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Lysophosphatidic Acid Receptor Gene *vzg-1/lp$_{A1}$/edg-2* Is Expressed by Mature Oligodendrocytes During Myelination in the Postnatal Murine Brain

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ABSTRACT

The growth-factor–like phospholipid lysophosphatidic acid (LPA) mediates a wide variety of biological functions. We recently reported the cloning of the first G-protein–coupled receptor for LPA, called *ventricular zone gene-1* (*vzg-1/lp$_{A1}$/edg-2*) because its embryonic central nervous system (CNS) expression is restricted to the neocortical ventricular zone (Hecht et al. [1996] J. Cell Biol. *135:*1071–1083). *Vzg-1* neural expression diminishes at the end of the cortical neurogenetic period, just before birth. Here, we have investigated the subsequent reappearance of *vzg-1* expression in the postnatal murine brain, by using in situ hybridization and northern blot analyses. *Vzg-1* expression was undetectable by in situ hybridization at birth, but reappeared in the hindbrain during the 1st postnatal week. Subsequently, expression expanded from caudal to rostral, with peak expression observed around postnatal day 18. At all postnatal ages, *vzg-1* expression was concentrated in and around developing white matter tracts, and its expansion, peak, and subsequent downregulation closely paralleled the progress of myelination. Double-label in situ hybridization studies demonstrated that *vzg-1*–expressing cells co-expressed mRNA encoding proteolipid protein (PLP), a mature oligodendrocyte marker, but not glial fibrillary acidic protein (GFAP), an astrocyte marker. Consistent with this, *vzg-1* mRNA expression was reduced by 40% in the brains of *jimpy* mice, which exhibit aberrant oligodendrocyte differentiation and cell death. Together with our characterization of *vzg-1* during cortical neurogenesis, these data suggest distinct pre- and postnatal roles for LPA in the development of neurons and oligodendrocytes and implicate lysophospholipid signaling as a potential regulator of myelination. J. Comp. Neurol. 398:587–598, 1998.

Indexing terms: glia; G-protein–coupled receptor; myelin; PLP; LPA

The mature mammalian central nervous system (CNS) develops from the comparatively simple embryonic neural tube. In the embryo, CNS neuroblasts proliferate in a zone adjacent to the lumen of the neural tube, termed the ventricular zone (VZ; Boulder Committee, 1970). Before birth, most postmitotic neurons making up the mature CNS are generated and migrate to more superficial zones where they differentiate and begin to establish axonal connections. These connections continue to mature in the perinatal period, which is also marked by the proliferation and differentiation of glial cells (Das, 1979; Jacobson, 1991). One type of mature glial cell, the oligodendrocyte, subsequently myelinates axons in the first weeks of postnatal life. Mechanistic understanding of how these various developmental stages proceed requires the identification

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and characterization of the molecules and signaling pathways involved.

We recently identified *ventricular zone gene-1* (*vzg-1;* also designated lp_{A1}, for "lysophospholipid A1 receptor"), a member of the G-protein–coupled receptor family expressed in the VZ of the cerebral cortex during the embryonic neurogenetic period (Hecht et al., 1996). Extensive characterization of *vzg-1* has indicated that it is the first cloned receptor for lysophosphatidic acid (LPA; Hecht et al., 1996; Fukushima et al., 1998), a phospholipid with a number of established bioactivities relevant to CNS development. LPA can induce cell proliferation, neurite retraction and cell rounding, inhibit differentiation, and disrupt gap-junctional communication (reviewed by Jalink et al., 1994; Moolenaar, 1995; Moolenaar et al., 1997). In cortical VZ neuroblasts, LPA induces both electrophysiological responses and morphological changes (Dubin et al., 1997; Fukushima and Chun, 1997). The identification of *vzg-1* as an LPA receptor (Hecht et al., 1996; An et al., 1997), along with the recent characterization of a related lysophospholipid receptor family (Chun et al., 1998; An et al., 1998; Lee et al., 1998), has implicated lysophospholipid signaling pathways in the control of nervous system development. Toward identifying further functional roles for LPA signaling in the CNS, we have examined patterns of *vzg-1* expression in the murine brain from birth to adulthood.

Here we show that postnatal *vzg-1* expression correlates temporally and spatially with oligodendrocyte differentiation and the progress of myelination throughout the brain. By using double-label in situ hybridization analyses, we show that *vzg-1*–expressing cells also express proteolipid protein (PLP) mRNA, which encodes the major protein constituent of CNS myelin (Griffiths et al., 1995), identifying them as mature oligodendrocytes. This postnatal localization of *vzg-1* expression implicates G-protein–coupled LPA signaling as a regulator of oligodendrocyte biology and nervous system myelination. A portion of this work has appeared in abstract form (Weiner et al., 1997).

MATERIALS AND METHODS

In situ hybridization

All animal protocols have been approved by the Animal Subjects Committee at the University of California, San Diego, and conform to NIH guidelines and public law. Balb/C mice between 1-day-old and 6-months-old were killed by swift decapitation (for younger animals) or cervical dislocation, and heads or isolated brains were frozen by using Tissue-Tek OCT (Miles, Elkhart, IN) and Histofreeze (Fisher, Pittsburgh, PA). Hemizygous male *jimpy* mice and matched controls (B6CBACa) were obtained from Jackson Laboratories (Bar Harbor, ME) and used at postnatal day 18 (P18). Parasagittal cryostat sections (20 μm) were cut, thaw-mounted onto charged microscope slides (Superfrost Plus, Fisher) and fixed and processed as previously described (Chun et al., 1991). Digoxigenin-labeled riboprobes were transcribed in the sense and antisense orientations from linearized plasmids containing full-length murine *vzg-1* or *png-1* (Weiner and Chun, 1997) cDNAs by using standard protocols (Boehringer Mannheim, Indianapolis, IN). Hybridization was carried out by using 2 ng/μl of labeled riboprobe in hybridization solution (50% formamide, 2× SSPE [standard sodium phosphate-EDTA; 2× = 300 mM NaCl, 20 mM NaH_2PO_4, 25 mM EDTA, pH 7.4], 10 mM dithiothreitol, 2 mg/ml yeast tRNA, 0.5 mg/ml polyadenylic acid, 2 mg/ml bovine serum albumin [fraction V], 0.5 mg/ml salmon sperm DNA) for 12–16 hours at 65°C. Slides were washed twice for 45 minutes at room temperature in 2× SSPE/0.6% Triton X-100, followed by three 30-minute washes at 65°C in high-stringency buffer (2 mM Na_4P2O_7, 1 mM Na_2HPO_4, 1 mM sodium-free EDTA, pH 7.2). Following washes, slides were incubated in a humidified chamber in blocking solution (1% blocking reagent (Boehringer Mannheim)/0.3% Triton X-100 in Tris-buffered saline [TBS]) for at least 1 hour, followed by overnight incubation with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer Mannheim) at 1:500 in blocking solution. Slides were washed in TBS and then processed for colorimetric detection (purple-brown) with nitroblue tetrazolium (NBT) and 5′-bromo, 4′-chloro, 3′-indolyl phosphate (BCIP; Boehringer Mannheim). Before coverslipping, sections were fluorescently counterstained with 0.35 μg/ml DAPI (4′, 6-diamidino-2-phenylindole, Sigma, St. Louis, MO).

