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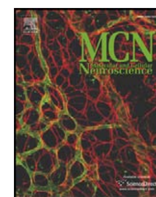

Deletion of lysophosphatidic acid receptor LPA_1 reduces neurogenesis in the mouse dentate gyrus

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ABSTRACT

Neurogenesis persists in certain regions of the adult brain including the subgranular zone of the hippocampal dentate gyrus wherein its regulation is essential, particularly in relation to learning, stress and modulation of mood. Lysophosphatidic acid (LPA) is an extracellular signaling phospholipid with important neural regulatory properties mediated by specific G protein-coupled receptors, $LPA_{1–5}$. LPA_1 is highly expressed in the developing neurogenic ventricular zone wherein it is required for normal embryonic neurogenesis, and, by extension may play a role in adult neurogenesis as well. By means of the analyses of a variant of the original LPA_1-null mutant mouse, termed the Malaga variant or "maLPA_1-null," which has recently been reported to have defective neurogenesis within the embryonic cerebral cortex, we report here a role for LPA_1 in adult hippocampal neurogenesis. Proliferation, differentiation and survival of newly formed neurons are defective in the absence of LPA_1 under normal conditions and following exposure to enriched environment and voluntary exercise. Furthermore, analysis of trophic factors in maLPA_1-null mice demonstrated alterations in brain-derived neurotrophic factor and insulin growth factor 1 levels after enrichment and exercise. Morphological analyses of doublecortin positive cells revealed the anomalous prevalence of bipolar cells in the subgranular zone, supporting the operation of LPA_1 signaling pathways in normal proliferation, maturation and differentiation of neuronal precursors.

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Introduction

It is well accepted that the vertebrate brain continues to produce new neurons throughout adulthood (Altman and Bayer, 1990; Eriksson et al., 1998; Alvarez-Buylla et al., 2002; Gage, 2002). Most of these cells are produced in the subgranular zone of the dentate gyrus (DG) in the hippocampus, with an estimated 9000 (rat) or 800–1600 (mouse) new cells generated per day (Cameron and McKay, 2001; Christie and Cameron, 2006). Neuronal DG progenitors proliferate, migrate, and differentiate into granular neurons and subsequently integrate into the hippocampal circuitry wherein they become functional. Though controversial (Leuner et al., 2006), numerous studies have provided evidence that newly formed neurons are significantly involved in cognition (Macklis, 2001; Shors et al., 2001; van Praag et al., 2002; Shors, 2004; Aimone et al., 2006). Accordingly, the clinical importance of this hippocampal neurogenesis becomes manifest in age-related deficiencies (West, 1993a; Persson et al., 2006), neural damage (Kuhn et al., 2001) or psychiatric disorders (Eisch and Nestler, 2002; Silva et al., 2006).

The survival of newly generated DG neurons is known to be positively modulated by a variety of external factors including learning tasks (Gould et al., 1999; Dobrossy et al., 2003), environmental enrichment (Kempermann et al., 1997; Olson et al., 2006) and exercise (van Praag et al., 1999a,b; Eadie et al., 2005). Postnatal neural progenitor cells are influenced by similar regulatory gene cascades and growth factors to those that control proliferation and differentiation during development (Vaccarino et al., 2001; Esposito et al., 2005). Identified neuronogenic molecules like IGF-I (Åberg et al., 2003; Sun et al., 2005), Sonic Hedgehog (Ahn and Joyner 2005), EGF or FGF-2

Abbreviations: BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; DCX, doublecortin; DG, dentate gyrus; GCL, granular cell layer; GFAP, glial fibrillary acidic protein; IGF-I, insulin growth factor 1; LPA, lysophosphatidic acid; ML, molecular layer; NeuN, neuron-specific nuclear protein; NGF, nerve growth factor; SGZ, subgranular zone; PSA-NCAM, polysialylated neuronal cell adhesion molecule.

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(Kuhn et al., 1997) and many environmental niche factors (Gage, 2002; Alvarez-Borda et al., 2004; Fabel et al., 2003; Battista et al., 2006) influence the overall process, while others are currently being elucidated.

Lysophospholids have recently emerged as important influences on normal nervous system development (Chun et al., 2002; Anliker and Chun, 2004; Moolenar et al., 2004; Chun, 2005, 2007). Lysophosphatidic acid (LPA) is a simple phospholipid that can act as an extracellular signal through at least 5 specific G protein-coupled receptors, LPA_{1-5} (Chun et al., 2002; Anliker and Chun 2004; Ishii et al., 2004; Lee et al., 2006). During development, LPA_1 is expressed in neural progenitor cells suggesting a regulatory function in neurogenesis (Hecht et al., 1996). In vivo LPA_1 expression has been detected in the hippocampus wherein it seems to be predominantly restricted to oligodendrocytes (Weiner et al., 1998; Handford et al., 2001) while only hardly detectable levels of LPA_1 mRNA were found in neurons (Allard et al., 1998). However, a reasonable level of neuronal hippocampal LPA_1 expression has been demonstrated under ex vivo circumstances (Tabuchi et al., 2000; Fujiwara et al., 2003; Pilpel and Segal, 2006) or in immortalized hippocampal progenitor cells (Rhee et al., 2006). Exogenous delivery of LPA has demonstrated LPA_1 receptor-mediated functions including morphophysiological changes in neural progenitors (Dubin et al., 1999; Kingsbury et al., 2003; Fukushima, 2004; Fukushima and Morita, 2006; Fukushima et al., 2000, 2007). Likewise, LPA delivery to hippocampal neurons is known to increase the tyrosine phosphorylation of FAK (Derkinderen et al., 1998), regulate cell death (Holtsberg et al., 1998), mimic neurotrophic effects (Fujiwara et al., 2003) or mediate synaptic changes associated with spatial memory (Dash et al., 2004). Effects of LPA_1 loss on interneuron-mediated rhythms in vivo (Cunningham et al., 2006) and over-expression gain on synapse formation (Pilpel and Segal, 2006) have been reported in the hippocampus. In addition, the presence of modulators of lysophosphatidic acid activity has been also demonstrated in the adult hippocampus (Brauer et al., 2003).

At present, null animals have been obtained for most of the known LPA receptors by targeted gene disruption, all of them being mice (Choi et al., 2008). Receptor loss-of-function studies using LPA_1-null or LPA_1-/LPA_2-double null mice have suggested centrally mediated behavioral defects and relatively minor morphological cerebral alterations, although the initially generated mutation was associated with ~50% perinatal lethality that may have a CNS component, along with defective olfaction (Contos et al., 2000, 2002; Harrison et al., 2003; Roberts et al., 2005). Recently, the propagation of the original mixed background strain of LPA_1-null mice (Contos et al., 2000) in our laboratories, led to a stable variant of LPA_1-null mice called the "*Malaga* variant" (reported as "$maLPA_1$-null" mice). These mutants exhibited improved perinatal viability and showed altered cortical neurogenesis and increased cell death during brain development that caused a reduction of cortical layer cellularity in adults, indicating the action of as yet unidentified genetic modifiers of LPA_1 that influence cortical neurogenesis (Estivill-Torrús et al., 2008). Here we report that $maLPA_1$-null mice display reduced postnatal DG neurogenesis under both basal and environmentally enriched conditions.

Results

Adult hippocampal formation of mice lacking the LPA_1 receptor

We first examined the patterning of the developed hippocampus from wild-type and $maLPA_1$-null mice at birth (P0). Haematoxylin-stained brain sections (Figs. 1A, B), showed no gross anatomical abnormalities in the hippocampal formation. The volume of the DG did not significantly differ in the $maLPA_1$-null mice (Fig. 1B).

By P0, the hippocampal CA1 and CA3 areas and the granule cell layer (GCL) of the dentate gyrus (DG) are clearly definable by examination expression of NeuN (Figs. 1C, D), commonly used for the labelling of mature hippocampal neurons (van Praag et al., 2002;

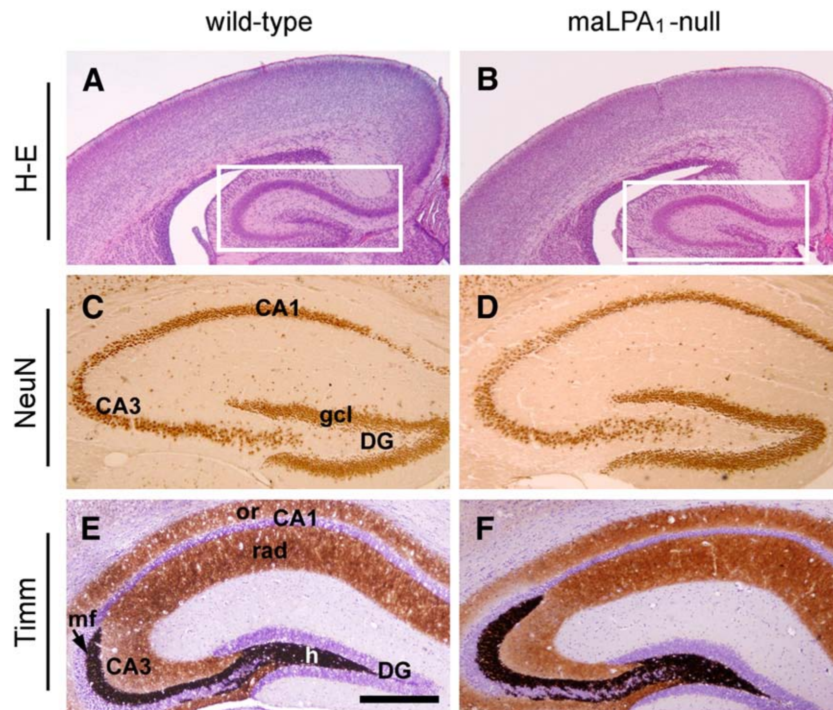

Fig. 1. Hippocampus formation in wild-type and $maLPA_1$-null mice. Comparable hippocampal DG coronal sections from wild-type (A, C, E) and $maLPA_1$-null (B, D, F) mice at postnatal P0 (A, B) and 12 week-old (E–F) ages. (A, B) Haematoxylin-stained wild-type (A) and $maLPA_1$-null (B) cerebral cortices. Hippocampal formation is marked in rectangles. (C, D) Neuronal expression of NeuN in postnatal P0 mice states clearly and evidences a normal granule cell layer (gcl) in dentate gyrus (DG), CA3 and CA1 hippocampal areas in wild-type (C) and $maLPA_1$ null (D) mice. (E, F) Timm's stained sections from wild-type (E) and maLPA1-null (F) mice. Reactive zinc labels the hilus (h), the mossy fibers (mf) into CA3 area and shows a lighter laminar staining in stratum radiatum (rad) and oriens (or) of CA1. Both genotypes exhibit a similar histochemical pattern. Scale bar in A, B=1000 μm; C, D=375 μm; E, F=300 μm.

