Ventricular Zone Gene-1 (vzg-1) Encodes a Lysophosphatidic Acid Receptor Expressed in Neurogenic Regions of the Developing Cerebral Cortex

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Abstract. Neocortical neuroblast cell lines were used to clone G-protein-coupled receptor (GPCR) genes to study signaling mechanisms regulating cortical neurogenesis. One putative GPCR gene displayed an in situ expression pattern enriched in cortical neurogenic regions and was therefore named ventricular zone gene-l (vzg-1). The vzg-1 mRNA transcript and encoded a protein with a predicted molecular mass of 41-42 kD, confirmed by Western blot analysis. To assess its function, vzg-1 was overexpressed in a cell line from which it was cloned, inducing serum-dependent “cell rounding.” Lysophosphatidic acid (LPA), a bioactive lipid present in high concentrations in serum, reproduced the effect seen with serum alone. Morphological responses to other related phospholipids or to thrombin, another agent that induces cell rounding through a GPCR, were not observed in vzg-1 overexpressing cells. Vzg-1 overexpression decreased the EC50 of both cell rounding and Gi activation in response to LPA. Pertussis toxin treatment inhibited vzg-1-dependent LPA-mediated Gi activation, but had no effect on cell rounding. Membrane binding studies indicated that vzg-1 overexpression increased specific LPA binding. These analyses identify the vzg-1 gene product as a receptor for LPA, suggesting the operation of LPA signaling mechanisms in cortical neurogenesis. Vzg-1 therefore provides a link between extracellular LPA and the activation of LPA-mediated signaling pathways through a single receptor and will allow new investigations into LPA signaling both in neural and nonneural systems.

A critical event in the formation of the mammalian cerebral cortex is the ordered generation of its neurons from a discrete proliferative region overlying the cerebral ventricles, the ventricular zone (vz) (6) (Fig. 1). In most mammalian species, neurogenesis occurs during fetal life when the vz can be delineated by histological stains, or by brief pulses of 5-Bromo-2′-deoxyuridine (BrdU) or [3H]thymidine, which identify neuroblasts undergoing S-phase (58, 64, 67). Cortical neuroblasts display a stereotyped change in their morphology that is linked to their proliferation. During S-phase of the cell cycle, vz neuroblasts appear bipolar, with the cell body at the supercicial margin of the vz and with processes oriented towards the ventricular and superficial (pial) surfaces of the cerebral wall. With the progression of the cell cycle, the neuroblast undergoes “interkinetic nuclear migration” whereby its nucleus descends to the ventricular surface during G2, its pial process is retracted, and the cell “rounds up” (62, 63). After rounding, the cell undergoes mitosis and then regains its bipolar morphology to complete the cell cycle (Fig. 1 a). In the mouse, cortical neurogenesis is limited to the period between embryonic day 11 (E11) and E18 (9, 10, 67). Beyond this period, cells are still produced within the cerebral wall; however, these cells are generally of glial rather than neuronal lineages (4, 10, 22, 32, 67).

The molecular mechanisms responsible for controlling vz neurogenesis are largely unknown. However, three observations suggest that it is regulated, at least in part, by interactions between a neuroblast and its environment, rather than through cell-autonomous mechanisms. First, a number of growth factors operating through receptor tyrosine kinases (RTKs) (3, 78), most notably basic fibroblast growth factor, promote proliferation of telencephalic cells in primary culture (8, 30, 44, 70). Second, uncharacterized serum factors appear to be required in addition to

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1 Abbreviations used in this paper: aa, amino acid; BrdU, 5-Bromo-2′-deoxyuridine; CNS, central nervous system; E, embryonic day; GPCR, G-protein-coupled receptor; LPA, lysophosphatidic acid; LPC, lysophosphatidylethanolamine; LPE, lysophosphatidylethanolamine; LPG, phosphatidylglycerol; PA, phosphatidic acid; PTX, pertussis toxin; RT, room temperature; RTK, receptor tyrosine kinase; TM, transmembrane; vz, ventricular zone; vzg-1, ventricular zone gene-1.
identified growth factors to promote cortical cell proliferation (43, 44), consistent with normal cortical development in mice with null mutations for known growth factors and/or their receptors (45). Third, cell membranes or cell–cell contacts also promote proliferation of telencephalic blasts (30, 69). These observations could be explained by the operation of novel receptors, distinct from RTKs, regulating cortical neurogenesis.