Double-label in situ hybridization

For double-label studies, fluorescein-labeled riboprobes were transcribed in the sense and antisense orientations as above from plasmids containing murine glial fibrillary acidic protein (GFAP; a 1.2-kb HindIII fragment containing most of the open reading frame) or rat PLP (full-length cDNA; probe detects both *PLP* and *DM20* transcripts). Each fluorescein-labeled riboprobe was hybridized together with the digoxigenin-labeled *vzg-1* riboprobe as above. After washes and detection of the *vzg-1* signal as above, slides were rinsed well in TBS and then heated at 70°C in TBS for 2 hours to inactivate the alkaline phosphatase enzyme. Slides were then blocked for at least 1 hour with 1% blocking reagent in TBS (without Triton X-100), followed by overnight incubation with alkaline phosphatase-conjugated anti-fluorescein Fab fragments (Boehringer Mannheim) at 1:500 in the same solution. Slides were then washed in TBS and incubated with Fast Red/Naphthol Phosphate (bright pink color; Research Genetics, Huntsville, AL). Double-labeled cells were clearly identifiable, appearing a reddish-brown color. Control experiments (e.g., see Fig. 6B) demonstrated that this protocol did not produce any spurious double-labeling, and that the 2-hour treatment at 70°C was sufficient to completely inactivate the alkaline phosphatase activity from the first color reaction.

Northern blotting

Northern blots of 20 μg of total (from brain tissues) or cytoplasmic (from cell lines) RNA were made by using standard protocols (Ausubel et al., 1994). Tissue samples included the entire brain, cut off at the caudal brainstem. Blots were probed with random-primed, ^{32}P-labeled *vzg-1* full-length cDNA, *PLP* 600-bp PstI fragment, or *cyclophilin* full-length cDNA at 5×10^6 cpm probe/ml of hybridization solution (25% formamide, 0.5 M Na_2HPO_4, 1% bovine serum albumin (BSA), 1 mM EDTA, 5% sodium dodecyl sulfate [SDS]) at 55°C, followed by standard saline citrate (SSC)/SDS washes of increasing stringency (final wash of 0.2× SSC/0.1% SDS at 65°C). Blots were then either exposed to film for autoradiography or radioanalytically scanned (AMBIS), followed by quantitation of *vzg-1*

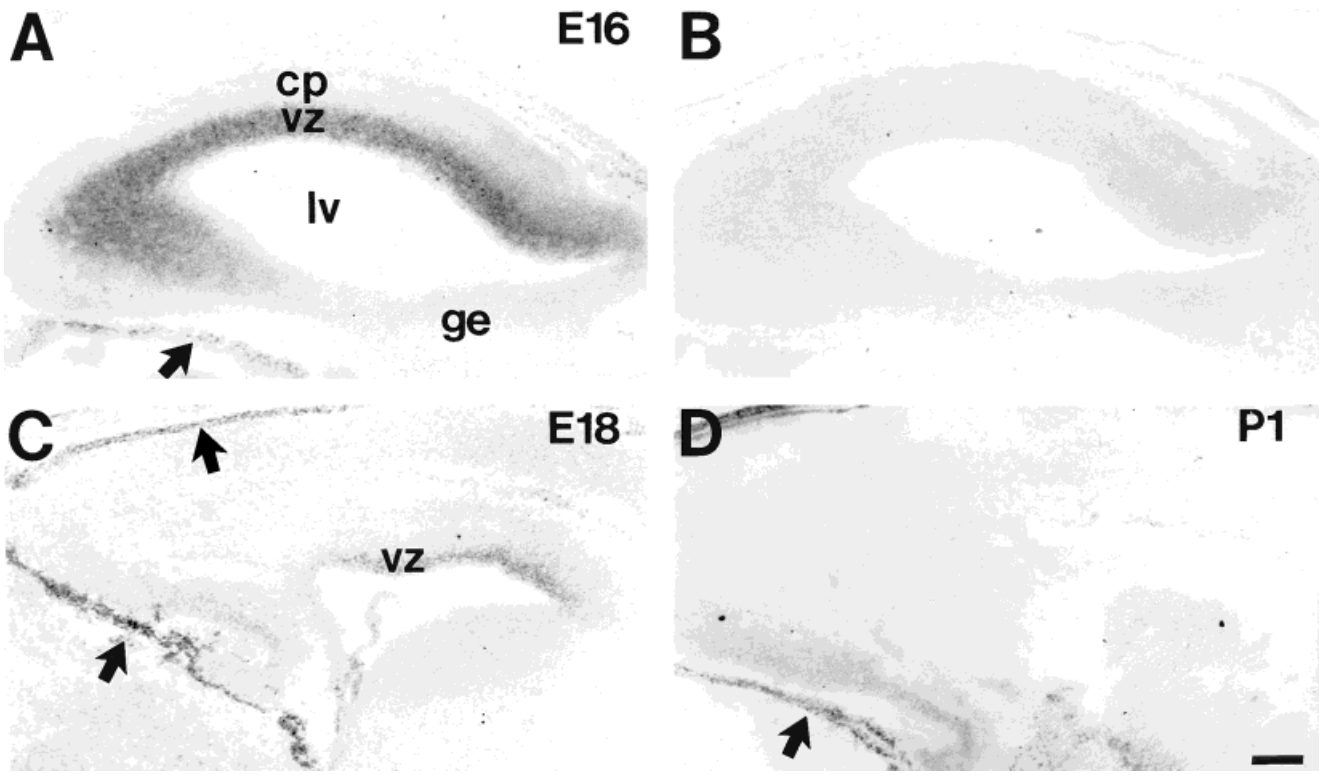

Fig. 1. *Vzg-1* expression in the embryonic cortex diminishes with the disappearance of the ventricular zone. In situ hybridization with a digoxigenin-labeled antisense riboprobe demonstrates *vzg-1* expression in the E16 cortex (**A**), restricted to the proliferative ventricular zone (vz). Expression is absent from the postmitotic neuronal cortical plate (cp), and from the proliferative zone of the basal ganglia, the ganglionic eminence (ge). Control sense riboprobes showed no hybridization on the adjacent tissue section (**B**), or on sections at any other age. At E18 (**C**), *vzg-1* expression is diminished, correlating with a decrease in the size of the vz with the end of cortical neurogenesis. By birth (P1; **D**) *vzg-1* expression is absent from the cortex and from the rest of the brain. At all three ages, however, expression is present in putative meningeal cells (arrows). lv, lateral ventricle. Dorsal, top; rostral, right. Scale bar = 200 μm.

expression, normalized to *cyclophilin* signal within each lane.

Cell culture

TR cortical neuroblast cells (Chun and Jaenisch, 1996) were grown in OPTIMEM (Gibco/BRL, Gaithersburg, MD) with 2.5% fetal calf serum (FCS), and 20 mM glucose. C6 rat glioma (ATCC, Rockville, MD) and RN2 rat Schwannoma (kind gift of Dr. Greg Lemke) cell lines were grown in DMEM (Gibco/BRL) with 10% FCS.

Figure production

Figures 4, 5, 7, and 8 were composed from autoradiographs or photographic negatives scanned into an Apple Macintosh computer by using a UMAX Power Look 2000 scanner. Color tone, contrast, and brightness were adjusted in Adobe Photoshop 4.0, and labels and arrows were added in Photoshop or in Adobe Illustrator 7.0.

