

# Survival of, and competition between, oligodendrocytes expressing different alleles of the *Plp* gene

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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., 1998b) 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* (*Plp<sup>jp</sup>*) and *rumpshaker* (*Plp<sup>jp-rsh</sup>*) mutations. *Plp<sup>jp</sup>* 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, *Plp<sup>jp-rsh</sup>* 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 *Plp<sup>jp/-</sup>* or *Plp<sup>jp-rsh/-</sup>* 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

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\*Abbreviations used in this paper: APC, adenomatous polyposis coli; E, embryonic day; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; P, postnatal day; PLP, proteolipid protein.

Key words: myelin protein; mutation; oligodendrocyte; proteolipid protein; heterozygote

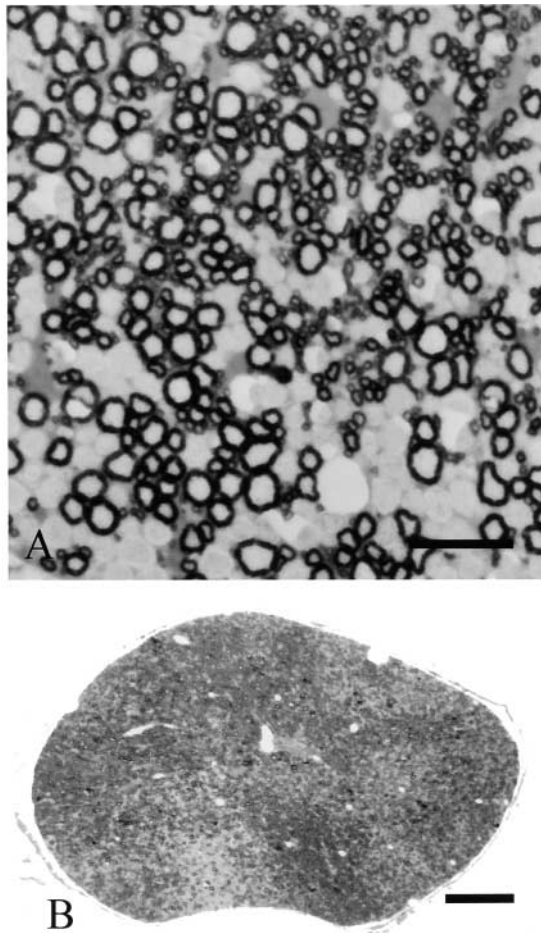


Figure 1. **Heterozygote, 4-mo-old mouse CNS, expressing wild-type and null alleles of the *Plp* gene, immunostained for PLP/DM20.**

(A) Ventral column of spinal cord to show mosaic of wild-type myelin (positively stained) and myelin formed by oligodendrocytes expressing a null allele (no staining); the former predominate. (B) Optic nerve to show mosaic of positive and negative sheaths in conjunction with patches predominantly of one immunotype. Bars: (A) 20  $\mu\text{m}$ ; (B) 50  $\mu\text{m}$ .

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 > null > *rumpshaker* > *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

