Mutations in the X-linked Plp gene lead to dysmyelinating phenotypes and oligodendrocyte cell death. Here, we exploit the X inactivation phenomenon to show that a hierarchy exists in the influence of different mutant Plp alleles on oligodendrocyte survival. We used compound heterozygote mice to study the long-term fate of oligodendrocytes expressing either the jimpy or rumpshaker allele against a background of cells expressing a Plp-null allele. Although mutant and null oligodendrocytes were generated in equal numbers, the proportion expressing the mutant allele subsequently declined, but whereas those expressing the rumpshaker allele formed a reduced but stable population, the number of jimpy cells fell progressively. The age of decline in the jimpy cells in different regions of the CNS correlated with the temporal sequence of myelination. In compound heterozygotes expressing rumpshaker and jimpy alleles, oligodendrocytes expressing the former predominated and were more abundant than when the rumpshaker and null alleles were in competition. Thus, oligodendrocyte survival is not determined solely by cell intrinsic factors, such as the conformation of the misfolded PLP, but is influenced by neighboring cells, possibly competing for cell survival factors.

Introduction

The highly conserved X-linked Plp gene encodes for proteolipid protein (PLP), the major CNS myelin protein, and its minor isoprotein DM20 (Nave et al., 1987). PLP/DM20 is believed to regulate the structure of the intraperiod line and may be involved in glial/axonal interactions (Nave et al., 1987; Boison and Stoffel, 1994; Klugmann et al., 1997; Griffiths et al., 1998) and oligodendrocyte survival. Mutations of the PLP/Plp gene in man and animals cause dysmyelinating diseases ranging in severity from mild to lethal (for reviews see Griffiths et al., 1998a; Werner et al., 1998; Garbern et al., 1999; Yool et al., 2000). In the mouse, the disparity in phenotype is illustrated by the jimpy (Plp1) and rumpshaker (Plp1rsh) mutations. Plp causes a lethal disorder with severe hypomyelination, premature death of many oligodendrocytes, and astrocytosis (Knapp et al., 1986; Vela et al., 1998). On the other hand, Plp1rsh is a relatively benign condition when expressed in the C3H/101 strain (Griffiths et al., 1990; Schneider et al., 1992). Rumpshakers have normal longevity, produce more myelin than jimpy mice, and have fewer numbers of dying oligodendrocytes.

The Plp gene, like the majority of X-linked genes, is subject to random inactivation of one allele in the female. Because X-linked genes in glia exhibit the predicted 50% maternal/paternal allele inactivation (Tan et al., 1995), 50% of the oligodendrocytes in Plp mutant heterozygotes should express the mutant allele. In reality, the defect, as assessed by the degree of dysmyelination, never affects 50% of the total oligodendrocyte population (Skoff and Montgomery, 1981; Bartlett and Skoff, 1986; Duncan et al., 1987; Fanarraga et al., 1991). This strongly suggests that cells expressing the mutant allele are at a disadvantage through their expression of the mutant allele or their failure to express the wild-type allele. Alternatively, neighboring cells expressing the wild-type allele may hinder the mutant cells’ development or survival. The exact fate of these mutant cells is uncertain because they are difficult to identify against the wild-type background.

We, therefore, used Plp knockout mice to generate Plp1−/− or Plp1rsh−/− compound heterozygotes in which the jimpy or rumpshaker cells can be identified against the PLP-negative background. This allowed us to assess the survival of oligodendrocytes expressing different mutant alleles, when in competition with those expressing the null allele. The results
demonstrate that in adult compound heterozygotes, fewer cells express the missense alleles compared with oligodendrocytes expressing the null allele. The number of cells expressing the jimpy allele decreases markedly over the first months of life, whereas the number of oligodendrocytes expressing the rumpshaker allele remains relatively constant for several months. When cells expressing either the jimpy or the rumpshaker allele are in direct competition, the latter predominate. Thus, the various alleles of the murine Plp gene demonstrate a hierarchy in their influence on oligodendrocyte survival and myelination from wild type /H11022 null /H11022 rumpshaker /H11022 jimpy. Our results show that survival depends not only on the “intrinsic severity” of an individual mutation but also on the allele being expressed by neighboring cells.

