Cocaine modulates both, glutaminase gene expression and glutaminase activity in the brain of cocaine-sensitized mice

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Abstract

Rationale Glutaminase is considered the main Glutamate (Glu) producing enzyme. Two isoforms, liver (LGA) and kidney (KGA) type glutaminases have been identified in neurons. The role of both enzymes in psychopharmacological responses to cocaine remains unknown.

Objectives We examined both, mRNA and protein expression of KGA and LGA in the brain of mice sensitized to cocaine. Additionally, total glutaminase activity was also measured.

Methods Total glutaminase activity and mRNA and protein expression of KGA and LGA were measured on the dorsal striatum, prefrontal cortex, hippocampus and cerebellum of cocaine-sensitized mice

Results Cocaine-sensitized animals (20 mg/kg x 5 days, followed of 5 drug-free days) exhibited a decrease of total glutaminase activity in both the dorsal striatum and the prefrontal cortex. This was associated with an increase in KGA mRNA expression in both brain areas,that was not observed when protein KGA levels were measured by western blot. LGA mRNA expression was increased as results of acute cocaine administration in sensitized animals, although protein levels were only enhanced in the prefrontal cortex of sensitized mice. These findings suggest that chronic cocaine administration modulates glutamate production through the regulation of glutaminase expression and activity. These actions are mainly observed in the prefrontal cortex-dorsal striatum circuit, the neuroanatomical target for the psychostimulant sensitization properties of cocaine.

Conclusions The present results indicates that glutaminase enzymes (mainly KGA) are modulated by cocaine in both, the prefrontal cortex and the dorsal striatum, as part of the neuroadaptions associated with behavioural sensitization to this drug of abuse.

Keywords: cocaine, glutamate, glutaminase, mice, sensitization, striatum, prefrontal cortex

Abbreviations

BS	behavioral sensitization
CL	conditioned locomotion
EAAC1	neuronal excitatory amino-acid carrier 1
GA	phosphate-activated glutaminase
Gln	glutamine
Glu	glutamate
KGA	kidney-type glutaminase isoform
LGA	liver-type glutaminase isoform
mGluR	metabotropic glutamate receptor
NAc	accumbens nucleus
PFC	prefrontal cortex
RT-PCR	reverse transcription-polymerase chain reaction
VTA	ventral tegmental area

Introduction

Cocaine, can induce long-term adaptive changes in brain circuits involved in the control of motivated behaviour. Repeated exposure to cocaine and amphetamines induced behavioural sensitization as part of the neuroplasticity changes derived of repeated exposure to these psychostimulants. Modification on both dopamine and glutamate signalling mediate these changes, as it has been described in recent years (Ferrario et al., 2010; Itol et al., 2002; Kalivas, 2004; Mohn et al. 2004). Repeated exposure to psychostimulants produces alterations in glutamatergic transmission within the mesolimbic dopaminergic reward system and associated limbic regions (Kauer and Malenka 2007). Recent studies have implicated glutamatergic synapsis in the prefrontal cortex and the dorsal striatum as the place where dynamic changes on glutamate signalling underlies behavioural sensitization. These changes involve both, ionotropic and metabotropic glutamate receptors (Engblom et al. 2008; Ghasemzadeh et al., 2009a and 2009b; Kim et al,, 2009). Despite the clear role for glutamate receptors in behavioural sensitization, there is scarce information on the role of biosynthetic enzymes responsible for Glu production (efferent presynaptic terminal) and Glu transport (Miguens et al., 2008) in the glutamatergic excitatory synapses of cocaine sensitized mice.

Glutamate (Glu) is the main excitatory neurotransmitter in the mammalian central nervous system (Collingridge and Lester 1989; Fonnum 1984), whereas glutamine (Gln) is considered an important precursor for its synthesis in brain through phosphate-activated glutaminase (GA) reaction (Kvamme 1984; Nicklas et al. 1987). GA is both an important contributor to transmitter pools of Glu (Nicklas et al. 1987) and the main Gln-utilizing enzyme in neurons (Kvamme 1984). For this reason, GA is regarded as the main glutamate-producer enzyme in the brain. Recently novel GA isoforms have been discovered in the brain of mammals, named as kidney-type glutaminase (KGA) and liver-type glutaminase (LGA), located in the mitochondria and neuronal nuclei respectively (Olalla et al. 2002). The identification of the function of each isozyme is essential for understanding the role of GAs in cerebral function. Some of the physiological functions of GA include synthesis of cerebral Glu, renal ammoniagenesis, nitrogen supply for hepatic urea biosynthesis, and energy supply for the bioenergetics of cells (Curthoys and Watford 1995; Kovacevic and McGivan 1983). In addition to those functions, Glu byosinthesis in the brain regulate synaptogenesis, synaptic plasticity, and participate in the pathogenesis of neuropsychiatric diseases (Conti and Weinberg 1999).

