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Chronic central modulation of LPA/LPA receptors-signaling pathway in the mouse brain regulates cognition, emotion, and hippocampal neurogenesis.

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Short title: LPA_1-signaling modulation on neurogenesis and behavior

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ABSTRACT

Several studies have demonstrated that lysophosphatidic acid (LPA) acts through its LPA receptors in multiple biological and behavioral processes, including adult hippocampal neurogenesis, hippocampal-dependent memory, and emotional regulation. However, analyses of the effects have typically involved acute treatments, and there is no information available regarding the effect of the chronic pharmacological modulation of the LPA/LPA receptors-signaling pathway. Thus, we analyzed the effect of the chronic (21 days) and continuous intracerebroventricular (ICV) infusion of C18:1 LPA and the LPA_{1-3} receptor antagonist Ki16425 in behavior and adult hippocampal neurogenesis. Twenty-one days after continuous ICV infusions, mouse behaviors in the open field test, Y-maze test and forced swimming test were assessed. In addition, the hippocampus was examined for c-Fos expression and α-CaMKII and phospho-α-CaMKII levels.

The current study demonstrates that chronic C18:1 LPA produced antidepressant effects, improved spatial working memory, and enhanced adult hippocampal neurogenesis. In contrast, chronic LPA_{1-3} receptor antagonism disrupted exploratory activity and spatial working memory, induced anxiety and depression-like behaviors and produced an impairment of hippocampal neurogenesis. While these effects were accompanied by an increase in neuronal activation in the DG of C18:1 LPA-treated mice, Ki16425-treated mice showed reduced neuronal activation in CA3 and CA1 hippocampal subfields. Treatment with the antagonist also induced an imbalance in the expression of basal/activated α-CaMKII protein forms.

These outcomes indicate that the chronic central modulation of the LPA receptors-signaling pathway in the brain regulates cognition and emotion, likely comprising hippocampal-dependent mechanisms. The use of pharmacological modulation of this pathway in the brain may potentially be targeted for the treatment of several neuropsychiatric conditions.

Keywords

Lysophosphatidic acid receptor, emotional behavior, working memory, adult hippocampal neurogenesis, c-Fos, α-CaMKII

1. Introduction

Lysophosphatidic acid (LPA) is a major member of the bioactive lysophospholipids with intercellular signaling properties that acts through a complex family of G-protein coupled receptors (LPA_{1-6}) widely distributed in peripheral organs and brain (Choi et al., 2010; Choi and Chun, 2013; Yung et al., 2014, 2015). This small lipid plays an essential role in a variety of cellular and developmental processes in both physiological and pathological conditions (Lin et al., 2010; Aikawa et al., 2015). Among all LPA receptors, LPA_1 is the best studied in relation to biological actions through LPA (Lin et al., 2010; Choi et al., 2010; Choi and Chun, 2013; Aikawa et al., 2015; Yung et al., 2014, 2015). In the central nervous system (CNS), LPA_1 receptor is expressed in the developing and adult brain in most neural cells, including neurons, precursors, oligodendrocytes, and astrocytes (Hecht et al., 1996; Weiner et al., 1998; Estivill-Torrús et al., 2008; Shano et al., 2008; García-Díaz et al., 2014; Walker et al., 2016), consistent with studies of functional autoradiography showing receptor activity not only in myelinated areas of white matter, such as corpus callosum and internal capsule, but also in the hippocampus, frontal cortex, amygdala and striatum (González San Roman et al. 2015, 2019).

Most of the studies performed to investigate the functional role of LPA and LPA_1 receptor in mature brain have been realized using LPA_1-null mice and restricted to the hippocampus, a key brain region involved in emotion and cognition. In mice, the deletion of the LPA_1 receptor causes several hippocampal and behavioral abnormalities in adulthood, resulting in an endophenotype that has allowed studies of its role in relation with this structure. Thus, mice lacking the LPA_1 receptor display a defective proliferation and maturation of newly born neurons and blunted increases in cell proliferation and survival in response to environmental enrichment and voluntary exercise (Matas-Rico et al., 2008; Castilla-Ortega et al., 2013). Moreover, the deletion of LPA_1 receptor aggravates the impairment in hippocampal neurogenesis (cell proliferation, apoptosis and neuronal maturation) induced by chronic stress (Castilla-Ortega et al., 2011). Interestingly, adult LPA_1-null mice undergoing chronic stress displayed enhanced hippocampal oxidative stress (García-Fernandez et al., 2012), an exacerbated corticosterone response to acute stressors (Pedraza et al. 2014) and significant reductions in both volume and neuronal density in the granular zone of the hippocampus

(Castilla-Ortega et al., 2011). Furthermore, the hippocampus of LPA_1-null mice exhibits a core of molecular abnormalities in the synaptic mechanisms at both the pre- and postsynaptic levels, with dysregulation of Ca^{2+}/calmodulin (CaM)-dependent kinase II (CaMKII) activity and phosphorylation (Musazzi et al., 2011), linked to elevated basal levels of glutamate release (Roberts et al., 2003) and altered glutamatergic synapsis and receptors (Musazzi et al., 2011; Blanco et al., 2012; Peñalver et al., 2019).

All these results have demonstrated that the absence of LPA_1 receptor induces profound abnormalities in the hippocampus, compromising the normal development of emotional and cognitive functions where this region is critically involved. Accordingly, behavioral studies have determined that mice lacking the LPA_1 receptor display altered exploration, increased anxiety-like behaviors, hypersensitivity to stress and impaired spatial memory in both the water maze and the hole-board test (Santín et al., 2009; Castilla-Ortega et al., 2010). LPA_1-null mice also exhibit deficits in episodic-like memory tasks (Castilla-Ortega et al., 2012), cocaine-induced place preference (Blanco et al., 2012), extinction of contextual fear memory (Pedraza et al., 2014) and prepulse inhibition of the startle reflex (Harrison et al., 2003).

To date, very few studies have tested the effect of central pharmacological modulation of LPA receptors in behavior, and most of them used acute administration. One of the first reports showed that the posttraining acute intrahippocampal infusion of low concentrations of 1-oleoyl-LPA (18:1 LPA) was able to improve spatial memory consolidation in the water maze task (Dash et al., 2004). In this line, the pretraining acute intracerebroventricular (ICV) injections of 18:1 LPA in rodents resulted in a reduction of the locomotor activity in the open field test, induced anxiety-like responses in the hole-board or elevated plus-maze test, impaired preference for novelty in the Y-maze, and an increase in immobility in the forced swimming test (Castilla-Ortega et al., 2014, Yamada et al., 2015). More recently, we demonstrated that acute central administration of LPA in mice increased hippocampal neurogenesis and weakened the acquired cocaine-associated memories (Ladrón de Guevara et al. 2019). In a related study, we have also reported that the continuous ICV delivery of LPA in chronically stressed animals potentiated the negative behavioral effects of stress, affecting the expression in genes encoding proteins involved in the excitatory/inhibitory balance in the ventral hippocampus (Moreno-Fernández et al., 2020).

Although different lines of research indicate that LPA, mainly through the LPA_1 receptor, regulates hippocampal function and behavior, there is currently no information available regarding the effect of the chronic central pharmacological modulation of the

LPA/LPA_1 receptor-signaling pathway on the hippocampal neurogenesis and behavior in a nonpathological state. Further considering that lipid phosphate phosphatase catalyzed hydrolysis of LPA resulting in functional inactivation at LPA receptors (Sciorra and Morris, 2002), it is necessary to minimize the effect of degradation of the LPA during continuous administration. Thus, the aim of the present study was to: (1) identify the behavioral effects of the continuous and chronic ICV administration of a stable and nonhydrolyzable LPA [1-(9Z-octadecenyl)-2-hydroxy-*sn*-glycero-3-phosphate (ammonium salt) (C18:1 LPA)] and the $LPA_{1\text{-}3}$-selective antagonist Ki16425 in the open field test, Y maze and forced swimming test, and (2) study the effects of those administrations in hippocampal neurogenesis. Moreover, we also studied the neuronal activation (c-Fos expression) in the hippocampal subfields dentate gyrus, CA1, and CA3, as well as the levels of the α-Ca2+/calmodulin (CaM)-dependent kinase II (α-CaMKII) and phospho-α-CaMKII, an intracellular protein involved in the regulation of glutamatergic transmission and neural maturation, which are critically affected in LPA_1-null mice (Musazzi et al., 2011).

2. Material and methods

2.1. Animals

All behavioral experiments were performed on 3-month-old male mice with a hybrid C57BL/6J x 129S1/SvImJ background (JAX stock #101045, The Jackson Laboratory, Bar Harbor, Maine, USA) (n = 40; 30 ± 2 g). Mice were housed under a 12-h light/dark cycle, with water and food provided *ad libitum*. Experiments were conducted in accordance with the ARRIVE guidelines and the European guidelines (European Council Directives 2010/63/UE and 90/219/CEE, Regulation (EC) No. 1946/2003) and Spanish national laws on laboratory animal welfare (Royal Decrees 53/2013 and 1386/2018, Law 32/2007) and approved by the Experimentation Ethics Committees of the University of Malaga and the Biomedical Research Institute of Málaga (CEUMA: 2012-0006-A; 2012-0007-A).