Results

PLP does confer an advantage for myelination

Our previous studies established that oligodendrocytes expressing a null allele of the Plp gene survived in normal numbers and elaborated sheaths so that the vast majority of larger-diameter axons were myelinated (Klugmann et al., 1997; Yool et al., 2001). Absence of PLP/DM20 resulted in ~26–30% of axons <2 µm remaining nonmyelinated (Yool et al., 2001). Because of X chromosome inactivation, heterozygotes are chimeras in which oligodendrocytes lacking PLP/DM20 are in competition with those expressing the proteins. If the presence of PLP/DM20 confers no advantage or disadvantage, then 50% of cells and their myelin sheaths should express each allotype. In fact, ~66% of sheaths were PLP positive, a value significantly different from 50% (Yool et al., 2001). The proportion of PLP+ sheaths was similar throughout the CNS and was stable between postnatal day (P) 20 and 1 yr of age. In general, the distribution of PLP+ and PLP− sheaths showed an intermingling mosaic pattern throughout the CNS, with the exception of the optic nerve, where, commonly, a large number of contiguous myelin sheaths were of a single immunotype, thus forming a “patch” (Fig. 1).

These studies established that although oligodendrocytes expressing a Plp-null allele survive, the presence of PLP/DM20 does confer an advantage for myelination, probably in the ability of the cell to associate with axons. The strategy for the present studies is to allow oligodendrocytes expressing a mutant allele to compete with those expressing a null allele, thus providing a “PLP-negative” background on which the former cells are detectable. A further benefit of this strategy is that because the null cell does not have the “advantage” conferred by wild-type PLP, any deviation from the predicted 50% survival can be linked to the mutant protein. If mutant alleles have a similar influence as the null allele on cell survival and myelination, the compound heterozygotes should contain equal numbers of oligodendrocytes and sheaths of each allotype.
No PLP is better than misfolded PLP

The *rumpshaker* allele causes the least severe phenotype of any of the murine Plp mutants and also results in a relatively mild disorder in humans (Kobayashi et al., 1994). Oligodendrocyte numbers in affected males (*Plp<sup>jimpy</sup>*/H<sup>11021</sup>* alleles were clinically normal; lacking the tremor associated with affected *rumpshaker* males. We used in situ hybridization to identify oligodendrocytes expressing the mutant allele in cervical spinal cord of compound heterozygotes and their affected male *rumpshaker* littermates at P20, P50, and P100. By visual inspection, the number of positive cells in the heterozygotes was <50% of those in the male littermates at all ages (Fig. 2). When we immunostained sections from the compound heterozygotes (*Plp<sup>jimpy</sup>*/*H<sup>11001</sup>), the majority of myelin sheaths throughout the CNS at ages from P20 to P200 were PLP<sup>+</sup>, indicating their origin from oligodendrocytes expressing the null allele (Fig. 3). In the white matter, the distribution of the PLP<sup>+</sup> fibers varied from single isolated sheaths through to large patches of immunopositive fibers, the latter being particularly prominent in the optic nerves (Fig. 3, C and D). Small islands of PLP<sup>+</sup> sheaths were present in the cerebral cortex, suggesting their origin from a single cell or a small cluster of oligodendrocytes expressing the *rumpshaker* allele (Fig. 3, G and H). By light microscopy, we detected small numbers of dysmyelinated axons with thin myelin sheaths, corresponding to the immunostained sheaths (unpublished data). We quantified the proportions of PLP<sup>+</sup> and PLP<sup>−</sup> myelin sheaths in 1-μm resin sections from the ventral columns of thoracic spinal cord in compound heterozygotes at P20, P50, and P100. The proportion of PLP<sup>+</sup> (*rumpshaker*) sheaths changed from 26 ± 5% (mean ± SEM, n = 4) at P20 to 19 ± 2% at P50 to 16 ± 3% at P100, values that were not significantly different (Fig. 4). However, at all ages, the percentage of PLP<sup>+</sup> myelin sheaths was significantly <50% (P = 0.0286) (Fig. 4).

Phenotypically severe mutations result in a progressive loss of oligodendrocytes

The *jimpy* allele causes one of the most severe phenotypes of any *Plp* gene mutation, one characteristic of which is the increased apoptosis of oligodendrocytes (Knapp et al., 1986). Indirect assessments suggest that in natural heterozygotes, there is a progressive loss of cells expressing the mutant allele, when in competition with oligodendrocytes expressing...
a wild-type allele (Skoff and Ghandour, 1995). To examine the long-term survival and distribution of such oligodendrocytes, we generated compound heterozygotes in which oligodendrocytes could express either a null or \textit{jimpy} allele.

