Developmental Biology **228,** 6–18 (2000)
doi:10.1006/dbio.2000.9930, available online at http://www.idealibrary.com on IDEAL®

Lysophosphatidic Acid (LPA) Is a Novel Extracellular Regulator of Cortical Neuroblast Morphology

Nobuyuki Fukushima, Joshua A. Weiner,*,[1] and Jerold Chun*,†

*Department of Pharmacology, *Neurosciences Program, and †Biomedical Sciences Program, School of Medicine, University of California at San Diego, La Jolla, California 92093-0636*

During cerebral cortical neurogenesis, neuroblasts in the ventricular zone (VZ) undergo a shape change termed "interkinetic nuclear migration" whereby cells alternate between fusiform and rounded morphologies. We previously identified *lp*$_{A1}$, the first receptor gene for a signaling phospholipid called lysophosphatidic acid (LPA) and showed its enriched expression in the VZ. Here we report that LPA induces changes in neuroblast morphology from fusiform to round in primary culture, accompanied by nuclear movements, and formation of f-actin retraction fibers. These changes are mediated by the activation of the small GTPase, Rho. In explant cultures, where the cerebral cortical architecture remains intact, LPA not only induces cellular and nuclear rounding in the VZ, but also produces an accumulation of rounded nuclei at the ventricular surface. Consistent with a biological role for these responses, utilization of a sensitive and specific bioassay indicates that postmitotic neurons can produce extracellular LPA. These results implicate LPA as a novel factor in cortical neurogenesis and further implicate LPA as an extracellular signal from postmitotic neurons to proliferating neuroblasts.

Key Words: **lysophosphatidic acid; LPA receptor; ventricular zone; neurogenesis; cerebral cortex; neuroblast; interkinetic nuclear migration; Rho.**

INTRODUCTION

Neurons of the mammalian cerebral cortex arise from neuroblasts in the embryonic telencephalon which proliferate in a zone overlying the lateral ventricles called the ventricular zone (VZ) (Boulder Committee, 1970). Neuroblasts undergo cell cycle progression that is associated with an actin cytoskeleton-based "to-and-fro" movement of neuroblast nuclei termed "interkinetic nuclear migration" (Sauer, 1935; Berry and Rogers, 1965; Messier and Auclair, 1974; Seymour and Berry, 1975; McConnell, 1995). Associated with the nuclear movement, neuroblasts concomitantly alter their morphology from fusiform to round where upon neuroblasts mitose at the ventricular surface to give rise to more blasts or newly postmitotic neurons.

The molecular mechanisms regulating neurogenesis and interkinetic nuclear migration are incompletely understood. Control of proliferation clearly involves peptide growth factors (e.g., basic fibroblast growth factor (bFGF)) acting through receptor tyrosine kinases (RTKs) (Ghosh and Greenberg, 1995; Qian *et al.*, 1997). It may also be influenced by the ionotropic actions of neurotransmitters (e.g., glutamate and GABA), which have been reported to inhibit DNA synthesis in cortical neuroblasts (LoTurco *et al.*, 1995). Factors regulating morphological changes that are also linked to cortical neuroblast proliferation have not been reported. This, along with mild cortical phenotypes in mice lacking peptide growth factors or their RTKs (Klein, 1994), suggests that as yet unidentified mechanisms can regulate specific aspects of cortical neurogenesis.

Toward identifying such mechanisms, we previously isolated a novel G-protein-coupled receptor (GPCR) gene, *ventricular zone gene-1*, or *vzg-1*, from a neuroblast cell line derived from the cortical VZ (Chun and Jaenisch, 1996; Hecht *et al.*, 1996; Chun *et al.*, 1999). Expression of this receptor gene was enriched within the cortical VZ during neurogenesis, suggesting that it might influence neurogenic events (Hecht *et al.*, 1996). Functional analyses identified lysophosphatidic acid (LPA), an extracellular lysophospho-

[1] Present address: Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 S. Euclid Avenue, Campus Box 8108, St. Louis, MO 63110.

0012-1606/00 $35.00

lipid (LP) signaling molecule, as the high-affinity ligand for VZG-1 (Hecht *et al.*, 1996; Fukushima *et al.*, 1998; Chun *et al.*, 1999), and this receptor is now termed lysophopholipid receptor A1 or LP_{A1} (Chun *et al.*, 1999; Fukushima *et al.*, 2001).

Lysophosphatidic acid is a simple phospholipid that nonetheless has properties of an extracellular growth factor, mediating diverse cellular responses through the activation of multiple signal transduction pathways (Moolenaar, 1995; Moolenaar *et al.*, 1997). These responses include stimulation of cell proliferation through a pertussis toxin (PTX)-sensitive pathway (van Corven *et al.*, 1989) and actin rearrangement through a PTX-insensitive pathway (Ridley and Hall, 1992). The PTX-insensitive pathway utilizes the small G protein, Rho, and a Rho-associated kinase downstream of Rho, whose activation leads to actin rearrangement. In neuroblastoma cell lines, the LPA-induced actin rearrangement results in retraction of processes and cell rounding (Jalink *et al.*, 1994; Hecht *et al.*, 1996; Fukushima *et al.*, 1998; Hirose *et al.*, 1998). We previously demonstrated that expression of lp_{A1} was sufficient to mediate actin rearrangement induced by LPA (Fukushima *et al.*, 1998). These morphological effects on neuroblastoma cell lines have suggested roles for LPA in the nervous system.

The VZ-enriched expression of lp_{A1} indicates that receptor-mediated actions of LPA may contribute to cellular phenomena associated with cortical neurogenesis. Indeed, we previously found that LPA can activate both chloride and nonspecific cation conductances to cause membrane depolarization in cortical neuroblasts (Dubin *et al.*, 1999). Here, we examine possible roles for LPA in the morphological changes associated with interkinetic nuclear migration and examine whether biologically active, extracellular LPA can be produced by cellular elements derived from the embryonic cerebral cortex.

MATERIALS AND METHODS

Materials

Oleoyl-LPA, lysophosphatidyl choline (LPC), lysophosphatidyl ethanolamine (LPE), and phosphatidic acid (PA) were from Avanti Polar Lipids. Sphingosine 1-phosphate (S1P) was from BioMol. Cell-Tak was from Collaborative Research. Opti-MEM, DMEM, and N2 supplement were from Gibco-BRL. Cresyl violet, tetramethylrhodamine isothiocyanate (TRITC)–phalloidin, 4′,6-diamidino-2-phenylindole (DAPI), anti-flag monoclonal antibody (M2), fatty acid-free bovine serum albumin (FAFBSA), and monoclonal anti-MAP2 antibody were from Sigma. Monoclonal anti-nestin antibody (RAT401) was from PharMingen. Polyclonal anti-glutamate transporter (GLAST) antiserum was from Chemicon. Bromodeoxyuridine (BrdU) labeling kit, anti-BrdU antibody, and *in situ* cell death detection kit were from Boehringer Mannheim. Biotinylated anti-mouse or guinea pig IgG antibody, FITC-labeled anti-mouse IgG antibody, FITC–avidin, and ABC kit were from Vector Labs. Cytochalasin D, colchicine, and pertussis toxin were from Calbiochem. Syto-11 was from Molecular Probes. His-C3 was prepared as described previously (Fukushima *et al.*, 1998). Y27632 was a generous gift from Welfide Pharmaceutical Industries, Saitama, Japan. Retroviruses expressing lp_{A1} were produced by transient transfection of the retroviral vector containing flag-tagged lp_{A1} into Phoenix cell lines (Dr. G. Nolan, Stanford University).

Cortical Primary Cultures

Embryos were obtained from timed-pregnant Balb/c mice (Harlan–Sprague–Dawley) with morning of vaginal plug designated embryonic day 0 (E0). Cell cluster cultures were produced essentially as described previously (Chun and Jaenisch, 1996; Dubin *et al.*, 1999). Briefly, the cerebral cortices from E12–13 mice were dissected, and the meninges were carefully removed. Tissues were transferred to culture medium (Opti-MEM containing 55 μM β-mercaptoethanol, 20 mM glucose and penicillin–streptomycin) and gently triturated with a fire-polished Pasteur pipette into small clusters (<100 cells). Cells were seeded onto glass coverslips (100–1000 clusters on 12-mm coverslips or 10–30 clusters on the center of 40-mm coverslips) precoated with Cell-Tak (2 $\mu g/cm^2$) and cultured at 37°C under 5% CO_2. For brain explant culture, brains (clipped at the caudal hindbrain or midbrain) were gently dissected from E14 mice, transferred to culture medium (DMEM with N2 supplements), and cultured at 37°C under 5% CO_2 for 14 h.

