Absence of LPA₁ Signaling Results in Defective Cortical Development

Lysophosphatidic acid (LPA) is a simple phospholipid with extracellular signaling properties mediated by specific G protein-coupled receptors. At least 2 LPA receptors, LPA₁ and LPA₂, are expressed in the developing brain, the former enriched in the neurogenic ventricular zone (VZ), suggesting a normal role in neurogenesis. Despite numerous studies reporting the effects of exogenous LPA using in vitro neural models, the first LPA1 loss-of-function mutants reported did not show gross cerebral cortical defects in the 50% that survived perinatal demise. Here, we report a role for LPA1 in cortical neural precursors resulting from analysis of a variant of a previously characterized LPA1-null mutant that arose spontaneously during colony expansion. These LPA1-null mice, termed maLPA1, exhibit almost complete perinatal viability and show a reduced VZ, altered neuronal markers, and increased cortical cell death that results in a loss of cortical layer cellularity in adults. These data support LPA₁ function in normal cortical development and suggest that the presence of genetic modifiers of LPA1 influences cerebral cortical development.

Keywords: brain development, cerebral cortex, LPA, lysophosphatidic acid, neurogenesis

Introduction

Development of the cerebral cortex requires a carefully coordinated sequence of events essential for the construction of a functional brain. Cortical cells arise mainly in the ventricular zone (VZ), a pseudostratified neuroepithelium of dividing neural progenitor cells (NPCs) lining the surface of the telencephalic lateral ventricles (Sidman et al. 1959; Bayer and Altman 1991). The cortical layers form in an inside-out sequence by migration of neurons in coordination with proliferative processes (Takahashi et al. 1999), including influences of cleavage planes and programmed cell death (Blaschke et al. 1996, 1998). Consequently, any alteration in this sequence of events could have a critical impact on normal brain development (Caviness et al. 1995; Rakic et al. 1995).

Neurogenesis is influenced by numerous factors including intrinsic mechanisms and extracellular signals such as growth factors (Reynolds et al. 1992; Qian et al. 1997; Jin et al. 2002). Of these, lysophospholipids have recently emerged as important influences on normal nervous system development (Chun et al. 2002; Anliker and Chun 2004; Chun 2005; Gardell et al. 2006). Lysophosphatidic acid (LPA) is a prominent endogenous lysoGuillermo Estivill-Torrús¹, Pedro Llebrez-Zayas¹, Elisa Matas-Rico¹, Luis Santín², Carmen Pedraza², Isabel De Diego³, Ignacio Del Arco¹, Pedro Fernández-Llebrez⁴, Jerold Chun⁵ and Fernando Rodríguez De Fonseca¹

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phospholipid that can act as an extracellular signal through specific G protein-coupled receptors, 5 of which have been identified, LPA_{1-5} (Chun et al. 2002; Anliker and Chun 2004; Ishii et al. 2004; Lee et al. 2006). LPA_1 was identified in the embryonic cortex, enriched in the VZ. LPA_1 and LPA_2 show spatio-temporally regulated gene expression, suggesting a role in regulation of cortical neurogenesis (Hecht et al. 1996; Contos and Chun 2001; Anliker and Chun 2004). Previous studies have demonstrated cortical functions for LPA receptors via exogenous delivery of LPA inducing morphophysiological changes in postmitotic neurons and cortical NPCs (Dubin et al. 1999; Lu et al. 1999; Fukushima et al. 2000, 2002; Fujiwara et al. 2003; Dash et al. 2004; Fukushima 2004).

LPA exposure in an ex vivo cortical culture increases NPC terminal mitosis, inducing cortical folding and thickening, effects which depend on LPA₁ and LPA₂ expression (Kingsbury et al. 2003, 2004). Receptor loss-of-function studies using LPA₁-null or LPA₁/LPA₂-double null mice have demonstrated ~50% perinatal lethality and, among survivors, behavioral deficits that suggest centrally mediated defects (Contos et al. 2000, 2002; Harrison et al. 2003). These mice are smaller and exhibit cranial dysmorphism and have increased apoptosis in the sciatic nerve (Contos et al. 2000, 2002; Kingsbury et al. 2003; Anliker and Chun 2004). Somewhat surprisingly, only minor abnormalities were observed in mutant cerebral cortices, with the exception of a variable reduction in embryonic cerebral wall thickness (Contos et al. 2000).

During propagation of the original mixed background strain of LPA₁-null mice (Contos et al. 2000) in our laboratories, a spontaneous, stable variant arose and led to the establishment of the LPA₁-null reported here as the "*Málaga* variant" (here referred to as "maLPA₁-null" mice). We show that their LPA₁ absence results in defects in cortical development including a reduction in proliferative populations, premature NPC maturation, and increased apoptosis. The observed alterations not only affected NPCs but also resulted in reduced adult cortical cellularity.

Material and Methods

Mice

Procedures were carried out with wild-type and maLPA₁-null heterozygous and homozygous females (on a mixed background C57Bl/6 \times 129SW) in compliance with European animal research laws (European Communities Council Directive 86/609/EEC and 2003/65/CE). A spontaneously established LPA₁-null mouse colony, termed maLPA₁-null (from *Málaga* variant of LPA₁-null), was derived from previously reported (Contos et al. 2000) during the original colony expansion by crossing heterozygous foundation parents within their original mixed background. More than 12 maLPA₁-null generations have been obtained exhibiting all the defects described in this work. MaLPA₁-null mice carrying an *lpa*₁ deletion were born at the expected Mendelian ratio and survived to adulthood exhibiting increased perinatal survival rates and absence of suckling defects when compared with original LPA₁-null mice (Supplementary Tables 1 and 2 and Supplementary Fig. 1).

For experiments, the day of the vaginal plug following mating was designated embryonic day 0.5 (E0.5). Research was performed on E11.5, E14.5, E15.5, E16.5, and E18.5 embryos, perinatal pups (postnatal day 0-P7), and 12-week-old male mice always obtained from heterozygous × heterozygous/homozygous maLPA₁-null mating and genotyped for *lpa*₁ deletion by polymerase chain reaction (Contos et al. 2000; see Supplementary Fig. 2). LPA₁ absence was also confirmed by immuno-histochemistry on embryonic cerebral cortices (Supplementary Fig. 2).