Cells expressing the \textit{jimpy} allele were detectable by a positive in situ hybridization signal using a probe recognizing \textit{Plp}/\textit{Dm20} and the absence of any signal when hybridized with a \textit{Plp} exon 5–specific probe (unpublished data). Spinal cords and hindbrains were examined by in situ hybridization at P5, 20, 35, 50, and 100 to determine the approximate proportion of positive cells (Fig. 5 A). The additional time points at P5 and P35 were taken because preliminary studies suggested a more dynamic change in the cell population in the \textit{jimpy} compared with the \textit{rumpshaker} mouse. At P5, the number of \textit{Plp} \textsuperscript{+/} cells appeared similar to those found in the \textit{Plp} \textsuperscript{−/−} heterozygotes. By P20, the number of \textit{Plp} \textsuperscript{+} (\textit{jimpy}) cells was reduced in the ventral and lateral columns compared with P5. In the dorsal columns, the positive cells were predominantly in the areas of the fasciculus gracilis and corticospinal tracts. At P35 and subsequently, the proportion of \textit{Plp} \textsuperscript{+} cells was markedly reduced throughout the entire transverse section compared with earlier ages, although there was little further change between P35 and P100. The surviving cells were present in white and gray matter and appeared random in distribution. A similar loss of positive cells was observed in hindbrain regions, although foci of \textit{jimpy} oligodendrocytes were present at P100 (Fig. 5 B). The additional time points at P5 and P35 were taken because preliminary studies suggested a more dynamic change in the cell population in the \textit{jimpy} compared with the \textit{rumpshaker} mouse. At P5, the number of \textit{Plp} \textsuperscript{+/} cells appeared similar to those found in the \textit{Plp} \textsuperscript{−/−} heterozygotes. By P20, the number of \textit{Plp} \textsuperscript{+} (\textit{jimpy}) cells was reduced in the ventral and lateral columns compared with P5. In the dorsal columns, the positive cells were predominantly in the areas of the fasciculus gracilis and corticospinal tracts. At P35 and subsequently, the proportion of \textit{Plp} \textsuperscript{+} cells was markedly reduced throughout the entire transverse section compared with earlier ages, although there was little further change between P35 and P100. The surviving cells were present in white and gray matter and appeared random in distribution. A similar loss of positive cells was observed in hindbrain regions, although foci of \textit{jimpy} oligodendrocytes were present at P100 (Fig. 5 B).

Using PLP-specific antibodies, we immunolabeled the cell bodies and major processes of oligodendrocytes expressing the mutant allele. As the mutant PLP is largely retained in the cell body (Gow et al., 1994; Gow and Lazzarini, 1996; Jung et al., 1996), any myelin sheaths formed by these oligodendrocytes remain unstained. For the purpose of quantification, the number of \textit{jimpy} (PLP \textsuperscript{+}) cells was expressed as a percentage of the total number of mature oligodendrocytes (adenomatous polyposis coli [APC] \textsuperscript{+}). The results mirrored those produced by in situ hybridization with an age-related decrease in \textit{jimpy} oligodendrocytes (Fig. 6). By analyzing three different CNS regions, we showed that the age at which the loss of cells occurred varied and appeared to correlate with the temporal differences in myelination pattern. Thus, in the cervical spinal cord, which normally myeli-nates early, the proportion of \textit{jimpy} cells declined significantly between P10 and P20 (Fig. 6, A, B, and E), whereas in the optic nerve and corpus callosum, where myelination occurs later, differences were delayed until P50 (Fig. 6, C, D, and E). As with the hybridization studies, \textit{jimpy} cells were still detectable in the optic nerve and forebrain at P100 and even P280 (unpublished data). The oligodendrocytes expressing the \textit{jimpy} allele were clearly identifiable within the myelinated white matter generated by the \textit{Plp-null} cells (Fig. 7 A). Foci of immunopositive oligodendrocytes were often ob-
served, particularly in the optic nerve (Fig. 7, B and C), and in some instances, the density of jimpy cells was sufficient to result in a focus of dysmyelination (Fig. 7 C).

Are the jimpy oligodendrocytes replaced?
The age-related decrease in the number of jimpy oligodendrocytes in the compound heterozygotes suggested that many were dying, as evidenced by examples of PLP$^+$ (jimpy) oligodendrocytes with pyknotic nuclei in brain and optic nerve at P20 (Fig. 7, D and E). Cells in white matter tracts, with a similar distribution to jimpy oligodendrocytes, immunostained for caspase 3 (Fig. 7 F), a key effector caspase in the apoptotic pathway (Casaccia-Bonnefil, 2000). As our anti-caspase and anti-PLP antibodies were raised in the same species, we were unable to double label for both markers.

