The Brain-Specific Protein MLC1 Implicated in Megalencephalic Leukoencephalopathy With Subcortical Cysts Is Expressed in Glial Cells in the Murine Brain

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ABSTRACT The human MLC1 gene (also known as KIAA0027 and WKL1) and its murine orthologue (Mlc1) encode a putative transmembrane protein expressed primarily in brain. Recessive mutations within human MLC1 cause megalencephalic leukoencephalopathy with subcortical cysts (MLC), whereas a missense mutation resulting in a methionine substitution within a transmembrane leucine string of MLC has been implicated in catatonic schizophrenia in a large pedigree. To gain insight into the function of the MLC protein and to elucidate the pathophysiology of these severe neurodegenerative disorders, information on the cellular and regional distribution of the murine Mlc1, as well as the developmental pattern of Mlc1 expression in brain, is required. Using in situ hybridization (ISH), Mlc1 mRNA was exclusively detected in glial cells of the adult murine brain, such as astrocytes, Bergmann glia, and ependymal cells. ISH, Northern blot analysis, and quantitative real-time polymerase chain reaction (PCR) demonstrated that Mlc1 mRNA is broadly distributed in the adult mouse brain, with highest concentrations of expression in the cerebellum and olfactory bulb. Furthermore, differential expression patterns during brain development were revealed. Overall brain Mlc1 mRNA concentrations exhibited a substantial increase in the perinatal period reaching adult concentrations at postnatal day 5. At the cellular level, highest Mlc1 expression was found during the pre- and perinatal period in multipotential neural precursor cells, especially in the subventricular zone of the lateral ventricle, whereas in adulthood highest Mlc1 mRNA concentrations were revealed in Bergmann glia cells. Because the temporal expression profile of Mlc1 indicates that, in contrast to developing and mature astrocytes, oligodendrocytes are devoid of Mlc1 expression, white matter tract abnormalities observed in these disorders may result from a primary astrocytic defect. Detailed information on Mlc1 expression in brain is likely to lead to a better understanding of Mlc1 involvement in the pathogenesis of both MLC and catatonic schizophrenia. © 2003 Wiley-Liss, Inc.

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INTRODUCTION

The human MLC (WKL1) gene was first described by Nomura et al. (1994) and originally designated KIAA0027. Leegwater et al. (2001, 2002) demonstrated that homozygous and compound heterozygous recessive mutations of MLC1 cause megalencephalic leukoencephalopathy with subcortical cysts (MCL, MIM 604004), a severe neurodegenerative disorder associated with motor symptoms and mental decline. Furthermore, a missense mutation resulting in methionine substitution within a transmembrane leucine string of MLC1 has been implicated in catatonic schizophrenia (Steinke et al., 2003). A severe neurodegenerative disorder possibly associated with motor symptoms and mental decline is also caused by homozygous and compound heterozygous recessive mutations of MLC1 (MCL, MIM 604004), a severe neurodegenerative disorder associated with motor symptoms and mental decline. Furthermore, a missense mutation resulting in methionine substitution within a transmembrane leucine string of MLC1 has been implicated in catatonic schizophrenia (Steinke et al., 2003).

Both the human MLC1 and the murine Mlc1 are almost exclusively expressed in brain, as demonstrated by Northern blot analysis (Meyer et al., 2001; Steinke et al., 2003). In addition, MLC1 expression was detected in all human brain regions investigated, including cortex, hippocampus, caudate nucleus, amygdala, thalamus, and cerebellum. The MLC1 protein consists of 377 amino acids and contains six to eight predicted transmembrane domains, while functional data are still lacking (Nomura et al., 1994; Meyer et al., 2001).

Since MLC1 appears to be involved in the pathogenesis of both MLC and catatonic schizophrenia, knowledge about the cellular and regional distribution of murine Mlc1 as well as the developmental pattern of Mlc1 expression in brain would be a useful tool to reveal the function of this protein and to gain insights into the pathophysiology of these severe neurodegenerative disorders.

In the present study, we used nonradioactive in situ hybridization (ISH) to detect Mlc1 mRNA in the adult and developing mouse brain. The aim was to identify unequivocally the neural cell types expressing Mlc1 and to provide detailed information about the distribution pattern of Mlc1 mRNA in various brain regions. Changes in the expression pattern of Mlc1 during brain development were also assessed. Finally, all results obtained by ISH in the adult and developing brain were confirmed by a quantitative analysis of Mlc1 expression using real-time polymerase chain reaction (PCR) methodology.