RESULTS

Expression of *vzg-1* in the perinatal brain is biphasic

Our previous work (Hecht et al., 1996) demonstrated that *vzg-1* expression in the embryonic brain is restricted to the VZ of the cerebral cortex during the neurogenetic period (E12–18). By the end of that period, expression diminishes along with the extent of the VZ. The expression of *vzg-1* in the perinatal cortex is demonstrated by in situ hybridization with *vzg-1* antisense riboprobes in Figure 1. Expression was still relatively high in the VZ at E16 (Fig. 1A; the sense strand hybridization control shown in Fig. 1B, and all other control sections, gave no signal), but was greatly diminished by E18 (Fig. 1C). In the newborn (P1; Fig. 1D), *vzg-1* expression was absent from the cortex; however, the expression in what appeared to be presumptive meninges in the embryo did continue into the postnatal period (Fig. 1A,C,D, arrows). The absence of *vzg-1* expression in the cortex continued into the 2nd postnatal week (Table 1).

During the 1st postnatal week, *vzg-1* expression reappeared, first detectable in the caudal hindbrain on P2–3 (Fig. 2A). By P6, many positive cells were detected throughout the medulla, with some cells aligned in a column at the ventral surface (Fig. 2B). By P9, *vzg-1*–expressing cells were also detected in the developing white matter of the cerebellum (Fig. 2C), but not in the internal or external granule layers.

Expression of *vzg-1* in the postnatal brain is correlated temporally and spatially with myelination

With the continued postnatal development of the brain, the extent of *vzg-1* expression expanded in a caudal-to-

TABLE 1. Relative Expression of *vzg-1* in Different Brain Regions During the Postnatal Period[1]

Region/tract	P0	P3	P6	P9	P12	P15	P18	P21	P28	Young adult	Aged adult
Medulla	+/−	1+	1+	2+	3+	4+	4+	4+	3+	+/−	+/−
Cerebellum—white matter	−	−	+/−	1+	2+	4+	3+	3+	3+	−	−
Pontine fibers	−	−	−	+/−	2+	3+	4+	4+	4+	1+	+/−
Corpus callosum	−	−	−	+/−	1+	3+	4+	4+	3+	2+	1+
Cerebral cortex—scattered cells	−	−	−	−	+/−	2+	2+	2+	1+	+/−	−
Anterior commissure	−	−	−	−	−	3+	3+	4+	3+	−	−
Internal capsule	−	−	−	−	1+	3+	3+	3+	3+	+/−	−
Fimbria	−	−	−	−	1+	3+	4+	4+	3+	1+	+/−
Basal ganglia	−	−	−	−	1+	2+	2+	1+	1+	−	−
Hypothalamus	−	−	−	−	+/−	1+	2+	3+	3+	−	−
Thalamus—anterior	−	−	−	−	ND	3+	2+	2+	1+	−	−
Thalamus—posterior	−	−	−	−	ND	1+	2+	1+	+/−	−	−

[1]Expression is rated from 1+ (least) to 4+ (most) based on approximate number of labeled cells and density of histochemical reaction product per cell. Regions marked with "−" had no appreciable labeling, whereas those marked with "+/−" had very light labeling that was not observed in all sections. "Young adult" indicates mice 6–10 weeks of age; "Aged adult" indicates mice over 3 months old. N.D., not determined.

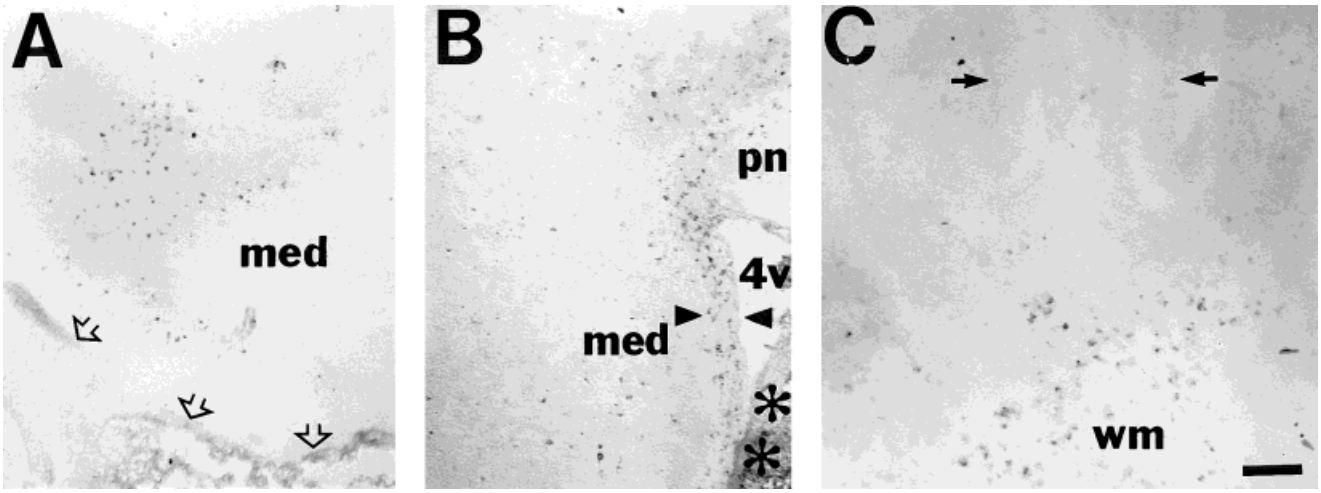

Fig. 2. *Vzg-1* expression reappears in caudal brain regions during the 1st postnatal week. *Vzg-1*–expressing cells reappear in the caudal hindbrain by P3 (**A**), and are particularly evident in ventral columns in the P6 medulla (med; **B,** arrowheads; asterisks mark non-CNS tissue). Positive cells are also detected in the developing white matter (wm), but not the neuronal internal and external granule layers of the cerebellum by P9 (**C**). pn, pons; open arrows in A mark the ventral border of the medulla; arrows in C mark the external granule layer of a cerebellar folium. 4v, fourth ventricle. A,C: dorsal, top; rostral, left; B: dorsal, top; rostral, right. Scale bars = 200 μm in A and B, 100 μm in C.

rostral fashion, with highest expression localized to emerging white matter tracts (Table 1). *Vzg-1* expression was detected in the forebrain between P9 and P12, with increasing expression between P12 and P15 in many regions (Table 1; Fig. 3), including the corpus callosum (Fig. 3A,B), the cerebellar white matter (Fig. 3C,D), and the hindbrain (Fig. 3E,F). Peak expression was attained between P18 and P21 (Fig. 4A; Table 1), with heavy labeling detected in cells within and surrounding major fiber tracts such as the anterior commissure (Fig. 4B), the internal capsule (Fig. 4C), the fimbria (Fig. 4D), and the cerebral peduncles (Fig. 4E). In some sections, *vzg-1*–positive cells were aligned in rows that appeared to follow fiber tracts coursing through the brain (e.g., Fig. 3F, arrows; Fig. 4C, arrowheads). *Vzg-1* expression did not appear to correlate with any major neuronal population: Only scattered labeled cells were detected in the cortex, and expression was absent from the prominent granule neuron layers of the hippocampus (Figs. 4A, 6A) and the cerebellum (Figs. 3C,D, 4A).

After the peak of *vzg-1* expression, levels throughout the brain declined gradually by P28, falling off more sharply by 6 weeks of age (Table 1). Expression remained clearly detectable in fiber tracts of older animals, but the fewer positive cells appeared to express a lower level of *vzg-1* transcript. A northern blot of brain RNA from E16 through the adult, probed for the 3.8 kb *vzg-1* transcript, showed an increase, peak, and decrease in expression of this single transcript corresponding to that observed with in situ hybridization (Fig. 5). The developmental expression levels observed in the northern blot analysis paralleled the levels of expression within the brain, but not the putative meningeal expression (which generally disappeared in the 2nd postnatal week), observed in situ.