Christie and Cameron, 2006). MaLPA$_1$-null mice containing the deletion of the main coding region of the murine *lpa*$_1$ gene (Contos et al., 2000) showed normal development of CA1, CA3 and DG areas (Fig. 1D) compared with wild-type mice (Fig. 1C). Hippocampal cytoarchitecture was relatively unaffected; both genotypes showed similar cell density and thickness in the cited regions from P0 up to 3 months old (see below). Since previous works had shown evidence of an LPA-mediated effect on synaptic or psychophysiological remodeling (Cunningham et al., 2006; Pilpel and Segal, 2006), we analyzed the adult hippocampus using the Timm's silver staining, a histochemical technique that selectively labels synaptic terminals because of their high zinc content (Danscher, 1981). Timm's method labels hippocampal mossy fibers, axons of granule cells that converge in the dentate hilus and run through the stratum lucidum to synapse with hilar and CA3 neurons (Fig. 1E). No obvious, altered synaptic primary pattern in the hippocampus was observed in null mutants compared to controls. Dentate granule cells projected mossy fiber axons that established their synaptic contacts with hilar and CA3 cells in a normal pattern (Fig. 1F). In maLPA$_1$-null mice the staining depicted the layered organized stratum radiatum (rad) and oriens (or) of CA1 with a similar pattern, though less intense staining, compared to wild-type mice.

LPA and its specific LPA$_1$ receptor pathway has been demonstrated to be involved in the regulation of the mitotic activity, differentiation and cell death of cerebral cortical neural progenitors (Kingsbury et al., 2003; Rehen et al., 2006; Fukushima et al., 2007; Estivill-Torrús et al., 2008). Analysis of adult granule cells in reference to their early development and apoptosis was performed to exclude possible delayed effects of altered cellular mechanisms generated during hippocampal development. Dentate cells were examined by their specific Prox-1 expression, a transcription factor that is expressed in granule cells starting at early proliferative stages when newly formed cells can be considered precursors (Pleasure et al., 2000; Brandt et al., 2003). Consequently, alterations in the granule cells development could be detected by altered Prox-1 expression. In the adult wild-type hippocampus, Prox-1 immunoreactivity mainly identified the entire granule cell layer of DG (Fig. 2A) where it was restricted to the cell nuclei (Fig. 2C). The patterning of Prox-1 expression was similar in adult maLPA$_1$-null hippocampus (Figs. 2B, D). In both genotypes the immunoreactive GCL showed comparable thickness (marked by brackets) and total positive cells number, as confirmed by stereological analysis (Fig. 2G). Concomitantly with Prox-1 expression, as the DG develops, mature NeuN-positive cells become integrated into the granule cell layer (Kempermann et al., 2003). Parallel analysis showed

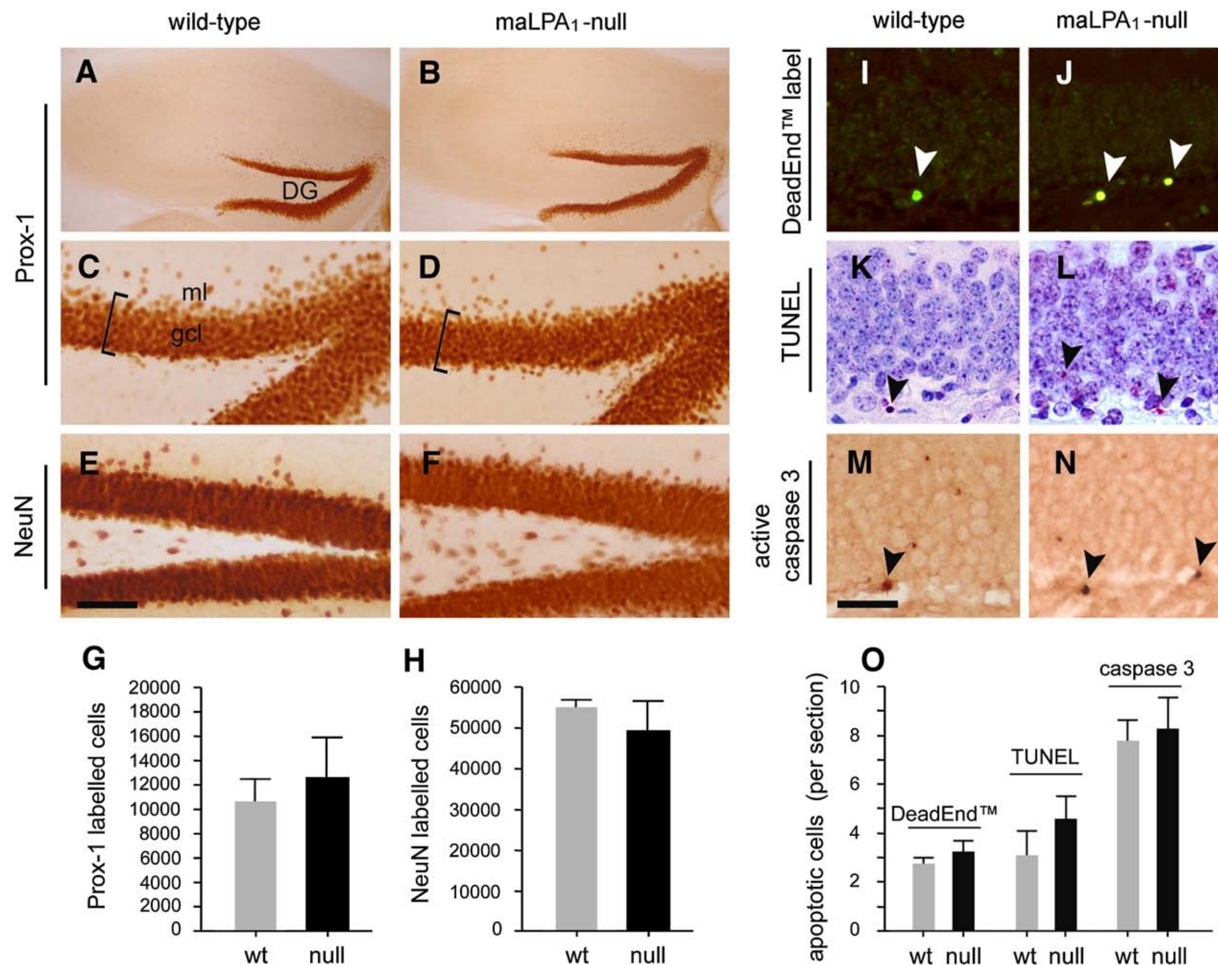

Fig. 2. Normal development and apoptosis of mature granule cell layer of maLPA$_1$-null mice. (A–D) Immunolabelling of granule cells with the specific marker Prox-1 in 3 month-old mice reveals no differences in coronal sections of the DG from maLPA$_1$-null hippocampus (B, detailed in D) when compared with wild-type (A, detailed in C). gcl, granule cell layer, bracket delimits layer thickness; ml, molecular layer. (E, F) Similar results were obtained after immunostaining in parallel sections with the neuronal marker NeuN in wild-type (E) or maLPA$_1$-null mice (F). (G, H) Total number of Prox-1 positive (G) or NeuN-positive (H) granule cells in DG of wild-type (wt) and maLPA$_1$-null (null) mice. Analysis did not find significant differences between both genotypes (n=6). (I–O) Cell death detection in wild-type and maLPA$_1$-null mice. Representative photographs of 3 month-old mice coronal hippocampal sections from wild-type (I, K, M) and maLPA$_1$-null (J, L, N) mice showing labelled nuclei (arrowheads, as examples) with DeadEnd™ Colorimetric Apoptosis Detection System (I, J), TUNEL (K, L) or anti-active caspase 3 (M, N). (O) Cell death detection in wild-type and maLPA$_1$-null DG expressed as number of apoptotic cells per section for each corresponding method. LPA$_1$-null DG exhibits a scarcely detectable increased levels of apoptosis although without statistical variation from observed in wild-type (n=8). In both graphs data expressed as mean±SEM. Scale bar in A, B=540 μm; C–F=90 μm; I–N=270 μm.

no differences between wild-type (Fig. 2E) and maLPA$_1$-null DG (Fig. 2F) based on NeuN expression as well as after quantification (Fig. 2H). Detection of apoptotic cells was performed at similar levels and ages of both wild-type (Figs. 2I, K, M) and maLPA$_1$-null mice (Figs. 2J, L, N) using either fluorometric DNA endlabelling (Figs. 2I, J) and TUNEL (Figs. 2K, L) identification of dying cells or active caspase 3 expression (Figs. 2M, N). After quantification, the percentages of positive nuclei (arrowheads in figures) did not significantly differ in mice lacking LPA$_1$ from those observed in wild-type (Fig. 2O). Combined, these results indicate a lack of obvious hippocampal defects in maLPA$_1$-null mice.