MATERIALS AND METHODS

Animals and Tissue Resources

Brains of C57BL/6 mice (n = 90) at different developmental stages (embryonic day [E]14 (n = 20), E16 (n = 12), E18 (n = 14), postnatal day [P]0 (n = 10), P5 (n = 12), P10 (n = 8), P20 (n = 7), and adult (n = 7)) were used for RNA preparation and ISH. In addition, different brain regions from adult mice, such as cerebral cortex, striatum, hippocampus, cerebellum, thalamus, brainstem, and olfactory bulb, were dissected and used for RNA preparation and ISH. In addition, different brain regions from adult mice, such as cerebral cortex, striatum, hippocampus, cerebellum, thalamus, brainstem, and olfactory bulb, were dissected and used for RNA preparation and ISH. In addition, different brain regions from adult mice, such as cerebral cortex, striatum, hippocampus, cerebellum, thalamus, brainstem, and olfactory bulb, were dissected and used for RNA preparation and ISH.

Generation of Digoxigenin-Labeled cRNA Probes

Digoxigenin (DIG)-labeled probes were generated from two cDNA fragments complementary to coding and noncoding regions of the murine Mlc1 sequence reported by Steinke et al. (2003) (GenBank accession AF449425). cDNA fragments were amplified and cloned into pGEM-T Vector (Promega, Madison, WI). The following primers were used for amplification: 5’-AAAATTCAAGAAGCTGTCG-3’ (complementary to nucleotides 358–377) and 5’-CAGGGCTTTCTCTTGTCAAACCTCC-3’ (nucleotides 1591–1615) for generation of a plasmid containing cDNA of the coding region (pMlc1-cds); and 5’-GAGGTGTTCCAGGCCCCT-3’ (nucleotides 1568–1588) and 5’-GCAAAGTGGAT- TATGCTCAAGC-3’ (nucleotides 2752–2776) for generation of a plasmid containing cDNA of the noncoding 3’-end (pMlc1-3’-UTR). For transcription, plasmid pMlc1-3’-UTR was linearized with SauII (antisense) or NcoI (sense) and plasmid pMlc1-cds was linearized with NcoI (antisense) and NotI (sense). Phenol-chloroform purified and linearized plasmids were ethanol precipitated, and DIG-labeled cRNA was synthesized using the appropriate RNA polymerases (SP6 or T7) according to the manufacturer’s manual (Roche, Mannheim, Germany). cRNA probes were analyzed on agarose gels (1%).

In Situ Hybridization

All in situ hybridization (ISH) procedures were performed as previously described (Kugler and Schmitt, 1999; Schmitt and Kugler, 1999; Schmitt et al., 2002). Hybridization on 14-μm pre-treated cryostat sections of mouse brain was carried out at 58°C for 16–18 h with 10–15 ng/μl DIG-labeled antisense cRNA in the hybridization solution. Substitution of the antisense cRNA probe by an equivalent amount of labeled sense cRNA, RNase A treatment before hybridization or omission of labeled cRNA led to a lack of staining (cf. Figs. 1D and 2K). From these findings it was concluded that the antisense probes were specific, DIG detection did not create labeling artifacts, and there was no endogenous alkaline phosphatase activity left in the sections. In some experiments, following alkaline phosphatase visualization, several sections were used for the immunocytochemical detection of glial fibrillary acidic protein (GFAP), a specific marker protein for astrocytes and maturing ependyma (Bignami et al., 1972), and of nestin, a marker for multipotential neural precursor...
Northern Blot Analysis

For Northern blot analysis, total RNAs were prepared from different brain regions and brains at different developmental stages by acid guanidinium-phenol-chloroform extraction. Each RNA sample (20 μg/lane) was denatured with formamide and size-separated by formaldehyde/agarose gel electrophoresis. RNAs were transferred and fixed to nylon membrane (Nytran, Schleicher & Schuell, Germany) and hybridized with the 32P-dCTP-labeled cDNA probes complementary to nucleotides 358–1615 (Mlc1-cds; see above). Conditions for hybridizing and washing were the same as described elsewhere (Multiple Tissue Northern Blot user manual; Clontech, Palo Alto, CA). For normalization, a cDNA probe for mouse β-actin gene was rehybridized to the membrane.