Glu is the precursor for the synthesis of aminobutyric acid (GABA) (Erecinska and Silver 1990). Our group have described the simultaneous expression of LGA and KGA mRNA transcripts in human brain (Aledo et al. 2000; Gomez-Fabre et al. 2000). The regional distribution of GA transcripts indicates that both isoforms co-localize in numerous cells throughout different brain regions (Olalla et al. 2002). Interestingly, LGA isoenzyme was not only expressed in neuronal cells, but also was found in astrocytes (Olalla et al. 2008). Furthermore, co-expression of KGA and LGA isoforms was also demonstrated in brain of other mammalian species like cow, mouse, rabbit, mouse and rat (Olalla et al. 2002). Various regions specifically involved in glutamatergic transmission, such as the cerebral cortex, hippocampus, striatum and cerebellum, were emphasized as those presenting the more intense GA immunolabeling (Marquez et al. 2006).

Understanding the mechanisms by which Glu plays its role in diverse processes requires not only a detailed analysis of the enzymes implicated in Glu synthesis on the presynaptic terminal, but also knowledge of the involved postsynaptic mechanisms. Neuronal excitatory amino acid carrier 1 (EAAC1, also called EAAT3 or SLC1A1) is a transporter capable of uptake extracellular Glu in cerebral cortex, hippocampus, cerebellum, thalamic nuclei, olfactory bulb and spinal cord (Kanai et al. 1995; Kanai and Hediger 1992; Shashidharan et al. 1994). In addition to transporting Glu, EAAC1 is involved in the neuronal uptake of cysteine for the synthesis and maintaining of glutathione homeostasis (Aoyama et al. 2006), and plays other roles in regulating GABA synthesis and supporting neuron viability. It is possible that EAAC1 may have alternative activities distinct from Glu removal because these functions are mainly carried out by transporters of the GLT1 and GLAST subtypes. *Early in situ hybridization* and immunocytochemical studies have shown different patterns of cellular distribution for GLT1, GLAST and EAAC1 (Kanai et al. 1995; Kanai and Hediger 1992; Lehre et al. 1995; Rothstein et al. 1994; Velaz-Faircloth et al. 1996). Comparing these studies can observe that GLT1 and GLAST are mostly located in glial cells, whereas EAAC1 is often expressed in neurons and we chose it for this reason. At the excitatory synapse, EAAC1 could contribute to limit diffusion of Glu from the synapse to extra-synaptic NMDA receptors, avoiding excessive activation of these receptors that could be deleterious in the case of over-stimulation (Nieoullon et al. 2006).

Neither the mRNA expression of glutaminase nor the mRNA expression of the EAAC1 transporter have been studied in the brain of cocaine-sensitized mice. In the present

work, we have studied whether the expression of both isoforms of GA and the EAAC1 transporter, as well as total GA activity may be modulated by cocaine in several brain areas of cocaine-sensitized mice. The results indicate that GA is modulated by cocaine in the prefrontal cortex-dorsal /dorsal striatum circuit as part of the neuroadaptions associated with psychostimulant-induced behavioural sensitization.

Material and methods

Animals and housing

We used male C57BL/6J mice (25±5 g; Charles Rivers Laboratories) for cocaine behavioural studies, genomic, proteomic and GA enzymatic activity. All animals were maintained at the central vivarium of the University of Malaga. They were housed in clear plastic cages and maintained in a temperature (20±2°C) and humidity (40±5%) controlled room on a 12-h light/dark cycle with food and water *ad libitum*. Moreover, all mice were handled for 5 min/day for at least 2 days prior to behavioural testing to reduce the effects on test behavior of the nonspecific stress of being handled. The maintenance of the animals as well as the experimental procedures were in accordance with the European animal research laws (European Communities Council Directives 86/609/EU, 98/81/CEE, 2003/65/EC and Commission Recommendation 2007/526/EC).