2.3. Drugs

C18:1 LPA [1-(9Z-octadecenyl)-2-hydroxy-*sn*-glycero-3-phosphate (ammonium salt), CAS Number 799279-68-8, Avanti Polar Lipids, Alabaster, AL, USA] and Ki16425 (CAS Number 355025-24-0, Selleck Chemicals LLC; Houston, TX, USA) were used for this study. Functional activity of nonhydrolyzable C18:1 LPA was evaluated by [^{35}S]GTPγS

functional autoradiography assay, allowing us to analyze the level of induced G protein stimulation. The [^{35}S]GTPγS binding assay measures the level of G protein activation following agonist occupation of a G protein-coupled receptor (GPCR) (Sim et al. 1997). The LPA_{1-3} antagonist Ki16245 was used as a control of the receptor specificity of the stimulations induced by C18:1 LPA (see Supplementary material).

2.3. Chronic pharmacological treatment

Mice were anesthetized under ketamine/xylazine anesthesia (80 mg/kg ip ketamine and 12 mg/kg ip xylazine (Sigma-Aldrich Co., St. Louis, MO, USA)) and positioned under a stereotaxic apparatus (Stoelting Co., Wood Dale, IL, USA). A 30-gauge stainless steel cannula was implanted into the left lateral ventricle of mice, using the following coordinates relative to the bregma and skull surface (Paxinos and Franklin, 2001): -0.34 mm antero-posterior, 2.3 mm dorso-ventral, +1.0 mm medio-lateral. The intracranial cannula was connected via polyethylene tubing to an Alzet® 1004 osmotic minipump (Durect Corporation, Cupertino, CA, USA). Minipumps were filled with vehicle (reservoir volume 100 µl/pump; 3% fatty acid-free bovine serum albumin (FAF-BSA) in saline; n = 10 mice), C18:1 LPA (0.036 µg/pump, 20 nM, n = 10 or 0.36 µg/pump, 200 nM, n = 10) and Ki16425 (0.75 µg/pump, 400 nM, n = 10) dissolved in vehicle. The minipumps were implanted subcutaneously, dorsal (left) to the scapulae, and the infusion was performed continuously over 21 days at a pumping rate of 0.11 µl/h. Under this flow rate, C18:1 LPA was dispensed at 0.93 ng/kg/day or 9.30 ng/kg/day, and Ki16425 at 20 ng/kg/day. The minipumps were weighed before and 21 days after implantation as a flow control (see Fig. 1 for general schedule). The functional activity of the LPA receptor agonist C18:1 LPA was confirmed in rodent brain slices using a [^{35}S]GTPγS autoradiography assay (see Supplementary Material).

2.4. Behavioral assays

At day 21, once the administration was concluded, the mice were sequentially subjected to open field (OF) test, Y-maze and forced swimming test (FST), with a 30-min interval between tests. Mice were handled and habituated to each experimental test in daily sessions of 5 min during the four days previous to the behavioral analysis. The analysis was performed using video tracking software (Ethovision XT, Noldus Information Technology bv., Wageningen, The Netherlands) for spatiotemporal parameters and observationally, when indicated.

Open field test (OF). OF was used as previously described (Santín et al., 2009) to assess the exploratory activity and the degree of anxiety-like behavior. The apparatus consisted

of a 50 cm × 50 cm area divided into central (8 cm × 8 cm) and peripheral areas with 38-cm-high walls. The mouse was placed in the center of the OF, and its behavior was recorded for 5 min. The following behaviors were assessed: locomotor activity (distance traveled), percentage of the time spent by the animal in the center of the maze, supported rearing (the mouse stood on its hind paws, with its forepaws on the walls), unsupported rearing (the mouse stood on its hind-paws stretching the forepaws up), grooming (licking, nibbling, and scratching of fur, limbs and tail, head-washing, and/or licking of genitals), risk assessment (stretched-attend posture with forward elongation of the head and shoulders toward the open arm, followed by retraction to the original posture) and defecation boli.

Y-maze test. This test was used to evaluate the spatial working memory on the basis of continuous spontaneous alternation behavior as previously described (Hughes, 2004). The Y-maze consisted of three equal arms (length 40 cm x height 14 cm x width 4.5 cm). The procedure involved allowing mice free access to the three arms of the maze for 8 min during which time sequences of entries of the arms were recorded. The following behaviors were analyzed for each mouse: locomotor activity (distanced traveled), arm entry sequence (triplets of explored arms assigning letters as arm codes) and the number of arm entries. Alternation was defined as triplets of explored arms and counted only if the mouse entered the three arms of maze (without revisiting the first arm at the third visit). The percentage of spontaneous alternation behavior (SAB) for each mouse was calculated as the ratio of successful overlapping alternations by the total possible triplets (defined as the total number of arm entries minus 2) multiplied by 100 (Hugues, 2004).

Forced swimming test (FST). A modified forced swimming test (FST), as described by Cryan et al. (2002), was used to study to assess depressive-like behavior. Mice were individually placed in glass cylinders (height 30 cm, diameter 10 cm) and subjected to swimming sessions in 23 ± 1ºC water with a height of 15 cm. The water of the tank was changed between each test. Each session had a duration of 6 min. The percentage of the time spent immobile (when the animal stopped struggling and moved only enough to remain floating in the water, keeping its head above water) and climbing (vigorous movements with forepaws in and out of the water, usually directed against the wall of the tank) were measured in the last four minutes.

2.5. Histological procedures

BrdU administration. Mice were treated with bromodeoxyuridine (BrdU) to label proliferating cells as previously described (Matas-Rico et al., 2008; Castilla-Ortega et al., 2011). Starting on day 17 after the implantation of the minipump, the mice were given a

daily intraperitoneal (IP) injection of BrdU (Sigma-Aldrich, Inc., St. Louis, MO, USA) at 100 µg/g of body weight and dissolved in saline, for three days (17-19). On day 20, three additional doses were administered every 3 h.

Immunohistochemistry and apoptosis determination. Animals were sacrificed at day 21 after the start of the infusion, 30 - 40 min following the last behavioral test. Six mice per group were intracardially perfused with 0.1 M phosphate-buffered saline pH 7.4 (PBS) and a periodate-lysine-paraformaldehyde solution (PLP, McLean and Nakane, 1974) in PBS as previously described (Matas-Rico et al., 2008; Castilla-Ortega et al., 2011). Brains were collected, fixed for 48 h in PLP and cryopreserved in 30% sucrose in PBS overnight at 4ºC. Each hemisphere was cut in coronal sections (50 µm width, coronal) using a freezing sliding microtome (HM 450, Thermo Fisher Scientific Inc., Waltham, MA, USA). Sections from the right hemisphere, i.e., contralateral to the side of infusion, were processed for immunohistochemistry using primary antibodies against BrdU (G3G4, mouse, 1:1000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA), doublecortin (DCX) (c-18, goat, 1:200, Santa Cruz Biotechnology Inc., Dallas, TX, USA), and c-Fos (sc-52, rabbit, 1: 2500, Santa Cruz Biotechnology). Rabbit anti-mouse, rabbit anti-goat, and swine anti-rabbit biotinylated secondary antibodies (Dako, Agilent Technologies, Inc., Santa Clara, CA, USA) were used as appropriate at a 1.800 dilution. Antibodies were detected using ExtrAvidin®-peroxidase (Sigma-Aldrich) and diaminobenzidine. Cell death was detected in right hemisphere sections by terminal UTP nick end labeling (TUNEL)-based procedures using NeuroTACS-II™ (Trevigen Inc., Gaithersburg, MD, USA), in accordance with the manufacturer's instructions.

Cell quantification was performed by a modified stereological method based on Kempermann et al. (2003) using an Olympus BX51 microscope equipped with an Olympus DP70 digital camera (Olympus, Glostrup, Denmark). Sections were arranged from rostral to caudal, covering the dorsal region of hippocampus, from -1.22 mm to -2.46 mm with respect to the bregma in the longitudinal axes. The total number of positive cells in the dentate gyrus was counted in one of every four (BrdU detection) or one of every eight (DCX, c-Fos, apoptotic cell detection) equally spaced sections of the hippocampus. The quantification of c-Fos immunoreactive cells was also carried out in CA3 and CA1 areas. Resulting numbers were multiplied by four or eight, as appropriate, to give an estimate of the total number of positively stained cells per hippocampus.

2.6. Western blotting

The expression levels of α-CaMKII and phospho-α-CaMKII were evaluated by western blotting. Four animals per group were sacrificed by decapitation after behavioral tests

and brain removal. The two hippocampi were dissected out and frozen independently in dry ice and stored at -80ºC until use. One right hippocampus per animal was homogenized in a buffer containing 125 mM NaCl, 20 mM HEPES pH 7.0, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol and a mixture of protease inhibitors (SigmaFAST™, Sigma-Aldrich). Homogenates were spun at 2,000 x g at 4ºC for 15 min, and the resulting supernatant was centrifuged at 100,000 x g at 4ºC for 1 h. The supernatant was aliquoted and stored at -20ºC. Samples containing 25 µg of protein as determined by Bradford assay (Sigma-Aldrich) were loaded in Laemmli buffer and separated on 12% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Trans-Blot® Mini PVDF Transfer Pack; Bio-Rad, Hercules, CA, USA) at 13 V for 20 min using the Trans-Blot® Turbo™ Transfer-System (Bio-Rad). The membranes were blocked by 1-h incubation at room temperature in Tris-buffered saline containing 3% nonfat dry milk, 0.1% Tween-20 and 1% bovine serum albumin. The membranes were exhaustively washed and incubated overnight at room temperature with the following primary antibodies: anti-α-CaMKII (M-176, rabbit, 1:1000, Santa Cruz), anti-phospho-α-CaMKII (Thr 286) (rabbit, 1:1000, Santa Cruz), and mouse anti-GAPDH (D-6, mouse, 1:1000, Santa Cruz). The immunoreaction was visualized with standard procedures using a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Bio-Rad) and 4-chloro-1-naphthol (Opti4CN™, Bio-Rad) in accordance with the manufacturer's protocol. Protein levels were normalized to GAPDH. Quantifications of proteins were carried out with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MA, USA, https://imagej.nih.gov/ij/, 1997-2018).

