Journal of Cellular Biochemistry 92:1004–1012 (2004)

Genetics and Cell Biology of Lysophosphatidic Acid Receptor-Mediated Signaling During Cortical Neurogenesis

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Abstract Lysophosphatidic acid (LPA) is a small lysophospholipid that signals through G-protein coupled receptors (GPCRs) to mediate diverse cellular responses. Two LPA receptors, LPA_1 and LPA_2, show gene expression profiles in mouse embryonic cerebral cortex, suggesting roles for LPA signaling in cerebral cortical development. Here, we review loss-of-function and gain-of-function models that have been used to examine LPA signaling. Genetic deletion of *lpa*$_1$ or both *lpa*$_1$ and *lpa*$_2$ in mice results in 50–65% neonatal lethality, but not obvious cortical phenotypes in survivors, suggesting that compensatory signaling systems exist for regulating cortical development. A gain-of-function model, approached by increasing receptor activation through exogenous delivery of LPA, shows that LPA signaling regulates cerebral cortical growth and anatomy by affecting proliferation, differentiation and cell survival during embryonic development. J. Cell. Biochem. 92: 1004–1012, 2004.

Key words: LPA; development; GPCR; knockout

Lysophosphatidic acid (LPA) is a simple lysophospholipid that is a metabolite in the biosynthesis of membrane phospholipids and a well-characterized signaling molecule. Through the activation of specific G-protein coupled receptors (GPCRs), LPA promotes proliferation, survival, death, gap-junction closure, Ca^{2+} mobilization, membrane depolarization, and cytoskeletal remodeling in numerous cell types [Moolenaar, 1995; Fukushima et al., 2001]. In addition, LPA has been implicated in processes such as angiogenesis, wound healing, tumor invasion, and nervous system development [Moolenaar, 1995, 1997; Fukushima et al., 2001; Ishii et al., 2004]. To date, four lysophospholipid receptors that bind LPA with high affinity have been identified and include LPA_1–LPA_4 [Ishii et al., 2004]. While LPA_1, LPA_2, and LPA_3 share high amino acid similarity with one another [Contos et al., 2000b], LPA_4 is a recently described receptor with divergent sequence from LPA_1–LPA_3, that likely has distinct signaling properties [Noguchi et al., 2003; Ishii et al., 2004].

The discovery of the first lysophospholipid receptor, LPA_1, implicated LPA signaling in nervous system development. This receptor is expressed in the neurogenic region of the embryonic cerebral wall, termed the ventricular zone (VZ) [Hecht et al., 1996], as well as in oligodendrocytes and Schwann cells during the postnatal period that coincides with myelination [Weiner et al., 1998; Weiner and Chun, 1999]. Subsequent studies identified LPA_2 expression in postmitotic cells of the embryonic cortex [Fukushima et al., 2002; McGiffert et al., 2002] and LPA_3 expression within the early

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Grant sponsor: National Institute of Mental Health and Human Frontiers Science Program (to J.C.); Grant sponsor: National Institute of Aging (Neuroplasticity of Aging Training Grant as a postdoctoral fellowship to M.A.K.); Grant sponsor: PEW Latin American Fellows in the Biomedical Sciences (postdoctoral fellowship to S.K.R.); Grant sponsor: The Helen L. Dorris Institute for the Study of Neurological and Psychiatric Disorders of Children and Adolescents (to J.C., M.A.K., S.K.R., X.Y.).

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Received 24 November 2003; Accepted 6 January 2004

DOI 10.1002/jcb.20061

postnatal brain [Contos et al., 2000b]. LPA, the ligand for these receptors, is present in the brain [Das and Hajra, 1989; Sugiura et al., 1999] and can be produced by postmitotic neurons and Schwann cells in culture [Weiner and Chun, 1999; Fukushima et al., 2000].

Following the identification of LPA_1, studies were directed towards understanding the role of LPA in cortical development [Dubin et al., 1999; Contos et al., 2000a; Fukushima et al., 2000]. Within the VZ, neural progenitor cells (NPCs) undergo proliferation, differentiation, and death, as well as unique morphological changes as they progress through the cell cycle. When NPCs are exposed to LPA in culture, they show cell rounding and the formation of retraction fibers [Fukushima et al., 2000]. These cytoskeletal changes are similar to those associated with the "rounding-up phase" of interkinetic nuclear migration in the VZ [Fukushima et al., 2000]. In addition, LPA stimulates depolarizing ionic conductances and modest proliferation in cultured NPCs [Dubin et al., 1999; Contos et al., 2000a]. However, it is unclear how these effects might relate to those mediated by LPA in vivo.

