Embryonic brain expression analysis of lysophospholipid receptor genes suggests roles for *s1p$_1$* in neurogenesis and *s1p$_{1-3}$* in angiogenesis

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Abstract In a comparison of embryonic brain expression patterns of lysophosphatidic acid and sphingosine 1-phosphate receptor genes (*lpa$_{1-3}$* and *s1p$_{1-5}$*, respectively), transcripts detected by Northern blot were subsequently localized using in situ hybridization. We found striking *s1p$_1$* expression adjacent to several ventricles. Near the lateral ventricle, *s1p$_1$* expression was temporally and spatially coincident with neurogenesis and overlapped with *lpa$_1$* in the neocortical area. We also observed a widespread diffuse pattern for *lpa$_{2-3}$* and a scattered punctate pattern for *s1p$_{1-3}$*. The punctate pattern colocalized with vascular endothelial markers. Together, these results suggest that *s1p$_1$* influences neurogenesis and *s1p$_{1-3}$* influence angiogenesis in the developing brain.

Key words: CD34; PECAM-1; EDG; G-protein coupled receptor; Cerebral cortex; Mesencephalon; LPA; S1P; lpB

1. Introduction

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are bioactive lysophospholipids widely distributed in mammalian tissues [1,2]. Three G-protein coupled receptors mediate cellular responses to LPA (termed LPA_{1-3}, or, formerly, LP_{A1-3}, or Edg2, Edg4, and Edg7, respectively), and five similar receptors mediate cellular responses to S1P (termed $S1P_{1-5}$, also termed LP_{B1-3}, LP_{C1}, and LP_{B4}, or Edg1, Edg5, Edg3, Edg6, and Edg8, respectively [3,4]). There are many cellular responses to LPA and S1P, including increased proliferation, suppression of apoptosis, neurite retraction, cell rounding, and cell migration [4–7]. Based on cellular responses and receptor expression patterns it has been proposed that LPA and S1P are involved in wound healing, neurogenesis, angiogenesis, and tumorigenesis [4–8].

Several LPA receptor genes are expressed in the embryonic brain. The first lysophospholipid receptor gene identified, *lpa$_1$*, was shown to have specific expression in the ventricular zone of the mouse embryonic cerebral cortex during the period of neurogenesis [9]. This suggested a role for *lpa$_1$* in nervous system development that was subsequently confirmed in deletion mutant mice [10]. Transcripts of the two additional LPA receptor genes, *lpa$_2$* and *lpa$_3$*, were found in the embryonic mouse brain using Northern blots and RT-PCR [11,12]. However, no in situ hybridization localization data exist, so specific brain regions potentially affected by *lpa$_2$* and *lpa$_3$* are not known.

Likewise, little is known regarding the expression of S1P receptors in the embryonic mouse brain. It is likely that the *s1p$_1$* transcript is present in the embryonic telencephalon based on β-galactosidase activity in mutant mice in which lacZ replaced the normal open reading frame [13]. Also, the zebra fish *s1p$_1$* homolog was expressed in the optic stalks, hypothalamus, hindbrain, and adjacent to several ventricles [14]. The *s1p$_2$* transcript was found in the rat embryonic brain using Northern blot [15], and immunohistochemistry localized the protein to neurons [16]. In addition, while the *s1p$_3$* transcript was localized by in situ hybridization to the embryonic mouse choroid plexus [17] and dot blots showed a very faint expression of *s1p$_4$* in the human fetal brain [18], little is known regarding *s1p$_5$* expression in the embryonic brain. No comparative analysis of the expression of all *s1p* genes in embryonic brain currently exists.

In this study, we first used Northern blots to determine which of the *s1p* receptor transcripts were expressed in the embryonic mouse brain (data on *lpa* transcripts have already been published [12]). We then used in situ hybridization to localize the *lpa* and *s1p* gene transcripts that showed prominent expression with Northern blots. The results suggest potential roles for lysophospholipid receptors in both neurogenesis and angiogenesis within the developing brain.

