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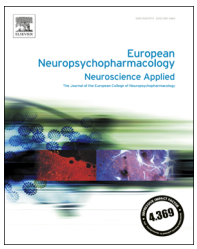

Cocaine-induced behavioral sensitization decreases the expression of endocannabinoid signaling-related proteins in the mouse hippocampus

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Abbreviations: CB1, cannabinoid type 1 receptor; CL, conditioned locomotion; LS, locomotor sensitization to cocaine; DAGLα/β, diacylglycerol lipase alpha/beta; ECS, endocannabinoid system; FAAH, fatty acid amino hydrolase; GluA1/2/3/4, AMPA-glutamate receptor subunits; KGA, kidney-type glutaminase isoform; LGA, liver-type glutaminase isoform; MAGL, monoacylglycerol lipase; mGluR, metabotropic glutamate receptor; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D; NMDAR, NMDA-glutamate receptor; GluN1/2A/2B/2C, NMDA-glutamate receptor subunits

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Abstract
In the reward mesocorticolimbic circuits, the glutamatergic and endocannabinoid systems are implicated in neurobiological mechanisms underlying cocaine addiction. However, the involvement of both systems in the hippocampus, a critical region to process relational information relevant for encoding drug-associated memories, in cocaine-related behaviors remains unknown. In the present work, we studied whether the hippocampal gene/protein expression of relevant glutamate signaling components, including glutamate-synthesizing enzymes and metabotropic and ionotropic receptors, and the hippocampal gene/protein expression of cannabinoid type 1 (CB1) receptor and endocannabinoid metabolic enzymes were altered following acute and/or repeated cocaine administration resulting in conditioned locomotion and locomotor sensitization. Results showed that acute cocaine administration induced an overall down-regulation of glutamate-related gene expression and, specifically, a low phosphorylation level of GluA1. In contrast, locomotor sensitization to cocaine produced an up-regulation of several glutamate receptor-related genes and, specifically, an increased protein expression of the GluN1 receptor subunit. Regarding the endocannabinoid system, acute and repeated cocaine administration were associated with an increased gene/protein expression of CB1 receptors and a decreased gene/protein expression of the endocannabinoid-synthesis enzymes N-acyl phosphatidylethanolamine D (NAPE-PLD) and diacylglycerol lipase alpha (DAGLα). These changes resulted in an overall decrease in endocannabinoid synthesis/degradation ratios, especially NAPE-PLD/fatty acid amide hydrolase and DAGLα/monoacylglycerol lipase, suggesting a reduced endocannabinoid production associated with a compensatory up-regulation of CB1 receptor. Overall, these findings suggest that repeated cocaine administration resulting in locomotor sensitization induces a down-regulation of the endocannabinoid signaling that could contribute to the specifically increased GluN1 expression observed in the hippocampus of cocaine-sensitized mice.

1. Introduction

In recent years, learning and memory processes have been identified as key players among mechanisms underlying cocaine addiction (Dong and Nestler, 2014). The hippocampus is one of the most important brain regions involved in learning and memory and, thus, its contribution in processes underlying cocaine addiction has not gone unnoticed, although it remains unclear (Blanco et al., 2012a, 2012b; Castilla-Ortega et al., 2015; Fole et al., 2014; Noonan et al., 2010; Rivera et al., 2013; Thompson et al., 2002, 2005; Yamaguchi et al., 2004).

When cocaine is repeatedly administered in the same setting, environmental stimuli become associated with the rewarding effects of the drug (Crombag et al., 2008; Miller and Marshall, 2005; Xie et al., 2013). These drug-paired contextual cue memories are long-lasting and are involved in two important phenomena relevant to relapse: cocaine-induced conditioned locomotion and locomotor sensitization (Alaghband et al., 2014; Steketee and Kalivas, 2011). Conditioned locomotion is observed when vehicle-treated mice or rats repeatedly pretreated with cocaine show an increased motor activity, compared to those pretreated with vehicle, when are re-exposed to the environment where the pretreatment has taken place (Blanco et al., 2012a; Blanco-Calvo et al., 2014). On the other hand, locomotor sensitization is defined as a progressive increment of cocaine-induced motor activity through repetitive and intermittent drug administration (Steketee and Kalivas, 2011). Locomotor sensitization to cocaine is often studied using a protocol in which cocaine-pretreated animals are administered a priming cocaine injection resulting in a magnified motor response compared to those receiving an acute cocaine administration (Galeano et al., 2013; Kalivas and Stewart, 1991). Conditioned locomotion is by definition influenced by the environment while locomotor sensitization is influenced by both the environment and drug stimuli. For instance, the magnified motor response induced by the priming cocaine injection is higher when the priming injection is administered in the same environment where animals were repeatedly pretreated with cocaine than when the environment is different (Kalivas and Stewart, 1991).

Since the dorsal hippocampus is critical for encoding contextual information required to form associations between contextual cues and drug-rewarding effects (contextual cue memories) (Smith and Bulkin, 2014), it could be hypothesized that this anatomical structure must be involved in cocaine-induced conditioned locomotion and locomotor sensitization. Moreover, one of the most important hippocampal neurotransmitter systems is the glutamatergic system, which is essential for memory consolidation through its regulation of synaptic plasticity (Mukherjee and Manahan-Vaughan, 2013; Park et al., 2013; Poncer, 2003). Furthermore, glutamatergic signaling plays a key role in cocaine-induced synaptic plasticity in the ventral tegmental area (VTA) and nucleus accumbens (NAc) (Mameli and Lüscher, 2011; Thomas and Malenka, 2003). On the other hand, glutamatergic transmission is retrogradely modulated by endocannabinoids through the stimulation of CB1 receptors

(Freund et al., 2003; Piomelli, 2003), located in the glutamatergic and GABAergic axon terminals and preterminals of the brain. It has also been demonstrated that the endocannabinoid system (ECS) plays an important role in cocaine addiction, synaptic plasticity and memory consolidation (De Oliveira Alvares et al., 2008; Castillo et al., 2012; Melis and Pistis, 2012; Sidhpura and Parsons, 2011). Despite this background, the possible role of the hippocampal glutamatergic system and endocannabinoid signaling in cocaine conditioning and sensitization processes is poorly understood.

The main aim of the present work was to study whether glutamate- and endocannabinoid-related gene and protein hippocampal expression might be altered following acute cocaine administration, cocaine-induced conditioned locomotion and/or locomotor sensitization. In addition, phosphorylation of the AMPA receptor GluA1 subunit, which is implicated in synaptic plasticity and learning and memory processes (Lee et al., 2003), was also measured.

2. Experimental procedures

2.1. Ethics statement

All experimental procedures with animals were performed in compliance with the European Communities directive of 24 November 1986 (86/609/ECC) and Spanish legislation (BOE 252/ 34367-91, 2005) regulating animal research. Research procedures included in the present study were approved by the Research and Ethics Committee of Universidad de Málaga and Hospital Regional Universitario de Málaga. All efforts were made to reduce the number of animals used and to minimize their suffering.

2.2. Animals and housing

Experimentally naïve male C57BL/6J mice (25 ± 5 g; Charles River Laboratories International, Wilmington, MA, USA) were used for behavioral procedures, gene and protein expression analyses. Animals were housed in the *vivarium* of the Universidad de Málaga in clear plastic cages, in a temperature (22 ± 2 °C)- and humidity ($65 \pm 5\%$) -controlled room with a 12-h light–dark cycle (lights on at 8:00 a.m.). Purina chow and tap water were available *ad libitum*.

2.3. Drugs

Cocaine-HCl was obtained from Alkaliber S.A. (Madrid, Spain), dissolved in sterile saline (0.9% NaCl) just before experimentation and administered i.p. at doses of 10 (acute administration and locomotor sensitization to cocaine) and 20 mg/kg (repeated cocaine administration phase).

2.4. Apparatus and general procedures

All mice were handled and habituated to injection procedures twice a day for 2 days prior to behavioral testing to reduce the effects of the nonspecific stress of being handled during the behavioral tests. All experiments were carried out between 08:00 and 15:00 h. Animals were acclimated to the experimental room for 30 min each day. Performance in the Open Field (OF) was recorded by a computer-based video tracking system (Smart v2.5®, Panlab, Barcelona, Spain). The maximum light intensity in the center of the OF was 100 lx. Four OF ($50 \times 50 \times 50$ cm, Panlab) with gray backgrounds were used. Animals were placed in the center of the arena, and horizontal locomotion was recorded for 30 min in time bins of 5 min. Horizontal locomotion was measured as the total distance traveled (cm).