Here, we review studies of $lpa_1^{(-/-)}$, $lpa_2^{(-/-)}$, and $lpa_1^{(-/-)}lpa_2^{(-/-)}$ knockout mice that represent loss-of-function models for LPA signaling in the intact organism [Contos et al., 2000a, 2002]. While $lpa_1^{(-/-)}$, $lpa_2^{(-/-)}$ and $lpa_1^{(-/-)}$ $lpa_2^{(-/-)}$ null mice do not display major abnormalities in cerebral cortical structure, they are characterized by various defects, including significant neonatal lethality, craniofacial deformities, frontal hematomas, and olfactory deficits that may have a CNS origin. Complementing receptor loss-of-function studies, LPA receptor gain-of-function can be addressed by increasing LPA receptor activation. Recent results from a gain-of-function model in which intact cerebral cortical hemispheres were exposed to exogenous LPA [Kingsbury et al., 2003] are also reviewed. In this paradigm, LPA receptor-mediated signaling rapidly alters cerebral cortical growth and anatomy and has multiple effects on neurogenic processes that, somewhat unexpectedly, operate through mechanisms other than cell proliferation.

LOSS OF FUNCTION: GENETIC DELETION OF LPA RECEPTORS

To date, mice null for LPA_1 and LPA_2, two of the four LPA-activated GPCRs, have been generated [Contos et al., 2000a, 2002]. The mouse lpa_1 gene consists of at least five exons with the majority of the coding region within exon 3 [Contos et al., 2000a]. In LPA_1 null mice, exon 3, which encodes transmembrane domains I–VI, was completely deleted using the Cre-loxP system [Contos et al., 2000a]. The mouse lpa_2 gene consists of three exons with exons 2 and 3 containing the coding region [Contos et al., 2002]. In LPA_2 null mice, part of exon 2, which encodes transmembrane domains IV–VI, and the intron donor site immediately downstream of exon 2, were replaced with the neomycin resistance gene in reverse orientation [Contos et al., 2002]. Mice null for both LPA_1 and LPA_2 (double knockout) were obtained through intercrosses of $lpa_1^{(-/-)}$ and $lpa_2^{(-/-)}$ null mice [Contos et al., 2002].

PHENOTYPES OF LPA RECEPTOR NULL MICE

Mice null for lpa_1 have ~50% neonatal lethality because of impaired suckling; in rodents, suckling is primarily driven by olfaction, indicating the presence of olfactory deficits [Contos et al., 2000a]. These mice are also characterized by craniofacial dysmorphism, smaller size, increased apoptosis in the sciatic nerve, and a small incidence of frontal hematoma (~2.5%) and exencephaly (~5%) [Contos et al., 2000a]. In contrast, $lpa_2^{(-/-)}$ mice do not have an obvious phenotype [Contos et al., 2002]. Deletion of both lpa_1 and lpa_2 results in no additional abnormalities relative to loss of lpa_1 alone except for an increase in neonatal frontal hematomas (26%) and a slight increase in lethality [Contos et al., 2002]. In the cerebral cortex, no obvious abnormalities are observed in $lpa_1^{(-/-)}$ or $lpa_1^{(-/-)}lpa_2^{(-/-)}$ mice [Contos et al., 2000a, 2002]. An exception is the occasional reductions in cerebral wall thickness in embryonic and neonatal $lpa_1^{(-/-)}$ mice [Contos et al., 2000a].

