Rapid communication

Lysophosphatidic acid stimulates neuronal differentiation of cortical neuroblasts through the LPA$_1$–G$_{i/o}$ pathway

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Abstract

Lysophosphatidic acid (LPA) is an extracellular lipid mediator that regulates cortical development. Here we examined how LPA influences the cell fate of cortical neuroblasts using a neurosphere culture system. We generated neurospheres in the presence of basic fibroblast growth factor (bFGF). Treatment with LPA throughout the culture period significantly reduced the number of cells in the neurospheres. When dissociated single cells derived from neurospheres were induced to differentiate by adherence on coverslips, the proportion of MAP2-positive neurons was higher in LPA-treated neurospheres than in those treated with bFGF alone, and the proportion of myelin basic protein-positive oligodendrocytes was lower. Consistent with this finding, LPA raised the ratio of β-tubulin type III-positive young neurons and reduced the ratio of CD140a-positive oligodendrocyte precursors in neurospheres. These effects of LPA were inhibited by pretreatment of neurospheres with pertussis toxin or an LPA$_1$-preferring antagonist, Ki16425. Moreover, LPA-induced enhancement of neuronal differentiation was not observed in neurospheres derived from lpa_1-null mice. These results suggest that LPA promotes the commitment of neuroblasts to the neural lineage through the LPA$_1$–G$_{i/o}$ pathway.
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1. Introduction

Lysophosphatidic acid (LPA) is a simple phospholipid that activates G protein-coupled LPA receptors to induce cellular responses (Moolenaar, 1999). To date, five types of LPA receptor genes (lpa_1–lpa_5) have been identified, and lpa_1, lpa_2, and lpa_3 are known to be structurally related (Fukushima et al., 2001; Ishii et al., 2004; Lee et al., 2006). Mouse embryonic brains express the lpa_1, lpa_2, and lpa_3 genes, as well as genes of LPA-metabolizing enzymes, suggesting that LPA signaling functions in early brain development (Fukushima et al., 2001; Ishii et al., 2004). LPA induces a variety of cellular responses in cortical neuroblasts, which predominantly express lpa_1, and young cortical neurons, which express lpa_1 and lpa_2. These LPA-induced responses include stimulation of changes in neuroblast shape, alterations of ionic conductances in neuroblasts, enhancement of neuroblast survival, inhibition

of neurite outgrowth, and stimulation of growth cone collapse (Dubin et al., 1999; Fukushima et al., 2000, 2002a,b; Kingsbury et al., 2003; Fukushima and Morita, 2006).

Mice lacking lpa_1, lpa_2, or both surprisingly show no severe morphological defects in their cerebral cortices (Contos et al., 2000, 2002). However, a gain-of-function analysis using an ex vivo system recently revealed that exposure of wild-type embryonic brains to LPA results in malformation of the cerebral cortex; this malformation does not occur in brains from lpa_1/lpa_2 double knockout mice (Kingsbury et al., 2003). This study has further shown that LPA promotes terminal mitosis of neuroblasts in the ventricular zone, where neurogenesis occurs and from where differentiated neurons migrate out toward the cortical plate.

Floating neurosphere cultures have been widely used to examine the cellular and molecular mechanisms of neurogenesis. A cortical neurosphere in culture is generated from a single cortical neuroblast under appropriate growth conditions and is thought to contain self-renewing, multipotent, and cell lineage-restricted neuroblasts. Extrinsic factors affect neuroblast growth and differentiation in the neurospheres (Kilpatrick

and Bartlett, 1995; Qian et al., 1997; Martens et al., 2000; Ciccolini, 2001). In the present study, we employed the neurosphere culture system to examine how neuroblast growth and differentiation are influenced by LPA.

2. Experimental procedures

2.1. Cell cultures

Embryos were obtained from timed-pregnant ICR females (Shimizu Labora-tory, Kyoto, Japan) or lpa_1 heterozygous females that were crossed with lpa_1 heterozygous males (Contos et al., 2000), with the morning of the vaginal plug designated embryonic day 0 (E0). Embryos from lpa_1 heterozygous females were genotyped by PCR using genomic DNAs prepared from a small part of tail. The cerebral cortices from E12 mice were dissected, and the meninges were com-pletely removed. Tissues were transferred to Opti-MEM medium (Invitrogen, Carlsbad, CA), and triturated with a fire-polished pasteur pipette to obtain single cells. The cell suspension was transferred to a non-coated plastic dish ($\sim 1 \times 10^6$ cells/60-mm diameter dish) and cultured in the presence of 2% B27 supplement (Invitrogen), 0.1% fatty acid-free bovine serum albumin (Sigma, St. Louis, MO), and growth factors (20 ng/ml basic fibroblast growth factor [bFGF] and/or 50 ng/ml epidermal growth factor [EGF], both from Wako Pure Chemicals, Tokyo, Japan) at 37 °C under a 5% CO_2 atmosphere.