2.5. Acute and repeated cocaine administration, conditioned locomotion and locomotor sensitization to cocaine

Locomotor sensitization to cocaine was conducted following a consecutive four-phase paradigm (Blanco et al., 2012a) (Figure 1A): cocaine conditioning (repeated cocaine administration), drug free period (no-treatment), conditioned locomotion (CL) probe and locomotor sensitization (LS) to cocaine. Firstly, two groups of mice were injected with cocaine (20 mg/kg) or vehicle (0.9% NaCl) for five consecutive days and exposed immediately to the OF for 30 min (cocaine conditioning, Figure 1A). During the next phase animals did not receive any treatment for five/six consecutive days (no-treatment, Figure 1A). On the following day (day 13, Figure 1A), half of the animals that were pretreated with vehicle and half of the animals that were pretreated with cocaine (20 mg/kg), were treated with a single injection of vehicle and exposed again to the OF for 30 min. It was expected that animals pretreated with cocaine showed an increased motor response in comparison with those pretreated with vehicle (CL). Finally, on the next day (day 14), half of the animals that were pretreated with vehicle and half of the animals that were pretreated with cocaine were treated with a primed injection of cocaine (10 mg/kg) and exposed again to the OF during 30 min. Thus, we tested whether animals pretreated with cocaine and treated with a primed cocaine injection showed an increased motor activity (LS) compared to those pretreated with vehicle and injected with a primed dose of cocaine (acute cocaine administration). According with this protocol, four groups of animals can be distinguished: (1) mice pretreated with vehicle and treated with vehicle (vehicle–vehicle group or V-V, $n=8$); (2) mice pretreated with vehicle and treated with cocaine (vehicle–cocaine group or V-C, $n=8$); (3) mice pretreated with cocaine and treated with vehicle (cocaine–vehicle group or C-V, $n=8$); and (4) mice pretreated with cocaine and treated with cocaine (cocaine–cocaine group or C-C, $n=8$). Animals from these four groups were employed for gene and protein expression analyses.

2.6. Tissue collection

On days 13 and 14 (Figure 1A), one hour after administration of vehicle or cocaine, animals were killed by decapitation, and their brains were immediately dissected, frozen on dry ice, and stored at -80 °C. Brains were sliced in coronal sections (1 mm thick), on dry ice, using razor blades and a mouse brain slicer matrix (Zivic Instruments). The dorsal hippocampus was precisely removed with fine surgical instruments according to Paxinos and Watson atlas (Paxinos and Watson, 2007). Samples were stored at -80 °C until further processing for gene and protein analyses.

2.7. RNA isolation and quantitative real-time PCR analysis

Real-time PCR was used to measure relative mRNA levels as previously described (Blanco et al., 2014). Total RNA was isolated using the Trizol® method, according to the manufacturer's instructions (Gibco BRL Life Technologies, Baltimore, MD, USA). Tissues were placed into 1 ml of Trizol Reagent (Invitrogen, CA, USA) and homogenized with an IKA-Ultra-Turrax®T8 (IKA-Werke GmbH, Staufen, Germany). To ensure the purity of the mRNA sequences and exclude molecules smaller than 200 nucleotides, RNA samples were isolated with a RNeasy Minelute Cleanup Kit (Qiagen, Hilden, Germany), which included digestion with DNase I column (RNase-free DNase Set, Qiagen), according to the manufacturers' instructions, and purified using RNeasy Mini Kit (Qiagen, Hilden, Germany).

The total mRNA concentrations were quantified using a spectrophotometer (Nanodrop 1000 Spectrophotometer, Thermo Scientific, Rochester, NY, USA) to ensure A260/280 ratios of 1.8-2.0. Reverse transcription was carried out from 1 mg of mRNA using the Transcriptor Reverse Transcriptase kit and random hexamer primers (Transcriptor RT, Roche Diagnostic GmbH, Manheim, Germany). Negative controls included reverse transcription reactions that omitted the reverse transcriptase. The obtained cDNAs were used as templates for quantitative real-time PCR with an iCycler system (BioRad, Hercules, CA, USA) using the Quanti-Test SYBR Green PCR kit (Qiagen, Hilden, Germany). The primers used are described in Table 1. Oligonucleotides were provided by Sigma-Proligo (Proligo France SAS, Paris, France). Quantification was carried out according to standard curves run simultaneously with the samples, and each reaction was run in duplicate. The PCR product was separated by electrophoresis in a 1% agarose gel to verify fragment size and the absence of contamination fragments, quantified by measuring the absorbance at 260 nm, and serially diluted to 10^{-5} pg/ml. Several 10-fold dilutions (10^{-1}-10^{-5}) were checked for optimal cycling on the iClycler system and three of them were selected for standard curves. Each reaction was run in duplicate and contained 2.5 μl of cDNA template, 8 μl of Master SYBR Green, 4.86 μl of PCR Ultra Pure Water and 0.64 μl of primers in a final reaction volume of 15 μl. Cycling parameters were the following: 95 °C for 15 min to activate DNA polymerase, then 30-40 cycles of 94 °C for 15 s, annealing temperature specific for each primer for 30 s and a final extension step of 72 °C for 30 s in which fluorescence was acquired. Melting curves analysis was performed to ensure that only a single product was amplified. Absolute values from each sample were normalized with regard to *Actb* (constitutive gene). This internal standard was chosen based on a first analysis of a panel of housekeeping genes that additionally included *CypA* and *Sp1*.

2.8. Western blot analysis

Western blotting was used to measure protein levels as previously described (Crespillo et al., 2011; Blanco et al., 2012b). To measure ECS-related proteins, samples were homogenized in 50 mM Hepes buffer (pH 8) and 0.32 M sucrose buffer in order to obtain membrane protein extracts. The homogenate was centrifuged at 800*g* for 10 min at 4 °C and the supernatant centrifuged at 40,000*g* for 30 min. The pellets were resuspended in 50 mM Hepes buffer (pH 8) and pulverized using a homogenizer. To measure glutamatergic system-related proteins, samples were homogenized and incubated in RIPA buffer 1 × (Thermo Scientific) containing a proteinase and phosphatase inhibitor cocktail (sodium fluoride 50 mM, sodium orthovanadate 1 mM, sodium pyrophosphate 10 mM, β-glycerophosphate 10 mM, NaF 5 mM, $NaOV_4$ 100 μM, NaH_2PO_4 1 mM, aprotinin 80 μM, pepstatin A 2 mM, trypsin inhibitor 1 μM, phenylmethylsulfonyl fluoride 50 μM; Merck) for 2 h at 4 °C, and then centrifuged at 10,000*g* for 15 min at 4 °C. Protein concentration was measured using the Bradford protein assay. For immunoblotting, protein samples (40 μg) were separated on 10% (w/v) SDS-PAGE gels, transferred to nitrocellulose membranes (BioRad) and controlled by Ponceau Red staining. After blocking with 5% (w/v) bovine serum albumin (BSA) in PBST buffer (0.1% Tween 20 in PBS) at room temperature for 1 h, membranes were incubated with the primary antibodies overnight at 4 °C, as previously described (Crespillo et al., 2011; Blanco et al., 2012a): anti-CB1 receptor (Cayman, cat. no. 101500) diluted 1:200, anti-DAGLα (produced in our laboratory) diluted 1:100, anti-DAGLβ (produced in our laboratory) diluted 1:100, anti-NAPE-PLD (produced in our laboratory) diluted 1:100, anti-FAAH (Cayman, cat. no. 101600) diluted 1:100, anti-MAGL (Cayman, cat. no. 100035) diluted 1:200, anti-mGluR5 (Thermo Scientific, cat. no. PA1-24637) diluted 1:500, anti-GluN1 (Millipore, cat. no. AB9864) diluted 1:500 and anti-phospho (pSer 845)-GluA1 (Thermo Scientific, cat. no. OPA1-04118) diluted 1:500. After incubation with a peroxidise-conjugated goat anti-rabbit IgG (H+L) antibody (Promega) diluted 1:2500 for 1 h at room temperature, membranes were revealed using the Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology). The specific protein bands were visualized and quantified by chemiluminescence using an imaging AutoChemi™ UVP BioImagin System (LTF Labortechnik). βactin was quantified and used as a loading control (anti-βactin, Sigma, cat. no. A5316, diluted 1:1000).