In addition to the aforementioned phenotypes in LPA receptor null mice, cells from these animals are characterized by signaling deficits. Both LPA_1 and LPA_2 activate similar intracellular signaling molecules, including pertussis toxin (PTX)-sensitive $G_{i/o}$, and PTX-insensitive $G_{q/11/14}$ and $G_{12/13}$, to regulate cellular functions such as proliferation, survival, differentiation, and morphogenesis [Ishii et al., 2004]. NPCs from the cortical VZ of $lpa_1^{(-/-)}$ mice show reduced cell rounding, migration and proliferation

in response to LPA exposure, compared to those from $lpa_1^{(+/+)}$ mice [Contos et al., 2000a]. Additionally, $lpa_1^{(-/-)}$ Schwann cells show a severe reduction in actin rearrangement following LPA exposure, relative to $lpa_1^{(+/+)}$ Schwann cells [Weiner et al., 2001]. Mouse embryonic fibroblasts (MEFs) from $lpa_1^{(-/-)}$ or $lpa_2^{(-/-)}$ mice show a partial reduction in phospholipase C activation, Ca^{2+} mobilization, and JNK and Akt activation following LPA treatment, while those from $lpa_1^{(-/-)}$ $lpa_2^{(-/-)}$ mice show an absence or severe reduction in these responses [Contos et al., 2002]. In contrast, adenylyl cyclase inhibition is almost completely abolished in $lpa_1^{(-/-)}$ MEFs but unaffected in $lpa_2^{(-/-)}$ MEFs. In fibroblast cells derived from the meninges, LPA-induced stress fibers are severely reduced in $lpa_1^{(-/-)}$ $lpa_2^{(-/-)}$ mice [Contos et al., 2002]. All together, these results indicate that LPA signaling is deficient in cells from LPA receptor null mice and that LPA_1 acts redundantly with LPA_2 to mediate many cellular responses to LPA.

GAIN-OF-FUNCTION: EX VIVO EXPOSURE OF CORTICAL HEMISPHERES TO LPA

While dissociated cells and organotypic slice cultures are useful models for understanding brain development, they only partially mimic in vivo neural processes since cerebral cortical anatomy is not fully preserved in these preparations. To bridge the gap between in vitro and in vivo paradigms, an ex vivo brain culture system was developed that permits the increased activation of LPA receptors in cortical hemispheres through exogenous LPA exposure [Kingsbury et al., 2003]. Importantly, neurogenesis is preserved in this gain-of-function model. An additional advantage of this system is that it internally controls for developmental stage differences that may exist amongst embryos of the same or different litters, since opposite hemispheres from the same animal are cultured in the presence or absence of LPA.

PHENOTYPE OF LPA-TREATED HEMISPHERES

Within this ex vivo system, LPA exposure rapidly alters the organization of the developing cerebral cortex [Kingsbury et al., 2003]. After just 17 h, LPA-treated hemispheres display striking cortical folds, compared to control hemispheres obtained from the same animals (Fig. 1A,B) [Kingsbury et al., 2003]. A time course analysis showed that this folding is evident at 6 h ex vivo, is progressive and is not reversible [Kingsbury et al., 2003]. In addition to cortical folding, LPA produces a widening of the cerebral wall (Fig. 1C). This expansion of cortical thickness is due to an increase in cells within both proliferative (VZ) and postmitotic regions (i.e., intermediate zone (IZ) and cortical plate (CP)) without a corresponding change in cell density [Kingsbury et al., 2003]. Whereas LPA is a strong mitogen for many cell types [Moolenaar, 1995, 1997], the observed increases are not due to increased proliferation since [^{3}H]thymidine incorporation is reduced in LPA-treated cortices, compared to controls [Kingsbury et al., 2003]. Instead, LPA induces terminal mitosis in NPCs based on an increase in both mitotic cells, as well as early differentiating cortical neurons, following LPA treatment (Fig. 1D,E) [Kingsbury et al., 2003]. In addition, LPA is a survival factor for cells within the VZ (Fig. 1F) [Kingsbury et al., 2003], a region characterized by substantial cell death during development [Blaschke et al., 1996, 1998; Pompeiano et al., 2000]. The increase in cortical thickness is, therefore, attributed to both the increased survival of NPCs, which sustains the proliferative population, and the promotion of NPC terminal mitosis, which increases the number of postmitotic neurons in developing cortex.