Immunocytochemistry and Histochemistry

Cultured cells were fixed with 4% paraformaldehyde (PFA) for staining with anti-nestin, anti-MAP2 (1:100), or anti-GLAST (1:500) or fixed with 70% ethanol for BrdU staining. The bound antibodies were visualized by successive incubation with biotinylated secondary IgG, ABC complex, and diaminobenzidine. In some experiments, cells fixed with PFA were subjected to fluorescent *in situ* end labeling (ISEL) labeling using an *in situ* cell death detection kit. In all staining, DAPI was used to visualize nuclei. For brain explant cultures, brains were fixed with 4% PFA for 24 h and frozen. Sagittal sections (20 μm) were cut and stained with cresyl violet or DAPI. Some sections were processed for BrdU staining as described previously (Blaschke *et al.*, 1996; Weiner and Chun, 1997).

In Situ Hybridization

In situ hybridization of cluster cultures at high stringency with DIG-labeled RNA probes of lp_{A1} was used as described previously (Weiner and Chun, 1997; Weiner *et al.*, 1998; Weiner and Chun, 1999).

Time-Lapse Video Microscopy

Time-lapse video-enhanced differential interference contrast (DIC) microscopy was used to examine cortical cells cultured on the center of 40-mm coverslips for 18–30 h. The coverslips were mounted onto a heat-controlled perfusion apparatus (BioOptiks) set at 37°C (the chamber volume was 700 μl) and observed with an inverted microscope (Axiovert 135, Carl Zeiss) using a 100× or 63× oil immersion objective lens. Clusters containing approximately 10–100 cells were selected and observed for at least 20 min to confirm that cells were healthy, and then the experiment was started. Replacement of culture medium containing 0.1% FAFBSA was manually performed with a syringe (approximately 2 ml/min).

DIC images were collected with a cooled charge-coupled device (CCD) color camera (DEI-47, Carl Zeiss). Images were collected every 15 s on a Power Macintosh G3 running Scion Image software (Scion Corp.) and Adobe Photoshop. Cluster area was determined using Scion Image software. See Fig. 3A for the determination of the percentage changes. For direct observation of nuclear movement, clusters were pretreated with 1 μM syto-11 for 5 min and subjected to time-lapse recording. Fluorescent images were collected every 1 min, as described above.

Scanning Electron Microscopy

Cultured whole brains were fixed with 3% PFA/3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for 1–3 days at 4°C. The cortical ventricular surface was exposed by cutting with tungsten microsurgery needles. The fixed brains were further treated with osmium tetroxide, dried in a critical point drying apparatus, coated with gold, and then observed with a Hitatchi S-520 scanning electron microscope.

Preparation of Conditioned Medium (CM)

Cerebral cortices were dissected from E13 mice and triturated to yield mostly clusters (for low- and medium-neuron cultures) or mostly single cells (for high-neuron cultures). Cells were plated at 0.5 cortex/10 cm^2 on Cell Tak-coated, six-well dishes and grown in culture medium (serum-free Opti-MEM containing 0.1% FAFBSA) for 3 days. For the production of low-neuron cultures, bFGF was added at 20 ng/ml for the duration of the culturing. The culture supernatant was harvested and centrifuged at 12,000*g* for 5 min, and the resulting supernatant (CM) was stored at −20°C until use. To concentrate CM, ultrafiltration membranes (Ultrafree-15 centrifugal filter units; molecular weight cutoff 10,000, Millipore) were used. Producer cells were washed 3× with PBS, scraped, and centrifuged at 1000*g* for 5 min. The pellet was resuspended in PBS containing 0.1% SDS and the protein concentration of cell extracts was determined by a protein assay kit (Bio-Rad) to normalize LPA activity of CM. In some experiments, CM was treated with phospholipase B (PLB, 2 U/ml for 24 h at 22°C) or proteinase K (50 μg/ml for 30 min at 37°C) or boiled (15 min at 100°C). At the end of the treatment with PLB or proteinase K, the enzymes were inactivated by boiling for 15 min at 100°C, and then the CM was used for a bioassay as below.

Determination of LPA Activity in CM

LPA-like activity was determined by measuring stress-fiber-forming activity in RH7777 cells expressing lp_{A1} (Fukushima *et al.*, 1998). Native RH7777 cells, which lack lp_{A1} expression and LPA responsivity, were plated on coverslips coated with Cell-Tak, infected with retroviruses expressing FLAG-tagged lp_{A1} for 18 h, and further cultured in 300 μl of serum-free medium for 1 day. An equal volume of CM was added for 30 min, and cells were fixed and double stained for FLAG and f-actin (Fukushima *et al.*, 1998). FLAG-positive cells with stress fibers were counted and the concentration of LPA-like activity in CM was estimated by comparison to a standard LPA dose–response curve (0.3–10 nM). LPA-like activity was normalized to the protein concentration of extracts of cell cultures from which each CM was collected and determined as picomole equivalents of LPA per milligram of protein of cell extracts.

Statistical Analysis

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, Berkeley, CA).

RESULTS

LPA-Dependent Effects on Cortical Neuroblast "Cluster Cultures"

We employed cluster cultures from mice at E12, an age when the cortex consists largely of neuroblasts (Weiner and Chun, 1997; Dubin *et al.*, 1999), to examine the effects of LPA on neuroblast morphology. These cultures consisted of hemispherical groups of approximately 10–100 cells, formed by gentle trituration of cortical tissue without proteolysis. Primary cluster cultures of neuroblasts have the advantage of maintaining many features observed *in vivo*, such as cell proliferation, gap junctional communication, and normal electrophysiological resting potentials (LoTurco and Kriegstein, 1991; Temple and Davis, 1994; Ghosh and Greenberg, 1995; Dubin *et al.*, 1999).

Through 30 h in culture, most cells (>85%) in clusters were positive for nestin (an intermediate filament protein expressed by neuroblasts) (Lendahl *et al.*, 1990), while only a small minority were positive for MAP2 (a marker for postmitotic neurons in the embryonic cortex and elsewhere) (Chun *et al.*, 1987) (Fig. 1A and Table 1). During the same period, the majority of cells in clusters underwent cell cycle progression, as determined by BrdU pulses and immunostaining with anti-BrdU antibody (Fig. 1B and Table 1). Consistent with our previous data (Hecht *et al.*, 1996), *in situ* hybridization demonstrated expression of lp_{A1} *in vitro* in these nestin-positive, proliferating cell clusters (Figs. 1C and 1D). Relatively few cells within clusters (but many cells present outside of the clusters) were apoptotic at 18 h (Table 1). The number of radial glial cells in the clusters was only 6.0 ± 2.5% of cells (n = 18 clusters) as identified by immunoreactivity for GLAST, which is specifically expressed by radial glial cells in the embryonic brain (Shibata *et al.*, 1997). Together, these results confirmed that most cells in cortical cluster cultures maintained under serum-free conditions were proliferative neuroblasts expressing lp_{A1}.

Time-lapse recordings of DIC images of cluster cultures demonstrated that LPA induced rapid changes in cell and cluster morphology (Fig. 2). In control cultures, the outlines of individual cells (cell margins) in clusters were distinct (Fig. 2A). Most cells possessed lamellipodial structures with a few filopodia or short processes in cells at the outer edge of the clusters (Fig. 2A, arrow). The morphology of the clusters was not changed by vehicle treatment, whereas exposure to medium containing 100 nM LPA resulted in pronounced cluster contraction. Neuroblasts at the periphery of the clusters retracted their lamellipodia, accompanied by centripetal cellular migration (toward the center of the clusters) as early as 2.5 min after LPA addition (Fig. 2B)

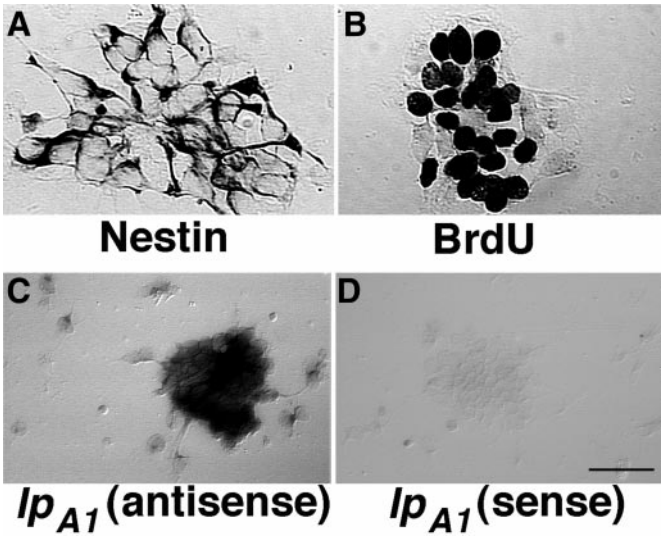

FIG. 1. Cortical clusters consist of proliferative neuroblasts expressing lp_{A1}. Cortical cluster cultures from E12 mice were maintained in serum-free medium for 18 h. (A) Nestin immunostaining. (B) BrdU immunostaining of clusters pulsed with 40 μM BrdU between 6 and 18 h after plating. (C) Lp_{A1} expression shown by *in situ* hybridization with an antisense lp_{A1} riboprobe. (D) *In situ* hybridization control with an lp_{A1} sense riboprobe. Bar, 20 μm in A and B; 32 μm in C and D.

and became more marked over time; the maximal LPA response was observed by 15 min, with complete cluster compaction. Within contracted clusters, individual cells appeared more rounded, and the contact sites between individual cells were obscured, as viewed using DIC microscopy. Such cluster compaction was maintained for at least 18 h after plating in the presence of a single exposure to 100 nM LPA stabilized with FAFBSA (data not shown). However, when LPA was removed, the LPA-induced shape changes were found to be reversible (data not shown).

