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LPA_1 receptor and chronic stress: Effects on behaviour and the genes involved in the hippocampal excitatory/inhibitory balance.

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Abstract

The LPA_1 receptor, one of the six characterized G protein-coupled receptors ($LPA_{1–6}$) through which lysophosphatidic acid acts, is likely involved in promoting normal emotional behaviours. Current data suggest that the LPA-LPA_1 receptor pathway may be involved in mediating the negative consequences of stress on hippocampal function. However, to date, there is no available information regarding the mechanisms whereby the LPA_1 receptor mediates this adaptation. To gain further insight into how the LPA-LPA_1 pathway may prevent the negative consequences of chronic stress, we assessed the effects of the continuous delivery of LPA on depressive-like behaviours induced by a chronic restraint stress protocol. Because a proper excitatory/inhibitory-inhibition balance seems to be key for controlling the stress response system, the gene expression of molecular markers of excitatory and inhibitory neurotransmission was also determined. In addition, the hippocampal expression of mineralocorticoid receptor genes and glucocorticoid receptor genes and proteins as well as plasma corticosterone levels were determined. Contrary to our expectations, the continuous delivery of LPA in chronically stressed animals potentiated rather than inhibited some (e.g., anhedonia, reduced latency to the first immobility period), though not all, behavioural effects of stress. Furthermore, this treatment led to an alteration in the genes coding for proteins involved in the excitatory/inhibitory balance in the ventral hippocampus and to changes in corticosterone levels. In conclusion, the results of this study reinforce the assumption that LPA is involved in emotional regulation, mainly through the LPA_1 receptor, and regulates the effects of stress on hippocampal gene expression and hippocampus-dependent behaviour.

1.- INTRODUCTION

Lysophosphatidic acid (LPA) is an important bioactive lipid species that is part of the lysophospholipid (LP) family, and it signals through G protein-coupled receptors, mainly the high-affinity LPA_1 receptor. This receptor has been recently implicated in emotion (Pedraza et al., 2014) and mood regulation, and its dysfunction may be associated with the development of anxious depression (Moreno-Fernández et al., 2017; 2018). Several lines of evidence have also suggested a role for the LPA_1 receptor in mediating the consequences of stress on the hippocampus. In fact, LPA_1 receptor deficiency exacerbates the reduction of neurogenesis and the increase of apoptosis induced by stress, confers vulnerability to chronic stress (Castilla-Ortega et al., 2011). Animals lacking the LPA_1 receptor exhibit exaggerated endocrine responses to emotional stimuli (Pedraza et al., 2014) and an impaired adaptation of the HPA axis after chronic stress (Castilla-Ortega et al., 2011), suggesting that the LPA-LPA_1-receptor pathway plays a role in regulating the HPA axis. Because these factors have also been strongly correlated with depression, it seems reasonable to assume that this receptor may be involved in the development of depression induced by stress. To date, there is no available data on the mechanism through which the LPA_1 receptor may be involved in this adaptation. The alteration of glutamate/GABA cycling is a possible mechanism. GABA and glutamate play a major role in the central integration of HPA stress responses (Herman et al., 2004). A proper excitatory/inhibitory balance seems to be key for controlling the stress response system, and changes in this balance are involved in the translation of stress effects (Sandi, 2011; Tzanoulinou et al., 2014; van der Kooij et al., 2014; Cordero et al., 2016).

Both genetic deletion and pharmacological approaches have indicated that LPA, mainly through the LPA_1 receptor, is involved in regulating excitatory (Blanco et al., 2012; Musazzi et al., 2011; Peñalver et al., 2017) and inhibitory (Cunningham et al., 2006) neurotransmission. Because the LPA_1 receptor colocalizes with excitatory (vGLUT2) and inhibitory (vGAT) presynaptic structures (García-Morales et al., 2015), these data suggest that the LPA/LPA_1-receptor pathway can play a key role in the regulation of the excitatory/inhibitory balance in the brain. Indeed, in the hippocampus of animals lacking the LPA_1 receptor, alterations in the expression of metabotropic mGLUR3 glutamate receptors (Blanco et al., 2012) and in the morphology of the dendritic spines of glutamatergic pyramidal cells have been observed (Peñalver et al.,

2017). Moreover, CaMKII, which is involved in strengthening glutamatergic synapses (Fink et al., 2002; Robison, 2014), and related synaptic mechanisms at glutamatergic synapses are strongly dysregulated in this genotype (Musazzi et al., 2011). Regarding GABAergic neurotransmission, the absence of LPA_1 causes a reduction in GABAergic parvalbumin-positive interneurons in the entorhinal cortex (Cunningham et al., 2006). On the other hand, LPA administration to excitatory synapses initiates the rapid and reversible depression of excitatory postsynaptic currents, an effect that is mediated by presynaptic $LPA_1/G_{\alpha i/o}$ proteins (García-Morales et al., 2015). Moreover, LPA administration induces the depression of GABAergic transmission, possibly through $GABA_A\gamma_2$ dephosphorylation (García-Morales et al., 2015).

To gain further insight into how the LPA-LPA_1 receptor pathway may be involved in the negative consequences of chronic stress, we assessed the effects of the continuous and chronic ICV administration of a stable and non-hydrolysable form of LPA (1-(9Z-octadecenyl)-sn-glycero-3-cyclic-phosphate (C18:1 LPA; ammonium salt)), which we will refer to henceforth as LPA and which has a high affinity for the LPA_1 receptor, on behaviours related to depression (anhedonia and passive stress-coping response) in chronically stressed animals. We then determined the gene expression of molecular markers of excitatory and inhibitory neurotransmission. In addition, the hippocampal expression of mineralocorticoid receptor and glucocorticoid receptor genes and proteins as well as serum corticosterone (CORT) levels were determined. Furthermore, the relationship between the variables was examined using a principal component analysis (PCA) multivariate approach to determine whether gene and protein expression and CORT levels account for anhedonia or passive stress-coping behaviour.

2.- MATERIALS AND METHODS

2.1.- Animals

For this study, 3-month-old male mice with a hybrid C57BL/6J129X1/SvJ background were used (N=37; 28 g± 2 g). In all cases, the mice were individually housed and maintained on a 12-h light/dark cycle (lights on at 08:00 a.m.), with water and food provided ad libitum. All procedures were carried out in compliance with European animal research laws (European Communities Council Directives

2010/63/UE, 90/219/CEE, Regulation (EC) No. 1946/2003) and the Spanish National Guidelines for Animal Experimentation (Real Decreto 53/2013) and were approved by the Ethics Committee of Malaga University (CEUMA 2013-0008-A / CEUMA: 1-2015-A, 08-7- 15-273) and Junta of Andalucia (08-7-15-273).

The experimental procedure is summarized in Fig. 1. For the behavioural tests, four groups were used. Each group underwent one of the two environmental treatments (control or chronic stress) and one of the two pharmacological treatments (vehicle or LPA). The molecular studies were performed on the following groups: the control group not submitted to stress or ICV administration (n=7); vehicle (n=7) or LPA (n=7), not submitted to stress and that received continuous ICV administration of either vehicle or LPA and the two stressed groups that received continuous ICV administration of either vehicle (n=8) or LPA (n=8). The animals were individually housed until surgery, and the fluid consumption of each individual mouse was assessed.

2.2.- Chronic pharmacological treatment

Using a stereotaxic instrument (Kopf, California, USA), a 30-gauge stainless steel cannula was implanted into the right lateral ventricle under ketamine/xylazine anaesthesia (80 mg/kg ip ketamine and 12 mg/kg ip xylazine (Sigma-Aldrich, Madrid, Spain)). The coordinates used were 0.34 mm posterior to bregma, 1 mm lateral to the midline, and 2.3 mm deep from the dura (Paxinos and Franklin, 2001). The intracranial cannula was connected via polyethylene tubing to an Alzet® 1004 osmotic minipump (Durect Corporation, Cupertino, California, USA). The minipumps were filled with vehicle (reservoir volume 100 µl/pump; 3% fatty acid-free bovine serum albumin (BSA)/phosphate buffer saline (PBS); N=15) or LPA (C18:1 LPA; Avanti Polar Lipids; Alabaster, EEUU) (0.36 µg/pump; 200 nM; N=15) dissolved in vehicle. The minipumps were implanted subcutaneously dorsal to and to the left of the scapulae, and the infusion was made continuously over 21 days at a flow rate of 0.11 µl/h. Under this flow rate, LPA was delivered at a dose of 0.93 ng/kg/day. The minipumps were weighed before and after the 21-days implantation period as a flow control.

2.3.- Stress protocol

Five days after surgery, on an unfixed schedule, the animals were restrained for 3.5 h per day for 21 days, excluding weekends (see Fig.1). For this purpose, 50 ml clear polystyrene conical centrifuge tubes modified with air holes for ventilation were used.

Previously, we used a similar protocol to induce hippocampal-dependent deficits, such as the reduced survival of new neurons and impaired hippocampal-dependent memory, with high efficacy (Castilla-Ortega et al., 2014). The control mice remained undisturbed in their home cages.

2.4.- Behavioural assessment

2.4.1.- Saccharin preference test.

Prior to testing, the mice were habituated to the presence of two drinking bottles for two periods of 24 h. The test was conducted on the last day of the stress protocol. During the test, the mice had the opportunity to choose between a saccharin solution (0.05%) and tap water for 24 h. No previous food or water deprivation was applied. The consumption of water and saccharin solution was estimated simultaneously in the control and experimental groups by weighing the bottles. Saccharin intake was calculated as the amount of saccharin consumed in mg per g of body weight. Saccharin preference was calculated according to the following formula: saccharin preference = (saccharin intake)/(saccharin intake + water intake) × 100, as previously described (Strekalova et al., 2004). Moreover, total liquid intake was recorded. An intake of saccharin as 65% of the total drinking liquid was considered an indicator of anhedonia (Strekalova et al., 2004).

2.4.2.-Forced swim test.