Since previous studies have shown that both LPA_1 and LPA_2 are co-expressed in a number of tissues [Contos et al., 2000b] and activate the same signaling pathways to interchangeably mediate many effects of LPA [Ishii et al., 2000; Contos et al., 2002], cortices from $lpa_1^{(-/-)}$ $lpa_2^{(-/-)}$ double null mice were examined in the ex vivo system to reduce possible compensation from either receptor. Neither folding, nor increases in cortical thickness, mitosis or cell survival are observed in $lpa_1^{(-/-)}$ $lpa_2^{(-/-)}$ mice (Fig. 2A–D) [Kingsbury et al., 2003], indicating that LPA's effects are receptor-mediated.

COMPARISON OF GAIN-OF-FUNCTION WITH PREVIOUS ACCOUNTS OF LPA'S EFFECTS

The finding that LPA promotes the survival of NPCs within the VZ is not surprising given that LPA is a survival factor for many other cell types, including ovarian cancer cells, renal proximal tubular cells, macrophages, 3T3 fibroblasts, T lymphoblasts, mesangial cells, cardiac

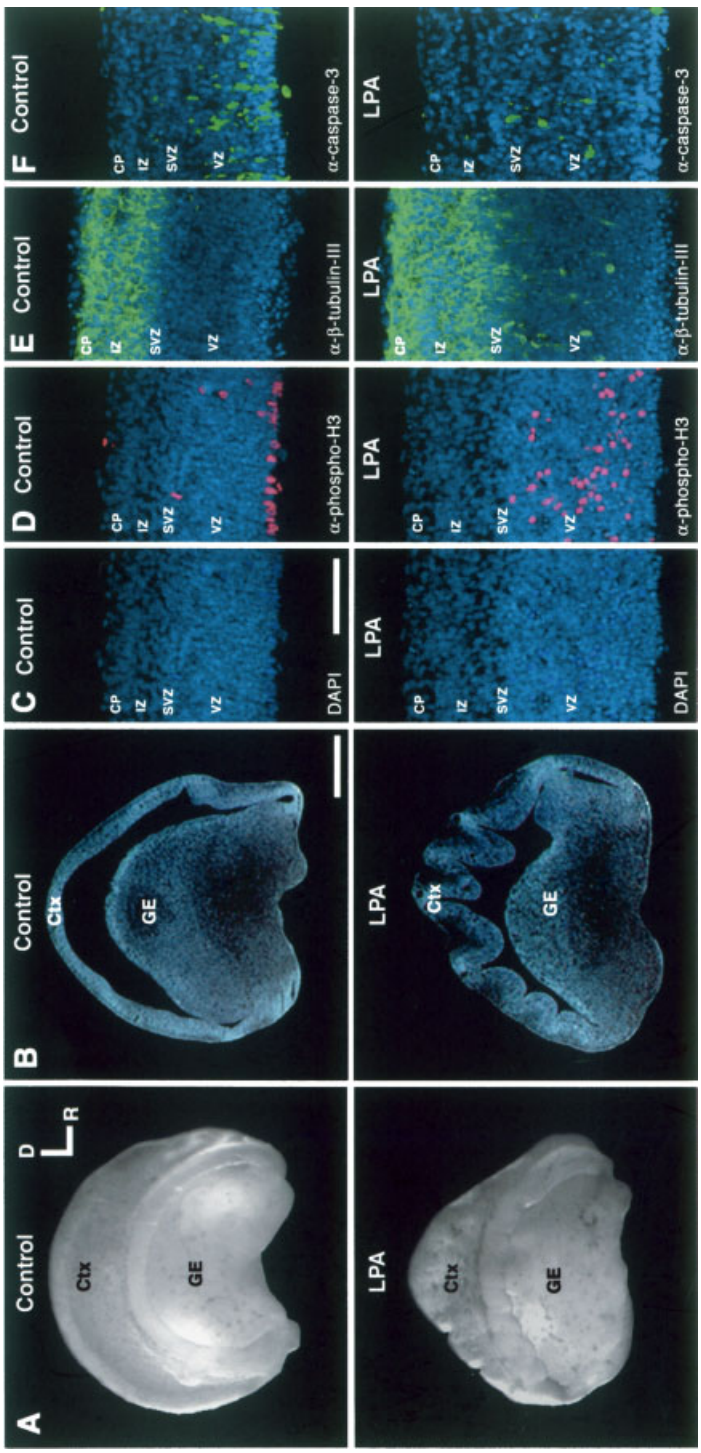

Fig. 1. Intact cerebral hemispheres exposed to LPA ex vivo exhibit cortical folding and widening of the cerebral wall compared to control hemispheres from the same animal. Whole-mount views (**A**) and sagittal sections (**B**) from embryonic day 14 (E14) hemispheres show dramatic cortical folding following culture with LPA, compared to control medium. D, dorsal; R, rostral; Ctx, cortex; GE, ganglionic eminence. Scale bar, 0.5 mm. **C**: E14 cortices labeled with the nuclear stain, 4′, 6′-diamino-2-phenylindole (DAPI) show increased thickness following LPA-treatment. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bar, 100 μm. LPA promotes terminal mitosis based on an increase in M-phase cells immunolabeled with α-phosphohistone H3 (**D**), a decrease in S-phase cells (data not shown) and an increase in differentiating neurons immunolabeled with α-β-tubulin III (**E**). LPA decreases the number of dying cells immunolabeled for α-active caspase-3 (**F**). Cortical width should be compared only within a given pair of control versus LPA-treated samples. Data are modified from Kingsbury et al. [2003], with permission. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