The localization of *vzg-1* expression in the postnatal brain, along with the time course of its caudal-to-rostral spread, peak, and diminution, was closely correlated with prior reports of the progress of myelination in the rodent brain. Classical studies relied upon morphological criteria of oligodendrocytes and various stains for the detection of the myelin sheath itself (Jacobson, 1963; Mitrova, 1967; Caley and Maxwell, 1968; Schonbach et al., 1968; Vaughn, 1969; Sturrock, 1980), whereas more recent work has employed in situ hybridization, immunohistochemistry, and transgenic mice to track the expression of the major proteins of CNS myelin, proteolipid protein (PLP, and its isoform DM-20)

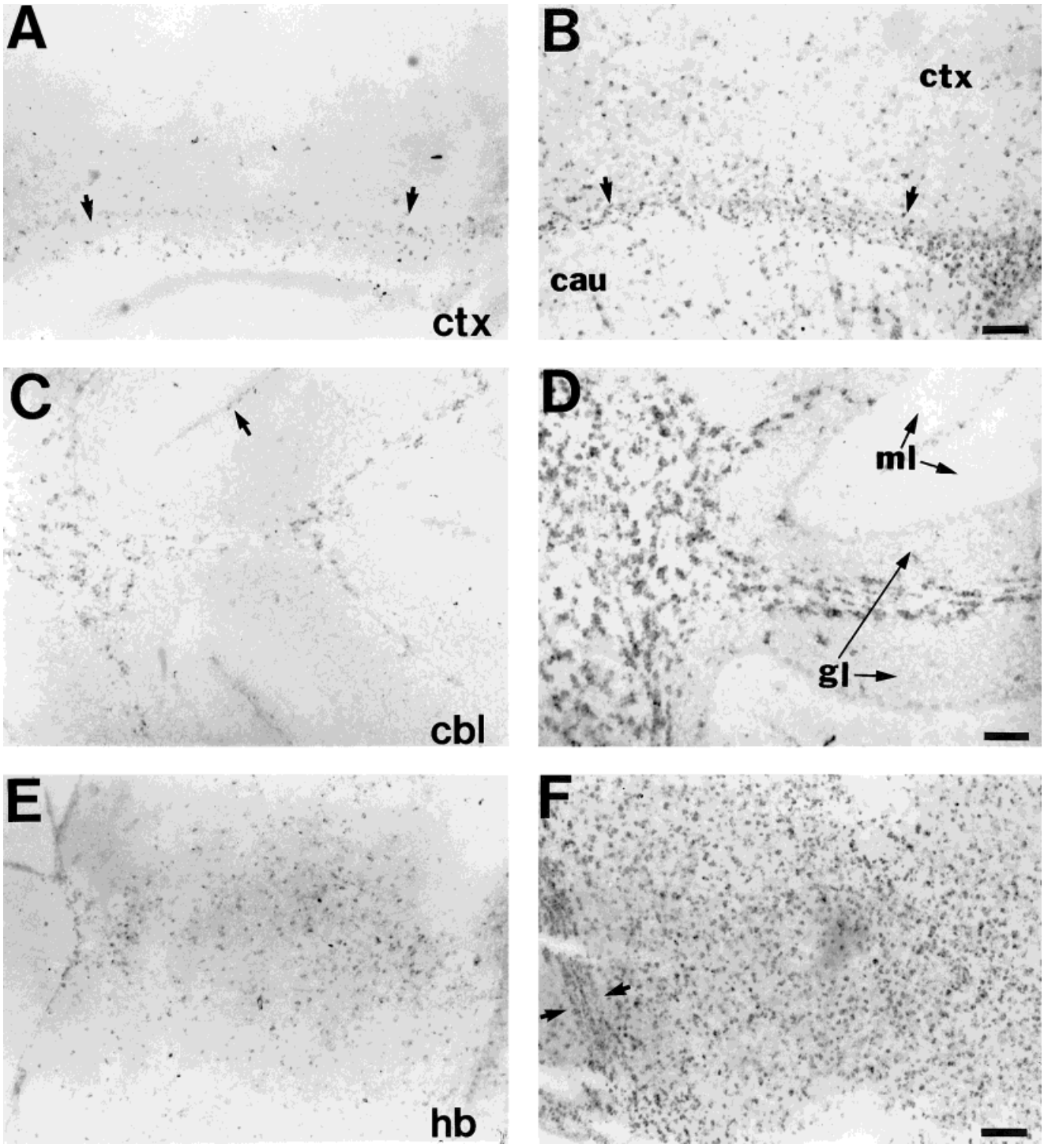

Fig. 3. *Vzg-1* expression increases at the end of the 2nd postnatal week. *Vzg-1* in situ hybridization signal increases appreciably between P12 (**A,C,E**) and P15 (**B,D,F**) in the cortex (ctx), cerebellum (cbl), and hindbrain (hb). In all regions, expression is closely associated with developing white matter tracts and is generally absent from or sparse in neuronal regions, including the cortex (ctx, A,B) and the cerebellar molecular and granule layers (ml and gl, C,D). Arrows in A and B mark the corpus callosum; arrows in F mark rows of positive cells along a fiber tract; arrow in C marks some residual putative meningeal labeling seen up to this age. Dorsal, top; rostral, left. Scale bars = 200 μm in A,B,E,F; 100 μm in C,D.

and myelin basic protein (MBP; Verity and Campagnoni, 1988; Shiota et al., 1989; Foran and Peterson, 1992). As shown in Table 2, the age of peak *vzg-1* expression in major white matter tracts closely parallels that of peak PLP and MBP expression and the appearance of morphologically observable myelin. PLP/MBP expression is diminished in