A day after finishing the stress protocol (27 days after starting continuous ICV administration), a modified version of the forced swim test (FST) described by Cryan et al. (2002) was used to assess depressive-like behaviour. The test was conducted in a testing room illuminated at 15 lux. The mice were placed individually in a vertical cylindrical tank made of Plexiglas (with a height of 30 cm and a diameter of 10 cm) and filled with 23 ± 1°C water to a depth of 25 cm. The FST was conducted once per animal for 6 min, and the duration of immobility was recorded. Immobility was defined as a mouse floating in the water and making only those movements necessary to keep its head above the water (Porsolt et al., 1977). The sessions were recorded using a video camera and analysed by EthoVision software (Noldus; Netherlands) using the parameters of floating behaviour adapted from Juszczak et al. (2008). The latency to the first immobility period and the total duration of immobility were recorded. The latency

to the first immobility period was the time between the introduction of the mouse to the pool and the first instance of complete immobility of the animal's entire body lasting at least 2 s.

After finishing the test, each animal was removed from the tank and dried with a towel before being returned to its home cage.

2.5.- Gene expression analysis

Two days after the last behavioural test, the mice were decapitated, and the brains were snap-frozen in isopentane at -40°C and stored at -80°C until further processing. Gene expression analysis was done in two different experiments (first control, vehicle and LPA with stress, and next vehicle and LPA without stress). For this reason, the data were normalized to 100% of the control in the first block of experiments and of the control vehicle in the second block of experiments and analyzed independently.

Using a cryostat (Leica CM3050S, Heerbrugg, Switzerland), bilateral punches of 150-µm-thick slices of the dorsal and ventral hippocampus were made. The tissue was stored at -80°C in RNase-free tubes until RNA and protein extraction and isolation. The extraction of mRNA and protein was done using Trizol, as done previously (Bacq et al., 2018).

For mRNA determination, quantitative polymerase chain reaction (PCR) was performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies, Warrington, Florida, USA) and an ABI Prism 7900 Sequence Detection system (Applied Biosystems, Life Technologies, Singapore). Two genes were used as internal controls: TATA-BOX binding protein (*tbp*) and eukaryotic elongation factor-1 (*eef1*). Primers for the genes of interest were designed using NCBI primer design tool.

Primers for the genes of interest, except for NR1.48, were designed using Assay Design Centre software from Roche Applied Science; Basel, Switzerland) Gene expression was analysed with qBase 1.3.5 software using the comparative cycle threshold method. The genes investigated were *Grin1*, *Slc17a7*, *Gad1*, *Slc32a1*, *Nr3c1*, and *Nr3c2* (for a more detailed description of the mouse genes names and the whole name of each gene, please see Table 1). The qPCR assays were validated and a melt curve was run to confirm that the primers were specific.

2.6.- Protein expression analysis

In parallel to the quantification of the mRNA levels, the relative protein expression levels of hippocampal glucocorticoid receptor in the dorsal hippocampus were investigated by Western blotting. Homogenates of the hippocampus were centrifuged at 2000 x g for 15 min at 4°C, and the supernatant was centrifuged at 100.000 x g for 1 h at 4°C. Protein extracts (25 µg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred onto PVDF membranes (Trans-Blot® Mini PVDF Transfer Pack; Bio-Rad, CA, USA) using Trans-Blot® Turbo™ Transfer-System (Bio-Rad, CA, USA). The membranes were blocked with 3% non-fat dry milk in Tris buffered saline containing 0.1% Tween-20 and 1% bovine serum albumin (TBST) at room temperature for 1 h. Then, the membranes were incubated at room temperature overnight with: rabbit anti-GR (Santa Cruz, sc-1004) and rabbit anti-Actin (1:1000) primary antibodies followed by the appropriate secondary anti-rabbit antibody (1:1000). The immunocomplexes were visualized using a chemiluminescence peroxidase substrate (SuperSignal West Dura Extended Duration Substrate), and the immunoreactivity was detected using the ChemiDoc XRS system (Bio-Rad Laboratories AG, Cressier, Switzerland). Densitometry analysis of the bands was performed using Quantity One 4.2.3 software (Bio-Rad Laboratories AG, Cressier, Switzerland). Each protein band was normalized to actin levels on the same membrane. Thus, the results are displayed as the relative level of GR to actin. The quantification of the proteins was carried out with ImageJ 1.41 software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

2.7.- Corticosterone assay

Because glucocorticoid hormones are consistently increased in rodent stress models (de Kloet et al., 2008) and a large body of evidence has demonstrated that chronically high concentrations of glucocorticoids impair hippocampal excitatory/inhibitory neurotransmission (Elizalde et al., 2010; Martisova et al., 2012; Saaltink and Vreugdenhil, 2014), serum corticosterone level determination was carried out in the control and chronically stressed animals (vehicle- or LPA-treated). Three samples of blood were collected from the lateral tail vein during treatment (basal measure, 15 days after starting the stress protocol) and 10 min after completing the FST (see Fig. 1). The last measure was used to evaluate HPA responsiveness to an acute stressor i.e., the FST (a heterotypic stressor in the chronically stressed group). Tubes

containing EDTA were centrifuged, and the supernatant was stored at -80°C. Serum corticosterone levels were determined in duplicate using a commercially available Enzyme Immunoassay Kit, sensitivity ca. 27.0 pg/ml, following the manufacturer's instructions (Assay Designs/Stressgen, Ann Arbor, Michigan, USA).

2.8.- Cellular cultures

Given that the administration of C18:1 LPA in chronically stressed animals has a very unexpected effect that opposes the effect of its administration in control environmental conditions (see behavioural and endocrine results and accumulated data from behaviour (Pedraza et al., 2014; Moreno-Fernández et al., 2018) and immunochemical studies (Ladrón de Guevara-Miranda et al., 2018), the intracellular trafficking of the LPA_1–receptor in cell culture was investigated. For this experiment SHSY-5Y cells were used and incubated with PBS, BSA/PBS as vehicle, LPA (200 nM), dexamethasone (DEX) (10μM), a synthetic corticosteroid, or a combination of both drugs, tried to emulate the experimental condition of 'in vivo' studies. For a detailed description of the methods please see supplementary material.

2.9.- Statistical analysis

The data from the behavioural tests were analysed using a factorial ANOVA (with two factor with two levels: pharmacological and environmental treatments). For PCR and Western blotting studies, one-way ANOVA was applied for normalized data. For molecular studies, the data of the first block (Control, vehicle and LPA with stress) and those of the second block (vehicle and LPA without stress) were analyzed independently. The corticosterone level data and LPA_1 receptor internalization over time in cell culture were analysed by a factorial repeated-measures ANOVA (with two factor pharmacological or environmental treatment for corticosterone levels or pharmacological treatment and time in the case of cellular culture). The analyses were followed by post hoc Fisher's least significant difference (LSD) analyses when required. For the benefit of clarity and brevity, only the relevant results of these statistical analyses are reported.

Principal component factorial analysis (PCA) with varimax rotation was subsequently performed to reduce the gene expression and CORT level data into

neurobiological modules underlying behaviour. Only a set of genes was selected to undergo PCA so that the analysed samples from the groups would meet statistical adequacy criteria (Balaban et al., 2014). The correlation matrix of the whole sample of animals (n=30 vehicle and LPA with and without stress) was used for the analysis and tested for sampling adequacy by the Bartlett sphericity and the Kaiser–Meyer–Olking (KMO) tests. The resulting factors with the eigenvalue>1were selected. 'Factor loading' (i.e. the contribution of each variable to a factor was considered significant when it was >0.50. Finally, considering that the component or factor scores represent the relative contribution or weight of each loading pattern for each case, factorial ANOVA (with two factor with two levels: pharmacological (vehicle and LPA) and environmental treatments (control and stress)) was used to determine whether differences existed between the groups in a given loading pattern.

The statistical significance was set at $p \leq 0.05$.

3.- RESULTS

3.1.- Behavioural effects of chronic LPA administration

3.1.1.- Effects of chronic LPA administration on hedonic behaviour

The data revealed a pharmacological x environmental treatment effect ($F(1, 26)=10.574$, $p=0.00317$). LPA administration induced a significant increase in hedonic behaviour, with chronically treated animals achieving a 93% preference of saccharin over water ($P \leq 0.05$ versus vehicle). Stress reduced the preference for saccharin to the level of the anhedonic criterion (64% preference over water) (Strekalova et al., 2004), although no significant differences were observed in comparison with that in the vehicle group ($P>0.05$ versus vehicle). However, in stressed animals, the continuous delivery of LPA induced anhedonic behaviour (49% preference over water) ($P \leq 0.005$ versus vehicle and LPA; $P \leq 0.05$ versus stress) (See Fig. 2A). No differences were observed in total liquid intake ($F(1, 26)=0.652$, $P>0.05$).

3.1.2.- Effects of chronic LPA_1 administration in the forced swim test

Classically, in the FST, immobility has been interpreted as reflecting depressive--like behaviour in rodents (Porsolt et al., 1977) and, more recently, as a passive coping strategy (Molendijk and Kloet, 2015). Factorial ANOVA revealed an effect of stress on immobility time ($F(1, 25)=10,141$, $P=0.00386$) and an interaction of pharmacological x

environmental treatment on the latency to the first period of immobility ($F(1, 26)=4,6222$, $P=0.04104$). Thus, although no significant effect of LPA treatment was observed on floating behaviour, chronic stress increased immobility behaviour regardless of treatment ($P\leq0.05$ versus vehicle or LPA without stress) (Fig. 2B). However, LPA administration in the chronically stressed animals reduced the latency to the first immobility period ($P\leq0.05$ versus all other groups) (Fig. 2C).

3.3. Analysis of the excitatory/inhibitory balance

The expression of genes related to the excitatory/inhibitory balance was examined in the hippocampus (dorsal and ventral) after chronic restraint stress and continuous LPA treatment.

In the dorsal hippocampus, LPA, stress alone or in conjunction with chronic LPA administration did not have any effect on GAD67, vGAT, vGlut1 or NR1 mRNA expression. The chronic administration of LPA (irrespective of the environmental treatment) did not produce any changes in the dorsal hippocampal excitatory/inhibitory balance (Fig. 3, for individual data points please see figure S3).