Immunostaining of clusters following time-lapse observation confirmed that all cells responding with cell migration were nestin positive and MAP2 negative (data not shown). In addition to migration, neuroblasts exposed to LPA formed fine retraction fibers that were accompanied by membrane withdrawal (Fig. 2B, arrowheads). MAP2-positive cells did show morphological changes following LPA exposure, but these cells were not associated with marked cell migration (Fig. 2B, arrow). Staining of the clusters with TRITC–phalloidin revealed that LPA induced actin rearrangement in neuroblasts, especially visible around the circumference of the clusters, and that the retraction fibers consisted of f-actin (Fig. 2C vs Fig. 2D).

To provide a quantitative description of the response of cells within clusters to varying LPA concentrations, cluster areas were measured before and after LPA exposure (Fig. 3A). LPA-induced cluster compaction was dose dependent, with an estimated EC_{50} of 10–20 nM (Fig. 3B). This was similar to the EC_{50} of LPA-induced neurite retraction reported previously for cerebral cortical neuroblast cell lines endogenously expressing lp_{A1} (Chun and Jaenisch, 1996; Hecht *et al.*, 1996). Vehicle controls never produced cluster compaction, in marked contrast to that produced by LPA. For example, absence of cluster compaction in the presence of vehicle solution over a 15-min period (Fig. 2A) was unaffected by increasing the duration of vehicle exposure using a second change of vehicle solution and an additional 15 min of exposure (vehicle, $-2.2 \pm 1.3\%$ from five clusters; LPA, $10.7 \pm 3.6\%$ from four different clusters). Cells in the center of the clusters, despite being more difficult to observe individually, also responded to LPA with both cell rounding and loss of distinct cell margins (Fig. 2B), thereby contributing to the reduction in cluster area. Other structurally related lipids, including LPC, LPE, and PA, failed to reproduce the morphological effects of LPA on cluster size (Fig. 3C). S1P, another signaling lysophospholipid that acts through GPCRs homologous to LP_{A1} (Lee *et al.*, 1998; Zondag *et al.*, 1998; Chun *et al.*, 1999; Zhang *et al.*, 1999), had no significant effect on cluster area (Fig. 3C), although it appeared to be capable of affecting the lamellipodia of a small number of neuroblasts (data not shown).

LPA-Dependent Signaling Pathways in Cluster Cultures

The signaling pathways involved in LPA-induced contraction of neuroblast clusters were examined to determine their relevance both to previously reported pathways activated by LPA in neuroblastoma cell lines and to previously identified pathways activated by LP_{A1} (Fukushima *et al.*, 1998). When clusters were preincubated with cytochalasin D, a reversible inhibitor of actin polymerization, LPA treatment failed to cause significant contraction (Fig. 3D). After washout of cytochalasin D, a second challenge with

TABLE 1
Characterization of Clusters in Cortical Cultures

	% of total cells in clusters	
	18 h	30 h
BrdU+	81.1 ± 1.8 (30)	68.6 ± 5.4 (21)
Nestin+	85.1 ± 3.0 (22)	85.0 ± 2.8 (17)
MAP2+	11.6 ± 1.7 (24)	16.5 ± 2.1 (24)
ISEL	3.7 ± 0.8 (24)	ND

Note. Cortical cells were fixed at the indicated time and immunostained. For the experiments with BrdU, cells were treated with 40 μM BrdU between 6 and 18 h or 18 and 30 h after plating and fixed at the end of the treatment. Immunostained cells (BrdU, nestin, and MAP2) were counted and the percentage of total cells in clusters (of 10–100 cells) was determined. Viability within clusters was high (based on cells unlabeled by ISEL), contrasting with overall culture cell death which averaged 50%. Data are means ± SEM. (*n*), number of clusters. ND, not determined.

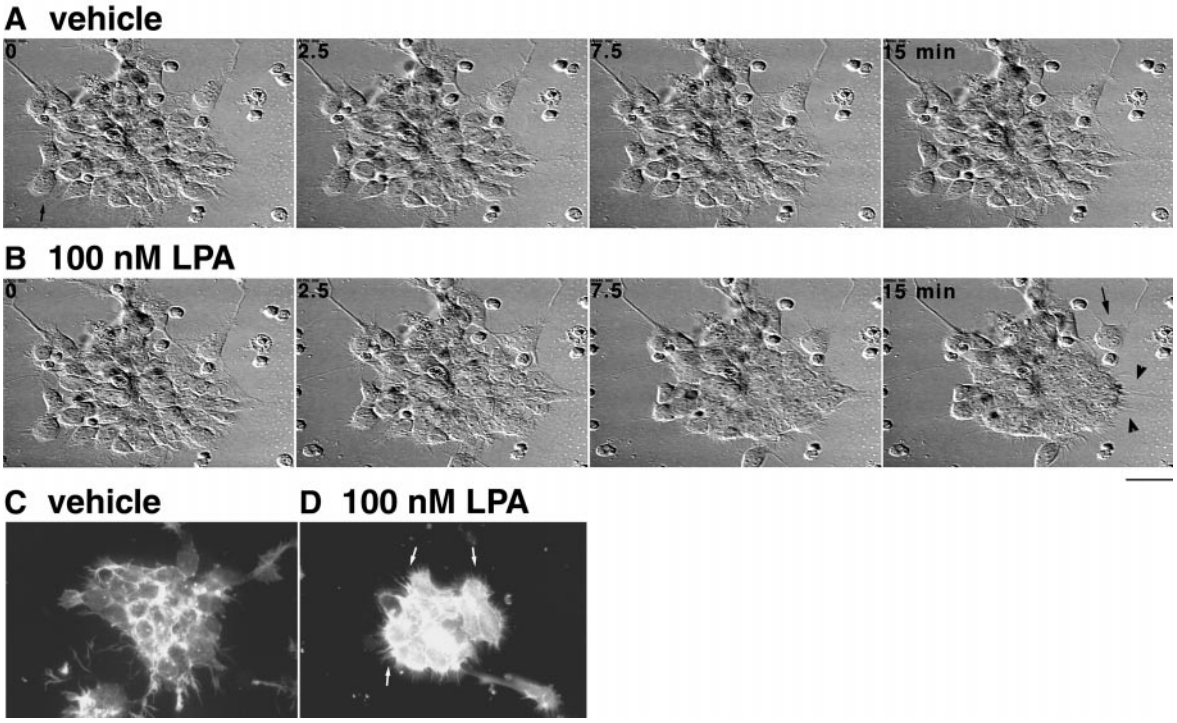

FIG. 2. Lysophosphatidic acid (LPA) causes rapid contraction of neuroblast clusters, accompanied by actin rearrangement. Cortical clusters from E12 mice were cultured in serum-free medium for 18 h and subjected to time-lapse recording. (A) Cluster treated with vehicle (fatty-acid-free bovine serum albumin). Arrow, lamellipodia with short processes. (B) Cluster treated with 100 nM LPA for 15 min. Arrow, MAP2-positive cell that showed no cell movement in response to LPA; arrowheads, retraction fibers. (C, D) F-actin staining of clusters treated with (C) vehicle or (D) 100 nM LPA for 15 min. Arrows, retraction fibers. Bar, 20 μm.

LPA again induced a significant reduction in cluster area. By contrast, colchicine, a tubulin polymerization inhibitor, did not alter the effects of LPA exposure (Fig. 3D). Pretreatment of cortical cells with PTX for 24 h, which ADP-ribosylates $G_{i/o}$ and inhibits receptor coupling, did not block LPA-induced cluster compaction (Fig. 3D). This result strongly contrasted with that observed in cells pretreated with C3 exoenzyme (his-C3 (Fukushima *et al.*, 1998)) which ADP-ribosylates and inactivates Rho and which abolished the effects of LPA on neuroblast morphology and cluster compaction (Fig. 3D). Y27632, a fast-acting Rho-associated kinase inhibitor (Uehata *et al.*, 1997; Hirose *et al.*, 1998), also inhibited LPA-induced cluster compaction. This inhibition was reversible (Fig. 3D). These results demonstrated that the Rho-to-actin pathway, but not the PTX-sensitive $G_{i/o}$ pathway, mediated LPA-induced changes.

LPA-Dependent Effects on Neuroblast Nuclear Movement

The cell cluster compaction in response to LPA included nuclear movement. This phenomenon was most pronounced for nuclei located in the cluster periphery that, following LPA exposure, moved toward the cluster center. To observe nuclear movement directly, nuclei of neuroblasts were prelabeled with syto-11, a vital fluorescent dye, and time-lapse recording was performed. As expected, LPA induced centripetal nuclear movement toward the center of the clusters (Figs. 4A and 4B, white arrows). Nuclei were observed to migrate within the cytoplasm of the neuroblast somata, independent of obvious cell migration (Fig. 4B, black arrowhead).