Immunobistochemistry

Experiments were performed from E11.5 to neonatal stages. At least 12 embryos from each age per genotype were used. Brains were dissected out into cold phosphate buffer, fixed in Bouin's solution overnight at room temperature, washed and embedded in paraffin. All steps were carried out at room temperature. Sections (10 µm) were first treated for 10 min with 0.1 M phosphate buffered saline (PBS) pH 7.4 containing 10% methanol and 10% hydrogen peroxide to inactivate endogenous peroxidase. After washing, they were exposed to anti-proliferating cell nuclear antigen (PCNA; Sigma, St Louis, MO), anti-\beta-tubulin-III (Promega Co., Madison, WI), anti-GAP43 (Sigma) mouse monoclonal or anti-phospho-histone-H3 (Upstate, Lake Placid, NY), anti-parvalbumin (Swant, Bellinzona, Switzerland), anti-Tbr2 (Abcam, Cambridge, UK), and anti-LPA1 (Affinity Bioreagents, Golden, CO) rabbit polyclonal antibodies for 18 h. Standardized detection used biotin-conjugated rabbit anti-mouse or swine anti-rabbit (as appropriate) immunoglobulins (Ig) (DakoCytomation, Glostrup, Denmark), ExtrAvidin-peroxidase (Sigma), and diaminobenzidine (DAB) (Sigma). All the antibodies were used according to the manufacturer's instructions and diluted in PBS containing 0.5% Triton X-100 and normal serum (rabbit or swine serum, depending on the source of the secondary reagent used). Omission of the primary antibody resulted in no detectable staining. Sections were counterstained with hematoxylin when required. Parallel sections of some brains were stained with hematoxylin-eosin for a general histological description.

For the analysis of phospho-histone-H3-positive cells, 4 sections per embryo and 8 embryos for each genotype were analyzed and at least 200 cells in the proliferative VZ/subventricular zone (SVZ) in each sectioned brain were assessed. At each section, cells were estimated from a 150 μ m × 150 μ m bin comprising the cortical VZ/SVZ. Percentages were expressed from the total hematoxylin counted cells.

In Situ Hybridization

In situ hybridization was carried out on E14.5 embryos. At least 8 embryos per genotype were used. Brains were dissected into cold phosphate buffer and fixed overnight in 4% paraformaldehyde at 4 °C and kept at 4 °C until dehydration in methanol/PBT (phosphate buffered saline, PBS, with 0.1% Tween 20) (PBS with 0.1% Tween 20). After rehydration, the telencephalon was embedded in a gelatin/ albumin mixture and cut 200 µm thick sections with a vibroslicer (Campden Instruments, Loughborough, UK). Finally, they were dehydrated in methanol/PBT and kept at -20 °C overnight. In situ hybridization used a digoxygenin-labeled riboprobe transcribed from a Pax6 cDNA clone (kind gift from D. Price, University of Edinburgh). TAG-1 (kindly provided by M. Wassef, Ecole Normale Superieure, Paris, and D. Karagogeos, University of Crete; see Supplementary Fig. 4) was produced as previously described (Denaxa et al. 2001). Probes were labeled with digoxigenin-uridine triphosphate (UTP) using commercial kits (Roche, Mannheim, Germany; Promega), and hybridization was performed at 70 °C, using a water bath, on previously rehydrated sections. An anti-digoxigenin alkaline phosphatase-conjugated antibody

(Roche; 1:2000) and NBT/BCIP (supplied as a solution of 18.75 mg/ml NBT [Nitro blue tetrazolium chloride] and 9.4 mg/ml BCIP [5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt] in 67% DMSO (v/v); Roche) were used for probe detection.

Cortical Precursor Cultures

NPCs from E14.5 cortices were enzymatically dissociated by incubation for 45 min at 37 °C in Earle's buffered salts solution containing 20 units/ml papain (Papain Dissociation System, Worthington Biochemical, Lakewood, NJ) and cultured as described by Estivill-Torrus et al. 2002). Briefly, cells in suspension were plated onto poly-L-lysine-coated wells (LabTek II, Nunc, Rochester, NY) at a density of 1.5×10^5 cells/ cm² and cultured in serum-free Dulbecco's modified Eagle's medium (Sigma) at 37 °C and 5% CO₂.

Cultures were analyzed at various times after plating (12.75-24 h, see below) and each time-point was repeated in 4 separate experiments. Viable cells were identified on the basis of morphology and Trypan Blue exclusion. For cell counts, 800-1500 viable cells per culture were assessed in 6 randomly selected microscope fields. Eight embryos were used per genotype.

To obtain the labeling index, 12 h after plating, bromodeoxyuridine (BrdU) was added to the culture medium (final concentration 10 μ M) for 45 min and then removed and fresh culture medium added. Cells were fixed in 4% paraformaldehyde either immediately after the pulse of BrdU, that is, 12.75 h after plating or at 18, 24, and 36 h after plating and processed as formerly reported (Estivill-Torrús et al. 2002). Detection of BrdU utilized a monoclonal anti-BrdU antibody (Sigma). Immunocytochemistry was carried out at room temperature as follows. Cultures were treated with 90% methanol prior to incubation in blocking solution (PBS containing 0.5% Triton X-100 and 2.5% normal rabbit serum). Cells were then exposed to the primary antibody for 18 h and subsequently processed with biotin-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark) and ExtrAvidin-peroxidase (Sigma). Reactions were visualized with DAB (Sigma).

Other immunocytochemical reactions were performed similarly using anti-p27^{kip1} (BD Biosciences, Franklin Lake, NJ), anti-PCNA (Sigma), anti-Cyclin A (Sigma), anti- β -tubulin-III (Promega), anti-MAP2 (Sigma), anti-GFAP (Sigma), or anti-CNPase (Sigma) monoclonal antibodies. All primary antibodies were diluted in blocking solution. For dual-labeling experiments, cultures were processed for the second staining using alkaline phosphatase conjugated rabbit anti-mouse, following standard procedures and visualized with NBT/BCIP (Roche). Percentages of labeled cells were expressed from the total cells as identified by phase contrast or histological conventional staining. Cell death was also analyzed using a DeadEnd Fluorometric Apoptosis Detection System (Promega) following the manufacturer's instructions.

BrdU Labeling for the Analysis of Migration Patterning

Pregnant dams were given a single injection of BrdU (70 µg/g in sterile saline intraperitoneally) on E14.5. Embryos were removed 45 min and 12 h later or on E18.5. After dissection, brains were fixed overnight in 4% paraformaldehyde at 4 °C, embedded in wax, serially sectioned coronally at 10 µm, mounted and immunoreacted to reveal BrdU labeling, as described (Gillies and Price 1993). BrdU cells were sorted into densely labeled cells, in which more than half of the nucleus is labeled and lightly labeled cells, which have lighter nuclear labeling, indicating that they had undergone more than 1 round of division after labeling. Telencephalic wall was analyzed in depth in nonadjacent coronal sections taken at the middle cortical portion. At each section, 100 µm wide radial strips were divided into different bins corresponding to cortical layering (see Fig. 5) and the position of each labeled cell assigned to a bin to generate histograms of average cell density against depth. The estimations combined results from 8 sections per embryo and 8 embryos per genotype. In each bin, the number of labeled cells is expressed as a percentage of the total number of labeled cells in the strip and means were compared statistically.

