Oligodendrocyte ablation impairs cerebellum development

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Summary

Oligodendrocytes (OLs) are the glial cells of the central nervous system and are classically known to form myelin sheaths around most axons of higher vertebrates. Whether these cells might have other roles, in particular during development, has not been studied. Taking advantage of a transgenic mouse model in which OLs can be selectively killed in a desired time-frame, we have investigated the impact of OL ablation on cerebellar development. OL ablation was induced during the first 3 postnatal weeks, a time at which cerebellum development is ongoing. Strikingly, OL ablation triggers a profound perturbation of the known cerebellum developmental program, characterized by the disorganization of the cortical layers, abnormal foliation and a complete alteration of Purkinje cell dendritic arborization and axonal fasciculation. This phenotype is accompained by decreased granule cell density, a disorganized Bergmann glia network and impaired migration of interneurons in the molecular layer. These results demonstrate a previously ignored role of OLs in the formation of the cerebellar cytoarchitecture.

Key words: Oligodendrocytes, TK ablation system, Transgenic mice, Cerebellum, Development

Introduction

Cerebellar development in the mouse initiates during embryogenesis but extends into postnatal development (Goldowitz and Hamre, 1998; Hatten and Heintz, 1995). The cerebellar cortex is a highly organized structure, composed of a set of well-characterized neurons whose development, organization and finely tuned circuitry has been extensively studied (Ramón y Cajal, 1889; Ramón y Cajal, 1911; Ramón y Cajal, 1960; Miale and Sidman, 1961; Palay and Chan-Palay, 1974; Rakic, 1971). Purkinje neurons are the master neurons of the cerebellar cortex, being the only efferent relay to the deep nuclei and hence to the central nervous system (CNS). These cells are born during embryogenesis and form a monolayer, which in the adult sets the limits between the molecular and the granule cell layers. Purkinje neurons have a bushy dendritic tree, which extends into the molecular layer (ML) of the cerebellar cortex. During postnatal development, concomitant with Purkinje dendrite extensions, connections are formed with the ingrowing afferent axons from pre-cerebellar nuclei (the climbing fibers), as well as directly or indirectly with cerebellar interneurons. Granule cells are the most abundant interneurons of the cerebellum. Progenitors of these neurons actively proliferate during the first 2 postnatal weeks in the most superficial layer of the developing cerebellar cortex, the external granular layer (EGL). As the inward migration of granule cells progresses to form the internal granular layer (IGL), the EGL disappears. In the ML, granule neurons make synaptic contacts with the dendritic trees of Purkinje cells, and with basket and stellate interneurons. In the IGL, granule cells contact Golgi interneurons, and both receive information from efferent mossy fibers. Through these series of synaptic contacts, Purkinje cells receive information from cerebellar and extracerebellar inputs, thus generating an integrated output to the CNS.

The participation of glial cells in cerebellum development is also very important. The Bergmann glia, a specialized type of astrocyte, provides a scaffold for cerebellar neuron migration and positioning (Rakic, 1971), as well as for Purkinje cell dendritic outgrowth and synapse formation (Yamada et al., 2000). Selective ablation of these cells in the early postnatal period strongly impairs cerebellum development (Delaney et al., 1996). Astrocytes have also been shown to induce and stabilize CNS synapses (Ullian et al., 2001). The role of oligodendrocytes, the CNS myelin forming cells, in cerebellum development has not been clearly addressed. However, neuron-oligodendrocyte interaction at the axonal level is required for the maturation of CNS neurons (Brady et al., 1999; Colello et al., 1994; Colello and Schwab, 1994; Mathis et al., 2000), and for the formation and maintenance of the nodes of Ranvier and the paranodal regions (Arroyo et al., 2002; Mathis et al., 2001). Furthermore, knockout of contactin, a protein localized both in neurons and oligodendrocytes (Koch et al., 1997), leads to ataxia consequent to an abnormal microorganization of the cerebellum (Berglund et al., 1999). These results suggest a possible role for oligodendrocytes in cerebellum development.

To explore this possibility, we used transgenic mice in which herpes simplex virus 1 thymidine kinase (HSV1-TK) was targeted to OLs under the control of the MBP promoter. In these mice, OLs can be ablated in a temporal inducible manner (Mathis et al., 2000) by injection of FIAU, a non-harmful nucleoside analog that is converted to a toxic compound by TK activity (Borrelli et al., 1988; Borrelli et al., 1989). In this way,

the effects of oligodendrocyte ablation can be studied within the context of cerebellar development.

Loss of oligodendrocytes during the first postnatal weeks results in a profound perturbation of the cerebellar cytoarchitecture. In particular, Purkinje cells do not align properly and fail to form dendritic arborizations. We suggest that oligodendrocytes may be crucial for the proper maturation of Purkinje cells due to a loss of oligodendrocytes-myelin/neuron interactions. As a consequence of this, the terminal differentiation of the complete cerebellar circuitry is aborted leading to a severe phenotype. Importantly, the effect of oligodendrocyte ablation on cerebellar development is time-dependent. Furthermore, loss of these cells was concomitantly associated with the disappearance of basket and stellate cell interneurons in the ML. This striking observation, together with the white matter origin of these cerebellar interneurons (Milosevic and Goldman, 2001; Zhang and Goldman, 1996b), raises the possibility of a key role for oligodendrocytes and/or myelin in the survival/migration of these interneurons. These results uncover a previously unknown and important role for oligodendrocytes during cerebellar development.

Materials and methods

Animals and tissue preparation

MBP-TK mice were generated as described (Mathis et al., 2000). In each experiment, wild-type and transgenic (MBP-TK) siblings were treated equally with the nucleoside analog FIAU [1-(2-deoxy-2-fluoro-β-δ-arabinofuranosyl)-5-iodouracil] by subcutaneous daily injections (40 mg/kg). The following treatments were performed 1-20 days (d), 1-3 d, 1-6 d, 3-20 d and 6-20 d. All animals were killed at the age of 21 days. For developmental studies, MBP-TK and wild-type mice treatments were started 24 hours after birth, and continued until the day of the test (2, 3, 6, 10 and 15 postnatal day). Each treatment has been repeated at least on 10 different litters, and a minimum of six different mice for each treatment and genotype have been analyzed. Animal genotypes were determined as previously described (Mathis et al., 2000). For histological studies, brain tissues were embedded in paraffin wax after fixation in BOUIN's fixative. Sagittal sections (10 μm thick) were made using the microtome and stained using Cresyl Violet.