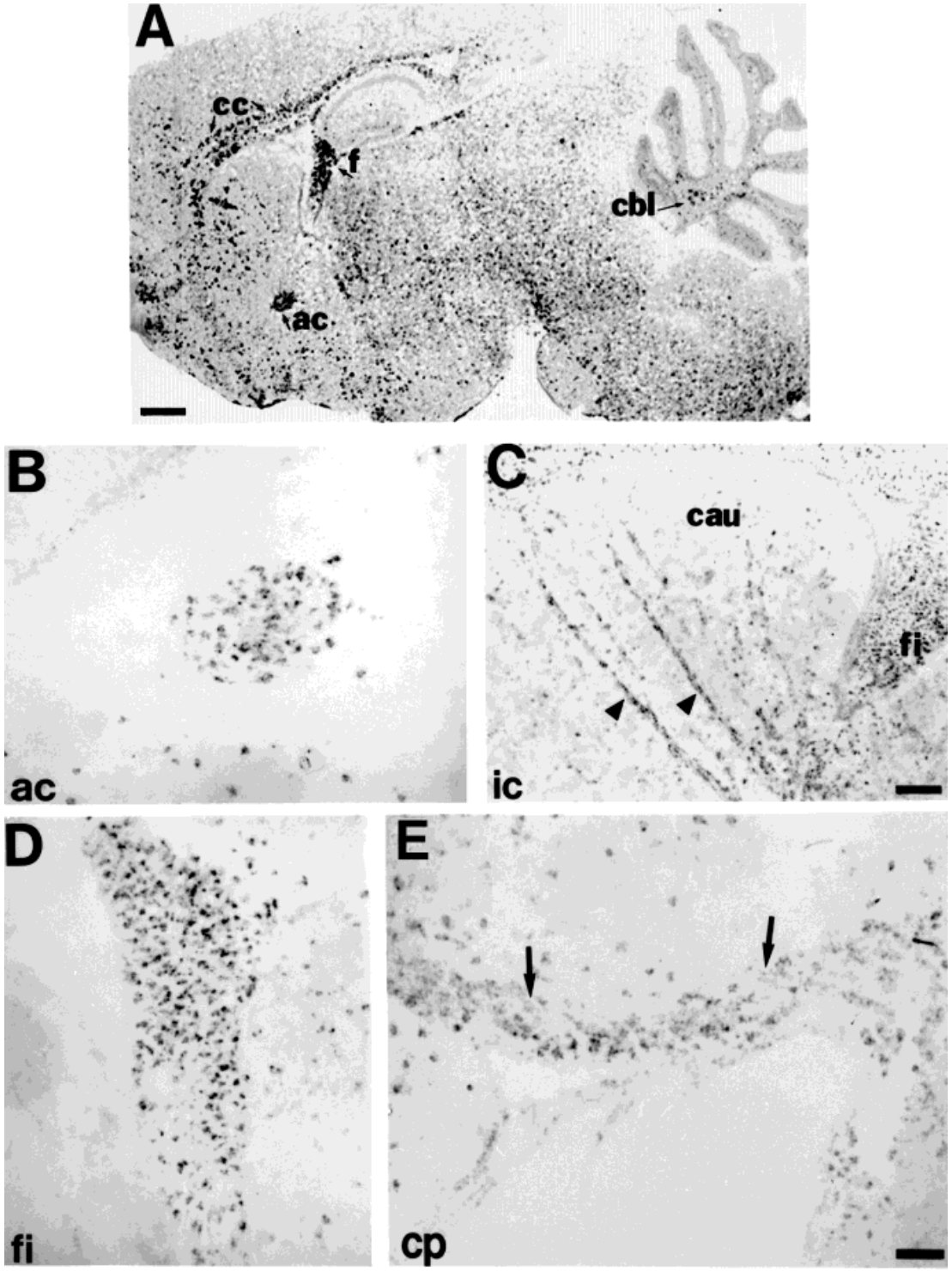

Fig. 4. Peak *vzg-1* expression in the 3rd postnatal week is localized to white matter regions. A low-magnification view of antisense *vzg-1* in situ hybridization to a sagittal section from P18 brain is shown in (**A**), with higher-magnification views of fiber tracts at P18 or P21 shown in (**B–E**). Peak expression is observed between P18 and P21 and is clearly localized to white matter tracts, including the corpus callosum (cc, A); fimbria (f, A; fi, C, D); anterior commissure (ac, A,B); cerebellar white matter (cbl, A); internal capsule (ic, C); and the cerebral peduncle (cp, E, arrows). Arrowheads in (C) indicate *vzg-1*–expressing cells radially aligned along fiber tracts of the internal capsule. cau, caudate/putamen. Dorsal, top; rostral, left. Scale bars = 900 μm in A, 100 μm in B,D,E, 200 μm in C.

the adult brain, being approximately 25% of peak expression (data not shown; Verity and Campagnoni, 1988; Shiota et al., 1989), and most myelination in the brain is complete by 6 weeks of age. The reduction of *vzg-1* expression in the mature adult brain (Table 1) parallels this slowing of the myelination process.

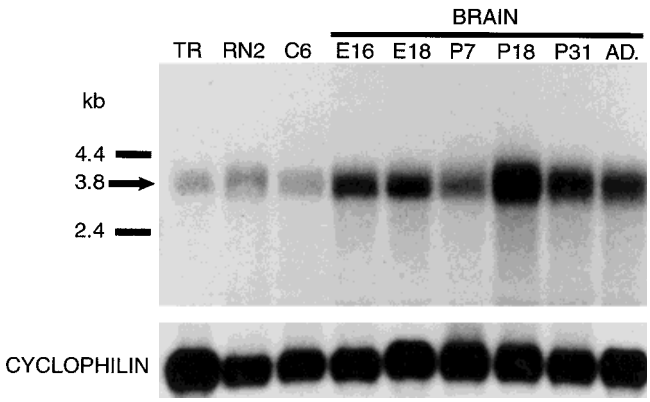

Fig. 5. Northern blot analysis detects the 3.8-kilobase *vzg-1* transcript in pre- and postnatal brain and in glial cell lines. Northern blot analysis of total or cytoplasmic RNA (20 μg) with a *vzg-1* probe demonstrates expression of the 3.8-kilobase (kb) *vzg-1* transcript (arrow) by the embryonic cortical neuroblast cell line TR (Chun and Jaenisch, 1996), the Schwannoma cell line RN2 (Pfeiffer and Wechsler, 1972), the glioma cell line C6 (Benda et al., 1968), and whole brain samples (cut off at the caudal brainstem) from E16, E18, P7, P18, P31, and adult. The approximate relative level of transcript observed at different ages parallels the intensity of the in situ hybridization signal in the brain. The prominence of the signal at E18 compared to the in situ hybridization (see Fig. 1C) is likely due to the continued expression by putative meningeal cells. Although this meningeal expression may contribute to the northern blot signal at these early ages, it is greatly diminished after birth as observed with in situ hybridization, and is thus likely only a minor contributor at later ages. A probing of the same blot for cyclophilin transcript is shown below as a loading and transfer control.

Vzg-1–expressing cells in the postnatal brain are oligodendrocytes

The temporal and spatial expression pattern described above suggested that the postnatal *vzg-1*–expressing cells were glia, and probably oligodendrocytes associated with myelination. Consistent with this, northern blot analysis indicated that *vzg-1* was expressed by the C6 rat glioma cell line, which has oligodendrocyte-like properties (Benda et al., 1968; Parker et al., 1980), and by RN2 (Pfeiffer and Wechsler, 1972), a tumor line with properties of Schwann cells, the myelinating glia of the PNS (Fig. 5). To confirm that *vzg-1*–expressing cells in the postnatal brain were oligodendrocytes, we employed a double-label, nonradioactive in situ hybridization method combining a digoxigenin-labeled *vzg-1* riboprobe with a fluorescein-labeled riboprobe to the *PLP* gene as a marker for oligodendrocytes (see Materials and Methods). The *PLP* gene gives rise to two protein isoforms: PLP, the complete protein, and DM-20, which has a 35-amino acid deletion (Griffiths et al., 1995). Although low expression of the *DM-20* transcript has been reported in some embryonic neural cell types (Timsit et al., 1992; Yu et al., 1994), the *PLP* gene is expressed solely by mature, myelinating oligodendrocytes in the postnatal brain (Griffiths et al., 1995; Yan et al., 1996).

In sections from all ages examined, essentially all *vzg-1*–expressing cells detected by in situ hybridization co-expressed PLP mRNA. Figure 6 shows results from experiments performed on brain sections from P18, the peak of *vzg-1* and *PLP* expression. In these experiments, the *vzg-1* hybridization signal appears purple-brown, whereas *PLP* hybridization signal appears bright pink. Double-labeled cells were clearly identifiable, appearing a brownish red color distinct from that produced by either label alone. A low-magnification view of the hippocampal region, double-labeled for *vzg-1* and *PLP* (Fig. 6A), demonstrated co-expression in the cells which make up the fimbria and the corpus callosum; as expected, neither transcript was detected in the neuronal hippocampus. An adjacent section (Fig. 6B), double-labeled with riboprobes to *PLP* (pink) and to *png-1* (purple-brown), a postmitotic neuronal marker (Weiner and Chun, 1997), demonstrated the expected separate populations of oligodendrocytes and neurons and served as a control, confirming that no spurious double-labeling was produced by our technique.