Quantitative Real-Time PCR

To generate cDNA from mouse brain samples, 2 μg of total RNA was reverse transcribed with oligo(dT)18 primers, using the Thermoscript™ RT-PCR-System from Life Technologies. Real-time PCR was performed using an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA), an iQ Supermix (Bio-Rad), and SYBR Green dye (Molecular Probes Europe BV, Leiden, the Netherlands), according to the manufacturer’s protocol. SYBR Green is a molecule that binds to double-stranded DNA and is fluorescent when bound. The primers (Mlc1-a: 5’-AGCCATCAGCAGCTCACTAC and Mlc1-b: 5’-GCAGCAGCAGCAGCAACAG) used in the real-time PCR procedure were designed using specific primer analysis software (Beacon Designer, Bio-Rad). Each SYBR Green reaction (25 μl total volume) contained either 2.5 μl of cDNA or standard plasmid. For quantification, a 10-fold serial dilution of purified pMlc1-cds (see above) was prepared in duplicate to generate a standard curve. After initial denaturation at 95°C for 4 min, the following iCycler iQ™ conditions were used: 38 cycles with denaturation at 95°C for 30 s, and annealing and extension at 60°C for 1 min. Because SYBR Green binds indiscriminately to double-stranded DNA, the samples were subjected to the heat dissociation protocol after the final cycle of the PCR, confirming the specificity of the amplification product. For this, the reactions were heat-denatured over a 15°C gradient at 0.5°C/10 s from 80–95°C. Heat dissociation of the amplified DNA detected differences in the melting temperature and produced a single dissociation peak for the Mlc1 PCR product. The presence of a single PCR product was also verified by 2% agarose gel electrophoresis. To control for differences in the efficiency of cDNA synthesis and subsequent real-time PCR, the entire procedure was done in triplicate. Quantities of specific mRNA were measured according to the corresponding gene-specific standard curve using the iCycler iQ™ Real-Time PCR Detection System software.

RESULTS

In Situ Hybridization

Application of the cRNA probes complementary to coding (Mlc1-cds) and 3′-noncoding (Mlc1-3′-UTR) regions of the mouse Mlc1 cDNA to cryostat sections resulted in an identical pattern of cellular and regional distribution of Mlc1 mRNA (e.g., Fig. 1C,E). The Mlc1-3′-UTR cRNA probe provided higher signal intensities compared to the Mlc1-cds cRNA probe. Therefore, the documentation of results is based essentially on ISH using the Mlc1-3′-UTR cRNA probe. Applying the sense cRNA, no labeling was observed (Figs. 1D and 2K).

Cellular and Regional Expression of Mlc1 in the Adult Mouse Brain

ISH showed Mlc1 expression exclusively in glial cells, which on the basis of their shape and distribution appeared to be Bergmann glia (Fig. 1A–E), astrocytes (Fig. 2B–D,F,H,I,L,M), and ependymal cells (Fig. 2J). Double-labeling with GFAP yielded conclusive evidence of Mlc1 mRNA localization in Bergmann glia (Fig. 1E,F) and astrocytes (Fig. 2B,D; F,I; L,M). In Bergmann glia, the reaction product extended into proximal processes (Fig. 1B,C,E), but finer processes were not labeled. Neurons, oligodendrocytes, tanyocytes, epithelial cells of the choroid plexus, and microglia showed no staining.

The strongest ISH reaction was found in Bergmann glia in the Purkinje cell layer of the cerebellar cortex (Fig. 1A,B). Astrocytes reacting positively for Mlc1 mRNA were detected throughout the CNS and showed different levels of reactivity. The highest density of moderately to strongly reacting astrocytes was observed in different layers of the olfactory tract (Fig. 2H), in the thalamus and in the hippocampus (Fig. 2C). In the hippocampus, ISH-positive cells were more densely localized in the subgranular zone of the dentate gyrus (arrows in Fig. 2C). The granule cell layer of the cerebellar cortex (Fig. 1B) and other brain regions (not shown) showed only faint astrocytic labeling. In white matter tracts of the brain, e.g., corpus callosum, fimbria of the hippocampus, and white matter tracts of the cerebellum, scattered astrocytes were stained faintly. Ependymal cells lining the third ventricle (Fig. 2J) as well as other ventricular regions displayed moderate to strong labeling.
Using a cDNA probe complementary to the coding region of Mlc1 (Mlc1-cds), a single band of the expected size of 2.8 kb was shown by Northern blots of different murine brain regions (Fig. 3A). Labeling intensities (Fig. 3A) varied between different brain regions. The strongest labeling was detected in RNA preparations of the cerebellum, whereas weakest labeling was detected in cerebral cortex, hippocampus, and striatum. Quantitative real-time PCR was used to quantify and verify these results (Fig. 3B). RNA preparations of the olfactory bulb were included in these investigations. Mlc1 mRNA concentrations per μg total RNA were determined by using standard curves. Real-time PCR results correlate well with Northern blot results. Figure 3B shows up to 2-fold higher Mlc1 mRNA concentrations in the cerebellum and the olfactory bulb, compared to Mlc1 expression levels in the whole brain, cerebral cortex, striatum, and hippocampus. In thalamus and brainstem, intermediate Mlc1 mRNA concentrations were detected.