Western Blot Analysis

Eight embryos per genotype were decapitated and telencephalic hemispheres dissected out and frozen sectioned. After scraping out layer I, upper and middle regions comprising cortical layer II/III were accurately dissected and homogenized in lysis buffer containing 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, 250 mM sucrose, 1mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 µg/ml pepstatin, pH 7.4. The homogenate was centrifuged for 10 min at 900 \times g and the supernatant spun for 20 min at 75 000 \times g. The pellet was resuspended in the lysis buffer containing 1% Triton X-100 and subsequently centrifuged for 10 min at 14 000 \times g to eliminate the insoluble material. Samples containing 25 µg of protein determined by Bradford reactive (Sigma) were loaded in Laemli buffer and separated on 10% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis. Proteins were electrotransferred to polyvinylidene difluoride membranes at 100 V for 1 h. Nonspecific binding was blocked by 1-h incubation in PBS containing 5% nonfat dry milk and 0.1% Tween 20. Membranes were then exhaustively washed in PBS and incubated overnight at 4 °C in mouse monoclonal anti-GAP43 (Sigma) diluted in blocking buffer. Immunoreaction was visualized with standard procedures using a biotin-labeled rabbit anti-mouse antibody, ExtrAvidinperoxidase (Sigma), and 4-cloronaftol. Omission of the primary antibody resulted in no specific staining. Optical density measurement for each band was estimated using OptiQuant v.4.00 software (Packard Instruments Co. Downers Grove, IL) after membrane scanning.

Apoptosis Detection

Estimation of apoptosis was performed for E11.5 embryos and P7 mice. Mice (8 per genotype and age) were transcardially perfused in 4% paraformaldehyde. Brains were dissected out, fixed overnight in the same solution at 4 °C, and cryosectioned. Coronal cortical sections of 30 μ m were obtained and mounted onto poly-L-lysine treated slides. Cell death was detected using a DeadEnd Fluorometric Apoptosis Detection System (Promega) following the manufacturer's instructions. Counterstain used ethidium bromide, and cell number estimations were measured on nonadjacent coronal sections from the middle cortical portion according to the cited bin scheme.

Stereological Studies

The numerical density of neurons in the primary motor cortex of 12week-old mice was determined by the optical disector method. Six animals per genotype were transcardially perfused with 4% paraformaldehyde and brains dissected out and overnight fixed at 4 °C. Analysis was performed on free-floating cryosections immunostained for a monoclonal mouse anti-NeuN (Chemicon, Temecula, CA) in accordance with the mentioned protocol, with minor modifications. After mounting, 7 sections of 30 µm equally spaced along the rostral-to-caudal primary motor cortex extension were used per animal. Cortical layers were delineated according to the criteria of Paxinos and Flanking. A random set of 25-70 sampling optical disectors was generated for each layer on each section using the C.A.S.T. Grid (Olympus, Albertslund, Denmark). Each optical disector was a 43.4 μ m × 43.4 μ m sampling frame with exclusion lines. Counting was set starting at 5 µm below the surface and considering focused labeled neurons through the 15-µm section optical plane. The number of cells per unit of volume (N_v) was calculated for each animal, as the number of applied disectors multiplied by V_{dis} ; V_{dis} = $Sd \times Hd$, where Sd = area of the disector grid (counting frame) and Hd = depth of disector. Data were analyzed statistically.

Mice Sample Size, Statistical Analysis, and Image Acquisition

Most experiments used at least 8 embryos per stage and genotype. No significant differences were found using different or same litters. Histological and densitometric analyses were tested for significance using the Student *t*-test. Stereological studies were analyzed using Kruskal-Wallis 1-way analysis of variance (ANOVA) on ranks and compared by post hoc analysis. In vitro data were compared by ANOVA (significance assigned when P < 0.05). Immunostaining and fluorescence were analyzed under an Olympus BX51 microscope equipped with UPLSAPO ×4 0.16 NA, ×10 0.4 NA, ×20 0.75 NA, ×40 0.90 NA, or ×100 1.40 NA objectives and an Olympus DP70 digital camera. Black/white image of cultured NPCs was acquired using a Canon Powershot digital camera coupled to a Leica DM IL inverted microscope equipped with ×10 0.20 NA or ×40 0.50 NA objectives. All pictures were analyzed using Adobe-Photoshop 7.0 software without modifying the content of the micrograph.

Results

Absence of LPA₁ Generates Anomalous Cortical Proliferative Patterning

In the original LPA₁-null mice, exon 3, which contains the majority of the coding region of the murine lpa_1 gene, is completely deleted (Contos et al. 2000). In accordance with their origin, maLPA₁-null mice show reduced size and body mass, craniofacial defects, including shorter snouts and wider-spaced eyes, and reduced brain volume and mass (Fig. 1, *A-D*). Likewise, maLPA₁-null mice cortical NPCs did not cluster in vitro after addition of LPA as wild type (Fig. 4 *A*).

MaLPA1-null mice reaching adulthood showed olfactory bulbs that were reduced in size compared with wild-type littermates and detectable changes in the shape of the cerebral hemispheres, appearing more compact and spherical (Fig. 1C). The gross morphology of subcortical structures, including the brainstem and cerebellum, were comparatively unaffected. Further analysis of the cortex of maLPA₁-null mice at different developmental ages revealed additional abnormalities that had not previously been described in the original LPA1 null. By E18.5, wild-type and maLPA₁-null mice could be distinguished based on gross structure of brain and snout (Fig. 1E). Coronal sections of E18.5 embryos demonstrated a slight reduction in brain size and cortical thickness (Fig. 1F) that by P0 appeared as a more condensed cortex with somewhat diffuse boundaries between different layers (Fig. 1G). Mutant cortices displayed a marked reduction in layer width and an altered cellularity, particularly at deeper levels. In some cases, disturbances in the cortical surface could be observed (arrow in Fig. 1G).

During brain development, expression of LPA₁ is enriched in the VZ synchronized with the neurogenic period (Hecht et al. 1996), and gain-of-function studies have shown NPCs are responsive to LPA signaling. To determine whether cortical defects in maLPA₁-null mice involve deficiencies in normal proliferation of NPCs, we analyzed neuronogenic parameters in the cortex from the start of cortical neurogenesis at E11.5 through the closing stages at E18.5 (Caviness et al. 1995). Immunostaining was performed using antibodies specific to PCNA, present in continually cycling NPCs (Ino and Chiba 2000) and to neuronspecific class III beta-tubulin, an early indicator of postmitotic neuronal differentiation (Menezes and Luskin 1994).