However, in the ventral hippocampus, significant differences in the expression of vGAT ($F_{(2,21)}=4.29$, $P=0.027$; Fig. 3H) and the VGlut1/vGAT ratio ($F_{(2,20)}=3.5$, $P=0.05$; Fig. 3J) were found between the groups. Thus, in the chronically stressed animals, LPA induced a significant reduction in the expression of vGAT (LSD: $P\leq0.05$ versus control and versus vehicle; Fig. 3H) that increased the vGlut1/vGAT ratio ($P\leq0.05$ versus control and versus vehicle; Fig. 3J). LPA without stress did not induce any effect in the expression of the examined genes in ventral hippocampus in comparison with the vehicle group.

3.4.- Expression of glucocorticoid receptors

The influence of stress and pharmacological treatment on hippocampal MR and GR mRNA expression was examined using quantitative PCR. Regarding the expression of the MR receptor, pharmacological treatment did not induce any changes ($F_{(2,20)} = 1.29$, $P>0.05$; Fig. 4A). With respect to GR mRNA expression, significant differences were observed ($F_{(2,20)}=5.478$, $P=0.012$; Fig. 4A and B) in the dorsal but not the ventral hippocampus. Stress induced a reduction in GR mRNA expression regardless of pharmacological treatment (vehicle or LPA). The ratio of MR/GR mRNA expression was not changed by treatments in dorsal hippocampus ($F_{(2,20)} = 1.73$, $P>0.05$; see Fig.

4C and D). However, in ventral hippocampus, the LPA administration in non-stressed animals reduced the MR:GR ratio in comparison with vehicle group ($F_{(1,12)}$=4.52; P= 0.05).

The data regarding GR gene expression were corroborated by Western blot analysis (Fig. 4E, F and S2). Thus, stress, irrespective of pharmacological treatment, reduced GR protein expression in the dorsal hippocampus ($F_{(2,20)}$=8.56, P=0.002 ; LSD: P≤ 0.01 and P≤0.001 versus vehicle and LPA, respectively, Fig. 4E). Although the chronic LPA-administered animals showed a greater reduction in the levels of this protein, statistically significant differences compared to those in the vehicle group were not observed (P>0.05). For individual data points please see figure S4.

3.5.- Effects of chronic LPA and stress on CORT levels.

To examine the changes induced by chronic LPA treatment and stress on corticosterone levels, the levels were measured at three time points: at baseline, after 15 days of chronic restraint stress (20 days after starting pharmacological treatment), and 10 min after the FST (which may be considered a heterotypic acute stressor in the chronically treated animals) (Fig. 1).

Factorial ANOVA indicated an interaction of pharmacological x environmental treatment 15 days after beginning the stress procedure (20 days after starting pharmacological treatment) ($F_{(1,25)}$=4.42; P=0.05) and 10 min after the FST ($F_{(1,24)}$=4.39; P≤0.05). Thus, in the chronically stressed animals, LPA markedly increased (by over two and a half and ten times compared with the responses of the stressed and non-stressed vehicle-treated groups, respectively), the levels of CORT (P≤0.05 versus vehicle, LPA and vehicle + stress). However, this treatment had no effect after acute heterotypic stress. On the other hand, the non-stressed animals chronically administered LPA showed the greatest reduction in CORT levels after the FST (P≤0.05 versus all of the other groups) (Fig. 5).

3.6.- Relationship between behavioural outcomes and biological factors.

A principal component analysis (PCA) was conducted to study the relationship among the molecular, endocrine and behavioural changes induced by treatments. PCA with variance-maximizing (varimax) rotation revealed a two-component solution accounting for 59.73% of the total variance. Component 1 showed a negative correlation with hedonic behaviour (-0.69) and the expression of GR in the dorsal

hippocampus (-0.60) and positive correlation in the *vGlut1/vGAT* mRNA ratio (0.70); CORT levels after chronic stress (0.76) and the MR/GR mRNA ratio in the ventral part of the hippocampus (0.80). Component 2 showed a positive correlation with the latency to the first immobility period (0.87) and a negative correlation with the time spent floating (immobile) (-0.70). Component 1 could be categorized as 'anhedonic behaviour' and component 2 as 'active stress-coping behaviour'. Two-way ANOVA revealed differences among the interaction pharmacological x environmental treatments in the scores of factor 1 ($F_{(1, 25)}$=7.67, P=0.010) and in the scores of factor 2 ($F_{(1, 25)}$=4.33, P=0.047). LSD revealed that stress affected the hedonic behaviour (P=0.009 vehicle versus vehicle + stress; P= 0.0000001 LPA versus LPA+stress). However, LPA-treated animals, under stress, showed a significantly higher 'anhedonic behaviour' score and a lower in 'active stress-coping behaviour' score. (see Fig. 6B and C).

In sum, this analysis sheds light on the relationship between the biological and behavioural variables assessed in this study and helps to identify which neurobiological measure has a stronger effect on or engagement in specific behaviours (El Rawas et al., 2012).

3.7.-Effect of DEX and LPA in the LPA_1 receptor internalization.

Among of the experimental condition, the incubation of cellular culture with LPA followed by DEX enhanced LPA_1 receptor accumulation and induced the greatest degree of the LPA_1 receptor intracellular immunofluorescence at the longer examined period, compared to the rest of the treatments ($P \leq 0.05$) (Fig. S1). These data suggest that this treatment extend the internalization of LPA_1 receptor. For a more detailed description please see supplementary results. for individual data points please see figure S5.

4.- Discussion

Our data showed that, contrary to our expectations, the continuous delivery of LPA in chronically stressed animals induced negative effects. Thus, the stress protocol alone did not lead to a clear behavioral phenotype. However, in combination with a second hit (LPA administration) induced anhedonia and a reduction in latency to the first immobility period. Moreover, unlike what was observed under conditions of stress (in vehicle group), LPA impaired the excitatory/inhibitory balance in the ventral hippocampus and induced a considerable increase in CORT levels after 15 days of

restraint stress. However, the administration of LPA under conditions of stress did not affect the hippocampal expression of MR or GR or the levels of CORT after acute stress in chronically stressed animals.

The continuous delivery of LPA affects reward sensitivity, which, interestingly, depends on environmental treatments; i.e., in stressed animals, the effect of LPA is opposite of that observed in non-stressed animals. Thus, under basal conditions, LPA treatment increased the preference for saccharin over water to just under 100%. Because anhedonia is a core symptom of mood disorders (Der-Avakian and Markou, 2012), the attention of many research studies has been on treatments that decrease the ability to experience pleasure. However, much less is known about procedures that increase hedonic behaviour. The identification of mechanisms involved in hedonic behaviour should greatly aid in understanding the neurobiological bases of mood disorders and identify novel therapeutic targets. Saccharin preference in the stressed animals was just under the anhedonic threshold of 65% (Strekalova et al., 2004). Conversely, the continuous delivery of LPA in the stressed animals reduced the preference for saccharin by nearly half that observed in the animals only treated with LPA and even potentiated the anhedonic effects of stress. The effects observed in the stressed animals that received a continuous delivery of LPA are in line with previous findings from studies that used the maLPA$_1$-null mouse line, an animal model of anxious depression that exhibits an anhedonic phenotype (Moreno-Fernández et al., 2017). These data, together with the data accumulated with the maLPA$_1$-null mice, reinforce the assumption that the LPA-LPA$_1$ pathway plays an essential role in the hedonic reactions to reward.

Passive stress-coping behaviour is characteristic of depressive disorders and can be assessed in animals using the FST. Although many behavioural reactions in the FST can be observed as reflecting defence mechanisms in response to an aversive environment (Cryan et al., 2002; Molendijk and Kloet, 2015; Moreno-Fernández et al., 2017; Anyan and Amir, 2018), classically, immobility has been interpreted as reflecting depressive-like behaviour in rodents (Cryan and Mombereau, 2004). The effect of stress on immobility has been widely documented (Becker et al., 2008; Bogdanova et al., 2013; Chiba et al., 2012; Veenaet al., 2009), supporting that stress can predispose an individual to develop depression (Pittenger and Duman, 2008). Chronic restraint stress, irrespective of pharmacological treatment, increased the total immobility time. However, while LPA did not have an effect on passive stress-coping behaviour, the

infusion of LPA in stressed animals reduced the latency to the first immobility period, which is a more sensitive measure of depressive-like behaviour than the duration of immobility (Castagné et al., 2009) and may be interpreted as a reduction in intrinsic motivation to escape the situation (Mosienko et al., 2012). These data indicate that LPA was not effective in mitigating the negative consequences of stress on passive stress-coping behaviour but rather increased the adverse consequences of the environmental treatment.

Stress can have a lasting impact on the structure and function of the brain and can result in changes in mood and motivation, among other behaviours (Pizzagalli, 2014). The hippocampus, in which a proper excitation-inhibition balance seems to be key for controlling the stress response system (Kim et al.,2016), is a target of stress (Orlovsky et al., 2014). Surprisingly, stress did not induce any changes in the expression of the genes used as markers of the excitation or inhibition in the hippocampus. Alterations in the expression of the genes examined in our study have not always been found after stress. With exceptions, recent studies conducted in adult animals have shown that chronic stress does not affect the expression of the genes used as markers of the excitation/inhibition balance in the hippocampus (Gilabert-Juan et al., 2011; Venzala et al., 2013), which supports our data. Changes in the expression of these genes depend on the experimental procedure and the period of life in which the stress protocol has been implemented. In particular, gene expression in periods during which significant brain re-organization occurs, such as the perinatal (Welberg and Seckl, 2001) and peripubertal phases (Márquez et al., 2013; Tzanoulinou et al., 2014), is more sensitive to stress effects than that in the adult period. However, in the ventral hippocampus of the stressed animals chronically administered LPA, decreased vGAT mRNA expression, which may alter excitatory signalling, was observed. In fact, in this group, an increase in the vGlut1/vGAT mRNA ratio was observed. An increased number of glutamatergic projections from the ventral hippocampus (vHIP) to the nucleus accumbens have been associated with depression (Bagot et al., 2015). Because the ventral hippocampus has been linked to emotional regulation (Schoenfeld et al., 2013), has been identified as playing a relevant role in regulating general affective states (Bannerman et al., 2004; Fanselow and Dong, 2010; Snyder et al., 2011), and has been related to anhedonic phenotype patterns induced by stress (Ritov et al., 2015), the excitatory/inhibitory imbalance observed in the chronically stressed LPA-treated

animals may explain, at least in part, the anhedonic and passive stress-coping behaviours induced by these treatments.