Drug administration

Cocaine–HCl was obtained from Sigma-Aldrich (Madrid, Spain) and dissolved in sterile 0.9% NaCl just before experimentation.

Acute cocaine dose-response curve

We injected different single-doses (0, 5, 10 and 20 mg/kg) of drug subcutaneously in C57BL/6J mice (n = 8 per group). After drug administration all animals were tested in the open field test for 30 minutes through videotracking system (Smart®, Panlab, Barcelona, Spain) and the total distance travelled (cm) was measured. The open field used was an opaque square cage with gray arena (40 x 40 x 40 cm) (Panlab, Barcelona, Spain).

Cocaine Sensitization

Cocaine sensitization was conducted following a consecutive four-phase paradigm: cocaine conditioning, drug free period (abstinence), conditioned locomotion (CL) probe and behavioural sensitization (BS) test. Firstly, two mice group were injected with cocaine (20 mg/kg) or vehicle during five consecutive days and exposed to the open

field (cocaine conditioning). Next five days, all animals rested without drug. Then, we evaluated the locomotor activity response induced by the association between repeated administrations of cocaine and the place where it exerted its stimulant effect by simulated administration (vehicle), CL response. Last day, we tested the presence of sensitization by lower-dose cocaine (priming: 10 mg/kg) administration and BS test was assessed. All animals were evaluated in the open field test to measure the distance travelled (cm) for 30 minutes, except in the drug free period. In this way, we had 4 four experimental groups: animals conditioned with cocaine (20mg/kg) and administered with vehicle (n = 8) or cocaine (10 mg/kg) (n = 8), and animals conditioned with vehicle (n = 8) and treated with vehicle or cocaine (10mg/kg) (n = 8). These independent groups of animals (chronic cocaine pretreatment + acute vehicle treatment, chronic cocaine pretreatment + acute cocaine treatment, chronic vehicle pretreatment + acute vehicle treatment, chronic vehicle pretreatment + acute cocaine treatment) were employed by gene, protein and enzymatic glutaminase activity studies.

Analysis of gene expression

One hour after acute and chronic cocaine administration, all animals were sacrificed and their brains removed, frozen (-80º C) and dissected in coronal brain slices (2 mm thickness) with razor blades in a mouse brain slicer matrix (Zivic Instruments). The discrete brain regions (striatum, hippocampus, prefrontal cortex and cerebellum) were picked up by free hand dissection using a scalpel. These brain regions were identified according to Mouse Brain in Stereotaxic Coordinates (Paxinos and Franklin 2001): prefrontal cortex from the bregma 2.46 to 1.34 mm, hippocampus from the bregma -1.22 to -3.52 mm, striatum from the bregma 1.54 to -0.46 mm, and cerebellum from the bregma -5.52 to -7.80 mm.

Reverse transcription and real-time PCR

Real-time PCR was used to measure relative quantification of synthesis enzymes and transporter mRNA expression involved in the glutamatergic (LGA and KGA synthesis enzymes; EAAC1 transporter) neurotransmission. Total RNA from selected brain regions was isolated using Trizol reagent (Gibco BRL Life Technologies, Baltimore, MD, USA) according to the manufacturer's instructions, and purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration and purity were determined by measuring optical density at 260 nm and 280 nm using a spectrophotometer (Biotech Photometer, UV 1101, WPA). In all cases, RNA samples showed A260/280 ratios between 1.8 and 2.0. Reverse transcription and first-strand synthesis from each sample

was carried out using random hexamer primers and M-MuLV reverse transcriptase (Roche Applied Science, Indianapolis, USA) according to manufacturer's instructions. Negative controls (omitting reverse transcriptase) were run in parallel. Resulting cDNAs were used as templates for quantitative real-time PCR with an iCycler system (Bio-Rad, Hercules, CA, USA) using the Quanti-Tect SYBR Green PCR kit (Qiagen, Hilden, Germany). The following primers were used for real-time PCR (accession no. from NCBI database): LGA (NM_001033264) forward: 5'-gcactcggatcatgacgcctcac-3', reverse: 5'-ttggaccatgcgctgcatcttg-3' (190 bp product); KGA (NM_001081081) forward: 5'-gcgagggcaaggagatggtg-3', reverse: 5'-ctctttcaacctgggatcagatgttc-3' (190 bp product); EAAC1 (BC065099.1) forward: 5'-caggctttctattggcagga-3', reverse: 5'-acccttttcccttcccctaaa-3' (216 bp product). Oligonucleotides were provided by Sigma-Proligo (Proligo France SAS, Paris, France).