2. Materials and methods

2.1. Materials, animals, and tissue processing

Unless otherwise noted, chemicals were purchased from Sigma. Three-month-old male (for Northern blots) or timed-pregnant female (for in situ hybridizations) BALB/c mice were purchased from Charles River Laboratories and sacrificed by cervical dislocation. The day after vaginal plug was designated the first embryonic day. Embryos were fresh-frozen in Tissue-Tek OCT compound (Sakura) and tissue sections were prepared as previously described [19]. Animal protocols conformed to NIH guidelines and were approved by the University of California, San Diego Animal Subjects Committee.

2.2. Northern blots, riboprobe preparation, and in situ hybridization

Northern blots were prepared and analyzed as described previously [12], using DNA fragments containing coding regions from each *s1p* gene. For riboprobe synthesis, linearized plasmids containing coding regions of mouse *lpa$_{1-3}$*, *s1p$_{1-3}$*, platelet/endothelial cell adhesion molecule-1 (*PECAM-1*), and *CD34* were transcribed in sense and antisense directions using T7, T3, or SP6 (*PECAM-1* sense only) RNA polymerase [9,11,12,20–23]. Hybridization of digoxigenin-labeled riboprobes and visualization using an alkaline phosphatase-conjugated

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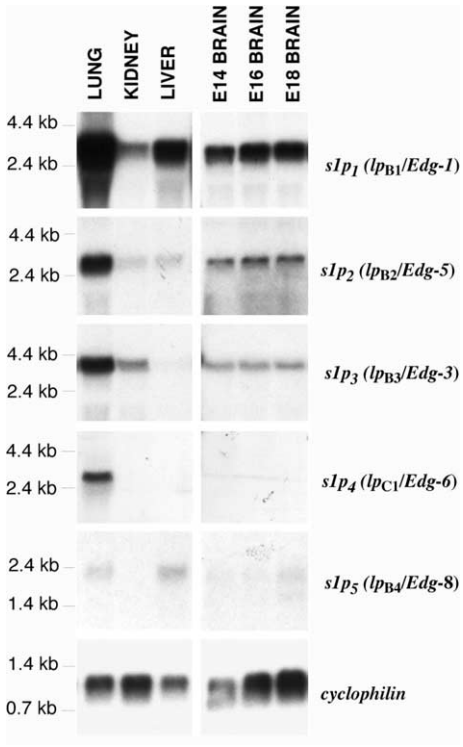

Fig. 1. Northern blots probed for *s1p* genes. Total RNA blots (20 μg per lane) are shown from adult lung, kidney, and liver (controls; left panel); and E14, E16, and E18 whole mouse brain (right panel). Molecular weight marker sizes and positions are indicated to the left. A cyclophilin probe was used to demonstrate mRNA loading quantity.

anti-digoxigenin antibody (Roche) were performed as previously described [24]. Sections were subsequently counterstained using 0.35 μg/ml DAPI (4′,6-diamino-2-phenylindole).

2.3. Double-labeling studies

For bromodeoxyuridine (BrdU) labeling and in situ hybridization, 1 h prior to sacrifice, timed-pregnant BALB/c mice were intraperitoneally injected with 20 μl 10 mM BrdU per gram body weight. Tissues were processed and in situ hybridization carried out as described above, then sections were processed for BrdU labeling as previously described [9].

2.4. Double in situ hybridization labeling

For double in situ hybridization labeling, fluorescein-labeled $s1p_1$ or $s1p_3$ riboprobes and digoxigenin-labeled *CD34* or *PECAM-1* riboprobes were used. Sections hybridized with both probes were washed as described above and processed sequentially per the TSA Plus Fluorescein System and TSA Cyanine 3 System protocols (New England Nuclear). Sections were subsequently counterstained with DAPI. Both anti-digoxigenin and anti-fluorescein POD antibodies (Roche) were used at a concentration of 1:2000.

3. Results

3.1. Northern blots demonstrate $s1p_{1-3}$ expression in embryonic brain

Northern blots were used to determine whether *s1p* genes were expressed in the embryonic mouse brain. Total brain RNA from embryonic days (E) 14, 16, and 18, as well as control adult tissues, were probed with unique DNA fragments from each *s1p* gene cDNA (Fig. 1). We found a high expression level of $s1p_1$ and lower but clearly detectable expression levels of $s1p_2$ and $s1p_3$ at each age. There was no detectable $s1p_4$ and only a barely detectable level of $s1p_5$. These results suggested that $s1p_{1-3}$ genes, in addition to lpa_{1-3} genes [11,12], may play roles in embryonic central nervous system (CNS) development.