2.9. Statistical analysis

Data were expressed as the mean ± standard error of the mean (S.E.M.) of eight determinations per experimental group. Statistical analyses of behavioral data were carried out by mixed two-way repeated measures ANOVA tests followed by Tukey's *post hoc* test, with group (V-V and C-V for CL and V-C and C-C for LS) as between-subject factor and time bin as within-subject factor. Gene and protein quantifications were analyzed by one or two-way ANOVA tests followed by *post hoc* Bonferroni test, with pretreatment (repeated administration of vehicle or cocaine for five days) and treatment (vehicle or cocaine administration during CL and LS) as between-subject factors. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Cocaine-induced conditioned locomotion and locomotor sensitization

Figure 1 depicts the experimental design (Figure 1A) and results of the expression of conditioned locomotion (Figure 1B, C) and locomotor sensitization to cocaine (Figure 1D, E) tests. Based on previous dose-response studies developed by our group (Blanco et al., 2012a, 2014), we selected a dose of cocaine of 20 mg/kg for the conditioning phase and 10 mg/kg for the acute cocaine administration and the primed injection during cocaine sensitization. A two-way mixed ANOVA test showed that the main effect of group was significant ($F_{1,14}=24.55$, $P<0.001$), indicating that the C-V group covered a significantly longer distance than the V-V group during the 30 min exposure to the OF (Figure 1B). The main effect of time bin was also significant ($F_{5,70}=59.39$, $P<0.001$) while the interaction group × time bin was not ($F_{5,70}<1$), indicating that the distance traveled decreased over time at the same ratio in both groups.

Figure 1 (A) Schematic representation of the different phases of the protocol employed to evaluate cocaine-induced conditioned locomotion (CL) and locomotor sensitization (LS). Following two days of handling mice were daily administrated i.p. with vehicle or cocaine (20 mg/kg) during five consecutive days. The next five/six days animals were kept in their home cages and they did not receive any treatment. On day 13, half of the mice pretreated with vehicle and half of the mice pretreated with cocaine were treated i.p. with vehicle to evaluate CL. One hour after vehicle administration mice were euthanized. Finally, on day 14, the remaining half of the mice pretreated with vehicle and the remaining half of the mice pretreated with cocaine were treated i.p. with a primed injection of cocaine (10 mg/kg) to assess LS response. (B-E) Effects of CL and LS on the cumulative total distance traveled and distance covered during 5-min time bins in a 30-min OF session. *$P<0.05$, **$P<0.01$, ***$P<0.001$ *vs.* vehicle-vehicle (V-V) group or vehicle-cocaine (V-C) group.

Post hoc pairwise comparisons showed that the C–V group covered a significantly longer distance than the V–V group across all time bins (Figure 1C). Regarding locomotor sensitization, a two-way mixed ANOVA test indicated that the C–C group

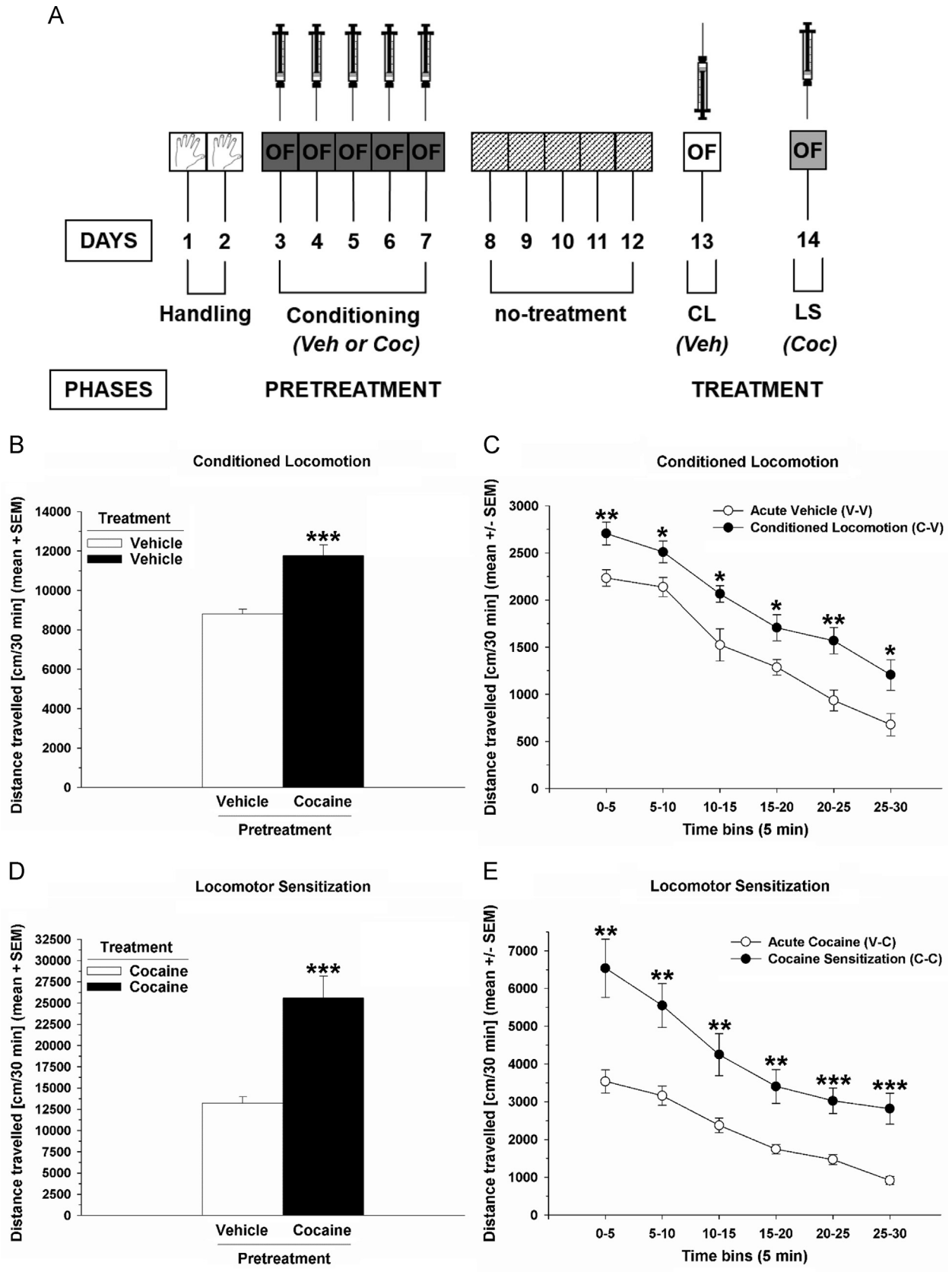

Table 1 Primers sequences used for gene expression analysis.

	Gene ID	GenBank accession numbers	Forward sense primers	Reverse antisense primers	Product size (bp)
	Actb (βactin)	NM_007393	tacagcttcaccaccacagc	aaggaaggctggaagagagc	206
ECS	*Cnr1* (CB1 receptor)	NM_007726.1	gctgcaatctgtttgctcag	ttgccatcttctgaggtgtg	201
	Faah (FAAH)	NM_010173.2	Cggagagtgactgtgtggtg	tcagtgcctaaacccagagg	220
	Napepld (NAPE-PLD)	AB_112350	gcgccaagctatcagtatcc	tcagccatctgagcacattc	223
	Mgll (MAGL)	NM_011844.3	Catggagctggggaacactg	Ggagatggcaccgcccatggag	240
	Dagla (DAGLα)	NM_198114.1	agaatgtcaccctcggaatg	gcaggttgtaagtccgcaaa	153
	Daglb (DAGLβ)	NM_144915.2	Aagcggccagatacattcac	ggataagcgacacgacaaag	246
Glutamate synthesizing enzymes	*Gls2* (LGA)	NM_001033264	Ttggaccatgcgctgcatcttg	Gcactcggatcatgacgcctcac	190
	Gls (KGA)	NM_001081081	Gcgagggcaaggagatggtg	Ctctttcaacctgggatcagatgttc	179
Metabotropic glutamate receptors	*Gmr3* (mGluR3)	NM_181850.2	Tgagtggtttcgtggtcttg	tgcttgcagaggactgagaa	153
	Gmr5 (mGluR5)	BC096533.1	Aagaaggagaaccccaacca	ttcggagactggagagtttg	179
NMDA ionotropic glutamate receptor subunits	*Grin1* (GluN1)	NM_008169	gtgcaagtgggcatctacaa	tgggcttgacatacacgaag	157
	Grin2a (GluN2A)	NM_008170	gtttgttggtgacggtgaga	aagaggtgctcccagatgaa	180
	Grin2b (GluN2B)	NM_008171	Atgtggattgggaggatagg	tcgggctttgaggatacttg	249
	Grin2c (GluN2C)	NM_010350	ggaatggtatgatcggtgag	ccgtgaggcacattacaaac	225
AMPA ionotropic glutamate receptor subunits	*Gria1* (GluA1)	NM_008165.2	Ttttctaggtgcggttgtgg	cctttggagaactgggaaca	210
	Gria2 (GluA2)	NM_001039195.1	Aaggaggaaagggaaacgag	ccgaagtggaaaactgaacc	217
	Gria3 (GluA3)	NM_016886.2	Caacaccaaccagaacacca	atcggcatcagtgggaaa	229
	Gria4 (GluA4)	NM_019691.3	ttggaatgggatggtaggag	taggaacaagaccacgctga	250