Floating neurospheres were harvested by centrifugation and dissociated into single cells by pipetting. Cells were counted using a hemocytometer. Disso-ciated cells were seeded onto glass coverslips (3000 cells per 12-mm coverslip) precoated with Cell-Tak (2 μg/cm², Becton Dickinson Labware, Bedford, MA) and cultured in Opti-MEM containing 1% B27 at 37 °C under 5% CO_2.

2.2. Bromodeoxyuridine labeling

To identify proliferating cells, neurospheres were treated with 20 μM bromodeoxyuridine (BrdU, Sigma) for 2 h. Dissociated cells were plated on coverslips as above and fixed with 4% paraformaldehyde (PFA) 1 h after plating. BrdU-positive cells were detected immunocytochemically, as pre-viously described (Fukushima et al., 2000).

2.3. TUNEL staining

To identify dead cells, dissociated cells from neurospheres were plated on coverslips for 1 h and fixed with PFA. The cells were subjected to a fluorescent TUNEL assay using a DeadEND TUNEL kit (Promega, WI), according to the manufacturer's protocol.

2.4. Immunocytochemistry

Immunocytochemistry was performed as previously described (Fukushima et al., 2002a). Dissociated cells were fixed with 4% PFA and permeabilized and blocked with 0.2% Triton X-100 and 10% normal goat serum, followed by incubation with primary antibodies. The following primary antibodies were used at the indicated concentrations: anti-nestin (1 μg/ml, Chemicon, CA), anti-microtubule-associated protein 2 (MAP2; 1:100, Sigma), anti-myelin basic protein (MBP; 1:40, Sigma), anti-glial fibrillary acidic protein (GFAP; 1:400, provided by Dr. Watanabe at Hokkaido University), anti-CD140a (1 μg/ml, eBioscience, San Diego, CA), and anti-β-tubulin type III (1:200, Sigma). The bound antibodies were visualized by successive incubations with a biotinylated secondary IgG and Alexa 488–streptavidin (0.5 μg/ml, Invitrogen). For all immunostaining, tetramethyl rhodamine isocyanate (TRITC)-phalloidin and DAPI were used as counterstains to visualize viable cells and nuclei, respec-tively (Fukushima et al., 2002b).

2.5. Materials

LPA was purchased from Avanti Polar Lipid (Alabaster, AL, USA). Pertussis toxin (PTX) was purchased from Wako Pure Chemicals. Ki16425 was provided by the Kirin Brewery Company (Gunma, Japan).

2.6. Statistics

Analysis of variance (ANOVA) followed by a post hoc test was applied to data to determine statistical significance by using the statistical software, StatView 4.5 (Abacus Concepts).

3. Results and discussion

Fetal calf serum (FCS) and B27 supplement have been widely used for neurosphere preparation (Svendsen et al., 1995). In the experiments, we used serum-free culture conditions because FCS normally contains LPA at the micromolar level. We supplemented the medium with 2% B27. Although B27 is known to contain bovine serum albumin, which is a carrier protein for serum lipids, we confirmed that the concentration of LPA in B27 was below 100 nM equivalent, as measured with bioassays; in the 2% B27, the LPA concentration was therefore below 2 nM, too low to exert its biological activity (Fukushima et al., 2002b).