LPA-Dependent Effects in Brain Explant Cultures

To investigate the effects of LPA on neuroblast morphology in intact tissue, whole brains (nervous system proximal to the medulla) of E14 embryos were explanted into defined, serum-free medium with or without LPA for 14 h. The brains were then fixed, sectioned, and stained with cresyl violet. Histologically stained sections of control explants demonstrated a prominent VZ and emerging cortical plate as expected at this age (Fig. 5A). Cellular morphologies in the cortical VZ were fusiform and well organized (Figs. 5A–5C). BrdU exposure during the last hour followed by immunostaining revealed that BrdU-labeled cells were localized to the superficial region in the VZ (Fig. 5D). When LPA (1 μM; the increased concentration was used to ensure

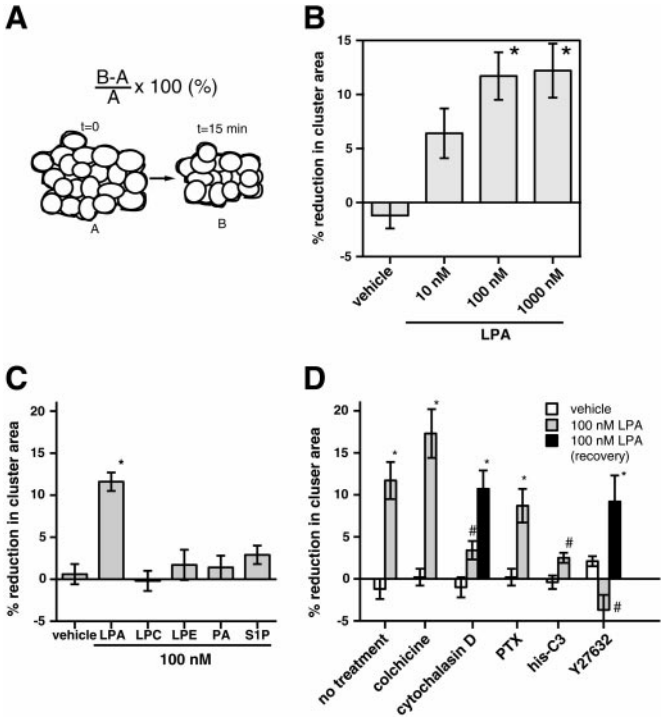

FIG. 3. Lysophosphatidic acid (LPA) induces cluster compaction through Rho-dependent actin polymerization. (A) A formula to determine the percentage reduction in cluster area as a quantitative description of cluster compaction. A and B are the cluster areas at $t = 0$ and 15 min, respectively. (B) LPA-induced reduction in cluster area. Percentage reduction in cluster area in each treatment was determined according to the formula in (A). Data are the mean ± SEM ($n = 8$). *$P < 0.05$ vs vehicle. (C) Effects of various lipids on cluster area. LPC, lysophosphatidyl choline; LPE, lysophosphatidyl ethanolamine; PA, phosphatidic acid; S1P, sphingosine 1-phosphate. Data are the mean ± SEM ($n = 3$). *$P < 0.05$ vs vehicle. (D) Effects of pharmacological inhibitors on LPA-induced morphological changes. The clusters were pretreated with specific inhibitors of cytoskeletal formation (colchicine, 10 μM, 5 min; cytochalasin D, 2 μM, 5 min) or Rho kinase (Y27632, 2 μM, 7.5 min) and then treated with 100 nM LPA for 15 min in the presence of each inhibitor. Recovery responses to 100 nM LPA were examined after 15 min washout of inhibitors by vehicle solution. For treatment with pertussis toxin (PTX, which blocks heterotrimeric G-proteins $G_{i/o}$) or his-C3 (which inactivates Rho), cultures were incubated with PTX (200 ng/ml) or his-C3 (30 μg/ml) overnight. Data are the mean ± SEM ($n = 3$–5). *$P < 0.05$ vs vehicle, #$P < 0.05$ vs LPA in no treatment.

that enough LPA could diffuse into the intact brain tissue) was present in the medium for the duration of culturing, the organization of the cerebral wall was clearly altered compared to vehicle controls. The morphology of the cells within the VZ appeared more generally "rounded" (Figs. 5F–5H). No difference in the relative position of BrdU-labeled cells was observed following LPA exposure (Fig. 5I), indicating that tissues were viable and that LPA did not affect the position of S-phase cells.

LPA-induced changes in cell morphology were further examined by scanning electron microscopy (SEM). In vehicle control cultures (Fig. 5E), cells within the VZ were radially oriented, as shown in Figs. 5A–5C and in previous reports (Seymour and Berry, 1975; LoTurco and Kriegstein, 1991). In contrast, SEM images confirmed that LPA-treated VZ cells assumed an imperfect, rounded shape with axes obliquely or horizontally oriented (Fig. 5J). In addition, many thin, short processes were observed to be emanating from the soma of these cells (Fig. 5J, arrows). The SEM results, combined with results obtained with histologically stained tissue, confirmed that exogenous LPA treatment broadly affected both the cell morphology of neuroblasts within and the overall structure of the cortical VZ.

A remarkable change observed in the VZ following LPA exposure was an increase in the number of nuclei positioned along the apical (ventricular) surface. To provide a quantitative estimate of this phenomenon, the number of nuclei in the VZ were counted in three different pial-to-ventricular zones, of constant area and section thickness, within the VZ itself from multiple brains. Zone "a" approximates the most superficial, basal region of the VZ; zone "c" approximates the most innermost, apical region of the VZ; and zone "b" approximates the intervening, medial region between a and c (Fig. 6A). In the basal and medial portions of the VZ (zones a and b, respectively), no significant difference in the number of nuclei/unit area was observed between vehicle and LPA treatment (Fig. 6B). In striking contrast, LPA-treated brains had a significant increase in the number of nuclei in the apical portion "c" compared to control brains.

Analyses of LPA Production by Cortical Cells

The *in vivo* sources of extracellular signaling LPA in the nervous system are currently unknown, despite the expectation that an endogenous source exists to interact with the expressed receptors. We thus sought to determine (1) whether cells from the embryonic cerebral cortex could produce extracellular LPA and (2), if so, the identity of those cells. Because LPA is also produced during membrane biosynthesis (van den Bosch, 1974), it was necessary to turn to a cell culture system in which hypothesized release of LPA into the medium could be discriminated from the LPA present in intracellular compartments.

To address this issue, embryonic cortical cells were cultured under three serum-free conditions, designed to produce "high-neuron" (mostly dissociated, single cells, which rapidly undergo neuronal differentiation *in vitro*), "medium-neuron" (our standard cluster cultures), or "low-neuron" cultures (our cluster cultures grown with bFGF to further inhibit neuronal differentiation). Immunostaining of these cultures with antibodies to MAP2 and nestin confirmed their enrichment: postmitotic neurons comprised ~31% of total viable cells in high-neuron cultures,

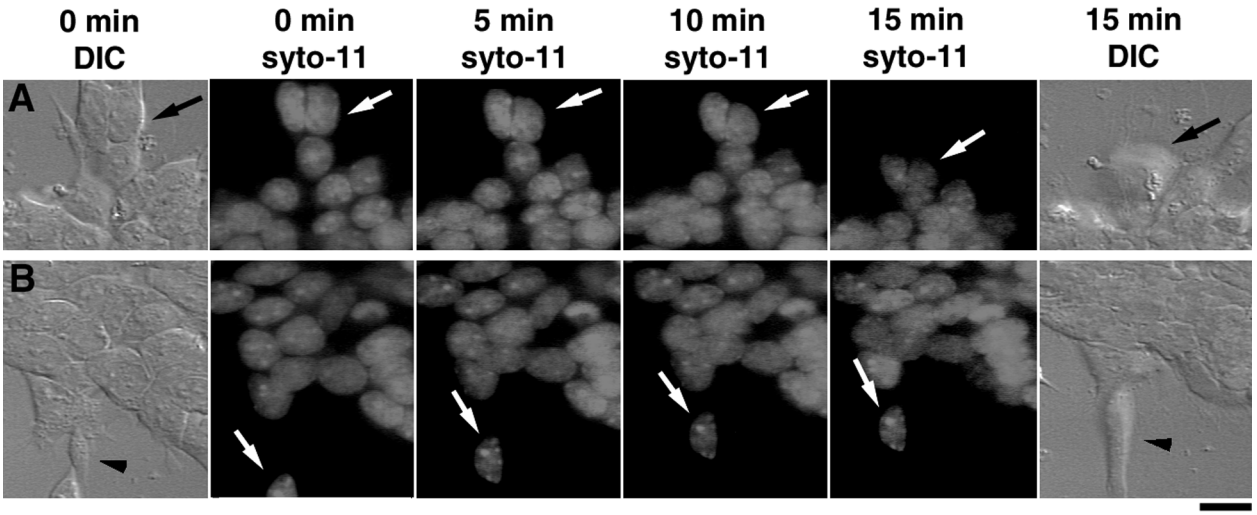

FIG. 4. Lysophosphatidic acid (LPA) induces nuclear movement. Cortical clusters from E12 mice were cultured in serum-free medium for 18 h and prelabeled with the vital dye syto-11. (A, B) Time-lapse recording of differential interference contrast (DIC) and fluorescent images of neuroblasts treated with 100 nM LPA. (A) and (B) are different areas of the same cluster. Black arrow, cell movement. Black arrowhead, nuclear movement without obvious cell movement. White arrows, nuclear movement. Bar, 10 μm.