**Differential Expression of Mlc1 During Development**

Mlc1 expression in sagittal and coronal sections of mouse brains from E18 up to P20 was analyzed by ISH.
In E18 brains, Mlc1 was expressed predominantly in the ependymal and subependymal layers of all ventricular regions, with highest ISH labeling intensity along the lateral ventricle (Fig. 4A). In addition, in E18 brains, ISH-positive cells scattered in the neuropil were detected throughout the brain, especially in brainstem, hippocampus, and cerebral cortex (Fig. 4A). As shown in Figure 5, there is an overall decrease in the number of Mlc1-positive cells in the subventricular zone of the lateral ventricle during development from E18 up to P20. These ependymal and subependymal Mlc1-expressing cells of E18, as well as of P5 brains, form multiple cell layers (Fig. 5B,C), which thin gradually during postnatal development until only a single layer of mature ependymal cells line the ventricular system (Fig. 5E,F,H,I,K,L). At E18, double-labeling with nestin yielded evidence of Mlc1 mRNA localization in immature cells of neuroepithelial origin (Fig. 6A,B). As shown in Figure 6B, and with higher magnification in Figure 6D–F, nestin-immunopositive cell bodies and processes are unevenly distributed along, and in the vicinity of, the lateral ventricle. Strongest nestin immunostaining was found in those parts of the lateral ventricle with less intense Mlc1 ISH labeling. Thus, an inverse relationship between the intensity of Mlc1-ISH staining and nestin staining was demonstrated. In addition, almost no GFAP-immunopositive structures appeared in this brain region at this developmental stage (data not shown). At P5, the number of nestin-positive cells is reduced in comparison with E18 (Fig. 7B,D). Cells double-labeled for Mlc1-mRNA and nestin are localized in the periventricular cell layers, as well as in the neuropil of surrounding brain regions, such as the caudate putamen (Fig. 7B,D). At P5, few of the Mlc1 mRNA-positive cells lining the lateral ventricle could already be identified as maturing ependymal cells using the double-labeling procedure with the GFAP antibody (Fig. 7A,C). In addition, only few scattered Mlc1-positive cells in the surrounding neuropil of the lateral ventricle showed immunoreactivity for GFAP. In contrast, in other brain regions, such as the fimbria hippocampi, dense groups of Mlc1 mRNA/GFAP-positive astrocytes became visible, serving as a positive control for the staining procedure (Fig. 7E,F).

In contrast to the decreased subventricular ISH signal intensity during development, ISH signals in the Bergmann glia increase from E18 until adulthood, in parallel with the perinatal and postnatal growth of the cerebellum (Fig. 4). With regard to astrocytes expressing Mlc1, there is an increase in number and intensity of ISH signals from E18 up to P5/P10, especially in white matter tracts, such as the white matter tracts of the cerebellar cortex (Fig. 4D,F) and the fimbria hippocampi (Fig. 5E,H). However, only weak ISH labeling could be detected in white matter astrocytes of P20 (Figs. 4H and 5K) and adult mice (Fig. 1B). Strong ISH labeling in brain tissue sections of P20 mice, as well as in the mature brain, was only detected in the Bergmann glia of the cerebellum.

Northern blots with RNA of mouse brains at different ages showed a perinatal increase in Mlc1 expression (Fig. 8A). Real-time PCR was used to quantify and verify these results. As shown in Figure 8B, Mlc1 expression levels increase continuously between E14 and P5 (about 20-fold) and decrease slightly between P5 and the adult brain (Fig. 8B).