3.2. In situ hybridization reveals distinct expression patterns for lysophospholipid receptors in embryonic brain

To compare spatial patterns of prenatal mouse brain expression of lysophospholipid receptor genes, parasagittal E14 embryonic brain sections were examined by in situ hybridization. One striking pattern of expression, observed for lpa_1 and $s1p_1$, was in bands of cells adjacent to one or more

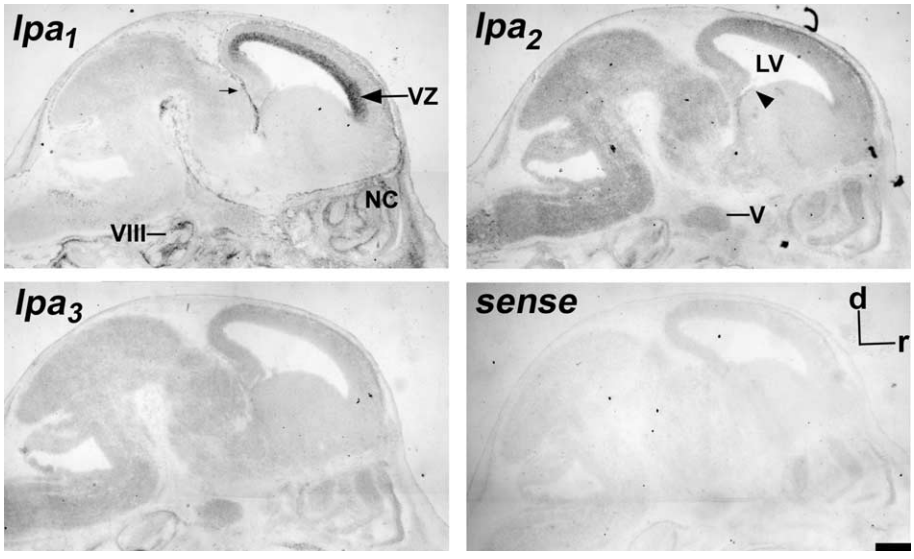

Fig. 2. E14 embryonic mouse brain localization of *lpa* receptors in sagittal sections. Signal for lpa_1 delimits meninges (small arrow) and the neocortical ventricular zone (VZ, large arrow), and is also obvious in the nasal cavity (NC) and vestibulocochlear ganglion (VIII). Signal for lpa_2 extends diffusely throughout the CNS, and is also apparent in the vestibulocochlear ganglion, trigeminal ganglion (V), and choroid plexus (arrowhead) of the lateral ventricle (LV). Like lpa_2, signal for lpa_3 also extends diffusely throughout the CNS and is apparent in the trigeminal ganglion. The lower right panel shows a section hybridized with a sense lpa_2 probe as a negative control. Scale bar, 500 μm, d = dorsal, r = rostral.