Immunohistochemistry

Immunofluorescence analyses were carried out on cerebellar sections, which were either 4% paraformaldhyde-fixed floating vibratome sections (100 μm) or cryosections (10 μm) fixed in formalin (Sigma), or on paraffin wax embedded tissue fixed in BOUIN (10 μm), depending on the primary antibody used, following the previously described protocols (Mathis et al., 2000). Antibody dilutions were as follows: rabbit anti-PAX2 (Zymed), 1:400; mouse anti-calbindin D-28K (SWant), 1:3000; rabbit anti-BLBP (a generous gift of Dr N. Heintz), 1:1500; rabbit anti-HSV1-TK (kindly provided by Dr P. Collins, Glaxo Wellcome, Beckenham, UK), 1:200; rabbit anti-PH3 (Upstate Biotechnology, Lake Placid, NY), 1:5000; rabbit anti-synaptophysin (Dako), 1:50; mouse anti-parvalbumin (Chemicon), 1:1000; mouse anti-MBP (Chemicon), 1:1000; mouse anti-GALC (Roche), 1:50; mouse anti-PLP (Chemicon), 1:800; and goat anti-rabbit Alexa 594 and goat anti-mouse Alexa 488 (Molecular Probes), 1:600. Negative controls were always performed by omitting the primary antibody. TUNEL experiments were performed on brain cryosections post-fixed in 1% paraformaldehyde in PBS, using the In Situ Cell Death Detection Kit (Roche, Germany) and dUTP-coupled with Alexa Fluor 488 (Molecular Probes). Immunolabeled sections were examined with a conventional microscope (Axiophot; Zeiss, Overbroken, Germany), and/or with a confocal microscope (DMRE; Leica, Nussloch, Germany).

In situ hybridization

In situ hybridization was performed as previously described (Mathis et al., 2000). ^{35}S-labeled RNA probes encoding MBP, PAX2, PAX6, GAD67 (GAD1 – Mouse Genome Informatics), zebrin-II (ALDO3 – Mouse Genome Informatics) sense and antisense riboprobes were synthesized using T3, T7 or SP6 polymerase in the presence of cytidine 5′-α[^{35}S]thiotriphosphate (10 mCi/ml, Amersham), according to the supplier's directions (Stratagene, Biolabs). After probe hybridization, slides were coated with Kodak NTB2 emulsion and stored at 4°C. Emulsions were finally developed in Kodak 19 and tissues were counter-stained with Toluidine Blue.

Basket and Stellate cells counts

Cresyl Violet stained sections from wild-type and MBP-TK mice treated with different protocols of FIAU injections (1-20 d, 1-6 d and 6-20 d) were used to estimate the proportion of interneurons located in the ML of the cerebellum. Anatomically matched cerebellum sections of wild-type and MBP-TK siblings identically treated were taken for this study. The number of fields of view (FOV=600 μm^2) examined varied between 25 and 35. Statistical significance was assessed by ANOVA (Bartlett's test).

Results

Myelination in the cerebellum

Myelin formation in the CNS occurs during the first weeks of postnatal development. The temporal and spatial characteristics of oligodendrocyte maturation and myelin formation have been extensively studied using different approaches and animal models (Akiyama et al., 2002; Dixon and Eng, 1984; Kristensson et al., 1986; Reynolds and Wilkin, 1988; Skoff et al., 1976; Skoff et al., 1980; Trapp et al., 1987). Thus, it has been proposed that myelination proceeds in a strictly rostrocaudal direction in the CNS, with the exception of the dorsal spinal cord, where myelination starts first in the cervical enlargement and continues in both rostral and caudal directions (Foran and Peterson, 1992).

Owing to its postnatal development, the cerebellum represents an ideal model system to analyze the impact of oligodendrocyte ablation on neighboring cells. MBP-TK transgenic mice expressing the HSV1-TK gene under the control of a 1.3 kb fragment of the myelin basic protein (MBP) promoter were used in this study. The MBP-TK transgene is specifically expressed in oligodendrocytes, as illustrated by the co-localization of MBP and TK immunostaining in the same cells (Fig. 1A-D) (Mathis et al., 2000). The normal white matter tract location and the density of TK-expressing oligodendrocytes is shown at lower magnification in cerebellar folia IX of p6 MBP-TK untreated mice (Fig. 1E). Upon nucleoside analog [i.e. FIAU (McLaren et al., 1985)] administration, these cells are selectively killed (Fig. 1F) (Borrelli et al., 1988; Mathis et al., 2001; Mathis et al., 2000). This property allowed us to induce oligodendrocyte cell death in vivo, upon FIAU treatment (Mathis et al., 2000). We chose a treatment protocol that was able to induce 95% oligodendrocyte ablation in the brain. MBP-TK and wild-type littermates were administered FIAU through subcutaneous daily injections (40 mg/Kg), starting 24 hours after birth until postnatal day 20 (1-20d) (Mathis et al., 2000). Wild-type and transgenic animals were killed at day 21 (P21) after birth and analyzed.