covered a significantly longer distance than the V-C group (main effect of group: $F_{1,14}=20.97$, $P<0.001$) (Figure 1D). The main effect of time bin was also significant ($F_{5,70}=41.57$, $P<0.001$) while the interaction group × time bin was not ($F_{5,70}<1$). *Post hoc* pairwise comparisons showed that this increased motor response was observed in all time bins (Figure 1E). Collectively, these results indicate that the behavioral protocol applied produced a robust conditioned locomotion and sensitized locomotor response to cocaine.

3.2. Gene and protein expression of glutamate signaling components in the hippocampus

We analyzed the gene expression of the glutamate-synthesizing enzymes LGA and KGA, the mGluR3/5 metabotropic receptors, the GluN1/2A/2B/2C NMDA metabotropic and GluA1/2/3/4-AMPA ionotropic receptor subunits, and protein expression of the mGluR5 metabotropic receptor, the GluN1 NMDA receptor subunit and phosphorylation of the GluA1 AMPA receptor subunit in response to vehicle administration (V-V), acute cocaine administration (V-C), conditioned locomotion (C-V) and locomotor sensitization to cocaine (C-C). Changes observed in gene expression were confirmed by protein expression analyses.

3.2.1. mRNA levels in the hippocampus

Two-way ANOVA tests showed significant pretreatment effects for the hippocampal gene expression of mGluR3, GluN1, GluN2A, GluN2C and GluA4 (mGluR3: $F_{1,28}=4.31$, $P<0.05$; GluN1: $F_{1,28}=13.94$, $P<0.001$; GluN2A: $F_{1,28}=5.19$, $P<0.05$; GluN2C: $F_{1,28}=24.52$, $P<0.001$; GluA4: $F_{1,28}=4.21$, $P<0.05$) (Figure 2C, E, F, H, L). A significant treatment effect was only detected for the KGA gene expression ($F_{1,28}=14.91$, $P<0.001$) (Figure 2B). Significant interactions between factors were observed in the gene expression of mGluR5, GluN1, GluN2A, GluN2C and GluA4 (mGluR5: $F_{1,28}=21.28$, $P<0.001$; GluN1: $F_{1,28}=10.13$, $P<0.01$; GluN2A: $F_{1,28}=5.87$, $P<0.05$; GluN2C: $F_{1,28}=19.48$, $P<0.001$; GluA4: $F_{1,28}=6.55$, $P<0.05$) (Figure 2D, E, F, H, L), indicating that cocaine treatment differentially affects gene expression of these glutamatergic receptors in a pretreatment-dependent manner.

When simple effects were analyzed by Bonferroni *post hoc* tests, the V-C group (acute cocaine administration) showed a significantly decreased gene expression of KGA ($P<0.01$), mGluR3 ($P<0.01$), mGluR5 ($P<0.001$), GluN1 ($P<0.01$), GluN2A ($P<0.01$), GluN2B ($P<0.05$), GluN2C ($P<0.01$), GluA2 ($P<0.05$), GluA3 ($P<0.05$) and GluA4 ($P<0.05$) compared to the V-V group (Figure 2B-H, J-L). The C-V group (conditioned locomotion) presented a significantly decreased gene expression of mGluR5 ($P<0.05$) compared to the V-V group (Figure 2D), but a significantly increased gene expression of GluN2B and GluN2C ($P<0.05$ for both comparisons) compared to the V-C group (Figure 2G, H). The C-C group (locomotor sensitization to cocaine) showed a significantly decreased gene expression of KGA ($P<0.01$), but a significantly increased gene expression of GluN1 ($P<0.05$) and GluN2C ($P<0.01$) compared to the V-V group (Figure 2B, E, H). Regarding the comparison between the C-C group with the V-C group, we detected a significantly increased gene expression of mGluR3 ($P<0.05$), mGluR5 ($P<0.001$), GluN1 ($P<0.001$), GluN2A ($P<0.01$), GluN2C ($P<0.001$), GluA2 ($P<0.05$) and GluA4 ($P<0.01$) (Figure 2C-F, H, J, L). Finally, when the C-C group was compared with the C-V group, we observed a significantly increased gene expression of KGA ($P<0.05$), mGluR5 ($P<0.05$), GluN1 ($P<0.05$) and GluN2C ($P<0.01$) (Figure 2B, D, E, H).

3.2.2. Protein levels in the hippocampus

Western blot analyses showed that the antibodies used against mGluR5, GluN1 and phospho-GluA1 revealed bands with the expected molecular weight in hippocampus, as was previously described (Blanco et al., 2012a). Thus, mGluR5 immunoblotting revealed a prominent band at about 132 kDa, GluN1 at 120 kDa and phospho-GluA1 at 100 kDa. Figure 3A illustrates representative immunoblots showing protein expression of the glutamatergic receptors analyzed in the hippocampus of the experimental groups V-V, V-C and C-C. One-way ANOVA tests indicated that the three measured proteins were modified by treatments (mGluR5: $F_{2,23}=9.84$; $P=0.001$; GluN1: $F_{2,23}=4.70$; $P=0.020$; phospho-GluA1: $F_{2,23}=6.08$; $P=0.008$). Bonferroni *post-hoc* tests indicated that the V-C group (acute cocaine administration) showed a significantly decreased level of phospho-GluA1 ($P<0.05$) compared to the V-V group (Figure 3D). The C-C group (locomotor sensitization to cocaine) showed a significantly increased protein expression of GluN1 ($P<0.01$) compared to the V-V group, and mGluR5 and GluN1 ($P<0.01$) compared to the V-C group (Figure 3B and C).

3.3. Gene and protein expression of ECS components in the hippocampus

To address whether the observed changes in the glutamatergic signaling components following the behavioral protocols were associated with alterations in the endocannabinoid signaling system, we analyzed the gene and protein expressions of several ECS components in response to vehicle administration (V-V), acute cocaine treatment (V-C), conditioned locomotion (C-V) and locomotor sensitization to cocaine (C-C). Furthermore, to estimate whether the differential expression of either endocannabinoid producing (NAPE-PLD, DAGLα/β) or degrading (FAAH, MAGL) enzymes may result in an altered endocannabinoid tone in the hippocampus, we also calculated NAPE-PLD/FAAH, DAGLα/MAGL and DAGLβ/MAGL ratios. These ratios can suggest possible changes in anandamide and 2-arachidonylglycerol levels, respectively.