An attractive candidate for this role is the G-protein–coupled-receptor (GPCR) family. It is crucial to central nervous system (CNS) function (20) and contains many diverse members (7, 57), some of which have known mitogenic effects (1, 41, 74). To isolate GPCR genes, we used novel clonal cell lines derived from the vz that resemble cortical neuroblasts (14). These cell lines provide a unique source of cDNA for cloning strategies and can further be used for functional studies of receptor genes, enabling an identified receptor to interact with signaling pathways approximating those found in vivo.

Here we report the complete sequence and developmental CNS expression pattern of a new member of the GPCR family named ventricular zone gene-1 (vzg-1) because of its restricted expression within the cerebral cortical vz (Fig. 1 b). Based on functional assays, ligand binding data, and its tissue distribution, we conclude that vzg-1 encodes a receptor for lysophosphatidic acid (LPA). LPA is a phospholipid signaling molecule that has a wide variety of effects on many different cell types (see Discussion; for recent reviews see 26 and 54). Although LPA almost certainly acts through a GPCR, a cDNA clone of this receptor has not been identified, in part reflecting the chemical characteristics of LPA that result in unacceptably high levels of nonspecific binding, making techniques such as expression cloning impractical. Therefore, the possibility...
that nonreceptor mechanisms could account for observed effects of LPA has been left open (26, 71, 74, 77). To overcome these difficulties, we have instead relied on well-defined functional characteristics of LPA signal transduction to identify the vzg-1 gene product as a receptor for LPA.

**Materials and Methods**

**Cell Culture**

Cell lines TSM and TR (14) were grown in OptiMEM I (GIBCO BRL, Gaithersburg, MD) with 5% FCS (HyClone Labs, Logan, UT), 20 mM glucose, 35 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO₂.

**Cell Transfection.** Transient transfection used calcium phosphate precipitation (12) with a 10:1 molar ratio of vzig-1 expression plasmid to β-galactosidase expression plasmid pCMVβ (Clontech, Palo Alto, CA). After 18 h, cells were refed, grown for 24 h, fixed in 4% paraformaldehyde in PBS for 10 min, and stained for β-galactosidase activity (2). Positive cells (200/plate) were counted “blind.” The statistical program Instat (Graphpad Software, San Diego, CA) was used for one-way analysis of variance and the Student-Newman-Keuls pairwise t-test. Stable transfection used a 10:1 precipitation (12) with a 10:1 molar ratio of vzig-1 expression plasmid to pSV2-puro (79) and selection in medium containing 10 μg/ml puromycin. After 2 wks of selection, single colonies of cells were picked using cloning cylinders, expanded, and then stored or processed for RNA isolation and Northern analyses as previously described (14, 19).

**Morphological Assay.** Stable cell lines (5,000/well in 24-well plates) were serum starved for 24 h, and then media containing the desired agents was added to the required final concentration. Cells were fixed in 4% paraformaldehyde in PBS to terminate incubation and examined. Experiments were performed in duplicate (200 cells counted/well) and representative samples were evaluated by multiple investigators. Statistical methods used were identical to transient experiments.

**cAMP Assay.** Cells (30,000) were plated, serum starved overnight, and then stimulated for 7 min at 37°C with serum-free medium containing 200 nM 3-isobutyl-1-methylxanthine, 10 μM isoproterenol, and LPA. cAMP accumulation was measured as described (55). Data were analyzed using Prism (Graphpad Software).

**Pertussis Toxin (PTX) Treatment.** Cells were treated with 200 ng/ml PTX for 16 h, followed by a 3-h treatment with fresh PTX.