2.7. Statistical analyses

Data from behavioral, immunohistochemistry and western blotting studies were analyzed using a one-way ANOVA followed by Newman-Keuls post-hoc test for intergroup comparisons. Only probabilities less than 0.05 were considered significant. Subsequently, a principal components factorial analysis (PCA) was performed on the OF variables to reduce them to a smaller set of dimensions that would underlie animals' behavior (Budaev, 2010; Castilla-Ortega et al., 2010, 2013). The correlation matrix of the whole sample of subjects (n = 40) was used for the analysis. PCA was followed by a varimax orthogonal rotation, which ensures that the extracted factors are independent of one another. The resulting factors with eigenvalues > 1 were selected, and the contribution of each behavioral variable to a factor (i.e., 'factor loading') was considered significant when it was more than 0.4 in absolute value. Finally, factor scores were

calculated for each animal by the regression method, and the results were analyzed using a one-way ANOVA followed by Newman-Keuls post-hoc test.

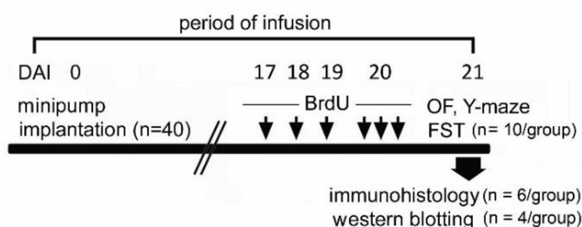


Fig. 1. General schedule of the experimental design. Osmotic minipumps with vehicle, C18:1 LPA and Ki16425 were stereotaxically implanted in the right lateral ventricle of the mice. Seventeen days after starting the infusion (DAI), BrdU was IP administered for four days consecutively (time points indicated by broken arrows). Then, 30-40 min after completing the behavioral analysis, at 21 DAI, two groups of animals were sacrificed and analyzed using immunohistochemistry and western blotting. OF, open field; FST, forced swimming test.

3. Results

3.1. Chronic administration of C18:1 LPA and Ki16425 differentially modulated exploration and anxiety-like behavior in the OF test.

The exploratory behaviors of mice were assessed in the OF test. ICV infusion of vehicle, C18:1 LPA and Ki16425 for 21 days induced behavioral changes in the OF test depending on the specific pharmacological treatment. One-way ANOVA indicated that mice treated with either 20 nM or 200 nM of C18:1 LPA showed increased locomotor activity compared with the control group ($F_{(3,36)} = 6.401$, $P \leq 0.01$; Newman-Keuls post-hoc test, $P = 0.010$ and $P = 0.024$, respectively) (Fig. 2A). In contrast, Ki16425 administration did not result in a significant change in locomotion compared with the vehicle group (Newman-Keuls test, $P > 0.05$). In addition, mice treated with C18:1 LPA did not differ significantly from vehicle-treated mice in the percentage of time spent in the central area of the OF ($F_{(3,36)} = 5.980$, $P \leq 0.01$; Newman-Keuls test, $P > 0.05$; Fig. 2B). However, the administration of the antagonist Ki16425 resulted in a significant reduction of the percentage of time spent by the mice in the central area of the OF when compared with the control group (Newman-Keuls test, $P = 0.043$; Fig. 2B). Furthermore, ethological measures revealed increased grooming ($F_{(3,36)} = 4.264$, $P \leq 0.05$; Newman-Keuls test, $P = 0.009$), risk assessment ($F_{(3,36)} = 9.323$, $P \leq 0.001$; Newman-Keuls test, $P = 0.001$) and defecation ($F_{(3,36)} = 2.342$, $P \leq 0.05$; Newman-Keuls test, $P = 0.046$) in Ki16425-treated

mice, supporting an anxiety-like effect induced by this antagonist (Table 1). In contrast, these parameters were unaltered in response to both 20 nM and 200 nM C18:1 LPA infusion, but not the unsupported rears, which were significantly increased ($F_{(3,36)}$ = 11,917, $P \leq 0.0001$; Newman-Keuls test, $P = 0.001$, $P = 0.0003$, respectively; Table 1).

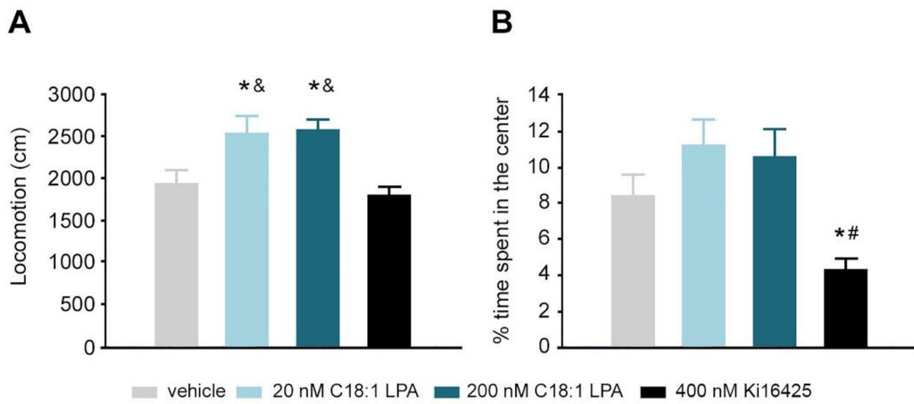


Fig. 2. Effect of 21 days of treatment with C18:1 LPA (20 nM and 200 nM) and Ki16425 (400 nM) on locomotor activity and anxiety-like behavior in the OF test. (A) The total distance moved (locomotion) increased in mice treated with both doses of C18:1 LPA, but not in those treated with the LPA_{1-3} receptor antagonist Ki16425. (B) The percentage of time spent in the central area of the OF was significantly reduced only in mice treated with Ki16425. Data are represented as the means ± SEM (10 animals per group). Data were analyzed through one-way ANOVA followed by the Newman-Keuls test. $^{*}P < 0.05$ compared with the control group; $^{\&}P < 0.05$ compared with the Ki16425 group; $^{\#}P < 0.05$ compared with the C18:1 LPA groups.

OF behavior					
Treatment	Sup.rearing	Uns. rearing	Grooming	Risk assessment	Defecation
Control	32.3 ± 3.5	2.4 ± 0.6	2.0 ± 0.7	2.9 ± 0.8	1.9 ± 0.6
20 nM C18:1 LPA	33.4 ± 4.2	10.1 ± 2.3 *&	1.9 ± 0.5	1.9 ± 0.5	1.7 ± 0.6
200 nM C18:1 LPA	25.9 ± 3.9	12.3 ± 1.8 *&	1.8 ± 0.5	1.6 ± 0.6	1.8 ± 0.4
400 nM Ki16425	23.9 ± 1.5	1.6 ± 0.4	5.1 ± 1.2 *#	6.4 ± 0.9 *#	3.3 ± 0.6 *

Table 1. Examined behavior in the OF test after 21 days of pharmacological treatment. Mice treated with C18:1 LPA exhibited an active behavior, increasing unsupported rearing (Uns. rearing). Mice treated with Ki16425 increased grooming, stretched-attend posture (risk assessment), and defecation. The table presents the number of behavior bouts per minute, except for defecation, indicated as the total number of boli in the arena at the end of the test. All data are expressed as the mean values ± SEM (10 animals per group). Data were examined by one-way ANOVA followed by the Newman-Keuls test. $^{*}P < 0.05$ compared with the control group; $^{\&}P < 0.05$ compared with the Ki16425 group; $^{\#}P < 0.05$ compared with the C18:1 LPA groups.

The relationship among the behaviors exhibited by animals in the OF was studied by a PCA. PCA followed by a varimax rotated solution yielded 2 independent factors with eigenvalues > 1 (Table 2). The variables best contributing to each factor were those loaded high (> 0.4) in that factor but low in the other. The first factor was composed of percentage of time spent in the central area of the OF, locomotor activity (negative), grooming, risk assessment and defecation (positive), which likely represented 'Anxiety' (Castilla-Ortega et al., 2010, 2013). The second factor was composed of locomotor activity, unsupported rearing, supported rearing (positive) and risk assessment (negative). It was named 'Exploration' because it suggested a dimension of general activity and investigation of the environment. Therefore, the measures best representing Anxiety were the time spent in the central area, grooming, and defecation, while the rearing measures purely represented exploratory activity that was independent of anxiety-like behavior. The measures of locomotion and risk assessment, instead, could be influenced both by Anxiety and Exploration. Finally, the factor scores were calculated and compared among groups. One-way ANOVA revealed differences in both Exploration ($F_{(3,36)} = 6.226$, $P \leq 0.01$) and Anxiety ($F_{(3,36)} = 7.267$, $P \leq 0.001$) among the groups. Newman-Keuls post-hoc test revealed that the treatment with Ki16425 increased Anxiety ($P = 0.031$), by comparison with the control group, and reduced Exploration, when compared with the LPA groups ($P = 0.001$ and $P = 0.004$, respectively). The treatments with C18:1 LPA showed a tendency to increase Exploration and reduce Anxiety (Fig. 3) in agreement with their OF data concerning locomotion and rearing (Fig. 2, Table 1).