~17% in medium-neuron cultures, and ~7% in low-neuron cultures; conversely, the neuroblasts comprised ~25% of total viable cells in high-neuron cultures, ~39% in medium-neuron cultures, and ~68% in low-neuron cultures. These values resulted in the highest postmitotic neuron:neuroblast ratio (1.23) in high-neuron cultures, an intermediate ratio (0.45) in medium-neuron cultures, and the lowest ratio (0.11) in low-neuron cultures. No significant difference in the percentage of dead cells among these cultures was observed (data not shown). CM from each of these cultures was collected after 3 days of culturing for detection of LPA-like activity.

A previously established bioassay based on heterologous expression of LPA receptors was used to identify and quantify LPA from CM. A hepatoma cell line, RH7777, was engineered to express lp_{A1}, expression of which is required to observe LPA-dependent actin stress fiber formation (Fukushima *et al.*, 1998). This bioassay is quantitative and sensitive, with a detection limit of ~0.3 nM. It is also specific, detecting the activity of LPA but not of other related phospholipids, including LPC, LPE, PA, or S1P (Fukushima *et al.*, 1998; and data not shown). In addition, it requires minimal perturbation of the medium sample (e.g., no concentration, extraction, or precipitation to recover active fractions). A LPA dose–response standard curve allowed estimation of the LPA concentrations in the CMs. Estimates of LPA from high-neuron cultures ranged from 2.4 to 6.2 nM (Fig. 7A). By contrast, CM from medium-neuron cultures produced LPA concentrations ranging from 0.8 to 2.4 nM. Little or no LPA-like activity was detected in CM from low-neuron cultures. The specific LPA-like activity (corrected for cellular proteins of the producing cultures) in the high-neuron CM was always substantially higher than that from medium- or low-neuron cultures (Fig. 7B).

The active CM was also subjected to treatment with PLB, which cleaves and inactivates LPA (Misaki and Matsumoto, 1978; Valet *et al.*, 1998), or to proteinase K digestion and/or boiling, which should eliminate proteinaceous factors but will not affect lipids such as LPA (Hecht *et al.*, 1996), followed by bioassay. PLB treatment completely removed the LPA activity from active CM, whereas other treatments had no effect (Fig. 7C). These findings, combined with the sensitivity and specificity of the bioassay based on heterologous expression of a specific LPA receptor (Fukushima *et al.*, 1998), indicated that LPA was present in CM from high-neuron cortical cultures.

We also examined whether CM was capable of inducing morphological changes in neuroblast clusters. CM was concentrated threefold and added to clusters. Time-lapse recording analyses revealed that CM induced cluster compaction equivalent to that produced by 10 nM LPA (Figs. 7D and 3B). The activity in the CM was completely lost following PLB treatment (Fig. 7D). These results demonstrated that extracellular LPA produced by high-neuron cultures could stimulate morphological changes indistinguishable from added LPA in cortical neuroblast clusters.

DISCUSSION

The data presented here show that extracellular LPA can induce morphological changes in cortical neuroblasts. This process utilizes signaling pathways that are known to be activated by the high-affinity LPA receptor, LP_{A1}. The

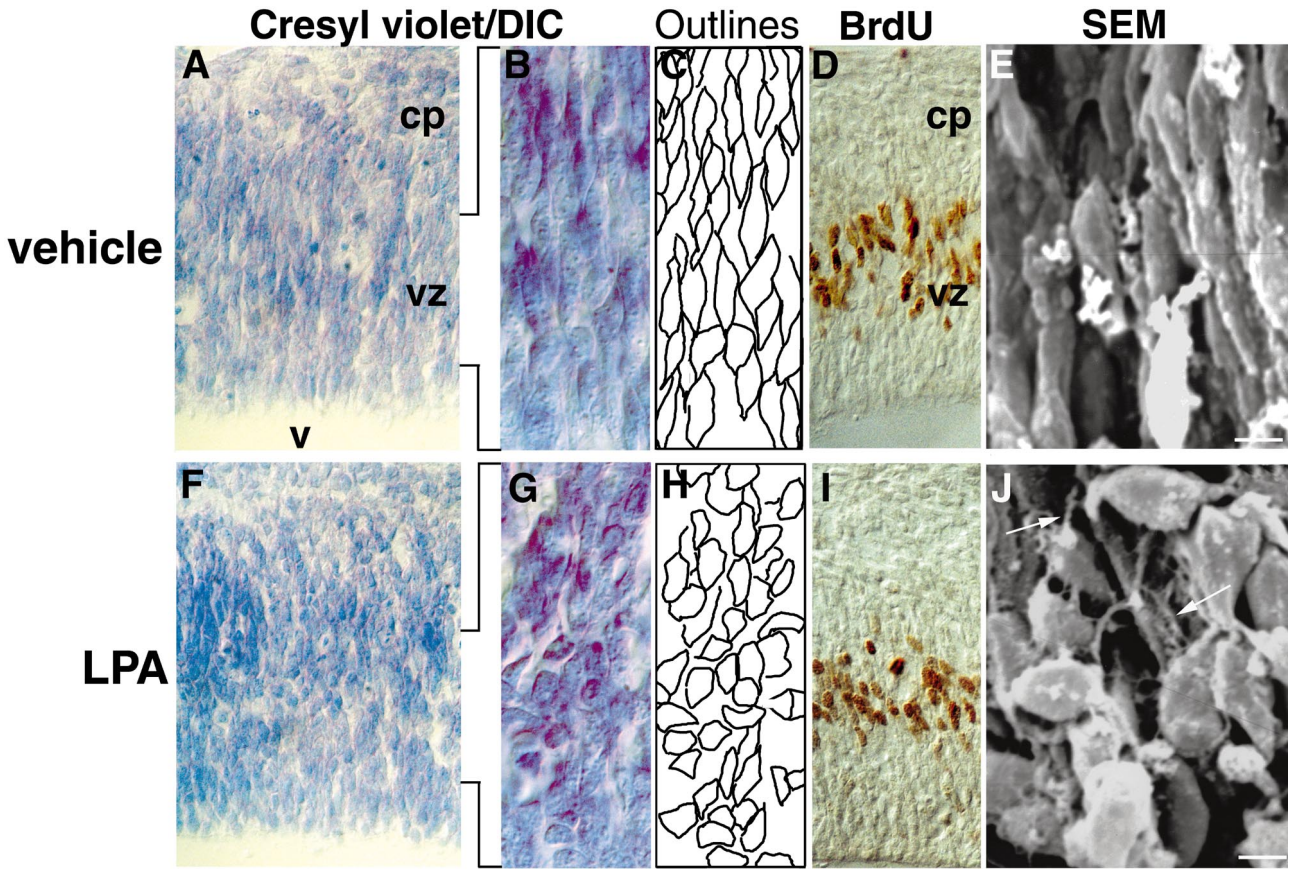

FIG. 5. Lysophosphatidic acid (LPA) induces rounded morphologies of cortical neuroblasts in E14 brain explant cultures. E14 mouse brain explants were cultured for 14 h under serum-free conditions in the absence (A–E) or presence (F–J) of 1 μM LPA and pulsed with BrdU for the last hour. (A, B, F, G) Sagittal sections of the cortex stained with cresyl violet. B and G show high magnification views of ventricular zone (VZ) neuroblasts. (C, H) Outlines of individual VZ cells in B and G, respectively. (D, I) Sagittal sections of the cortex stained for BrdU. (E, J) Scanning electron microscopy of the VZ cells. Arrows, short processes that appear to be "intramitotic filopodia." DIC, differential interference contrast; SEM, scanning electron microscopy; cp, cortical plate; vz, ventricular zone; v, ventricle. Bar, 24 μm in A, D, F, and I; 10 μm in B and G; and 3.5 μm in E and J.

presence of LPA in CM from high-neuron cultures identifies postmitotic cortical neurons as a likely endogenous source of extracellular LPA. These results are indicative of a physiological role for LPA signaling in cortical neurogenesis and additionally raise the possibility that LPA serves as an extracellular signal from postmitotic neurons to proliferating neuroblasts (Fig. 8).