When cortical cells were dissociated and cultured in the absence of exogenous growth factors or in the presence of 50 ng/ml EGF, neurosphere formation was not evident after 5 days in vitro (Fig. 1B). In contrast, the addition of 20 ng/ml bFGF induced the formation of large numbers of neurospheres with 30–80 μm in diameter (Fig. 1A). This bFGF-dependency and EGF-independency is in good accord with previous reports showing that EGF-dependency appears late in bFGF-treated cultures of cortical neuroblasts (Ciccolini and Svendsen, 1998; Maric et al., 2003). There were no marked differences between bFGF-treated cultures and EGF/bFGF-cotreated cultures in the neurosphere size or in the number of cells in the spheres (Fig. 1B), indicating that 5 days in culture were not sufficient to induce EGF-dependent cell growth. Treatment with 1 μM LPA alone did not result in neurosphere formation (data not shown), nor did cotreatment with LPA and EGF (Fig. 1B). However, LPA inhibited neurosphere growth in bFGF-treated cultures, reducing both sphere size and cell numbers (Fig. 1A and B). LPA suppressed neurosphere growth in cultures cotreated with bFGF and EGF to a similar degree as in cultures treated with bFGF alone (Fig. 1B).

We examined whether the reduction in neurosphere cell numbers caused by LPA could be attributed to inhibition of cell proliferation or to stimulation of cell death. To measure cell proliferation, neurosphere cultures treated with bFGF were pulsed with BrdU for 2 h. The cells were dissociated, plated on coverslips, and BrdU-positive cells were detected immunocy-tochemically. A slight but non-significant decrease in BrdU-positive cells was observed in LPA-treated cultures on days 3 and 5 (Fig. 2A). By contrast, the TUNEL method revealed that LPA significantly increased the percentage of dead cells on day 3, but not day 5 (Fig. 2B). The same profile was seen in cultures cotreated with bFGF and EGF (data not shown). Taken together, these results suggest that LPA induced cell death during a limited period of culture. However, this increase in cell death might not entirely account for the LPA-induced reduction in the number of cells in the neurospheres; the possibility cannot be excluded that a slight and continuous decrease in cell
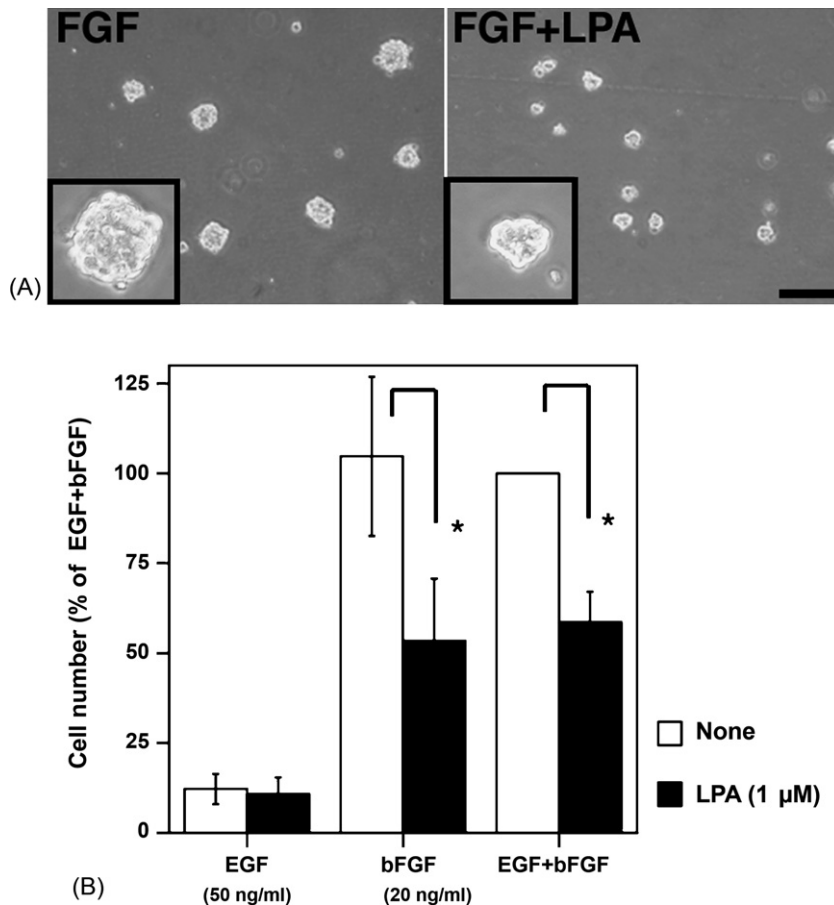
Fig. 1. LPA reduces neurosphere growth. (A) Phase contrast microscopy of cortical neurospheres. Cortical cells were cultured in the presence of bFGF without (left panel) or with (right panel) 1 μM LPA for 5 days. LPA was added daily. Inset; three-fold magnified pictures of neurospheres. (B) Cell numbers in neurosphere cultures. Neurospheres were dissociated into single cells and cells were counted. Data are means ± S.E.M. ($n > 6$). $^*p < 0.05$, and bar: 100 μm.

proliferation during culture may also be involved in the inhibition of neurosphere growth by LPA.