To determine whether jimpy cells were continuing to be generated in mice older than 1 mo, we injected animals with BrdU between P30 and P32 and immunostained for PLP at P34. Only a small minority of cells in brain white matter were PLP$^+$/BrdU$^+$ (Fig. 7 G). In the optic nerve, for example, 2.8 ± 1.5% (mean ± SEM, n = 3) of jimpy oligodendrocytes had incorporated BrdU. Numerous PLP$^+$/BrdU$^+$ cells were present throughout the CNS (Fig. 7 G); in the optic nerve, 51 ± 10% (mean ± SEM, n = 2) of BrdU-labeled cells immunostained for NG2 (Fig. 7 H), 26 ± 6% for APC, and 12.7 ± 5% stained for CD45. We were unable to identify any BrdU-labeled cells costained for caspase 3.

A hierarchy in the survival of oligodendrocytes expressing different Plp alleles
The results from the various compound heterozygotes indicated that oligodendrocytes expressing the rumpshaker allele fared better than those expressing the jimpy allele when in competition with PLP-deficient cells. This suggested a possible hierarchy for survival of oligodendrocytes expressing these Plp alleles. To test this further, we generated compound heterozygotes whose oligodendrocytes could express either rumpshaker or jimpy alleles, with the expectation that the former cells would survive better. Female mice (Plpjimpy/H11001$^-$, H11021$^+$) developed a marked tremor that persisted for at least 100 d (the longest time point used in the study) and appeared more severe than that seen in rumpshaker female homozygotes (Plpjimpy/H11006$^-$, H11001$^+$). The prolonged survival of these compound heterozygotes expressing jimpy and rumpshaker alleles is in marked distinction to jimpy males (Plpjimpy/H11005$^-$, H11001$^+$), which die around P30; this provides further evidence for the dominance of the rumpshaker allele in the heterozygotes. Tissue from mice aged from P20 to P100 was immunostained with a PLP COOH-terminal antibody that recognizes the rumpshaker, but not the jimpy, products. At all ages and locations, the vast majority of the myelin sheaths and oligodendrocyte processes were positively stained, indicating a rumpshaker origin (Fig. 8 A), and contrasted markedly with the paucity of rumpshaker cells and myelin when they competed with those expressing a null allele (Fig. 8 B).

Competing alleles are generated in equal proportions but have nonuniform distribution
The above data obtained from juvenile and adult animals suggest that the various Plp alleles affect oligodendrocyte survival differentially in competitive situations. To confirm that cell survival and not cell production was compromised, we determined whether the initial populations of oligodendrocytes expressing the competing alleles were equal in the heterozygotes. To achieve this, we examined the PLP$^+$ cell population in the ventral cervical spinal cord, the earliest region to myelinate, on the first day of life (P1) in compound heterozygotes expressing null and rumpshaker alleles and com-
pared this with the male rumpshaker littermates. This age is before when apoptosis of mutant oligodendrocytes occurs; the rumpshaker PLP is partially retained in the cell bodies, making them easy to distinguish from myelin sheaths or cell processes. The number of PLP$^+$ cells in the compound heterozygote was 125 ± 25 cells/mm$^2$ (mean ± SEM, n = 4), a value not significantly different from 50% of the population in male littermates, 112 ± 16. We used the same method to show that the number of PLP$^+$ cells in wild-type/null heterozygotes was not different from the 50% value in wild-type male littermates at P1. Visual appraisal of tissue from early myelinating optic nerve (Fig. 9, A–F) and spinal cord (Fig. 9, G–I) of compound heterozygotes expressing null and rumpshaker alleles also suggested that starting cell populations were approximately equal. We also examined the spinal cord of compound heterozygotes expressing jimpy or null alleles at P5 (jimpy) cells are detected with a PLP-specific antibody [Table I] as opposed to wild-type or rumpshaker cells, which are detected with an anti-PLP/DM20 antibody; the low level of the PLP isoform in the cells at P1 precluded quantification of the jimpy cells at this age. The number of PLP$^+$ jimpy oligodendrocytes was 133 ± 13 (mean ± SEM, n = 3), compared with 121 ± 5, which represented 50% of the total APC$^+$ cells; these values are not significantly different.

Although the populations of competing oligodendrocytes are generated equally, their final pattern of distribution is clearly uneven, particularly in relation to the patches in the optic nerve. To determine whether the basis for this was established early in development or subsequently, we examined optic nerve and spinal cord from compound heterozygotes, expressing null and rumpshaker alleles, at various time points through the initial myelination process. In the optic nerve at P9 and subsequently, definite PLP$^+$ patches were present in the heterozygotes. In contrast, the deposition of the myelinating PLP$^+$ cells was uniform in male rumpshaker littermates, and the myelin basic protein (MBP) staining, which represents both mutant and null cells, was uniform in the heterozygotes (Fig. 9, A–D). In the heterozygote spinal
cord at P1, an indication of smaller patches was present, although less consistent than in the optic nerve (Fig. 9, G–I).