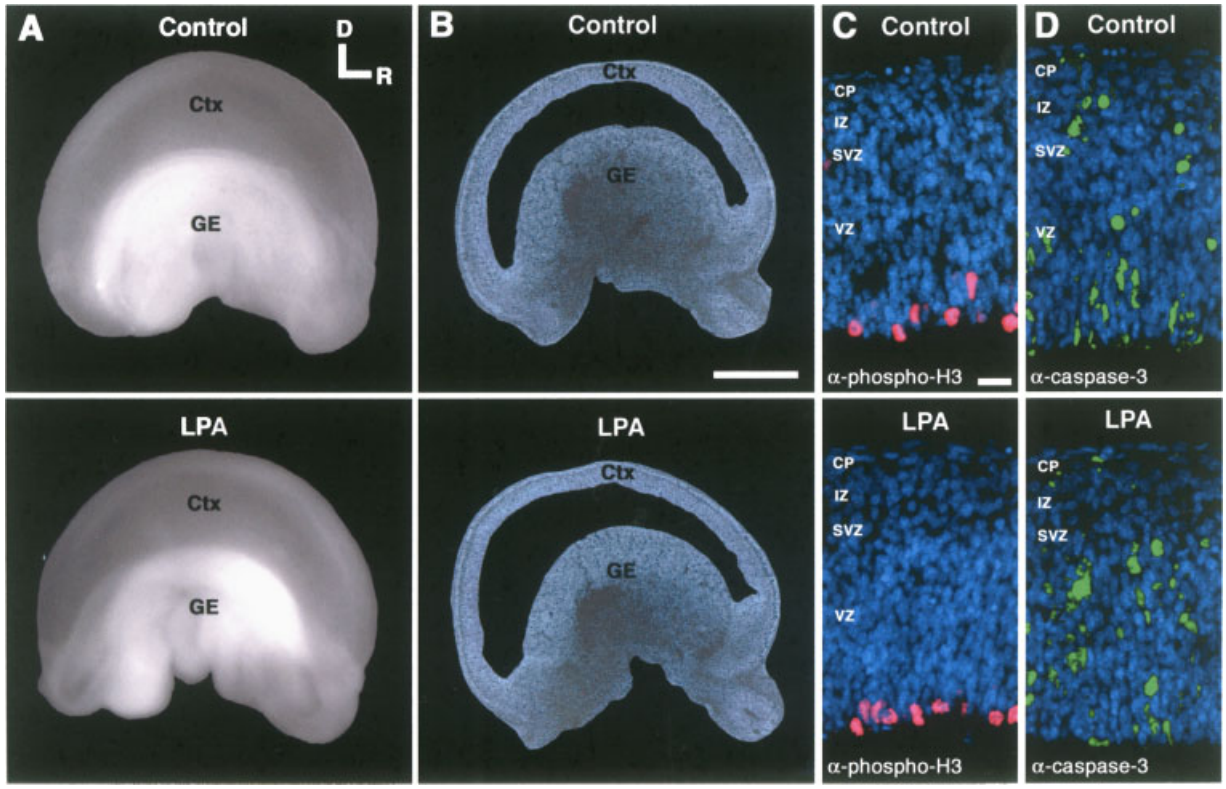

Fig. 2. LPA's effects are absent in cerebral hemispheres from $lpa_1^{(-/-)}$ $lpa_2^{(-/-)}$ double null mice cultured ex vivo. Whole-mount views (**A**) and sagittal sections (**B**) from E14 $lpa_1^{(-/-)}$ $lpa_2^{(-/-)}$ hemispheres are similar following culture with LPA or control medium. D, dorsal; R, rostral; Ctx, cortex; GE, ganglionic eminence. Scale bar, 0.5 mm. **C**, **D**: LPA exposure has no effect on the number of mitotic NPCs (immunolabeled with α-phosphohistone H3; (C) or the number of dying cells (immunolabeled for α-active caspase-3; (D) in DAPI-stained E14 $lpa_1^{(-/-)}$ $lpa_2^{(-/-)}$ cortices. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bar, 25 μm. Data are modified from Kingsbury et al., [2003], with permission. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

myocytes, Jurkat cells, HeLa cells, and Schwann cells (reviewed in [Ye et al., 2002]).

The observation that LPA increases the number of mitotic NPCs within the VZ of cultured hemispheres is also not unexpected. Previous work has shown that LPA exposure triggers cell rounding and process retraction in VZ NPCs through actin cytoskeletal rearrangements [Fukushima et al., 2000]. These shape changes are comparable to those that occur during the rounding up phase of interkinetic nuclear migration that immediately precedes mitosis [Fukushima et al., 2000]. Thus, the increased number of mitotic NPCs following LPA treatment [Kingsbury et al., 2003] supports the hypothesis that LPA stimulates morphological changes that facilitate mitotic division.

The increase in NPC mitosis in LPA-treated ex vivo cultures is accompanied by an increase in neuronal differentiation, suggesting that LPA induces NPC cell cycle exit in the developing brain [Kingsbury et al., 2003]. It is currently unclear how LPA regulates the differentiation of NPCs; however it may be related to the displacement of mitotic NPCs away from the bottom of the VZ (Fig. 1D), which may in turn disturb lateral inhibition mediated by delta/notch signaling [Murciano et al., 2002]. Nevertheless, this displacement of mitotic profiles suggests that the nuclei of NPCs need not undergo mitosis at the bottom of the VZ in order to form a stratified cerebral wall [Kingsbury et al., 2003].

