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Effects of osmolality on PLP-null myelin structure: Implications re axon damage

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Abstract

In order to test the adhesiveness of PLP-null compact myelin lamellae we soaked aldehyde-fixed CNS specimens from PLP-null and control mice overnight in distilled water, in Ringer's solution or in Ringer's solution with added 1M sucrose. Subsequent examination of the tissue by EM showed that both PLP-null and control white matter soaked in Ringer remained largely compact. After the distilled water soak, control myelin was virtually unchanged, but PLP-null myelin showed some decompaction, i.e., separation of myelin lamellae from one another. After the sucrose/Ringer soak, normal myelin developed foci of decompaction, but the great majority of lamellae remained compact. In the PLP-null specimens, in contrast, many of the myelin sheaths became almost completely decompacted. Such sheaths became thicker overall and were comprised of lamellae widely separated from one another by irregular spaces. Thus, in normal animals, fixed CNS myelin lamellae are firmly adherent and resist separation; PLP-null myelin lamellae, in contrast, are poorly adherent and more readily separated. Mechanisms by which impaired adhesiveness of PLP-null myelin lamellae and fluctuations in osmolality in vivo might underlie slowing of conduction and axon damage are discussed.

Keywords

demyelination; dysmyelination; proteolipid protein; Pelizaeus-Merzbacher disease; spastic paraplegia type 2; multiple sclerosis

1. Introduction

Inactivation of the proteolipid protein (PLP) gene results in the formation of CNS myelin of near normal structure and abundance despite the complete lack of PLP and its smaller isoform, DM20 (Klugmann et al., 1997). These animals survive well, but develop progressive degeneration of CNS axons beginning at ~6–8wk (Griffiths et al., 1998), reminiscent of the axon loss thought to underlie secondary progression in the relapsing/remitting form of multiple sclerosis (Trapp, et al., 1998).

Despite its relatively normal appearance, previous studies have noted inconspicuous defects in PLP-null myelin consisting primarily of widening and irregularity of the interlamellar spaces

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(Rosenbluth et al. 2006). This subtle decompaction, visible in electron micrographs of fixed tissue, corresponds closely to the greater disorder and wider extracellular spaces between lamellae found in X-ray diffraction studies of unfixed PLP-null myelin (Yin et al, 2006) and is consistent with defective adhesion of myelin lamellae along their external surfaces due to loss of homophilic bonding of PLP molecules between adjacent myelin lamellae. Chemical modification of PLP, thought to interfere with homophilic bonding, also results in decompaction (Bizzozero et al. 2001, 2004).

We have suggested previously that defective compaction of PLP-null myelin could result in axon damage from leakage of ions and other solutes through extracellular pathways in the internodal myelin (Rosenbluth et al 1996). In the present paper we present further evidence that PLP-null myelin lamellae are poorly adherent in that they are more readily separable than normal in non-isosmotic solutions.

Our results provide an explanation for the marked differences in PLP-null myelin structure reported in studies that have used different methods for preparing the tissues. In addition, they introduce the possibility that variations in osmolality could increase the leakiness of PLP-null myelin sheaths in vivo as a potential basis for conduction defects and axon damage.

2. Results

Compact myelin

Ringer's soak—An overnight soak in Ringer's solution (R) following aldehyde fixation corresponds to the conditions used in preparing the specimens used in our previous study of PLP-null myelin compared with control (Rosenbluth et al. 2006). Data obtained from the current Ringer's soaked group confirmed the earlier observations, which will be reviewed briefly here but not illustrated.

Cross sections through control specimens showed myelinated nerve fiber profiles whose outlines were primarily in the form of rounded polygons. 'Splits' in myelin sheaths occurred infrequently. Ferricyanide, which acts as an extracellular tracer (Aguas, 1982), was found between lamellae in some of the thin sheaths, but in thicker sheaths it was confined to the inner and outer regions and did not extend between lamellae in the middle. Myelin generally appeared compact with a regular periodicity.