**Discussion**

Female heterozygotes associated with most missense mutations of the X-linked Plp gene exhibit few, if any, clinical signs, suggesting that the majority of oligodendrocytes express the wild-type, rather than the mutant, allele. A progressive loss of oligodendrocytes expressing the mutant allele in jimpy heterozygotes has been proposed, based indirectly on total cell counts or levels of the jimpy transcript (Kagawa et al., 1994; Skoff and Ghandour, 1995). By generating compound heterozygotes, we can identify the mutant cells unequivocally. This approach demonstrates that oligodendrocytes expressing the rumpshaker allele. The vast majority of the cells and myelin are of rumpshaker origin. In contrast to the previous animal, the majority of cells and sheaths express the null allele. Bar, 100 μm.

**A hierarchy in survival of oligodendrocytes expressing different Plp alleles**

Spontaneous missense mutations of the Plp gene are associated with increased numbers of dead oligodendrocytes in affected males. The magnitude of cell death varies between different mutations, being greater in those associated with severe phenotypes (Knapp et al., 1986; Schneider et al., 1992). Misfolded PLP is retained within the RER and has been implicated in the death of oligodendrocytes (Gow and Lazzarini, 1996; Gow et al., 1998), although formal proof is currently lacking. Abnormal oligodendrocyte death is not a feature of PLP-deficient mice, suggesting that this protein is not essential for cell survival (Klugmann et al., 1997; Yool et al., 2001) and cell death in the mutants is not due to a lack of functional PLP. However, in adult Plp<sup>−/−</sup> heterozygotes,

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<th>Table 1. Strategy for detecting oligodendrocytes expressing different alleles of the Plp gene in the various heterozygotes</th>
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<td><strong>Genotype of heterozygotes</strong></td>
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<tr>
<td>Plp&lt;sup&gt;Dm20&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>null</td>
</tr>
<tr>
<td>null</td>
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<td>rumpshaker</td>
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The genotype indicates the two alleles at the X-linked Plp locus. The riboprobe for Plp<sup>Dm20</sup> detects the majority of the coding region for the two transcripts. Exon 5 of the Plp<sup>Dm20</sup> transcript is deleted in jimpy. When used in conjunction with the Plp<sup>Dm20</sup> probe, it provides confirmation that jimpy cells are being detected. The PLP-CT antibody detects both PLP and DM20. The PLP-specific antibody is specific for that isoform and is used to detect jimpy cells.
wild-type oligodendrocytes always predominate when in competition with those expressing a null allele, suggesting that the presence of PLP/DM20 confers a definite advantage. In similar competitive situations, the cells expressing a null allele always predominate over those with a mutant allele, and jimpy oligodendrocytes fare better than jimpy oligodendrocytes. Using the amount of jimpy myelin as an indicator, <25% of cells expressing the jimpy allele survive when competing with PLP-deficient oligodendrocytes, whereas a majority survive when matched against jimpy cells. Thus, the survival of the cell appears to depend not only on the intrinsic severity of a particular mutation but also on the allele being expressed by competitor cells. This variability in cell survival strongly suggests that oligodendrocyte death must be influenced by factors additional to any intrinsic toxicity of the misfolded PLP or the absence of normal PLP.

Why do some mutant oligodendrocytes survive?
The proportion of jimpy oligodendrocytes decreased over time in the compound heterozygotes expressing jimpy and null alleles. The age at which a significant reduction occurred varied according to the region of CNS and correlated with the temporal pattern of myelination. A similar regional variation in cell death has been reported in male jimpy mice (Knapp et al., 1986), although such mice survive for only ~4 wk. The use of the long-surviving compound heterozygotes demonstrated that a small number of jimpy cells was present at P100 or older. The low number and scattered distribution of such cells suggested that they had survived from the period of postnatal gliogenesis. We attempted to address this aspect by labeling compound heterozygotes with BrdU between embryonic day (E) 17 and P9, when gliogenesis is taking place, and then detecting the label in the mature mice. We were, however, unable to detect any BrdU-labeled oligodendrocyte nuclei after this lengthy period and formal proof of prolonged survival is still lacking. We did show by BrdU labeling that some jimpy oligodendrocytes are generated in mice older than 1 mo and that half of the BrdU-labeled cells were NG2 - and therefore potentially competent to give rise to oligodendrocytes. This finding is not unexpected, as the normal adult CNS contains a population of slowly proliferating oligodendrocyte progenitors (Levson et al., 1999; Nishiyama et al., 1999; Horner et al., 2000; Levine et al., 2001) and a marginal increase in proliferation rate occurs in older jimpy heterozygotes (Rosenfeld and Friedrich, 1986). The present study did not distinguish which Plp allele was activated in these progenitors, but presumably only 50% are potential jimpy oligodendrocytes.