During early corticogenesis, the wild-type cerebral wall was composed almost entirely of PCNA-labeled NPCs forming the germinative VZ (Fig. 2A,G). Mature neurons reached the upper level to form the initial preplate consisting of a thin layer of tangentially oriented cells and fibers, together with the radially oriented processes of underlying VZ cells (Fig. 2B, J). At this stage, maLPA1-null embryos displayed a minor reduction of PCNA expression (Fig. 2E,I) and, most significantly, early and ectopic expression of class III beta-tubulin in the VZ, including some cells lining the ventricular surface (Fig. 2F,L). By E14.5 the developing wild-type cortex had a laminar structure comprising the proliferative layers (VZ and SVZ), an intermediate zone (IZ) and the cortical plate (CP) containing differentiating neurons. Similarly, coronal sections of E14.5 wild-type brain showed a telencephalic wall delineated by PCNA-labeled NPCs forming the germinative layer (Fig. 2M,S) and a complementary upper layer of cells expressing neuronal beta-tubulin forming the IZ/CP levels (Fig. 2N,V). By contrast, and corresponding to the above observations, maLPA1-null mice had a clearly smaller



Figure 1. Gross anatomy and cerebral morphological defects in maLPA₁-null mice. (A) Three-month-old homozygous malpa₁^{-/-} mice are smaller in size and exhibit craniofacial deformities and shortened snouts when compared with wild-type malpa1+ + littermates. Note the spherical and reduced aspect of the mutant head. (B) Total body mass estimations (according to Contos et al. 2000) for male (left) and female (right) malpa₁^(-/-) mice (\bullet) and malpa₁^(+/-) littermates (\hat{x}) from malpa₁^{+/-}×malpa₁⁺ matings. Data are expressed as means \pm standard deviation (n = 14). *P, 0.05 (repeated measures were analyzed with ANOVA). Mass data for malpa₁^{+/} littermates that overlapped those shown for malpa $_1^{+/-}$ are not presented for clarity. (C) Dorsal and side views of 3-month-old wild-type, malpa $_1^{+/-}$ and malpa₁^{-/-} brains, showing that deleting lpa_1 results in a rounding and reduction of telencephalic vesicles and olfactory bulb (arrowhead). (D) Brain wet weight (means ± standard error of the mean [SEM]; n = 14) at cited age for male , and control ma/pa, + / + littermates. *P, 0.05 (analyzed with ANOVA). Significant differences were detected between maLPA1-null brain and their wildmalpa. ` mice, ma*lpa*₁⁺ type and heterozygous counterparts. (E) Hematoxylin-eosin stained sagittal sections of E18.5 wild-type and maLPA1-null littermates showing similar gross anatomy. (F) Histological coronal brain sections stained with hematoxylin showing the reduction in size and cortical thickness of the maLPA1-null brain (right panel) when compared with wild-type control (at left) at E18.5. (G) Sections showing normal cortical thickness and 6-layered cortex from P0 wild-type mice and equivalent for maLPA1-null brain. Cortical layers, as deduced from cell size and packing density, are indicated by Roman numerals I through VI. Undersized telencephalic wall from maLPA1-null mice exhibits a reduced layer I and there is no clear layering at deeper levels. Arrow (G, right) indicates cortical surface disruption. Scale bars in (F) represent 600 µm, whereas in (G) they represent 100 µm.

cortex by E14.5 and displayed a strongly reduced PCNA expression (Fig. 2Q,U). The reduction of the proliferative area was coupled with a proportional increase in beta-tubulin-III expression (Fig. $2R_{,X}$). Furthermore, most maLPA₁-null NPCs showed increased numbers of beta-tubulin-III positive cells in the putative VZ/SVZ, and many atypical fibers above this region running parallel to the ventricular surface (arrow and arrowhead, respectively, in Fig. 2X). Heterozygous embryos showed a graded pattern of immunostaining between that observed for wild-type and homozygous genotypes (see middle column in Fig. 2). Defects in cortical proliferation/differentiation patterning affected, in similar terms and proportions, both cerebral hemispheres throughout the rostral-caudal axis and including the olfactory bulb. Further analysis of mitotic activity was performed using an antibody against the phosphorylated form of histone H3 (anti-phospho-H3) that identifies mitotic chromosome condensation (Hendzel et al. 1997). Phospho-H3 immunolabeling at E14.5 revealed a marked decrease (32%) in the number of mitotic VZ cells in the maLPA1-null cortices, as compared with wild-type controls (P = 0.01; Fig. 3A,B) that correlated with the reduction in the proliferative PCNA-stained population. Parallel analysis of abventricular mitosis in SVZ

showed similar reduced phospho-H3 immunolabeling in the absence of LPA1 (Fig. 3B). No ectopic phospho-H3 staining was found in the maLPA1-null cortex. Previous studies have shown the transcription factor Pax6 to be involved in the regulation of early neural proliferation in the developing brain (Warren et al. 1999; Estivill-Torrús et al. 2002), where the sequential expression of Pax6 and Tbr2 transcription factors leads the differentiation of NPCs into intermediate SVZ progenitors (Englund et al. 2005; Quinn et al. 2007). To see if the observed changes in the proliferative region correlated with an altered pattern of Pax6 or Tbr2 expression, they were analyzed by in situ hybridization and immunohistochemistry in coronal brain sections at similar embryonic ages. During early neurogenesis, dorsal telencephalic expression of Pax6 was restricted to the VZ/SVZ, where it was expressed by most NPCs (Walther and Gruss 1991) and strongly delimited the germinative region at E14.5 (Fig. 3C). Examination of E14.5 maLPA1-null cortex showed that the region expressing Pax6 was noticeably reduced (Fig. 3C, right panel) reflecting the observed PCNA immunostaining pattern. Similarly, the region exhibiting premature expression of beta-tubulin-III lacked Pax6, consistent with the loss of progenitor identity. At similar age, Tbr2 is known



Figure 2. Cortical proliferative patterning is affected in mice lacking LPA₁. Expression patterns of PCNA and beta-tubulin-III in E11.5 sagittal (A-L) and E14.5 coronal (M-X) sections from the cerebral cortex of wild-type (A, B, G, J, M, N, S, and V), ma/pa₁ heterozygous (C, D, H, K, O, P, T, and W) and ma/pa₁-null (E, F, I, L, O, R, U, and X) embryos. Images are false color-converted from originally obtained. By E11.5 the absence of Ipa_1 resulted in a slight reduction of PCNA immunoreactive area (I) and early expression of beta-tubulin-III in VZ cells (arrows in K and L), predominantly next to the ventricular surface. At E14.5, an anomalous germinative layering resulted when the receptor was absent in comparison with wild-type patterning, demonstrated by a reduction of PCNA in VZ/SVZ and a premature strong expression of beta-tubulin-III in that area (arrows in W and X). At this age beta-tubulin-III immunodetection showed a disturbance in cortical radial organization with fibers running parallel to ventricular surface (arrowheads in W and X). Broken lines delimit marker expression. Depicted squares (A-F, M-R) are magnified 10× in the next images; cp, cortical plate; pp, preplate. Scale bars in (A-F), 250 µm; (G-L), 55 µm; (M-R), 300 µm;

to be expressed in both basal SVZ progenitors undergoing abventricular mitosis, and some early postmitotic neurons delimiting the SVZ and in close association to Pax6-expressing cells underneath (Englund et al. 2005; Quinn et al. 2007; see Fig. 3D). According to a decrease of the PCNA-positive presumed VZ/SVZ of maLPA₁-null mice, it would be also expected a reduction in the region expressing Tbr2. Interestingly, with the slight variations, we did not found significant differences in Tbr2 expression in the maLPA₁-null embryonic cortex (Fig. 3D). Because the acquirement of intermediate progenitor SVZ cell identity is associated with upregulation of Tbr2 and downregulation of Pax6 (Englund et al. 2005), our results suggest an early cell-cycle exit into the acquisition of partial SVZ identity.