**Molecular Biological Techniques**

**PCR Amplification of GPCR Family Members.** Poly-A+ RNA was isolated from TR and TSM cells (twice selected on oligo-dT celltose [Phar...
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Results

Vzg-1 Encodes a Novel Seven-Transmembrane Domain Molecule

Degenerate oligonucleotide primers against amino acid (aa) sequences from TM II and TM VII of the GPCR family (7, 48) were used in PCR amplification of neocortical cell line cDNA (lines TR and TSM (14); Materials and Methods). A total of 154 DNA fragments representing 58 distinct PCR products in the expected size range from 600 to 1,300 bp were cloned. One fragment, "513," was localized to the vz (Fig. 1 b) by in situ hybridization and was represented by 13 independently derived clones having GPCR aa sequence homology. Northern blot analysis of embryonic brain detected a single 3.8-kb transcript. Library screening led to isolation of a 2.4-kb cDNA, termed vzg-1, containing an open reading frame (Fig. 2 a) encoding a 41-kD protein with seven hydrophobic membrane spanning domains (Fig. 2 b), as well as other features of the GPCR family. Comparisons to published sequences (data not shown) demonstrated that Vzg-1 shared homology with the melanocortin receptor (32% aa identity) (23), cannabinoid receptor (30% aa identity) (52), and the orphan receptor gene edg-1 (37% aa identity) (36).

Vzg-1 Expression Correlates with Cortical Neurogenesis

The full-length vzg-1 clone hybridized to a transcript iden-
Vzg-1 overexpression induced neurite retraction and cell rounding, with a sustained morphological response to LPA (Fig. 5). As this wall thickens with further development, vzz-g-I expression remained primarily restricted to the proliferative vz (Fig. 5; see also Fig. 1 b). The vzz-g-I hybridization signal appeared most intense at E12 and E14, somewhat less at E16, and was barely detectable at E18. Thus, vzz-g-I expression is primarily restricted to the vz of the cerebral cortex during neurogenesis.

Vzg-1 Overexpression Induces Sustained, LPA-dependent Cell Rounding

To determine possible functions of vzz-g-I, cell line TSM, chosen for its comparatively low expression of the endogenous transcript (Fig. 3), was transiently transfected with expression vectors containing vzz-g-I in the sense or antisense orientation. Transfection with the vzz-g-I sense expression vector induced neurite retraction and cell rounding, which was maintained for at least 24 h (Fig. 6 a, "sustained cell rounding"). This morphological change required the presence of serum. Sense transfected cells exposed to serum had 48 ± 3.6% round morphology, compared to 22 ± 5.0% without serum (Table I).

The reproducibility of cell rounding allowed its use as a bioassay to identify putative ligands for vzz-g-I. Boiling the serum did not abolish its ability to mediate cell rounding (data not shown), indicating that the ligand was a heat-stable molecule that might be associated with (a) cytoskeletal changes and (b) cell proliferation, since vzz-g-I expression was restricted to the vz. A molecule present in serum that met these criteria (see Discussion) was LPA. Since endogenous vzz-g-I should be active in the cell lines from which it was identified, untransfected TSM cells were first assayed for their ability to respond to LPA. Indeed, TSM cells responded with a rapid increase in the percentage of round cells. However, this response was reversible such that by 3 h, the percentages of round cells returned to their baseline values (Fig. 6 b).

Transfection of TSM cells with vzz-g-I in the sense orientation sustained the rounding response to LPA such that at 3 h, 49 ± 4.9% of treated transfected cells still displayed a round morphology, compared to 29 ± 1.9% of untreated transfected cells (Table I). Importantly, thrombin, a serum component which also induces cell rounding in some neural cell lines (38, 66), did not induce sustained cell rounding in cells transfected with vzz-g-I, although it did induce sustained cell rounding in cells transfected with the thrombin receptor (Table II). Thus, transient overexpression of vzz-g-I specifically alters LPA-mediated changes in cell morphology.