PLP-null specimens differed in that the outlines of the sheaths were more varied in shape, with frequent redundant folds involving all or some of the lamellae extending away from the axon. As a result the external contour of the fibers often looked scalloped or irregular, as if the sheath was too large for the enclosed axon. Where ferricyanide density could be seen, it more often infiltrated between layers throughout even thicker sheaths in an irregular manner (Fig. 3b in Rosenbluth et al., 2006). The width of the ferricyanide-labeled spaces between layers was variable, resulting in corresponding variation in the repeat period of the compact lamellae. Nevertheless, at low magnification, PLP-null myelin sheaths were close to normal in appearance and compaction at all ages examined.

Distilled water soak—After an overnight soak in distilled water (DW) following aldehyde fixation, myelin sheaths in control specimens had typical rounded polygonal contours and, aside from occasional splits, showed normal myelin compaction (Fig. 1A and Table 1). The great majority of myelin lamellae displayed typical periodic structure with regular alternation of major dense lines and intermediate (intraperiod) lines.

Some of the PLP-null myelin sheaths also looked superficially compact, but others, especially thicker sheaths around larger caliber axons, showed moderate decompaction such that

individual or small groups of lamellae were widely and irregularly separated from their neighbors around part of the circumference of the fiber (Fig. 1B), thus increasing the overall thickness of those sheaths. Quantification showed that 41% of the sheaths were decompacted over $>3/4$ of their circumference (Table 1) compared with ~25% for sheaths soaked in Ringer ($p<0.0001$). Changes were comparable in all of the equivalent specimens analyzed. It was occasionally possible to find decompacted sheaths side by side with normally compacted sheaths (* in Fig. 1B). The overall appearance of the myelin did not show any obvious variation with age, and results were comparable in optic nerve and spinal cord.

We assume that the narrow, irregular spaces between lamellae in the mutant myelin sheaths normally contain solutes and thus swell when the sheaths are soaked in distilled water, thereby increasing the width of the spaces between lamellae.

In a previous study, McIntosh and Robertson (1976) soaked unfixed rat optic nerve in distilled water for 2 to 24 hr at 4 degrees C. Subsequent EM examination showed separation along every *other* intermediate line, resulting in the formation of lamellar pairs containing two major dense lines. We have seen this only occasionally in our distilled water-soaked specimens, which were aldehyde-fixed prior to soaking. Robertson also noted that swelling with separation of lamellae did not occur in regions that displayed the radial component and concluded that these elements bound the lamellae together, a conclusion that has been borne out by more recent studies (Gow et al., 1999; Chow et al., 2005).

Sucrose/Ringer soak—An overnight soak in sucrose/Ringer's solution (1MS) following aldehyde fixation produced the most striking differences between control and PLP-null sheaths. The respective specimens were readily distinguished in electron micrographs. Control myelin was relatively unaffected (Fig. 2A and Table 1) but in some places showed interlamellar splits that appeared as clear spaces between single or small groups of lamellae and extended across much of the width of the sheath (Fig. 2B), sometimes producing an appearance similar to that of a Schmidt-Lanterman cleft. These splits were confined to focal regions around the circumference of the fiber, occupying no more than one fourth of the sheath circumference in 63% of cases (Table 1). But in the intervening regions, comprising most of the circumference, all or most of the myelin lamellae remained adherent. Examples of PNS-like split intermediate lines, separated by narrow (2–4nm) clefts were also seen in the sucrose/Ringer-soaked controls, comparable to those reported in edematous CNS myelin (Hirano et al., 1966) These showed slight variation in interlamellar width where ferricyanide penetrated between lamellae (Fig. 2C).

The effects of the sucrose/Ringer soak on the PLP-null sheaths were quite different. In survey views, some of the sheaths, particularly those of small-caliber fibers, showed only focal decompaction, while in larger-caliber fibers the extent of decompaction was much more extensive. Overall, $>3/4$ of the circumference was decompacted in 82% of fibers (Table 1) compared with ~25% in specimens soaked in Ringer ($p<0.00001$) but without apparent change in the periaxonal space (Fig. 3A). As a result, individual or small groups of myelin lamellae were widely and irregularly separated from one another (Fig. 3B). We saw no evidence for age-related differences in PLP-null myelin from mice 1 to 22mo old, and the differences between PLP-null and control specimens were clear-cut at all ages examined and in all of the samples analyzed.