Reduced adult hippocampal neurogenesis in the absence of LPA$_1$

After anatomical examination, we analyzed adult hippocampal neurogenesis in 12 week-old mice under two different conditions: 1) normal cage environment, and 2) a combination of both environmental enrichment and voluntary exercise, conditions that are known to increase hippocampal neurogenesis in mice. Basal cell proliferation was monitored by BrdU incorporation (reviewed in Taupin, 2007) 24 h after pulse injections of BrdU over 4 days (standard housing in Fig. 3). In both wild-type and maLPA$_1$-null mice, BrdU-positive cells were observed in the SGZ, the GCL, the hilus and molecular layer (ML) of the DG of the hippocampus (Figs. 4A, D). However, a significant decrease (54%) in the total number of BrdU labelled dentate cells was observed in maLPA$_1$-null DG (Figs. 4C, E, basal columns; 1050.1±129.4 and 568.1±47.2 in absolute numbers for wild-type and null DG, respectively).

Adult hippocampal neurogenesis is stimulated by environmental enrichment (Kempermann et al., 1997, 2002) and voluntary physical activity (Van Praag et al., 1999a, 1999b; Eadie et al., 2005). Both pathways lead to an increase in hippocampal neurogenesis although they are dissociable (Olson et al., 2006). Combinations of both experimental conditions have shown evidence for reciprocal interactions between enrichment and exercise in the behavioral responses (Pietropaolo et al., 2006) and have been suggested to be most beneficial on cognitive performance (Nichol et al., 2007) and increase the number of endogenous progenitor cells (Hicks et al., 2007). To test the induced neurogenic response, we exposed the animals to a protocol of combined environmental enrichment (20 days) and voluntary exercise (14 days) following the schedule depicted in Fig. 3. Over the last four days, the mice received pulse injections of BrdU following the method mentioned above. As expected, enrichment and exercise condition generated a significant two-fold increase (103%) of labelled cells in the DG of wild-type mice (Fig. 4B, central set in E; 2144.5±127.1 total labelled cells) compared with standard housing.

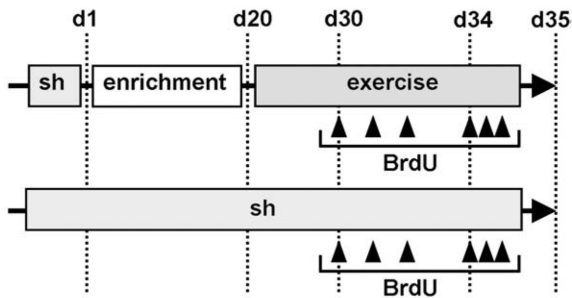

Fig. 3. Training schedule and BrdU administration. Schematic representation of the experimental design (see Experimental methods for details). BrdU was i.p. injected (arrowheads) during four days (days 30–34) after a period of exposure to environmental enrichment (d1 to d20) followed by voluntary exercise (days 20–30). Exercise was maintained during days of injection. Naïve animals were under standardized housing (Sh) and receive appropriate BrdU doses likewise on an equivalent period. On day 35 animals were processed for fixation.

Under the same conditions, the total numbers of BrdU labelled cells in the DG of maLPA$_1$-null mice were also increased with respect to the basal condition but visibly in less proportion (59%) compared with wild-type mice (Fig. 4D, central set in E; 903.6±120.5 total labelled cells). Thus, in the absence of LPA$_1$, the hippocampus exhibits a lower neurogenic ground state, newly formed neurons are minor in number and, in addition, their proliferative competence is reduced. This result indicates a requirement for LPA$_1$-mediated signaling in the proliferation of hippocampal DG cells.

Further analysis demonstrated that the absence of LPA$_1$ also affected the survival of newly formed cells. To test this, parallel experiments were performed in which mice exposed to enrichment and exercise were sacrificed one month after the last BrdU injection. This date of sacrifice was estimated as appropriate given that most of hippocampal neurons that survive the first 2 weeks after birth are stable through several months (Kempermann et al., 2003). Stereological analysis of labelled DG cells indicated that the numbers of surviving cells (Fig. 4E, columns at right) was almost 50% of the total number of newly formed cells in wild-type hippocampus while in maLPA$_1$-null mice, the survival rate did not rise above 30%.

In order to identify which population of newly formed cells might be particularly vulnerable to the absence of LPA$_1$ signaling, we analyzed the BrdU labelled cells for doublecortin (DCX) or glial fibrillary acidic protein (GFAP) expression one day after the last BrdU injection. Doublecortin is a microtubule-associated protein expressed specifically and transiently in young neurons during adult hippocampal neurogenesis (Kempermann et al., 2003) used as a valuable marker to evaluate neurogenesis (Rao and Shetty, 2004; Couillard-Despres et al., 2005). GFAP is an intermediate filament protein predominantly expressed by differentiated astrocytes (Eliasson et al., 1999) and expressed by multipotent hippocampal neural progenitors as well (Garcia et al., 2004). However, GFAP-positive glial cells can be distinguished from multipotent neural cells based on their distinctive multipolar astrocytic morphology versus the bipolar or unipolar morphology (Garcia et al., 2004).

Colocalization by confocal and conventional fluorescent microscopy of BrdU and neuronal (DCX) or glial (GFAP) antigens in the DG of wild-type animals (Figs. 5A, C) demonstrated a preponderance of DCX-positive cells amongst BrdU labelled cells (41%±3.6%; n=10; Fig. 5E). A small percentage of the remaining BrdU labelled cells were multipolar GFAP immunoreactive (9.9%±0.7%; n=10; Fig. 5F). By comparison, the DCX/GFAP ratio was proportionally maintained in maLPA$_1$-null DG although the percentages decreased significantly in both DCX/BrdU (27.2%±2.5%; n=10) and multipolar GFAP/BrdU labelled cells (5.6%±0.4%; n=10). These results were in accordance with the observed reduced proliferation but, therefore, demonstrated a higher percentage of cells without an observable phenotype in the absence of LPA$_1$ suggesting a cell missfate or delay in fate choice.

Structural plasticity and maturation are defective in maLPA$_1$-null granule precursors

During adult neurogenesis, neuroblasts undergo cellular modifications that result in structural changes and adaptations, which involve actin cytoskeleton regulation (Luo, 2002). Numerous studies have demonstrated LPA-mediated cell morphology reorganization (Fukushima, 2004; Fukushima and Morita, 2006; Fukushima et al., 2000, 2007). Induction of cellular restructuring by LPA and actin filaments has also been showed in hippocampal neurons (Fujiwara et al., 2003; Dash et al., 2004; Pilpel and Segal, 2006). To examine whether structural remodeling is related to the effects of LPA signaling in hippocampal neurogenesis, we hence analyzed the expression of two molecules commonly associated with morphological and plastic features of hippocampal neuroblasts, PSA-NCAM and doublecortin. PSA-NCAM, the polysialylated form of the neuronal cell adhesion molecule, is specifically expressed by neuroblasts in cerebral areas