Vzg-1 Overexpressing Cell Lines Show Morphological Responses Specific to LPA

Vzg-1 function was characterized in TSM cells stably transfected with the vzz-g-I sense construct, using the antisense construct and empty vector as transfection controls. (Stably transfected cell lines will be referred to as "sense, empty vector, or antisense cells.") Overexpression of vzz-g-I in sense cells was at least fivefold over endogenous expression, and in antisense cells at least twofold over endogenous expression, determined by Northern analysis and scanning densitometry (data not shown). Vzg-1 protein in sense cells (line 3) was overexpressed relative to empty vector (line 33) or antisense (line 17) cells (Fig. 7 a). Sense cells, but not empty vector or antisense cells, responded to LPA by sustained cell rounding at 3 h (Fig. 7 b).

The specificity of the LPA response in vzz-g-I sense cells was tested using a range of structurally related phospholipids. Two forms of LPA, 1-oleoyl-LPA and 1-stearoyl-LPA, were examined along with four other structurally related lipids: 1-oleoyl-LPC, 1-oleoyl-LPE, 1-oleoyl-LPG, and 1,2-dioleoyl-PA (Table III). Compared to the other phospholipids, only LPA lipids produced sustained cell rounding after 3 h of treatment, and thus the response was specific to LPA.

Vzg-1 Overexpression Decreases the EC50 of LPA-induced Cell Rounding

The cell rounding bioassay was used to determine the effect of LPA concentration on sense, antisense, and empty vector cells. Concentration-response experiments defined the EC50, minimal, and maximal response values of the morphological response to LPA (Fig. 7 c and Table IV). Sense cells displayed a similar maximal response to LPA as empty vector cells, but were 6.9-fold more sensitive, as demonstrated by a shift in the concentration-response curve to the left. In contrast, antisense cells displayed decreased basal and maximal responses but equal sensitivity.

Figure 3. Vzz-g-I is expressed in the embryonic mouse brain and in neocortical neuroblast cell lines. Total RNA (10 μg) from mouse brains at E13-E17 and cytoplasmic RNA from cell lines TSM and TR were analyzed by Northern blot analysis using a vzz-g-I probe. The vzz-g-I transcript is 3.8 kb (γ-actin loading control is shown).
Figure 4. Vzg-1 expression in the embryonic CNS is enriched within the vz. Low magnification view of adjacent sagittal (a, c, and e) and coronal (b, d, and f) sections of E14 mouse embryo hybridized to antisense (a and b) or sense control (c and d) digoxigenin-labeled riboprobes, or stained with cresyl violet (e and f). The hybridization pattern produced by the antisense probe is restricted to the neuroproliferative vz of the embryonic cortex. Ctx, cortex; LV, lateral ventricle; OB, olfactory bulb; SA, striatum; Th, thalamus; T, tectum; P, pons; Cb, cerebellum; M, medulla; GE, ganglionic eminence; TV, third ventricle; POA, preoptic area; d, dorsal; r, rostral; m, medial. Bar, 1 mm.
Figure 5. Developmental expression of vzg-1 parallels the anatomical location and period of cortical neurogenesis. Sagittal sections of the cortex from E12-E18 hybridized to antisense vzg-1 riboprobe (a, c, e, and g) compared to an adjacent section stained with cresyl violet (b, d, f, and h). Vzg-1 expression is largely confined to the vz throughout this period. Vzg-1 appears highly expressed at E12 and E14, less so at E16 and at minimal levels at E18. vz, ventricular zone; iz, intermediate zone; cp, cortical plate; mz, marginal zone; v, ventricle. Bar, 50 μm.

as control cells. Two other sets of independently derived cell lines showed similar results (data not shown). Thus, overexpression of the sense vzg-1 construct specifically increased the potency of LPA, a result consistent with vzg-1 encoding a receptor for LPA.

Vzg-1 Overexpression Decreases the EC50 of LPA-stimulated G\textsubscript{i} Activation