In the 'wide spaced' myelin resulting from intercalation of IgM molecules into the sheath (Rosenbluth et al. 1996a), the individual lamellae are very regularly spaced. In osmotically swollen PLP-null myelin, in contrast, the lamellae are not held at a fixed distance from each other. Rather they look as though they have simply come apart and are no longer interconnected, even indirectly.

Radial/Diagonal component

The radial component of normal CNS myelinated fibers has been shown to consist of stacks of tight junctions extending over multiple layers of compact myelin. These interlamellar junctions are aligned such that they overlie each other forming a roughly 90 degree angle with the lamellae themselves (Peters, 1961). These junctions have been shown to be associated with OSP/Claudin 11 (Gow et al., 1999) and thus have a dual function. On the one hand they occlude the intercellular gap between lamellae. On the other, they bind the adjacent membranes together mechanically.

In control specimens soaked in sucrose/Ringer (Fig. 3C), the radial component is readily seen in typical form, consisting of groups of radially aligned densities between successive lamellae. In these regions, the lamellae remain compact and display the normal repeat period both at the sites of the radial component and in between them. Equivalent structures were also seen in control specimens soaked in distilled water (not shown), and in that case too the lamellae remained compact

For reasons that are not clear, the counterpart structures in transverse sections of PLP-null myelin align at a 45 degree angle to the myelin lamellae (Rosenbluth et al., 2006). Here too the contact points appear to be firmly attached together. In contrast to normal myelin lamellae, however, the membrane regions *between* adjacent attachment sites along the same pair of lamellae tend to separate from one another and often become filled with ferricyanide, resulting in a brickwork pattern of narrow intercellular densities (Fig. 4d in Rosenbluth et al., 2006).

Soaking PLP-null myelin overnight in distilled water had little effect on the appearance of these structures, but the sucrose/Ringer soak produced a clear distortion in which the width of the intercellular space between attachment sites increased in the radial direction, thus changing the shape of the space from a narrow sliver to something much more rounded (Fig. 3D). We saw no evidence of rupture of the membranes or disjunction at the attachment sites, however.

The appearance of the swollen interlamellar spaces between diagonal component attachment sites can be likened to the appearance of a partially buttoned sweater. If the sweater fits, then the unbuttoned regions overlap one another even though not attached together (Fig. 3E). If the sweater is too small, then the unbuttoned regions gape apart (Fig. 3F).

We interpret these results as evidence that in PLP-null myelin, sucrose is able to diffuse into the gaps between attachment sites over the course of an overnight soak, but cannot readily diffuse out again. Dilution of the medium outside the myelin sheath during further tissue processing then results in a movement of water into these sucrose-containing spaces, causing them to round up, thus increasing their volume more than their surface area.

3. Discussion

Our principal finding is that compact myelin from mutant mice lacking PLP and DM20 looks relatively normal under standard preparative conditions but undergoes more decompaction than normal myelin when soaked in solutions of either high or low osmolality at the step after aldehyde fixation. The myelin lamellae separate everywhere except at the tight junctions that form the diagonal component.

We suggest that the basis for the decompaction is twofold:

1. Defective *occlusion* of the interlamellar spaces in the absence of PLP, resulting in greater penetrability of those spaces by solutes in the surrounding medium.

2. Defective *adhesion* between adjacent lamellae resulting in diminution in their ability to withstand radial forces tending to separate them.

There has been disagreement in the literature about the appearance of PLP-null myelin, some authors claiming that it looks nearly normal (Klugmann et al., 1997) and others that it tends to look 'exploded' (Boison and Stoffel, 1995). Since the mutants used in the respective studies are not identical, the structural differences seen could reflect underlying differences in protein expression that could affect myelin ultrastructure. Our results suggest, however, that these differences could simply reflect differences in preparative methods. Thus, adhesion between CNS myelin lamellae may be defective in both mutants, but the myelin lamellae separate from one another under some preparative conditions, while under others they remain more closely apposed.