Variable	Factor 1: Anxiety	Factor 2: Exploration
Time spent in the center	*-0.877*	0.008
Locomotion	*-0.541*	*0.405*
Unsupported rearing	-0.245	*0.661*
Supported rearing	0.167	*0.742*
Grooming	*0.502*	-0.356
Risk assessment	*0.502*	*-0.522*
Defecation boli	*0.740*	-0.003
Variance explained	38.05%	15.403%
Eigenvalues	2.664	1.078

Table 2. Behavioral factors resulting from the Principal Component Analysis (PCA) for the OF test. The PCA was followed by a varimax rotation to ensure that the extracted factors were independent of one another. The importance of a factor to explain the total variance was explored by means of the eigenvalues, so only factors with eigenvalue > 1 were selected. A variable was considered as included in a factor when its loading was > 0.4 in absolute value (shown in italics).

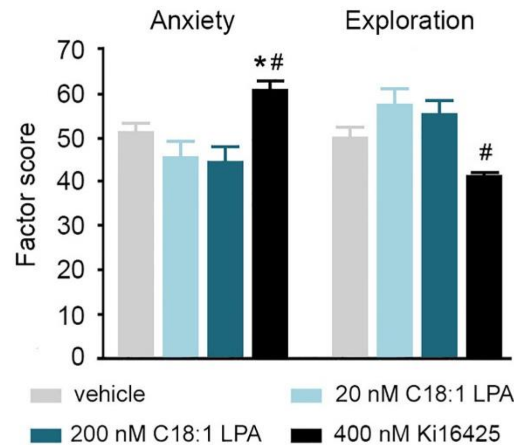


Fig. 3. PCA factorial scores in the OF test. Ki16425 treatment increased Anxiety and reduced Exploration. Data are represented as the mean values ± SEM (10 mice per group). Data were analyzed by one-way ANOVA followed by the Newman-Keuls test. *P < 0.05 compared with the control group; #P < 0.05 compared with the C18:1 LPA groups.

3.2. Chronic administration of C18:1 LPA and Ki16425 induced opposite effects in the Y-maze.

Spontaneous alternation behavior in the Y-maze is widely used to study the spatial working memory in rodents (Hughes, 2004). In our study, the one-way ANOVA test revealed significant differences among groups in the spontaneous alternation displayed in the Y maze ($F_{(3,36)}$= 7.124, $P \leq 0.01$; Fig. 4A). Administration of 20 nM and 200 nM C18:1 LPA induced an increase in alternation performance compared with the control group (Newman-Keuls test, $P = 0.030$ and $P = 0.036$, respectively; Fig. 4A). In contrast, Ki16425 treatment impaired performance on the Y-maze, and mice exhibited a lower percentage of SAB than the control mice (Newman-Keuls test, $P = 0.006$). Furthermore, no differences were found in locomotion assessed as the total distanced travelled ($F_{(3,36)}$ = 1.094, $P > 0.05$; data not showed), supporting that the reported effects induced by C18:1 LPA and Ki16425 were due to the modulation of the spatial working memory.

3.3.Chronic administration of C18:1 LPA and Ki16425 exerted opposite effects in the FST.

FST is the most extensively used tool for assessing the antidepressant activity of drugs. FST immobility is assumed to reflect depressive-like behavior either as behavioral

despair or passive coping (Porsolt et al., 2000). The effects of treatment with C18:1 LPA and Ki16425 on immobility in the FST are shown in Fig. 4B. Our results indicate that chronic ICV infusion of C18:1 LPA and Ki16425 exerted opposite effects in the performance of the animals in the FST. One-way ANOVA revealed a significant difference in the percentage of immobility among groups ($F_{(3,36)}$ = 19.173, $P \leq 0.0001$; Fig. 4). C18:1 LPA-treated groups significantly decreased immobility compared to vehicle, as revealed by Newman-Keuls test ($P = 0.020$ and $P = 0.001$, for 20 nM and 200 nM C18:1 LPA, respectively), while Ki16425-treated mice increased this behavior (P = 0.001). Correspondingly, the reduction of immobility in the 20 nM and 200 nM C18:1 LPA groups were accompanied by active coping and increased climbing behavior ($F_{(3,36)}$ = 4.4061, $P \leq 0.01$; Newman-Keuls test, $P = 0.031$ and $P = 0.023$; not showed).

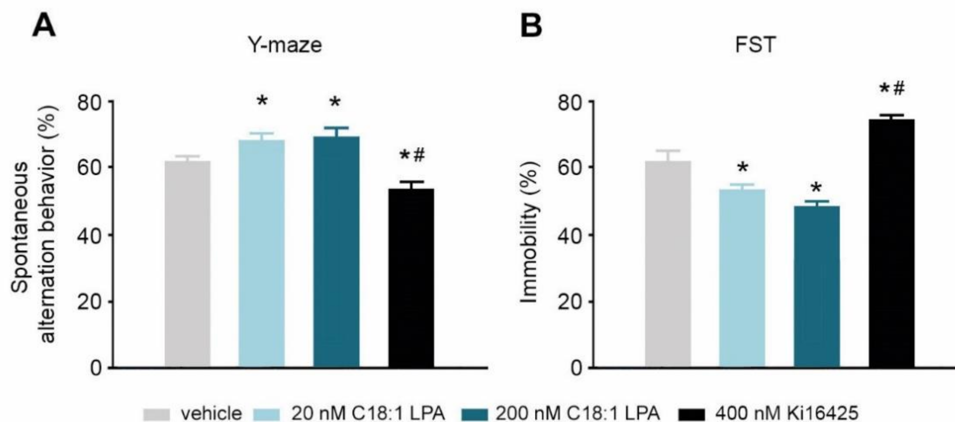


Fig. 4. Effect of treatment with C18:1 LPA (20 nM and 200 nM) and Ki16425 (400 nM) on Y-maze and FST. (A) Percentage of spontaneous alternation in the Y-maze. Spontaneous alternation increased in C18:1 LPA-treated mice and decreased in Ki16425-treated mice. (B) Percentage of immobility in the FST. Both doses of C18:1 LPA caused reductions in immobility. In contrast, Ki16425-treated mice increased the percentage of immobility. Data are represented as the mean values ± SEM (10 animals per group). Data were analyzed by one-way ANOVA followed by the Newman-Keuls test. $^{*}P < 0.05$ compared with vehicle; $^{\#}P < 0.05$ compared with the C18:1 LPA groups.

3.4. Chronic administration of C18:1 LPA and Ki16425 induced different effects on adult hippocampal neurogenesis.

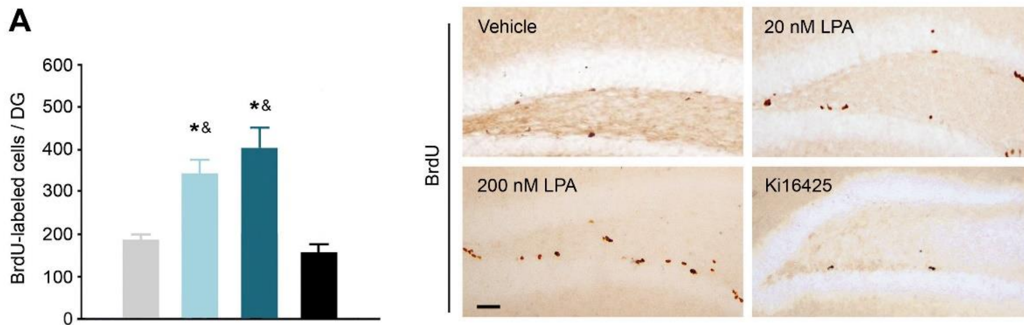
Despite the fact that previous studies highlighted different aspects of LPA regulating hippocampal function and behavior, none of them focused on the effect of the chronic central modulation of the LPA/LPA_1 receptor-signaling pathway, even more using a stable LPA, on the regular hippocampal neurogenesis and behavior. We analyzed the

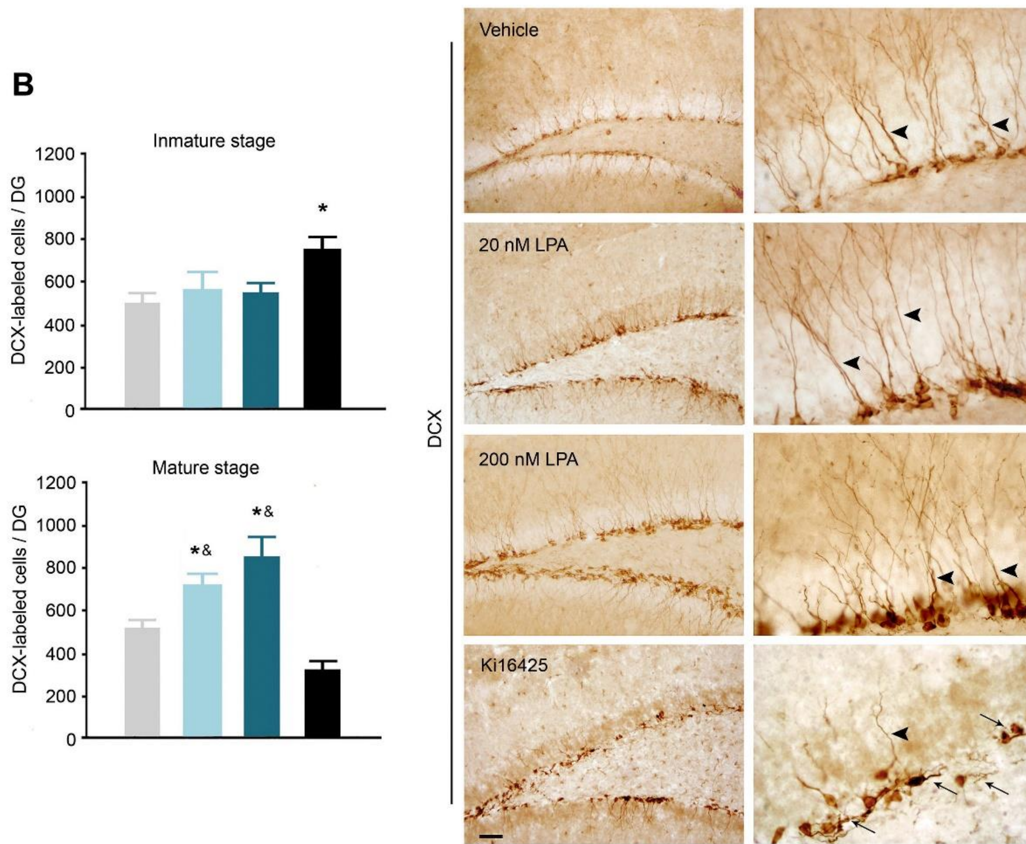
effect of chronic central administration on adult hippocampal neurogenesis and confirmed that both C18:1 LPA and the LPA_{1-3} receptor antagonist Ki16425 were able to modulate hippocampal neurogenesis in opposite ways to each other. Analysis of the number of BrdU-labeled cells in the dorsal hippocampus showed that C18:1 LPA-treated mice (20 nM and 200 nM) significantly increased cell proliferation in the dentate gyrus compared to the control (approximately 50%) ($F_{(3,20)} = 7.092$, $P \leq 0.01$; Newman-Keuls test, $P = 0.037$ and $P = 0.011$, respectively; Fig. 5A). In contrast, the number of BrdU-labeled dentate cells after treatment with Ki16425, although not differing significantly from the control ($P > 0.05$), showed a tendency to decrease (Fig. 5A).