The finding that LPA is anti-mitogenic in the ex vivo culture, however, is surprising. LPA is a potent mitogen for a variety of cultured cells, including fibroblasts and smooth muscle cells [van Corven et al., 1989; Tokumura et al., 1994]. In regards to the nervous system, LPA stimulates the proliferation of dissociated astrocytes, retinal pigment epithelial cells and NPCs from the VZ [Keller et al., 1997; Thoreson and Chacko, 1997; Contos et al., 2000a; Rao et al., 2003]. However, exposing NPCs in intact cerebral

cortices to LPA decreased their levels of proliferation [Kingsbury et al., 2003]. These opposing effects of LPA on NPC proliferation in dissociated cells versus intact hemispheres suggest that LPA's effects within organized tissues can be distinct from those in dissociated cell culture and emphasize the importance of examining lipid effects in models most akin to the living tissue in situ.

POSSIBLE SIGNALING PATHWAYS FOR LPA'S EFFECTS IN EX VIVO CULTURE

While LPA's effects on terminal mitosis are mediated through LPA receptors, it is currently unclear which G-protein coupled pathways ($G_{i/o}$, G_q, and $G_{12/13}$) [An et al., 1998; Ishii et al., 2000; Fukushima et al., 2001; Rao et al., 2003] are involved. One possibility is that LPA's effects are mediated through $G_{12/13}$ pathways. In VZ NPCs, LPA activates Rho-dependent cytoskeletal changes [Fukushima et al., 2000], as well as depolarizing ionic currents [Dubin et al., 1999] that have been linked to decreased DNA synthesis when induced by γ-aminobutyric acid or glutamate [LoTurco et al., 1995]. In N1E-115 and NG105-18 neuronal cells, LPA produces similar changes in cell morphology and ionic conductance that are mediated by $G_{\alpha 12}$ and $G_{\alpha 13}$, respectively [Postma et al., 2001]. Thus, by inducing $G_{\alpha 12/13}$ cytoskeletal changes to facilitate entry into mitosis, and depolarizing currents to reduce re-entry into S-phase (i.e., DNA synthesis), LPA could promote terminal cell division in the current ex vivo model.