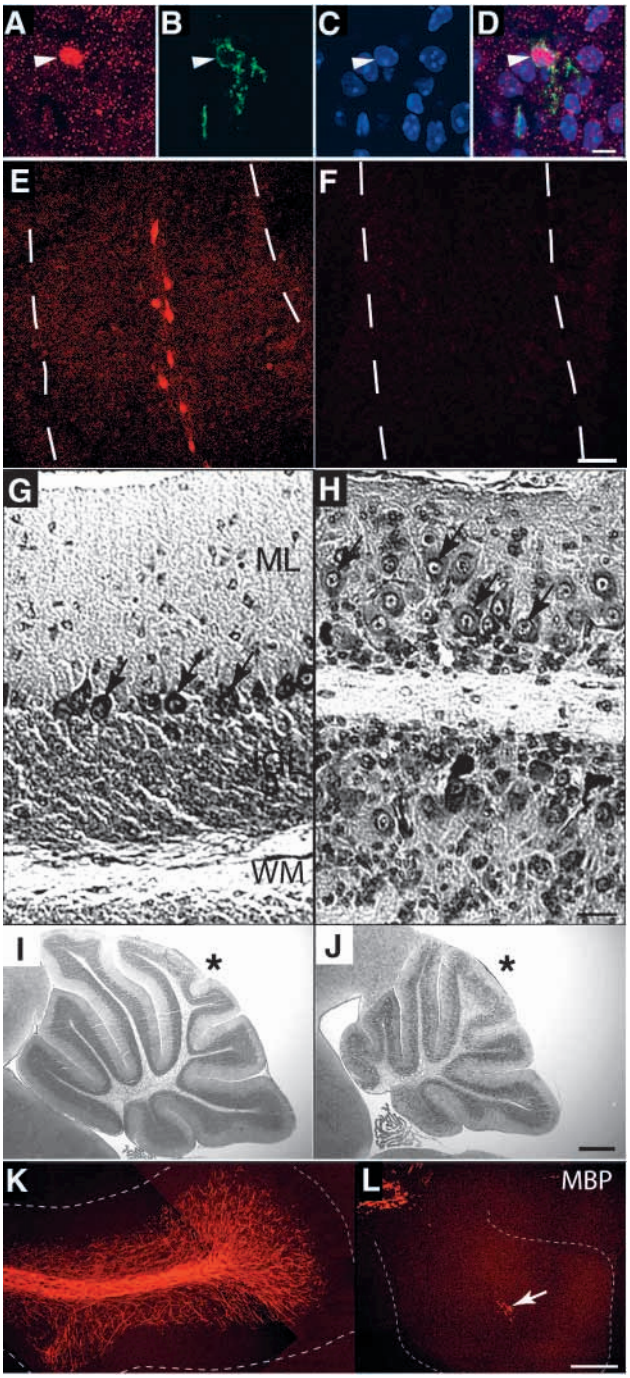

Fig. 1. Ablation of oligodendrocytes alters cerebellum development. (A-D) HSV1-TK expression is restricted to MBP-positive oligodendrocytes (arrowheads). Cerebellar sections from a 2-day-old (P2) untreated MBP-TK mouse were double stained with anti-HSV1-TK (red) and MBP (green) antibodies. Nuclei were stained with DAPI (blue). (D) Merging of the different stains shows nuclear localization of TK in MBP-positive cells. Sections were analyzed by confocal microscopy. (E,F) Immunofluorescence analysis of TK-positive oligodendrocytes in cerebellar folia IX of P6 untreated (E) versus treated (F) MBP-TK mice, using the anti-HSV1-TK antibody. (G-J) Cresyl Violet staining of paraffin wax-embedded cerebella sagittal sections from wild-type (G,I) and MBP-TK (H,J) mice treated from day 1 to 20. The cerebellum of the treated wild-type animal presents a normal architecture both at high (G) and low (I) magnification. The cerebellum of the treated MBP-TK mice is smaller with an abnormal foliation (J; star) and a highly disorganized cortex (H). The fissura intercruralis in both wild-type and MBP-TK mice is indicated by a star (I,J). Arrows (G,H) show Purkinje cell bodies. ML, molecular layer; IGL, internal granular layer; WM, white matter tract. (K,L) Cerebellar sagittal sections immunostained with an anti-MBP antibody from 3-week-old wild-type (K) and MBP-TK (L) mice treated from day 1 to 21. Note rare MBP-positive fibers in treated MBP-TK mice (arrow; L). Scale bars: 5 μm (A-D), 80μm (E-F), 30 μm (G-H), 300 μm (I-J), 100 μm (K-L).

Oligodendrocyte ablation strongly affects cerebellar architecture

Macroscopic observation of the cerebellum of transgenic treated mice revealed a significant reduction in the size of this organ compared with that of treated wild-type littermates. To further characterize the cerebellar phenotype of these animals, histological analyses were performed using Cresyl Violet staining (Fig. 1G-H).

Sagittal sections from wild-type treated cerebella (1-20d) showed the classical morphology (Fig. 1G,I). In wild-type mice cerebellar folia are well formed and are separated by deep fissures. The classical cortical organization is identifiable by the three well known layers (Fig. 1G). The ML containing fibers and interneurons are underlined by the large soma monolayer of Purkinje neurons (PCL). Just beneath this layer is the IGL, composed of very densely packed soma of small neurons, the GCs, and the soma of some scattered larger neurons, the Golgi cells. Thus, FIAU treatment does not alter the structure and composition of the cerebellum in treated wild-type mice, as previously reported in the brain and other organs (Borrelli et al., 1988; Borrelli et al., 1989; Heyman et al., 1989; Mathis et al., 2000). Similarly, untreated MBP-TK transgenic mice did not show any sign of cerebellar abnormality (data not shown).

By contrast, 1-20d FIAU-treated MBP-TK mice present an outstanding phenotype, associated with the almost complete ablation (95%) of oligodendrocytes (Mathis et al., 2000). As shown in Fig. 1J, the fissura intercruralis, which normally separates folia VI and VII in the wild type (Fig. 1I), is absent in MBP-TK mice (Fig. 1J, star). At higher magnification, the laminar distribution of cerebello-cortical neurons is highly altered (Fig. 1H) when compared with wild-type mice (Fig. 1G), and the size of the folia is greatly reduced. This phenotype is accompanied by a misalignment of the Purkinje cell soma, which appears dispersed among a reduced population of GCs. In addition, the ML is not clearly defined in these animals. These results indicate that the postnatal ablation of oligodendrocytes and consequent dysmyelination results in a major impairment of cerebellar development.

Cellular abnormalities in the cerebellum of treated MBP-TK mice

The white matter tract of the cerebellum is composed of Purkinje cell axons and the two afferent pathways of the cerebellum, the climbing and the mossy fibers. Mossy fibers are connected to GC and Golgi neurons. Climbing fibers have synaptic contacts with Purkinje cells and their primary dendrites in the ML. Myelinated fibers are visualized (Fig. 1K,L) by immunofluorescence with an antibody raised against

MBP. At P21, all of these fibers are fully myelinated in treated wild-type mice (Fig. 1K). Oligodendrocyte ablation, in treated MBP-TK mice, results in a drastic reduction of myelinated fibers in the white matter tract, where only a few spared MBP-positive fibers can still be observed (Fig. 1L, arrow). These fibers are myelinated by residual oligodendrocytes that escaped ablation; they were probably postmitotic at the time the treatment was started (Mathis et al., 2000). Quantification by mRNA analysis of myelin markers shows a very strong reduction of these proteins. Indeed, the MBP mRNA level, analyzed at P21, was reduced by 90±10% (n=15) with respect to treated wild-type mice.