Assuming that a small proportion of jimpy oligodendrocytes is capable of a prolonged survival raises the question as to what differentiates such cells from those that die. As surviving cells express readily detectable amounts of misfolded PLP, their longevity cannot be due to an absence of this potentially damaging product. One possible reason could relate to an association with axons. During normal myelination, in excess of 50% of oligodendrocytes generated may die, probably as a result of failure to associate with axons and secure necessary survival factors (Barres et al., 1992; Barres and Raff, 1994, 1999). Mature oligodendrocytes appear much less dependent on axonal contact (Ludwin, 1990; McPhilemy et al., 1990). We suggest that the surviving mutant oligodendrocytes may have established sufficient axonal support during the critical early period. This hypothesis would not require such cells to maintain axonal contact to ensure their survival indefinitely.

A Darwinian model explains the differential survival of oligodendrocytes and the optic nerve patches
Our data fit well with a “Darwinian” model in which developing oligodendrocytes expressing the various Plp alleles compete to survive. Those cells more adept at associating with axons gain the essential survival factors, whereas the less competent die. This model readily explains the differential
survival of both the *rumpshaker* and null oligodendrocytes when set against disparate competitor cells and can account for the disproportionate patches of allotypes in the optic nerve. In the early neonatal period, there are clearly areas in the optic nerve, and to a lesser extent in the spinal cord, with clusters of mutant cells adjacent to zones where these cells are absent. Equally, there are areas in both regions where the two cell populations are intermingled. To generate a patch of mutant cells suggests prolonged clonal expansion from a common progenitor or the fortuitous contiguous settlement of progenitors expressing the mutant allele or a combination of both processes. If clonal expansion is the sole reason for a patch, it is difficult to envisage why the entire length of both optic nerves is not involved. To determine the extent of clonal expansion will require the recognition of progeny of individual progenitors, possibly through retroviral labeling before their migration into the nerve. Whatever process leads to a patch of cells with a single allotype, there appears to be a difference between the optic nerve and the remainder of the CNS in that the former region is more markedly affected. Once a patch of mutant oligodendrocytes has been laid down, we propose that there are two likely fates. Mutant cells capable of establishing an association with axons survive and generate thin myelin sheaths, as evidenced by the patches of *rumpshaker* myelin in the optic nerves of the compound heterozygotes. In contrast, mutant cells failing to establish this contact will be eliminated. If the two competing populations of cells are initially intermingled, as in the majority of the CNS, the more dominant cell will largely take over the territory of the “less fit” oligodendrocyte. Evidence for such a repair mechanism is found in the spinal cord and brain stem of heterozygotes with spontaneous mutations of the *Plp* gene (Knapp et al., 1986; Cuddon et al., 1998). If mutant cells originally forming a patch are eliminated, as in the optic nerves, there appears to be minimal repair and the end result is a zone of amylinated axons, as found in heterozygotes with the *jimpy* and *myelin-deficient* mutations (Skoff and Montgomery, 1981; Duncan et al., 1993).

**Implications for heterozygotes of spontaneous mutations of the *Plp* gene**

Large amylinated patches are a feature of the optic nerve of animals heterozygous for the *jimpy, myelin-deficient*, and *shaking* mutations of the *Plp* gene (Skoff and Montgomery, 1981; Duncan et al., 1987, 1993), whereas patches of naked axons are much rarer in spontaneous *rumpshaker* heterozygotes (Fanarraga et al., 1991). The patches appear random and show marked variation in number and size, even between the two nerves of a single heterozygote (Duncan et al., 1993). The basis of this phenotype is revealed by our studies, as discussed above. However, it is not clear why there is little repair of the amylinated patches in the optic nerve in contrast to other regions of the CNS. Recent studies have emphasized the large number of oligodendrocyte progenitors that populate the CNS in normal adults (Levison et al., 1999; Nishiyama et al., 1999), and our study has demonstrated numerous proliferating NG2+ cells in the compound heterozygotes. One might anticipate that such progenitors expressing the wild-type (or null) allele would myelinate the bare axons. Why this does not occur is a topic we are currently pursuing and has relevance to remyelination in disorders, such as Multiple Sclerosis.