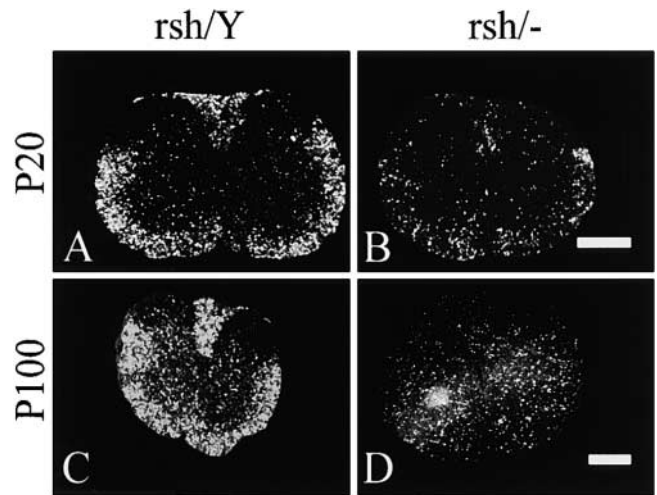
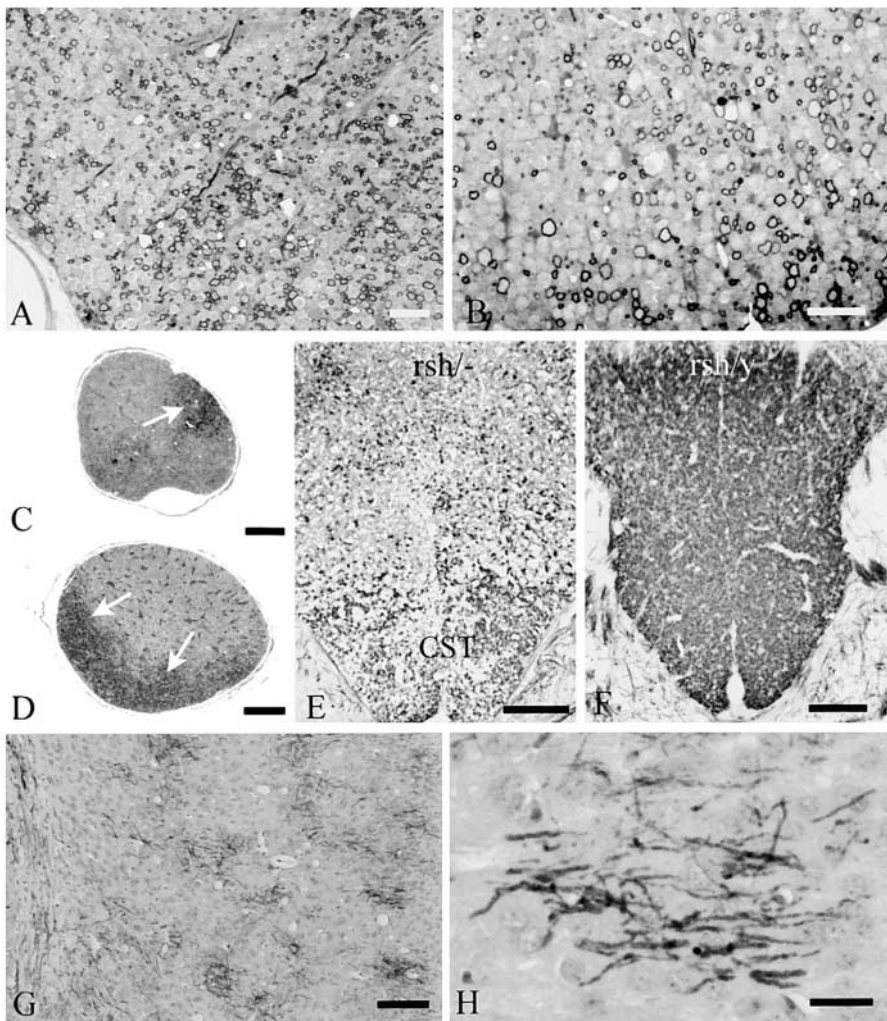


Figure 2. **In situ hybridization for *Plp/Dm20* in spinal cords of *rumpshaker* male mice (*rsh/Y*) and female littermates expressing a *rumpshaker* and a null allele of the *Plp* gene (*rsh/-*).** Animals aged P20 and P100 are shown. The affected males indicate the total number of oligodendrocytes capable of expressing the *rumpshaker* allele. At both P20 and P100 the number of positive cells in the heterozygotes is considerably <50% of those in the male littermates. However, the proportion of positive cells appears very similar at both ages in the heterozygotes. The bright signal to the lower left of the P100 heterozygote cord is an artifact. Bars, 0.5 mm.

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  $\mu\text{m}$  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<sup>+</sup> 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<sup>+</sup> and PLP<sup>-</sup> 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.





**Figure 3. Female compound heterozygotes expressing *rumpshaker* and null alleles of the *Plp* gene, immunostained for PLP/DM20.** Myelin sheaths formed by oligodendrocytes expressing the *rumpshaker* allele are positively stained, whereas those supported by cells expressing the null allele remain unstained. (A and B) Resin sections of ventral columns of spinal cord from mice aged P20 and P100, respectively. There is a small reduction in the number of positive sheaths between the two ages. Bars, 20  $\mu\text{m}$ . (C and D) Resin sections of optic nerves from mice aged P20 and P50, respectively. Both mice show patches of *rumpshaker* myelin (arrows) comprising a minority of the myelin sheaths. Bars, 50  $\mu\text{m}$ . (E and F) Paraffin sections of the dorsal columns of spinal cord from a heterozygote aged P200 (*rsh*<sup>-/-</sup>) and her male *rumpshaker* littermate (*rsh*<sup>/Y</sup>), respectively. The affected male (F) indicates the amount of PLP/DM20 that can be present if all oligodendrocytes express the *rumpshaker* allele. The heterozygote contains considerably <50% positive sheaths although their concentration is higher in the corticospinal tracts (CST) than elsewhere in the dorsal columns. Bars, 50  $\mu\text{m}$ . (G) Paraffin section of cerebral cortex from a heterozygote aged P200 to show several small regions containing *rumpshaker* myelin, suggesting single oligodendrocytes or a small clone of cells expressing the mutant allele. Bar, 100  $\mu\text{m}$ . (H) Higher magnification of one of the patches of *rumpshaker* myelin shown in G. Bar, 20  $\mu\text{m}$ .