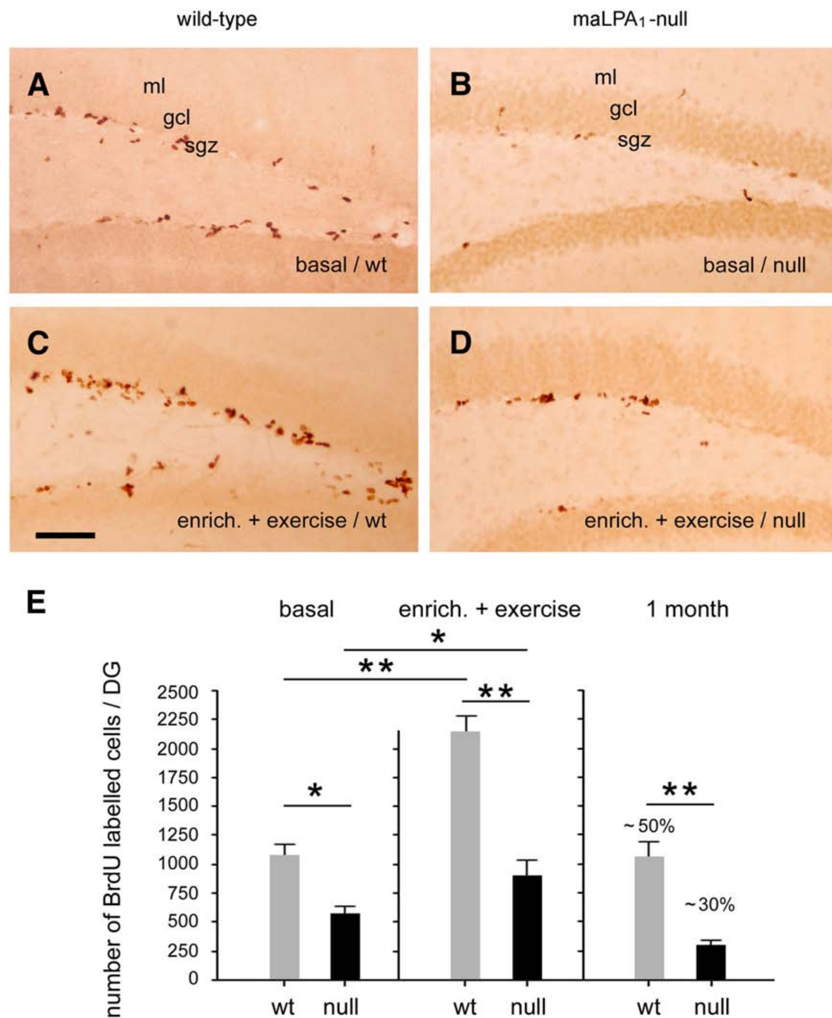

Fig. 4. Reduced cell proliferation and production of neural cells in the dentate gyrus in the absence of LPA_1. (A–D) Representative immunohistochemical staining for BrdU detection in adult hippocampus. The basal number of BrdU-positive cells (A) is increased in the wild-type mice after exposure at enriched environment for 20 days followed by 14 days of activity in the running-wheel (C). By contrast, basal neural precursor proliferation is reduced in the DG of maLPA$_1$-null mice (B) in almost 50% from the total label as compared with the wild-type. This reduction is maintained even in housing conditions of environmental enrichment and exercise (D). (E) Estimation of numbers of total BrdU labelled cells in wild-type (wt) and maLPA$_1$-null (null) adult mice for i) basal condition (basal), ii) after the exposure at enriched environment and exercise (enrich.+exercise), and iii) one month later of last BrdU injection, to evaluate the survival of generated cells. Bars represent mean±SEM. Reduction of cell proliferation was significant between both genotypes in all conditions ($n=8$; *, $P<0.05$; **, $P<0.01$). Significantly, while wild-type mice increase the number of BrdU-positive cells under environmental complexity and exercise, ($n=8$; **, $P<0.05$) maLPA$_1$-null mice do not initiate a significant proliferation under these conditions. Likewise, survival of newly generated cells is clearly minor in the absence of LPA_1 receptor, with values estimated in about 20% less than wild-type ($n=8$; *, $P<0.01$). Abbreviations as in previous figures. Scale bar=100 µm.

which retain structural plasticity and provides an appropriate neuronogenic microenvironment (Seki, 2002; Bonfanti, 2006). Doublecortin (DCX) has also been related to structural plasticity because of its effects on microtubule reorganization in axonal outgrowth or synaptogenesis (Nacher et al., 2001; Corbo et al., 2002). Recently, a correlation between the growth grade of DCX-immunoreactive dendritic trees and the status of differentiation of newly generated hippocampal neurons has been demonstrated (Rao and Shetty, 2004; Couillard-Despres et al., 2005).

Immunohistochemical analysis was performed on sections parallel to those used in the previous experiment. In agreement with the BrdU findings, maLPA$_1$-null adult hippocampi showed a considerable reduction in PSA-NCAM and DCX expressing neurons relative to wild-type (Fig. 6). Both PSA-NCAM and DCX stained cells can be visualized at the SGZ and the innermost part of the GCL. The number of PSA-NCAM immunoreactive cells was decreased in maLPA$_1$-null mice, particularly in the lower blade of DG (Fig. 6B) compared with wild-type (Fig. 6A) although PSA-NCAM expression delineated the entire precursor cell body and growing processes in both genotypes (insert in Figs. 6A, B). Similarly, DCX was highly expressed in the soma and dendrites of the majority of SGZ/GCL neuroblasts (Figs. 6C, E), while the number of cells expressing DCX and the degree of dendritic arborization of neurons expressing DCX was reduced in mice lacking LPA_1 (Figs. 6D, F). Notably, in the lower blade of the DG in maLPA$_1$-null mice, there was a nearly complete absence of both DCX labelled cells and dendritic branching (Fig. 6D). Quantification agreed with PSA-NCAM expression images and confirmed a significant reduction (almost 50%) of DCX labelled cells in the maLPA$_1$-null hippocampus (Fig. 6G). Additionally, there was a slight trend towards reduced cell arborization with primary dendritic branches emanating from the apical dendrites as in wild-type (Fig. 6E), but with less secondary branching originated from other branches (Fig. 6F). These results indicate that LPA_1-mediated signaling is involved in both proliferation of neural precursors and may also contribute to dendritic morphology.

Finally, in order to distinguish DCX labelled early postmitotic neurons vs. precursor daughter cells, we analyzed cell morphology in the upper blade of the DG and expression of calretinin. According to previous studies, DCX expression during adult hippocampal neurogenesis reveals bipolar daughter cells which act as transient or

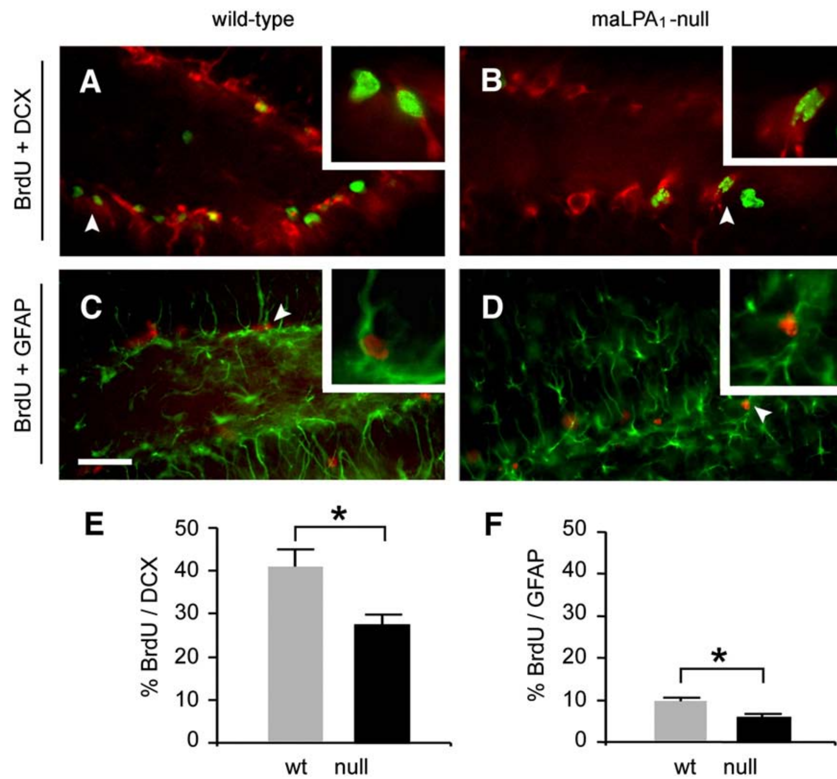

Fig. 5. Phenotypic characterization of newly generated dentate gyrus cells. (A, B) Descriptive images of double immunofluorescence labelling for newly born DG cells (BrdU-positive, Alexafluor® 488) with early neuronal phenotype (doublecortin, DCX-positive, Alexafluor® 568) in wild-type (A) and maLPA$_1$-null (B) mice after exposure to environmental enrichment and exercise. (C, D) Equivalent DG sections double immunolabelled for newly born cells (BrdU-positive, Alexafluor® 568) with glial phenotype (GFAP-positive, Alexafluor® 488) in wild-type (C) and maLPA$_1$-null (D) mice. Arrowheads magnified in squares. (E, F) Analysis of the percentages of double-labelled cells (expressed as mean±SEM, $n=10$; $^*P<0.05$) shows similar neuronal/glial proportions in newly born DG cells from wild-type and maLPA$_1$-null mice. Data for neuronal (DCX-positive, E) or glial (GFAP-positive, F) phenotype show equally preferential neuronal fate against glial although the percentages differ significantly between both genotypes with less amount of double DCX or GFAP BrdU labelled cells in the absence of LPA$_1$. Scale bar=200 μm; small images in A–D, 3× magnified.

intermediate precursors (Kempermann et al., 2004; Seri et al., 2001, 2004) During the earlier period early postmitotic granule cells show irregular soma close to the border of the hilus and GCL, with or without processes of short length running parallel to the GCL. As a neuron develops it exhibits an oval-shaped soma and longer processes that finally turn transverse toward the GCL in order to acquire their final radially oriented pattern (Kempermann et al., 2004; Esposito et al., 2005; Overstreet-Wadiche and Westbrook, 2006). This early postmitotic period is defined by the transient expression of calretinin (Brandt et al., 2003; Kempermann et al., 2004; Overstreet-Wadiche and Westbrook, 2006).

Analysis of newly formed DG cells after neurogenic stimulation showed that the majority of wild-type DCX labelled SGZ/GCL cells (73.2%±2.1%) possessed an evident dendritic tree running transverse to the GCL and molecular layer as reported (Figs. 6E, I), while bipolar cells with processes parallel to GCL occupied the smaller fraction (26.8%±2.3%). In contrast, in the maLPA$_1$-null DG, the proportion of immature granule cells showing radially oriented processes was reduced (53.5%±3.1%) and the amount of bipolar tangentially oriented cells was significantly elevated (46.5%±3.2%) (Figs. 6F, I). Analysis of calretinin expression in SGZ/GCL area confirmed these results showing that in the absence of LPA$_1$ a majority of postmitotic cells were restrained to migrate correctly toward upper GCL positions retaining their processes parallel to GCL. Fig. 6H shows the correct disposition of newly formed granule cells, most of them (82.3% ±2.6%; Fig. 6I) with transverse-oriented processes extending to the GCL. Only a small fraction of SGZ/GCL cells displayed parallel processes (17.7%±2.6%; Fig. 6I). By contrast, maLPA$_1$-null SGZ/GCL cells expressing calretinin predominantly retained their processes parallel to GCL (Fig. 6J) raising their percentage up to 89.2%±3.3% (Fig. 6I). Thus, combined results from BrdU and DCX/PSA-NCAM/ calretinin labelling suggest deficits in the balance between proliferation and maturation processes and they are in agreement with the prevalence of newly born granule cells without obvious neural phenotype in the absence of LPA$_1$.