On the other hand, considering that chronic high levels of CORT have often been associated with neuropsychiatric disorders, such as depression (Gao et al., 2014; Saaltink and Vreugdenhil, 2014), CORT levels were also determined in our study. Although, after 15 days of chronic stress, no changes were observed in the group treated with vehicle, the administration of LPA increased CORT levels. Regardless of pharmacological treatment, chronic stress did not affect the CORT response to acute stress in the FST (a heterotypic acute stressor), probably reflecting a ceiling effect of CORT levels due to the aversiveness of the FST (Piras et al., 2010). However, LPA administration in the non-stressed animals, reduced CORT release, mitigating the impact of acute stress on the HPA axis. Together, these data indicate that the LPA-LPA_1–receptor pathway may play a role in regulating the HPA axis response to stress. For this reason, and given what is known about the essential role of the hippocampus in the modulation of the HPA axis to regulate negative feedback, damper circulating CORT and stop the stress response (McEwen and Gianaros, 2011), the gene and protein expression of MR and GR in the hippocampus of the control and chronically stressed animals was determined.

In our study, MR mRNA expression was not affected by chronic stress (Fig. 4A and B), which is in agreement with the findings of a previous study that also used a 21-day restraint stress model (Touyarot and Sandi, 2002). Regarding the stress-induced regulation of MR in the hippocampal formation, there is no consistent data in the literature; MR mRNA has been reported to be elevated (Meyer, et al., 2001), not affected (Füchsl and Reber, 2016) and downregulated (Herman et al., 1999). In the stressed animals, LPA did not induce any effect on MR mRNA expression in either the dorsal or ventral hippocampus.

In contrast, the reduced expression and function of GRs have been proposed as a relevant mechanism of the pathogenesis of stress-related psychiatric disorders (Kloet et al., 2005; Holsboer, 2000). Consistent with previously described data (Chen et al., 2003; Gądek-Michalska et al., 2013; Meyer et al., 2001; Mizoguchi et al., 2003), our data revealed that chronic stress decreased GR gene and protein expression in the dorsal hippocampus. However, because no differences were observed between the vehicle- and LPA-treated animals, different mechanisms may be involved in the exaggerated increase in CORT levels observed after 15 days of stress in the animals that received a

continuous delivery of LPA. Additional studies are needed to clarify the underlying mechanisms responsible for this increase in CORT concentration after restraint stress. However, the LPA treatment without stress induced a reduced MR/GR mRNA ratio in the ventral part of the hippocampus, that may explain, at least partially the effect on anhedonic behaviour. Thus, although with exception, an increased MR/GR ratio (or reduced GR/MR balance) in ventral hippocampus has been associated with a reduced stress coping ability (reviewed in Barr and Forster, (2011)). Moreover, BCPT, a compound with antidepressant potential, improved the reward reaction as measured by increasing sucrose consumption and reduced serum CORT possibly due to a reduction of the ratio of MR/GR in the hippocampus (reviewed in Yin et al., (2007). Taking all these data into account, LPA affects reward sensitivity, which, interestingly, depends on environmental treatments. This opposite effect may be explained by different impact at molecular levels depending on whether LPA is administered together with stress.

To better understand how different biological factors, interact with each other and possibly contribute to the observed phenotype, PCA was used to condense the correlated neurobiological measures into principal components reflecting independent dimensions underlying the behaviour profile observed in the chronically stressed animals. Thus, saccharin preference, GR expression and MR/GR ratio in dorsal hippocampus, CORT levels after chronic stress and the vGlut1/vGAT mRNA ratio in the ventral hippocampus, load in the first factor. The latency to the first immobility period and the duration of immobility load in the second factor. Thus, a lower expression of the GR gene and increased MR/GR mRNA ratio in dorsal hippocampus, high plasma levels of corticosterone and an increased excitation/inhibition ratio in the ventral part of hippocampus are related to anhedonic behaviour. However, an increased latency of the first immobility period is associated with a reduced time of the immobility. In this sense, anhedonic-like behaviour in the CMS model has been associated with an alteration in the ventral part of the hippocampal formation (Jayatissa et al., 2006) and the dysfunction of the GABAergic inhibitory system in the rat hippocampus caused by a reduction in potential-dependent GABAergic release (Holm et al., 2011). Moreover, the reduced expression of GRs in the hippocampus causes decreased pleasure-seeking behaviour (anhedonia) in mice (Boyle et al., 2005). Chronic CORT elevations may predispose individuals to affective disorders that induce anhedonia (Malisch et al., 2009). Moreover, 3-week CORT injections cause depressive-like behaviour in rats, as indicated by a reduction in sucrose consumption (Huang et al.,

2011). Overall, the increased vGlut1/vGAT ratio in ventral hippocampus, the increased CORT levels after chronic stress and the reduced GR expression in dorsal hippocampus may explain the anhedonic behaviour induced by LPA under chronic stress. By contrast, because an increased MR/GR ratio (or reduced GR/MR balance) has been related with anhedonic behaviour (reviewed in Yin et al., (2007)), the hedonic behaviour exhibited by the LPA-treated animals under basal condition may be related, at least in part, to reduced MR/GR ratio in ventral hippocampus. Furthermore, LPA, under stress, induced a passive stress-coping behaviour, suggesting that LPA treatment in stressful situation precipitates the onset of depressive-like symptoms.

Overall, chronic LPA administration under conditions of chronic stress partly mimicked the phenotype of LPA_1-null mice. The internalization of LPA_1 receptors, which is followed by the recycling of the receptors back to the cell surface (Mirendil et al. 2015), has been observed after LPA administration (Mirendil et al., 2015) and is dependent on LPA concentration (Murph et al., 2003). Therefore, it is reasonable to assume that the continuous delivery of LPA under conditions of stress may further prolong the internalization effect induced by LPA and induce a reduction in the availability of LPA_1 receptors for ligand binding. In fact, in cell culture, LPA rapidly increased the intracellular accumulation of the LPA_1 receptor when normalized to basal conditions at 240 min, suggesting the induction of receptor internalization. However, surprisingly, DEX after LPA exposure produced an increase in LPA_1-receptor intracellular accumulation at 240 min, prolonging the effect of LPA (see supplementary materials). The internalization of the LPA_1 receptor may be an initial mechanism leading to more permanent changes, which may explain, at least in part, why the continuous delivery of LPA in chronically stressed animals recapitulates the phenotype observed in maLPA_1-null mice. However, other pathways have not been excluded and require further investigation.

In summary, we have determined here the effects of the continuous delivery of LPA, which induces different effects under basal and stress conditions, on depressive- like behaviour. Together, these data revealed that, under conditions of stress, an agonist of the LPA_1 receptor exacerbated the behavioural effect of stress and induced a higher VGLUT1/VGAT ratio in the ventral hippocampus, possibly through the LPA_1 receptor. However, the action of this lipid on other LPA receptors should not be dismissed.

The results of this study, together with the data accumulated in studies of LPA_1-null mice, reinforce the assumption that LPA regulates hippocampal-dependent behaviour and functions, mainly through the LPA_1 receptor. Our data support a role for LPA signalling in emotional regulation likely via the LPA_1 receptor, which, if impaired, may confer vulnerability to depression and may potentially be targeted for the development of antidepressants.

Author Contributions

C.P; C.S and R.D.M-F. conceived and designed research; R.D.M-F., A.B., C.R-V., O.Z., M.C., A.L. G and M.G-F: have performed the experiments and contributed to the data collection; R.D.M-F. and C.P: performed statistical analyses; C.P., C.S., R.D.M-F., L.J.S., M.C, M.P-M. F.R-F. and G.E-T: interpreted results of experiments; R.D.M-F., C.R-V., and M-C.: have prepared figures; R.D.M-F. and C.P.: Writing - Original Draft with contributions from the other authors; C.P., C.S., M.C, M.P-M., L.J.S., F.R-F. and G.E-T: edited and revised manuscript; all authors read and approved final version of manuscript.

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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References

Bacq, A., Astori, S., Gebara, E., Tang, W., Siva, B.A., Sanchez-Mut, J., Grosse, J., Gullot de Suduiraut, I., Sanoletti, O., Maclachlan, C, Knott, G.W., Gräff, J., Sandi, C. (2018). Amygdala GluN2B-NMDAR dysfunction is critical in abnormal aggression of neurodevelopmental origin induced by St8sia2 deficiency. Molecular Psychiatry, doi.org/10.1038/s41380-018-0132-3

Barr, J.L., Forster, G.L. (2011). Serotoninergic neurotransmission in the ventral hippocampus is enhanced by corticosterone and altered by chronic amphetamine treatment. Neuroscience, 182: 105-114.

Becker, C., Zeau, B., Rivat, C., Blugeot, A., Hamon, M., & Benoliel, J.-J. (2008). Repeated social defeat-induced depression-like behavioral and biological alterations in rats: involvement of cholecystokinin. *Molecular Psychiatry*, 13(12), 1079–92, https://doi.org/10.1038/sj.mp.4002097

Biedler, J. L., Roffler-Tarlov, S., Schachner, M., & Freedman, L. S. (1978). Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Research*, 38(11 Pt 1), 3751–7.