Newly born neurons (DCX immunoreactive cells) in the adult DG also exhibited treatment-dependent differences. In adult hippocampal neurogenesis, DCX is transiently expressed during a period that extends from a proliferative progenitor cell phase to a postmitotic stage with extended dendrites. DCX-expressing cells exhibit a range of morphologies reflecting neuronal differentiation (Plümpe et al., 2006). Accordingly, DCX-immunoreactive cells were classified into two populations as previously described (Plümpe et al., 2006; Beauquis et al., 2010; Castilla-Ortega et al., 2011). Therefore, the immature population corresponded to DCX-expressing cells with absent or short dendritic processes, while the mature population corresponded to at least one long vertical strong dendrite, clearly penetrating the granular cell layer, as well as subtle dendritic tree branching. The chronic treatment with C18:1 LPA (20 nM and 200 nM) increased the population of newly born neurons in the adult DG (DCX immunoreactive cells) with mature morphology ($F_{(3,20)} = 7.803$, $P \leq 0.01$; Newman-Keuls test, $P = 0.046$ and $P = 0.010$, accordingly; Fig. 5B). Interestingly, Ki16425-treated mice displayed a significant increase of the number of DCX-expressing cells with immature morphology ($F_{(3,20)} = 3.403$, $P \leq 0.05$; Newman-Keuls test, $P = 0.025$; Fig. 5B), but this effect was not observed on those with mature morphology which, conversely, tended notably to be reduced (Fig. 5C).

In parallel, cell death as assessed by TUNEL procedure revealed differences in the group of mice chronically treated with Ki16425 ($F_{(3,20)} = 3.196$, $P \leq 0.05$), with enhanced apoptosis in the DG in comparison with the control group (Newman-Keuls test, $P = 0.046$) and with the C18:1 LPA groups ($P = 0.012$ and $P = 0.026$, for 20 nM and 200 nM C18:1 LPA treatments, respectively) (Fig. 5C). This augmented apoptosis may reflect death of the adult newly born neurons, considering that most of the apoptotic nuclei were localized in the neurogenic zone of the DG, i.e., the subgranular zone.

vehicle 20 nM C18:1 LPA 200 nM C18:1 LPA 400 nM Ki16425





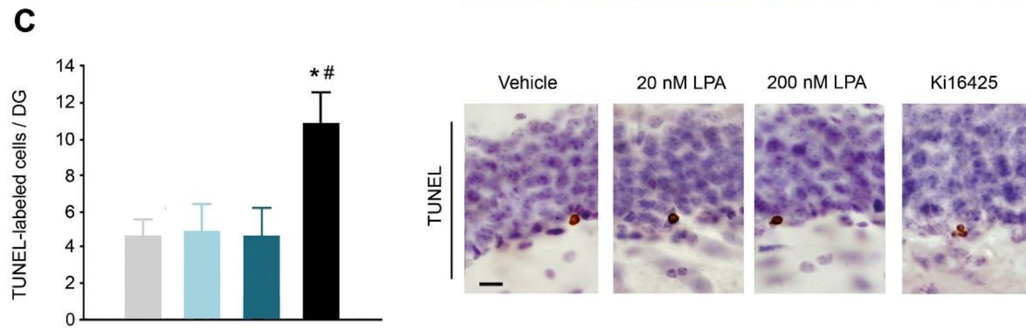


Fig. 5. Effects of chronic treatment with C18:1 LPA (20 nM and 200 nM) and Ki16425 (400 nM) on adult neurogenesis in the dentate gyrus (DG) at the dorsal region of the hippocampus. (A) Total number of DG cells expressing BrdU and corresponding representative images of the immunostaining for BrdU in coronal sections of the adult hippocampal DG of treated mice. (B) Total number of DCX-expressing DG cells classified according to maturation stage and corresponding representative images (magnified at right) of the DCX immunostaining in the adult DG of treated mice. Arrowheads indicate characteristic strong dendrites at the maturing postmitotic stage, evident in vehicle- and LPA-treated mice, and atypical in Ki16425-treated mice. Arrows indicate the aberrant organization of newborn neurons and dendritic arborization observed after treatment with the antagonist Ki16425. (C) Total number of dentate TUNEL-labeled cells and representative images of the apoptotic nuclei in the adult DG of treated mice. Scale bars: (A) 100 μm; (B) 100 μm (left panel) and 20 μm (right panel); (C) 20 μm. All data are represented as the mean values ± SEM (6 animals per group). Data were analyzed through one-way ANOVA followed by the Newman-Keuls test. $^{*}P < 0.05$ compared with the control group; $^{\&} P < 0.05$ compared with the Ki16425 group; $^{\#}P < 0.05$ compared with the C18:1 LPA groups.

3.5. Effects of chronic administration of C18:1 LPA and Ki16425 on α-CaMKII and phospho-α-CaMKII levels.

Deletion of LPA_1 in mice results in abnormalities of glutamatergic transmission at the both pre- and postsynaptic levels in the hippocampus (Roberts et al., 2003; Musazzi et al., 2011; Blanco et al., 2012; Peñalver et al., 2019), including the dysregulation of the activity and phosphorylation of postsynaptic α-Ca2+/calmodulin (CaM)-dependent kinase II (α-CaMKII) (Musazzi et al., 2011), involved in the regulation of glutamatergic transmission and neural maturation. Given of the effect of chronic treatment with C18:1 LPA and Ki16425 on hippocampal neurogenesis and behavior, we also analyzed their influence on α-CaMKII and phospho-α-CaMKII levels in the hippocampus by western blot analysis. ANOVA revealed significant changes for α-CaMKII ($F_{(3,12)} = 11.150$, $P \leq 0.001$). As revealed by Newman-Keuls post-hoc test, a significant reduction in comparison with control was present for the expression levels of α-CaMKII in the hippocampus of mice treated with Ki16425 ($P = 0.0002$; Fig. 6), with no changes in the rest of the groups. Albeit the treatment with 200 nM C18:1 LPA tended to slightly increase the phosphorylation levels in α-CaMKII, no significant differences were found between any of the groups ($F_{(3,12)} = 0.625$, $P > 0.05$; Fig. 6). These results revealed a requirement for LPA signaling for the right expression of α-CaMKII, in line with previous observations (Mussazzi et al., 2020), and suggest its role in plasticity maintenance rather than induction, given the absence of effects on α-CaMKII phosphorylation (Irvine et al., 2005; Chang et al., 2017).

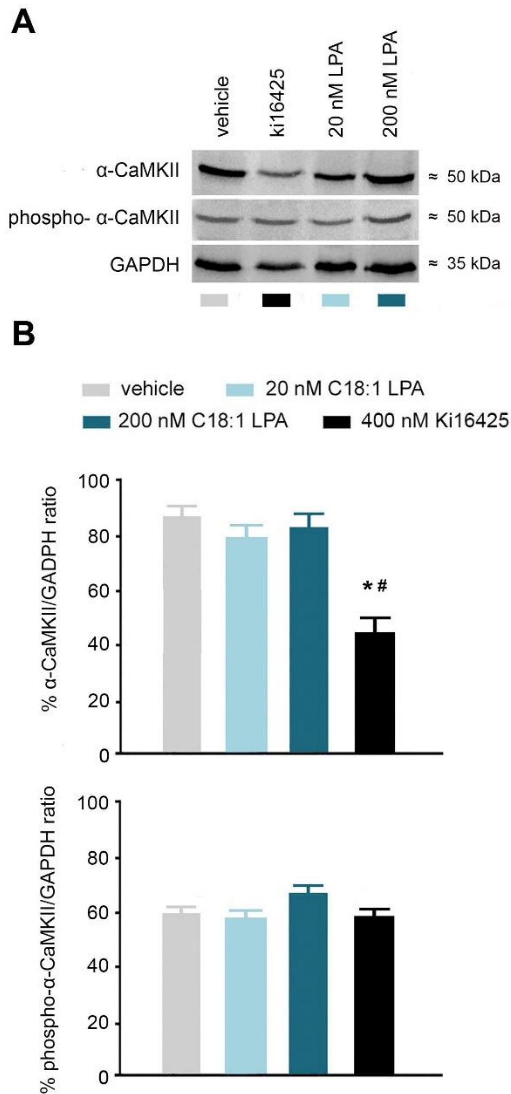


Fig. 6. Effects of treatment with C18:1 LPA (20 and 200 nM) and Ki16425 (400 nM) on α-CaMKII and phospho-α-CaMKII expression in the hippocampus. Representative western blot (A) and quantifications after normalizing with GAPDH (B). The percentage of α-CaMKII levels was lower in mice treated with Ki16425 than in the rest of the groups (B). Phospho-α-CaMKII levels were not affected by the pharmacological treatments (C). Data are represented as the means ± SEM (4 animals per group). Analyses were performed by one-way ANOVA followed by the Newman-Keuls test. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the C18:1 LPA groups.