The activation of PI3K via $G_{i/o}$ may be involved with the decrease in VZ cell death following LPA treatment since PI3K is activated in several other systems of LPA-mediated survival [Levine et al., 1997; Koh et al., 1998; Weiner and Chun, 1999]. In neonatal Schwann cells, LPA mediates survival through the activation of the $G_{\alpha i}$/PI3K/Akt pathway [Weiner and Chun, 1999]. LPA also promotes the survival of mouse renal proximal tubular cells via PI3K [Levine et al., 1997] and macrophages via PI3K and a downstream effector, $p70^{S6K}$ [Koh et al., 1998]. Thus, $G_{i/o}$ and PI3K are likely candidates for LPA-mediated survival of NPCs, yet future experiments are needed to clarify this possibility.

The activation of a G-protein coupled pathway that decreases gap junction communication amongst NPCs may provide a link between LPA's promotion of terminal mitosis and cell survival. In Rat-1 cells, not only does LPA stimulate similar cytoskeletal remodeling and Cl^--mediated membrane depolarization as in NPCs, it also induces gap junction closure via a Src tyrosine kinase pathway [Postma et al., 1998]. Importantly, reducing gap-junction communication correlates with cell cycle exit and neuronal differentiation in NPCs [Bittman et al., 1997] and increased cell survival in retinal and hippocampal preparations [Linden, 2000; Frantseva et al., 2002]. Future studies involving the inhibition of specific G-proteins and downstream effectors in cortical progenitors should lend insight into how receptor-mediated LPA signaling promotes terminal mitosis and cell survival in the developing cerebral cortex.

COMPENSATORY SIGNALING SYSTEMS IN CORTICAL DEVELOPMENT

One possible explanation for the absence of obvious cortical abnormalities in $lpa_1^{(-/-)}$, $lpa_2^{(-/-)}$, and $lpa_1^{(-/-)}$ $lpa_2^{(-/-)}$ knockout mice in vivo is a redundancy in signaling systems that are important for cell differentiation and survival during neurogenesis. Like LPA, glutamate, GABA, bone morphogenetic protein-4, pituitary adenylate cyclase-activating polypeptide, and neurotrophin-3 all promote cell cycle exit/differentiation of VZ NPCs [Ghosh and Greenberg, 1995; LoTurco et al., 1995; Lu and DiCicco-Bloom, 1997; Li et al., 1998]. In addition, sphingosine 1-phosphate (S1P), another simple lysophospholipid that binds the related lysophospholipid receptors $S1P_1$–$S1P_5$ to activate identical or similar signaling pathways as LPA (reviewed in [Moolenaar, 1999; Ishii et al., 2004], may provide compensation. The $S1P_1$ receptor has high expression in the embryonic cortical VZ that coincides with the temporal and spatial expression of LPA_1 [McGiffert et al., 2002]. In addition, S1P promotes changes in the N1E-115 cell line that are remarkably similar to those induced by LPA in VZ NPCs, such as cell rounding, neurite retraction, and Cl^--mediated membrane depolarization [Postma et al., 1996; Postma et al., 2001]. Furthermore, S1P is involved in nerve growth factor-mediated differentiation and survival in cells of neural origin [Edsall et al., 1997]. Finally, sphingosylphosphorylcholine (SPC), a lysophospholipid and possible high affinity ligand for the orphan G-protein-coupled receptor, GPR12, may have currently unrecognized roles in cortical development