Extracellular LPA Can Be Produced by Postmitotic Neurons

A requisite condition for considering receptor-mediated LPA signaling as neurobiologically relevant is identification of an endogenous source of LPA. There are several phospholipases or lipid kinases that are potentially involved in LPA production, including phospholipases A_1, A_2, and D, as well as monoacylglycerol kinase (Gaits *et al.,* 1997). Whether these enzymes function to produce extracellular LPA *in vivo*, in the nervous system or elsewhere, is currently unknown.

To determine the identity of cortical cells producing extracellular LPA, we utilized three types of cortical cultures designed to result in a range of postmitotic neuron: neuroblast ratios (high-, medium-, and low-neuron cultures). The composition of the cultures was established by assessing standard markers for proliferating neuroblasts (nestin immunoreactivity and BrdU incorporation (data not shown)) or for postmitotic neurons (MAP2 immunostaining), which revealed a greater than 10-fold difference between the ratios of high- vs low-neuron cultures (Fig. 7B). The amount of LPA-like activity in these conditioned media was positively correlated with the postmitotic neuron:neuroblast ratio such that high-neuron cultures produced the highest specific activity. No such correlation was

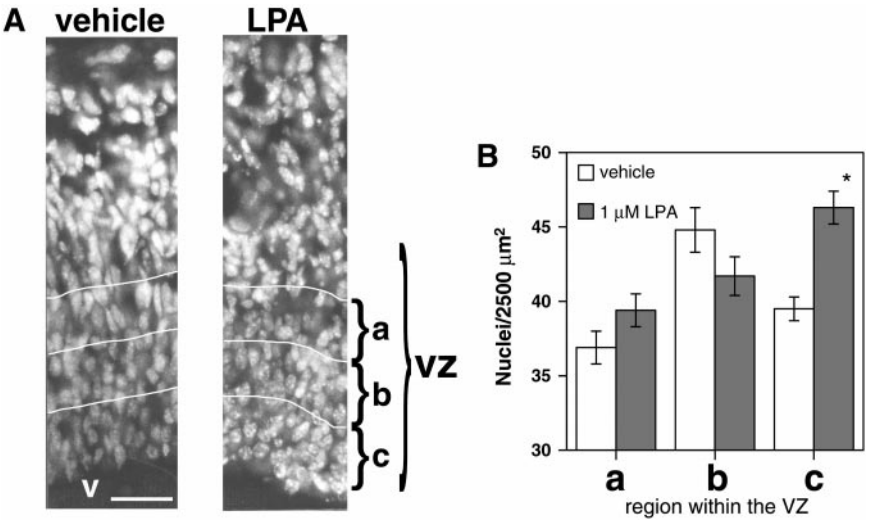

FIG. 6. Lysophosphatidic acid (LPA) affects nuclear morphology of neuroblasts and induces the accumulation of neuroblast nuclei at the apical surface of the ventricular zone (VZ). E14 mouse brain explants were cultured for 14 h in the absence or presence of 1 μM LPA. (A) Sagittal sections of the cerebral wall stained with DAPI. (B) Effects of LPA on the number of nuclei in three different regions within the VZ. The cortical VZ of the cultured brain was divided into three zones (a, b, and c; each zone has a width of 25 μm (A)) and the number of nuclei was counted in each zone (area and section thickness were held constant). Data are the mean ± SEM ($n = 8$). *$P < 0.05$ vs vehicle. Bar, 25 μm.

observed between dead cells or nonimmunostained cells. From this analysis, we conclude that postmitotic neurons can produce an extracellular LPA-like factor(s).

The identification of LPA as the active factor in CM is supported by several points. First, we used an established, sensitive bioassay utilizing cells heterologously expressing a single high-affinity LPA receptor (LP_{A1}). These cells respond specifically to LPA, but not to other related phospholipids (LPC, LPE, PA, or S1P) (Fukushima *et al.*, 1998). Use of this bioassay (Fig. 7) identified the activity in CM from high-neuron cultures as LPA. Second, consistent with this identification, the active factor (1) resisted proteinase K treatment or boiling, as previously reported for LPA (Hecht *et al.*, 1996); (2) was inactivated by PLB, which cleaves fatty acids from the 1- or 2-position of glycerol-based (e.g., LPA), but not sphingosine-based (e.g., S1P) lipids (Misaki and Matsumoto, 1978; Valet *et al.*, 1998); and (3) induced actin cytoskeletal changes indistinguishable from those produced by LPA in other cell types (neurite retraction in neuroblast cell lines; data not shown). In addition, preliminary chemical analyses by HPLC have identified oleoyl-LPA in our CM samples (data not shown). These data lead us to conclude that the active, extracellular factor released into medium conditioned by high-neuron cortical cultures is LPA.

LPA Induces Neuroblast Shape Changes That Resemble Cell Rounding of Interkinetic Nuclear Migration

LPA exposure induced morphological changes in neuroblasts, including cell rounding, membrane retraction, retraction fiber formation, and cellular and nuclear migration. Cell rounding and membrane retraction observed here are likely analogous to LPA-induced process retraction and cell rounding in immortalized neuroblast or neuroblastoma cell lines (Jalink *et al.*, 1993; Hecht *et al.*, 1996; Tigyi *et al.*, 1996; Sayas *et al.*, 1999). However, process retraction in cell lines has not been reported to be associated with the production of f-actin retraction fibers as observed here. More striking is the cellular and nuclear movement that was a prominent aspect of primary neuroblast responses to LPA exposure. These responses have not been reported previously for LPA-induced effects on neuronal cell lines, although some peripheral tumor cell lines respond to LPA with stimulation or inhibition of cell migration (Yamamura *et al.*, 1997; Sakai *et al.*, 1999). Taken together, these findings suggest that cellular responses of primary neuroblasts share some aspects of LPA signaling observed in cell lines, but also show distinct responses that likely underscore functional roles for LPA signaling *in vivo*.

Extension of these analyses to explant cultures produced consistent results supporting a role for LPA in the regulation of neuroblast shape *in vivo*. In explant cultures, LPA not only stimulates cell rounding but also increases the number of nuclei at the apical surface of the VZ where rounded cells are normally located during interkinetic nuclear migration. Because LPA has documented mitogenic properties (van Corven *et al.*, 1989; Fukushima *et al.*, 1998), one possible explanation for increases in nuclear number (nuclei in zone "c", at the ventricular surface, Fig. 6) is increased cell proliferation. However, morphological

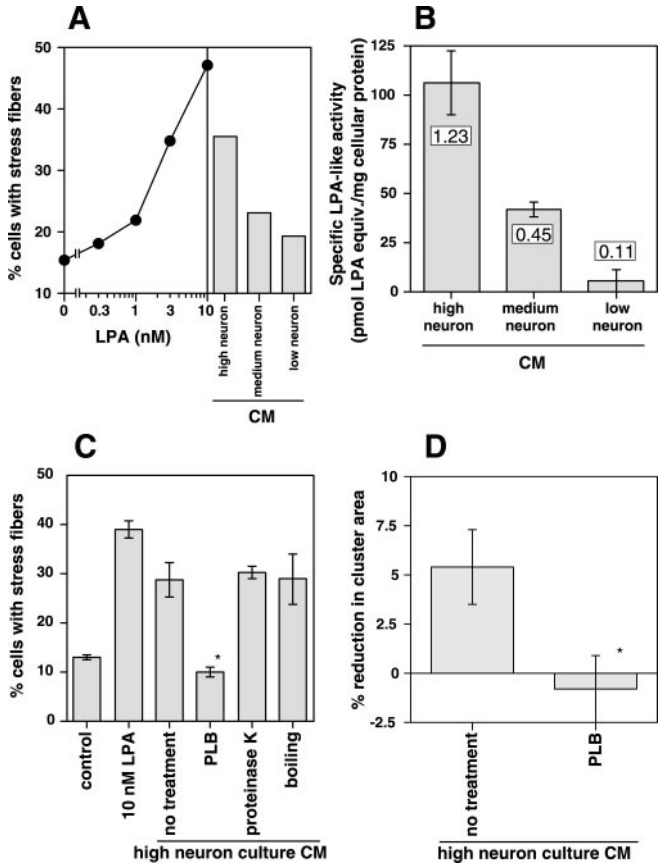

FIG. 7. Postmitotic cortical neurons can produce an LPA-like activity. (A) LPA-like activity in media conditioned by three types of cortical cultures. RH7777 cells infected with FLAG epitope-tagged lp_{A1}-expressing retroviruses were treated with 0–10 nM LPA or with conditioned medium (CM) (50% v/v) from high-, medium-, or low-neuron cultures (see text for details). Cells were fixed and stained with TRITC–phalloidin and anti-FLAG antibody, and the number of infected cells with stress fibers was determined; results from one representative experiment are shown. bFGF, present in the low-neuron CM, had no effect on stress fiber formation (data not shown). (B) Specific LPA-like activity in CM from three types of cultures. The specific activity was determined as described in the text. Data are the mean ± SEM ($n = 5$). The boxed values inside the bars are the postmitotic neuron:neuroblast ratios in the cultures from which the CM was harvested. (C) Effects of various treatments on LPA-like activity in high-neuron CM. CM was treated with phospholipase B (PLB), proteinase K, or boiling, as described in the text, and subjected to the bioassay. Data are the mean ± SEM ($n = 3$). *$P < 0.05$ vs no treatment. (D) Effects of high-neuron CM on cluster area in neuroblast cultures. Concentrated CM or PLB-treated CM was used for time-lapse analyses. Data were determined as described in the legend of Fig. 3 and are the mean ± SEM ($n = 8$). *$P < 0.05$ vs no treatment.

change is observed within 4 h of LPA treatment in explant cultures, and estimates of cell-cycle duration at the age examined in this study are approximately 14 h (Caviness *et al.*, 1995). Combined with the observation that neuroblast cluster contraction occurs within 15 min, the increase in VZ nuclei at the ventricular surface is most simply explained by cell and nuclear movement rather than increased proliferation of neuroblasts per se. The long-lasting effects of LPA producing nuclear accumulation at the ventricular surface observed in the present study are likely due to the continued presence of LPA during culturing.