### No PLP is better than misfolded PLP

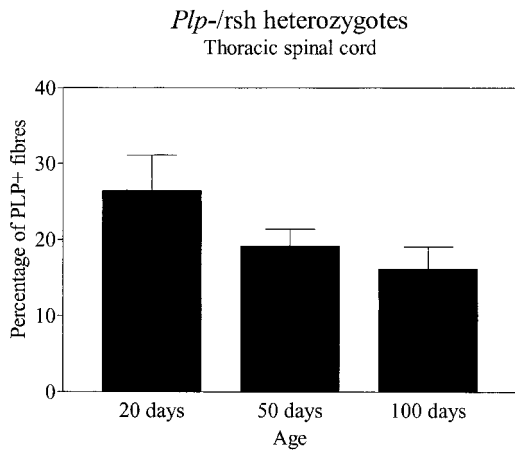
The *rumpshaker* allele causes the least severe phenotype of any of the murine *Plp* mutants and also results in a relatively mild disorder in humans (Kobayashi et al., 1994). Oligodendrocyte numbers in affected males (*Plp*<sup>*jp-rsh*</sup>/*Y*) are not reduced compared with wild type, and, with time, the majority of axons in the spinal cord acquire a PLP/DM20<sup>+</sup> myelin sheath (Fanarraga et al., 1992, 1993).

Females heterozygous for both the *rumpshaker* and null 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>*jp-rsh*</sup><sup>-/-</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- $\mu\text{m}$  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  $26 \pm 5\%$  (mean  $\pm$  SEM,  $n = 4$ ) at P20 to  $19 \pm 2\%$  at P50 to  $16 \pm 3\%$  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

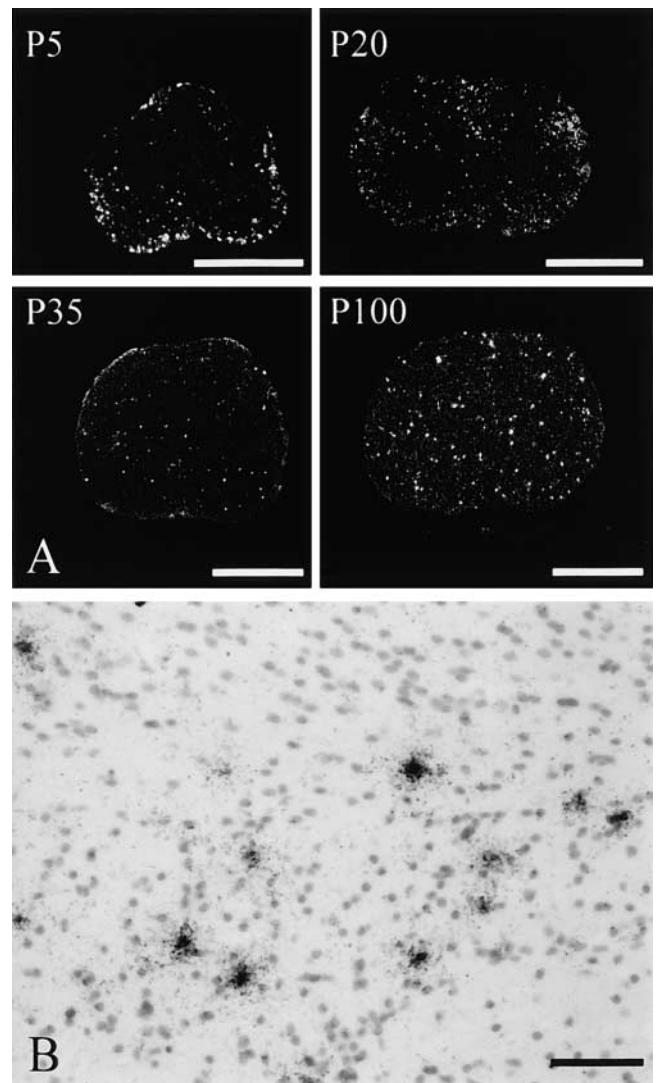


**Figure 4. Thoracic spinal cord from compound heterozygotes expressing a *rumpshaker* and a null allele of the *Plp* gene was immunostained for PLP/DM20.** The proportion of myelin sheaths formed by oligodendrocytes expressing the *rumpshaker* allele (PLP/DM20<sup>+</sup>) was determined in the ventral funiculi at different ages. At all ages, the proportion of *rumpshaker* myelin sheaths is significantly <50% ( $P = 0.0286$ , Mann-Whitney test). There is no significant difference between the groups at the three ages ( $P = 0.1498$ , ANOVA). Data are mean  $\pm$  SEM,  $n = 4$ .

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 *jimpy* allele.