Blanco, E., Bilbao, A., Luque-Rojas, M. J., Palomino, A., Bermúdez-Silva, F. J., Suárez, J., … de Fonseca, F. R. (2012). Attenuation of cocaine-induced conditioned locomotion is associated with altered expression of hippocampal glutamate receptors in mice lacking LPA1 receptors. *Psychopharmacology*, 220(1), 27–42, https://doi.org/10.1007/s00213-011-2446-6

Bogdanova, O. V, Kanekar, S., D'Anci, K. E., & Renshaw, P. F. (2013). Factors influencing behavior in the forced swim test. *Physiology & Behavior*, 118, 227–39, https://doi.org/10.1016/j.physbeh.2013.05.012

Castilla-Ortega, E., Hoyo-Becerra, C., Pedraza, C., Chun, J., Rodríguez De Fonseca, F., Estivill-Torrús, G., & Santín, L. J. (2011). Aggravation of chronic stress effects on hippocampal neurogenesis and spatial memory in LPA_1 receptor knockout mice. *PloS One*, 6(9), e25522, https://doi.org/10.1371/journal.pone.0025522

Chen, J. X., Tang, Y. T., & Yang, J. X. (2008). Changes of glucocorticoid receptor and levels of CRF mRNA, POMC mRNA in brain of chronic immobilization stress rats. *Cellular and Molecular Neurobiology*, 28(2), 237–244, https://doi.org/10.1007/s10571-007-9170-0

Chiba, S., Numakawa, T., Ninomiya, M., Richards, M. C., Wakabayashi, C., & Kunugi, H. (2012). Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, 39(1), 112–9,

https://doi.org/10.1016/j.pnpbp.2012.05.018

Cryan, J. F., Markou, A., & Lucki, I. (2002). Assessing antidepressant activity in rodents: recent developments and future needs. *Trends in Pharmacological Sciences*, 23(5), 238–245, https://doi.org/10.1016/S0165-6147(02)02017-5

Cryan, J. F., & Mombereau, C. (2004). In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. *Molecular Psychiatry*, 9(4), 326–57, https://doi.org/10.1038/sj.mp.4001457

Cunningham, M. O., Hunt, J., Middleton, S., LeBeau, F. E. N., Gillies, M. J., Gillies, M. G., … Racca, C. (2006). Region-specific reduction in entorhinal gamma oscillations and parvalbumin-immunoreactive neurons in animal models of psychiatric illness. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 26(10), 2767–76, https://doi.org/10.1523/JNEUROSCI.5054-05.2006

de Kloet, E. R., de Jong, I. E. M., & Oitzl, M. S. (2008). Neuropharmacology of glucocorticoids: focus on emotion, cognition and cocaine. *European Journal of Pharmacology*, 585(2–3), 473–82, https://doi.org/10.1016/j.ejphar.2008.03.011

de Kloet, E. R., Joëls, M., & Holsboer, F. (2005). Stress and the brain: from adaptation to disease. *Nature Reviews. Neuroscience*, 6(6), 463–75, https://doi.org/10.1038/nrn1683

Der-Avakian, A., & Markou, A. (2012). The neurobiology of anhedonia and other reward-related deficits. *Trends in Neurosciences*, 35(1), 68–77, https://doi.org/10.1016/j.tins.2011.11.005

El Rawas, R., Klement, S., Kummer, K. K., Fritz, M., Dechant, G., Saria, A., & Zernig, G. (2012). Brain regions associated with the acquisition of conditioned place preference for cocaine vs. social interaction. *Frontiers in Behavioral Neuroscience*, 6, 63, https://doi.org/10.3389/fnbeh.2012.00063

Elizalde, N., García-García, A. L., Totterdell, S., Gendive, N., Venzala, E., Ramirez, M. J., … Tordera, R. M. (2010). Sustained stress-induced changes in mice as a model for chronic depression. *Psychopharmacology*, 210(3), 393–406, https://doi.org/10.1007/s00213-010-1835-6

Fanselow, M. S., & Dong, H.-W. (2010). Are the dorsal and ventral hippocampus functionally distinct structures? *Neuron*, 65(1), 7–19, https://doi.org/10.1016/j.neuron.2009.11.031

Fink, K., Dooley, D. J., Meder, W. P., Suman-Chauhan, N., Duffy, S., Clusmann, H., & Göthert, M. (2002). Inhibition of neuronal Ca(2+) influx by gabapentin and pregabalin in the human neocortex. *Neuropharmacology*, 42(2), 229–36.

Füchsl, A. M., & Reber, S. O. (2016). Chronic Psychosocial Stress and Negative Feedback Inhibition: Enhanced Hippocampal Glucocorticoid Signaling despite Lower Cytoplasmic GR Expression. *PLOS ONE*, 11(4), e0153164, https://doi.org/10.1371/journal.pone.0153164

Gądek-Michalska, A., Spyrka, J., Rachwalska, P., Tadeusz, J., & Bugajski, J. (2013). Influence of chronic stress on brain corticosteroid receptors and HPA axis activity. *Pharmacological Reports : PR*, 65(5), 1163–75.

Gao, J., Wang, H., Liu, Y., Li, Y.-Y., Chen, C., Liu, L.-M., … Yang, C. (2014). Glutamate and GABA imbalance promotes neuronal apoptosis in hippocampus after stress. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, 20, 499–512, https://doi.org/10.12659/MSM.890589

García-Morales, V., Montero, F., González-Forero, D., Rodríguez-Bey, G., Gómez-Pérez, L., Medialdea-Wandossell, M. J., … Moreno-López, B. (2015). Membrane-derived phospholipids control synaptic neurotransmission and plasticity. *PLoS Biology*, 13(5), e1002153, https://doi.org/10.1371/journal.pbio.1002153

Gilabert-Juan, J., Castillo-Gomez, E., Pérez-Rando, M., Moltó, M. D., & Nacher, J. (2011). Chronic stress induces changes in the structure of interneurons and in the expression of molecules related to neuronal structural plasticity and inhibitory neurotransmission in the amygdala of adult mice. *Experimental Neurology*, 232(1), 33–40, https://doi.org/10.1016/j.expneurol.2011.07.009

Goodkin, H. P., Joshi, S., Mtchedlishvili, Z., Brar, J., & Kapur, J. (2008). Subunit-specific trafficking of GABA(A) receptors during status epilepticus. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 28(10), 2527–38, https://doi.org/10.1523/JNEUROSCI.3426-07.2008

Herman, J. P., Mueller, N. K., & Figueiredo, H. (2004). Role of GABA and glutamate circuitry in hypothalamo-pituitary-adrenocortical stress integration. *Annals of the New York Academy of Sciences*, 1018, 35–45, https://doi.org/10.1196/annals.1296.004

Herman, J. P., Watson, S. J., & Spencer, R. L. (1999). Defense of adrenocorticosteroid receptor expression in rat hippocampus: effects of stress and strain. *Endocrinology*, 140(9), 3981–91, https://doi.org/10.1210/endo.140.9.6962

Holm, M. M., Nieto-Gonzalez, J. L., Vardya, I., Henningsen, K., Jayatissa, M. N., Wiborg, O., & Jensen, K. (2011). Hippocampal GABAergic dysfunction in a rat chronic mild stress model of depression. *Hippocampus*, 21(4), 422–433, https://doi.org/10.1002/hipo.20758

Holsboer, F. (2000). The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, 23(5), 477–501, https://doi.org/10.1016/S0893-133X(00)00159-7

Juszczak, G. R., Lisowski, P., Sliwa, A. T., & Swiergiel, A. H. (2008). Computer assisted video analysis of swimming performance in a forced swim test: simultaneous assessment of duration of immobility and swimming style in mice selected for high and low swim-stress induced analgesia. *Physiology & Behavior*,

95(3), 400–7, https://doi.org/10.1016/j.physbeh.2008.07.003

Kim, H. K., Nunes, P. V., Oliveira, K. C., Young, L. T., & Lafer, B. (2016). Neuropathological relationship between major depression and dementia: A hypothetical model and review. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, 67, 51–57, https://doi.org/10.1016/j.pnpbp.2016.01.008

Márquez, C., Poirier, G. L., Cordero, M. I., Larsen, M. H., Groner, a, Marquis, J., … Sandi, C. (2013). Peripuberty stress leads to abnormal aggression, altered amygdala and orbitofrontal reactivity and increased prefrontal MAOA gene expression. *Translational Psychiatry*, 3(October 2012), e216, https://doi.org/10.1038/tp.2012.144

Martisova, E., Solas, M., Horrillo, I., Ortega, J. E., Meana, J. J., Tordera, R. M., & Ramírez, M. J. (2012). Long lasting effects of early-life stress on glutamatergic/GABAergic circuitry in the rat hippocampus. *Neuropharmacology*, 62(5–6), 1944–53, https://doi.org/10.1016/j.neuropharm.2011.12.019

McEwen, B. S., & Gianaros, P. J. (2011). Stress- and allostasis-induced brain plasticity. *Annual Review of Medicine*, 62, 431–45, https://doi.org/10.1146/annurev-med-052209-100430

Meyer, U., van Kampen, M., Isovich, E., Flügge, G., & Fuchs, E. (2001). Chronic psychosocial stress regulates the expression of both GR and MR mRNA in the hippocampal formation of tree shrews. *Hippocampus*, 11(3), 329–36, https://doi.org/10.1002/hipo.1047

Mirendil, H., Thomas, E. A., De Loera, C., Okada, K., Inomata, Y., & Chun, J. (2015). LPA signaling initiates schizophrenia-like brain and behavioral changes in a mouse model of prenatal brain hemorrhage. *Translational Psychiatry*, 5, e541, https://doi.org/10.1038/tp.2015.33

Mizoguchi, K., Ishige, A., Aburada, M., & Tabira, T. (2003). Chronic stress attenuates glucocorticoid negative feedback: involvement of the prefrontal cortex and hippocampus. *Neuroscience*, 119(3), 887–97.