3.3.1. mRNA levels in the hippocampus

Two-way ANOVA tests revealed significant pretreatment effects for the hippocampal gene expression of CB1, DAGLα and DAGLβ ($F_{1,28}=16.04$, $P<0.001$; $F_{1,28}=12.01$, $P=0.001$; $F_{1,28}=11.6$, $P<0.01$, respectively) (Figure 4A, D, E). These effects on gene expression also resulted in significant pretreatment effects for NAPE-PLD/FAAH ($F_{1,28}=10.94$, $P<0.01$) and DAGLα/MAGL ($F_{1,28}=26.4$, $P<0.001$) ratios (Figure 4G, H). A significant treatment effect was only observed for the NAPE-PLD/FAAH ratio ($F_{1,28}=8.95$, $P<0.01$) (Figure 4G). Significant interactions between factors (pretreatment and treatment) were detected for the gene expression of NAPE-PLD and DAGLα ($F_{1,28}=5.12$, $P<0.05$; $F_{1,28}=11.6$, $P<0.01$, respectively) (Figure 4B, D), indicating that cocaine treatment differentially affects

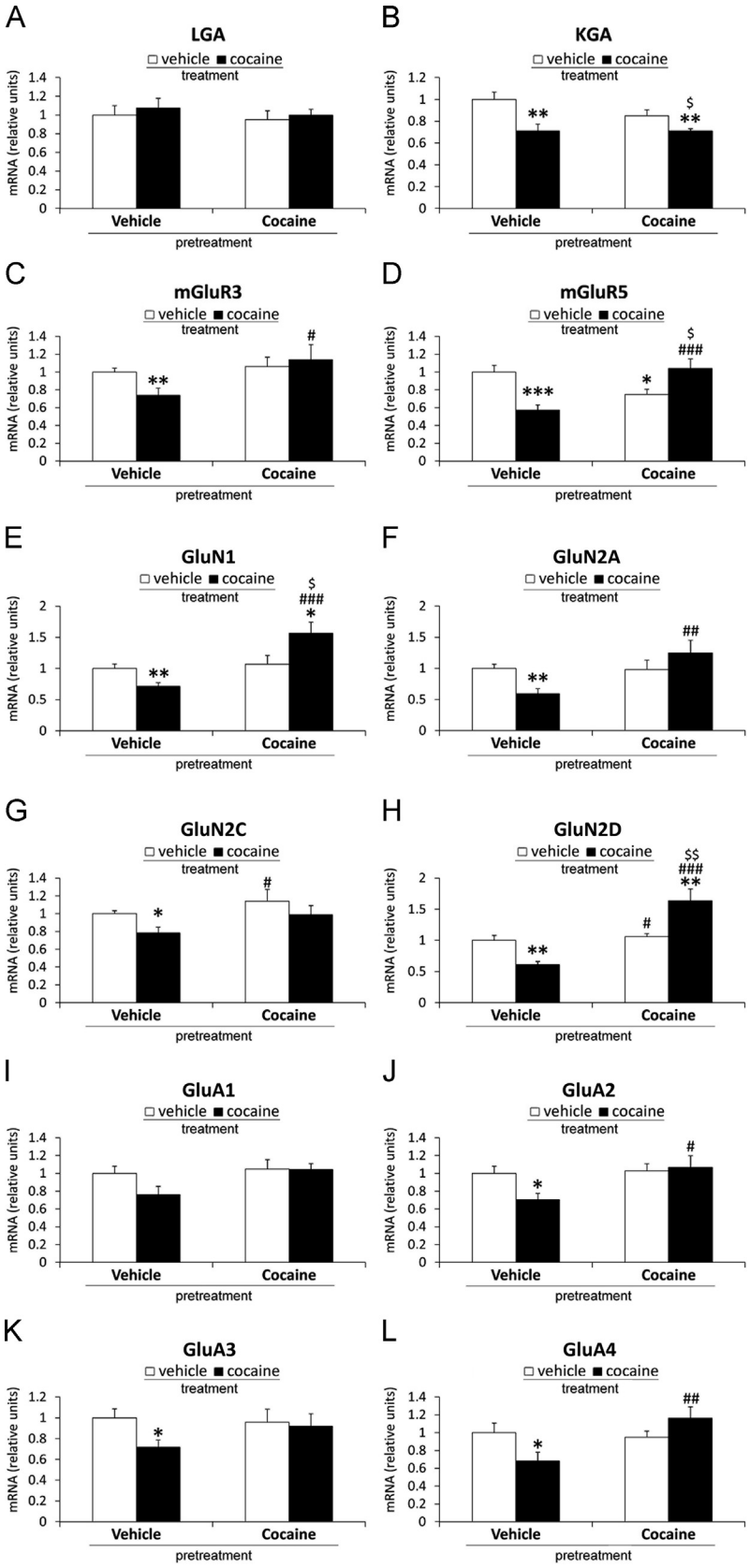

Figure 2 Gene expression of glutamate signaling components (LGA, KGA, mGluR3/5, GluN1/2A/2B/2C NMDA receptor subunits and GluA1/2/3/4 AMPA receptor subunits) in the mouse hippocampus following pretreatment (vehicle or cocaine) and treatment (vehicle or cocaine). Histograms represent the mean+S.E.M. of $n=8$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* V–V group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* V–C group; $^{\$}P<0.05$, $^{\$\$}P<0.01$ *vs.* C–V group.

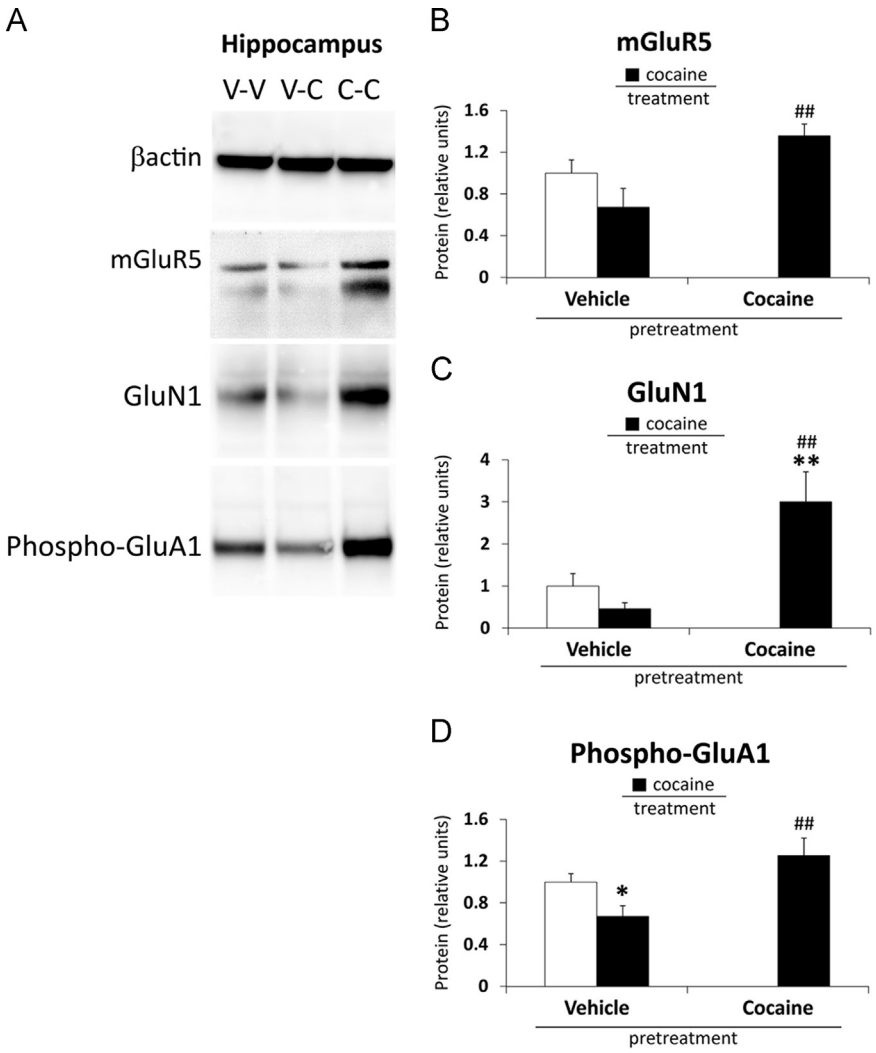

Figure 3 Representative immunoblots (A) and protein expression (B–D) of glutamatergic receptor mGluR5, GluN1 subunit and phospho-GluA1 in the mouse hippocampus after pretreatment (vehicle or cocaine) and treatment (cocaine). Histograms represent the mean+S.E.M of $n=8$. *$P<0.05$, **$P<0.01$ *vs.* V-V group; $^{\#\#}P<0.01$ *vs.* V-C group.

NAPE-PLD and DAGLα gene expression in a pretreatment-dependent manner.