Cells expressing the *jimpy* allele were detectable by a positive in situ hybridization signal using a probe recognizing *Plp/Dm20* and the absence of any signal when hybridized with a *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 *jimpy* compared with the *rumpshaker* mouse. At P5, the number of *Plp*<sup>+</sup> cells appeared similar to those found in the *Plp*<sup>-/+</sup> heterozygotes. By P20, the number of *Plp*<sup>+</sup> (*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 *Plp*<sup>+</sup> 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 *jimpy* oligodendrocytes were present at P100 (Fig. 5 B).

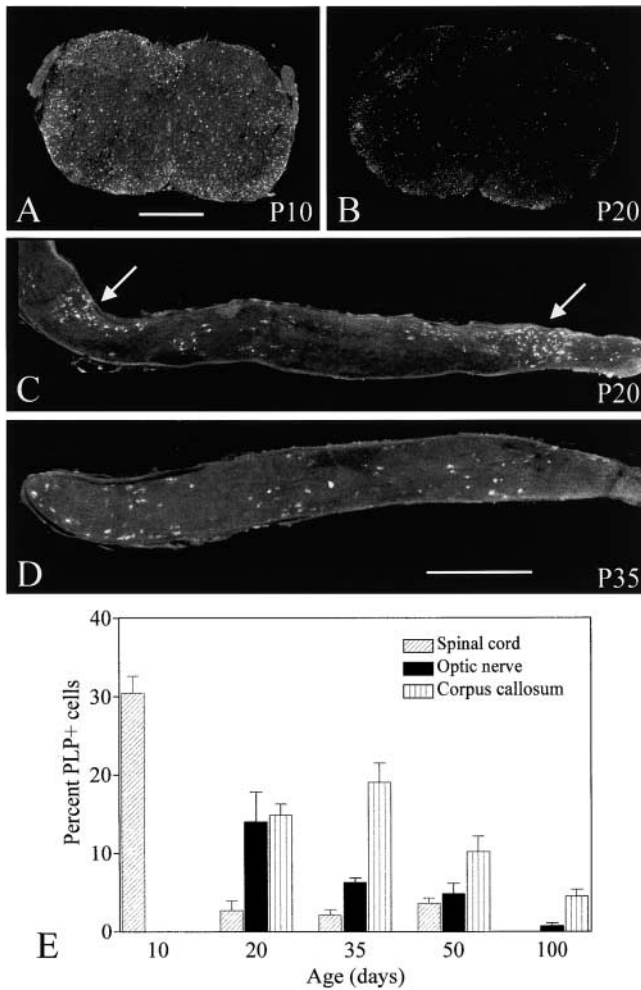
Using PLP-specific antibodies, we immunolabeled the cell bodies and major processes of oligodendrocytes expressing the mutant allele. As the mutant PLP is largely retained in the cell body (Gow et al., 1994; Gow and Lazzarini, 1996; Jung et al., 1996), any myelin sheaths formed by these oligodendrocytes remain unstained. For the purpose of quantification, the number of *jimpy* (PLP<sup>+</sup>) cells was expressed as a percentage of the total number of mature oligodendrocytes (adenomatous polyposis coli [APC]<sup>+</sup>). The results mirrored



**Figure 5. Female compound heterozygotes expressing *jimpy* and null alleles of the *Plp* gene hybridized for *Plp/Dm20* mRNA.** (A) Dark field autoradiograms of cervical spinal cord from mice at P5, P20, P35, and P100. At P5 and P20, many positive cells are present, but by P35 and P100, their numbers have reduced considerably. Bars, 1 mm. (B) Brightfield autoradiogram, counterstained with haematoxylin, from the corpus callosum of a P100 mouse with a several positive cells present. Bar, 50  $\mu$ m.

those produced by in situ hybridization with an age-related decrease in *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 myelinates early, the proportion of *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, *jimpy* cells were still detectable in the optic nerve and forebrain at P100 and even P280 (unpublished data). The oligodendrocytes expressing the *jimpy* allele were clearly identifiable within the myelinated white matter generated by the *Plp*-null cells (Fig. 7 A). Foci of immunopositive oligodendrocytes were often ob-