Influence of vascularity and trophic factors

Adult hippocampal neurogenesis has been demonstrated to be modulated by local microvasculature that innervates close to proliferating cells (Palmer et al., 2000; Alvarez-Buylla and Lim, 2004). In addition to vascular influences, the dentate neuronogenic niche is regulated by neurotrophins and trophic factors, particularly brain-derived neurotrophic factor (BDNF) or insulin-like growth factor-1(IGF-I), which are abundantly expressed in the hippocampus and have known roles in differentiation and survival of newly born neurons (O'Kusky et al., 2000; Lee et al., 2002; Åberg et al., 2003; Barnabe-Heider and Miller, 2003; Sairanen et al., 2005). It has been suggested that both influences are interrelated factors in the promotion of dentate neurogenesis and neuron survival in enriched or exercised animals (Trejo et al., 2001, 2008; Ding et al., 2006; Rossi et al., 2006). We analyzed DG vascularization to investigate if the absence of LPA$_1$ also influenced the vessel distribution and the proportion of newborn cells associated with the vasculature. Blood vessels were visualized using lectin staining in DG sections from BrdU injected mice. Examination of the amount and disposition of dentate vessels did not reveal neither qualitative or morphological differences between both genotypes nor differences in the quantities of BrdU-positive SGZ cells adjacent to endothelial cells between wild-type (Fig. 7A) and maLPA$_1$-null mice (Fig. 7B) as observed from percentages

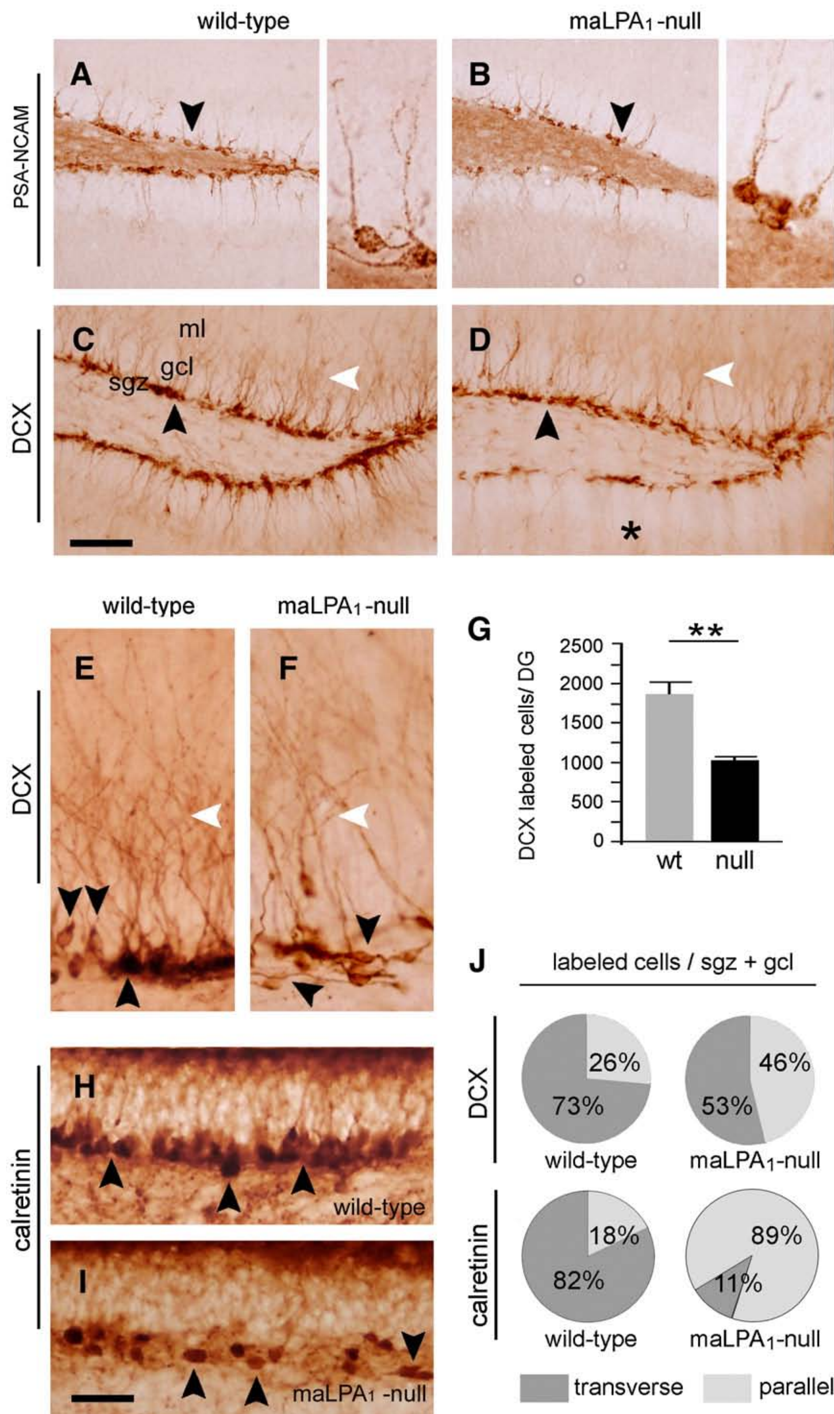

Fig. 6. Reduced PSA-NCAM and doublecortin expression and altered expression pattern in the absence of LPA_1 receptor (A, B). Illustrations of PSA-NCAM labelled cells in the dentate gyrus of wild-type (A) and $maLPA_1$-null (B) adult mice following enrichment and voluntary exercise. Expression of PSA-NCAM by newly born cells correlates with the reduced proliferation in mice lacking LPA_1 receptor showing less number of stained cells (arrowheads). Adjacent images in A and B are 6× detailed from arrowhead-pointed. PSA-NCAM immunolocalization delimits the cell body and extending process in both genotypes. (C–F) Photomicrographs illustrates the doublecortin expression and the morphology of early generated DG neurons of wild-type (C, E) and $maLPA_1$-null (D, F) adult mice in same conditions. A lesser amount of DCX-positive cells (black arrowheads) and dendritic processes extending into the molecular layer through GCL (white arrowheads) are observed in $maLPA_1$-null mice, being particularly reduced in the lower blade of hippocampus (asterisk). Enlarged images of SGZ/GCL (E, F) show a reduced cellular branching in $maLPA_1$-null DG as well as a preference of bipolar tangential cells with cell processes running parallel to SGZ/GCL (black arrowheads) against radially oriented that predominate in same wild-type area (black arrowheads). (G) Estimation of numbers of total dentate DCX labelled cells in wild-type (wt) and $maLPA_1$-null (null) adult mice (n=8; **, P<0.01). (H, I) Calretinin expression at SGZ/GCL of wild-type (H) and $maLPA_1$-null (I) mice defines the different disposition of cell processes. Arrowheads point the soma of immunoreactive cells. (J) Estimation of percentages of SGZ/GCL DCX and calretinin labelled cells in wild-type (wt) and $maLPA_1$-null (null) adult mice showing transverse (radial) or parallel (tangential) to GCL cell processes (n=10; significance was found among wild-type and $maLPA_1$-null mice, P<0.05). gcl, granule cell layer; ml, molecular layer; sgz, subgranular zone. Scale bar in A, B=200 µm; C, D=175 µm; E, F, H, I=35 µm.

of vascular-associated BrdU labelled cells (Fig. 7C). These results suggest that the decreased hippocampal neurogenesis observed in $maLPA_1$-null mice is not related to vascular changes.

Finally, trophic factors levels were measured in the hippocampus because of their influence in the genesis and survival of new hippocampal granule cells and dendritic branching. LPA signaling