Moreno-Fernández, R.D., Pérez-Martín, M., Castilla-Ortega, E., Rosell Del Valle, C., García-Fernández, MI., Chun, J., Estivill-Torrús, G., Rodríguez de Fonseca, F., Santín, L.J., Pedraza, C. (2017). maLPA1-null mice as an endophenotype of anxious depression. Translational Psychiatry. 7(4):e1077. Retrieve from https://doi.org/10.1038/tp.2017.24

Moreno-Fernandez, R.D., Nieto-Quero, A., Gómez-Salas, FJ., Chun, J., Estivill-Torrús, G., Rodriguez de Fonseca, F., Santín, L.J., Pérez-Martín, M., Pedraza, C. (2018). Effects of genetic deletion versus pharmacological blockade of the LPA_1receptor on depression-like behaviour and related brain functional activity. Disease Models and Mechanisms, 11: dmm035519.

Mosienko, V., Bert, B., Beis, D., Matthes, S., Fink, H., Bader, M., & Alenina, N. (2012). Exaggerated aggression and decreased anxiety in mice deficient in brain

serotonin. *Translational Psychiatry*, 2(5), e122-9. https://doi.org/10.1038/tp.2012.44

Murph, M. M., Scaccia, L. A., Volpicelli, L. A., & Radhakrishna, H. (2003). Agonist-induced endocytosis of lysophosphatidic acid-coupled LPA1/EDG-2 receptors via a dynamin2- and Rab5-dependent pathway. *Journal of Cell Science*, 116(Pt 10), 1969–80. https://doi.org/10.1242/jcs.00397

Musazzi, L., Di Daniel, E., Maycox, P., Racagni, G., & Popoli, M. (2011). Abnormalities in α/β-CaMKII and related mechanisms suggest synaptic dysfunction in hippocampus of LPA1 receptor knockout mice. *The International Journal of Neuropsychopharmacology / Official Scientific Journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)*, 14(7), 941–53, https://doi.org/10.1017/S1461145710001240

Orlovsky, M. A., Dosenko, V. E., Spiga, F., Skibo, G. G., & Lightman, S. L. (2014). Hippocampus remodeling by chronic stress accompanied by GR, proteasome and caspase-3 overexpression. *Brain Research*, 1593, 83–94, https://doi.org/10.1016/j.brainres.2014.09.059

Pedraza, C., Sánchez-López, J., Castilla-Ortega, E., Rosell-Valle, C., Zambrana-Infantes, E., García-Fernández, M., … Estivill-Torrús, G. (2014). Fear extinction and acute stress reactivity reveal a role of LPA(1) receptor in regulating emotional-like behaviors. *Brain Structure & Function*, 219(5), 1659–72, https://doi.org/10.1007/s00429-013-0592-9

Pittenger, C., & Duman, R. S. (2008). Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, 33(1), 88–109, https://doi.org/10.1038/sj.npp.1301574

Pizzagalli, D. A. (2014). Depression, stress, and anhedonia: toward a synthesis and integrated model. *Annual Review of Clinical Psychology*, 10, 393–423, https://doi.org/10.1146/annurev-clinpsy-050212-185606

Porsolt, R. D., Le Pichon, M., & Jalfre, M. (1977). Depression: a new animal model sensitive to antidepressant treatments. *Nature*, 266(5604), 730–2.

Rannals, M. D., & Kapur, J. (2011). Homeostatic strengthening of inhibitory synapses is mediated by the accumulation of GABA(A) receptors. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 31(48), 17701–12, https://doi.org/10.1523/JNEUROSCI.4476-11.2011

Ritov, G., Boltyansky, B., & Richter-Levin, G. (2015). A novel approach to PTSD modeling in rats reveals alternating patterns of limbic activity in different types of stress reaction. *Molecular Psychiatry*, (September), 1–12, https://doi.org/10.1038/mp.2015.169

Robison, A. J. (2014). Emerging role of CaMKII in neuropsychiatric disease. *Trends in Neurosciences*, 37(11), 653–62, https://doi.org/10.1016/j.tins.2014.07.001

Ross, R. A., Spengler, B. A., & Biedler, J. L. (1983). Coordinate morphological and biochemical interconversion of human neuroblastoma cells. *Journal of the National Cancer Institute*, 71(4), 741–7.

Saaltink, D. J., & Vreugdenhil, E. (2014). Stress, glucocorticoid receptors, and adult neurogenesis: A balance between excitation and inhibition? *Cellular and Molecular Life Sciences*, 71(13), 2499–2515, https://doi.org/10.1007/s00018-014-1568-5

Schoenfeld, T. J., Rada, P., Pieruzzini, P. R., Hsueh, B., & Gould, E. (2013). Physical exercise prevents stress-induced activation of granule neurons and enhances local inhibitory mechanisms in the dentate gyrus. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 33(18), 7770–7, https://doi.org/10.1523/JNEUROSCI.5352-12.2013

Snyder, J. S., Soumier, A., Brewer, M., Pickel, J., & Cameron, H. A. (2011). Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature*, 476(7361), 458–61, https://doi.org/10.1038/nature10287

Strekalova, T., Spanagel, R., Bartsch, D., Henn, F. a, & Gass, P. (2004). Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration. *Neuropsychopharmacology*, 29(11), 2007–2017, https://doi.org/10.1038/sj.npp.1300532

Touyarot, K., & Sandi, C. (2002). Chronic restraint stress induces an isoform-specific regulation on the neural cell adhesion molecule in the hippocampus. *Neural Plasticity*, 9(3), 147–59, https://doi.org/10.1155/NP.2002.147

Tzanoulinou, S., Riccio, O., de Boer, M. W., & Sandi, C. (2014). Peripubertal stress-induced behavioral changes are associated with altered expression of genes involved in excitation and inhibition in the amygdala. *Translational Psychiatry*, 4, e410, https://doi.org/10.1038/tp.2014.54

Veena, J., Srikumar, B. N., Raju, T. R., & Shankaranarayana Rao, B. S. (2009). Exposure to enriched environment restores the survival and differentiation of new born cells in the hippocampus and ameliorates depressive symptoms in chronically stressed rats. *Neuroscience Letters*, 455(3), 178–82, https://doi.org/10.1016/j.neulet.2009.03.059

Venzala, E., García-García, A. L., Elizalde, N., & Tordera, R. M. (2013). Social vs. environmental stress models of depression from a behavioural and neurochemical approach. *European Neuropsychopharmacology*, 23(7), 697–708, https://doi.org/10.1016/j.euroneuro.2012.05.010

Welberg, L. A., & Seckl, J. R. (2001). Prenatal stress, glucocorticoids and the programming of the brain. *Journal of Neuroendocrinology*, 13(2), 113–28.

Yinn, Y.Y., Ming, L., Zheng, L.F., Kan, H.W., Li, C.R., Li, W.P. (2007). Bioactive compounds from Paecilomyces tenuipes regulating the function of the hypothalamo-hypophyseal system axis in chronic unpredictable stress rats.

Chinese Medical Journal, 120: 1088-1092.

Bagot, R. C., Parise, E. M., Peña, C. J., Zhang, H.-X., Maze, I., Chaudhury, D., … Nestler, E. J. (2015). Ventral hippocampal afferents to the nucleus accumbens regulate susceptibility to depression. *Nature Communications*, 6(1), 7062, https://doi.org/10.1038/ncomms8062

Balaban, C. D., Ogburn, S. W., Warshafsky, S. G., Ahmed, A., & Yates, B. J. (2014). Identification of neural networks that contribute to motion sickness through principal components analysis of fos labeling induced by galvanic vestibular stimulation. *PloS One*, 9(1), e86730, https://doi.org/10.1371/journal.pone.0086730

Bannerman, D. M., Rawlins, J. N. P., McHugh, S. B., Deacon, R. M. J., Yee, B. K., Bast, T., … Feldon, J. (2004). Regional dissociations within the hippocampus--memory and anxiety. *Neuroscience and Biobehavioral Reviews*, 28(3), 273–83, https://doi.org/10.1016/j.neubiorev.2004.03.004

Figure Legends

Figure 1. Experimental procedures. The procedures for the continuous delivery of drugs and chronic stress are shown. The experimental groups are also indicated. Alzet minipump implantation refers to the day of vehicle (100 µl/pump; BSA-FAF; control) or LPA (0.36 µg/pump; 200 nM by Alzet® osmotic minipump) ICV infusion. Five days after Alzet minipump implantation, the animals were submitted to 21 days of the chronic and intermittent restraint procedure (15 days of restrain and 6 without restraint). On the last day of the stress procedure, the animals were tested for saccharin preference. A forced swim test was carried out a day after the stress procedure was completed. Two days after completing all behavioural tests, the animals were sacrificed by decapitation for PCR and Western blot analysis. Blood samples for corticosterone determination were collected 3 times during the experimental procedure: at the beginning of the procedure, in the middle of the procedure (after 15 days of stress) and 10 min after completing the FST. The ICV administration groups received the same treatment as the stressed animals, with the exception of the stress procedure. The control animals underwent the same molecular procedures but not ICV administration or stress and instead remained undisturbed in their home cages while the vehicle- and chronic LPA-treated animals received such treatments.

Figure 2. Behavioural tests. A) Hedonic behaviour in the saccharin preference test. Under basal conditions, LPA increased the preference for saccharin over water to just under 100%. However, stress reduced the preference for saccharin to just under the anhedonic behaviour threshold. The continuous delivery of the LPA in the stressed animals reduced the preference for saccharin by nearly 50%. B) and C) Stress-coping responses assessed in the FST. B) Stress increased the total time of immobility, but the continuous delivery of LPA in the non-stressed animals did not induce any effect. C) The continuous delivery of the LPA in the stressed animals decreased the latency to the first immobility period. LSD * $P<0.05$ *** $P<0.005$ compared to the unstressed vehicle group; # $P<0.05$ compared to the vehicle-treated group submitted to chronic stress; &&& $P<0.005$ compared to the unstressed LPA-treated group.

Figure 3. Excitation/inhibition balance in the dorsal and ventral hippocampus. A, C E, G, I. No significant differences were found in the expression of any of the genes examined in the dorsal hippocampus. B, D, F. Although the experimental treatments did not induce changes in the expression of *NR1, GAD67* (and v*Glut1* mRNA in the ventral hippocampus, (H) chronic LPA administration reduced the mRNA expression of *vGAT* compared to the vehicle group in this area (M) and increased *vGlut1/vGAT* mRNA ratio in these animals. * $P \leq 0.05$ compared to the control group; # $P \leq 0.05$ compared to the vehicle group.