Simple effects analyzed by Bonferroni *post hoc* tests showed that the V-C group (acute cocaine administration) showed a significantly increased CB1 gene expression ($P<0.05$) and DAGLβ/MAGL ratio ($P<0.01$) (Figure 4A, I), as well as a significantly decreased gene expression of NAPE-PLD ($P<0.05$), DAGLα ($P<0.01$), and NAPE-PLD/FAAH ratio ($P<0.05$), compared to the V-V group (Figure 4B, D, G). The C-V group (conditioned locomotion) showed a significantly increased CB1 gene expression ($P<0.01$) (Figure 4A), along with a significantly reduced gene expression of NAPE-PLD ($P<0.05$), DAGLα ($P<0.001$), and DAGLβ ($P<0.05$), which resulted in significant decrements of NAPE-PLD/FAAH ($P<0.05$) and DAGLα/MAGL ($P<0.01$) ratios compared to the V-V group (Figure 4B, D, E, G, H). When we compared the C-V group to the V-C group, we also detected a significant decrement in the gene expression of DAGLα ($P<0.001$) and DAGLβ ($P<0.01$) that resulted in a significant reduction of DAGLα/MAGL and DAGLβ/MAGL ratios ($P<0.01$ in both cases) (Figure 4D, E, H, I). The C-C group (locomotor sensitization to cocaine) showed a significantly increased CB1 ($P<0.001$) and FAAH ($P<0.05$) gene expression, as well as a significantly decreased gene expression of DAGLα ($P<0.001$) (Figure 4A, C, D). These changes resulted in significant decrements of NAPE-PLD/FAAH ($P<0.001$) and DAGLα/MAGL ($P<0.05$) ratios compared to the V-V group (Figure 4G, H). We also observed significant decrements for DAGLα ($P<0.01$) gene expression and for DAGLα/MAGL and DAGLβ/MAGL ratios ($P<0.05$ in both cases) compared to the V-C group (Figure 4D, H, I), and a significant reduction of the NAPE-PLD/FAAH ratio ($P<0.05$) compared to the C-V group (Figure 4G).

3.3.2. Protein levels in the hippocampus

Western blot analyses showed that the antibodies used against the ECS components revealed bands with the expected molecular weight in hippocampus, as was previously described (Suárez et al., 2008; Blanco et al., 2014). CB1 immunoblotting revealed a prominent band at about 60 kDa, NAPE-PLD at 46 kDa, FAAH at 63 kDa, DAGLα at 120 kDa, DAGLβ at 76 kDa

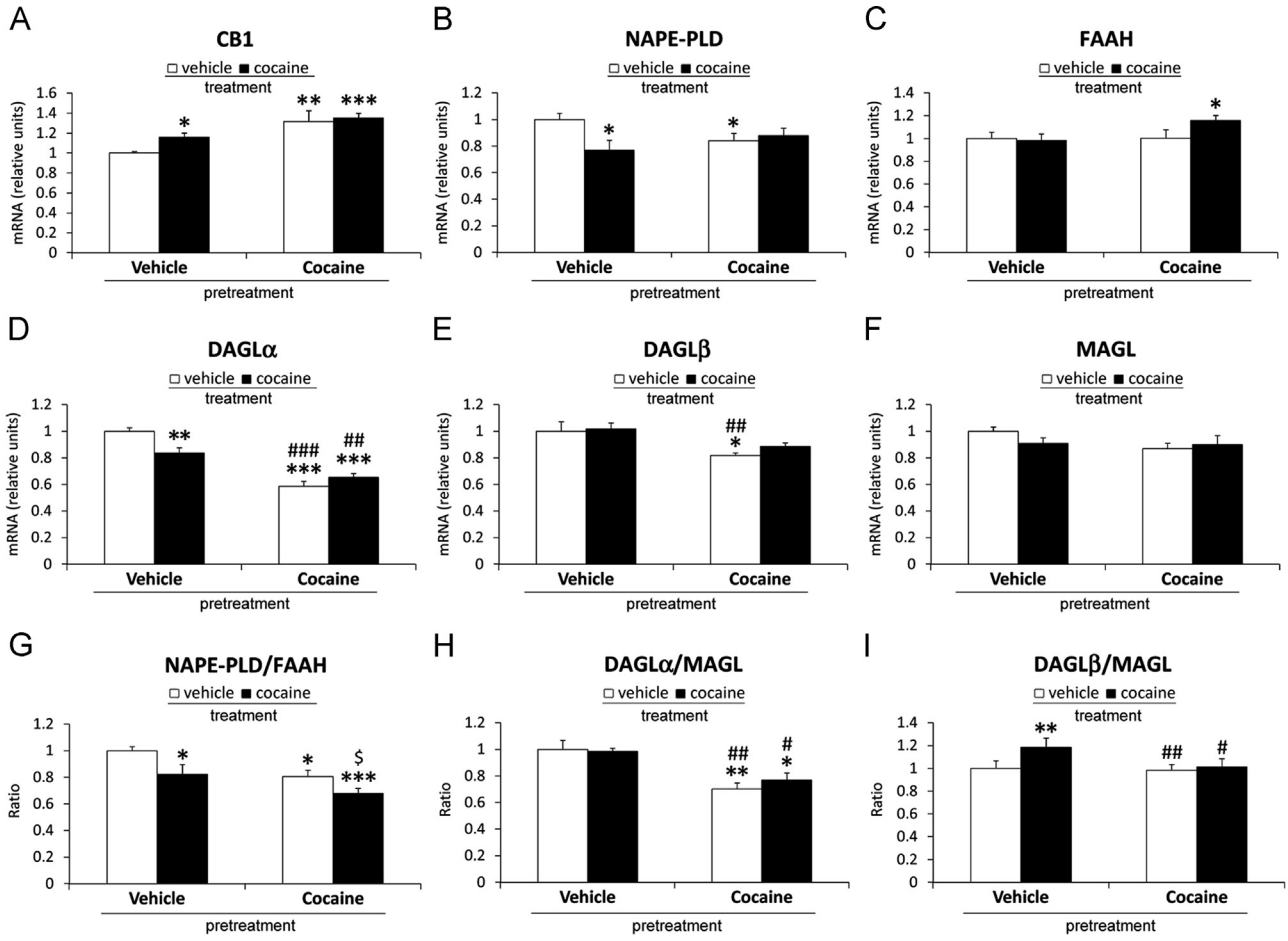

Figure 4 Gene expression of ECS components (CB1, NAPE-PLD, FAAH, DAGLα, DAGLβ and MAGL) in the mouse hippocampus after pretreatment (vehicle or cocaine) and treatment (vehicle or cocaine). Histograms represent the mean+S.E.M of $n=8$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* V-V group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* V-C group; $^{\$}P<0.05$ *vs.* C-V group.

and MAGL at 35–37 kDa. Figure 4A illustrates representative immunoblots showing the protein expression of the ECS components analyzed in the hippocampus of the four experimental groups (V-V, V-C, C-V and C-C).

Two-way ANOVA tests showed significant pretreatment effects for the hippocampal protein expression of CB1, NAPE-PLD and DAGLα ($F_{1,28}=5.28$, $P<0.05$; $F_{1,28}=6.5$, $P<0.05$; $F_{1,28}=7.3$, $P<0.05$, respectively) (Figure 5B, C, E), resulting in significant pretreatment effects for NAPE-PLD/FAAH and DAGLα/MAGL ratios ($F_{1,28}=4.84$, $P<0.05$; $F_{1,28}=6.35$, $P<0.05$, respectively) (Figure 5H, I). A significant treatment effect was only observed for DAGLα expression ($F_{1,28}=4.99$, $P<0.05$) and, as a consequence, for the DAGLα/MAGL ratio ($F_{1,28}=5.33$, $P<0.05$) (Figure 5E, I). Significant interactions between factors were detected for NAPE-PLD and DAGLα protein expression ($F_{1,28}=7.4$, $P<0.05$; $F_{1,28}=5.27$, $P<0.05$, respectively) (Figure 5C, E) and, consequently, for NAPE-PLD/FAAH and DAGLα/MAGL ratios ($F_{1,28}=6.08$, $P<0.05$; $F_{1,28}=8.81$, $P<0.05$, respectively) (Figure 5H, I), indicating that treatment with cocaine differentially affects NAPE-PLD and DAGLα protein expression in a pretreatment-dependent manner.

Analyses of simple effects by Bonferroni tests indicated that the V-C group (acute cocaine administration) showed a significantly decreased DAGLα protein expression ($P<0.05$), resulting in a significantly decreased DAGLα/MAGL ratio compared to V-V group ($P<0.05$) (Figure 5E, I). The C-V group (conditioned locomotion) presented significant decrements of the NAPE-PLD ($P<0.01$) and DAGLα ($P<0.05$) protein expression and, as a consequence, in NAPE-PLD/FAAH ($P<0.01$) and DAGLα/MAGL ($P<0.05$) ratios compared to V-V group (Figure 5C, E, H, I). The C-C group (locomotor sensitization to cocaine) showed a significantly reduced expression of DAGLα, as well as NAPE-PLD/FAAH and DAGLα/MAGL ratios compared to V-V group ($P<0.05$) (Figure 5E, H, I).