**DISCUSSION**

We have investigated Mlc1 expression in adult murine brain by ISH and detected specific Mlc1 ISH signals in Bergmann glia, ependymal cells, and astrocytes. In other types of neural cells, including neurons, oligodendrocytes, epithelial cells of the choroid plexus, and microglia, Mlc1 expression was not detectable. With regard to this cellular distribution of Mlc1 mRNA, labeled cells were unequivocally identified as astrocytes and Bergmann glia by combining ISH and immunolabeling of GFAP. Ependymal cells were also identified by their typical localization lining the ventricles. Analysis of regional distribution of Mlc1, analysis by ISH, Northern blot, and real time-PCR revealed high Mlc1 expression levels in cerebellum, olfactory tract, brainstem and thalamus. Conversely, only moderate to weak Mlc1 expression was found in cerebral cortex, striatum, and hippocampus.

To ensure specificity of the ISH, two different cRNA probes complementary to different domains of Mlc1 mRNA were used for ISH. Both antisense cRNA probes (Mlc1-cds and Mlc1-3′-UTR) displayed the same pattern of cell labeling, which confirms specificity of both probes in detecting Mlc1 mRNA. No staining was observed with the cRNA sense probe. Mlc1 expression levels analyzed using Northern blot and quantitative real-time PCR methodology supported the findings of ISH. However, because of its sensitivity and the need for smaller amounts of tissue than are required by conventional methods such as Northern blotting, real-time PCR has several advantages in comparison to other quantification methods (Estellés et al., 1994).

In addition to the cellular and regional distribution of Mlc1 in the adult brain, we have investigated Mlc1 expression during brain development. Northern blots and quantitative real-time PCR showed an increase in Mlc1 mRNA concentrations from E14 to P5, which then decreased slightly until adulthood. ISH studies in the developing brain revealed signals in the same cell types as in the adult brain. However, the pattern of Mlc1-positive cells situated alongside the lateral ventricle of E18 and P5 mice suggests that other than ependymal cells in this region are expressing Mlc1. Double-labeling with the intermediate filament protein nestin revealed Mlc1 expression in multipotent neural precursor cells, as defined by the expression of nestin. Nestin-positive cells are known to include radial glia, bipotential glial/neuronal progenitor cells, maturing astrocytes, and maturing ependymal cells. Notably, we observed inverse relationship between the intensity of

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Mlc1 ISH- and nestin-labeling in the ependymal and subependymal layers along the lateral ventricle. This raises the question of whether these variations in nestin labeling and Mlc1 ISH labeling represent distinct stages of neural cell differentiation. In this regard, double-labeling of the subventricular zone of P5 mice with nestin antibodies showed a reduction in the number of Mlc1/nestin double-labeled cells compared to E18 mice. Moreover, using anti-GFAP antibodies, it was found that some Mlc1-positive cells were already positive for this marker protein. As shown by Sultana and coworkers (2000), there is an inverse relationship between the amount of nestin expression and the amount of GFAP expression as a function of brain development.

In addition, nestin can form copolymer with either vimentin (marker for radial glia) or GFAP (Stewart, 1993). Therefore, it may be assumed that this intermediate filament protein nestin is expressed not only in multipotent precursor cells but also during their transition (from radial glia or bipotential glial/neuronal precursors) to astrocytes or other neural cell types like ependymal cells. With transition to mature astrocytes and ependyma, the number of Mlc1-expressing cells decreases to adulthood. This expression pattern of Mlc1 indicates that Mlc1 serves a function during the maturation of astrocytes and ependymal cells. In contrast, the intensity of Bergmann glia-Mlc1 signals increases from E18 to adulthood, in parallel with the perinatal and postnatal growth of the cerebellum.

In the mouse, ependymal precursor cells first arise on E12 and continue to proliferate until E18 (Rakic and Sidman, 1968). Proliferation turnover declines significantly after birth, and ependymal cells in human do not proliferate in adulthood and do not have the ability to regenerate (Sarnat, 1995). Ependymal cells serve a secretory function and may act as a cellular and axonal guidance system, particularly during fetal development. Thus, secretory proteins of ependymal cells appear to be important in the guidance of axonal growth cones (Sarnat, 1992). In the mature mammalian brain, ependymal cells possess the structural and enzymatic characteristics necessary for scavenging and detoxifying a wide variety of substances from the cerebrospinal fluid (CSF), thus forming a metabolic barrier at the brain-CSF interface (Del Bigio, 1995; Bruni, 1998).