Although early cortical neuroblasts are multipotent, the bFGF concentration has been demonstrated to regulate cell fate. Low bFGF concentrations maintain the neuronal cell fate of neuroblasts, while a high level of bFGF (over 10 ng/ml) stimulates production of neuroglial precursors that generate both neurons and oligodendroglia (Qian et al., 1997). Thus, the concentration (20 ng/ml) used in this study may preferentially produce neuroglial precursors rather than neuron-restricted neuroblasts in neurospheres. To examine whether neurospheres in this study contain neuroglial precursors, neurospheres were dissociated and plated on coverslips to induce cell differentiation, and then the phenotype of the cells was characterized immunocytochemically. Antibodies against MAP2, MBP, and GFAP were used to identify mature neurons, oligodendrocytes, and astrocytes, respectively. MAP2-immunopositive cells were detected in bFGF-treated neurospheres. The percentage of MAP2-positive cells increased with duration in culture and reached about 50% of total cells at day 8 after plating (Fig. 3), indicating that neuronal differentiation progressed with time. LPA treatment significantly increased the MAP2-positive population on day 8 in culture (Fig. 3).

MBP-positive cells were also detected, although at a lower percentage than MAP2-positive neurons. The proportion of MBP-positive cells from bFGF-treated neurospheres also increased with time in culture (Fig. 3), indicating the progression of oligodendroglial differentiation. In contrast to the case of MAP2-positive neurons, however, LPA exposure reduced the percentage of MBP-positive cells. No GFAP-positive cells were detected until day 8 after plating (data not shown), probably due to the lack of other extrinsic factors required for astrocyte generation (Qian et al., 1997).

Next, we examined the phenotype of cells in neurospheres using immunocytochemistry to verify whether the numbers of early differentiated neurons (or neuron-restricted neuroblasts) increase and those of early differentiated oligodendroglia (or oligodendroglia-restricted neuroblasts) decrease in neurospheres treated with LPA. Antibodies against nestin were used to identify total neuroblasts, antibodies against β-tubulin type III were used to identify early differentiated neurons, and antibodies against CD140a were used to identify early differentiated oligodendroglia. At day 5 in neurosphere culture, approximately 80% of cells expressed nestin, which was not affected by LPA treatment (data not shown). A relatively low number of cells (∼20% of total cells) expressed β-tubulin type III (Fig. 4A); as expected, LPA treatment increased the
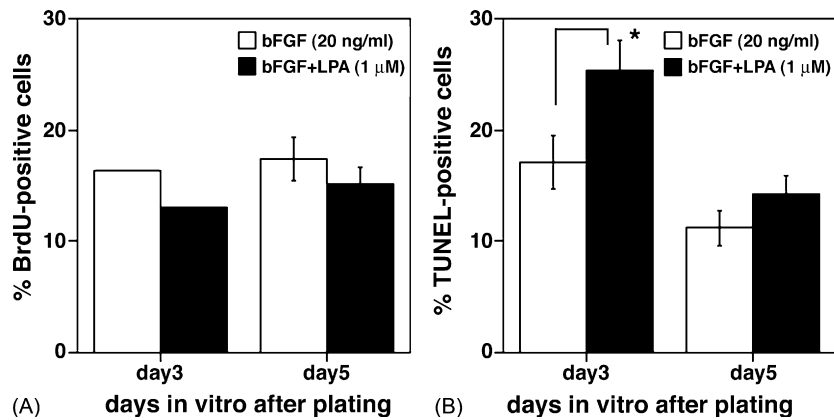
Fig. 2. LPA inhibits neurosphere growth by stimulating cell death. Cortical cells were cultured in the presence of bFGF without or with 1 μM LPA for 5 days. LPA was added daily. (A) Proliferating cells in neurosphere cultures. Proliferating cells were identified by immunostaining with an anti-BrdU antibody. (B) Dead cells in neurosphere cultures. TUNEL-positive cells were counted as a measure of dead cells. Data are means ± S.E.M. ($n \geq 4$) and $^*p < 0.05$.

