermates, a male and female, for more than 13 y. The male had a more severe and persistent tremor and ataxia than the female. In both dogs, however, no obvious tremor could be detected by 6 mo, and the male was no longer ataxic. Both dogs were neurologically normal for up to 13 y. Each was killed at around 13 1/2 y because of nonneurologic disease. Samples of the cervical and thoracic spinal cord were collected from the male immediately after death and immersion fixed in 2.5% glutaraldehyde. Tissue from the female dog was only available 3–4 h after death and immersion fixed, but the delay resulted in poorer fixation. For controls for the mature affected dogs, we used sections from the cervical and thoracic cord of 2-y-old normal dogs (WT; n = 2), as age-matched controls were not available. By 2 y of age, myelination is complete in the canine spinal cord, and myelin sheath thickness is representative of all mature canines (33). To illustrate the developmental defect on early myelination of the superficial tracts of the spinal cord and the change that occurs with time, we examined the thoracic spinal cord of a 2-wk-old affected Weimaraner with tremor and an age-matched control dog.

Blocks from the cervical and thoracic cord were trimmed and processed for plastic embedding and preparation of 1-μm toluidine blue-stained sections. One-micrometer sections were then used to measure the g ratios of ~250–500 μm of myelinated axons in both the LCs and VCg of both affected and control cord at two adjacent sites, using ImageJ software (NIH). The mean difference between case and control dogs was analyzed using a linear mixed-effects model (52), with two levels of random effects: one for measurements from the same dog and one for measurements taken within the same 1-μm slide. This model is considered a mixed-effect model with nested factors.

The second model used to study the persistence of thinly myelinated axons was a unique feline disorder in which feeding irradiated food resulted in profound and generalized demyelination and remyelination of the CNS (1). The disorder has been named FIDID. Two cats were studied as part of the original study at University of Wisconsin–Madison (1) and were killed over the course of 2 y after complete neurologic recovery. The third cat was part of an outbreak of clinical disease seen in Sydney, Australia (53). While on an irradiated diet, the cat developed severe neurolologic disease (tetraparesis, ataxia) but recovered to almost normal ambulation and was studied 14 mo after recovery. In this animal, pieces of the cervical and thoracic spinal cord were immersion fixed in 2.5% glutaraldehyde after euthanasia, processed, and embedded in plastic. Plastic blocks from spinal cord and optic nerve of the first two cats were also studied.

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