Mlc1 was shown to be highly expressed in premature and mature astrocytes of the pre- and perinatal brain. Astrocytes as the major glial cell type play different roles at different stages of the life span of the brain. During neurogenesis and early development, astrocytes provide a scaffold for the accurate migration of neurons and growth cones, provide guidance cues, and may also be involved in neuronal proliferation. In adulthood, astrocytes maintain neuronal homeostasis and synaptic plasticity (Vernadakis, 1996). It is interesting that a peak in Mlc1 mRNA concentrations in

Fig. 2. Cellular and regional distribution of Mlc1 mRNA using Mlc1-3′-UTR cRNA probes. A-D: Hippocampus. A: Nissl-stained coronal section of the hippocampus. C: In situ hybridization (ISH) labeling with moderate staining intensity is observed in astrocytes scattered loosely throughout the neuropil layers of the hippocampus. A higher density of these labeled cells is seen in the subgranular zone of the dentate gyrus, as indicated by arrows. B,D: Micrograph pair showing a section of the hippocampal radiatum layer with high magnification after ISH using the Mlc1-3′-UTR cRNA probe (B) and after additional glial fibrillary acidic protein (GFAP) immunostaining (D). GFAP-immunoreactive processes (arrowheads in D) permit identification of ISH-reactive cells (arrowheads in B) as astrocytes. E-I,M: olfactory tract. E,G: Nissl-stained coronal section of the olfactory bulb showing the different layers of the olfactory bulb with low (E) and higher (G) magnification. H: Scattered ISH labeling of moderate to low intensity, with highest labeling intensities in the glomerular layer, is seen in all layers of the olfactory bulb. F,I and L,M are micrograph pairs showing sections of two different layers of the olfactory tract (F,I, olfactory nerve layer; L,M, glomerular layer) with high magnification after ISH using the Mlc1-3′-UTR cRNA probe (F,L) and after additional GFAP immunostaining (I,M). GFAP-immunoreactive cell bodies with processes (arrowheads in I and M) permit identification of ISH-reactive cells (arrowheads in F,L) as astrocytes. J: Strong ISH labeling is also observed in ependymal cells (arrows) of the third ventricle (3V) adjacent to the arcuate hypothalamic nucleus. K: Hippocampus. No labeling is observed using the sense cRNA probe. C, the human genomic sequence of the Mlc1 gene was hybridized with a 1257-bp cDNA fragment corresponding to 11003 bp of the coding region of Mlc1. The detected transcript has the expected size of ~2.8 kb. High levels of Mlc1 mRNA were observed in cerebellum and thalamus. The equal loading and quality of the RNA were tested by performing the hybridization with a β-actin probe, shown in each bottom panel. The position of 28S and 18S rRNA subunits are indicated on the left side. B: Quantification of Mlc1 mRNA using real-time SYBR Green polymerase chain reaction (PCR). Data are expressed as means ± SE values of three independent experiments, each performed in duplicate amol (10^-15 mol).