**Figure 6. Female compound heterozygotes expressing *jimpy* and null alleles of the *Plp* gene.** (A and B) Cervical spinal cords at P10 and P20, respectively, immunostained for PLP to identify those oligodendrocytes expressing the *jimpy* allele. There is a marked decrease in cells between the two ages. Whereas at P10 most of the positive cells are distributed in white matter, at P20, there are relatively more in the gray matter. (C and D) Optic nerves from mice aged P20 and P35, respectively, also immunostained to show the oligodendrocytes expressing the *jimpy* allele. The P20 nerve (C) shows two clusters of *jimpy* cells (arrows) together with more dispersed cells, whereas at P35, the cells are mainly dispersed. Bars, 0.5 mm. (E) Quantification of oligodendrocytes expressing the *jimpy* allele in different regions of the CNS of the compound heterozygotes. The results (mean  $\pm$  SEM) are expressed as the percentage of PLP<sup>+</sup> cells relative to the number of APC<sup>+</sup> cells. The APC antigen marks mature oligodendrocytes. The age of the decline in *jimpy* cells varies between regions, corresponding to the order in which the regions normally myelinate. In the spinal cord, there is a significant decrease between P10 and P20 ( $P < 0.001$ ), in the optic nerve between P20 and P50 ( $P < 0.05$ ), and in the corpus callosum between P35 and P50 ( $P < 0.05$ ). Group sizes are between four and six.

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<sup>+</sup> (*jimpy*) oligo-

dendrocytes 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-Bonnel, 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<sup>+</sup>/BrdU<sup>+</sup> (Fig. 7 G). In the optic nerve, for example,  $2.8 \pm 1.5\%$  (mean  $\pm$  SEM,  $n = 3$ ) of *jimpy* oligodendrocytes had incorporated BrdU. Numerous PLP<sup>-</sup>/BrdU<sup>+</sup> cells were present throughout the CNS (Fig. 7 G); in the optic nerve,  $51 \pm 10\%$  (mean  $\pm$  SEM,  $n = 2$ ) of BrdU-labeled cells immunostained for NG2 (Fig. 7 H),  $26 \pm 6\%$  for APC, and  $12.7 \pm 5\%$  stained for CD45. We were unable to identify any BrdU-labeled cells costained for caspase 3.

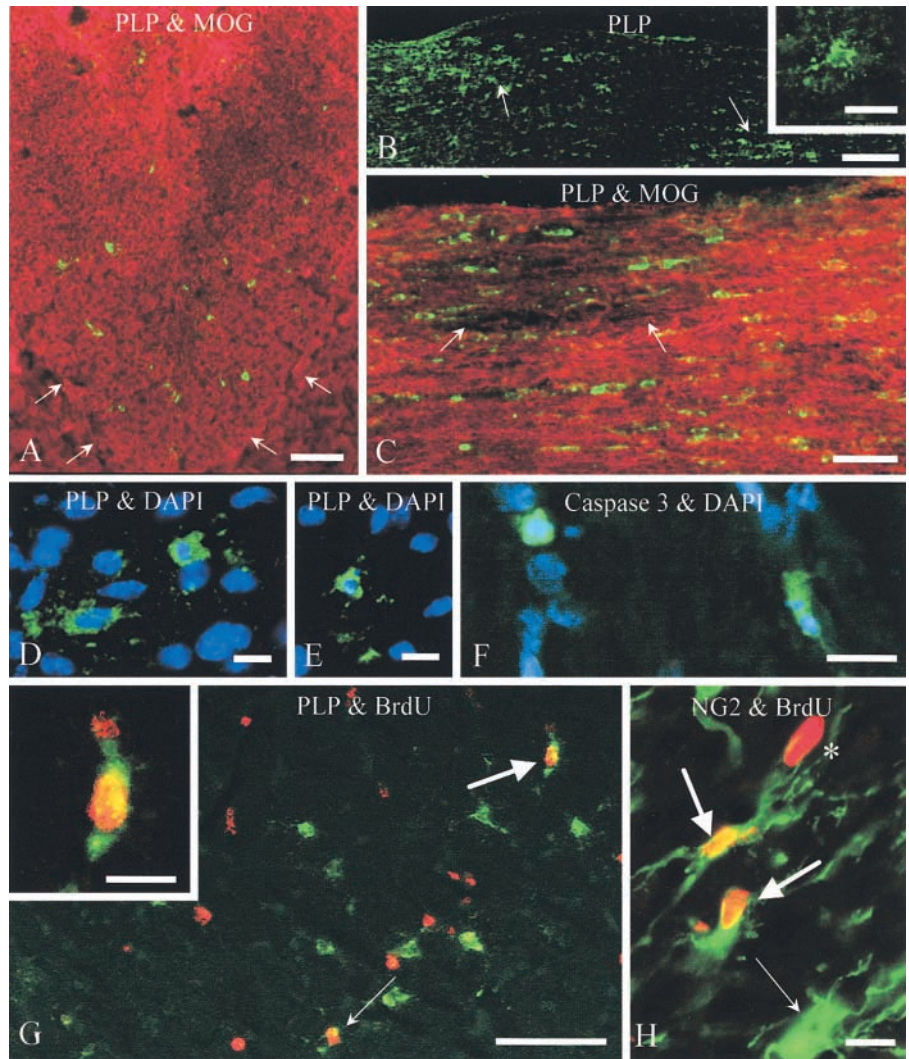
#### A hierarchy in the survival of oligodendrocytes expressing different *Plp* alleles