Quantification was carried out according to standard curves run simultaneously as the samples with each reaction run in duplicate. The PCR product was separated by electrophoresis in a 1% agarose gel to verify fragment size and the absence of contaminant fragments, quantified by measuring the absorbance at 260 nm, and serially diluted to 10-5 pg/ml. Several 10-fold dilutions (10-1 to 10-5) were checked for optimal cycling on the iCycler system (Bio-Rad, Hercules, CA, USA) 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 95ºC for 15 min to activate DNA polymerase, then 30-40 cycles of 94ºC for 15 s, annealing temperature for 30 s (LGA: 55ºC, KGA: 60ºC, EAAC1: 51,4ºC and β-actin: 57,1ºC) and a final extension step of 72ºC 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 normalised with regard to β-actin (constitutive gene) mRNA used as reference standard. This internal standard was chosen based on a first analysis of a panel of housekeeping genes that additionally included cyclophyllin and SP1.

Western blotting

To measure the levels of GA proteins in each brain region, western blotting with purified specific polyclonal antibodies against KGA or LGA, obtained as described by Olalla et al. (2002), were performed. Protein samples (40 µg) were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking at room

temperature for 1 h with 5% BSA in TBST buffer (0.1% Tween 20 in TBS), membranes were incubated with primary antibodies in blocking buffer with 5% BSA, overnight at 4ºC. After incubation with secondary antibody, the blots were developed by enhanced chemiluminescense technique as recommended by the supplier (Pierce) and bands quantified using the Chemi Doc System (BioRad). β-actin was quantified and used a as loading control.

GA enzymatic activity

The discrete brain regions from dissections were resuspended in TES buffer (25 mMTris-HCl, 0.2 mM EDTA, 0.33 M sucrose, pH 8.0) containing the complete protease inhibitor cocktail (Roche), homogenized and solubilised with TX-100 at a final concentration of 1% (V/V). After centrifugation at 100000 x g for 30 min at 4 ºC, the supernatants were divided into aliquots and kept at -80 ºC until analysis. The protein content in each sample was determined by the Bradford method. Glutaminase activity was assayed by measuring the ammonia produced in the catalytic reaction as described by Heini (1987). Samples of 25 µL were added to 35 µL of a mixture of 100 mM potassium phosphate, 171 mM L-glutamine and 1.5 mM NH_4Cl, pH 8.0, and incubated for 1 h at 37 ºC. The reaction was terminated by adding 10 µL of TCA 10 %, kept on ice for 15 min and centrifuged at 12000 x g. Aliquots of 5 µL were then mixed with 150 µL of *o*-phthalaldehyde/mercaptoethanol reagent (10 mL 0.2 M potassium phosphate, pH 7.4, 0.56 mL of 72 mM mercaptoethanol in ethanol and 0.56 mL of 186 mM*o*-phthalaldehyde in ethanol). The samples were kept at room temperature in the dark and their absorbance measured at 410 nm after 45 min together with a NH_4Cl standard. For blanks, samples and substrate solution were incubated separately and mixed after the addition of TCA.

Statistical analysis

Results are expressed as the mean ± SEM (Standard Error of the Mean) of at least ten determinations per experimental group. Statistical significance of behavioral results was assessed by One-way/RM (repeated measured) ANOVA and *post hoc* Newman-Keuls test. Statistical significance of gene quantifications was analyzed by two-way ANOVA and *post hoc* Bonferroni test, with acute treatment (vehicle or cocaine for 1 day) and chronic pretreatment (conditioning with vehicle or cocaine for 5 days) as two main factors. In all cases, we considered a statistically significant difference from chance at $p<0.05$. All data were analyzed with statistical software SPSS for Windows 11.5 (Lead Technologies).