Fig. 3. Regional distribution of the Mlc1 transcript in the mouse brain. A: Northern blot of total RNA (20 μg per lane) from different brain regions was hybridized with a 1257-bp cDNA fragment corresponding to 358–1615 bp of the coding region of Mlc1. The detected transcript has the expected size of ~2.8 kb. High levels of Mlc1 mRNA were observed in cerebellum and thalamus. The equal loading and quality of the RNA were tested by performing the hybridization with a β-actin probe, shown in each bottom panel. The position of 28S and 18S rRNA subunits are indicated on the left side. B: Quantification of Mlc1 mRNA using real-time SYBR Green polymerase chain reaction (PCR). Data are expressed as means ± SE values of three independent experiments, each performed in duplicate amol (10^-15 mol).
Fig. 4. Regional distribution of the Mlc1 transcript in the developing mouse brain. Sagittal sections of whole brains of mice at four developmental stages (E18, P5, P10, P20) were used for in situ hybridization (ISH) with the Mlc1-3'-UTR cRNA probe. A,C,E,F: Ependymal and subependymal cells of the lateral ventricle (LV) and the fourth ventricle (4V) show moderate to strong ISH staining. Note the decreasing labeling intensity of these cells from E18 through P20. Scattered ISH labeling of moderate to low intensity, which seemed to be astrocytes, is seen in all brain regions, e.g., cerebral cortex (c), thalamus (th), hippocampus (h), caudate putamen (cpu). B,D,F,H: Higher magnification of ISH labeling in the cerebellum (cb), as shown in A,C,E,G. The intensity of Bergmann glia-ISH signals increases from E18 until P20, in parallel with the perinatal and postnatal growth of the cerebellum. Note ISH-positive astrocytes in the granule cell layer and white matter tract of the cerebellar cortex of P5 and P10 old mice. In P20 old mice, astrocytes in these layers are only faintly labeled. E, embryonic day; P, postnatal day; gr, granule cell layer; mo, molecular layer; w, white matter. Scale bars = 825 μm in A; 1,200 μm in C,E; 1,300 μm in G; 450 μm in B,D,F,H. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].
Fig. 5. Distribution of the Mlc1 transcript in the developing mouse hippocampus. Sagittal sections of whole brains of mice at four developmental stages (E18, P5, P10, P20) were used for ISH with the Mlc1-3'-UTR cDNA probe. A,D,G,J: Cells in the subventricular zone of the lateral ventricle (LV) show moderate to strong ISH staining. B,E,H,K: Higher magnification of ISH labeling of the fimbria hippocampi (fi), as shown in A,D,G,J. Note the decreasing labeling intensity and decreasing number of subependymal cells from E18 through P20. The highest density of ISH-positive astrocytes in the fimbria hippocampi is seen in P5 and P10 old mice. C,F,I,L: Higher magnification of the periventricular region shown in B,E,H,K. Note the strong ISH labeling in ependymal as well as in subependymal cells of E18 and P5. Mlc1-positive cells in P10 and P20 form more a single layer of epithelial cells. E, embryonic day; P, postnatal day; cp, choroid plexus; CA1, CA3, cornu ammonis sector; DG, dentate gyrus. Scale bars = 500 μm in A,D,G,J; 100 μm in B,E,H,K; 30 μm in C,F,I,L. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].
astrocytes was observed at P5-P10 and could be localized to the deep white matter of cerebellum, to the corpus callosum, and to certain hippocampal fiber tracts. From P20 through adulthood, Mlc1 expression in astrocytes declines to a moderate level, and the Mlc1 mRNA level in the white matter is reduced to a low level in the adult brain. The transient increase in Mlc1 message at P5-P10 appears to correspond to the time

Fig. 6. Cellular distribution of Mlc1 mRNA in the periventricular region of mice at E18 and double labeling with a marker for immature cells of neuroepithelial origin. A–F: Coronal sections of the forebrain were used for ISH with the Mlc1-3’-UTR cRNA probe. A: In situ hybridization (ISH) labeling in the forebrain is observed with different labeling intensities in the neuroepithelial layers surrounding the lateral ventricle. Note the highest ISH-labeling intensities of periventricular cells in the middle descending part of the lateral ventricle as indicated by arrows. A,B and C,E are micrograph pairs showing sections after ISH (A,C) and after additional Nestin immunostaining (B,E). A,B: Note that the highest Nestin-immunoreactivity is localized in the dorsal and ventral end of the periventricular region (indicated by arrowheads), an inverse pattern of different labeling intensities compared to ISH-labeling intensities shown in A. C,E: Nestin-immunoreactive process-bearing cell bodies (arrows in E) permit identification of ISH-reactive cells (arrows in C) as immature cells of neuroepithelial origin. D,F: Higher magnification of double labeled sections of the LV region as shown in A, E, embryonic day; cpu, caudate putamen; LV, lateral ventricle. Scale bars = 300 μm in A,B; 35 μm in C,E; 40 μm in D,F. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].
point of high proliferation of oligodendrocytes during initial events of myelination (Landry et al., 1990). As shown by Mathis et al. (2000) in a transgenic mouse model for inducible and reversible dysmyelination, oligodendrocyte proliferation occurs mainly during the first 9 days after birth. In addition, dysmyelination induced in these mice is always accompanied by astrocystosis, which points to a possible role of astrocytes in

Fig. 7. Cellular distribution of Mlc1 mRNA in the periventricular and hippocampal region of mice at P5 and double labeling with marker for immature cells of neuroepithelial origin and astrocytes. A–F: Coronal sections of the forebrain were used for in situ hybridization (ISH) with the Mlc1-3′-UTR cRNA probe. A,C and E,F are micrograph pairs showing sections after ISH (A,E) and after additional glial fibrillary acidic protein (GFAP) immunostaining (C,F). A,C: Lateral ventricle. GFAP-immunoreactive processes (arrowheads and arrows in C) permit identification of some ISH-reactive cells in the neuropil (arrows in A) as astrocytes and some ISH-reactive cells beneath the LV (arrowheads in A) as ependymal cells. E,F: Fimbria hippocampi with lateral ventricle. GFAP-immunoreactive processes (arrowheads and arrows in F) permit identification of ISH-reactive cells in the fimbria hippocampi (arrows in E) as astrocytes and ISH-reactive cells lining the LV (arrowheads in C) as ependymal cells.