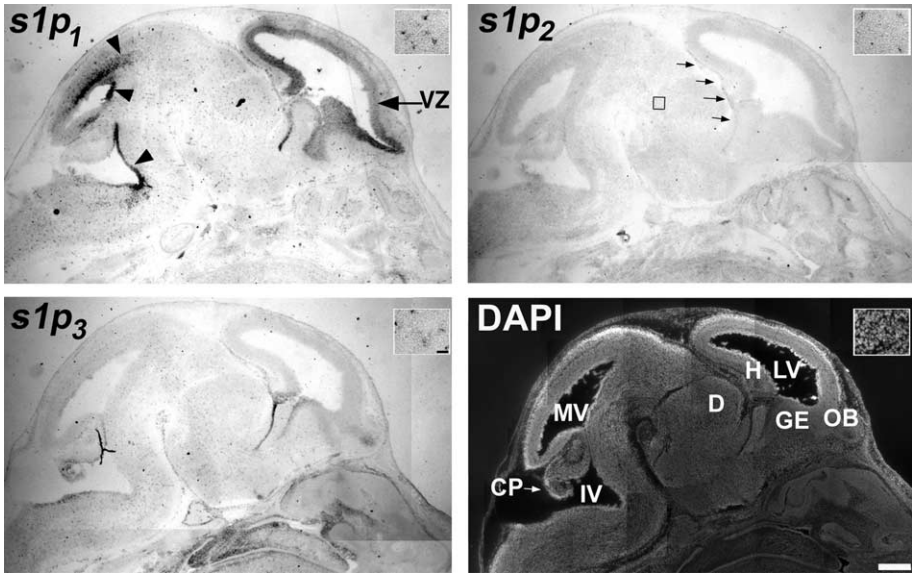

Fig. 3. E14 embryonic mouse brain localization of *s1p* receptors in sagittal sections. Signal for $s1p_1$ delimits the ventricular zone (VZ, large arrow) around the lateral ventricle extending into the hippocampal region (H), olfactory bulb (OB), and ganglionic eminence (GE). Intense $s1p_1$ signal is also seen by the mesencephalic vesicle (MV) and fourth ventricle (IV), denoted by two lower arrowheads. The uppermost arrowhead denotes the pretectum. Insets show magnified views of part of the diencephalon (D) and demonstrate punctate $s1p_{1-3}$ signals in scattered cells (a small box in the $s1p_2$ panel depicts magnification region and insets in other panels are chosen from a similar position in the diencephalon). Signals for $s1p_{1-3}$ are also present in the choroid plexus (CP) of the fourth ventricle and in meninges (small arrows). Lower right panel shows fluorescence image of DAPI-stained section. Scale bars, 500 μm low magnification view; 100 μm insets, LV = lateral ventricle.

ventricles (Figs. 2 and 3). By the lateral ventricle, both lpa_1 and $s1p_1$ overlapped in the ventricular zone of the neocortex. However, $s1p_1$ also extended into the hippocampal primordia, olfactory bulb, and ganglionic eminence (Fig. 3). In addition, $s1p_1$ was expressed adjacent to the mesencephalic vesicle and the fourth ventricle, and in a band of pretectal area cells (Fig. 3). We also observed lpa_1 in putative meninges, as previously shown [9].

A second pattern characterized lpa_2 and lpa_3, where diffuse expression was found in cells throughout the brain (Fig. 2). For lpa_2, relatively low signal intensity was apparent in the ganglionic eminence compared to cortex, midbrain, and hindbrain (Fig. 2), with more intense labeling present in the superficial cortical layers.

A third punctate expression pattern in scattered cells characterized $s1p_{1-3}$ (Fig. 3). For $s1p_1$ and $s1p_3$, these signals were relatively intense and scattered throughout the brain, while for $s1p_2$, they were fainter and most easily discerned in the midbrain/diencephalon region. Also found for all three *s1p* transcripts was expression in the choroid plexus of the fourth ventricle, and the putative meninges between the telencephalon and diencephalon.

Outside of the CNS, several other expression areas were evident. In the nasal cavity, transcripts for all six of the examined receptors were found. In addition, lpa_1 and lpa_2 were found in the vestibulocochlear ganglion, and lpa_2 and lpa_3 in the trigeminal ganglion.

3.3. Cellular localization of $s1p_1$ mRNA in proliferating cell populations in prenatal mouse brain

The expression of $s1p_1$ in the ventricular areas of the E14 mouse brain suggested that it delineated neuroproliferative zones. In order to determine if the $s1p_1$ transcript selectively colocalized with proliferating cells, sections were prepared from E14 embryos pulsed for 1 h with BrdU, a thymidine analog incorporated into newly synthesized DNA (i.e. proliferating cells in S-phase).

Two patterns were observed in sections stained for both BrdU and $s1p_1$. The first pattern, observed in the ventricular zone of the cerebral cortex and ganglionic eminence, is one where the BrdU and $s1p_1$ signals overlapped (Fig. 4A), and was highly evident in the hippocampal primordia (Fig. 4C). A second pattern of BrdU and $s1p_1$ labeling was observed near the fourth ventricle and mesencephalic vesicle. Here, the BrdU labeling and the $s1p_1$-expressing cells were largely segregated, with $s1p_1$ label located more rostrally (Fig. 4B,D).

3.4. Temporal colocalization of $s1p_1$ mRNA with proliferating cell populations in prenatal mouse brain

To further determine the temporal extent of $s1p_1$ expression in the cerebral cortical proliferative zone, we examined parasagittal sections at four developmental time points: E12, E14, E16, and E18 (Fig. 5). Similar to lpa_1 expression [9], $s1p_1$ expression in the neurogenic ventricular zone progressively decreased in expression level at E16 and E18. By E18, when neurogenesis had largely ceased, $s1p_1$ expression was barely detectable.