Results

Acute cocaine dose-response effect in C57BL/6J mice

We measured acute cocaine dose-response curve with different doses (0, 5, 10 and 20 mg/kg) in C57BL/6J mice (see Figure 1.A.). Our results showed a typical progressive enhancement on the distance travelled as cocaine doses were gradually higher (One-way ANOVA, doses effect: (F (3,44)=27.0265, p=0.000), with 10 (Tukey. 10 *vs.* 0, p=0.002) and 20 mg/kg dose producing the highest effect (Tukey. 20 *vs.* 0, p=0.000; 20 *vs.* 5, p=0.000; 20 *vs.* 10, p=0.008).

Cocaine Sensitization

All mice injected with vehicle saline exhibited a standard baseline control, whereas acquisition of conditioned response was progressively increased in cocaine exposed animals (20 mg/kg) for 5 days (One-way RM ANOVA, within-subjects factor: (F (4,120)=7.761, p=0.001) (see Figure 1.B.). Cocaine treated group showed a significant increase in locomotor activity compared to control group (One-way RM ANOVA, between-groups factor: (F (1,30)=208.21, p=0.001) caused by repeated drug injections, every day during treatment (post hoc Newman-Keuls: days 1, 2, 3, 4 and 5; $p \leq 0.001$).

Five days after cocaine conditioning, all groups were subjected to the cocaine CL test with vehicle administration. In this form, chronic cocaine-conditioned group showed a CL response compared to non-conditioned control group (One-way ANOVA, chronic pretreatment effect: (F (1,50)=6.5354, p=0.0137) (see Figure 1.C.). This effect was produced by association between the features of environment and cocaine-rewarding properties after 5 consecutive injection-days. One day later, we measured the BS response with animals conditioned with cocaine (20 mg/kg) and administered with vehicle or cocaine (10 mg/kg), and animals conditioned with vehicle and treated with vehicle or cocaine (10 mg/kg). Our results revealed that the groups who had received an acute-cocaine dose (10 mg/kg) showed increased locomotor activity compared to their control groups (One-way ANOVA, acute treatment effect respect to vehicle conditioning group: (F (1,15)=30.3958, p=0.001; acute treatment effect respect to cocaine conditioning group: (F (1,15)=33.1728, p=0.001). Moreover, this increase was significantly higher in mice that had previously been conditioned with cocaine (20 mg/kg) than whose had received the first dose (One-way ANOVA, cocaine sensitization effect: (F (1,15)=20.9737, p=0.001) as result of cocaine-induced sensitization (see Figure 1.D.). BS was established on these mice as a model of addiction for the gene

expression study, and thus identifies the neuroadaptive changes in the expression of genes associated with the effects of acute/chronic consumption and the induction of cocaine sensitization.

Cocaine may produce changes in glutamatergic transmission that have been associated with brain alterations in mice after chronic drug administration. The genomic dysfunctions induced by acute and chronic cocaine administration in the glutamatergic system were shown studying the expression of genes involved in biosynthesis and transport of Glu in the mouse brain. We assessed short/long-term mRNA modifications caused by differences in acute treatment (vehicle or cocaine for 1 day), chronic pretreatment (conditioning with vehicle or cocaine for 5 days) and its interaction (acute treatment x chronic pretreatment). To evaluate these factors, we analyzed the expression profiles of the genes coding for the enzymes of Glu synthesis (LGA, liver-type glutaminase; KGA, kidney-type glutaminase), and neuronal Glu transporter (EAAC1) in prefrontal cortex, hippocampus, striatum and cerebellum of treated-mice (dosing procedures were as described in the before experimental section).

Before analyzing the data region to region, the first relevant result found was that the KGA mRNA expression levels were much higher compared to LGA in the whole brain from mouse by qPCR using poly(A+)mRNA. The number of copies to KGA was $46.96 \times 10^4 \pm 9.48 \times 10^4$, while to LGA was only $3.021 \times 10^4 \pm 0.33 \times 10^4$ (authors' unpublished results, manuscript in preparation).