Note that almost all cells of the fimbria hippocampi lining the lateral ventricle show strong GFAP-immunoreactivity and therefore seem to be maturing ependymal cells in contrast to the medial lining of the LV, where only few cells show GFAP immunoreactivity. B,D is a micrograph pair showing sections after ISH in the first micrograph and after additional nestin immunostaining in the second micrograph. Nestin-immunoreactive process-bearing cell bodies (arrowheads and arrows in D) permit identification of ISH-reactive cells in the neuropil (arrows in B) and beneath the LV (arrowheads in B) as immature cells of neuroepithelial origin. Note, that at P5 only few ISH-reactive cells are double labeled for either GFAP or Nestin in this brain region. LV, lateral ventricle. Scale bars = 37.5 μm in A–F. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].
dysmyelination and as well as in the myelination processes. It is also interesting to note that the time pattern of Mlc1 expression in white matter is comparable to that of GFAP expression (Landry et al., 1990). Therefore, this transient increase in GFAP as well as Mlc1 message appears to reflect a transitional period of astrocyte specialization that occurs in white matter during stages of myelination.

Little is known about the function of Mlc1, although it has been speculated that MLC1 may encode a membrane protein with weak similarity to transporters, namely ABC-2 type transporters and sodium/galactoside symporters (Leegwater et al., 2001; Meyer et al., 2001). Recent work suggests involvement of MLC1 in severe neurological and psychiatric disorders, namely megalencephalic leukoencephalopathy with subcortical cysts (MLC) (Leegwater et al., 2001, 2002) and catatonic schizophrenia (Meyer et al., 2001). Leegwater et al. (2001, 2002) demonstrated recessive mutations in MLC1 causative for MLC, characterized by diffuse white matter abnormalities, the invariable presence of subcortical cysts, and an onset in early infancy. In addition, we recently reported a L309M missense mutation within a transmembrane leucine string of the MLC1 protein, possibly linked with catatonic schizophrenia in a large pedigree (Meyer et al., 2001). Interestingly, an impairment or alteration in motor function is common to patients with either disorder.

Analysis of cellular as well as spatiotemporal distribution of Mlc1 indicates that Mlc1 may be important in the development of the brain, especially for initial events of myelination. The time pattern of Mlc1 expression levels in astrocytes, especially in white matter tracts, provides an idea of the relevance of Mlc1 expression in astrocytes for oligodendrocyte function and the myelination process, as discussed above. These cell functions may be impaired by mutated Mlc1, and thus may be related to density abnormalities of white matter tracts in MLC brains. Moreover, Mlc1 expression in the ependyma could play a role in ependymal cell functioning as a cellular or axonal guidance system. Interestingly, both MLC and schizophrenia are thought to reflect neurodevelopmental disorders (van der Knaap et al., 1995; Lobato et al., 2001; Wassmer et al., 2001; Lewis and Levitt, 2002), further supporting the notion of a critical role of Mlc1 during development.

Significant Mlc1 expression in the mature brain was only detected in the Bergmann glia, a specialized group of cerebellar astrocytes. Little is known about the function of Bergmann glia. This type of glial cell was reported to be involved in several disorders, including the ataxic form of Creutzfeldt-Jacob disease (Lafarga et al., 1993) and autosomal recessive cerebellar hypoplasia (Mathews et al., 1989). Moreover, mice lacking vimentin, an intermediate filament protein almost exclusively expressed by Bergmann glia in the adult brain, show poorly developed abnormal Bergmann glia and Purkinje cells with a loss of spiny branchlets, accompanied by impaired motor coordination (Colucci-Guyon et al., 1999).

Taken together, our results show that expression of Mlc1 is developmentally regulated in a region- and cell type-specific manner. Studies of the distribution of Mlc1 expression in brain may yield clues to the better understanding of Mlc1 involvement in the pathogenesis of both MLC and catatonic schizophrenia. Future efforts are aimed at the study of Mlc1 expression at the protein level. Unfortunately, antibodies against Mlc1 are not yet available.

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