To examine whether vzg-1 overexpression altered LPA-mediated G\textsubscript{i} activation, sense, antisense, and empty vector cells were tested for LPA-stimulated inhibition of cAMP accumulation. As shown in Table IV, the EC50 in sense cells was reduced 6.6-fold relative to empty vector cells. Antisense cells had a similar EC50 as empty vector cells. The effect of LPA on cAMP accumulation was reversed by PTX treatment, indicating activation of G\textsubscript{i}; however, PTX treatment did not affect LPA-induced cell rounding (Table V). The effect of vzg-1 on LPA-mediated G\textsubscript{i} activation was specific to LPA since adenylate cyclase inhibition...
Table I. Serum and LPA Induce Sustained Cell Rounding in TSM Cells Transiently Transfected with Vzg-1

| Expression vector | 5% FCS (percent round, mean ± SEM) | 1 μM LPA (percent round, mean ± SEM) |
|-------------------|-----------------------------------|-------------------------------------|
|                   | +                                 | -                                   |
| Vzg-1 sense       | 48 ± 3.6*                         | 49 ± 4.9*                           |
| Control: vzg-1 antisense | 20 ± 0.88                      | 27 ± 2.1                            |
| Control: empty vector | 21 ± 6.0                         | ND                                  |

Cells were transiently transfected with the indicated expression vectors, followed by treatment with either FCS for 24 h or LPA for 3 h. Data represent the mean ± SEM for three to four independent experiments, each done in duplicate.

*Indicates statistically significant differences from control conditions (P < 0.001).

Figure 6. LPA induces cell rounding and neurite retraction in TSM cells. (a) Transfected cells, identified by β-galactosidase staining, either have round morphologies (arrows) or pyramidal or bipolar morphologies. Bar, 50 μm. (b) LPA response of untransfected TSM cells. Within 15 min, LPA caused an increase in the proportion of round cells. Note that the proportion of round cells returns to baseline after 3 h, allowing transfection experiments to be carried out on this time scale. Values represent the mean ± SEM of three independent experiments, each done in duplicate.

Discussion

LPA Meets Criteria Expected for a Vzg-1 Ligand

Functional analyses of vzg-1 required ligand identification, a difficulty underscored by the huge variety of molecules recognized by the GPCR family (80). Ligand identification was aided by three observations from our study. First, vzg-1-mediated by UK14304, an α2-adrenergic receptor agonist, did not show differential effects on sense and antisense overexpressing cells (data not shown).

Vzg-1 Overexpression Increases LPA Binding in Cell Membranes

The use of binding assays with [3H]LPA to study ligand-receptor interactions is complicated by the lipophilic and detergent properties of LPA (26, 71, 74, 77), resulting in substantial nonspecific and background binding (see Materials and Methods). Nevertheless, [3H]LPA binding assays were performed on membrane preparations from sense and antisense cells to determine whether overexpression of vzg-1 increased the number of specific [3H]LPA binding sites. Membrane preparations derived from sense cells showed a statistically significant increase in specific [3H]LPA binding compared to membranes from antisense lines (Fig. 7 d). This result demonstrated that vzg-1 overexpression is associated with an increase in the number of binding sites for [3H]LPA.

Adult Expression of Vzg-1 Parallels Prior Estimates of LPA Receptor Distribution

Prior studies examining the distribution of LPA receptors demonstrated that in adult tissues, receptors are present at very high levels in the brain, are virtually absent from the liver, and are present at intermediate levels in other tissues (71, 77). Additionally, high levels of receptor are expressed in NIH-3T3 cells. Northern blot analysis (Fig. 8) demonstrated that vzg-1 was expressed at maximal levels in the adult brain, absent from liver, and present at intermediate levels in other tissues, a pattern paralleling these previous results. Vzg-1 was also highly expressed in NIH-3T3 cells (data not shown).

Table II. LPA, but Not Thrombin, Stimulates Vzg-1-dependent Sustained Cell Rounding

| Expression vector | 1 μM LPA (percent round, mean ± SEM) | 0.5 U/ml thrombin (percent round, mean ± SEM) | Control: no treatment (percent round, mean ± SEM) |
|-------------------|-------------------------------------|-----------------------------------------------|-----------------------------------------------|
| vzg-1 sense       | 47 ± 2.2*                           | 23 ± 4.1                                      | 23 ± 4.3                                      |
| Thrombin receptor | 32 ± 2.0                            | 76 ± 2.1*                                     | 28 ± 2.3                                      |

TSM cells were transiently transfected with the indicated expression vectors followed by treatment with either LPA for 3 h or thrombin for 2 h. Data represent the mean ± SEM for three independent experiments, each done in duplicate.