4. Discussion

The present study reveals that repeated cocaine administration results in neuroadaptations in the expression of both the glutamatergic and the endocannabinoid signaling systems in the hippocampus. The issue of the neurobiological alterations underlying cocaine conditioning and cocaine-induced behavioral sensitization is a key factor for understanding the mechanisms that sustain the rewarding properties of cocaine and promote craving and relapse. Under this scope, the role of the hippocampus has been much less explored than other nodes of the reward system. Although the hippocampus is not considered, *stricto sensu*, to belong to the brain reward system, it is believed to play a critical role in the formation

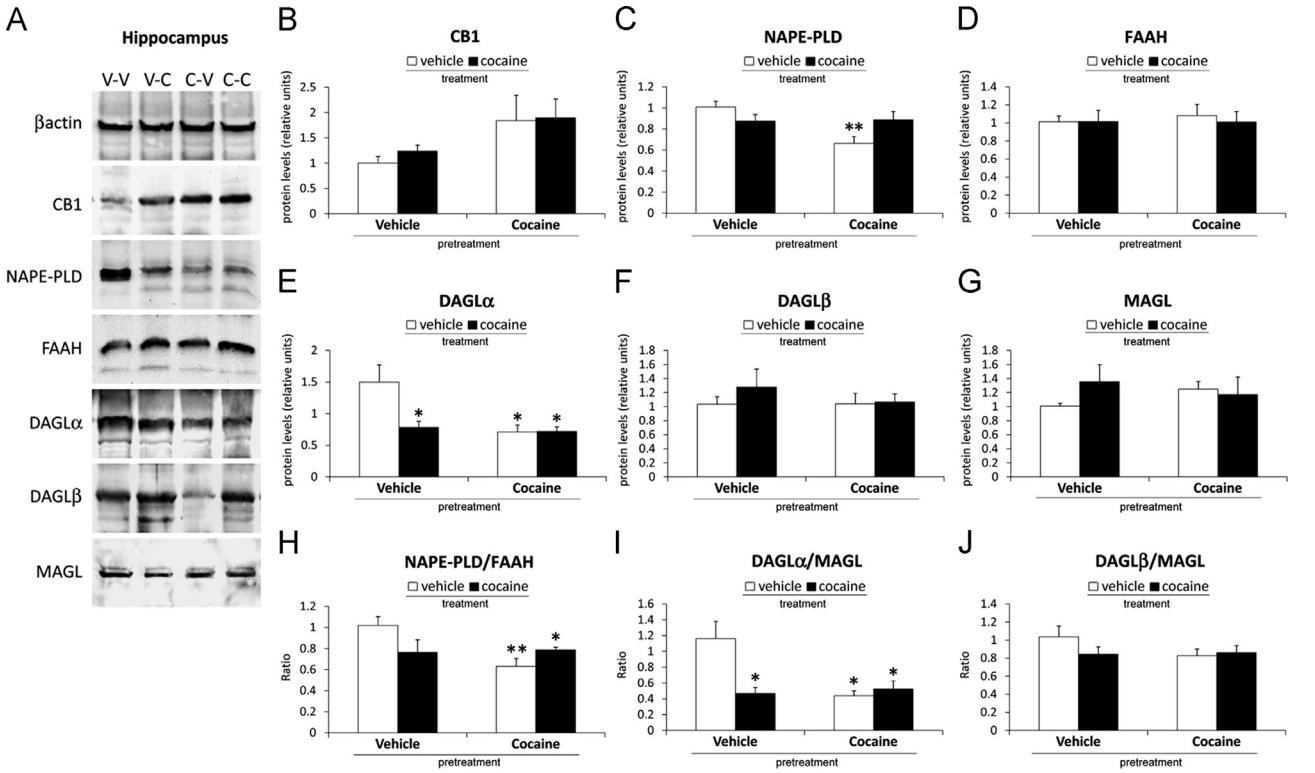

Figure 5 Representative immunoblots (A) and protein expression (B–J) of ECS components (CB1, NAPE-PLD, FAAH, DAGLα, DAGLβ and MAGL) in the mouse hippocampus after pretreatment (vehicle or cocaine) and treatment (vehicle or cocaine). Histograms represent the mean+S.E.M of $n=8$. *$P<0.05$, **$P<0.01$ *vs.* V-V group.

of enduring memories that associate environmental stimuli with the reinforcing effects of drugs, and trigger drug-seeking behaviors (Blanco et al., 2012a; Crombag et al., 2008; Xie et al., 2013). These drug-associative memories are involved in the expression of cocaine-induced conditioned locomotion and behavioral sensitization (Alaghband et al., 2014; Badiani and Robinson, 2004; Steketee and Kalivas, 2011). The hippocampal glutamatergic system is crucial for the formation of different types of memory through its modulation of synaptic plasticity (Connor and Wang, 2015), but its involvement in conditioned locomotion and cocaine sensitization remains to be conclusively determined, although a previous work developed by our group has already described this hippocampal-glutamate component in a mouse model (Blanco et al., 2012a). Moreover, even though endocannabinoids retrogradely modulate glutamatergic synaptic transmission through CB1 receptors (Piomelli, 2014), it is unknown whether the hippocampal endocannabinoid signaling participates in cocaine-induced conditioned locomotion and sensitization processes. The results obtained in the present study could shed light on these questions.

4.1. Hippocampal glutamatergic system is involved in acute cocaine administration and cocaine-induced locomotor sensitization

Acute cocaine administration (V-C group) induced a decrement in the mRNA levels of the glutamate-synthesizing enzyme KGA. Cocaine-sensitized mice (C-C group) also showed a reduced gene expression of KGA; however, since non-statistically significant differences were found between both groups, this reduced expression could not be ascribed to cocaine sensitization (see Table 2 for summary results). We have previously reported that the gene expression of KGA was also reduced in the prefrontal cortex after acute cocaine administration (Blanco et al., 2014). The decrement of hippocampal KGA gene expression can be linked to the lower gene expressions of metabotropic mGluR3 and GluR5 glutamate receptors and GluN1, GluN2A, GluN2B and GluN2C NMDA receptor subunits following acute cocaine administration. Moreover, the gene expressions of GluA2, GluA3 and GluA4 AMPA receptor subunits were also reduced after acute cocaine administration. Interestingly, locomotor sensitization to cocaine was specifically associated with an increased gene expression of GluN1 and GluN2C NMDA receptor subunits (Table 2). Collectively, these results indicate that acute cocaine administration is associated with a reduced gene expression of KGA and glutamate receptors, while the increased gene expression of GluN1 and GluN2C NMDA receptor subunits suggests an up-regulation of the hippocampal NMDA receptor, following locomotor sensitization to cocaine. These results are of particular interest because seminal works by Karler et al. (1989) and Pudiak and Bozarth (1993) have demonstrated that repeated co-administration of the NMDA receptor antagonist, MK-801, with cocaine attenuates or blocks cocaine-induced locomotion sensitization. Moreover, this effect is not attributable to the potential ability of MK-801 to prevent the acute locomotor stimulatory effects of

cocaine (Wolf et al., 1994). Since then, these results were extensively replicated. Due to the fact that repeated cocaine administration is known to induce synaptic plasticity in the hippocampus (Barr et al., 2014; Keralapurath et al., 2014; Perez et al., 2010) and some types of LTP are NMDA-dependent (Bliss and Collingridge, 2013; Granger and Nicoll, 2013), we hypothesized that the specific increment in gene expression of the GluN1 and GluN2C NMDA receptor subunits following cocaine sensitization could be linked to hippocampal synaptic plasticity, which in turn could be partially responsible for the expression of locomotor sensitization to cocaine. When we analyzed the protein expression of the mGluR5 receptor and GluN1 receptor subunit, we found that these results further support the conclusions drawn from the analyses of gene expression. Both, mGluR5 and GluN1 were significantly increased after repeated cocaine administration resulting in locomotor sensitization (Figure 3A-C). This increment was high and specific for GluN1 (Figure 3A, C). Since the phosphorylation of the AMPA receptor GluA1 subunit has been shown to be implicated in synaptic plasticity and learning and memory processes (Lee et al., 2003), we also studied the phosphorylation of this subunit. In this case, we observed an increment in the phosphorylation of GluA1 after cocaine sensitization while a reduction was observed after acute cocaine administration (see Figure 3A, D). These results might further support our suggestion that the hippocampus could play a critical role in cocaine sensitization through its relevant participation in synaptic plasticity and memory and learning processes.