It is of interest that the sensitivity of PLP-null myelin to decompaction is demonstrable even in aldehyde-fixed specimens, where, presumably, myelin lamellae are, to some extent, cross-linked to one another by the fixative. Clearly, despite the multitude of proteins present in compact myelin (Taylor et al., 2004), which could be associated with either the major dense line or the intermediate line, when PLP is absent aldehyde linkage is insufficient to keep the lamellae firmly attached to one another. This could reflect increased permeability of the sheath, permitting solutes such as sucrose to penetrate into the interlamellar spaces to a greater degree than normal and there cause swelling in the presence of a concentration gradient, an effect akin to osmotic swelling of a cell in a hypotonic solution. It could also reflect a paucity of molecules in successive lamellae capable of being interlinked by the fixative, given the absence of PLP/DM20 which normally comprises half the myelin protein (Nave, 1995). However, in shiverer mice loss of another major CNS myelin protein, MBP, which comprises 30% of the total, results in marked reduction in the amount of CNS myelin formed, but at the level of individual groups of myelin lamellae, extracellular appositions are normal (Rosenbluth, 1980) with no sign of decompaction regardless of the preparative method used. Thus, in shiverer mice, in which PLP is present, the absence of a different major myelin protein, and the corresponding loss of non-specific, fixative-mediated linkage, does not lead to obvious decompaction.

To what extent do changes of the kind seen in fixed PLP-null myelin occur in *living* animals or patients with PLP defects? Clearly the osmolality range of the test solutions used in this study is beyond any variations that would normally occur *in vivo*. These extreme conditions were used to bring out defects that might otherwise be so subtle as to make detection difficult but that might nevertheless have significant pathological consequences that develop slowly over time, comparable to the effects of some toxic agents, e.g. cigarette tar, that were not apparent until tested at much higher-than-normal concentrations.

Blood and CSF are regulated via CNS osmoreceptors, and the posterior pituitary hormone vasopressin acting on water reabsorption in the kidney. Blood and CSF osmolality can vary depending on water and salt intake and glucose level as well as other parameters. In view of our observations on normal myelin, minor variations are not likely to affect normal myelin sheaths significantly, although larger changes are known to contribute to 'osmotic myelinolysis' (Brown, 2000). In PLP-null myelin, in contrast, there is a greater chance that even minor shifts could affect myelin compaction, resulting in widening of the intercellular spaces within the sheaths and increased permeability of the sheaths to materials that normally have limited access to the axonal environment.

Studies of CNS conduction velocity in some PLP-mutant mice have shown a reduction to approximately 50% of normal (Boison and Stoffel, 1995). Rotarod performance in other PLP-mutants is not significantly affected at 6mo and 1yr (Klugmann et al. 1997) but declines after 1yr (Stecca et al., 2000). To what extent these physiological and behavioral parameters would be affected by changes in the osmolality of the cerebrospinal fluid (CSF) is not known. Narrow

variations in CSF osmolality within the normal range would probably not lead to significant decompaction in normally myelinated fibers. Even with the large changes we introduced, normal myelin remained largely compact. Our data suggest, however, that changes in CNS osmolality could cause decompaction of PLP-null myelin sufficient to affect sheath resistance and conduction velocity, possibly resulting in conduction block.

Whether 'leaky' myelin sheaths might also underlie axon damage is a provocative question that could possibly be relevant to the axon degeneration seen in PLP-null mice (Griffiths et al., 1998), Pelizaeus-Merzbacher disease (Garbern et al., 2002) and spastic paraplegia type 2 (Lee et al., 2006). Increased current flux across the internodal axon during activity could result in increased axonal sodium channel activity along the internodal axolemma and a corresponding increase in both sodium influx and, since the selectivity of the sodium channel is imperfect (Hille, 2001), calcium influx as well. This could potentially raise intra-axonal calcium concentration, normally maintained in the submicromolar range, enough to trigger intracellular protease activity and axon damage. Such a scenario could underlie gradual, progressive axon damage. Thus, although the susceptibility to decompaction is there from the outset, the consequent axon damage may accumulate slowly and progressively, resulting in neurological signs that appear only after some threshold proportion of axons has been damaged.