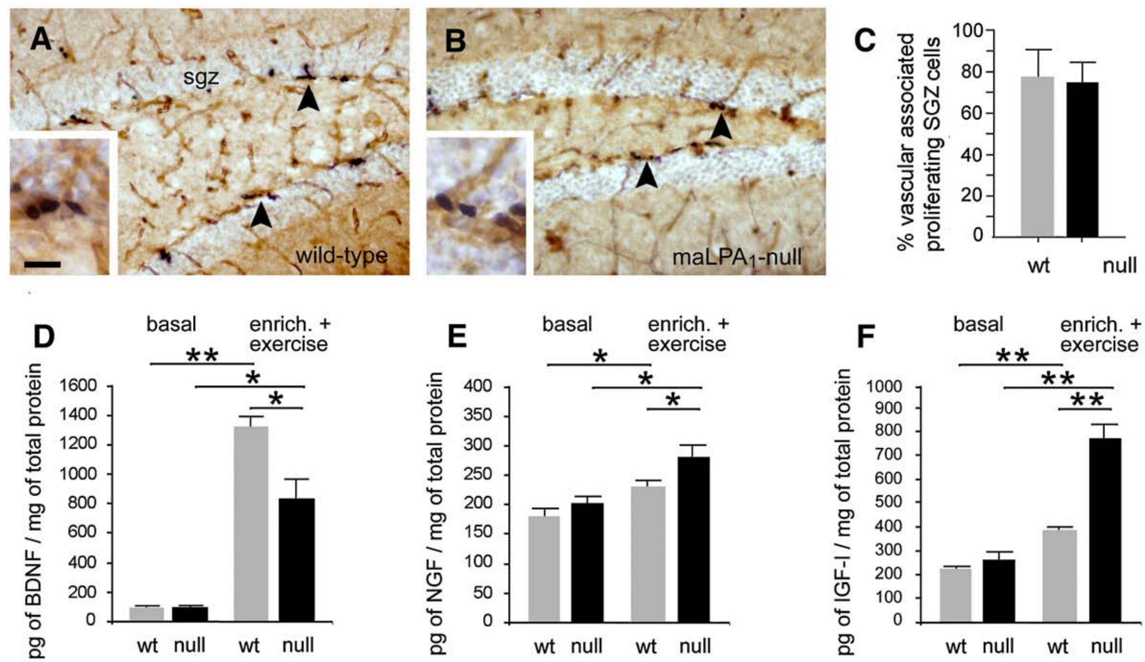

Fig. 7. Vascularity and analysis of the trophic factors in the maLPA$_1$-null hippocampus. (A, B) Double immunodetection for BrdU-positive proliferating cells (revealed with DAB plus nickel; black-purple reaction) and lectin histochemistry for blood vessels (DAB; brown reaction) in the dentate gyrus of wild-type (A) and maLPA$_1$-null (B) mice. Subgranular zone (sgz) proliferating cells (arrowheads) mostly associate with vessels in both genotypes as seen in corresponding magnified image at left squares. (C) Graph shows the quantified percentage of subgranular cells proliferating cells associated to vessels, expressed as mean ± SEM (n = 10). No significant differences were found in the absence of LPA$_1$ receptor (null) with almost 80% of cells adjacent to vascular in the same way to wild-type hippocampus (wt). (D) BDNF protein levels in hippocampus measured by ELISA for wild-type (wt) and maLPA$_1$-null (null) mice in basal and following enrichment and voluntary exercise. Data, indicated as mean ± SEM, do not differ between both groups at basal condition showing normal BDNF levels in maLPA$_1$-null mice as compared with control. Exposure to enriched environment and voluntary exercise raised the protein levels in both wild-type and maLPA$_1$-null mice, though this increase was significantly superior in wild-type mice (n = 8; *P < 0.05;**P < 0.001). (D) NGF protein levels in hippocampus measured by ELISA for wild-type (wt) and maLPA$_1$-null (null) mice in basal and following enrichment and voluntary exercise. Data, indicated as mean ± SEM, show the differences between both groups at basal condition and experimental conditions. Exposure to enriched environment and voluntary exercise made significant the increase of NGF levels in the absence of LPA$_1$. (n = 8; *P < 0.05). (E) Likewise, the enzimoimmunoassay for IGF-I protein level determination in similar extracts showed increase of IGF-I and differences between both genotypes just after experience and exercise. Remarkably, the increase of IGF-I levels in maLPA$_1$-null protein samples doubled the observed for wild-type samples (n = 8; **P < 0.01). Scale bar in A, B = 135 μm (small images 4× augmented).

has been shown to interact with receptor tyrosine kinase signaling that includes the neuregulins (Weiner and Chun, 1999) and neurotrophins. The latter are a family of secreted neuronal survival and plasticity factors comprising NGF, BDNF, neurotrophin-3 (NT-3), and NT-4,expressed endogenously in the hippocampal area and acting through p75 neurotrophin and Trk tyrosine kinase receptors (for review, see Lessmann et al., 2003). BDNF and NGF have been involved in regulating morphogenic events (Danzer et al., 2002; Lein et al., 2005) and promoting survival (Barnabe-Heider and Miller, 2003; Lee et al., 2002; Sairanen et al., 2005; Olson et al., 2006; Frielingsdorf et al., 2007) of new neurons in the adult hippocampus. The BDNF protein level in the wild-type hippocampus (Fig. 7D), was 93.4 ± 7.9 (pg/mg total), similar to maLPA$_1$-null mice, at 91.6 ± 12.7 (pg/mg total). In accordance with evidences for increased expression of BDNF in rodents in response to exercise (Berchtold et al., 2002; Gomez-Pinilla et al., 2002; Berchtold et al., 2005) or enriched environment (Ickes et al., 2000; Gobbo and O'Mara, 2004), wild-type mice in the enriched condition had elevated hippocampal BDNF levels, 1316.6 ± 66.6 (pg/mg total), correlating with enhanced neurogenesis and survival. In contrast, the enriched experimental conditions resulted in only a mild increase in LPA$_1$ deficient mice (828.1 ± 138.1 pg/mg total protein; P < 0.05 vs. wild-type) correlating with the lack of neurogenic response to environmental stimulation. Although without statistical significance, the analysis of basal NGF levels (Fig. 7E) showed an upward trend in maLPA$_1$-null hippocampus (176.5 ± 13.5 pg/mg total in wild-type vs.196.4 ± 8.9 pg/mg total in null mice; P < 0.05). Stimulation of neurogenesis increased the NGF levels in both groups of animals, according to reported data (Zhu et al., 2006). In this condition the differences were more evident and maLPA$_1$-null mice exhibited a significant increase of NGF levels (276.6 ± 13.5 pg/mg total) contrasting with wild-type mice (218.2 ± 7.6 pg/mg total; P < 0.05 vs. null mice) and indicating an imbalance of neurotrophin levels in the absence maLPA$_1$-null mice. Finally, analysis of basal IGF-I levels did not show differences between both genotypes (Fig. 7F; 229.1 ± 9.1 pg/mg total in wild-type vs. 266.2 ± 33.8 pg/mg total in null mice). Consistent with reported increased hippocampus uptake of IGF-I after exercise (Trejo et al., 2001, 2008) wild-type and null mice showed elevation of IGF-I levels. However, whereas wild-type experienced a natural moderate increase (389.2 ± 8.6 pg/mg total; P < 0.01) the lack of LPA$_1$ resulted in a markedly rise of IGF-I levels (778.5 ± 32.5 pg/mg total; P < 0.01).

Discussion

The present study was designed to determine whether targeted deletion of LPA$_1$ affects adult hippocampal neurogenesis. We found that DG neurogenesis, differentiation and survival of newly formed neurons are defective in LPA$_1$ deficient mice. In addition, the neurogenic response to environmental enrichment and voluntary running-wheel exercise was also impaired in the absence of LPA$_1$. These data demonstrate for the first time, a reduction of adult dentate neural progenitors in maLPA$_1$-null mice and a role for receptor-mediated LPA$_1$ signaling in adult neurogenesis.

These data complement prior studies on embryonic development in the cerebral cortex (Kingsbury et al., 2003; Fukushima, 2004; Fukushima et al., 2000, 2007; Fukushima and Morita, 2006 Rehen et al., 2006; Estivill-Torrús et al., 2008). MaLPA$_1$-null mice displayed a significant reduction in proliferating dentate cells that correlated with DCX and PSA-NCAM expression. The described lower neurogenic

ground state does not appear to be a consequence of anomalous development. In spite of deficiencies of adult neurogenesis, the present results indicate an apparently normal embryonic development of maLPA$_1$-null hippocampus. As a probable hypothesis our data support the view that adult and embryonic neurogenesis differs in substantial ways with respect to LPA signaling. Thus, adult neurogenesis is an individualized process, not a programmed and orchestrated event of sequential steps, unlike neuronal development which requires maintenance of a permissive microenvironment (Kempermann et al., 2004). Concomitant cell interactions and coupling processes promotes permissive conditions or intrinsic regulation mediated by sequential secretion of endogenous cues by cortical cells themselves, LPA among these (Fukushima et al., 2000). Other factors, i.e. adhesion molecules like PSA-NCAM can function differentially between embryonic and adult brain wherein it is also expressed by other cells already integrated in adult circuitry (Rutishauser, 2008). Moreover, newborn DG cells exhibit distinct functional properties compared with their mature neighbors in the adult brain and contrasting with neonatally born GCL cells that predominate in the adult (Muramatsu et al., 2007) arguing against a role for the LPA$_1$-null mutation in developmental defects. These results suggest that LPA$_1$ effects on neurogenesis may be context dependent, reflecting developmental period and involved cell types. Considering in addition the dissimilarities between embryonic ventricular zone and dentate subgranular zone architecture, it is also possible that indirect effects involving LPA$_1$ in other adult hippocampal cells could influence the neurogenic niche, particularly through astrocytes that are present in neonatal and adult germinal zones but are not participants in embryonic ventricular zone (vs. the embryonic presence of radial glia). Prior reports highlighted a role for postnatal astrocyte-derived cues in the specification of neurons (Seri et al., 2001; 2004; Song et al., 2002). Notably, astrocytes have been recently reported as influencing neuronal progenitor differentiation at least via LPA$_1$ (De Sampaio e Spohr et al., 2008). However, further analyses are necessary to determine the real contribution of astrocytes in LPA$_1$-dependent effects on adult hippocampal neurogenesis. Additionally, other brain areas could act as extrinsic modulators of hippocampal neurogenesis, as is the case for the olfactory system that is affected in LPA$_1$-null mice (Contos et al., 2000; Estivill-Torrús et al., 2008) and demonstrated to influence hippocampal cell survival (Pope and Wilson, 2007).

Besides impairment of neurogenesis, our BrdU data showed that the survival of newly formed neurons is also reduced in the absence of LPA$_1$. The decrease in survival of maturing neurons in maLPA$_1$-null mice occurs without the expected concurrent increase in cell death, as the three employed methods were similar in results for both genotypes. Although it is difficult to reconcile reduced cell survival in the face of no increase in cell death the results obtained by use of BrdU labelling in combination with DCX or PSA-NCAM markers, widely used to study the new neurons formation (Gould, 2007; Taupin, 2007) argue that maLPA$_1$-null mice indeed do have reduced cell survival. A possible explanation is that the rate of cell death is below our detection limit at this stage of development, or taking place during a short window of time missed by the employed approaches. This explanation is consistent with those studies that showed a discordance between cell birth and cell death rates that was attributed to technical issues in detecting of cell death (Cameron and Gould, 1996; Cameron and McKay, 1999; Biebl et al., 2000; Heine et al., 2004; Harburg et al., 2007).