On the other hand, our results do not support an association between cocaine-induced conditioned locomotion and the expression of the mRNA coding for hippocampal glutamatergic system-related genes because none of the glutamate-synthesizing enzymes, receptors and receptor subunits mRNAs were specifically modified by the re-exposition of vehicle treated-mice to the environment when cocaine conditioning took place (group C-V). However, as we will discuss below, a potential over-activation of the glutamatergic system could be underlying this conditioned response since the inhibitory retrograde endocannabinoid signaling is profoundly inhibited in the conditioned locomotion group, facilitating enhanced glutamatergic output without the need of overexpressing its components.

Table 2 Summary of the effects of acute cocaine treatment, conditioned locomotion and locomotor sensitization to cocaine on the gene expression of the glutamate signaling components.[a]

	Acute cocaine	Conditioned locomotion	Locomotor sensitization
LGA/KGA	ns/↓	ns/ns	ns/↓
mGluR3/5	↓/↓	ns/↓	ns/ns
GluN1/2A/2B/2C	↓/↓/↓/↓	ns/ns/ns/ns	↑/ns/ns/↑
GluA1/2/3/4	ns/↓/↓/↓	ns/ns/ns/ns	ns/ns/ns/ns

[a]Arrows indicated the direction of the change compared with V-V group (Bonferroni test). ns means no statistical significance.

4.2. The involvement of the hippocampal endocannabinoid signaling in acute and repeated cocaine administration

When the hippocampal endocannabinoid system was analyzed, it was found that NAPE-PLD/FAAH and DAGLα/MAGL ratios were decreased following acute (V-C group) and repeated cocaine administration resulting in conditioned locomotion or sensitization (C-V and C-C groups) (see Table 3 for summary results). The down-regulation of the endocannabinoid-production/degradation enzymes suggested a likely reduction of the endocannabinoid tone in the hippocampus. Since it was previously reported that NAPE-PLD and DAGLα-expressing fiber terminals were selectively found surrounding granular and pyramidal cells in the dentate gyrus and the hippocampal CA1 field respectively (Rivera et al., 2014a, 2014b), it can be hypothesized that the putative endocannabinoid reduction could be directly affecting the modulation of the glutamatergic neurotransmission of the hippocampal principal neurons. Moreover, in a previous study by our group (Rivera et al., 2013), we demonstrated that the hippocampal endocannabinoid system presents differences in its immunohistochemical expression in response to the sensitivity to cocaine self-administration. For instance, we showed a reduction of the NAPE-PLD/FAAH ratio in the hippocampus of cocaine self-administered Lewis rats, suggesting a lower anandamide tone (Rivera et al., 2013). Supporting this assertion, Orio et al. (2009) showed that anandamide levels in the NAc shell are decreased in rats with limited access to cocaine self-administration. Moreover, other works have shown that endocannabinoid neurotransmission enhancer AM404 reduces cocaine-induced hyperlocomotion (Vlachou et al., 2008), while administration of FAAH inhibitors, which increases anandamide levels, attenuates cocaine-induced reinstatement and decreases cocaine-seeking behavior and cue- and stress-induced relapse (Adamczyk et al., 2009; Chauvet et al., 2014).

On the other hand, the hippocampal CB1 receptor levels increased after repeating cocaine administration, which resulted in conditioned locomotion or sensitization (Table 3). This over-expression of the CB1 receptor could be a compensatory response linked to the down-regulation of the endocannabinoid-production/degradation enzymes that suggests a reduced endocannabinoid tone. Interestingly, a recent study by Adamczyk et al. (2012) demonstrated that chronic cocaine self-administration leads to significant elevations in the binding density of CB1 receptor in numerous rat brains areas, including CA1, CA2 and CA3 hippocampal fields and dentate gyrus. It should be mentioned that CB1-expressing fiber terminals were mainly localized surrounding most pyramidal glutamatergic cells of the hippocampus (Rivera et al., 2014a). These results agree with a previous study by Orio et al. (2009), in which total CB1 protein expression was up-regulated in the NAc and the amygdala of rats with extended access to cocaine self-administration. In addition, Soria et al. (2005) showed that the deletion of a CB1 receptor is linked to a reduction of the acute cocaine-induced locomotor activity and a failure in cocaine self-administration. Our group has previously

Table 3 Summary of the effects of acute cocaine treatment, conditioned locomotion and locomotor sensitization to cocaine on the gene and protein expression of the ECS components.[a]

	Acute cocaine mRNA/protein	**Conditioned locomotion** mRNA/protein	**Locomotor sensitization** mRNA/protein
CB1 receptor	↑/ns	↑/ns	↑/ns
NAPE-PLD/ FAAH ratio	↓/ns	↓/↓	↓/↓
DAGLα/ MAGL ratio	ns/↓	↓/↓	↓/↓
DAGLβ/ MAGL ratio	↑/ns	ns/ns	ns/ns

[a]Arrows indicated the direction of the change compared with V–V group (Bonferroni test). ns means no statistical significance.

demonstrated that the pharmacological blockade of the CB1 receptor prevents cocaine-induced conditioned locomotion, but not locomotor sensitization, after repeated cocaine administration (Blanco-Calvo et al., 2014). Interestingly, in this study we showed that the administration of the CB1 receptor antagonist Rimonabant also blocked the cocaine-induced reduction of cell proliferation in the hippocampus, a plasticity response related to persistent cocaine-associated memories. It is also worth mentioning that other studies reported opposite results. For instance, Martin et al. (2000) demonstrated that cocaine-evoked CPP is preserved in CB1-null mice. Additionally, our group has shown that the prevention of cocaine-induced conditioned locomotion after repeated cocaine administration is also achieved by the blockade of the cannabinoid CB2 receptors (Blanco-Calvo et al., 2014). These contradictory results suggest the additional participation of CB1-independent mechanisms in behavioral effects of cocaine.

Since endocannabinoids modulate glutamatergic synaptic transmission through CB1 receptors, the probable decreased endocannabinoid tone and the up-regulation of CB1 receptors after repeated cocaine administration could be linked to the up-regulation of mRNA levels of the GluN1 and GluN2C NMDA receptor subunits and to an increased protein expression of the GluN1 subunit observed in cocaine-sensitized mice as a compensatory mechanism against the likely increase in glutamatergic transmission. Thus, the putative decrement in the endocannabinoid tone, suggested by reduced NAPE-PLD/FAAH and DAGLα/MAGL ratios, could be altering NMDA-dependent synaptic plasticity in the hippocampus that underlies cocaine-induced locomotor sensitization. This hypothesis needs to be tested using electrophysiological models of hippocampal plasticity in cocaine-exposed animals.

In conclusion, the results of the present study indicate that cocaine administration induces a down-regulation of hippocampal endocannabinoid-production/degradation enzymes which could be linked to CB1 over-expression in hippocampus and up-regulation of the hippocampal GluN1 subunit. Since the increased levels of GluN1 subunit were specifically associated with locomotor sensitization to cocaine, while the reduced expression of endocannabinoid-production/degradation enzymes and increased CB1 expression were not restricted to the cocaine sensitized group of mice (C–C groups), it could be suggested that other non-endocannabinoid mediators could be influencing the NMDA hippocampal receptor during cocaine-evoked locomotor sensitization. These results provide new evidence about the interaction between the endocannabinoid and glutamatergic systems in the hippocampus, a brain region whose relevance in addiction-related processes has been rising in the last years. This hypothesis has to be confirmed by analyzing electrophysiologically the synaptic dynamics of hippocampal glutamatergic synapses in sensitized animals *vs.* animals receiving a single administration of cocaine.

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Contributors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Authors E.B., F.R.F. and J.S. designed the study and concept. Authors E.B., A.P., F.J.P., P.R., A.S., L.R., E.C.O. and J.D. acquired the data: Authors E.B., P.G., A.B., F.R.F. and J.S. analyzed and interpreted the data. E.B., P.G., F.R.F. and J.S. drafted and revised the manuscript for important intellectual content, obtained funding and study supervision. The authors declare that the data contained therein has not previously been published and that they have been approved by the responsible authorities in the laboratory where the work was carried out. The manuscript has been reviewed by all listed authors; they have concurred with the submission and approved the final manuscript.

Conflict of interest statement

The authors declare no competing financial interests or other conflict of interest that in relation to the work described.

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