Higher-magnification views of three adjacent sections through the anterior commisure, hybridized with riboprobes to *vzg-1* (Fig. 6C), PLP (Fig. 6D), or both (Fig. 6E), clearly demonstrated the different appearance of the single- and double-labeled cells, and showed that essentially all of the *vzg-1*–expressing cells co-expressed *PLP* (and vice versa; co-expression was near 100% in more than 1,000 cells counted from various areas). In the P18 cortex, the few *vzg-1*–expressing cells also expressed PLP mRNA (Fig. 6F) and appeared to have small, irregularly shaped nuclei distinct from those of many of the surrounding cortical neurons (Fig. 6G). Cells expressing *vzg-1* also expressed PLP mRNA in every region examined at P18, including the internal capsule (Fig. 6H) and the corpus callosum (Fig. 6I), and at every age examined (data not shown). Even in sections from P3 brain, around the time the first *vzg-1*– (see Fig. 2) and *PLP*– (data not shown; Verity and Campagnoni, 1988; Shiota et al., 1989) expressing cells were observed, the two transcripts were co-expressed.

Astrocytes are also observed in some portions of white matter tracts. Although the expression pattern of *vzg-1* did not appear to overlap with that reported for the astrocyte marker gene *GFAP* (Landry et al., 1990), it remained possible that a very small number of *vzg-1*–expressing cells were astrocytes. To examine this possibility, double-label experiments were performed using a fluorescein-labeled riboprobe to *GFAP*, along with the digoxigenin-labeled *vzg-1* riboprobe. *Vzg-1* expression did not overlap with GFAP mRNA expression at any age examined, as shown in a section from the P18 fimbria (Fig. 7B). In this and in other white matter tracts, the few *GFAP*-positive cells (pink) clearly did not co-express *vzg-1* (purple-brown), and vice versa. We also used this double-labeling procedure on sections of the P1 brain, to determine whether the *vzg-1*–expressing cells observed around the meninges (see Fig. 1) were astrocytes, which form the glial limitans in these regions (Landry et al., 1990; Jacobson, 1991). Co-expression of *vzg-1* and *GFAP* was also not observed in these sections (Fig. 7A), suggesting that the *vzg-1*–expressing cells at these early ages may be mesenchymal or neural crest-derived (Jacobson, 1991).

Vzg-1 expression is reduced in the brains of *jimpy* mice

In the *jimpy* mutant mouse, a mutation in the X-linked *PLP* gene leads to aberrant oligodendrocyte differentiation and to increased oligodendrocyte cell death in hemizygous males (Knapp et al., 1986; Griffiths et al., 1995). Because *vzg-1* is expressed by differentiated oligodendrocytes, we asked whether *vzg-1* expression was reduced in *jimpy* brain. Northern blot analysis using a *vzg-1* probe (Fig. 8A) demonstrated that expression is in fact reduced in the brains of 3-week-old *jimpy* mice, as compared to litter-

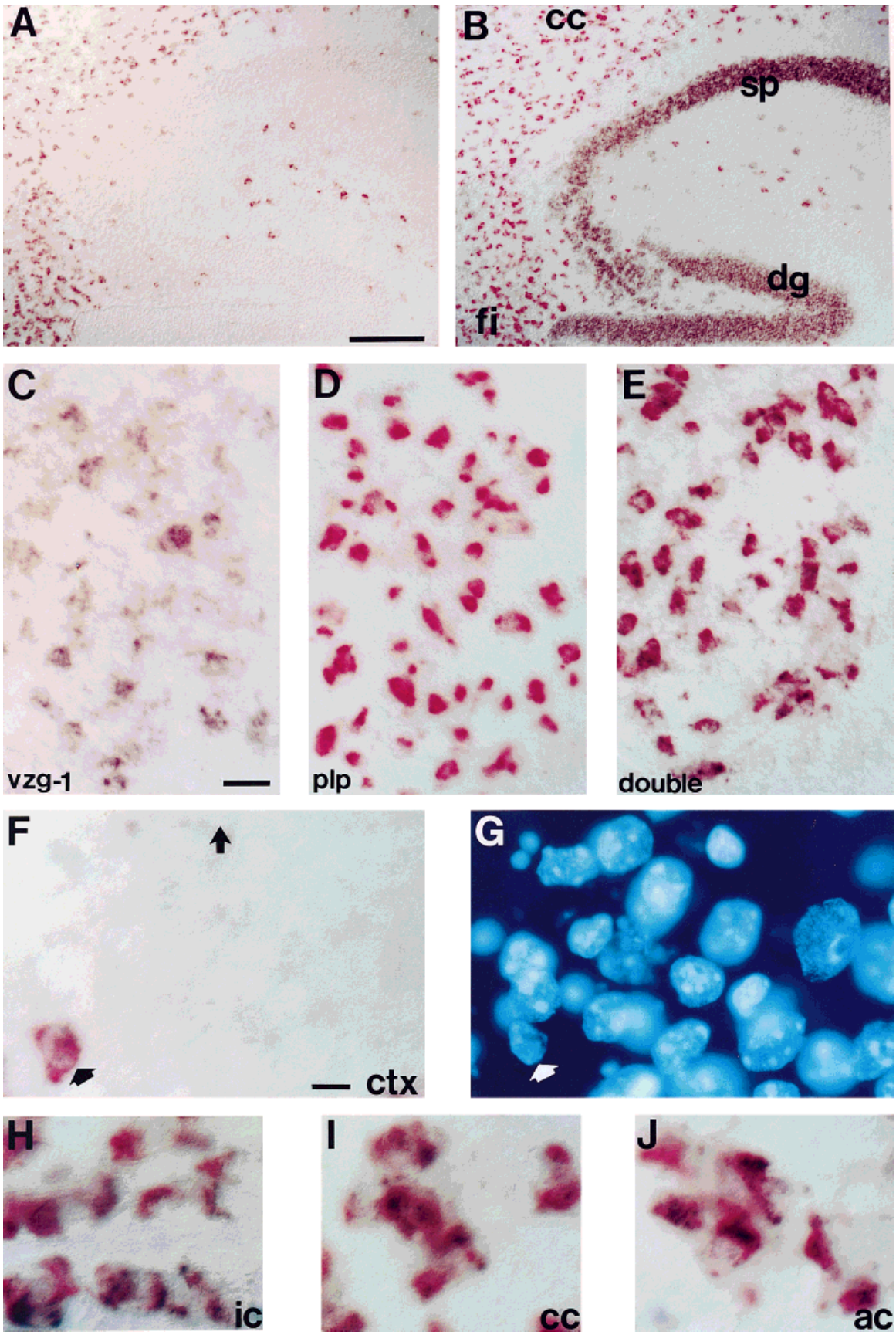

Figure 6

TABLE 2. Correlation of Postnatal Age of Peak *vzg-1* Expression in Various Tracts With That of Peak Myelin Gene Expression and Observable Myelination[1]

Region/tract	Peak *vzg-1* expression	Peak PLP/MBP expression[2]	Myelination observed morphologically
Cerebellum—white matter	P15	P14	Peak between P13 and P16[3]
Corpus callosum	P18–P21	P20	Peak at P20[4]
Anterior commisure	P21	P20	P17 start, sharp increase by P24[5]
Fimbria	P18	P20	P12 start, peak a few days thereafter[5]
Internal capsule	P15–P18	P14–P20	P10 start, sharp increase P14–P22[5]
Pontine fibers	P18	P20	P14 start, peak thereafter[5]

[1]PLP, proteolipid protein; MBP, myelin basic protein.
[2]Mouse: Verity and Campagnoni (1988), Shiota et al. (1989), Foran and Peterson (1992), present study.
[3]Rat: Mitrova (1967).
[4]Mouse: Sturrock (1980).
[5]Rat: Jacobson (1963).

mates and to wild-type Balb/C mice. A duplicate northern blot probed for *vzg-1* was quantitated radioanalytically, with signal intensity normalized to that of the ubiquitously expressed *cyclophilin* gene in each lane (Fig. 8B). The *vzg-1* signal in *jimpy* brain was only 60% of that in wild-type littermates, consistent with the reduced number of mature oligodendrocytes in the mutant brains. In situ hybridization experiments (not shown) on sections from P18 *jimpy* brains demonstrated fewer *vzg-1*–positive cells and further suggested that the expression level per cell was reduced.