Figure 4. The analysis of corticosteroid receptor expression in the hippocampus revealed reduced GR expression after chronic stress. A) Dorsal and B) ventral GR mRNA levels. In dorsal hippocampus, stress reduced the GR mRNA levels in both the LPA- and vehicle-treated groups. C) No differences were observed in the mRNA expression of MR in the dorsal and D) ventral hippocampus. E) In dorsal hippocampus, no significant differences were found in the MR:GR mRNA ratio. F) However, LPA in non-stressed animals, reduced the MR:GR mRNA ratio in the ventral part of the hippocampus. G) At the protein level, a statistically significant reduction in the expression of GR in the dorsal hippocampus was observed in both the vehicle- and LPA-treated animals under stress. H) In the ventral hippocampus, stress did not affect the expression of the GR protein. LSD: * $P<0.05$; ** $P \leq 0.01$ compared to their control group. GR: glucocorticoid receptor MR: mineralocorticoid receptor.

Figure 5. Corticosterone response. Measurement of CORT levels over time (prior to stress, after chronic stress and 10 min after completing the FST). The continuous delivery of LPA in the chronically stressed animals induced high CORT levels, as measured 15 days after the start of the stress procedure. However, the continuous delivery of LPA in the non-stressed animals may have mitigated the impact of acute stress (i.e., the FST) on the HPA axis. _*_ $P<0.05$ difference with respect to the rest of the experimental groups.

Figure 6. PCA representation. A) Principal component analysis of depressive-like behaviours and underlying biological measures. The interpretable factor loadings are in bold. The variables with negative scores are inversely related to the factor.

Rotation method: varimax with Kaiser normalization. Rotation converged in 3 iterations. KMO=0.70; χ^2=49,373; P<0.0005. B) Representative figure of the factor scores 1. C) Representative figure of the factor scores 2. The LPA-treated animals, exhibited the lowest or the highest scores on 'anhedonic behaviour' under basal or stress condition, respectively. The LPA-treated animals, under stress showed the lowest scores on 'active stress-coping behaviour'. *****P≤0.001 with respect to the control group; # P≤0.05 with respect the vehicle-treated group.

Table 1. Primer sequences for qPCR. In blue, the reference genes.

Table(s)

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Table 1. Primers sequences for qPCR

Gene	*Protein*	*Name*	*Forward primer (5'-3')*	*Reverse primer (5'-3')*	*RefSeq (NCBI)*
Grin1	NR1	glutamate receptor, ionotropic, NMDA1 (zeta 1)	TGGTACCCATGTCATCCCAA	GCCATCACTCATTGTGGGCT	NM_008169.2
Gad1	GAD67	glutamate decarboxylase 1	GTTCTTCAGGCTCTCCCGTG	CAGGAACAGGCTCGGTTCAG	NM_008077.4
Slc32a1	vGAT	solute carrier family 32 (GABA vesicular transporter), member 1	ACATTCATTATCAGCGCGGC	GCACGAACATGCCCTGAATG	NM_009508.2
Slc17a7	vGlut1	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	TTGTGGCTACCTCCACCCTA	GCATAGGAACCGCAAAAGGC	NM_182993.2
Nr3c1	GR	nuclear receptor subfamily 3, group C, member 1	CAAAGATTGCAGGTATCCTATGAA	TGGCTCTTCAGACCTTCCTT	NM_008173.3
Nr3c2	MR	nuclear receptor subfamily 3, group C, member 2	TTCGGAGAAAGAACTGTCCTG	CCCAGCTTCTTTGAGTTTCG	NM_001083906.1
Eef1a1	EEF1	eukaryotic translation elongation factor 1 alpha 1	TCCACTTGGTCGCTTTGCT	CTTCTTGTCCACAGCTTTGATG A	NM_010106.2
Tbp	TBP	TATA box binding protein	CTGGAATTGTACCGCAGCTT	CAGTTGTCCGTGGCTCTCTT	NM_013684.3

Table 1

Figure(s)

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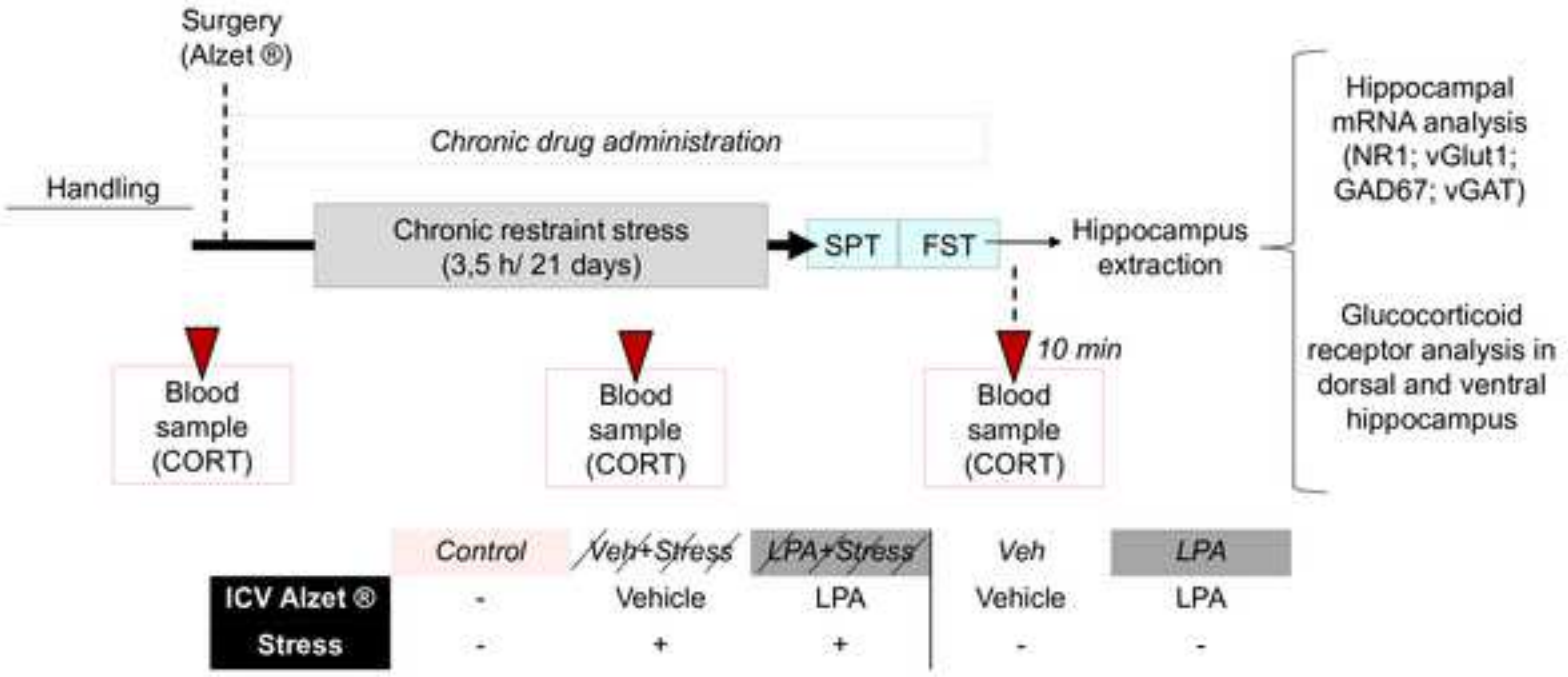

Figure 1. Experimental procedure.

Figure(s)

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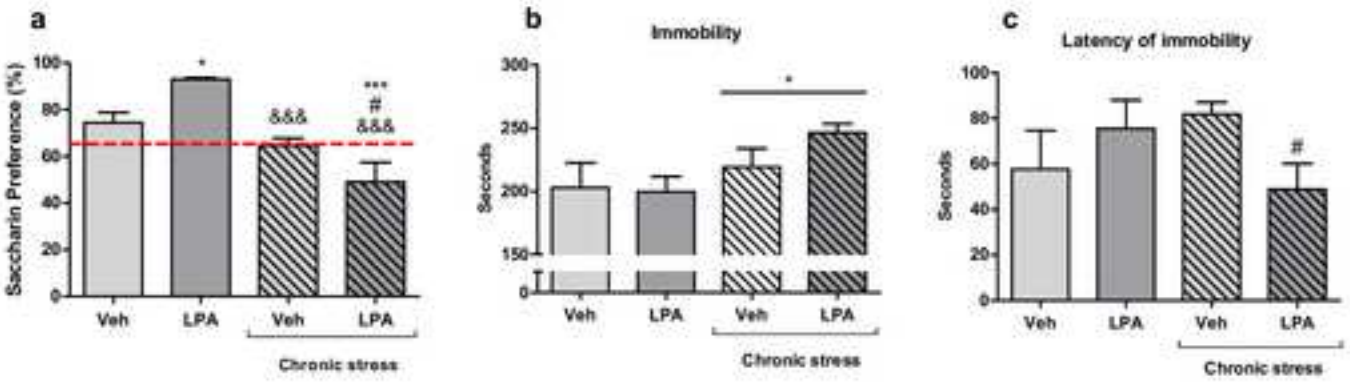