percentage about 1.5-fold (Fig. 4A). About 40% of cells expressed CD140a at day 5 (Fig. 4A), indicating the existence of early differentiated oligodendroglia co-expressing nestin and CD140a. Exposure to LPA during culturing significantly diminished the percentage of CD140a-expressing cells (Fig. 4A). Taken together, these results from Figs. 3 and 4A suggested that our neurospheres contained neuroglial precursors, and that LPA treatment promoted neuronal differentiation and suppressed oligodendroglial differentiation. These findings also supported a previous report that LPA induces terminal mitosis in cortical neuroblasts (Kingsbury et al., 2003). Although this enhanced neuronal differentiation in LPA-treated neurospheres presumably produces more neurons, these neurons might not survive due to the lack of an appropriate
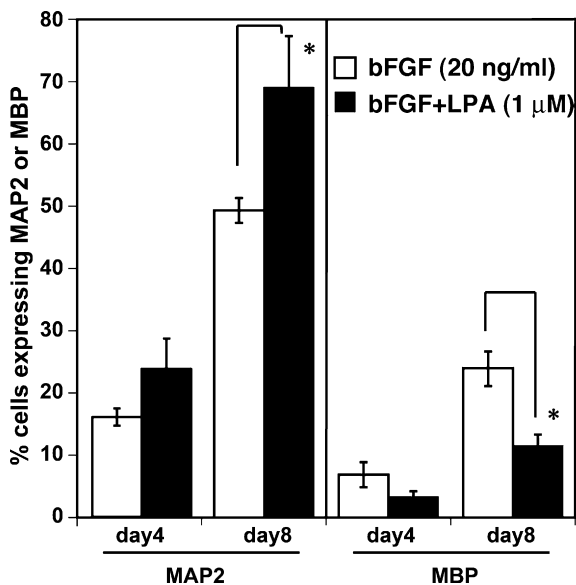

Fig. 3. The proportion of neurons increases in cultures derived from LPA-treated neurospheres. Cortical cells were cultured in the presence of bFGF without or with 1 μM LPA for 5 days. Cells dissociated from the neurospheres were further cultured for 4 or 8 days. MAP2- or MBP-positive cells were counted, and their percentage of total cells was determined. Data are means ± S.E.M. ($n \geq 3$) and $^*p < 0.05$.

scaffold in the floating neurospheres. This may account for the increased cell death and inhibited neurosphere growth shown in Figs. 1 and 2.

Finally, we explored the signaling pathway through which LPA stimulates neuronal differentiation. Neuroblasts predominantly express LPA_1, which couples to the PTX-sensitive G protein $G_{i/o}$ (Hecht et al., 1996). $G_{i/o}$ is highly expressed in cortical neuroblasts and is involved in neurogenesis (Shinohara et al., 2004). We therefore examined whether the effects of LPA would be blocked by PTX. Pretreatment of neurospheres with PTX significantly inhibited the effects of LPA on both neuronal and oligodendroglial differentiation (Fig. 4B). We next tested a recently developed, LPA_1-preferring antagonist, Ki16425, which completely inhibits the binding of LPA to LPA_1 at 10 μM (Ohta et al., 2003). When neurospheres were treated with 10 μM Ki16425, LPA failed to stimulate neuronal differentiation and suppress oligodendroglial differentiation (Fig. 4C). To further confirm the involvement of LPA_1 in LPA-dependent cortical neuroblast differentiation, neurospheres were prepared from lpa_1-null embryos and the effects of LPA were examined. Population of β-tubulin type III- and CD140a-positive cells from lpa_1-null embryos were significantly unchanged, compared with those from wild type littermates. However, lpa_1-null cells failed to respond to LPA with enhanced neuronal differentiation and decreased oligodendroglial differentiation (Fig. 4D). These results indicated that the effects of LPA on cortical neuroblast differentiation were mediated through the LPA_1–$G_{i/o}$ pathway.