The simplest biological explanation for these LPA-dependent effects is that these changes represent activation of the "rounding-up" phase of interkinetic nuclear migration (Fig. 8). During this phase, fusiform neuroblasts retract their basal process and round-up, accompanied by nuclear migration to the ventricular surface (Sauer, 1935; Berry and Rogers, 1965; Seymour and Berry, 1975). These morphological changes have been shown to require the actin cytoskeleton (Messier and Auclair, 1974). Seymour and Berry (1975) actually suggested that the "intramitotic filopodia" exhibited by neuroblasts in the rounding-up phase were retraction fibers. In the present study, the LPA-induced cellular responses observed in neuroblasts in cluster and explant cultures included cell and nuclear rounding, nuclear translocation to the ventricular surface of the VZ, retraction fiber formation, and actin cytoskeletal changes. All of these morphological changes are also observed in the rounding-up phase of interkinetic nuclear migration.

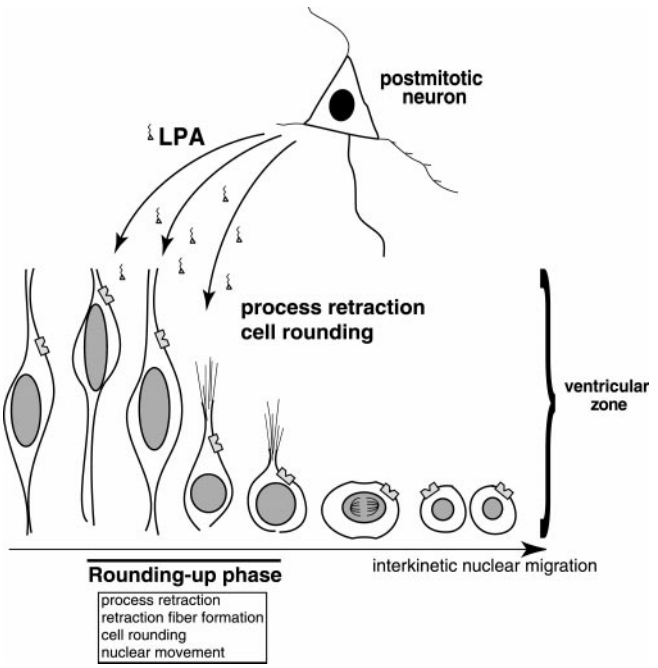

FIG. 8. Schematic model of LPA signaling in the developing cortex. Extracellular LPA produced by postmitotic neurons interacts with high-affinity LPA receptors (such as LP_{A1} or related LP family members) present on VZ neuroblasts. This activates G-protein signaling pathways leading to process retraction, cell rounding, and nuclear migration.

LPA as an Extracellular Lipid Signal from Neurons to Neuroblasts?

Combined with the previously described enriched expression LP_{A1} in the VZ (Hecht *et al.*, 1996; Chun *et al.*, 1999), and the observation in this study that LPA can be preferentially produced by postmitotic neurons (Fig. 7), we propose that LPA could serve as an extracellular signal from postmitotic neurons to proliferating neuroblasts as illustrated in Fig. 8. This model does not exclude LPA production from other cellular sources; at the earliest stages of cortical development, preceding postmitotic neuron production, other cell types such as neuroblasts themselves or perhaps overlying meningeal cells could also produce LPA. The identity of all involved LPA receptors remains to be determined, as it appears that at least one other LPA receptor, lp_{A2}, is also expressed in the VZ, as well as lp_{A1} (McGiffert *et al.*, unpublished observation). The previously observed link between cell cycle and interkinetic nuclear migration predicts that cell proliferation should be affected. However, the requirement for prolonged assays in assessing LPA-dependent cell proliferation limit the certainty that any observed effect is directly due to LPA, and for this reason, the question of LPA signaling in proliferation was not pursued in this study. Nevertheless, combined with the documented mitogenic effects of LPA (van Corven *et al.*, 1989; Fukushima *et al.*, 1998), and anti-apoptotic properties in other neural cell types (Weiner and Chun, 1999), it would be surprising if LPA signaling were not a neurogenic influence on cortical cells.

In view of its effects on neuroblasts, how might LPA signaling affect neurogenesis? Based on data presented here, LPA-induced morphological changes could potentially modify the orientation of cleavage planes of mitotic neuroblasts, as well as participate in the subcellular localization of molecules associated with this process like *numb* or *notch* (McConnell, 1995; Doe *et al.*, 1998; Jan and Jan, 2000; Redmond *et al.*, 2000) that can be associated with the actin cytoskeleton (Knoblich *et al.*, 1997; Shen *et al.*, 1998). Receptor-mediated actions of LPA also include activation of transcription via specific response elements (Fukushima *et al.*, 1998) or modification of signaling pathways activated by peptide growth factors via transactivation mechanisms (Daub *et al.*, 1996; Burrows *et al.*, 1997), all of which could influence neurogenesis. Ionic changes have been implicated in the control of neurogenesis (LoTurco *et al.*, 1995), and LPA has been shown to induce stimulation of two types of ion conductance changes in cortical neuroblasts (Dubin *et al.*, 1999). Each of these functions could potentially influence cortical neurogenesis.

In summary, our data suggest that receptor-mediated LPA signaling is a new and unanticipated influence on brain development that may in part underlie the initial, rounding-up phase of interkinetic nuclear migration and perhaps other aspects of neurogenesis. Future studies based on experimentally altering LPA signaling during development should provide additional insights into the roles for lysophospholipids during neurogenesis.

ACKNOWLEDGMENTS

We thank Ms. Carol Akita for expert laboratory assistance, Ms. Yuka Kimura for making FLAG-tagged lp_{A1} retroviruses, Mr. Lance Washington for technical assistance with the scanning electron microscope, Drs. Shuh Narumiya (Kyoto University, Kyoto, Japan) and Shu Murakami (Welfide Pharmaceutical Industries, Saitama, Japan) for generously providing Y27632, Dr. Gary Nolan (Stanford University) for the retroviral vector and Phoenix cell lines, and Drs. Joseph Gleeson, James Contos, and Dhruv Kaushal (UCSD) for critically reading the manuscript. This work was supported by the National Institute of Mental Health (J.C.), the Uehara Memorial Foundation (N.F.), and by an NRSA individual predoctoral fellowship through the NIMH (J.A.W.).

REFERENCES

Berry, M., and Rogers, A. W. (1965). The migration of neuroblasts in the developing cerebral cortex. *J. Anat.* **99,** 691–709.

Blaschke, A. J., Staley, K., and Chun, J. (1996). Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. *Development* **122,** 1165–1174.

Boulder Committee. (1970). Embryonic vertebrate central nervous system: revised terminology. *Anat. Rec.* **166,** 257–261.

Burrows, R. C., Wancio, D., Levitt, P., and Lillien, L. (1997). Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. *Neuron* **19,** 251–267.

Caviness, V. S., Jr., Takahashi, T., and Nowakowski, R. S. (1995). Numbers, time and neocortical neuronogenesis: A general developmental and evolutionary model. *Trends Neurosci.* **18,** 379–383.

Chun, J., Contos, J. J. A., and Munroe, D. (1999). A growing family of receptor genes for lysophosphatidic acid (LPA) and other lyso-phospholipids (LPs). *Cell Biochem. Biophys.* **30,** 213–242.

Chun, J., and Jaenisch, R. (1996). Clonal cell lines produced by infection of neocortical neuroblasts using multiple oncogenes transduced by retroviruses. *Mol. Cell. Neurosci.* **7,** 304–321.

Chun, J. J. M., Nakamura, M. J., and Shatz, C. J. (1987). Transient cells of the developing mammalian telencephalon are peptide-immunoreactive neurons. *Nature* **325,** 617–620.

Daub, H., Weiss, F. U., Wallasch, C., and Ullrich, A. (1996). Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379,** 557–560.