Reactive astrocytes and disorganized Bergmann glia network are observed in treated MBP-TK mouse cerebellum

Next, we analyzed a second type of glial cell contained in the cerebellum, the Bergmann glia. These cells are a specialized type of astrocyte that express two well characterized astrocytic markers: BLBP (brain lipid binding protein), during development, and GFAP (glial fibrillary acidic protein) in adult mature cells (Feng et al., 1994). Projections from these cells extend radially through the ML toward the cerebellar pial surface. These glial extensions are known to interact with migrating GCs and mature Purkinje neurons. Several studies have suggested that Bergmann glia are involved in the guidance of GC migration from the EGL to the IGL throughout the ML (Hatten, 1990; Hatten and Heintz, 1995; Rakic, 1971). In addition, the formation of the Bergmann glia network accompanies the formation of the Purkinje cell dendritic tree during development and synaptogenesis (Yamada et al., 2000). Using BLBP and GFAP, we assessed how loss of oligodendrocytes would affect Bergmann glia development. BLBP was analyzed at P6, as this marker has been reported to decrease in the second week after birth. As shown in Fig. 2A and C, these two astrocytic markers in treated wild-type mice show the classical palisade of Bergmann glia extensions in the ML, with glial cell bodies closely associated with Purkinje cell bodies. BLBP-positive cells are also present in the IGL, corresponding to astrocytes. In treated MBP-TK mice (Fig. 2B,D), BLBP-positive Bergmann glia cells were detected but their radial extensions into the ML were completely misaligned (Fig. 2D) with respect to those of wild-type siblings (Fig. 2C). As in the wild-type cerebella, BLBP-positive cells were also present in the IGL. GFAP immunostaining performed at P21 on cerebella from wild-type and MBP-TK mice confirmed the results obtained by BLBP staining in younger animals (Fig. 2C,D). We also quantified *Gfap* mRNA expression levels in the cerebella of treated wild-type versus treated MBP-TK mice. As previously reported, a 20±5% (n=15) increase of *Gfap* mRNA expression was noticed in treated MBP-TK mice when compared with wild-type littermates, which is in agreement with a normal astrocytic response to brain injuries.

Multicellular layer of Purkinje cells and altered dendritic formation in treated MBP-TK mice

Purkinje cells are born embryonically. During the first phase of postnatal development, these neurons are found in a pluricellular layer. Toward the end of cerebellar cortical development, Purkinje cells are aligned into a monolayer that

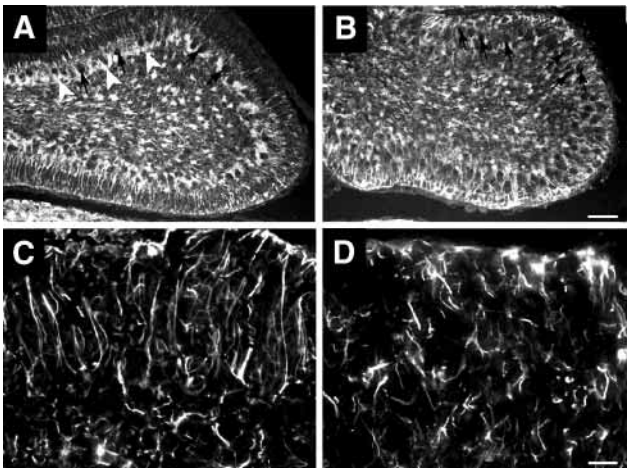

Fig. 2. Disorganization of the Bergmann glial network in young treated MBP-TK mice. (A-D) Immunofluorescence analysis of cerebellar sections from treated 6-day-old (A,B) or 21-day-old (C,D) wild-type (A,C) and MBP-TK (B,D) mice. Astrocytes were identified using specific antibodies directed against brain lipid binding protein (BLBP; A,B) and glial fibrillary acidic protein (GFAP; C,D). In the wild-type, the BLBP-positive soma of Bergmann glial cells (A; arrowheads) are located at the level of the Purkinje cell layer and their radial fibers extend across the ML toward the pial surface (C). In MBP-TK, Bergmann glial cells are dispersed throughout the folia (B) and their radial scaffold is strongly perturbed (D). Scale bars: 75 μm (A,B); 12 μm (C,D).

separates the ML from the IGL. The formation of this monolayer is generated by the coincident growth of the white matter tract and the migration of the GCs from the EGL into the IGL. At the same time, Purkinje neurons extend a dendritic tree toward the pial surface of the cerebellar folia. We visualized Purkinje cells on cerebellar sections of wild-type and MBP-TK treated mice by in situ hybridization and immunofluorescence using a zebrin-II probe (Fig. 3A,B) and the anti-calbindin antibody (Fig. 3C-F), respectively. A clear monolayer of Purkinje cell soma was observed in both approaches in wild-type 1-20d treated mice (Fig. 3A,C). In addition, these cells extended their bushy dendritic trees throughout the ML (Fig. 3C) and their axons were gathered together into bundles in the white matter tract (Fig. 3E), as observed by calbindin immunostaining in wild-type animals. Conversely, in transgenic 1-20d treated animals, Purkinje cells were packed into a multicellular layer (Fig. 3B,D). More importantly, their dendritic arborization was not ordered, appearing shorter and clearly misoriented, with defasciculated axons (Fig. 3D,F). This observation, probably accounts for the reduced size of the ML observed in the histological studies shown in Fig. 1 and demonstrates that Purkinje cell maturation is dependent on the presence of oligodendrocytes.

Reduction of the IGL density in the treated MBP-TK mice reflects the early disappearance of the EGL

Under normal conditions, the formation of the IGL results from the inward migration of postmitotic GCs, which are generated by active proliferation of their progenitors in the EGL. This germinal zone is observed only during the first 2 postnatal

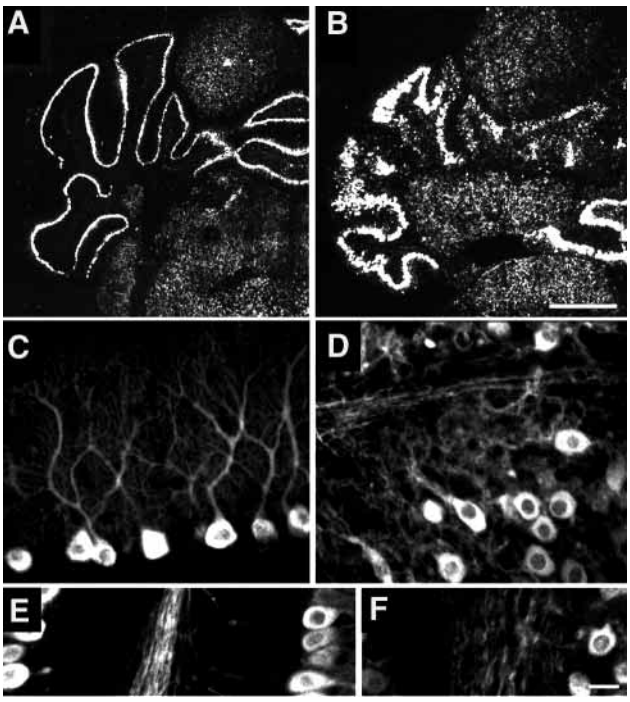

Fig. 3. Alteration of the Purkinje cell monolayer and dendritic formation in treated MBP-TK mice. (A,B) In situ hybridization using a zebrin-II antisense probe. (C-F) Immunofluorescence using an anti-calbindin antibody to visualize soma and dendritic extensions of Purkinje neurons. (A,B) Cerebellar coronal, and (C-F) sagittal sections of 3-week-old wild-type (A,C,E) and MBP-TK treated (B,D,F) (1-20d) mice. In wild type, Purkinje cell soma are aligned in a monolayer (A) and their dendritic tree extends throughout the ML (C). In MBP-TK cerebella, Purkinje cells form a multicellular layer (B) and the formation of their dendritic arborization is clearly altered (D). (E) Purkinje axons in wild type converge in the white matter tract to form bundles of myelinated fibers. (F) In MBP-TK mice, Purkinje axons are defasciculated. Scale bars: 75 μm (A,B); 25 μm (C-F).