3.6. Neuronal activation of hippocampal regions was differently modulated by C18:1 LPA and Ki16425.

Neuronal activation was studied by the expression of the immediate early gene c-*fos* in the three main regions of the hippocampus (DG, CA1 and CA3). The analysis of neuronal

activity revealed distinctive neuroadaptations after each treatment. One-way ANOVA showed that both 20 nM and 200 nM concentrations of C18:1 LPA increased the total number of c-Fos-labeled cells in the DG ($F_{(3,20)} = 9.592$, $P \leq 0.01$; Newman-Keuls test, $P = 0.009$ and $P = 0.012$, respectively), not differing from control treatment in CA1 ($F_{(3,20)} = 5.045$, $P \leq 0.01$; Newman-Keuls test, $P > 0.05$) or CA3 ($F_{(3,20)} = 7.028$, $P \leq 0.01$; Newman-Keuls test, $P > 0.05$) regions of the hippocampus (Fig. 7). In contrast, mice treated with Ki16425 displayed reductions of the total numbers of c-Fos-expressing cells in CA1 ($F_{(3,20)} = 5.045$, $P \leq 0.01$; Newman-Keuls test, $P = 0.040$) and CA3 ($F_{(3,20)} = 7.028$, $P \leq 0.01$; Newman-Keuls test, $P = 0.008$) areas, but not in the DG ($F_{(3,20)} = 9.592$, $P \leq 0.01$; Newman-Keuls test, $P > 0.05$) (Fig. 7).

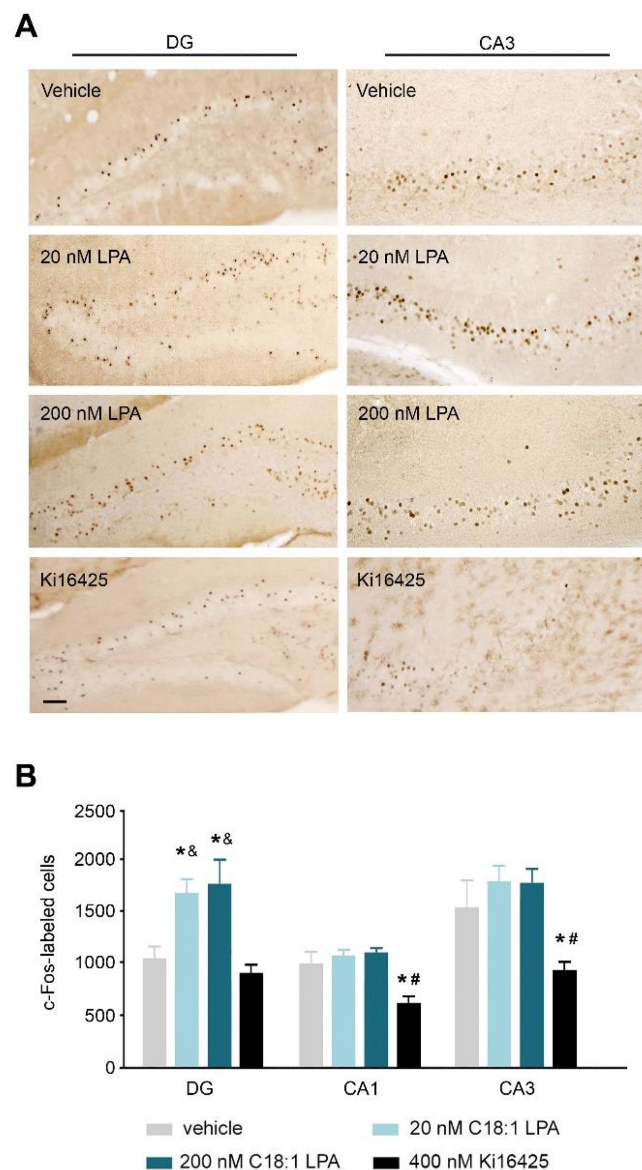


Fig. 7. Effects of treatment with C18:1 LPA (20 and 200 nM) and Ki16425 (400 nM) on c-Fos expression in the dentate gyrus (DG), CA1 and CA3 areas of the hippocampus. (A) Representative

images of the immunostaining for c-Fos expression in coronal sections of the hippocampal DG and CA3 areas of treated mice. (B) Total numbers of c-Fos-labeled cells in the hippocampal DG, CA1 and CA3 areas of treated mice. Mice treated with C18:1 LPA increased the expression of c-Fos in the hippocampal DG, while Ki16425-treated mice drastically reduced the numbers of c-Fos-expressing cells in both the CA3 and CA1 subfields. Scale bar: 200 µm. All data are represented as the mean values ± SEM (6 animals per group). Data were analyzed through one-way ANOVA followed by the Newman-Keuls post-hoc test. $^{*}P < 0.05$ compared with the control group; $^{\&}P < 0.05$ compared with the Ki16425 group; $^{\#}P < 0.05$ compared with the C18:1 LPA groups.

4. Discussion

Here, we provide information about the effects of the chronic pharmacological modulation of the LPA/LPA receptors-signaling pathway on hippocampal function and behavior. In particular, the chronic manipulation of the LPA receptors appears to affect exploratory activity, emotional regulation and spatial working memory. These effects were accompanied by changes in hippocampal neurogenesis, neuronal activation of the hippocampal subfields DG, CA3 and CA1, and α-CaMKII and phospho-α-CaMKII levels. In this work, we used a nonhydrolyzable LPA to minimize the effect of degradation of the LPA during continuous administration (Sciorra and Morris, 2002). Nonhydrolyzable C18:1 LPA has been previously used by other authors in in vitro studies on cell lines, as an activator of the RhoA/ROK pathway (Murányi et al., 2005), or in in vivo analysis for continuous ICV delivery in chronically stressed mice, as we have recently reported (Moreno-Fernández et al., 2020). The functional autoradiography analysis (supplementary material) confirmed the anatomical localization of the C18:1-induced LPA receptor activity and its pharmacological inhibition by Ki16425, in agreement with previous functional studies in rodents (González de San Román et al., 2015, 2019). Potentially, C18:1 LPA can activate all the LPA receptor subtypes expressed in the brain. Similarly, Ki16425 can block both LPA_1 and LPA_3 receptors and, with a lesser affinity, at higher concentration than that used here, the LPA_2 receptor. However, while the expression of LPA receptors in adult brain has been determined (reviewed in Choi et al., 2010), no data support the involvement of $LPA_{2\text{-}4}$ receptors in behavior. Thus, the present results, together with data accumulated from LPA_1-null mouse studies, reinforce the assumption that C18:1 LPA, most likely mainly through the LPA_1 receptor, regulates hippocampal-dependent behavior and functions.

Behaviorally, animals chronically treated with Ki16425 showed decreased exploratory activity and anxiogenic-like behavior in the OF test, indicative of high emotional arousal (Castilla-Ortega et al., 2014). Impaired exploration could be a consequence of

concomitant anxiety levels during the test (Kameda et al., 2007; Ramos and Mormede, 1998). However, PCA confirmed the independence of exploration and anxiety-related factors. In addition, the treatment with C18:1 LPA induced, at least partially, an anxiolytic response, facilitating locomotor activity in the OF test and increasing the unsupported rearing, a hippocampus-dependent behavior that increases if the environment becomes safer/less aversive (Sturman et al., 2018). In the forced swimming test, C18:1 LPA also induced a concentration-dependent reduction of the immobility, while opposite effects were seen upon treatment with Ki16425. The FST estimates behavioral despair in stressful and inescapable situations, reflecting depression-like behavior, and it is widely used as a screening test to assess the antidepressant effects of drugs (Porsolt et al., 2000; Castagné et al., 2011). This premise implies that, because of their effects on immobility, the chronic pharmacological modulation by C18:1 LPA could have potential antidepressant properties, whereas Ki16425 abolished the signal from receptor pathways inducing depression-like behavior. These results are consistent with our previous observations describing a comorbid phenotype between depression and anxiety-like behaviors in LPA_1-null mice (Moreno-Fernández, 2017, 2018). The interpretation of active versus passive coping strategies has been a topic of discussion over the last years. We cannot ignore the increasingly accepted argument that qualifies the rodent's floating response as the expression of a coping strategy through an adaptive learned response rather than a depressive-like behavior (Molendijkt and De Kloet, 2019). However, we should be cautious in this sense, not least considering that escape-directed behaviors could be driven by anxiety (Anyan and Amir, 2018).