Of interest in this regard is the observation that the presence of DM20 without PLP in one experimental system (Stecca et al., 2000) resulted in a ~20% increase in CNS myelin period and progressive axon degeneration comparable to that seen in PLP-null mice. In contrast, a different method for introducing DM20 without PLP (Sporkel et al., 2002) resulted in a normal CNS myelin period with only minimal axon degeneration. Assuming that the increased myelin period in the first case reflects widening of the interlamellar extracellular space, these results are consistent with the premise that leaky myelin sheaths lead to gradual axon degeneration.

In conclusion, this study makes two points:

1. Fixed PLP-null myelin is more susceptible than normal to decompaction in non-isosmotic solutions, which probably accounts for the marked disparity in its structure in various published reports.
2. Although this study used extreme conditions in order to bring out differences from normal, the results imply that myelin with PLP mutations could undergo subtler changes in vivo that might not be apparent but might still have significant physiological consequences over time. Subtle decompaction of PLP-null myelin may underlie not only slowed conduction but also increased activation of internodal axolemmal sodium channels and penetration of extracellular elements normally excluded from the internodal periaxonal space, both of which could cause cumulative, gradually progressive axon damage. Periodic fluctuations in CSF osmolality could contribute to this process by bringing about repeated transient increases in the permeability of PLP-null myelin sheaths.

4. Experimental procedure

Specimens used were obtained from PLP-null mice (Klugmann et al. 1997) at ages ~1, 2, 6–7, 15 and 22mo and control mice at ages ~3 and 6mo. Our PLP-null colony was begun with male and female heterozygote breeders kindly sent by Dr. R. Skoff of Wayne State University School of Medicine, courtesy of Dr. K. Nave. In view of the well-known susceptibility of fresh CNS myelin to hypoxic damage, which can produce marked structural changes, we carried out our tests on aldehyde-perfused specimens, which we knew were well fixed, varying only the osmolality of the solutions to which specimens taken from the same animals were subsequently exposed.

Mice were anesthetized with pentobarbital, as approved by the NYU School of Medicine IACUC, and the CNS was fixed by transcardiac perfusion of 3% glutaraldehyde + 2% formaldehyde (freshly made up from paraformaldehyde) in 0.1M cacodylate buffer (pH 7.3). Specimens of spinal cord and optic nerve were dissected out the following day and placed in either distilled water, Ringer's solution, or 1M sucrose in Ringer and left overnight at room temperature.

Ringer's solution (Hospira, Inc.) contains 309 milliosmoles/L of electrolytes, which approximates the composition of mammalian tissue fluid, and is considered to be *isosmotic* with mammalian cells. The soak containing Ringer plus 1M sucrose has an additional 1000 mosmoles/L of solute and is thus markedly *hyperosmotic*. In the short term, it would be expected to draw water from compartments containing solutes at lower concentration. After it infiltrates tissues, however, it would render them hyperosmotic and thus cause them to swell in subsequent rinse solutions of lower osmolality. The distilled water rinse is lower than normal in solutes by more than 300 mosmoles/L and is thus markedly *hypoosmotic*. It would be expected to cause swelling of compartments containing solutes at higher concentration.

Post-fixation was carried out as follows: The distilled water-soaked specimens were immersed in a solution of 1% osmium tetroxide, 1.5% potassium ferricyanide and 0.1M sodium cacodylate buffer in distilled water. The Ringer-soaked specimens were immersed in 1:4 Ringer's solution containing the same ingredients. The sucrose/Ringer-soaked specimens were immersed in 1:4 Ringer's containing the same ingredients plus 0.5M sucrose. Post fixation was allowed to proceed for ~2hr, after which the specimens were dehydrated in a series of solutions beginning with 30% methanol/water through propylene oxide and finally infiltrated and embedded in Araldite.