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 ( $Plp^{jp/jp-rsh}$ ) 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 ( $Plp^{rsh-rsh}$ ). The prolonged survival of these compound heterozygotes expressing *jimpy* and *rumpshaker* alleles is in marked distinction to *jimpy* males ( $Plp^{jp/Y}$ ), which die at 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<sup>+</sup> 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-

**Figure 7. Female compound heterozygotes expressing *jimpy* and null alleles of the *Plp* gene.** (A) Dorsal column from P20 mouse immunostained for PLP (green) to show oligodendrocytes expressing the *jimpy* allele and for MOG (red). Scattered *jimpy* oligodendrocytes are present in the white matter, although they represent considerably <50% of the expected total oligodendrocyte population. The base of the dorsal columns is outlined by arrows. Bar, 50  $\mu$ m. (B) Optic nerve from P20 mouse immunostained for PLP to show clusters of oligodendrocytes (arrows) expressing the *jimpy* allele. Bar, 100  $\mu$ m. (Inset) Higher magnification of individual *jimpy* oligodendrocyte showing immunostained cell body and processes but no obvious labeling of any myelin sheaths. Bar, 20  $\mu$ m. (C) Optic nerve from P20 mouse stained for PLP (green) and MOG (red) to show a small dysmyelinated region (arrows) of deficient MOG staining, associated with oligodendrocytes expressing the *jimpy* allele. Other *jimpy* cells are scattered through the myelinated region of the nerve. Bar, 50  $\mu$ m. (D and E) Optic nerve from P20 mouse immunostained for PLP (green) to identify oligodendrocytes expressing the *jimpy* allele and DAPI (blue) to show three *jimpy* cells with pyknotic nuclei indicating apoptosis. Bars, 10  $\mu$ m. (F) Corpus callosum from P20 mouse immunostained for caspase 3 (green) to show two dying putative *jimpy* oligodendrocytes. Nuclei are stained with DAPI. Bar, 10  $\mu$ m. (G) Corpus callosum from P34 mouse that was injected with BrdU on three successive days from P30 to P32 and the tissue was immunostained for PLP (green) and BrdU (red). Many PLP<sup>+</sup> *jimpy* oligodendrocytes are present, only two of which have incorporated BrdU into their nuclei (arrows). One of these (larger arrow) is shown at higher magnification in the inset. Many other cells of unknown phenotype have also incorporated BrdU. Bar, 50  $\mu$ m. Inset bar, 10  $\mu$ m. (H) Optic nerve from P34 mouse injected with BrdU, as above, and coimmunostained for NG2; two double-labeled cells (large arrows) are present and an NG2<sup>+</sup> cell with an unlabeled nucleus (small arrow). A BrdU-labeled nucleus from another cell type (\*) is also evident. Bar, 10  $\mu$ m.