DISCUSSION

We have examined the developmental expression of the LPA receptor gene *vzg-1/lp$_{A1}$/edg-2* in the postnatal murine brain. *Vzg-1* expression in the embryonic cerebral cortical VZ diminishes at the end of the neurogenetic period (E18) and is absent from the brain at birth. Expression reappears in the hindbrain during the first few postnatal days and expands in a caudal-to-rostral manner over the next 3 weeks, reaching a peak at P18. At all ages, *vzg-1* expression is concentrated in and around developing white matter tracts. Double-labeling experiments demonstrate that *vzg-1*–expressing cells co-express mRNA encoding PLP but not GFAP, identifying them as mature oligodendrocytes. Consistent with this, *vzg-1* mRNA expression is reduced by 40% in the brains of *jimpy* mutant mice, which are known to exhibit aberrant oligodendrocyte differentiation and increased oligodendrocyte cell death.

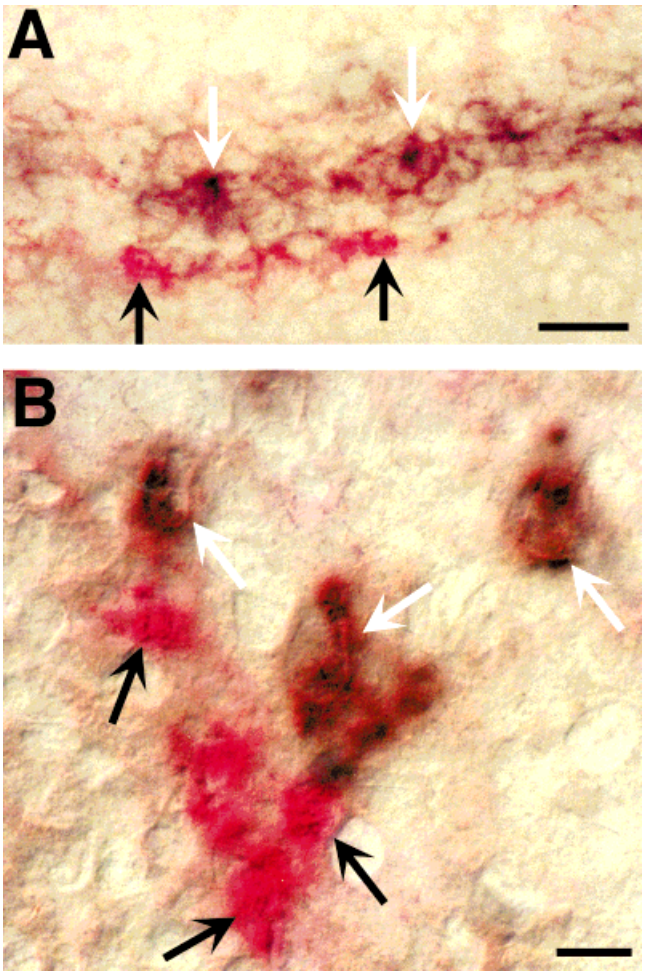

Fig. 7. *Vzg-1* is not expressed by astrocytes. Double-label in situ hybridization experiments on P1 (**A**) and P18 (**B**) brain sections employing a digoxigenin-labeled *vzg-1* riboprobe (detected with an anti-digoxigenin antibody; purple-brown precipitate) and a fluorescein-labeled *glial fibrillary acidic protein* (*GFAP*) riboprobe (detected with an anti-fluorescein antibody; bright pink precipitate) demonstrate that *vzg-1* is not expressed by astrocytes. In the P1 forebrain (A), *vzg-1* is expressed by cells which are likely to be meningeal (white arrows; see also Fig. 1). *GFAP*-positive astrocytes forming the glial limitans (black arrows) are closely apposed to the *vzg-1*–positive cells; however, double-labeled cells are not observed. *GFAP*-positive astrocytes line the outer edge of the P18 fimbria (B, black arrows), whereas *vzg-1*–positive cells lie within the tract itself (white arrows); again, double-labeled cells are not observed. Scale bars = 30 μm in A, 10 μm in B.

Fig. 6. *Vzg-1* is expressed by mature oligodendrocytes. Double-label in situ hybridization experiments on P18 brain sections employing a digoxigenin-labeled *vzg-1* riboprobe (detected with an anti-digoxigenin antibody; purple-brown precipitate) and a fluorescein-labeled *PLP* riboprobe (detected with an anti-fluorescein antibody; bright pink precipitate) demonstrate that *vzg-1*–expressing cells are oligodendrocytes. **A:** A low-magnification view of the P18 hippocampal area shows double-labeled cells (reddish-brown) in the white matter regions surrounding the unlabeled hippocampal granule neurons; in contrast, double-labeling with riboprobes to *PLP* (pink cells) and to *png-1*, a neuronal marker (Weiner and Chun, 1997; purple-brown cells), reveals the distinct oligodendroglial and neuronal cell populations (**B**). Adjacent sections through the anterior commissure demonstrate the appearance of *vzg-1* detection alone (**C**), *PLP* detection alone (**D**), and combined double-labeling (**E**). Double-labeled cells are clearly distinguishable by their dark reddish-brown color. A double-labeled cell in the cortex (**F,** short arrow; long arrow points to pial surface) has a small and irregularly shaped nucleus (**G,** 4′,6-diamidino-2-phenylindole [DAPI] fluorescence of the same section). **H–J:** High-magnification views of double-labeled cells in the internal capsule (ic), corpus callosum (cc), and anterior commissure (ac) clearly demonstrate the co-localization of the two reaction products. fi, fimbria; sp, stratum pyramidale; dg, dentate gyrus. A–G: dorsal, top, rostral, left. Scale bars = 200 μm in A,B, 30 μm in C–E, 10 μm in F–J.

Expression of *vzg-1* is biphasic in the perinatal murine brain

During the embryonic development of the CNS, *vzg-1* expression is restricted to the cortical VZ (Hecht et al., 1996; see Fig. 1A). This first phase of expression correlates