In adult hippocampal neurogenesis, the patterns of DCX and PSA-NCAM expression coincided and revealed maturation of proliferative progenitors to a postmitotic phase with the development of the polarity and dendritic trees (Kempermann et al., 2004; Rao and Shetty, 2004). Some authors have proposed that the expression of DCX in newly formed cells represents a developmental stage of migration and differentiation rather than cell division (Jessberger et al., 2005) in which the development of dendrites would be independent of precursor proliferation (Plümpe et al., 2006). Interestingly, in agreement with promoting maturation, LPA enhanced mature morphological changes in neural precursors (Fukushima, 2004; Fukushima and Morita, 2006; Fukushima et al., 2000, 2007). DCX and calretinin expression in newly born dentate cells from maLPA$_1$-null mice showed impairment of maturation reflected in part by the prevalence of bipolar immature cells at the SGZ/GCL border with diminished arborization. Consistent with this result, reduced expression of PSA-NCAM could influence the microenvironment of immature neurons (Seki et al., 2002).

It remains to be shown whether newly formed neurons migrate and functionally integrate in the absence of LPA$_1$. At present, several studies have shown that loss of LPA$_1$ causes neurochemical, morphological, or physiological changes in the hippocampus (Harrison et al., 2003; Cunningham et al., 2006; Pilpel and Segal, 2006) although none of them analyzed the neurogenic processes. Interestingly, no differences have been reported in the number of hippocampal GABA, parvalbumin or calretinin positive interneurons from LPA$_1$ deficient mice (Cunningham et al., 2006). On the other hand, the reduced neurogenesis and impairment of neuronal maturation in the maLPA$_1$-null DG could affect overall network functioning since young granule cells can receive synaptic input and generate action potentials when they are still expressing immature markers (Overstreet et al., 2004; Christie and Cameron, 2006; Karten et al., 2006), and this may have behavioral consequences (Macklis, 2001; Shors et al., 2001; van Praag et al., 2002; Shors 2004; Aimone et al., 2006). In this sense, the behavioral characterization of maLPA$_1$-null mice has allowed us to identify learning deficits associated with spatial memory (unpublished observations) in agreement with data from experimental LPA cerebral infusion (Dash et al., 2004). Some psychological disorders are associated with specific regional loss of neurons related to hippocampal topography (Silva et al., 2006). Particularly suggestive for the development of behavioral studies is the almost complete absence of DCX expression in the maLPA$_1$-null lower DG blade, since this region has been demonstrated to be susceptible to stress (Sunanda et al., 1995; Galea et al., 1997; McEwen, 1999).

Another feature of maLPA$_1$-null hippocampus is the absence of obvious vascular alterations. LPA has been demonstrated to promote the permeability of brain endothelial cells (Schulze et al., 1997) and the stability of vessels (Tanaka et al., 2006). The original LPA$_1$-deficient mouse fetuses exhibited hemorrhaging and the development of frontal hematomas (Contos et al., 2000). The analysis of null mice for autotaxin, an enzyme responsible for LPA production, showed that ATX and LPA are required for the proper development of embryonic vasculature (Tanaka et al., 2006). These effects appear to be mediated by LPA receptors, although identification of the involved LPA receptors has not been determined. The existence of multiple LPA receptors only one of which was removed in our studies could explain the apparently normal vascular phenotype of maLPA$_1$-null mice and the absence of irregularities of DG vascularization. It remains possible that other factors acting on adult neurogenesis via the vasculature could be affected by LPA$_1$ deletion, especially in view of the trophic support and BDNF production provided by the endothelium (Leventhal et al., 1999).

In addition to constitutive secretion, release of neurotrophins from cells is regulated by multiple factors such as exercise and environmental enrichment, synaptic networks, or calcium (Lessmann et al., 2003). In the present study we found that the failure of neuronal production and survival was accompanied by a reduction of BDNF levels only in animals subjected to environmental enrichment and exercise, in accordance with data showing similar changes in response to wheel-running exercise (Berchtold et al., 2002; Gomez-Pinilla et al., 2002; Berchtold et al., 2005) or exposure to enriched environment (Ickes et al., 2000; Gobbo and O'Mara, 2004; Rossi et al., 2006). BDNF signaling has been shown to play a role in the differentiation and survival of neuronal progenitor cells rather than proliferation (Barnabe-Heider and Miller, 2003; Sairanen et al., 2005; Olson et al.,

2006). Thus, it is possible that the defective survival of new neurons would be a consequence of the failure to upregulate BDNF from newly born cells, rather than a primary LPA_1-dependent effect since regulation of granule cell survival is independent of the regulation of proliferation (Abrous et al., 2005). Precedence for this view comes from studies on astrocytes, which can release neuroactive factors in an LPA1-dependent manner (De Sampaio e Spohr et al., 2008). Alternatively, synergistic and compensatory effects between BDNF and LPA are possible given that BDNF cooperates with other trophic factors to enhance neuronal differentiation (Gratacos et al., 2001; Lessmann et al., 2003) and LPA has been demonstrated to activate signaling pathways similar to some neurotrophins (Fujiwara et al., 2003; Moughal et al., 2006). Increased NGF protein levels observed here can be interpreted in pathophysiological situations as both endogenous neuroprotective mechanism or as contribution to accelerated neuronal death by activation of p75-dependent pathway (Snider, 1994; Schulte-Herbrüggen et al., 2007). Recently, NGF has been shown to promote the survival of newly born granule neurons (Frielingsdorf et al., 2007). Notably, the increased IGF-I levels observed in enriched and exercised animals in the absence of LPA_1 could also be interpreted as a neuroprotective compensatory mechanism. Thus, it has been found that IGF-I is able to counteract the loss of neurons or the impairment of neurogenesis (Azcoitia et al., 2005; Chen et al., 2007). It seems reasonable that this LPA_1-dependent deficit in proliferation and survival would generate compensatory mechanisms and instability in neurotrophin levels. This could involve influences on proliferation, survival or apoptosis, involving not only neurons but also glial cells such as oligodendrocytes and astrocytes, which can express LPA_1 (Allard et al., 1998; Weiner et al., 1998; Sorensen et al., 2003; De Sampaio e Spohr et al., 2008) and produce trophic factors in vivo and in vitro (Lessmann et al., 2003). In addition to neurogenic responses, an LPA_1-deficient microenvironment could also show altered neuronal–glial interactions and exhibit altered or imbalanced trophin production. The intriguing relevance of neurotrophin modulation through interactions with LPA receptors requires additional work to identify the mechanisms involved and their influences on the maturation and survival of newly formed cells.

Together, these results demonstrate that normal hippocampal adult neurogenesis requires LPA_1-mediated signaling for normal function. Neuronogenic stimulation in maLPA$_1$-null mice following environmental enrichment combined with voluntary exercise was impaired, independent of morphological changes in the vascular niche. This study adds to the growing list of lysophospholipid-dependent functions that impact health (Gardell et al., 2006). These data suggest that modifying LPA signaling may be useful in developing potential future therapies involving manipulation of endogenous neural precursors or potentially antidepressant therapies, considering the hypothesized involvement of adult hippocampal neurogenesis in antidepressant action (Sairanen et al., 2005; Warner-Schmidt and Duman, 2006).

Experimental methods

Mice

Procedures were carried out with wild-type and maLPA$_1$-null heterozygous and homozygous males (on a mixed background C57Bl/6×129SW) in compliance with European animal research laws (European Communities Council Directives 86/609/EU, 98/81/CEE, 2003/65/EC and Commission Recommendation 2007/526/EC). The maLPA$_1$-null (from *Málaga variant of* LPA_1-null; Estivill-Torrús et al., 2008) mouse colony arose spontaneously from the initially reported LPA_1-null mouse line (Contos et al., 2000) while crossing heterozygous foundational parents within their original mixed background. More than fourteen maLPA$_1$-null generations have been obtained by backcrossing, exhibiting the defects described in this work. Research was performed on perinatal pups (postnatal day 0 to P7) and 12 week-old male mice obtained from heterozygous×heterozygous/homozygous maLPA$_1$-null mating and genotyped for lpa_1 deletion by PCR (Contos et al., 2000) or immunohistochemistry (Estivill-Torrús et al., 2008).

Housing conditions and BrdU injections

Adult mice (12 week-old) were randomly assigned (eight per cage) to the group of animals kept in standard cages with free access to water and food (naïve group, eight mice) or to the group of mice trained in an enriched environment followed by voluntary exercise. Fig. 3 illustrates the training and injection schedule. The enriched environment consisted of standard cages, with food and water accessible, supplemented with various toys, small plastic objects, a spin wheel and ladders. Items were re-arranged daily and completely renewed after ten days. At day 20, they were replaced by stainless steel running wheels and ladders. Daily wheel-running activity was maintained for ten days (day 30) and monitored by blind observers. Starting on day 30, animals and naïve group were given a daily i.p. injection of bromodeoxyuridine (BrdU; Sigma; St.Louis, MO, USA) at 100 μg/g of body weight and dissolved in saline, for three days (days 31–33). On day 34, three additional doses were administered, every 5 h. Animals were sacrificed by perfusion the following day or one month after the last injection to estimate the survival of newly formed neurons.