LGA expression is activated by acute-cocaine treatment in the prefrontal cortex and acute-cocaine priming in striatum

We analyzed regional pattern of LGA gene expression after cocaine administration. In prefrontal cortex, LGA mRNA expression levels showed significant changes in acute (Two-way ANOVA, acute treatment effect: (F (1,27)=4.2880, p=0.0481), but neither chronic administration (Two-way ANOVA, chronic pretreatment effect: (F (1,27)=3.4110, p=0.0758), nor interaction effect (Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,27)=0.0982, p=0.7564) were found (see Figure 2.C.). In this case, levels of LGA mRNA in acute-cocaine/sensitized mice were increased in the prefrontal cortex. Otherwise, LGA expression in the striatal region (see Figure 2.A.) did not show any significant difference when cocaine was injected with acute (Two-way ANOVA, acute treatment effect: (F (1,24)=3.275, p=0.0829) or chronic injection protocols (Two-way ANOVA, chronic pretreatment effect: (F (1,24)=1.399, p=0.2485). However, we found an interaction effect between both variables (Two-way ANOVA,

acute treatment x chronic pretreatment effect: (F (1,24)=8.418, p=0.0078) that could indicates that part of this effect might be due to a robust cocaine-induced neuronal sensitization. On the other hand, the expression profile of LGA mRNA was similar in hippocampus and cerebellum (see Figure 2.B-D.). Levels of LGA expression did not show changes after different treatments, chronic pretreatment (Hippocampus. Two-way ANOVA, chronic pretreatment effect: (F (1,27)=0.5214, p=0.4765; Cerebellum. Two-way ANOVA, chronic pretreatment effect: (F (1,25)=0.1109, p=0.7419) and acute treatment (Hippocampus. Two-way ANOVA, acute treatment effect: (F (1,27)=0.4346, p=0.5153; Cerebellum. Two-way ANOVA, acute treatment effect: (F (1,25)=4.185, p=0.0514) with cocaine, nor interaction (Hippocampus. Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,27)=0.0246, p=0.8764; Cerebellum. Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,25)=0.2993, p=0.5891).

KGA expression is activated by chronic-cocaine pretreatment in striatum but is inhibited by acute-cocaine treatment in hippocampus

In striatal region, the expression of KGA synthesis enzyme showed statistically significant differences caused by chronic pretreatment (Two-way ANOVA, chronic pretreatment effect: (F (1,27)=5.369, p=0.0283) (see Figure 3.A.). Repeated administration of cocaine increased KGA expression basal levels in striatum. Chronic pretreatment produced long-term overstimulation throughout the increased neuronal excitability of glutamatergic striatal neurons. This neural excitation may be caused by an enhanced KGA enzymatic activity related to an augmented locomotion induced by cocaine conditioning. Instead KGA did not show significant differences in acute treatment (Two-way ANOVA, acute treatment effect: (F (1,27)=0.0251 p=0.8751) or interaction (Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,27)=3.273, p=0.0816). In the other way, our data show that acute treatment diminished KGA expression basal levels in hippocampus (Two-way ANOVA, acute treatment effect: (F (1,27)=15.840, p=0.0005), but we did not find differences produced by chronic pretreatment (Two-way ANOVA, chronic pretreatment effect: (F (1,27)=1.980, p=0.1708) or interaction (Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,27)=1.980, p=0.1708) (see Figure 3.B.). These results suggest that, under acute conditions, cocaine may downregulate the transcription of KGA. Moreover, the measurement of KGA expression in prefrontal cortex and cerebellum did not show variations induced by chronic pretreatment (Prefrontal cortex. Two-way ANOVA, chronic pretreatment effect: (F (1,24)=2.384, p=0.1357; Cerebellum. Two-

way ANOVA, chronic pretreatment effect: (F (1,26)=3.303, p=0.0807), acute treatment (Prefrontal cortex. Two-way ANOVA, acute treatment effect: (F (1,24)=3.432, p=0.0763; Cerebellum. Two-way ANOVA, acute treatment effect: (F (1,26)=1.107, p=0.3024), or interaction (Prefrontal cortex. Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,24)=0.678, p=0.1484; Cerebellum. Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,26)=1.626, p=0.2135) (see Figure 3.C-D.).