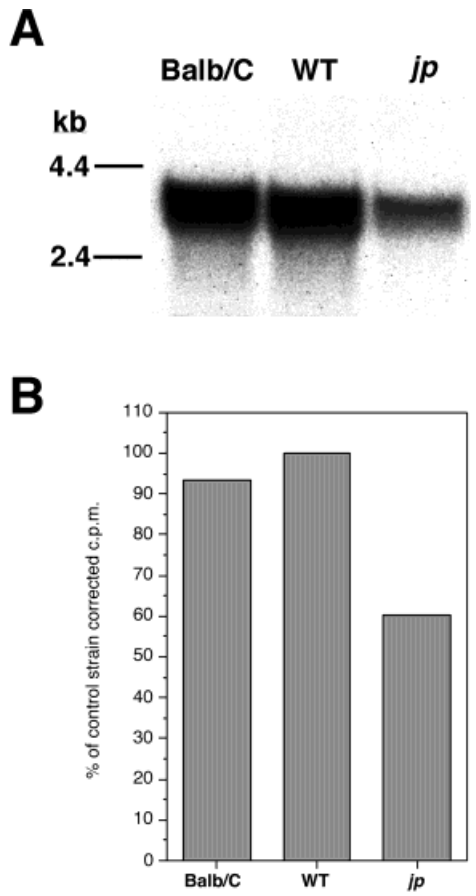

Fig. 8. *Vzg-1* expression is reduced by 40% in the brains of *jimpy* mutant mice. Northern blot analysis (**A**) of brain RNA from 3-week-old *jimpy* (*jp*), sibling control (WT), and Balb/C mice with a *vzg-1* probe demonstrates a greatly reduced amount of the 3.8-kb transcript in the *jp* brain. A duplicate blot was probed with both *vzg-1* and *cyclophilin* probes, and signal was quantitated by using a radioanalytic scanner. *Vzg-1* signal was normalized to *cyclophilin* signal in each lane to control for any unequal loading and graphed (**B**). This analysis demonstrates that *vzg-1* expression in *jp* brain is only 60% of that in WT control brain. c.p.m., counts per minute.

temporally with the cortical neurogenetic period, diminishing along with the extent of the VZ by E18. At birth, *vzg-1* expression is undetectable in the brain by in situ hybridization. A second distinct phase of expression begins in the first few postnatal days, in a population of hindbrain oligodendrocytes, with continual rostral expansion over the next 3 weeks. This caudal-to-rostral expansion of *vzg-1* expression correlates with the appearance of differentiated oligodendrocytes as monitored by PLP and MBP mRNA and protein expression (Verity and Campagnoni, 1988; Shiota et al., 1989; Foran and Peterson, 1992) and with the development of observable brain myelination (Jacobson, 1963; Mitrova, 1967; Caley and Maxwell, 1968; Schonbach et al., 1968; Vaughn, 1969; Sturrock, 1980).

These two phases of *vzg-1* expression are clearly distinct, not only temporally (with a clear pause around birth), but with regard to the cell lineages involved. During the first phase of *vzg-1* expression, neurons are the predominant cells being produced in the cortical VZ (Bayer and Altman, 1991). The expression of *vzg-1* disappears as the neuron-producing VZ diminishes in size near the end of the embryonic period. Glia, including oligodendrocytes, are generally produced from the adjacent subventricular zone after the end of neurogenesis, when *vzg-1* expression is absent (Das, 1979; Jacobson, 1991; Levison and Goldman, 1993). Therefore, the embryonic and postnatal phases of *vzg-1* expression in the brain likely represent two different cell lineages, rather than the same lineage before and after differentiation. This biphasic expression pattern suggests differential regulation of the *vzg-1* gene pre- and postnatally, which may involve distinct promoters (J.J.A. Contos and J. Chun, unpublished observations).

Vzg-1 encodes a novel oligodendrocyte-associated LPA receptor

Cells of the oligodendrocyte lineage pass through a number of different stages during their differentiation, which can be identified by the sequential expression of various molecular markers (Compston et al., 1997). The O2-A progenitor cell, which can give rise in vitro to both oligodendrocytes and type-2 astrocytes (Raff et al., 1983, 1984), can be identified by the A2B5 monoclonal antibody, whereas subsequently produced oligodendrocyte progenitors express the O4 antigen (Miller, 1996). Expression of galactocerebroside marks the terminal differentiation of oligodendrocytes and is followed by the nearly coincident appearance of the major myelin proteins MBP and PLP with the onset of myelination (Compston et al., 1997). The double-labeling studies reported here demonstrate that *vzg-1*–expressing cells co-express PLP mRNA in all regions and at all ages examined, beginning with the first *vzg-1*–positive cells in the early postnatal hindbrain. These data thus identify *vzg-1* expression as a novel marker for differentiated oligodendrocytes and suggest that initiation of *vzg-1* expression may contribute to the myelinating oligodendrocyte phenotype. This is supported by the observation that *vzg-1* expression, as detected by in situ hybridization, is downregulated in the oligodendrocytes of older animals, after myelination has been completed. The possibility exists that residual *vzg-1* expression in some post-myelinating oligodendrocytes could confer an ability to respond to survival or remyelination signals in response to injury.

We have demonstrated that *vzg-1* encodes a G-protein coupled receptor for lysophosphatidic acid (LPA; Hecht et al., 1996; Fukushima et al., 1998), an identification confirmed independently (An et al., 1997; Erickson et al., 1998). The expression of *vzg-1* by differentiated oligodendrocytes therefore implies that lipid-stimulated G-protein–coupled signaling pathways are active in these cells during myelination. This is notable in that previous studies of oligodendrocyte development and survival have focussed prominently on peptide growth factors such as PDGF and bFGF, CNTF, and ARIA (neuregulin), that bind to receptor tyrosine kinases or activate cytoplasmic tyrosine kinases (Barres et al., 1992; Louis et al., 1993; Vartanian et al., 1994; Bhat, 1995; Segal and Greenberg, 1996; Vartanian et al., 1997). The G-protein–mediated signaling pathways activated by LPA involve components distinct from such tyrosine kinase–activated pathways, although some convergence can be expected. LPA, via VZG-1, can act through at least two types of G proteins: pertussis toxin–sensitive G_i, which inhibits adenylyl cyclase and activates the Ras-MAP kinase cascade, and a pertussis toxin–insensitive G protein

that activates the Rho cytoskeletal pathway (Hecht et al., 1996; Fukushima et al., 1998). It will be important in future studies to identify which of these signaling pathways are active in oligodendrocytes and to determine how they interact with those employed by other growth or survival factors.

LPA is a novel potential mediator of myelination

LPA produces a wide variety of effects on many different cell types, including activation of proliferation, inhibition of gap junction communication, and morphological changes such as stress fiber formation and neurite retraction (Jalink et al., 1994; Hecht et al., 1996; Moolenaar et al., 1997; Fukushima et al., 1998). Studies examining the effects of LPA on oligodendrocytes or their PNS counterparts, Schwann cells, have yet to be reported. However, our demonstration of *vzg-1* expression by oligodendrocytes predicts that LPA can act on these cells. Some potential functions may be inferred from the effects of serum, which contains high concentrations of LPA (Eichholtz et al., 1993), on oligodendrocytes in vitro. For example, serum is able to prolong the survival of oligodendrocytes derived from the O2-A cell line CG-4 (Louis et al., 1992), and a serum factor, possibly a lipid, has been shown to increase galactocerebroside levels in primary oligodendrocyte cultures (Bologa et al., 1988). Serum also has a variety of effects on cultured sciatic nerve Schwann cells; these peripheral myelinating cells also express *vzg-1* both in vivo and in vitro (not shown; Weiner and Chun, unpublished observations).

A potential relationship of LPA signaling to the elaboration of the myelin sheath is suggested by the fact that lipids, including in large part complex phospholipids, make up approximately 75–80% of the dry weight of CNS and PNS myelin (Gould et al., 1992; Stoffel and Bosio, 1997). Lysophospholipids such as LPA and the structurally and functionally similar sphingosine-1-phosphate could be produced during the elaboration of myelin, and could act as an autocrine "feedback" signal for oligodendrocytes. Not mutually exclusive is the possibility that axons could also produce these signaling lysophospholipids. Future studies on receptor protein localization, in vitro and in vivo function, and LPA production in the nervous system will clarify the role of lysophospholipid signaling in oligodendrocytes and its relationship to known peptide growth factor signaling mechanisms.

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