In vitro proliferative behavior of NPCs isolated from wild-type and maLPA₁-null dissociated dorsal cortices support these differences in proliferation and maturation cellular patterning. No differences were found in survival or cell death during the experiment. BrdU labeling experiments showed fewer BrdU-labeled NPCs from maLPA₁-null mice (Fig. 4*A*,*D*) accompanied by a reduction in the proportion of cells containing the nuclear protein Cyclin A (Fig. 4*B*,*E*), which is selectively expressed during the S phase of the cell cycle (Darzynkiewicz et al. 1996), and a correlative increase in the proportion of cells expressing $p27^{Kip1}$ (Fig. 4*C*,*F*), a cyclin-dependent kinase inhibitor required for VZ neural cells to exit the cell-cycle and start terminal differentiation (Van Lookeren-Campagne and Gill 1998)

An explanation of this anomalous proliferative patterning accompanied with early expression of neuronal and differentiation markers may well be a modification in the mode of cell division. Asymmetric cell divisions correlate with neurogenesis



Figure 3. Absence of LPA₁ signaling alters the proportion of mitosis and Pax6 expression area. (*A*) Mitotic figures detected in E14.5 cortical coronal VZ by immunolabeling with anti-phospho-H3 for wild-type (wt) and maLPA₁-null (null) mice. (*B*) Histogram depicts further analysis of cross-sections showing the percentage of phospho-H3-stained cells from the total and the significant decrease in the proportion of mitosis observed for heterozygous and homozygous maLPA₁-null embryos (*t*-test, n = 8; **P = 0.01). Plots represent VZ and SVZ (abventricular) percentages. (*C*, *D*) Coronal brain sections (normal and magnified squares) from E14.5 wild-type and homozygous mutant littermates processed for in situ hybridization for *Pax6* expression (*C*) and Tbr2 immunostaining (*D*). The loss of LPA₁ generates a VZ/SVZ showing reduction of *Pax6* expression and exhibiting remarkable levels of Tbr2 expression (*D*). Scale bars in (*A*), 45 µm; (*C*, *D*) 400 µm.

and frequently take place with a horizontal orientation division (Chenn and McConnell 1995; Haydar et al. 2003). In this sense, abventricular mitosis associated with the horizontal mode of division in SVZ (Stricker et al. 2006) was not significantly increased in the maLPA₁-null cerebral cortex (Fig. 3B). To obtain a more comprehensive representation of the cortical cell division pattern, we quantified the mitotic cleavage orientations within VZ at different stages of neurogenesis. Coronal crosssections from embryonic brains were stained with hematoxylin, which clearly identifies cells in late anaphase and telophase and facilities the observation of the cleavage plane. Sections were cut through the full rostral-caudal extent of the cortex, but no significant effect on the incidence of division was found in the different regions. The number of divisions was counted and classified by plane-of-division orientation, either vertical, horizontal, or oblique, as previously described (Chenn and McConnell 1995; Estivill-Torrús et al. 2002) (Supplementary Fig. 3A). The analysis of cleavage orientations revealed a remarkable increase in the proportion of horizontal divisions in the cortex of LPA1-null embryos through cortical development (Supplementary Fig. 3B) indicating a premature conversion in the mode of cell division of LPA₁-null VZ as compared with wild type.

maLPA_T Null Mutant Reveals Defects in Cortical Formation

To characterize the function of LPA₁ in the developing cortex, we examined maLPA₁-null cortices for defects that could

influence postnatal cortical organization. First, since early cytoarchitectural analysis (Fig. 1G) suggested cortical layer defects in maLPA1-null mice, we studied the migratory behavior of NPCs using a BrdU pulse at E14.5 to label cortical cells born at that time and examined their distribution at E18.5. The percentage of labeled cells in each location were given from the total number of exclusively labeled cells, giving an estimation of migration (Gillies and Price 1993). Immediately after the BrdU pulse, both wild-type and maLPA₁-null E14.5 embryos showed qualitatively similar labeling patterns with the totality of labeled cells located in the VZ/SVZ area (data not shown). Twelve hours after BrdU administration, maLPA1-null mutants showed few labeled cells in the IZ (Fig. 5A-C), contrasting with wild-type cortices, where most BrdU-labeled cells covered twothirds of the telencephalic wall with a significant proportion of labeled cells in the IZ. By the end of the neurogenic period (E18.5), both wild-type and maLPA1-null embryos had a considerable number of E14.5-born cells (densely labeled) in the most superficial layers of the telencephalic wall (Fig. 5D-F), indicating that cells are capable of migration in the absence of LPA1. However, further quantification of BrdU-labeled cells throughout the cortical wall demonstrated that a considerable number of both densely and lightly labeled cells accumulated in the deeper layers of the maLPA₁-null cortex (Fig. $5E_{,}F_{)}$, initially suggesting a positioning defect.

To determine the abnormalities in NPC migration and subsequent differentiation, additional analysis was performed. From



Figure 4. In vitro analysis of maLPA₁-null cortical precursors. (*A*) Histograms representing the percentages of BrdU-positive cells from the total after a 45-min pulse given 12 h after plating. Significant reduction in BrdU incorporation (P < 0.05; ANOVA tested) was observed between cultures of wild-type and maLPA₁-null cells at all time-points. *Significance versus wild-type control; **significance between null homozygous and heterozygous NPC cultures. (*B*, *C*) Percentages of cells expressing Cyclin A (*B*) and p27^{kip1} (*C*) throughout culture period. Wild-type colls showed no significant changes in the number of cells expressing Cyclin A and a small increase in p27^{kip1} expression over the culture period. By contrast, maLPA₁-null NPCs display a significant (P < 0.05) decrease in the percentage of cells expressing Cyclin A and a substantial increase in p27^{kip1} expressing cells 24 h after plating. (*D*-*F*) Immunocytochemically detected differences in BrdU labeling (*D*), Cyclin A expression (*E*) and p27^{kip1} (*F*) in wild-type (left panel) and maLPA₁-null (right panel) NPC cultures 18 h after plating. Specific staining is detected as brown label with beta-tubulin-III counterstaining (blue label). Scale bars in (4). 20 um and (*G*-*I*). 25 um.