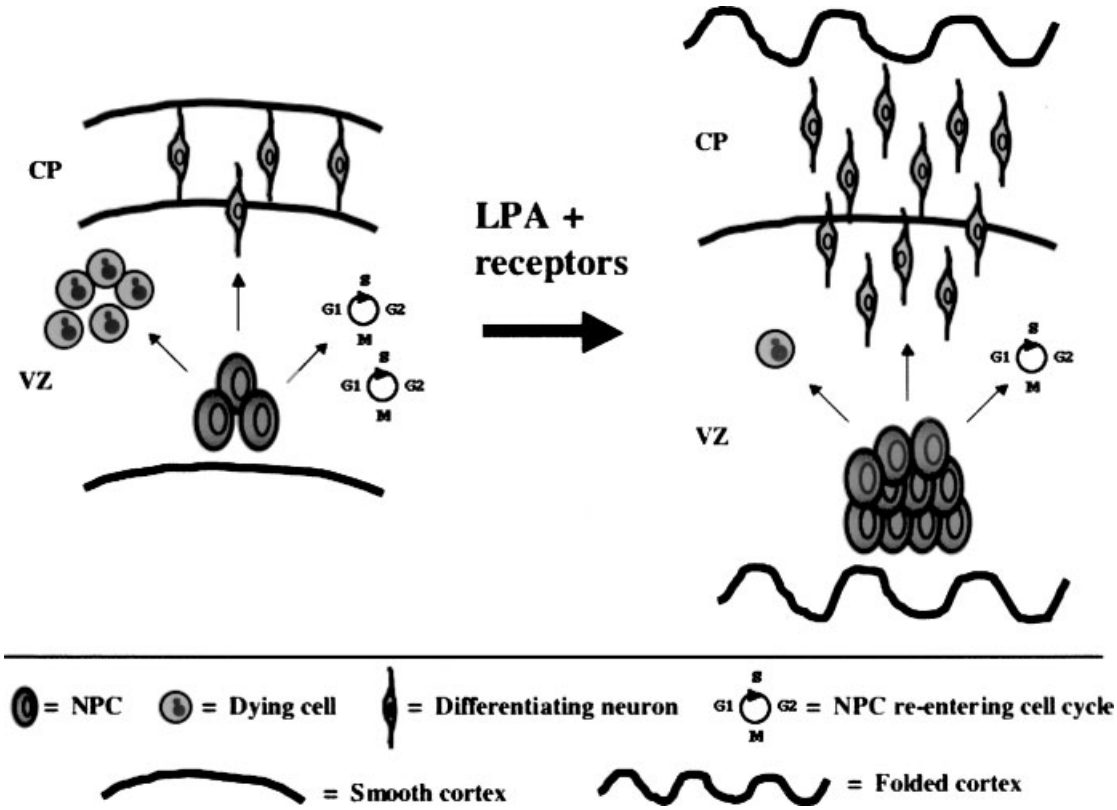

Fig. 3. Schematic model showing new functions for LPA signaling during cerebral cortical development based on results from ex vivo hemisphere culture. LPA alters cerebral cortical anatomy by producing regular cortical folds. In addition, LPA increases VZ thickness by reducing NPC cell death rather than by increasing cell cycle re-entry/proliferation. LPA increases CP thickness by promoting differentiation of NPCs.

since transcripts of GPR12 are present in the postmitotic region of the developing cortex [Ignatov et al., 2003], an area in which LPA_2 is also expressed [McGiffert et al., 2002]. Thus, in addition to growth factors, multiple lysophospholipids likely contribute to the control of various neurogenic processes during the formation of the adult cerebral cortex.

CONCLUSION

An understanding of lysophospholipid signaling in vivo has been greatly advanced by the identification of cognate receptors and the generation of LPA receptor null mice. The recent study of LPA signaling within a gain-of-function culture model that preserves the anatomy of the developing cerebral cortex combined with null-mutants has allowed definitive assignment of receptor-dependent functions. These new functions for LPA signaling during brain development include the promotion of cell survival and differentiation within the embryonic cerebral wall, and the generation of cortical folds (Fig. 3). The time course for increasing cortical thickness, as well as the LPA-mediated pathways involved in all of the effects mentioned above, remain to be determined. Future directions also include the examination of additional lysophospholipids and their receptors during cortical development, as well as the use of agonists/antagonists to pharmacologically manipulate lipid signaling within in vivo and ex vivo settings.

ACKNOWLEDGMENTS

We thank A. Yang and C. Higgins for critical reading of this manuscript.

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