3.5. Cellular localization of $s1p_1$ mRNA in blood vessels of prenatal mouse brain

The punctate expression pattern of $s1p_{1-3}$ in prenatal brain parenchyma, their expression in the choroid plexus, as well as their established association with endothelial cells [7], suggested that they might be localized to developing blood vessels. In order to test this hypothesis, we first stained sections for expression of the endothelial-specific genes, *CD34* or *PECAM-1*, which have previously been shown to localize to embryonic vasculature [22,23]. As expected, a similar punctate

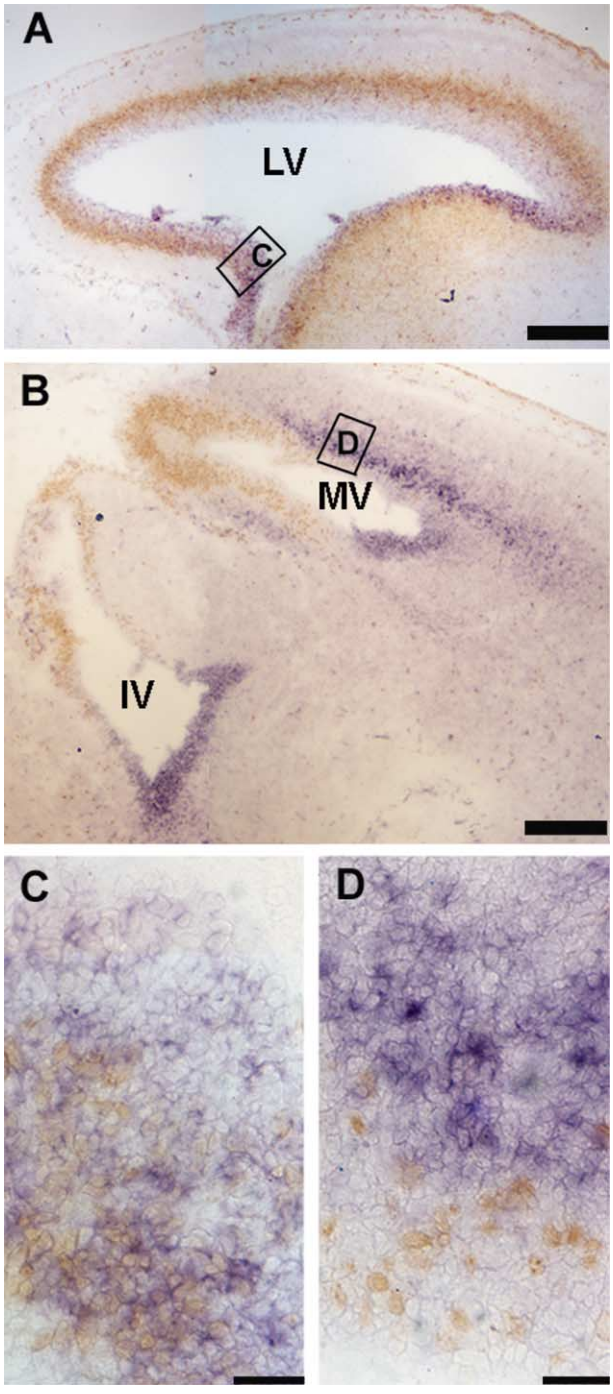

Fig. 4. Combined BrdU labeling and *s1p1* in situ hybridization in sagittal E14 sections. A: In the cortical ventricular zone by the lateral ventricle (LV), BrdU signal (gold) is apparent in a band of basal cells overlapping *s1p1* signal (purple), which is easily discerned in the boxed region of the hippocampal primordia at high magnification (C). B: In contrast, regions by the mesencephalic vesicle (MV) and fourth ventricle (IV) display BrdU signal largely segregated from *s1p1* signal, apparent in the boxed region at high magnification (D). Scale bars, 300 μm (A,B), 30 μm (C,D).

prenatal mouse brain expression pattern was observed, with sparse distribution in the ventricular areas (Fig. 6A). Next, we examined sections for expression of both *s1p1* and *CD34* using double in situ hybridization, which demonstrated coexpression of *s1p1* and *CD34* throughout the brain and body (not shown) of the embryo (Fig. 6B–D). Double labeling similarly demonstrated colocalization of *s1p3*/*CD34*, *s1p1*/*PECAM-1*, *s1p3*/*PECAM-1*, and *s1p1*/*s1p3* (data not shown).