E14.5 and during cortical development, ganglionic eminences are the major source of cortical interneurons, including parvalbumin-positive interneuron subtypes which travel to their final destination in the cortex via tangential migration (Xu et al. 2004; Wonders and Anderson 2005). A cumulative labeling of BrdU-positive cells at deeper layers could well be explained by an anomalous incorporation of neurons from tangential pathways. Immunohistochemical analysis for GAP43-a protein expressed in early development that is involved in axonal pathfinding, neurotransmitter release, and synaptic plasticity (Jacobson et al. 1986)-and in situ hybridization for axonal TAG-1, expressed in developing nervous system (Yamamoto et al. 1986), showed no alterations in axonal and tangential pathways when LPA1 was absent (Supplementary Fig. (4A-H). By contrast, parvalbumin-positive neurons were reduced in the adult maLPA1-null cortex, suggesting local defects in their cortical destination rather than migration. This reduction affected particularly deep layers (Supplementary Fig. 41-L) which tend normally to be enriched in parvalbumin-expressing neurons (Xu et al. 2004; Wonders and Anderson 2005).

Despite normal patterning during early stages of cortical development, the adult expression of the growth-associated protein GAP43 revealed a noticeable defect in the postnatal maLPA₁-null cortex. Coronal sections from P0 cortices showed GAP43 expression throughout the cortical layers in wild-type cortex (Fig. 6*A*). By contrast, maLPA₁-null cortices displayed less GAP43 immunoreactivity, with almost no labeling in cortical layer II (Fig. 6*B*), suggesting a defective neuronal differentiation. The neuronal identity of this GAP43-negative cortical population was confirmed with immunodetection of beta-tubulin-III (Fig. 6*C*,*D*). Analysis of upper cortical areas by western blot corroborated the immunohistochemical data. Figure 6*E* shows

a typical western blot from dissected cortical samples from wild-type and maLPA₁-null P0 mice stained for GAP43. Immunoreactivity mainly detected a single protein band of approximately 46 kDa. Densitometric quantitation of band intensities showed a significant (n = 8, P < 0.001) reduction in GAP43 protein levels in the maLPA₁-null layer II cortex (Fig. 6*F*).

Taken together, these findings indicate that early embryonic maLPA₁-null defects in proliferation would affect normal differentiation and later cortical formation.

Increased Cortical Apoptosis and Neuronal Reduction

Previously reported receptor-mediated LPA effects on cortical folding and thickness demonstrated an antiapoptotic role for LPA signaling (Ye et al. 2002; Kingsbury et al. 2003, 2004). A possible corollary of this function would be increased apoptosis following loss of LPA receptor signaling that could explain the smaller maLPA1-null brain. Detection of apoptosis was performed at E15.5, after the peak in cell death in embryonic cortex, using a fluorometric apoptosis detection system based on a modified terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling. A more than 2-fold increase in the percentage of apoptotic nuclei was clearly and consistently detected in maLPA1-null cortices when compared with wild type, predominantly at SVZ level (Fig. 7A,D,G). This embryonic cell death is distinct from that occurring among mature neurons associated with synaptogenesis (Blaschke et al. 1998). To test whether the altered pattern of the plasticity protein GAP43 and altered differentiation correlates with later apoptotic processes, postnatal analysis was performed. Increased apoptosis was detected in maLPA1-null cortex in both upper cortical areas, layers II/III (Fig. 7*E*), and the deeper layers V/VI (Fig. 7*F*) compared with wild-type levels (Fig. 7B,C). Quantitative analysis revealed that



Figure 5. Cortical cells accumulate at deeper CP layers in maLPA₁-null mice. Contribution of neural precursor migration in cortical layering was analyzed by examining the patterns of BrdU labeling in the E18.5 cortex (middle position) after embryos were BrdU injected on E14.5. Data were analyzed at 12 h after injection (A-C) and by E18.5 (D-F). (A, D) Coronal cortical sections of E14.5 (A) and E18.5 (D) wild-type (wt) and maLPA₁-null (null) embryos immunostained for BrdU. E18.5 sections were counted in 100 µm width radial strips further subdivided by depth, comprising the CP and IZ. Boundaries between layers are indicated by broken lines; MZ, marginal zone. (B, F) Percentages of dense (B, E) and light (C, F) labeled cells in each cortical bin (75 × 100 µm) are represented against depth. Graphs show the average densities (\pm SEM) of BrdU-labeled cells. In contrast with the wild-type pattern, the proportion from both densely and lightly labeled cells in maLPA₁-null cortex (null) is dramatically reduced in the IZ 12 h after the single-BrdU pulse. By E18.5, maLPA₁-null mice show a significant increase in BrdU-labeled cells (n = 8; **P < 0.001) in those bins corresponding to deeper levels of the cortical area immediately past the IZ, as compared with wild-type pattern. Abbreviations as previously mentioned. Scale bar in (A, D), 100 µm.

the percentage of cell death was significantly higher in maLPA₁-null cortex, particularly in deeper cortical levels (Fig. 7*G*).

To verify whether the decrease in cerebral cortex size was due to neuronal loss, NeuN-positive neurons from primary motor cortex were counted through its rostral-caudal extension (Fig. 8A). The numerical density of neurons (N_v) in the primary motor cortex was determined in wild-type and maLPA1 heterozygous and homozygous 12-week-old mice using the optical dissector method (West 1993). Neuronal cell types were clearly identifiable with NeuN labeling (Fig. 8B). In coronal sections, the primary motor cortex was characterized by a prominent layer V, with a relatively low density of large pyramidal neurons, and a narrow layer IV, sparsely populated by granular neurons (Fig. 8C, left panel). In the absence of the LPA₁ receptor, the cerebral cortex was thinner than normal but cortical layers were usually similarly definable although less precise than normal (Fig. 8C, panel at right). The $N_{\rm v}$ of neurons did not differ significantly among groups in layers I and IV (Table 1), although maLPA1-null mice exhibited a higher density in layer I. However, the N_v in layers II/III, V, and VI was significantly lower in LPA1-null motor cortex compared with wild type, demonstrating that LPA₁dependent cell reduction is markedly different at deeper cortical levels, in agreement with early neuronal defects and cell death data. In addition, post hoc comparisons showed significant differences between heterozygous and homozygous animals in layer V and between heterozygous and wild type in

layers II/III and VI. These results indicate that LPA₁ is required for maintenance of normal cortical cell number.