*Indicates statistically significant differences from control conditions (P < 0.001).
was highly expressed in the vz, suggesting a function in some aspect of cortical neurogenesis (see below). Second, vzg-1-dependent, serum-induced, sustained cell rounding was highly reproducible, allowing its use as a bioassay. Third, the serum factor inducing cell rounding was heat stable. Prior studies reported that cortical blast proliferation required (a) cell membrane extracts or cell–cell contact (30, 69) and (b) serum (43, 70). Thus, ligand(s) for Vzg-1 could (a) be involved in cell proliferation or differentiation, (b) promote morphological changes like cell rounding, (c) be heat stable, and (d) be a component of serum or cell membrane extracts.

All of these criteria were met by the bioactive lipid LPA (54). LPA is mitogenic for nonneural cells (72, 75, 81, 82), produces actin-based stress fiber formation in fibroblasts (61), and results in the retraction of “neurites” and cell rounding in neuroblastoma-derived cell lines (40). It is a heat-stable component of serum (61), present at a concentration of 2–20 μM, that is bound to albumin (27). It can also be produced from cell membrane phospholipids by phospholipase A2 (29).

Vzg-1 Has Predicted Properties of a Receptor for LPA

The vzg-1 gene product meets the expected criteria of a receptor for LPA. Vzg-1 encodes a GPCR based on se-

Figure 7. Vzg-1 sense overexpression enhances LPA-induced cell rounding and increases [3H]LPA binding to cell membranes. (a) Vzg-1 sense strand–transfected cell lines express more protein than control cells. Sense-transfected cells (shown for line 3) expressed more Vzg-1 than empty vector (33) or antisense (17) cells, using an α-Vzg-1 antiserum that specifically detects a 41–42-kD protein not detected by preimmune serum. An identically sized protein is detected in adult brain. Equivalent amounts of membrane protein (5.5 μg) were loaded in each well as determined by Bradford assay of the sample, and confirmed by Coomassie staining after gel electrophoresis (data not shown) (2). (b) Response of stably transfected cell lines after 3 h treatment with 1 μM 1-oleoyl LPA. Cell lines stably transfected with the sense vzg-1 expression construct (lines 3, 7, and 14) showed enhanced responses to LPA compared to empty vector control line 33. Antisense cell lines (lines 17, 18, and 24) have lower responses to LPA than the empty vector control line 33. * indicates statistically significant differences between experimental cells and control line 33 in the presence of LPA, with individual pairwise P values ranging from P < 0.05 to P < 0.001. # indicates statistically significant differences between experimental cells and control line 33 without LPA stimulation, with individual pairwise P values ranging from P < 0.05 to P < 0.001. All values represent the mean ± SEM of three to five independent experiments, each done in duplicate. (c) Concentration-response relationship of stably transfected cells to a three to five min treatment with LPA. Sense transfected cells show an increased sensitivity to LPA compared to empty vector control cells as demonstrated by a leftward shift of the EC50. Antisense transfected cells have identical EC50 as controls, but lowered minimal and maximal responses (Table IV). Curves were fitted to data points representing the mean ± SEM of three independent experiments, each done in duplicate. (d) Vzg-1 overexpression results in increased [3H]LPA binding in membranes from sense-transfected cells (shown for line 3), compared to membranes from antisense-expressing cells (shown for line 17). * indicates a significant difference from control (P = 0.021), data expressed as the mean ± SEM of four experiments, done in triplicate.
Phospholipid  

Sustained Cell Rounding  

24 h, and then treated with 1 I~M concentrations of the indicated phospholipids for 3 h.

Join the receptor to bind [3H]LPA was tested using membrane binding assays. Binding studies with LPA are complicated by the lipophilic detergent properties of the radioligand, which favor partitioning into phospholipid bilayers and micelle formation, resulting in substantial levels of nonspecific binding, inefficient separation of bound ligand from free, and high levels of background. As a result of these technical limitations, binding assays cannot be used to make quantitatively rigorous measurements of LPA binding sites, as noted in previous studies (26, 71, 74). Based on receptor theory (56), the increase in potency levels detected by Western blot analysis (Fig. 7). Prior studies on LPA receptor distribution implicated a 38-40-kD protein highly expressed in the adult brain and absent from liver (71, 77). Vzg-1 tissue distribution parallels these prior estimates, and, furthermore, vzg-1 encodes a predicted protein of consistent size (41 kD), confirmed by anti- Vzg-1 Western blot analysis.