weeks in the mouse, and its disappearance correlates with the formation of the IGL (Goldowitz and Hamre, 1998; Hatten and Heintz, 1995). Abnormal foliation is often associated with defects in EGL formation (Karagogeos and Pavlou, 1999).

Histological analyses of the cerebellar phenotype of treated MBP-TK mice (1-20d) showed an abnormal pattern of foliation (loss of fissura intercruralis) and a reduced IGL compared with wild-type littermates (Fig. 1G,H). A more in depth analysis of GCs in wild-type and MBP-TK 1-20d treated mice cerebella was performed using two markers, PAX6 and RU49. PAX6 is an early marker of these cells, which starts to be expressed when GCs are still proliferating in the EGL, and expression is maintained following migration into the IGL (Engelkamp et al., 1999). In situ hybridizations were performed on sagittal cerebellar sections from wild-type and MBP-TK treated mice at P6, using a PAX6 antisense riboprobe (Fig. 4A,B). These experiments showed that the EGL thickness in treated MBP-TK animals is reduced in the depth of the fissure as well as in the extremities of the folia (Fig. 4B; arrowheads) in comparison to that of treated wild-type siblings (Fig. 4A; arrowheads). In particular, a greater decrease of EGL depth is observed in the future position of the fissura intercruralis (Fig. 4B). This might explain the absence of this fissure in treated MBP-TK mice observed at P21. By P10, almost all of the EGL has disappeared in treated MBP-TK mice cerebella, with the exception of the fissura secunda and paraflocularis (data not shown). The decreased thickness of the EGL in MBP-TK mice is suggestive of an altered rate of proliferation of these cells in these mice compared with in wild-type animals. This hypothesis was confirmed by immunofluorescence studies using the anti-phosphorylated histone 3 (PH3) antibody that specifically labels mitotic cells (Fig. 4C,D). Moreover, TUNEL experiments performed on sections from these mice revealed a larger number of GC cells undergoing apoptosis in treated MBP-TK animals (Fig. 4F) in comparison to in treated wild-type mice (Fig. 4E). These data indicate that the reduced EGL size is the result of both a decrease of GC progenitor proliferation and increased apoptosis. These findings were confirmed at P21 by in situ hybridization using RU49 (Yang et al., 1996) as a probe (Fig. 4G,H), which labels GCs in the IGL. To exclude the possibility that the decrease of GCs could be due to an ectopic expression of the transgene in GC precursors, we performed double immunostaining using anti-PAX6 (Fig. 4I,L) and HSV1-TK (Fig. 4J,L) antibodies. This analysis showed the absence of TK expression in PAX6-positive cells in MBP-TK mice (Fig. 4I-L). These analyses were repeated at different time points and always gave negative results (data not shown).

Oligodendrocyte ablation is associated with the loss of interneuron migration

Interneurons have previously been shown to originate from a population of precursors proliferating in the white matter of the developing cerebellum (Zhang and Goldman, 1996b). Three types of GABAergic interneurons are present in the cerebellar cortex: the Golgi cells localized in the IGL, and the basket and stellate cells in the boundary and inside the ML. Golgi cells are the first interneurons to be generated and to migrate into the cerebellar cortex followed by basket and stellate cells (Zhang and Goldman, 1996a). To analyze the fate of interneurons in treated MBP-TK mice we used in situ hybridization and immunofluorescence analyses. Three types of markers were utilized: PAX2 to identify these cells during the first week of cerebellar development (Fig. 5A-F); glutamic acid decarboxylase (GAD67) (Fig. 5G,H); and parvalbumin in P21 mice (Fig. 5I,J). PAX2 immunofluorescence identified the presence of interneurons in the central region of the cerebellar folia in both wild-type and MBP-TK treated mice analyzed 6 days after birth (Fig. 5E,F). However, whereas in the wild-type sections a stream of PAX2-positive cells following the white matter tract was observed (Fig. 5E, arrowheads), in MBP-TK mice these cells were dispersed in the folia (Fig. 5F, arrowhead).

The analysis of sections of P21 mice using GAD67 in situ hybridization and parvalbumin immunofluorescence, respectively, showed a complete absence of interneuron migration in the ML of transgenic mice (Fig. 5H,J) versus wild-type littermates (Fig. 5G,I). The number of interneurons was then quantified in the ML of treated cerebella of P21 wild-type (15.88±1.26 cells/FOV) and MBP-TK (0.875±0.29 cells/FOV) mice. These results indicate that only a minimal number (5.5%) of interneurons are able to reach the ML in transgenic mice compared with in their treated wild-type siblings.