Discussion

Our results show that LPA1-mediated signaling is important during normal embryonic cortical neurogenesis, revealed by a new LPA₁ null-mutant variant (maLPA₁) derived from the original (Contos et al. 2000) after breeding and expansion of the colony. The absence of LPA1 in the maLPA1-null mice results in a reduction of cortical NPCs, increased apoptosis, and alterations in the formation of the cerebral cortex. Earlier gain-of-function studies were essential to understand LPA receptor-mediated mechanisms, particularly in combination with mice lacking LPA1 and LPA₂ (Kingsbury et al. 2003, 2004); however, these original null mutants have not themselves shown major cortical phenotypes (Contos et al. 2000, 2002). Thus, the main finding of this study is that there is an essential in vivo requirement of LPA₁ during cortical development, as shown through the spontaneous generation of an LPA1-null variant. In vivo observations in the developing maLPA₁-null cortex showed a reduced proliferative zone with premature expression of neuronal markers in the VZ/SVZ and IZ. Cortical maLPA1-null NPCs isolated in culture displayed reduced proliferation and a relative overproduction of differentiated cells, supporting the changes reported previously (Kingsbury et al. 2003; Rehen et al. 2006) and in this



Figure 6. Cortical maldevelopment in maLPA₁-null mice results in GAP43 deficiency. (*A*, *B*) Coronal sections of P0 wild-type (*A*) and maLPA₁-null (*B*) cortex immunostained for GAP43. The insert is magnified $3 \times$ from each section and illustrates the reduction in immunoreactivity in maLPA₁-null superficial layers relative to wild type. (*C*, *D*) Beta-tubulin-III immunostaining at corresponding levels in *A* and *B*. Cortical layering is indicated by Roman numerals. Scale bar in (*A*, *B*), 300 µm; (*C*, *D*), 175 µm. (*E*) A representative western blot of GAP43 in P0 wild-type and maLPA₁-null samples, specially in area 1 corresponding to the upper medial-dorsal portion of cortical layer II. (*F*) Densitometric analysis of band intensities confirmed the reduced expression in the absence of LPA₁, almost absent in area 1 (t-test, n = 8, as separated experiments; **wt1 vs. null1, P < 0.001; *wt2 vs. null2, P < 0.001).

study in vivo. These data indicate that these effects are intrinsic to the cortical cells rather than secondary indirect effects.

In the VZ, LPA released from postmitotic neurons may induce the "rounding up" phase of interkinetic nuclear migration through cytoskeletal changes (Fukushima et al. 2000, 2002; Fukushima 2004). Accordingly, an absence of LPA₁ could be expected to result in an increase in the proportion of fusiform neurons, consistent with the presence of misfated maLPA₁-null cells, as was observed in this study. A concomitant reduction in mitotic figures was also observed. Furthermore, LPA signaling affects the normal development of the SVZ, by virtue of the clear effects on NPCs. The proportions of symmetric nonterminal, symmetric terminal, and asymmetric cell divisions overlap during the entire developmental period (Cai et al. 2002; Noctor et al. 2004). Our studies here presented describe an increase in the proportions of apical NPCs exhibiting horizontal cleavage plane in absence of LPA1 receptor. Even though the orientation of the cleavage plane is an insufficient criterion to predict the mode of division, oblique and horizontal cleavage plane are still the most likely to produce asymmetric divisions by generating unequal separation of apical components (Haydar et al. 2003; Götz and Huttner 2005; Buchman and Tsai 2007). It has been recently demonstrated that LPA-induced enhancement of neuronal differentiation via LPA1 is mediated by its interaction with G protein subunit Gi/o (Fukushima et al. 2007). Interestingly, overexpression of Gi subunit has been suggested to result in mitotic spindle destabilization (Du and Macara 2004). Last studies on VZ/SVZ division have called attention to intermediate SVZ progenitors division of which mostly generates 2 neurons in symmetrical way in neurogenic phase (Haydar et al. 2003; Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004; Götz and Huttner 2005; Kriegstein et al. 2006). In view of that, the observed preponderance of horizontal cleavages in maLPA1-null apical NPCs would correlate with a subsequent increase of intermediate SVZ progenitors undergoing mitosis. However, and quite opposite, abventricular division is not significantly increased in favor of early maturation and apoptosis. In this sense, we should consider as a possible explanation the apparent changes in cell polarity and restructuring of apical/basal processes (as seen by the increase in horizontally oriented fusiform cells) because of their relevance in the government of cell division and fate determination (Miyata et al. 2004; Götz and Huttner 2005; Cappello et al. 2006; Buchman and Tsai 2007). Thus, LPA1-induced deficient proliferation and morphological changes, not mutually exclusive, would lead to an anomalous incomplete asymmetry which produces mispositioned premature VZ and SVZ cells and early apoptosis as immediate consequences.

Programmed cell death plays an important role in cortical development (Blaschke et al. 1996, 1998) and the increased apoptosis in maLPA₁-null embryos agrees with the proposed role for LPA in neuronal survival and normal cortical formation (Ye et al. 2002; Kingsbury et al. 2003, 2004). From our results, the effect of LPA1 absence would result as a cooperative summation of altered proliferation and apoptosis. Although in vitro and in vivo observations have showed consistency respecting the effect of LPA absence on survival/apoptosis, its consequences in proliferation continues under some discrepancies because LPA have been demonstrated to increase the number of mitotic cells without increasing proliferation by itself (Kingsbury et al. 2003, 2004). Significantly, maLPA₁-null mice exhibit a decrease of the number of mitoses in both ventricular and abventricular location. However, it seems difficult to determinate how this reduction may influence directly the final apoptosis rate rather than be this a secondary effect attributable to an early postmitotic state, especially considering that neuroproliferative apoptosis does not become evident until differentiating postmitotic cells are present (Blaschke et al. 1998).

As above mentioned, SVZ cells play an important role in the maintenance of cortical expansion, and novel regulators are now being known to influence the fate of apical VZ NPCs into SVZ, acquiring division and fate determinants of SVZ progenitors (Cappello et al. 2006). LPA would suitably be included



Figure 7. Cortical apoptosis is increased in maLPA₁-null mice. (*A*–*F*) Representative photographs of E15.5 (*A*, *D*) and P7 (*B*, *C*, *E*, *F*) cortical coronal sections from wild-type (wt, *A*–*C*) and maLPA₁-null (null, *D*–*F*) mice showing labeled nuclei with DeadEnd Colorimetric Apoptosis Detection System in E15.5 cortical (*A*, *D*) and P7 cortical layers II/III (*B*, *E*) and VI (*C*, *F*) at the dorsal-medial level of the cortex. (*G*) Graph shows the quantified percentage of labeled nuclei for mentioned cortical areas. Although significant differences between genotypes are observed for both cortical areas, note the remarkable increase in the proportion of apoptotic nuclei at developmental age E15.5 and in postnatal deeper cortical regions (**P* < 0.005 for comparison wt vs. null for layer II/III, *P* < 0.001 when tested in E15.5 cortex and cortical layer VI, *t*-test, *n* = 8). Scale bars, 150 µm (*A*, *D*) and 110 µm (*B*, *C*, *E*, *F*).

among these because it has been proposed to be released by postmitotic neurons and influence the nuclear organization, morphology, and normal migration of VZ NPCs (Fukushima et al. 2000, 2002). Both, in vitro LPA addition and LPA₁ absence generate a direct effect onto subsequent SVZ organization and maturation. However, whereas LPA produces in vitro a displacement of mitotic cells into SVZ (Kingsbury et al. 2003, 2004; Rehen et al. 2006) and morphological changes in cell processes (Fukushima et al. 2000, 2002; Rehen et al. 2006) not in discrepancy with a concomitant postmitotic LPA release on demand of NPCs ratio (Fukushima et al. 2000), the deletion of LPA₁-signaling pathway in maLPA₁-null mice would result in the real blockage of this postmitotic control on VZ NPCs generating a failure of cell cycle and a reduction in proliferation with the subsequent cited effects.