Therefore, seven different lines of evidence identify the vzg-1 gene product as a lysosphosphatidic acid receptor: (1) identification of vzg-1 as a GPCR gene, (2) vzg-1–dependent increases in specific [3H]LPA membrane binding, (3) vzg-1–dependent decrease of the EC50 for Gt-independent LPA-mediated cell rounding, (4) vzg-1–dependent decrease of the EC50 of LPA-mediated activation of Gt, (5) correspondence between the predicted size of the vzg-1 gene product and prior estimates of LPA receptor size, (6) detection of a gene product of the correct size in brain, with increased levels of expression in sense transfected cells, and (7) correlation between tissue expression of vzg-1 and previous measurements of LPA receptor distribution.

Vzg-1 and LPA Signaling Have Potential Functions in Cortical Neurogenesis

In situ hybridization and BrdU double labeling experiments demonstrate that vzg-1 is expressed within the embryonic vz and is both temporally and spatially related to the period of cortical neurogenesis (67, 68) (Figs. 1b and 5). The identification of the vzg-1 gene product as a receptor for LPA suggests that LPA signaling mechanisms may be important in regulating cortical neurogenesis.

### Table III. LPA Specifically Induces Vzg-1–dependent and Sustained Cell Rounding

| Phospholipid | Line 3 (percent round, mean ± SEM) | Line 7 (percent round, mean ± SEM) | Line 14 (percent round, mean ± SEM) |
|--------------|----------------------------------|-----------------------------------|-----------------------------------|
| 1-Oleoyl LPA | 50 ± 2.3*                        | 57 ± 2.8*                         | 54 ± 1.3*                         |
| 1-Stearoyl LPA | 33 ± 1.2                        | 55 ± 3.1*                         | 35 ± 2.6*                         |
| 1-Oleoyl LPE | 29 ± 1.3                        | 44 ± 1.1                           | 27 ± 1.1                           |
| 1-Oleoyl LPG | 22 ± 0.71                       | 37 ± 1.2                           | 25 ± 0.85                          |
| 1-Oleoyl LPC | 24 ± 2.5                        | 42 ± 2.0                           | 29 ± 0.25                          |
| 1,2-Dioleoyl PA | 27 ± 1.0                       | 43 ± 0.85                          | 31 ± 2.1                           |
| Control: medium alone | 27 ± 2.8                       | 39 ± 1.7                           | 24 ± 1.6                           |

Cell lines stably transfected with the vzg-1 sense construct were serum deprived for 24 h, and then treated with 1 uM concentrations of the indicated phospholipids for 3 h. Data represent the mean ± SEM for four independent experiments, each done in duplicate. *Indicates statistically significant differences from control conditions (P < 0.01) to P < 0.001.

### Table IV. Vzg-1 Sense Overexpression Increases the Potency of LPA to Induce Cell Rounding and Inhibit Adenylate Cyclase

| Treatment | cAMP percentage of maximum response (mean ± SEM) | Percent round cells (mean ± SEM) |
|-----------|--------------------------------------------------|----------------------------------|
| Control: medium alone | 100                                              | 40 ± 3.2                         |
| LPA       | 45 ± 3.4                                         | 77 ± 2.1*                        |
| LPA + PTX | 84 ± 3.5                                         | 81 ± 3.5*                        |

Vzg-1 sense overexpressing cells (cell line 3) were serum deprived 24 h and then stimulated with 100 nM LPA for 7 min for cAMP assays or with 50 nM LPA for 15 min rounding assays. Data represent the mean ± SEM for three independent experiments done in triplicate for cAMP assays or duplicate for rounding assays. *Indicates statistically significant differences from control conditions (P < 0.001).