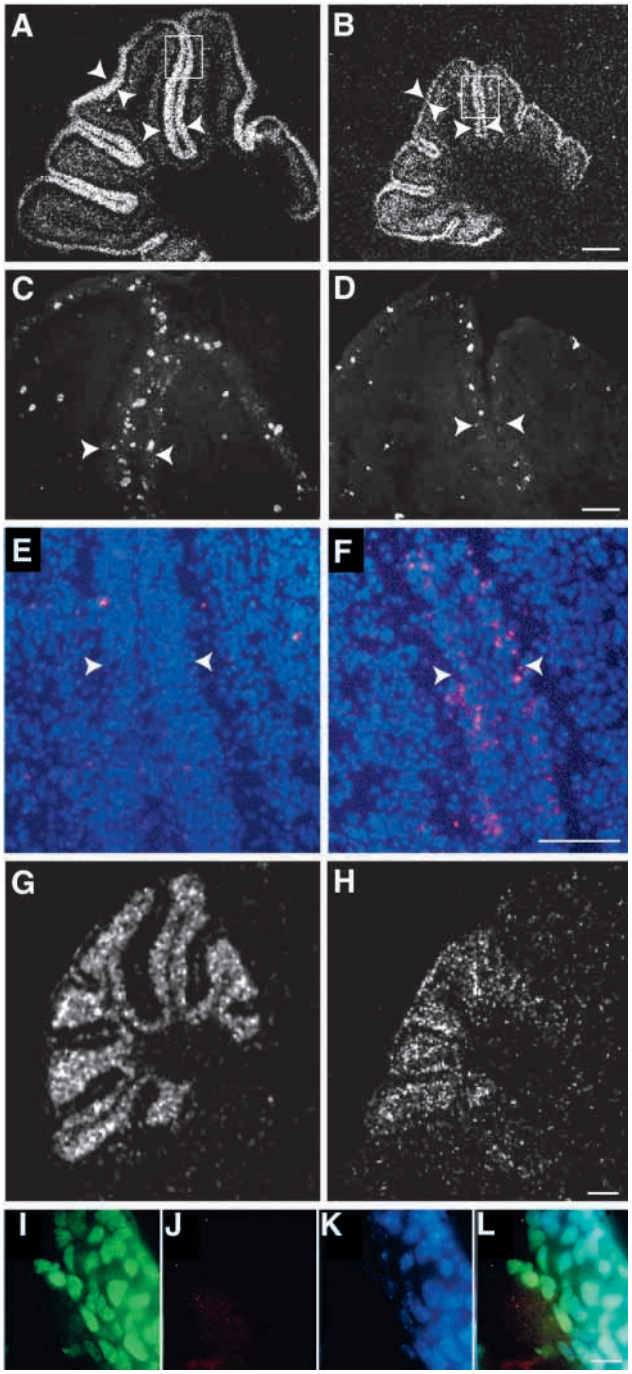

Fig. 4. The postnatal development of granule cells is altered in treated MBP-TK mice. Granule cells were analyzed in sagittal cerebellar cryosections from (A,C) wild-type and (B,D) MBP-TK 6-day-old treated mice. (A,B) In situ hybridization using a PAX6 antisense riboprobe to label granule cells in the EGL, and (C,D) immunofluorescence using an anti-phosphorylated histone 3 (PH3) antibody to reveal proliferating cells in this layer. Arrowheads delimit the thickness of the EGL in wild-type (A,C) and MBP-TK (B,D) cerebella. The arrowhead external to the cerebellum in D also indicates the reduced thickness of the EGL in the region in which the fissura intercruralis should have formed. Squares (A,B) indicate the regions that have been analyzed at higher magnification (C-F). (E,F) TUNEL fluorescent labeling of apoptotic nuclei in the EGL of treated 6-day-old (E) wild-type and (F) MBP-TK mice. Nuclei were counterstained with DAPI. (G,H) Dark-field micrographs of in situ hybridization experiments using a RU49 antisense riboprobe in cerebellar sections from 3-week-old (G) wild-type and (H) MBP-TK treated (1-20d) animals. (I-L) Double immunostaining using anti-PAX6 (I) and anti-HSV1-TK (J) revealed, as expected, a complete absence of TK-positive granule cell precursors. Nuclei were counterstained with DAPI. Scale bars: 100 μm (A,B), 35 μm (C,D), 35 μm (E,F), 100 μm (G,H), 15 μm (I-L).

FIAU-induced cerebellar abnormalities in MBP-TK mice are time-dependent

The biggest advantage of the TK-ablation system resides in the ability to kill cells in an inducible manner. We therefore investigated the status of the cerebellar cortex and of interneuron migration into this area following different FIAU treatment protocols. We thus compared the structure of the cerebellar cortex of transgenic animals treated from day 1 to 20, from day 1 to 6 and finally from day 6 to 20 (Fig. 6). In agreement with previous data, we found that ablation of oligodendrocytes during the first postnatal week resulted in a great impairment of the development of the cerebellar cortex. Indeed, Cresyl Violet staining of the cerebella of 1-6d treated MBP-TK mice (Fig. 6C,G) showed a phenotype very similar to that of 1-20d chronically treated mice (Fig. 6B,F). In wild-type animals, no difference (Fig. 6A,E) was observed in the organization of the cerebella cortex of animals that had followed the 6-20d FIAU treatment protocol (Fig. 6D,H).

In situ hybridization using the GAD67 antisense probe showed an absence of interneurons in the putative ML in 1-6d treated mice (6.5% of wild type) (Fig. 6C), which was similar to 1-20d treated mice (Fig. 6B). These results were confirmed by parvalbumin immunostaining of these cells (Fig. 6F,G). By contrast, transgenic animals that were treated from day 6 to 20 did not show any defects in the cerebellum cytoarchitecture (Fig. 6D,H), as shown by these markers. These data strongly suggest that the presence of oligodendrocytes during the first postnatal week is crucial for the migration of basket and stellate cells into the ML.

This observation prompted us to analyze the presence of these cells at early stages of postnatal cerebellar development. To do this, we examined cerebella of P3 and P6 treated MBP-TK and wild-type mice receiving FIAU from 24 hours after birth (Fig. 5A-D). At early stages of development, interneurons can be traced using the PAX2 marker. Cerebella of treated P3 MBP-TK mice already showed a reduction in interneurons when compared with wild-type siblings (Fig. 5A,B, arrowheads). Interneuron reduction became even more evident at day 6, where few PAX2-positive interneurons localized in the white matter tract were detectable, despite the presence of IGL localized Golgi cells (Fig. 5C,D, arrowheads).

Absence of interneurons could be related to their migratory pathway through the white matter tract in the wild type (Fig. 5E, arrowheads). Indeed, ablation of oligodendrocytes leads to a reduced development of this structure and only rare PAX2-positive interneurons in migration are found in the treated MBP-TK animals (Fig. 5F, arrowhead). This suggests that oligodendrocytes could be required for a trophic and/or migratory support of interneurons during the first postnatal week of cerebellum development.