During normal embryonic development, differences in NPC proliferation may underlie the variations in laminar thickness in the cerebral cortex (Caviness et al. 1995; Rakic 1995). Both cellintrinsic and-extrinsic factors contribute to changes in cell production affecting cortical growth and neuron survival. The genetic and molecular mechanisms underlying these processes are not completely understood. Recently, it has been demonstrated that extrinsic cues in the VZ environment of NPCs are able to restrict their fate (Campbell 2005). The NPC alterations described here in the maLPA₁-null mice provide evidence for the in vivo participation of LPA₁ signaling in these neurogenic processes, particularly those concerning maturing and apoptosis.

In addition to abnormal proliferation and survival, many maLPA₁-null forebrain neurons appear to arrest in the deeper cortical layers rather than migrating to more superficial sites, consistent with the initial abnormal positioning of mutant cells and increased postnatal apoptosis in layers V/VI. Whereas lpa_1 is enriched in the VZ, lpa_2 is enriched in the embryonic postmitotic regions at the end of neurogenesis (Hecht et al. 1996; Contos and Chun 2001). Later in development, partial compensation by LPA₂ (Contos and Chun 2001) may rescue some of these mispositioned neurons postnatally, giving rise



Figure 8. Stereological analysis of cerebral cortex for neuronal quantification in maLPA₁ null mice. (*A*, *B*) Representative sections (30 µm thick) of immunostaining against the neuron-specific protein NeuN throughout the cerebral hemispheres of homozygous maLPA₁-null adult littermates. Sections illustrate the rostral (*A*, left image) and more caudal (*A*, at right) sections through the primary motor cortex in the maLPA₁-null mice wherein the lamination is still definable to demarcate the layers. Predicted bregma is given. Similar sections were considered in wild-type and heterozygous mice. Boxes show the measured regions. (*B*) Representative image of layer V from the previous picture. White arrowheads show neurons positive for NeuN immunolabeling. (*C*) Photomicrographs of the primary motor cortex (NeuN immunostained) showing the pattern of usual cortical lamination in the wild-type and maLPA₁-null mice. Cortical layers indicated by Roman numerals I through VI. Scale bars in (*A*, *B*), 1mm; (*C*), 200 µm; (*D*), 25 µm.

to the abnormal but discernible conventionally layered cortex seen in the maLPA₁ null, although this remains to be determined experimentally. Conversely, analysis of axonal and tangential cortical pathways as well as parvalbumin-positive cell location suggested intriguing local defects in their destination rather than migration. Further analysis would be necessary to discriminate precisely a failure in cells to reach their destination from an impaired normal differentiation or the occurrence of both combined defects.

The reduced perinatal expression of GAP43 in the maLPA₁null cortex suggests LPA₁-dependent sequels linked to the function of GAP43, such as synapse formation and neural plasticity and the development of normal serotoninergic in-

Table 1

Neuronal quantification in control and $maLPA_1\mbox{-null}$ mice

Motor cortex

Genotype	malpa1 ^{+/+}	malpa1 ^{+/-}	ma <i>lpa</i> 1 ^{-/-}
Layer I Layer II/III Layer IV Layer V Layer VI	$\begin{array}{r} 30.9 \ \pm \ 2.21 \\ 250 \ \pm \ 30.4 \\ 215 \ \pm \ 6.16 \\ 148 \ \pm \ 3.79 \\ 228 \ \pm \ 7.65 \end{array}$	$\begin{array}{c} 24.6 \ \pm \ 17.7 \\ 172 \ \pm \ 8.02* \\ 185 \ \pm \ 18.6 \\ 136 \ \pm \ 9.58 \\ 184 \ \pm \ 2.28* \end{array}$	$\begin{array}{rrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrr$

Estimation of the numerical density of neurons (N_V , neurons/mm3; expressed as the mean \pm SEM) in individual layers of the primary motor regions of the cerebral cortex for wild-type (ma/pa1^(+/-)), heterozygous (ma/pa1^(+/-)), and homozygous (ma/pa1^(-/-)), 12-week-old mice. n = 6.

*P < 0.05.

**P < 0.005 as compared with wild-type group.

***P < 0.05 as compared with heterozygous group.

nervation in the cortex (Donovan et al. 2002); thus, anomalous synaptic connectivity could well result in both wiring defects and increased apoptosis. Further insights into the consequences of these cortical defects on brain function are necessary.

The new maLPA₁-null variant demonstrates a role for LPA₁ in normal cortical development and for maintaining normal cohorts of NPCs that was not seen in the original null mutant. The cortical abnormalities of the maLPA₁-null variant support the existence of unidentified, brain-specific LPA-signaling genetic modifiers, in view of the otherwise shared phenotype of maLPA₁-null and the original LPA₁-null mutants.

The reason for the modified phenotype of the original LPA₁null mice that appeared in Málaga is not known. This, that is, the presence of unknown modifiers on a single mutant allele, is a common fact in studies addressing the spontaneous variance of mutant phenotypes. Variant mutant phenotypes are not uncommon, as documented by major influences of background strain (Sibilia and Wagner 1995). Well-known examples that affect the brain include caspase-deficient animals as well as the Orl allele of reeler (Goffinet 1990; Rice and Curran 2001; Leonard et al. 2002; Nadeau 2002; Bergren et al. 2005). In both these cases, and the vast majority of reported strain-dependent differences, the full range of modifiers is not known. Genetic modifiers, such as other genes, viruses, retrotransposons, etc, may contribute to the new phenotype and identifying them exceed the aims of the present studies. However, because maLPA1-null mice have allowed the establishment of a highly penetrant and reproducible phenotype, this should serve as a starting point toward understanding and identifying interacting genes or nongene elements in future studies.

These findings may have relevance to developmental disorders of the central nervous system, such as dysregulation of apoptosis, microcephaly, and mental health (Haydar et al. 2000; Kadota et al. 2002; Bérubé et al. 2005). These data also add to a growing number of lysophospholipid-dependent functions that impact on normal organism development and function. It will thus be of interest to compare the more pronounced phenotype of maLPA₁-null mice with the phenotypic defects related to psychiatric diseases observed in nonvariant mutants (Harrison et al. 2003).

Supplementary Material

Supplementary material can be found at: http://www.cercor. oxfordjournals.org/.

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