Immunohistochemical procedures

At least ten animals from each experimental condition and per genotype were used. Mice were transcardially perfused with 0.1 M phosphate buffered saline (PBS) containing 4% paraformaldehyde, 0.08% glutaraldehyde and 15% (v/v) saturated picric acid (Somogyi and Takagi, 1982). Brains were dissected out into cold phosphate buffer and fixed overnight at 4 °C. For double labelling analysis using anti-doublecortin, fixation procedures used a periodate-lysine-paraformaldehyde (PLP) solution in 0.1 M sodium phosphate buffer (McLean and Nakane, 1974) instead. All steps were carried out at room temperature on free-floating vibratome sections (30 μm). Hippocampal coronal sections were first treated for 10 min with 0.1 M phosphate buffered saline pH 7.4 (PBS) containing 10% methanol and 10% hydrogen peroxide to inactivate endogenous peroxidase. Single-immunolabelling of BrdU required a 10 min digestion of sections in 5 μg/ml proteinase K (Sigma) followed by denaturation of the DNA in 2 N HCl for 30 min at 37 °C and neutralization in 0.1 M boric acid (pH 8.5). After washing in Tris-phosphate buffered saline pH 7.4 (TPBS), they were exposed to the following antibodies: monoclonal mouse anti-BrdU (DSHB, University of Iowa, USA), monoclonal mouse anti-NeuN (Chemicon, Temecula, CA, USA), monoclonal rat anti PSA-NCAM (clone 12F8, BD Biosciences, Franklin Lake, NJ, USA), polyclonal goat anti-doublecortin (DCX) (c-18; Santa Cruz Biotechnology Inc., CA, USA), anti-GFAP (DakoCytomation, Glostrup, Denmark), or anti-Prox1 (Reliatech, Braunschweig, Germany) antibodies. Standardized detection used biotin conjugated rabbit anti-mouse, rabbit anti-goat, or swine anti-rabbit (as appropriate) immunoglobulins (DakoCytomation, Glostrup, Denmark), ExtrAvidin®-peroxidase (Sigma) and DAB (Sigma). All the antibodies were used according manufacturers' instructions and diluted in TPBS containing 0.5% Triton X-100 and normal serum (rabbit or swine serum, depending of the source of the secondary reagent used). Omission of the primary antibody resulted in no detectable staining. Sections were haematoxylin counterstained when required. Parallel sections of some brains were stained with haematoxylin-eosin for a general histological description.

For double labelling of BrdU and phenotypic markers (DCX, GFAP) confocal analysis along with double light microscopy was used. Accordingly, secondary antibodies utilized for immunofluorescence

were donkey Alexafluor® 488 or Alexafluor® 568 anti-mouse, anti-goat, or anti-rabbit antibodies (Invitrogen, Carlsbad, CA, USA) determined by the primary used. After washing with TPBS, the slides were coverslipped with fluorescence mounting medium (DakoCytomation) before viewing on a Leica laser TCS NT confocal fluorescence microscope. All analyses were done in sequential scanning mode in order to rule out cross-bleeding between detection channels. Cells were considered double-labelled if both fluorophores were seen within the same cell in three consecutive 1 μm optical sections in the z axis. For each experiment, three series of 60 μm spaced hippocampal sections per animal were analyzed, and at least 30 BrdU-positive cells in the SGZ were sampled to determine whether they were positive for DCX or GFAP. Images were processed with Adobe Photoshop 7.0 (Adobe Systems). In order to increase the number of animals in our stereological analysis, quantification was also performed using double immunohistochemistry with conventional fluorescence or under light microscopy using DAB and DAB plus nickel chloride intensification. Results were analyzed using an Olympus BX51 microscope equipped with an Olympus DP70 digital camera and confirmed the results obtained by confocal analysis.

Vascular analysis

Lectin histochemistry was performed on sections adjacent to BrdU immunostained sections to analyze blood vessels (*Lycopersicon esculentum*; biotin conjugated, 1:200; Sigma). Visualization was obtained via DAB and DAB plus nickel chloride intensification. Quantification was performed using Image J 1.37v software (NIH, USA). In each section, proliferating cells associated with vessels were estimated within a 200 μm × 200 μm counting frame positioned within the dorsal dentate gyrus.

Stereology

For stereological analysis, sampling of BrdU or NeuN (as applicable) positive cells was performed throughout the hippocampal DG in the rostrocaudal dimension using both the optical dissector (West, 1993b, Gundersen et al., 1988) and the optical fractionator method which combines the optical dissector with a fractionator sampling scheme (Gundersen et al., 1988) to exclude volume divergences. Counterstaining with cresyl violet or phase contrast allowed delineation of the DG area in each section as defined by Paxinos and Flanklin (2001). For optical dissector, quantification, DG immunoreactive cells were counted in 12 representative 40 μm, evenly-spaced sections per animal. Two series per animal and 6 animals per genotype were analyzed. A random set of sampling optical dissectors was generated for each section using the C.A.S.T. Grid (Olympus; Albertslund, Denmark) and 50% of the total of sampling optical dissectors was measured. The number of cells per unit of volume (Nv) was calculated for each animal, as the number of applied dissectors multiplied by Vdis; Vdis = Sd × Hd, where Sd = area of the dissector grid (counting frame) and Hd = depth of dissector. When fractionator method was used, sections were sampled every 100 μm, starting at the most dorsal part of the hippocampus, where DG blades become fully established (approximately 1.06 mm posterior to Bregma). Sampling ended at approximately 2.46 mm posterior to Bregma. Numbers of NeuN, BrdU, Prox1, DCX or GFAP immunolabelled cells were quantified in 12 representative 100 μm, evenly-spaced sections per animal. Two series per animal and 6 animals per genotype were analyzed. A random set of sampling frames with a known area (a_{frame}) was generated for each section using the C.A.S.T. Grid (Olympus; Albertslund, Denmark). After having counted the objects (ΣQ^{-}), the total number of positive granular cells was estimated as: $N = \Sigma Q^{-} \times fs \times fa \times fh$ (Gundersen et al., 1988), where fs is the numerical fraction of the section used, fa is the areal fraction and fh is the linear fraction of section thickness. The coefficient of error (CE) for each estimation and animal ranged from 0.05 to 0.1. The total CE of each group ranged from 0.07 to 0.08. Counting of labelled neurons was set starting at 5 μm below the surface and focusing through the 15 μm section optical plane.

Timm's staining

Timm's silver staining is a histochemical technique that selectively labels axons due to their high zinc content (Danscher, 1981). It has been widely used as an indicator of hippocampus function because of the high concentration of Zinc in hippocampus, particularly in the mossy fibers of the dentate gyrus, which has suggested a role in hippocampal processing (Fredrikson and Danscher, 1990). From each genotype, eight hippocampal equivalent sections per animal and per six mice were used for Timm's staining. After animal sacrifice, freshly obtained brain pieces comprising the hippocampus were immersed in 0.1% sodium sulphide solution in 0.1 M phosphate buffered saline pH 7.4 for 10 min, subsequently fixed overnight in 0.1 M phosphate buffer containing 4% paraformaldehyde, and cryopreserved in 20% sucrose. After freezing, 10 μm thick coronal hippocampus cryosections were mounted and processed for Timm's staining in a developing solution consisting of 50% gum Arabic, 2 M sodium citrate, 0.5 M hydroquinone and 0.5% silver lactate for 60 min in darkness at room temperature. Sections were then rinsed in water and counterstained with haematoxylin before mounting.

Apoptotic DNA endlabelling and immunodetection

Mice (eight per genotype and age) were transcardially perfused in 4% paraformaldehyde. Brains were dissected out, fixed overnight in the same solution at 4 °C, and processed either for cryosectioning (30 μm) or paraffin embedding and sectioning (10 μm). Hippocampal coronal sections (eight equivalent hippocampal sections per animal) were obtained and mounted onto poly-L lysine treated slides. Cell death was detected using a DeadEnd™ Fluorometric Apoptosis Detection System (Promega) on cryosections and terminal UTP nick endlabelling (TUNEL) procedure (Roche Diagnostic, Manheim, Germany) on wax sections, following the manufacturer's instructions. Pretreatment of sections with DNAse served as a positive control for the enzymatic procedures; omission of the enzyme served as a negative control. Non-adjacent coronal sections from hippocampus were counterstained with ethidium bromide and cell numbers were estimated. Calculations were performed using Image J 1.37v software (NIH, USA). For each section, labelled cells were estimated from a 200 μm × 200 μm counting frame positioned within the dorsal dentate gyrus. Parallel sections were exposed to polyclonal rabbit anti-active caspase 3 (Affinity Bioreagents, Golden CO, USA) and processed for immunohistochemistry as before mentioned.

Neutrophin and growth factor quantification

Endogenous neurotrophin levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in hippocampal homogenates were determined by a colorimetric ELISA in an antibody sandwich format using BDNF Emax® and NGF Emax® ImmunoAssay Systems (Promega Corporation, Madison, WI, USA) with a detection limit of 15.6 pg BDNF/ml and 7.8 pg NGF/ml respectively. For obtaining hippocampal homogenates, 12 week-old mice (eight per genotype) were decapitated and bilateral hippocampi dissected out, weighed, and homogenized in 200 μl of lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1% NP40, 10% glycerol and 1 mM PMSF, 10 μg/ml aprotinin and 1 μg/ml leupeptin as protease inhibitors. Samples were sonicated and centrifuged at 16,000 ×g for 30 min at 4 °C. The resulting supernatants were removed (aliquots of 100 μl) and used in the immunoassay system after they were diluted 1:4 in Promega's sample buffer and serially diluted per column in ELISA plates.

Quantitative determination of insulin-like growth factor 1 (IGF-1) protein expression levels was performed in the same hippocampal homogenates. Supernatants from the different samples were analyzed by a colorimetric ELISA in an antibody sandwich design using Quantikine Mouse IGF-I Immunoassay (R and D Systems, Inc., MN, USA) which has a minimum detection of 3.5 pg/ml. Samples were diluted and serially added to a 96-well ELISA plate and incubated according the package instructions.

BDNF, NGF and IGF-1 ELISA data were obtained in triplicate for each sample, and triplicates were averaged to obtain one value per sample. Immunoassay protocol and colorimetric reaction followed the manufacturer's instructions. Results were obtained in a microplate reader. The value of the sample was normalized to the total soluble protein concentration, and the data were expressed as mean ± SEM.

Statistics

Statistical significance was determined using Student's *t*-test or ANOVA (as appropriate). Stereological studies were analyzed using Kruskal–Wallis One-Way Analysis of Variance on Ranks and compared by post hoc analysis. All statistical analyses were conducted with Sigma Stat 3.0 and Sigma plot 8.0 software.

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