One prediction from the present study is that female patients heterozygous for a mutant allele associated with a “mild” Pelizaeus-Merzbacher disease phenotype in affected males may be more severely affected than those heterozygotes with an allele causing a “severe” phenotype in the respective male patients. The cells expressing the mild allele survive in competition with wild-type cells, whereas those with the severe allele die and are replaced by normal cells or myelin. There is indeed evidence for this in some cases of Pelizaeus-Merzbacher disease/Spastic Paraplegia type 2 (Garbern et al., 1999; Sivakumar et al., 1999). Similarly, in the animal mutants *shaking pup* and *jimpy*, there is evidence for progressive replacement of mutant oligodendrocytes in the spinal cord and brain stem by wild-type cells (Knapp et al., 1986; Cuddon et al., 1998).

**Materials and methods**

**Generation of and characterization of mice**

Female *jimpy* heterozygotes (*Plp*<sup>jp-rsh</sup>) were maintained on a C3H/101 background without the tabby linkage. Female *rumpshaker* heterozygotes (*Plp*<sup>jp-rsh</sup>) or homozygotes (*Plp*<sup>jp-rsh</sup><sup>/*</sup>) were on an identical background (the benign nature of the *rumpshaker* mutation allows generation of homozygous female mutants). The derivation and characterization of the *Plp*-null mutants, which are maintained on a C57BL/6 background, has been previously described (Klugmann et al., 1997; Griffiths, 1998b). The *Plp* knockout mice do not express *Plp* mRNA, as detectable by in situ hybridization, nor PLP protein, as detectable by Western blotting and immunocytochemistry (Klugmann et al., 1997; Yool et al., 2001). Female mice carrying the *jimpy* or *rumpshaker* mutation were crossed with *Plp*<sup>Y</sup> to generate compound heterozygotes carrying null and mutant alleles (*Plp*<sup>r</sup>- and *Plp*<sup>Y</sup>-). *Jimpy* heterozygotes were mated with *rumpshaker* males to generate female mice expressing both mutant alleles (*Plp*<sup>r</sup>-<sup>r</sup>).

Mice were genotyped by PCR, as described previously (Schneider et al., 1992; Klugmann et al., 1997; Thomson et al., 1999), using DNA derived from tail biopsies, or identified by immunostaining of cryosections of spinal cord with a PLP COOH-terminal antibody (see below).

**BrdU labeling**

BrdU (Sigma-Aldrich) was used to identify mitotic cells. Mice aged 30 d were injected intraperitoneally with 50 μg BrdU in 0.9% saline at midday on three consecutive days and tissues were sampled on the fifth day.

**Tissue sampling**

Animals were killed at various ages between P1 and 12 mo. Mice were perfused by intracardiac injection of paraformaldehyde–glutaraldehyde mixture (Griffiths et al., 1981), and cervical spinal cord and optic nerve were processed for resin embedding or perfused with buffered neutral formalin and processed for paraffin embedding. For double staining with BrdU and NG2, APC, or CD45, mice were perfusion fixed with periodeate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974) and cervical cord, brain, and optic nerves were transferred to 20% sucrose and then embedded in OCT compound (Sakura Finetek), snap frozen, and prepared for cryosectioning. Cervical and thoracic spinal cord, brain, and optic nerve from other mice were embedded, unfixed in OCT compound, snap frozen in isopentane cooled in liquid nitrogen, and prepared for cryosectioning.

**In situ hybridization**

Cryosections (15 μm) of transverse cervical spinal cord or sagittal sections of the brain were hybridized with the <sup>35</sup>S-labeled or DIG-labeled PMP-22 probe, detecting both Plp and Dm20 transcripts as previously described (Griffiths et al., 1989; Vouyoudakis et al., 2000). Exon 5 is deleted from the final transcript of jimpy Plp mRNA; a riboprobe specific to exon 5 was generated for studies involving these mutants. Autoradiograms were counterstained with haematoxylin and examined using darkfield and transmitted light. DIG was detected using an alkaline phosphatase conjugate and BCIP/NBT or HRP and diaminobenzidine. A summary of the riboprobes used and the alleles they detect is shown in Table I.
Immunostaining