Quantification was carried out on EM prints of transversely sectioned myelinated fibers in each experimental group (R, 1MS, DW) of PLP-null and control specimens. Each group comprised 4–10 prints obtained from 3 PLP-null and 3 control mice. Axons were marked individually, and for each the proportion of the myelin sheath circumference displaying decompaction was assessed visually. Scores from 0% (no decompaction) to 100% (complete decompaction) were tabulated and binned into four groups: 0–25% (0–1/4), 26–50% (>1/4–1/2), 51–75% (>1/2–3/4) and 76–100% (>3/4). Results were analyzed statistically using a two-tailed t-test.

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Figure 1. Myelin after overnight soak in distilled water

A. Control optic nerve. The fibers appear as rounded polygons surrounded by compact myelin. Bar = 0.1 micron.

B. PLP-null optic nerve. The outer contour of the myelin shows the protrusions characteristic of this mutant. A moderate degree of lamellar separation is visible in the sheaths at center and upper right. The thinner sheath around the axon at the lower right (*) appears compact. Bar = 0.1 micron.

Figure 2. Control myelin after overnight soak in sucrose/Ringer

A. Optic nerve. Myelin sheaths are compact, by and large. Bar = 0.1 micron.

B. Spinal cord showing a focal region in a thick myelin sheath where the lamellae are widely and irregularly separated. On either side the myelin is compact through the full thickness of the sheath. Bar = 0.1 micron.

C. Spinal cord showing ferricyanide between lamellae in a thin myelin sheath. In several places the interlamellar space widens slightly, but most lamellae show a consistent spacing. Bar = 0.1 micron.

Figure 3. Myelin after overnight soak in sucrose/Ringer

A. PLP-null spinal cord showing 'exploded' myelin sheaths in which lamellae are extensively separated by clear, irregular spaces. Bar = 1 micron

B.. PLP-null spinal cord in which major dense lines appear singly, in twos or in threes. Bar = 0.1 micron.

C. Control spinal cord showing the radial component (arrows) consisting of intermediate line densities aligned at ~90 degrees to the myelin lamellae. No separations between lamellae are visible either at the intermediate line densities or between them. Bar = 0.1 micron.

D. PLP-null optic nerve. Diagonal component elements (arrows), where adjacent lamellae are fused together, are aligned at 45 degrees to the myelin lamellae. The interlamellar spaces between fusion sites gape apart, in some cases forming wide, elliptical spaces filled with ferricyanide. Bar = 0.1 micron.

E. Partially buttoned sweater in which the edges overlap each other.

F. Same as E but with increased bulk inside causing the edges to gape apart. Bar (E and F) = 20cm.

Table 1

After an isosmotic soak (R), 4% of control myelin sheaths and ~25% of PLP-null myelin sheaths showed decompaction around >3/4 of their circumference. However, the p value for this apparent difference was 0.19 (n.s.) In contrast, comparison of the >3/4 bin for PLP-null specimens soaked in R (~25%) with PLP-null specimens soaked in IMS (82%) showed a highly significant difference (p<0.00001), as did comparison of the >3/4 bin for PLP-null specimens in R (~25%) with PLP-null specimens soaked in DW (41%) (p <0.0001). Under all three conditions, control myelin showed relatively little decompaction.

Extent of Decompaction in Fixed PLP-null and Control Myelin Under Different Osmotic Conditions

	R/		IMS/		DW	
	PLP-null (n)	control (n)	PLP-null (n)	control (n)	PLP-null (n)	control (n)
0-1/4	44.5% (89)	80% (223)	10% (28)	63% (94)	45% (209)	90% (311)
>1/4-1/2	24.5% (43)	15% (43)	5% (13)	19% (29)	10% (47)	10% (31)
>1/2-3/4	5.5% (11)	1% (3)	3% (8)	11% (16)	4% (18)	0% (0)
>3/4	25.5% (51)	4% (11)	82% (226)	7% (10)	41% (188)	0% (0)