Moreover, C18:1 LPA modified the percentage of SAB in the Y-maze. SAB is dependent on optimal levels of anxiety, as the rates decrease in mice with higher levels of anxiety (Bats et al., 2001). The enhanced anxious-like behavior under novel conditions observed in Ki16425-treated mice could explain the reduction of SAB. However, this behavior is also dependent on spatial working memory and relies on the need to remember which of the maze arms was recently visited and to allow animals to alternate their choice on a following entry. Pharmacological manipulation could primarily modify memory processes that are reflected by changes in the percentage of SAB (Hughes, 2004). Thus, chronic stimulation of the LPA receptors with C18:1 LPA was followed by an increment in the percentage of SAB without concomitant changes of anxiety levels during the OF test, supporting the effects of the pharmacological modulation in spatial working memory.

The LPA_1 receptor, mainly in the hippocampus, is a strong candidate to mediate the central effects induced by C18:1 LPA and Ki16425 on anxiety, emotional regulation, and spatial working memory. The LPA_1 receptor activity is located in the hippocampus,

among other structures, in accordance with present and previously reported data (Gonzalez San Roman et al., 2015, 2019). Moreover, unlike LPA_{2-4} receptors, the LPA_1 receptor has been extensively involved in behavior and hippocampal-dependent tasks (Santín et al. 2009; Castilla-Ortega et al., 2010, 2011, 2012; Musazzi et al., 2011; Moreno-Fernández et al., 2017, 2018; Peñalver et al., 2019). Taking into account that the dose of drug used in our study is near the Ki value (Ohta et al., 2003) and considering that, in the presence of the antagonist, the increment of [^{35}S]GTPγS induced by C18:1 LPA is blocked, we could infer that, in our model, the behavioral effects of the pharmacological treatment are specifically mediated by the LPA_1 receptor, likely in the hippocampus, a key substrate for regulating the behavioral processes assessed in our study (Lalonde et al., 2002).

It is intriguing to note that treatment with Ki16425 mimics the behavioral deficits of LPA_1-null mice, supporting that the LPA_1 receptor is involved in memory and emotion. In keeping with these deficiencies, animals lacking the LPA_1 receptor have a behavioral phenotype consistent with hippocampal dysfunction, deficit in spatial working memory, anxiety-like responses, and reduced exploratory activity after exposure to a novel environment (Santín et al. 2009; Castilla-Ortega et al., 2010). LPA_1-null mice also exhibited emotional deregulation (Pedraza et al., 2014) and dysfunctional coping of chronic stress (Castilla-Ortega et al., 2011). We have also reported the involvement of this receptor in additional hippocampal-dependent memories such as spatial learning (Santin et al., 2009; Castilla-Ortega et al., 2010), episodic-like memory tasks (Castilla-Ortega et al., 2012), drug-context association (Blanco et al., 2012, Ladrón de Guevara et al., 2019) and extinction of contextual conditioned fear (Pedraza et al., 2014).

Nevertheless, while studies using mice lacking the LPA_1 receptor support our findings, some, though not all, of the pharmacological studies produced apparently contradictory results. Therefore, whereas acute ICV infusion of 18:1 LPA was able to improve spatial memory consolidation in the water maze (Dash et al., 2004) and posttraining ICV administration of Ki16425 prevented the extinction of contextual fear-conditioned memory (Pedraza et al., 2014), anxiogenic changes and emotional deregulation after acute 18:1 LPA infusion were also reported (Castilla-Ortega et al., 2014; Yamada et al., 2015). Thus, acute ICV injections of 18:1 LPA reduced locomotor activity in the OF test and induced anxiety-like responses in the elevated plus maze, impaired preference for novelty in the Y-maze, and increased the immobility in the FST (Castilla-Ortega et al., 2014). Similarly, acute ICV 18:1 LPA infusion in mice showed increase anxiety-like behaviors in both the elevated plus maze and the hole-board test (Yamada et al., 2015). Those apparently inconsistent findings might most likely be attributed to differences in

the procedures applied, with different patterns of acute administration, and accentuate the necessity of studies, as shown here, covering the effect of the chronic pharmacological modulation of the LPA/LPA receptors-signaling pathway. Compared to the concentration used here, the previous studies from Castilla et al. (2014) and Yamada et al. (2015) utilized considerably higher concentrations of LPA, introducing the possibility of the occurrence of internalization (Murph et al., 2003; Mirendil et al., 2015) or the involvement of other LPA receptors. As far as we know, there is no evidence supporting a role for LPA_{2-4} receptors in behavior. Nonetheless, although the expression of the LPA_5 receptor remains to be determined, the possible participation of this receptor in emotion has been suggested, given the anxiolytic-like phenotype exhibited by the LPA_5-null mice (Callaerts-Vegh et al., 2012).

With exceptions, most strategies to disrupt neurogenesis induce reminiscent effects of either anxiety or depression. Moreover, neurogenesis is required for at least some of the beneficial effects of antidepressants (reviewed by Lino de Oliveira et al., 2020). To the best of our knowledge, only one study tested the negative consequences of pharmacological depletion of hippocampal neurogenesis in spontaneous alternation (Hazane et al., 2009). Since chronic manipulation of the LPA receptors induced neurogenic changes, it is possible that at least some of the behavioral consequences could be the result of the neurogenic effects. The chronic stimulation with C18:1 LPA not only produced an increment of cell proliferation but also promoted the differentiation and dendritic development of the newly born cells. In Ki16425-treated mice, no changes could be observed in cell proliferation. However, in accordance with our previous observations in LPA_1-null mice, the antagonist induced an impairment in maturation, reflected in part by the prevalence of neurons with diminished arborization (Matas-Rico et al., 2008; Castilla-Ortega 2011), and an increase of apoptosis, probably due to the former. Considering that apoptosis is detected in a limited timeframe (Elmore et al., 2007) and that newly born neurons may die long before the apoptosis detection, after 21 days of pharmacological treatment, apoptosis might also be underestimated in the antagonist-treated animals. Cell proliferation and apoptosis are critical factors in regulating adult neurogenesis facilitating cellular remodeling (Kim et al., 2009). In this scenario, an incremented apoptosis along with altered differentiation, as observed in the antagonist group, could affect the incorporation of the newborn cells into pre-established mature hippocampal circuits inducing their functional impairment. In contrast, continuous C18:1 LPA infusion could facilitate the integration of the new neurons into the hippocampal circuit, “fine-tuning" this process and improving its function.

Considering that the α isoform of CaMKII is deregulated in hippocampal synaptosomes from LPA_1-null mice (Musazzi et al., 2011) and its key roles in the modulation of synaptic activity, neurogenesis, neuronal maturation and neuroplasticity (Lisman et al., 2002), it is not surprising that the effects of the pharmacological modulation of LPA receptors are associated with changes of expression between inactive/active forms of α-CaMKII. In the adult hippocampus, α-CaMKII contributes to glutamatergic synaptic structural and functional plasticity (reviewed in Robison, 2014), which is a crucial survival signal and is suggested to play a key role in the integration of newly generated granular cells shaping hippocampal circuits (Arruda-Carvalho et al., 2014). As observed, chronic infusion of Ki16425 affected α-CaMKII expression but not α-CaMKII phosphorylation. Interestingly, Thr^{286} autophosphorylation of α-CaMKII has been demonstrated not be required for the maintenance of memory once it is established (Irvine et al., 2005). Similarly, studies of the CaMKII activity in spines have shown that autophosphorylation is essential for the induction of spine plasticity, but dispensable for plasticity maintenance (Chang et al., 2017). Very recently, Andersen et al. (2020) has shown that α-CaMKII autophosphorylation is not essential for the establishment of opioid-induced conditioned place preference (CPP). Consequently, and given the reduction in neuronal activity in CA1 and CA3, Ki16425 may well induce maladaptive learning because of structural and plasticity alterations in the neurogenic niche in addition to neuronal loss. Together, these data suggest that the changes induced by Ki16425 in the α-CaMKII expression could be related to behavioral and neuroplastic actions observed in our study. Because α-CaKMII activation is thought to strengthen glutamatergic synapsis, and in absence of the LPA_1 receptor, disruptions of the glutamatergic system have been observed (Harrison et al., 2003; Roberts et al., 2005; Musazzi et al., 2011; Blanco et al., 2012; Peñalver et al., 2019), it is possible that glutamatergic receptors may mediate the effects of the chronic antagonism of the LPA receptors. Nevertheless, this study was not designed to directly test this hypothesis. Therefore, future experiments will be aimed at identifying the effects of C18:1 LPA pharmacological treatment on glutamatergic receptors, leading toward understanding the relationship between these neurotransmitter systems.

In relation to this last aspect, although the specific weight of the the different neurotransmission systems on the effects mediated by the pharmacological modulation of LPA receptors has not been experimentally addressed in this work, we have no doubt that those could facilitate, to a great extent, the observed changes in neurogenesis and in behavior. Of note, activation of LPA receptors by gintonin, a ginseng-derived LPA receptor ligand, has been demonstrated to stimulate gliotransmitter and neurotransmitter release and enhance both excitatory and inhibitory transmission (Park et al., 2015).

Gintonin has been demonstrated to stimulate hippocampal glutamate release and the increase of acetylcholine and choline acetyltransferase expression, enhancing synaptic transmission in mouse hippocampus, besides resulting in beneficial consequences for hippocampal neurogenesis and contextual memory formation (Kim S et al., 2016; Kim HJ et al., 2016; Kim HJ et al., 2018). Furthermore, the oral administration of this LPA ligand in mice increased plasma 5-HT levels and attenuated depressive-like behaviors (Kim et al., 2017), an effect which, added to those mentioned, provides a rough guess of the influence that neurotransmission systems can have on the effects mediated by the modulation of LPA receptors and opens the need to address future studies of this nature.