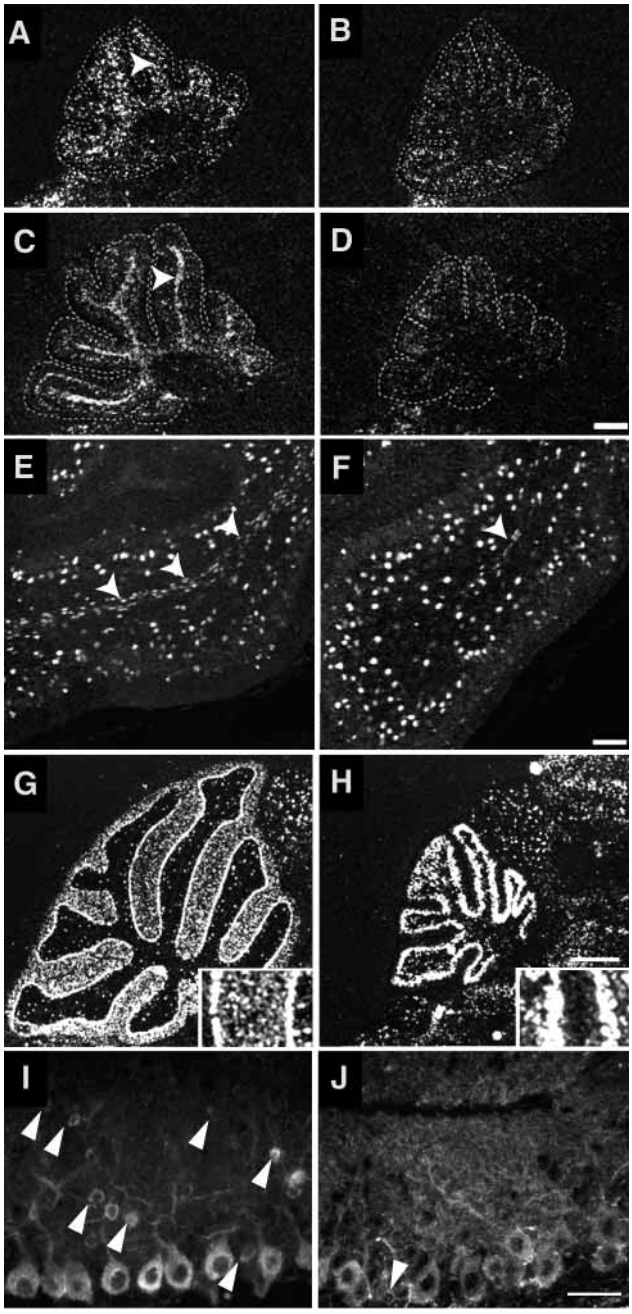

Fig. 5. Interneuron migration to the cerebellar cortex is impaired in treated MBP-TK mice. Cerebellar sections from 3- (A,B) and 6-day-old (C-F) wild-type (A,C,E) and MBP-TK (B,D,F) treated mice were analyzed using a PAX2 antisense riboprobe (A-D). (E,F) Serial sections from the same 6-day-old treated mice were immunostained with an anti-PAX2 antibody (E, wild type; F, MBP-TK). In the wild type, migrating PAX2-positive cells are found in the white matter tract (arrowheads; A,C,E). In MBP-TK cerebella, a strong reduction in migrating PAX2-positive cells (arrowhead in F) is observed (B,D,F). (G,H) In situ hybridization using a GAD67 riboprobe. Insets show higher magnification of the ML of each section. (I,J) Immunofluorescence using an anti-parvalbumin antibody on cerebellar sections of 21-day-old wild-type and MBP-TK (1-20d) treated mice. (G) In wild-type mice, the GAD67 probe labels Purkinje cell bodies and interneurons localized in the ML and in the IGL. (H) In MBP-TK mice, only the Purkinje multicellular layer and interneurons in the IGL can still be detected. Higher magnification of the ML (insets in G,H) shows the presence and absence of GAD-positive interneurons in wild-type and MBP-TK mice, respectively. (I) Basket and stellate cells (arrowheads) visualized by anti-parvalbumin immunostaining within the ML of the wild-type cerebella. (J) Interneurons are absent in the ML of the MBP-TK cerebella. Arrowhead in J indicates parvalbumin-positive interneurons. Scale bars: 170 μm (A-D); 70 μm (E,F); 200 μm (G,H); 50 μm (I,J).

Discussion

Oligodendrocytes are glial cells devoted to the formation of myelin in the CNS. Their degeneration leads to devastating neurological diseases in humans. The role of glial cells has recently been re-evaluated based on evidence of a functional implication of these cells in neural development. The generation of MBP-TK animals has allowed us to delineate the time frame of oligodendrocyte proliferation (Mathis et al., 2000), and to assess the role of oligodendrocyte-neuron contact in the establishment of the node of Ranvier (Mathis et al., 2001). In this respect, it appears clear that these cells, in addition to forming myelin, have a crucial importance in the functional organization of the adult CNS.

We have analyzed the impact of oligodendrocyte loss on the development of the cerebellum, which occurs mainly in the postnatal period. Strikingly, ablation of oligodendrocytes following daily treatment with FIAU during the first three weeks after birth results in a dramatic phenotype characterized by an unstructured cerebellar cortex.

The cerebellum is composed of different cell types that are closely and orderly interconnected. Several studies have demonstrated the importance of cell-cell interactions in the normal development of this structure (Baptista et al., 1994; Changeux and Mikoshiba, 1978).

Purkinje cell differentiation depends on local epigenetic factors, provided partly by granule neurons (Baptista et al., 1994). Interestingly, we show that oligodendrocyte ablation leads to the misalignment of Purkinje cell bodies, accompained by poorly developed dendritic arborizations and loose fasciculation of their axons. This suggests that the presence of oligodendrocytes, and possibly myelination of Purkinje axons, during the final maturation of these cells is crucial for the completion of their differentiation program.

Oligodendrocyte ablation also affects granule neuron proliferation and migration. Indeed, in treated MBP-TK cerebella, we observed reduced proliferation of GC progenitors, paralleled by an increased apoptotic rate of these cells. This results in a smaller cerebellum, abnormal foliation (loss of fissura intercruralis) and a sparse IGL in transgenic treated mice (1-20d and 1-6d). It is known that proliferation of GC progenitors requires factors including sonic hedgehog, which is secreted by Purkinje neurons (Dahmane and Ruiz-i-Altaba, 1999). The defective maturation of Purkinje neurons in treated MBP-TK mice could thus directly affect the generation of granule neurons. However, future in depth studies are required to trace the series of molecular events leading to the reduced numbers of granule cells.