Figure 2

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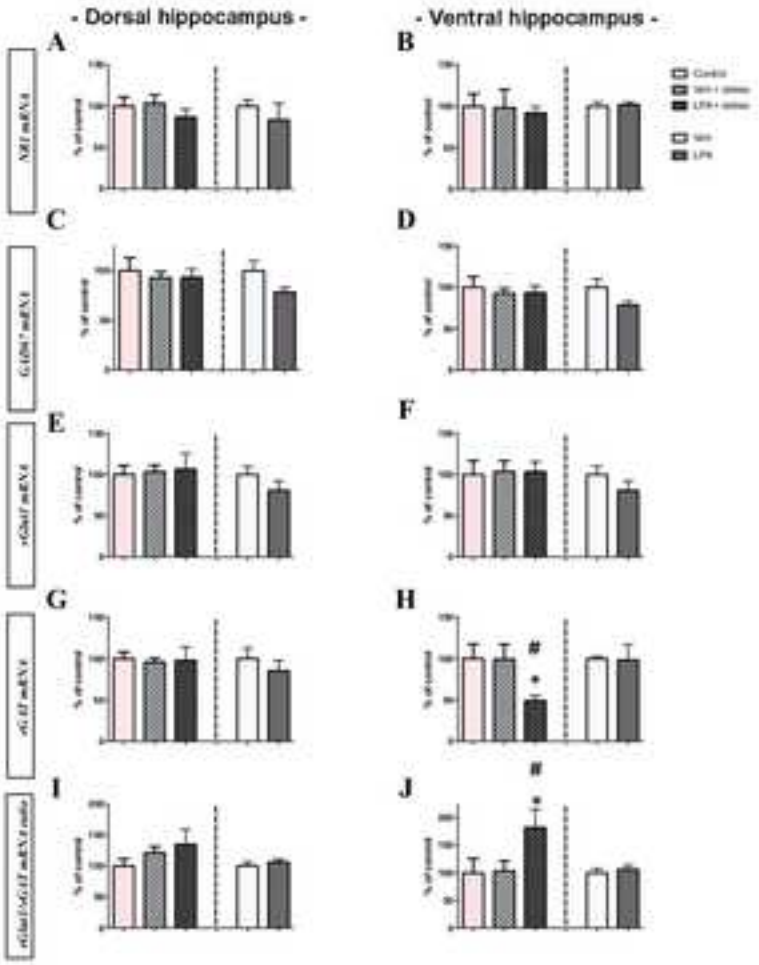

Figure 3

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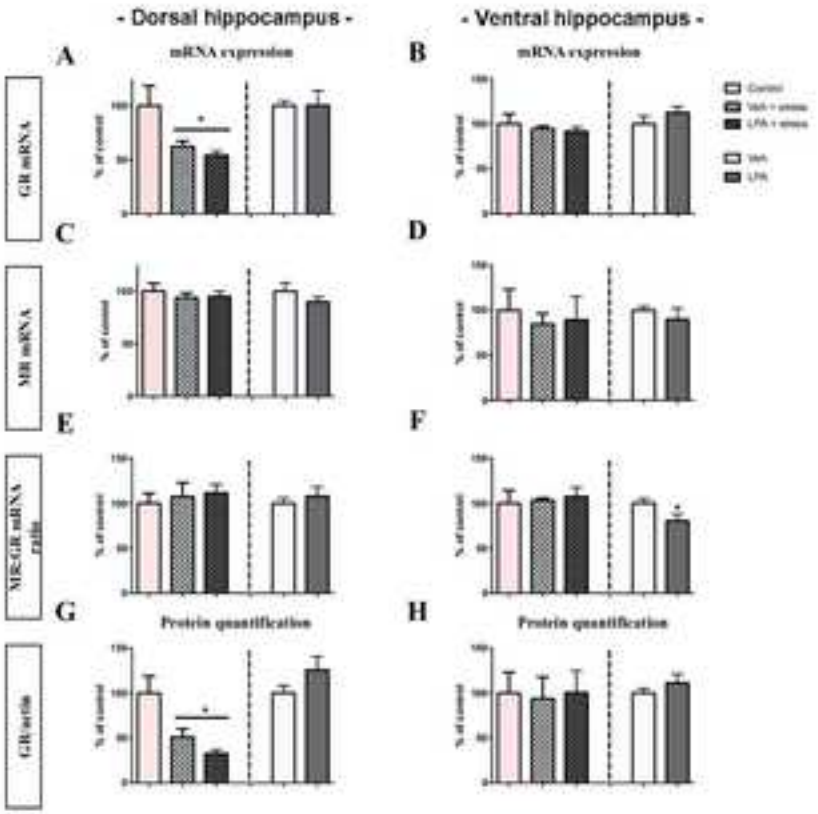

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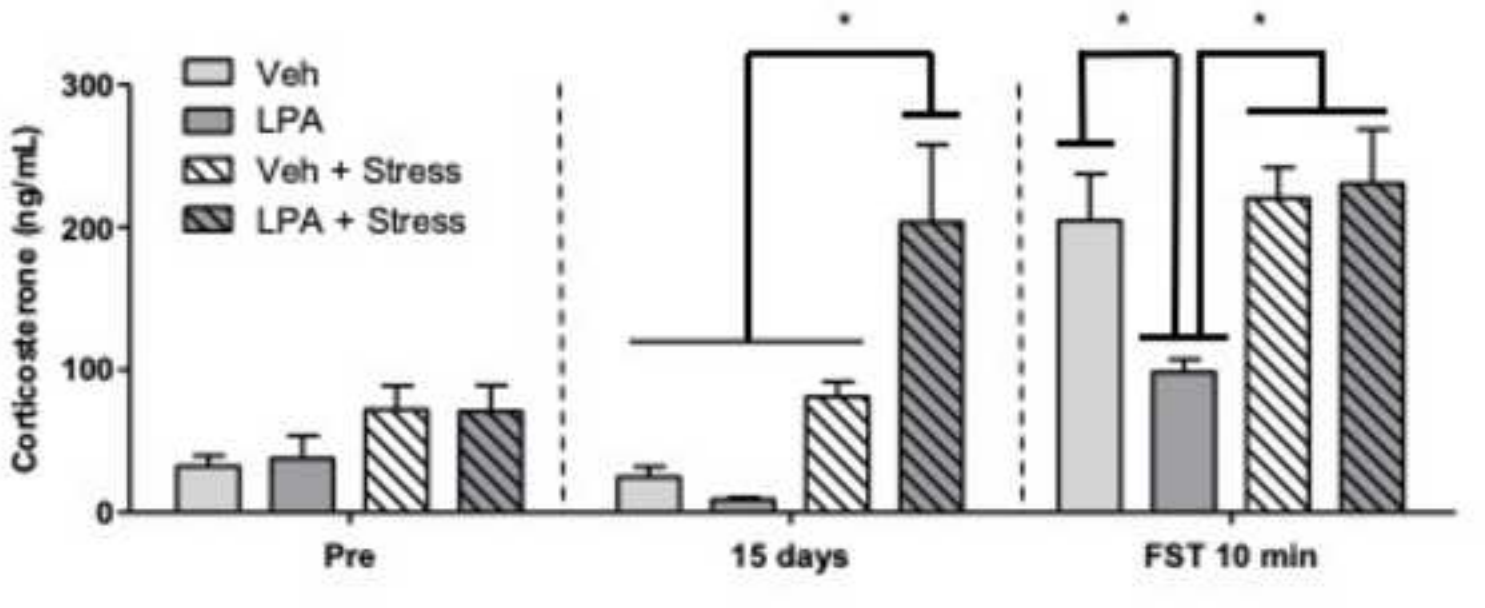

Figure 5

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A

Measure	Principal components	
	Factor 1	Factor 2
Saccharin preference (%)	-0.695	0.391
Latency of immobility (s)		0.871
Immobility (s)		-0.765
GR mRNA levels in the dorsal hippocampus	-0.604	
CORT levels after chronic stress	0.760	-0.343
vGlut1/vGAT mRNA ratio in ventral hippocampus	0.702	
MR/GR mRNA ratio ventral hippocampus	0.797	
Eigenvalue	2.8	1.377
% Variance (Cumulative)	35.25	59.73

B

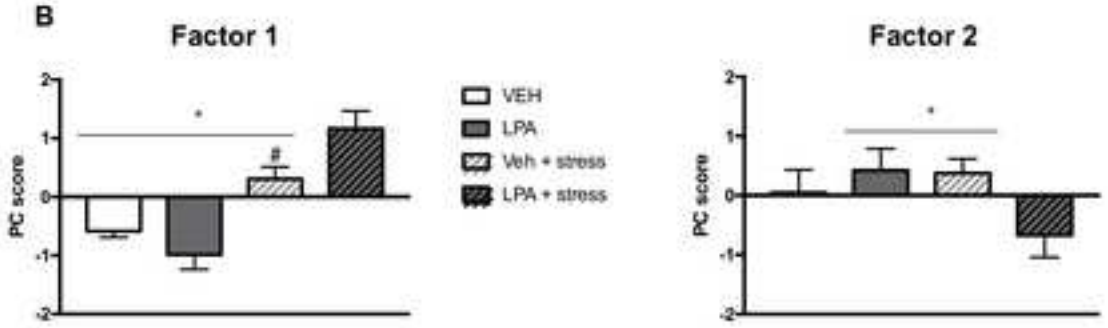

Figure 6

Supplementary Material

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Author Contributions

Carmen Pedraza; Carmen Sandi and Román D. Moreno-Fernández conceived and designed research; **Román D. Moreno-Fernández, Alexandre Bacq; Cristina Rosell-Valle; Olivia Zanoletti; Manuel Cifuentes; Ana L. Gavito, María García-Fernández:** have performed the experiments and contributed to the data collection; **Román D. Moreno-Fernández and Carmen Pedraza:** performed statistical analyses; **Carmen Pedraza, Carmen Sandi**, **Román D. Moreno-Fernández; Luis J. Santín; Manuel Cifuentes, Margarita Pérez-Martín: Fernando Rodriguez de Fonseca, Guillermo Estivill-Torrús,** interpreted results of experiments; **Román D. Moreno-Fernández; Cristina Rosell-Valle and Manuel Cifuentes:** have prepared figures; **Román D. Moreno-Fernández and Carmen Pedraza**: Writing - Original Draft with contributions from the other authors; **Carmen Pedraza; Carmen Sandi**, **Luis J. Santín; Manuel Cifuentes, Margarita Pérez-Martín: Fernando Rodriguez de Fonseca, Guillermo Estivill-Torrús:** edited and revised manuscript; all authors read and approved final version of manuscript.