Overall, our data suggest that chronic pharmacological manipulation of the LPA receptors modifies hippocampal function. Determination of neuronal activation after behavioral tests could contribute to the characterization of the functional status of hippocampus induced by treatments that, if modified, may be related to the behavioral effects. By analyzing c-Fos expression in antagonist-treated mice, we found reductions of neuronal activation in both CA1 and CA3 regions, whereas C18:1 LPA enhanced neuronal activation in DG. As no animals were assessed in basal conditions, we cannot rule out that a potential modification by treatments in the basal activity could contribute to our c-Fos results.

In summary, we have identified, for the first time, the effects of the continuous and chronic ICV administration of a stable and nonhydrolyzable LPA and the LPA_{1-3} receptor Ki16425 antagonist in behavior. These effects were accompanied by changes in neuronal activation, neurogenesis as well as α-CaMKII and phospho-α-CaMKII levels in the hippocampus. In broader terms, we observed that the effects of the chronic and continuous stimulation or blockade of the LPA/LPA receptors-signaling pathway can promote or affect hippocampal-dependent behaviors and functions, respectively. As hippocampal dysfunction has a crucial role in the pathophysiology of both depression- and anxiety-related disorders, our data support the role for LPA signaling, most likely via the LPA_1 receptor, in cognition and emotional regulation as a vulnerability factor for anxiety and depression (Moreno-Fernández et al., 2017, 2018) and may potentially be targeted for antidepressant and anxiolytic development.

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Ethical statement

All the experiments described in this paper were conducted in accordance with the ARRIVE guidelines and the European guidelines (European Council Directives 2010/63/UE and 90/219/CEE, Regulation (EC) No. 1946/2003) and Spanish national laws on laboratory animal welfare (Royal Decrees 53/2013 and 1386/2018, Law 32/2007) and approved by the Experimentation Ethics Committees of the University of Malaga and the Biomedical Research Institute of Málaga (CEUMA: 2012-0006-A; 2012-0007-A).

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Supplementary Methods

Chemicals

[^{35}S]GTPγS (initial specific activity 1250 Ci/mmol) was purchased from Perkin Elmer (Boston MA, USA). Nonhydrolyzable LPA, C18:1 LPA [1-(9Z-octadecenyl)-2-hydroxy-*sn*-glycero-3-phosphate (ammonium salt), CAS Number 799279-68-8] and LPA [1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (sodium salt), CAS Number 325465-93-8] were acquired from Avanti Polar Lipids (Alabaster, AL, USA). Ki16425 (CAS Number 355025-24-0) was purchased from Selleckchem (Houston, TX, USA).

Animals

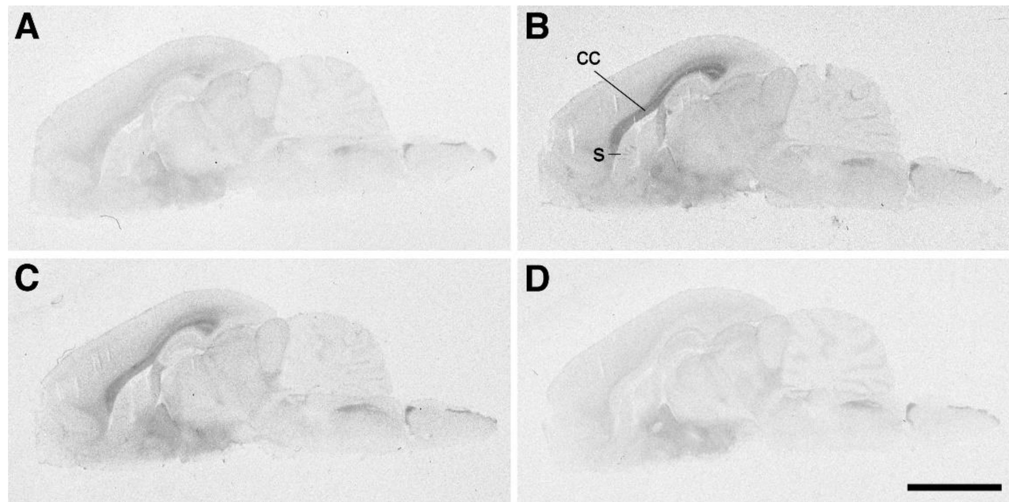
Male Sprague–Dawley rats (225–275 g) were used. Rats were housed under a 12 : 12 h light : dark cycle, at 22°C and 65% humidity, with food and water provided *ad libitum*. Experimental protocols were approved by the Local Ethics Committee for Animal Research at the University of the Basque Country (CEBA/91/2010/Rodriguez Puertas). All procedures were performed in accordance with European guidelines (European Council Directives 2010/63/UE and 90/219/CEE, Regulation (EC) No. 1946/2003) and Spanish national laws on laboratory animal welfare (Royal Decrees 53/2013 and 1386/2018, Law 32/2007). Rodent brains were quickly removed by dissection after anesthesia. Then, tissues were frozen on dry ice and kept at -80°C. The brains were cut on a Microm HM550 cryostat (Thermo-Fisher Scientific, Walldorf, Germany) to obtain 20-μm sections that were mounted onto gelatin-coated slides and stored at -20°C until use.

[^{35}S]GTPγS-binding assay

Sections were air-dried for 15 min, followed by preincubation in HEPES-based buffer (50 mM HEPES, 100 mM NaCl, 3 mM $MgCl_2$ and 0.2 mM EGTA, bovine serum albumin 0.5%, pH 7.4) for 30 min at 30°C in a water bath. The preincubation was repeated a second time in a new buffer. The samples were then incubated for 2 h at 30°C in a similar buffer that was supplemented with 2 mM guanosine-50-diphosphate (GDP), 1 mM DL-dithiothreitol, adenosine deaminase (3 mU/mL), and 0.04 nM [^{35}S]GTPγS. The evaluation of [^{35}S]GTPγS basal binding was duplicated by using consecutive slices on different slides. Agonist-stimulated binding was measured under the same conditions in the presence of the selective LPA receptor agonists C18:1 LPA (10 μM) and LPA (10 μM). The LPA_{1-3} antagonist, Ki16425 (10 μM) was used together with C18:1 LPA (10 μM) on a consecutive slide. Nonspecific binding was determined in the presence of 10 μM of nonlabelled GTPγS on another consecutive slide. Sections were washed twice in 50 mM HEPES buffer (pH 7.4), dipped in distilled water and air-dried. Sections were exposed to Kodak Biomax MR autoradiography film, together with [^{14}C]-microscale (GE-Healthcare, Chicago, IL, USA) standards for 48 h at 4°C in a hermetically closed cassette.

Image analysis of film autoradiograms

Films were scanned and quantified by transforming the optical densities (255 level grayscale, 0 white and 255 black) of each anatomical area into nCi/g tissue equivalent (nCi/g t.e.) using NIH-image software (NIH-IMAGE, Bethesda, MA, USA; developed at the U.S. National Institutes of Health and freely available on the Internet at http://rsb.info.nih.gov/nih-image/). Coexposed [^{14}C]-microscale standards were used to calibrate the optical densities with the level of radioactivity labeled in the sections.



Supplementary Fig. 1. Representative autoradiograms of sagittal rat brain sections showing [^{35}S]GTPγS binding in the absence of agonist, i.e., basal binding (A) and stimulated by 10 μM LPA (B), 10 μM C18:1 LPA (C), and C18:1 LPA(10 μM) in the presence of Ki16425 (10 μM) (D). Both LPA and C18:1 LPA stimulation result in similar specific patterns. cc, corpus callosum; s, striatum. Scale bar: 5 mm

	Stimulation over basal (%)		
Brain region	LPA	C18:1 LPA	C18:1 LPA + Ki16425
Anterior commissure	139 ± 35	105 ± 52	-29 ± 9
Basal prosencephalon	72 ± 22	42 ± 26	25 ± 8
Cerebellum			
White matter	124 ± 64	33 ± 35	-2 ±17
Gray Matter	134 ± 68	44 ± 30	-27 ± 68
Cingulate cortex	26 ± 2	-5 ± 15	-3 ± 50
Corpus callosum	162 ± 26	113 ± 13	-65 ± 13
Hippocampus			
CA1	95 ± 49	29 ± 3	4 ± 30
Dentate gyrus	25 ± 3	16 ± 25	0 ± 49
Hypothalamus	42 ± 13	13 ± 8	4 ± 11
Motor cortex	15 ± 12	13 ± 8	-15 ± 42
Striatum	31 ± 21	13 ± 1	-15 ± 42
Superior colliculus	48 ± 31	19 ± 34	4 ± 33
Thalamus			
Anterior nuclei	51 ± 11	39 ± 29	9 ± 52
Central nuclei	53 ± 6	39 ± 32	1 ± 50

Supplementary Table 1. [^{35}S]GTPγS binding induced by LPA (10 μM) and C18:1 LPA (10 μM) in different areas of rat brain, calculated as percentage of stimulation over basal (mean ± SEM values). Although C18:1 LPA stimulation values seemed lower than those induced by LPA in several regions, differences between LPA and C18:1 LPA percentages were not significant (Student's *t*-test; $P \leq 0.05$; n = 4 mice per genotype).

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

I sign, on behalf of all the authors of this work.

Guillermo Estivill Torrús

Málaga, 28th September 2020.

Ethical statement

All the experiments described in this paper were conducted in accordance with the ARRIVE guidelines and the European guidelines (European Council Directives 2010/63/UE and 90/219/CEE, Regulation (EC) No. 1946/2003) and Spanish national laws on laboratory animal welfare (Royal Decrees 53/2013 and 1386/2018, Law 32/2007) and approved by the Experimentation Ethics Committees of the University of Malaga and the Biomedical Research Institute of Málaga (CEUMA: 2012-0006-A; 2012-0007-A).

Author statement

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