### Table V. LPA-induced Adenylate Cyclase Inhibition, but Not Cell Rounding, is Blocked By PTX

| Treatment | EC50 (nM) 95% confidence interval | -logEC50 ± SEM | Minimum response (Percent round, mean ± SEM) | Maximum response (Percent round, mean ± SEM) |
|-----------|----------------------------------|----------------|-----------------------------------------------|-----------------------------------------------|
| Control: medium alone | 100 |  | 38 ± 2.1 | 75 ± 1.2 |
| LPA       | 45 ± 3.4 |  | 22 ± 1.4 | 54 ± 1.3 |
| LPA + PTX | 84 ± 3.5 |  | 35 ± 1.9 | 77 ± 1.8 |

Vzg-1 sense overexpressing cells (cell line 3) were serum deprived 24 h and then stimulated with 100 nM LPA for 7 min for cAMP assays or with 50 nM LPA for 15 min rounding assays. Data represent the mean ± SEM for three independent experiments done in triplicate for cAMP assays or duplicate for rounding assays. *Indicates statistically significant differences from control conditions (P < 0.001).
functions of LPA in cortical neurogenesis, based on known bioactivities of LPA and biological events occurring within the vz, include regulation of cytoskeletal events such as interkinetic nuclear movement, cell rounding, and cleavage plane orientation (13, 40), mitogenesis (discussed below), gap junction regulation (34, 49), and influence on the binding and assembly of fibronectin (11, 83), which is expressed in the embryonic cortex (15, 65). Additionally, regulation of apoptosis, recently shown to occur in the vz (5), may also be influenced by LPA signaling (47). The actual roles of LPA signaling in the vz, mediated through vzg-1, and the source of endogenous ligand remain to be
determined.

Vzg-1 Expression Implicates Distinct GPCR-mediated Signaling Pathways in Cortical Neurogenesis

The major signal transduction elements currently implicated in cortical neurogenesis operate through peptide ligand stimulation of the RTK pathway (70). By contrast, the bioactive lipid LPA operates through Vzg-1, a GPCR family member. Prior studies on LPA signaling have demonstrated that LPA activates heterotrimeric G proteins, as well as small GTPases such as Rho that are positioned downstream in the signaling pathway (54). Therefore, many of the initial signaling steps mediated by LPA are distinct from the RTK pathway. The presence of vzg-1 expression within the cerebral cortical vz indicates the existence of a specific role for heterotrimeric G protein signaling in cortical neurogenesis.

In view of vzg-1 expression in the zone of neuroblast proliferation, there is a possible role for mitogenic signaling mediated by LPA in cortical neuron generation. Mitogenic effects of LPA appear to involve activation of two signaling pathways. The PTX-sensitive pathway, involving activation of G, and Ras (74, 76), is probably mediated through βγ subunit signaling (21, 50, 51). The PTX-insensitive pathway depends on functional Rho and might lead to cell proliferation through activation of a mitogen-activated protein kinase cascade (33, 35). Functional Rho is also necessary for actin-based cytoskeletal changes (40, 61). Our results directly demonstrate the activation of the PTX-sensitive G-linked pathway, and indirectly demonstrate the activation of the PTX-insensitive pathway leading to Rho activation, by vzg-1 mediated LPA signaling in neocortical neuroblast cell lines. Thus, a single LPA receptor appears to mediate distinct signaling pathways. The possible interactions between RTK pathways and these G protein mediated events during cortical development will be addressed in future studies. Understanding these and related factors controlling cortical neurogenesis may provide new insights into the observed increase in cerebral cortical size with phylogenetic ascension.

In addition to roles in neural development, this LPA receptor may participate in the functioning of many other developing and adult tissues and cell types, based on the diversity of effects known to be stimulated by LPA. Availability of a cDNA encoding a receptor for LPA should facilitate the molecular genetic characterization of LPA receptor-dependent interactions in various cell and tissue types and allow the development of agents that can be used to manipulate LPA receptor signaling pathways. The physiological consequences of LPA receptor elimination in mice, the sites of LPA production in vivo, and the possible role of this receptor in pathological states remain to be determined.

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