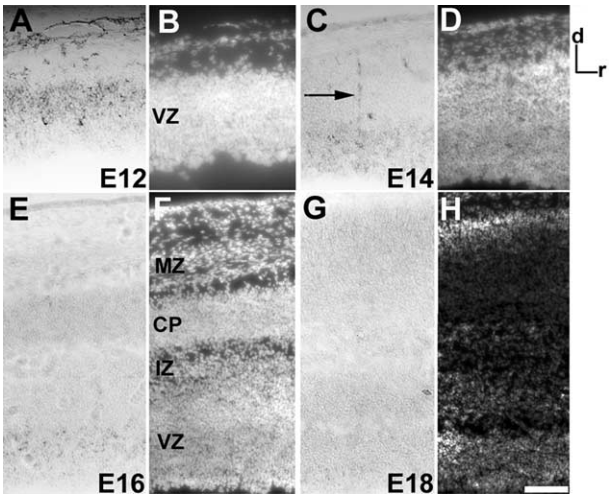

Fig. 5. Temporal expression of *s1p1* in cerebral cortex. Sagittal sections through the neocortex of E12 (A,B), E14 (C,D), E16 (E,F) and E18 (G,H) embryos showing localization of *s1p1* riboprobe (A,C,E,G) and DAPI fluorescence (B,D,F,H). Note that *s1p1* signal in the ventricular zone (VZ) is high at E12 and E14, moderate at E16, and minimal at E18. The arrow in C denotes punctate expression in the cerebral cortical preplate. MZ, marginal zone; CP, cortical plate; IZ, intermediate zone. Scale bar, 300 μm, d = dorsal, r = rostral.

4. Discussion

One of the most intriguing findings here is the prominent cerebral cortical proliferative zone expression of *s1p1*, which is temporally coincident with the period of neurogenesis and with the expression of *lpa1*. What might the $S1P_1$ receptor be doing in the cerebral cortical ventricular zone? Several possibilities can be gleaned from studies of $S1P_1$-receptor mediated signaling in cells in culture. Stimulation of the $S1P_1$ receptor activates the $G_{i/o}$ subfamily of G proteins, which leads to at least three responses in fibroblast/endothelial cells: increased proliferation, suppression of apoptosis, and cell migration [4,7]. In the embryonic cerebral cortex, neuroblast proliferation, apoptosis, and migration of postmitotic neurons into the cortical plate are all normal developmental events [25,26]. The $S1P_1$ receptor may be regulating one or more of these processes. Furthermore, there may be interactions between LPA and S1P in the neocortical ventricular zone, since *lpa1* expression temporally and spatially overlaps with *s1p1* here.

We also found prominent *s1p1* expression in the ventricular areas of the mesencephalon, in areas that give rise to the superior/inferior colliculi and pons. While it is possible that *s1p1* is affecting proliferation in these regions, at least at E14 it is more likely playing a role in cell migration and/or survival because *s1p1* expression was segregated from BrdU labeling in these areas. The functions of *s1p1* in ventricular areas are presumably conserved across vertebrates, since fish embryos also show ventricular *s1p1* expression [14]. One would thus expect an abnormal brain phenotype in animals with *s1p1*