The present study showed that LPA regulates the cell fate of cortical neuroblasts (i.e. neuroglial precursors). However, whether LPA stimulates only neuronal differentiation, leading to complementary reduction of glial differentiation, is unclear. Alternatively, LPA might independently stimulate two signals: one for the neural lineage and the other for the oligodendroglial lineage. Another question to be resolved is the mechanisms of LPA action downstream of $G_{i/o}$. Because FGF receptor expression is unlikely to be altered by LPA exposure (data not shown), LPA_1-mediated signaling may modify pathways
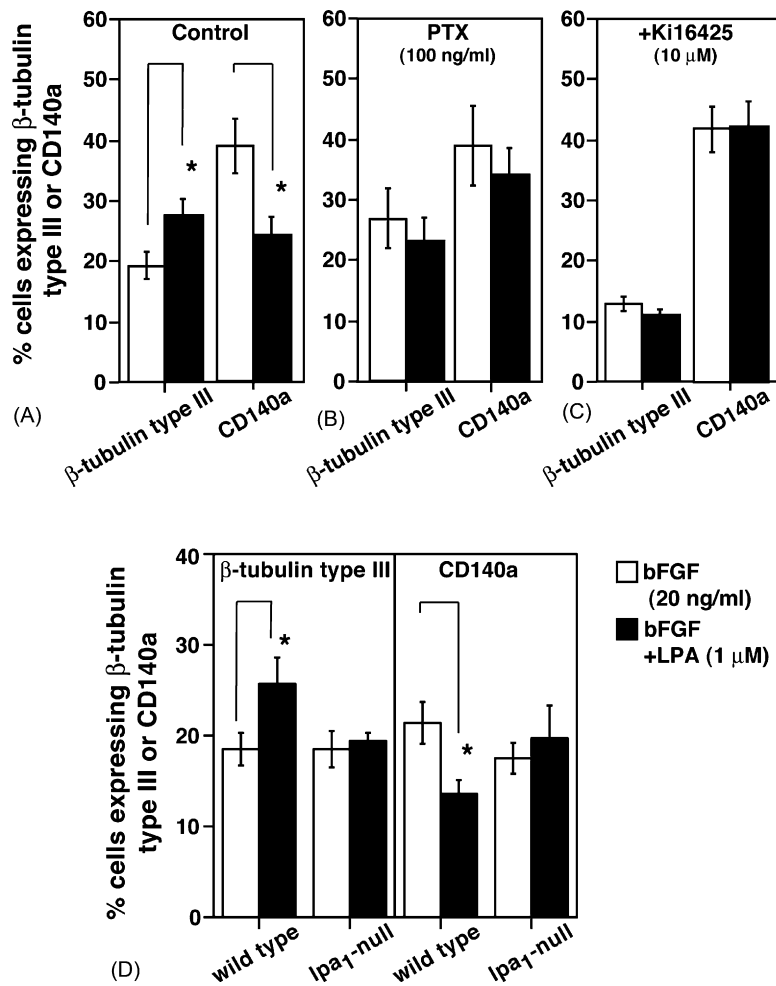
Fig. 4. LPA increases the percentage of neuronal precursors in neurospheres. (A) Effects of LPA on neuroblast differentiation in neurospheres. Cortical cells were cultured in the presence of bFGF for 5 days. LPA was added at 1 μM on days 2 and 4. Cells were dissociated, plated, and immunostained for nestin, β-tubulin type III, or CD140a 1 h after plating. The percentage of total cells positive for β-tubulin type III or CD140a was determined. Data are means ± S.E.M. ($n = 8$–10), $^*p < 0.05$. (B) Effects of pertussis toxin (PTX) on LPA-induced stimulation of neuroblast differentiation. PTX (100 ng/ml) was added on days 1 and 3, and LPA was added on days 2 and 4. Cells were dissociated on day 5, and cells immunopositive for β-tubulin type III or CD140a were counted. Data are means ± S.E.M. ($n = 6$). (C) Effects of Ki16425 on LPA-induced stimulation of neuroblast differentiation. Ki16425 (10 μM) was added 10 min before the addition of 1 μM LPA on days 2 and 4. Cells were dissociated on day 5 and cells immunopositive for β-tubulin type III or CD140a were counted. Data are means ± S.E.M. ($n = 4$). (D) Effects of LPA on neuroblast differentiation in neurospheres derived from lpa_1-null mice or wild type littermates. Cortical cells were cultured in the presence of bFGF for 5 days. LPA was daily added at 1 μM. Cells were dissociated on day 5, and cells immunopositive for β-tubulin type III or CD140a were counted. Data are means ± S.E.M. ($n = 4$–8) $^*p < 0.05$.

activated by the FGF receptor. Further analyses of cell fate determinant genes, LPA-responsive neuroblast types, and intracellular signal interactions between LPA$_1$ and the FGF receptor are needed.

Acknowledgments

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