Doe, C. Q., Fuerstenberg, S., and Peng, C. Y. (1998). Neural stem cells: From fly to vertebrates. *J. Neurobiol.* **36,** 111–127.

Dubin, A., Bahnson, T., Weiner, J. A., Fukushima, N., and Chun, J. (1999). Lysophosphatidic acid (LPA) stimulates neurotransmitter-like conductance changes that precede GABA and L-glutamate in early, presumptive cortical neuroblasts. *J. Neurosci.* **19,** 1371–1381.

Fukushima, N., Ishii, I., Contos, J. A., Weiner, J. A., and Chun, J. (2001). Lysophospholipid receptors. *Annu. Rev. Pharmacol. Toxicol.* **41,** in press.

Fukushima, N., Kimura, Y., and Chun, J. (1998). A single receptor encoded by *vzg-1/lp*$_{A1}$*/edg-2* couples to G proteins and mediates multiple cellular responses to lysophosphatidic acid. *Proc. Natl. Acad. Sci. USA* **95,** 6151–6156.

Gaits, F., Fourcade, O., Le Balle, F., Gueguen, G., Gaig, B., Gassama-Diagne, A., Fauvel, J., Salles, J. P., Mauco, G., Simon, M. F., and Chap, H. (1997). Lysophosphatidic acid as a phospholipid mediator: Pathways of synthesis. *FEBS Lett.* **410,** 54–58.

Ghosh, A., and Greenberg, M. E. (1995). Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. *Neuron* **15,** 89–103.

Hecht, J. H., Weiner, J. A., Post, S. R., and Chun, J. (1996). *Ventricular zone gene-1* (*vzg-1*) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J. Cell Biol.* **135,** 1071–1083.

Hirose, M., Ishizaki, T., Watanabe, N., Uehata, M., Kranenburg, O., Moolenaar, W. H., Matsumura, F., Maekawa, M., Bito, H., and Narumiya, S. (1998). Molecular dissection of the Rho-associated protein kinase p160ROCK-regulated neurite remodeling in neuroblastoma N1E-115 cells. *J. Cell Biol.* **141,** 1625–1636.

Jalink, K., Eichholtz, T., Postma, F. R., van Corven, E. J., and Moolenaar, W. H. (1993). Lysophosphatidic acid induces neuronal shape changes via a novel, receptor-mediated signaling pathway: Similarity to thrombin action. *Cell Growth Differ.* **4,** 247–255.

Jalink, K., van Corven, E. J., Hengeveld, T., Morii, N., Narumiya, S., and Moolenaar, W. H. (1994). Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *J. Cell Biol.* **126,** 801–810.

Jan, Y.-N., and Jan, L. Y. (2000). Polarity in cell division: What frames thy fearful asymmetry? *Cell* **100,** 599–602.

Klein, R. (1994). Role of neurotrophins in mouse neuronal development. *Faseb J.* **8,** 738–744.

Knoblich, J. A., Jan, L. Y., and Jan, Y. N. (1997). The N terminus of the *Drosophila* Numb protein directs membrane association and actin-dependent asymmetric localization. *Proc. Natl. Acad. Sci. USA* **94,** 13005–13010.

Lee, M. J., Van Brocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzeleev, R., Spiegel, S., and Hla, T. (1998). Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* **279,** 1552–1555.

Lendahl, U., Zimmerman, L. B., and McKay, R. D. (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* **60,** 585–595.

LoTurco, J. J., and Kriegstein, A. R. (1991). Clusters of coupled neuroblasts in embryonic neocortex. *Science* **252,** 563–566.

LoTurco, J. J., Owens, D. F., Heath, M. J., Davis, M. B., and Kriegstein, A. R. (1995). GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron* **15,** 1287–1298.

McConnell, S. K. (1995). Constructing the cerebral cortex: Neurogenesis and fate determination. *Neuron* **15,** 761–768.

Messier, P. E., and Auclair, C. (1974). Effect of cytochalasin B on interkinetic nuclear migration in the chick embryo. *Dev. Biol.* **36,** 218–223.

Misaki, H., and Matsumoto, M. (1978). Purification of lysophospholipase of *Vibrio parahaemolyticus* and its properties. *J. Biochem.* **83,** 1395–1405.

Moolenaar, W. H. (1995). Lysophosphatidic acid signalling. *Curr. Opin. Cell Biol.* **7,** 203–210.

Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. C. (1997). Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr. Opin. Cell Biol.* **9,** 168–173.

Qian, X., Davis, A. A., Goderie, K. S., and Temple, S. (1997). FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. *Neuron* **18,** 81–93.

Redmond, L., Oh, S. R., Hicks, C., Weinmaster, G., and Ghosh, A. (2000). Nuclear Notch1 signaling and the regulation of dendritic development. *Nature Neurosci.* **3,** 30–40.

Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70,** 389–399.

Sakai, T., de la Pena, J. M., and Mosher, D. F. (1999). Synergism among lysophosphatidic acid, beta1A integrins, and epidermal growth factor or platelet-derived growth factor in mediation of cell migration. *J. Biol. Chem.* **274,** 15480–15486.

Sauer, F. C. (1935). Mitosis in the neural tube. *J. Comp. Neurol.* **62,** 377–405.

Sayas, C. L., Moreno-Flores, M. T., Avila, J., and Wandosell, F. (1999). The neurite retraction induced by lysophosphatidic acid increases Alzheimer's disease-like *Tau* phsophorylation. *J. Biol. Chem.* **274,** 37046–37052.

Seymour, R. M., and Berry, M. (1975). Scanning and transmission electron microscope studies of interkinetic nuclear migration in the cerebral vesicles of the rat. *J. Comp. Neurol.* **160,** 105–125.

Shen, C. P., Knoblich, J. A., Chan, Y. M., Jiang, M. M., Jan, L. Y., and Jan, Y. N. (1998). Miranda as a multidomain adapter linking apically localized Inscuteable and basally localized Staufen and Prospero during asymmetric cell division in *Drosophila. Genes Dev.* **12,** 1837–1846.

Shibata, T., Yamada, K., Watanabe, M., Ikenaka, K., Wada, K., Tanaka, K., and Inoue, Y. (1997). Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. *J. Neurosci.* **17,** 9212–9219.

Temple, S., and Davis, A. A. (1994). Isolated rat cortical progenitor cells are maintained in division in vitro by membrane-associated factors. *Development* **120,** 999–1008.

Tigyi, G., Fischer, D. J., Sebök, A., Yang, C., Dyer, D. L., and Miledi, R. (1996). Lysophosphatidic acid-induced neurite retraction in PC12 cells: Control by phosphoinositide-Ca^{2+} signaling and Rho. *J. Neurochem.* **66,** 537–548.

Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389,** 990–994.

Valet, P., Pages, C., Jeanneton, O., Daviaud, D., Barbe, P., Record, M., Saulnier-Blache, J. S., and Lafontan, M. (1998). Alpha2-adrenergic receptor-mediated release of lysophosphatidic acid by adipocytes: A paracrine signal for preadipocyte growth. *J. Clin. Invest.* **101,** 1431–1438.

van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T., and Moolenaar, W. H. (1989). Lysophosphatidate-induced cell proliferation: Identification and dissection of signaling pathways mediated by G proteins. *Cell* **59,** 45–54.

van den Bosch, H. (1974). Phosphoglyceride metabolism. *Annu. Rev. Biochem.* **43,** 243–277.

Weiner, J. A., and Chun, J. (1997). *Png-1,* a nervous system-specific zinc finger gene, identifies regions containing postmitotic neurons during mammalian embryonic development. *J. Comp. Neurol.* **381,** 130–142.

Weiner, J. A., and Chun, J. (1999). Schwann cell survival mediated by the signaling phospholipid lysophosphatidic acid. *Proc. Natl. Acad. Sci. USA* **96,** 5233–5238.

Weiner, J. A., Hecht, J. H., and Chun, J. (1998). Lysophosphatidic acid receptor gene *vzg-1/lp$_{A1}$/edg-2* is expressed by mature oligodendrocytes during myelination in the postnatal murine brain. *J. Comp. Neurol.* **398,** 587–598.

Yamamura, S., Yatomi, Y., Ruan, F., Sweeney, E. A., Hakomori, S., and Igarashi, Y. (1997). Sphingosine 1-phosphate regulates melanoma cell motility through a receptor-coupled extracellular action and in a pertussis toxin-insensitive manner. *Biochemistry* **36,** 10751–10759.

Zhang, G., Contos, J. J. A., Weiner, J. A., Fukushima, N., and Chun, J. (1999). Comparative analysis of three murine G-protein coupled receptors activated by sphingosine-1-phosphate. *Gene* **227,** 89–99.

Zondag, G. C., Postma, F. R., Etten, I. V., Verlaan, I., and Moolenaar, W. H. (1998). Sphingosine 1-phosphate signalling through the G-protein-coupled receptor Edg-1. *Biochem. J.* **330,** 605–609.

Received for publication July 25, 2000
Revised September 7, 2000
Accepted September 7, 2000
Published online October 25, 2000