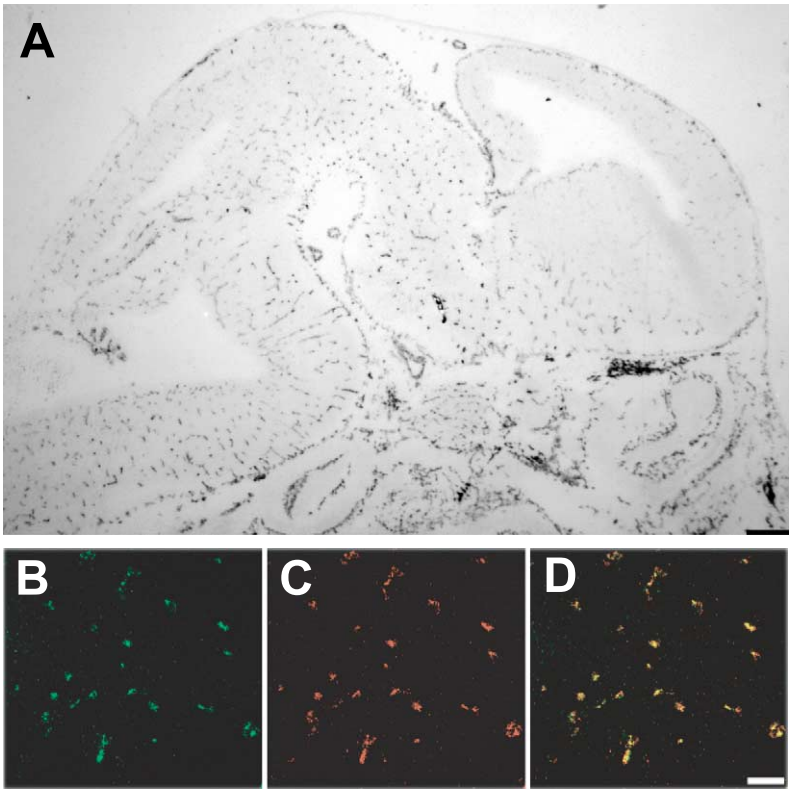

Fig. 6. Expression of *s1p1* in blood vessels. A: Sagittal E14 section showing the *PECAM-1* blood vessel transcript expressed in a widespread punctate pattern. B–D: Magnified view of the tectum showing cells expressing *s1p1* in green (B), the blood vessel marker *CD34* in red (C) and an overlay of the two, with yellow denoting cells expressing both signals (D). Scale bars, 300 μm (A), 50 μm (B–D).

deletions. Unfortunately, analysis of the nervous system in *s1p1*(−/−) mice is complicated because they die embryonically before most neurons are generated [13].

The colocalization of blood vessel markers with the punctate expression pattern of *s1p1* and *s1p3*, as well as the expression of these receptors in highly vascular choroid plexus, is consistent with previous findings indicating functions for S1P signaling in angiogenesis and vascular maturation [7]. First, *s1p1* expression is dramatically increased when endothelial cells are induced to differentiate in vitro [27]. Second, both $S1P_1$ and $S1P_3$-regulated signaling pathways were shown to be required for endothelial cell morphogenesis into capillary-like networks, as well as for adhesion and migration [28,29]. Third, the embryonic lethality in *s1p1*(−/−) mice was due to a blood vessel maturation defect [13]. Thus, the finding of *s1p1* and *s1p3* expression in developing brain blood vessels suggests that S1P signaling is critical for normal angiogenesis in the embryonic brain.

Although we did not specifically attempt to colocalize *s1p2* transcript with blood vessel markers (due to the faint *s1p2* signal), its expression in a punctate pattern and in the choroid plexus suggests that it too is localizing to embryonic blood vessels. We did not observe an embryonic neuronal expression pattern of the mouse *s1p2* transcript like that observed for $S1P_2$ protein in the rat [16]. This is consistent with the observation that *s1p2*(−/−) mice show no developmental CNS abnormalities [30,31], and might reflect a very low transcript level in embryonic neurons or be attributable to rat–mouse species differences. The latter explanation is not unprecedented, since rat and mouse have been shown to express differentially two lysophospholipid receptor genes (*lpa1* and *lpa3*) in cultured microglia [32].

Our in situ hybridization data have confirmed the expression of *lpa1* in the neocortical ventricular zone and meninges and have additionally demonstrated expression in the nasal cavity. This novel finding may have relevance to the deficient suckling behavior in *lpa1*(−/−) neonatal mice, a behavior that is likely due to defects in olfactant perception [10].

The roles of LPA and S1P signaling in the developing nervous system are emerging. Determination of the precise spatial and temporal expression profiles of each receptor gene member gives clues as to where one might expect aberrant function should the gene be inactivated. Our results implicate *s1p1* in cerebral cortical neurogenesis and midbrain development, as well as *s1p1*, *s1p2*, and *s1p3* in brain angiogenesis.

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