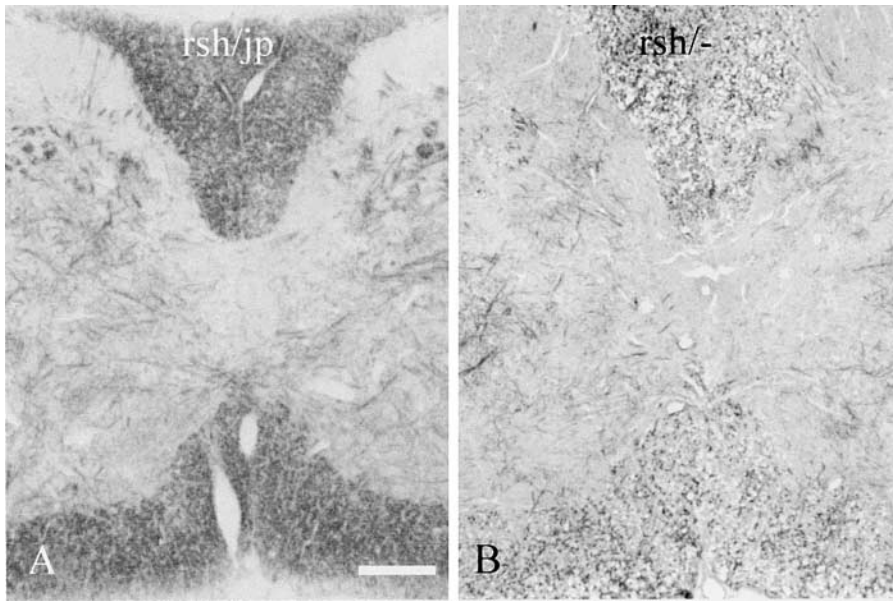


pared this with the male *rumpshaker* littermates. This age is before when apoptosis of mutant oligodendrocytes occurs; the *rumpshaker* PLP is partially retained in the cell bodies, making them easy to distinguish from myelin sheaths or cell processes. The number of PLP<sup>+</sup> cells in the compound heterozygote was  $125 \pm 25$  cells/mm<sup>2</sup> (mean  $\pm$  SEM,  $n = 4$ ), a value not significantly different from 50% of the population in male littermates,  $112 \pm 16$ . We used the same method to show that the number of PLP<sup>+</sup> 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<sup>+</sup> *jimpy* cells was  $133 \pm 13$  (mean  $\pm$  SEM,  $n = 3$ ), compared with  $121 \pm 5$ , which represented 50% of the total APC<sup>+</sup> 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<sup>+</sup> patches were present in the heterozygotes. In contrast, the deposition of the myelinating PLP<sup>+</sup> 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





**Figure 8. Female compound heterozygotes expressing either *rumpshaker* and *jimpy* alleles or *rumpshaker* and null alleles of the *Plp* gene.** (A) Compound female heterozygote mouse aged P50 expressing *rumpshaker* and *jimpy* alleles of the *Plp* gene (*rsh/jp*). A section of spinal cord showing the dorsal and ventral columns and central gray matter is immunostained with an antibody against the COOH terminus of PLP/DM20. As this region of the protein is deleted in the *jimpy* product, the staining detects only the myelin sheaths and oligodendrocytes expressing the *rumpshaker* allele. The vast majority of the cells and myelin are of *rumpshaker* origin. (B) Similarly stained section from a P50 compound heterozygote expressing *rumpshaker* and null alleles (*rsh/-*). In contrast to the previous animal, the majority of cells and sheaths express the null allele. Bar, 100  $\mu$ m.

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 *jimpy* allele are indeed lost over time and introduces the concept of a competitive hierarchy between cells expressing different alleles of the *Plp* gene.

### The starting populations of mutant cells in the heterozygotes are not reduced

Our results show that in compound heterozygotes, oligodendrocytes expressing *rumpshaker* or *jimpy* alleles of the *Plp* gene are generated in approximately equal numbers as those expressing the null allele. Similarly, in heterozygotes express-

ing wild-type or null alleles, the number of wild-type cells is normal; so from this we infer that those expressing the null allele are not reduced. This finding is not unexpected, as studies of affected male animals with *Plp* mutations indicate that the generation and initial populations of oligodendrocytes are normal (Pringle et al., 1997; Thomson et al., 1999). Because the starting populations of mutant oligodendrocytes are not reduced, subsequent cell loss is likely to account for the diminished proportions of mutant cells in the older mice.

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

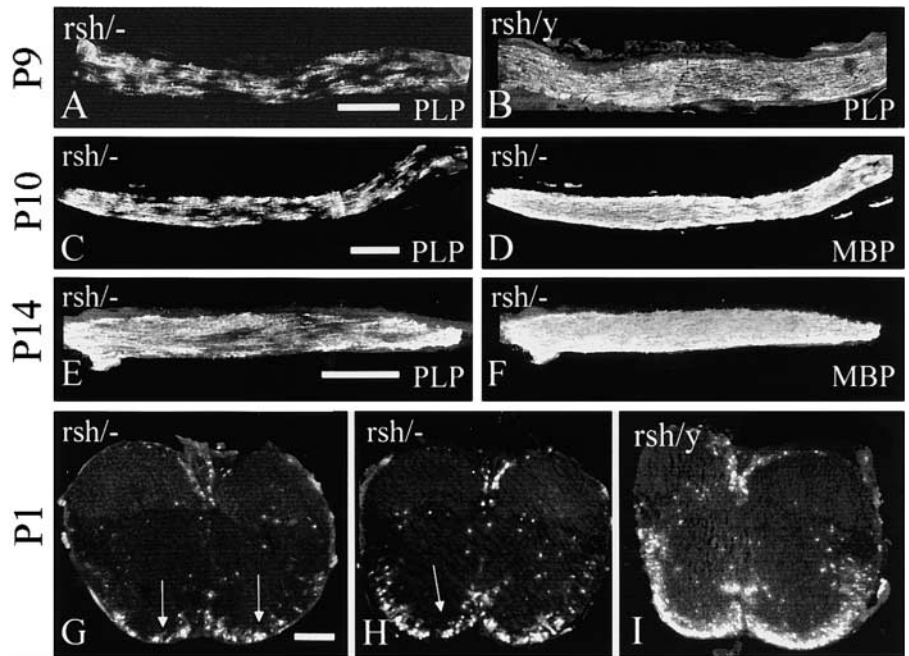
Spontaneous missense mutations of the *Plp* gene are associated with increased numbers of dead oligodendrocytes in affected males. The magnitude of cell death varies between different mutations, being greater in those associated with severe phenotypes (Knapp et al., 1986; Schneider et al., 1992). Misfolded 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,