Antibodies. PLP/DM20 was detected using a polyclonal antibody to COOH-terminal residues 271–276, which are common to both isoforms (N.P. Groome, Oxford Brookes University, Oxford, UK). PLP was identified with an antibody raised against the PLP-specific region, residues 117–129 (E. Trifilieff, University of Strasbourg, Strasbourg, France). A summary of the PLP antibodies used and the alleles they detect is shown in Table I. MBP was detected using a rat monoclonal (clone 12; N.P. Groome). Myelin oligodendrocyte glycoprotein (MOG) was identified with a mouse monoclonal antibody (S. Piddlesden, University of Wales, Cardiff, Wales). Caspase 3 was detected with a rabbit polyclonal antibody (R&D Systems). BrdU was labeled with a mouse monoclonal antibody (Sigma-Aldrich). The APC antigen, which marks mature oligodendrocytes, was identified with a mouse mouse monoclonal antibody (CC1 clone; Ommge Research Products). NG2 was detected with a rabbit polyclonal antibody (Chemicon International Ltd.) and CD45 with a rat polyclonal antibody (Serotec Ltd.).

Immunostaining. Resin sections (1 µm) and paraffin wax sections (8 µm) were immunostained with the anti-PLP COOH-terminal antibody using the peroxidase antiperoxidase technique (Sternberger et al., 1970; Trapp et al., 1981). Cryosections (15 µm) were stained with antibodies to the PLP COOH-terminal and the PLP-specific region and with the caspase 3 and MOG antibodies, using indirect immunofluorescence. Sections were fixed in 4% paraformaldehyde in PBS for 20 min followed by 0.5% Triton X-100/PBS for 30 min at room temperature, and blocked in 0.1% Triton X-100, 0.2% pig skin gelatin in PBS for 30 min at room temperature. Primary antibodies were applied overnight at 4°C, and the secondary conjugates for 30 min at room temperature in the blocking buffer. Sections labeled with BrdU were treated with 50% HCl/1% Triton X-100 for 10 min at room temperature, after the paraformaldehyde fixation. The anti-BrdU antibody was applied for 2 h at room temperature in 0.2% Triton X-100/PBS. After labeling with the secondary conjugate, the sections were incubated overnight with appropriate antibodies, followed by the secondary conjugate. Sections were then fixed in 50% acetic acid/50% ethanol before continuing with the BrdU stain, as described. Some cryosections were labeled with 2.2 µg/ml DAPI in H2O for 1 min at room temperature. Cryosections were mounted in Citifluor antifade medium.

Quantification of PLP+ myelin sheaths and cells

To determine the proportion of PLP+ and PLP− myelin sheaths in PLP+/− mice, resin sections of thoracic spinal cord were immunostained for PLP/DM20. Two regions of the ventral columns on each side of the ventromedian fissure were photographed and printed at a final magnification of 2,800. A lined grid was placed on the prints and all fibers touching the lines were scored for their PLP status. Over 1,000 fibers were counted per animal and groups of four mice were analyzed at each age. The numbers of PLP+ cells were determined in various regions of the CNS in PLP+/− mice. The optic nerve and corpus callosum were sectioned longitudinally and the cervical cord cut transversely. Cryosections were immunostained with a PLP-specific antibody (to detect the oligodendrocytes expressing the (jumpy) allele) and with an anti-APC antibody to label mature oligodendrocytes. Nuclei were labeled with DAPI. Images of antibody and DAPI-stained cells were merged (Adobe Photoshop 6.0; Adobe Systems) and all immunopositive cell bodies containing a nucleus, within a defined area, were counted (Image-Pro Plus; Media Cybernetics). As PLP+ cells occurred randomly, the fields for imaging were selected under phase optics (<200 objective) to avoid bias. Fields were selected along the lengths of the optic nerve and corpus callosum and in the ventral columns of the spinal cord. Groups of four to six animals were analyzed at various ages from P10 to P100. Quantification of PLP+ cells in the spinal cord of compound heterozygotes aged P1 was performed as above, except that comparisons were made with affected male littermates. We found that the APC reaction was capsular at this age and could not be used to quantify the total oligodendrocyte count, which was estimated from the male littermates.

Statistical analysis

In compound heterozygotes expressing a null or a rumpshaker allele, each allele should be expressed in 50% of the oligodendrocytes and their myelin sheaths, if neither allele confers an advantage. To test the hypothesis that cells expressing the rumpshaker allele are not at a disadvantage, we determined whether the percentage of PLP-positive rumpshaker myelin sheaths, as determined above, was less than the theoretical 50% value (represented by the total number of PLP-positive and PLP-negative sheaths × 0.5) using a one-tailed Mann-Whitney test. Comparison of the proportions of PLP-positive myelin sheaths or PLP-positive cells at different ages was performed using ANOVA with Bonferroni’s Multiple Comparison Test as the post-test. Significance was P ≤ 0.5. Analyses were performed using the GraphPad Prism software (GraphPad Software).

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