In addition, GCs use the Bergmann glia scaffold to reach their final location in the IGL (Rakic, 1971). In treated MBP-

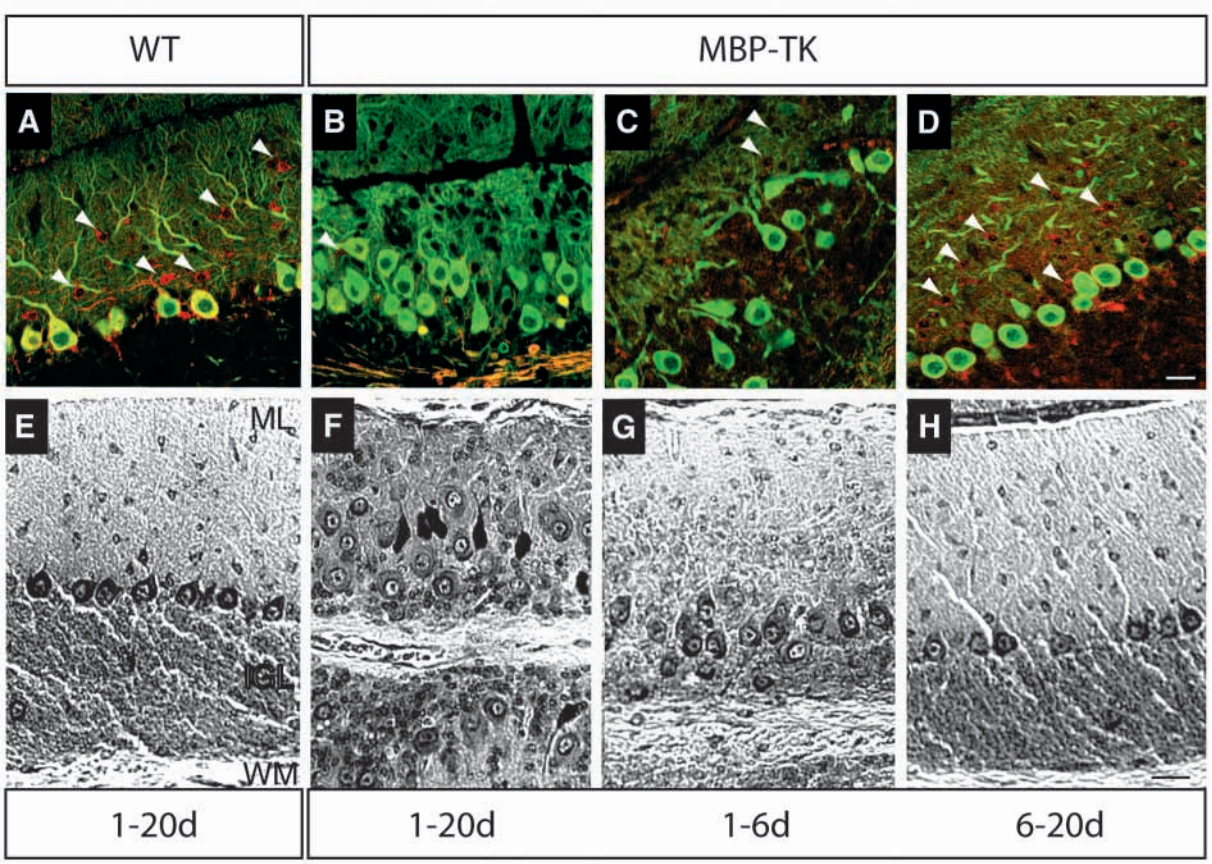

Fig. 6. The cerebellar phenotype of MBP-TK mice is dependent on oligodendrocyte ablation timing. Cerebella of 3-week-old wild-type (A,E) and MBP-TK (B-D,F-H) mice treated with three different FIAU injection protocols (1-20d: A,B,E,F; 1-6d: C,G; 6-20d: D,H) were used for double immunostaining with anti-calbindin (green) and anti-parvalbumin (red) antibodies (A-D), and histological analysis of Cresyl Violet stained sections (E-H). Parvalbumin-positive interneurons are indicated by arrowheads (A-D). ML, molecular layer; IGL, internal granular layer; WM, white matter. Scale bars: 25 μm (A-D); 50 μm (E-H).

TK cerebella, the Bergmann glia are disorganized, which probably has an impact on the migration and maturation of the GC precursors, leading to their premature death and, consequently, to the formation of a sparse IGL. Lack of organization of Bergmann fibers is also very likely to be dependent upon the lack of maturation of Purkinje cells, as the transformation of Bergmann fibers is synchronized with the dendritic differentiation of these cells (Yamada et al., 2000). These events combine to result in an unstructured ML.

It should also be taken into account that afferent pre-cerebellar and pontine climbing and mossy fibers are in contact with, and myelinated by, oligodendrocytes (Palay and Chan-Palay, 1974). Thus, we cannot exclude the possibility that oligodendrocyte ablation may affect the connections of these two afferent pathways with their cerebellar targets, thus contributing to the severe phenotype observed in treated MBP-TK mice.

Importantly, loss of myelin in genetic myelin mutants (*shiverer, jimpy, quaking*) was never reported to affect cerebellum development as has been observed in MBP-TK mice (Mikoshiba et al., 1979; Nagaike et al., 1982). In these mutants, and in contrast with MBP-TK mice, the oligodendrocytes are present and are able to myelinate axons at early postnatal stages of development. However, during the second postnatal week, oligodendrocytes and myelin eventually degenerate in these mutants (Knapp et al., 1986; Nave, 1994; Readhead and Hood, 1990). Thus, it appears that the presence of oligodendrocytes during the first postnatal week is an absolute requirement for the normal development of the cerebellum. In agreement with this, MBP-TK mice treated from day 6 to 20, despite a myelin reduction close to 50% in the cerebellum, do not present any structural cerebellar abnormality.

We can exclude the possibility that the cerebellar phenotype might be due either to an ectopic expression of TK in other cell types, or to FIAU non-specific toxic effects as TK expression has never been observed in other cerebellar cells and FIAU treatment does not alter cerebellar development in wild-type controls. We can also exclude a potential toxic cell-autonomous effect of TK expression in oligodendrocytes, as in untreated MBP-TK mice cerebellar development is normal. Similarly, the observed phenotype is not due to a non-specific toxicity on granule cells. Indeed, FIAU treatments started at postnatal day 6 does not have any effect on cerebellar structure, despite the still active proliferation of GCs during the second postnatal week.

This study also shows that the absence of oligodendrocytes impairs basket and stellate cell proliferation and/or migration into the ML. This finding suggests that oligodendrocytes might secrete a factor(s) that helps their survival and migration,

and/or that serves as a substrate for the migration of interneurons to their final location. A recent study on interneuron progenitors located in the white matter of postnatal cerebellum suggests that these cells begin to differentiate during migration (Milosevic and Goldman, 2001). Thus, another explanation for the disappearance of cortical interneurons might be that oligodendrocytes are involved in the differentiation process of these progenitors into basket and stellate cells. This is an important contribution of oligodendrocytes to cerebellar development that has not been previously observed, and which warrants further future investigations.

In conclusion, oligodendrocytes appear to have a crucial role in the establishment of the cerebellar cytoarchitecture. Our findings clearly show that the role of glial cells in brain development should be re-evaluated. New tools, represented by genetically engineered mice, are of great importance to analyze the relevance not only of genes, but also of cell populations, in the development, function and organization of tissues in vivo.

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