Table I. Strategy for detecting oligodendrocytes expressing different alleles of the *Plp* gene in the various heterozygotes

Genotype of heterozygotes		Riboprobe or antibody			
		ISH		Immunostain	
		<i>Plp/Dm20</i>	<i>Plp/Dm20</i> exon 5	PLP-CT	PLP specific
WT	null	WT <sup>+</sup>	ND	WT <sup>+</sup>	ND
null	<i>rumpshaker</i>	<i>rumpshaker</i> <sup>+</sup>	ND	<i>rumpshaker</i> <sup>+</sup>	ND
null	<i>jimpy</i>	<i>jimpy</i> <sup>+</sup>	Both negative	<i>jimpy</i> <sup>-</sup>	<i>jimpy</i> <sup>+</sup>
<i>rumpshaker</i>	<i>jimpy</i>	ND	ND	<i>rumpshaker</i> <sup>+</sup>	ND

The genotype indicates the two alleles at the X-linked *Plp* locus. The riboprobe for *Plp/Dm20* detects the majority of the coding region for the two transcripts. Exon 5 of the *Plp/Dm20* transcript is deleted in *jimpy*. When used in conjunction with the *Plp/Dm20* 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.

**Figure 9. Distribution of competing cell populations in compound heterozygotes expressing null and *rumpshaker* alleles during early myelination of the optic nerve and spinal cord.** (A and B) Optic nerves at P9 from compound heterozygote (*rsh*<sup>-/-</sup>) and affected male littermate (*rsh*<sup>/Y</sup>) stained for PLP/DM20, showing non-uniform distribution of PLP<sup>+</sup> (*rumpshaker*) cells in the heterozygote. (C–F) Optic nerves from compound heterozygotes at P10 and P14, respectively, showing the patches of PLP<sup>+</sup> *rumpshaker* cells compared with the uniform MBP staining of the same nerve. (G–I) Spinal cords from compound heterozygotes and an affected male littermate at P1, stained for PLP. The heterozygotes show small clumps (arrows) of *rumpshaker* oligodendrocytes and myelin, in contrast to the male, in which the distribution is relatively uniform in the ventral white matter. The size and number of patches are less marked than in the optic nerves. Bars, 200  $\mu$ m.



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 *rumpshaker* cells fare better than *jimpy* oligodendrocytes. Using the amount of *rumpshaker* myelin as an indicator, <25% of cells expressing the *rumpshaker* 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<sup>+</sup> 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 (Levison 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 myelinogenesis, 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 amyelinated 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 amyelinated 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 amyelinated 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<sup>+</sup> 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 cur-

rently 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/+</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/jp-rsh</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 C57BL6 background, has been previously described (Klugmann et al., 1997; Griffiths et al., 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>jp/-</sup> and *Plp*<sup>jp-rsh/-</sup>). *jimpy* heterozygotes were mated with *rumpshaker* males to generate female mice expressing both mutant alleles (*Plp*<sup>jp/jp-rsh</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/g 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 periodate-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 Fintek), 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 saggital sections of the brain were hybridized with the <sup>35</sup>S-labeled or DIG-labeled *PLP-1* riboprobe detecting both *Plp* and *Dm20* transcripts as previously described (Griffiths et al., 1989; Vouyiouklis 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 monoclonal antibody (CC-1 clone; Oncogene 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  $\mu$ m) and paraffin wax sections (8  $\mu$ m) were immunostained with the anti-PLP COOH-terminal antibody using the peroxidase antiperoxidase technique (Sternberger et al., 1970; Trapp et al., 1981). Cryosections (15  $\mu$ m) 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 immunostained for PLP or caspase 3. For NG2, APC, or CD45 double label with BrdU, cryostat 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  $\mu$ g/ml DAPI in H<sub>2</sub>O for 1 min at room temperature. Cryosections were mounted in Citifluor antifade medium.

## Quantification of PLP<sup>+</sup> myelin sheaths and cells

To determine the proportion of PLP<sup>+</sup> and PLP<sup>−</sup> myelin sheaths in *Plp<sup>jp-rsh1</sup>* 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<sup>+</sup> cells were determined in various regions of the CNS in *Plp<sup>jp-rsh1</sup>* 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 *jimpy* 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<sup>+</sup> cells occurred randomly, the fields for imaging were selected under phase optics ( $\times 20$  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<sup>+</sup> 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 capricious 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 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  $\times$  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  $P \leq 0.5$ . Analyses were performed using the GraphPad Prism software (GraphPad Software).

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