EAAC1 glutamate transporter expression is decreased in striatum after both acute- and chronic-cocaine treatments but increased in cerebellum by chronic pretreatment

Regarding the EAAC1 Glu transporter in striatum, our data showed statistically significant differences in both forms of cocaine administration. The levels of EAAC1 transporter in striatum were affected by cocaine acute treatment (Two-way ANOVA, acute treatment effect: (F (1,24)=13.610, p=0.001) and chronic pretreatment (Two-way ANOVA, chronic pretreatment effect: (F (1,24)=8.386, p=0.008), but not their interaction (Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,24)=1.548, p=0.225) (see Figure 4.A.). This means that cocaine conditioning as well as the acute treatment and relapse to the drug diminished the levels of EAAC1 transporter mRNA expression in striatum. In case of the EAAC1 mRNA expression levels in cerebellum, we observed a chronic effect induced by cocaine pretreatment (Two-way ANOVA, chronic pretreatment effect: (F (1,27)=4.520, p=0.043), but not by acute treatment (Two-way ANOVA, acute treatment effect: (F (1,27)=0.322, p=0.575) or interaction (Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,27)=1.034, p=0.318) (see Figure 4.C.). This enhancement in the levels of Glu transporter mRNA could be due to initial chronic pretreatment with cocaine in Purkinje's cells where EAAC1 transporter is especially abundant. The remaining regions studied, hippocampus and prefrontal cortex, did not present differences in the rest of comparisons for Glu transporter (see Figure 4.B-D.). Levels of EAAC1 expression did not show modifications after diverse treatments, chronic pretreatment (Hippocampus. Two-way ANOVA, chronic pretreatment effect: (F (1,27)=1.655, p=0.209; Prefrontal cortex. Two-way ANOVA, chronic pretreatment effect: (F (1,26)=3.276, p=0.082) and acute treatment (Hippocampus. Two-way ANOVA, acute treatment effect: (F (1,27)=1.212, p=0.281; Prefrontal cortex. Two-way ANOVA, acute treatment effect: (F (1,26)=0.138, p=0.713) with cocaine, nor interaction (Hippocampus. Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,27)=3.400, p=0.076;

Prefrontal cortex. Two-way ANOVA, acute treatment x chronic pretreatment effect: (F (1,26)=1.483, p=0.234).

Western blotting results

After ending the analysis of genomic changes, LGA and KGA protein levels were measured on brain regions in where had been revealed changes in mRNA glutaminase expression (see Figure 5.A-D), by western blotting. In this sense, we only observed a significant interaction effect between acute x chronic cocaine pretreatment (Two way ANOVA: Interaction effect F(1,20)= 12.80, p<0.01) in LGA protein levels measured in Prefrontal Cortex (see Figure 5.B.).

GA activity results

Our results showed a reduced GA activity in Striatum (Two way ANOVA: Pre-treatment effect F(1,20)=7.787, p<0.05) and Prefrontal Cortex (Two way ANOVA: Pre-treatment effect F(1,20)= 26.17, p<0.001) after chronic cocaine pretreatment, but this effect did not found in Hippocampus (Two way ANOVA: Pre-treatment effect F(1,20)= 0.8354, p<0.3716) after cocaine conditioning (see Figure 6.A-C). This functional measured did not show significant differences after acute treatment in the brain regions evaluated (Striatum, Two way ANOVA: Treatment effect F(1,20)= 0.01884, p<0.8922; Hippocampus, Two way ANOVA: Pre-treatment effect F(1,20)= 0.8354, p<0.3716; Prefrontal Cortex, Two way ANOVA: Treatment effect F(1,20)= 1.947, p<0.1782) (see Figure 6.A-C). In summary, we observed a reduction of enzymatic GA activity in the striatum and prefrontal cortex after 5 days of abstinence.

Discussion

Three main findings arise from the present studies. First, glutaminase (GA) activity is decreased after repeated exposure to cocaine, using a dose regimen that induces behavioural sensitization to this psychostimulant. Second, there is a clear temporal dissociation between the effects on the expression of the mRNAs for GA isoforms and the EAAC1 glutamate transporter, which are mainly affected after acute cocaine challenge, and the total GA activity, which is affected mainly by repeated exposure to the psychostimulant. Third, main changes were observed on the prefrontal cortex-dorsal striatum circuit, which are the anatomical site for the neuroplasticity events that underlie cocaine-induced behavioural sensitization (Dong et al., 2005; Engblom et al. 2008; Everitt and Wolf, 2002; Ghasemzadeh et al., 2009a; Kim et al,, 2009). Overall, the results set in place GA as a relevant protein involved in the neuroadaptions associated to repeated cocaine exposure.

Glutaminase activity changes can be attributed to alterations on KGA since in the mouse brain there is a differential expression of L- and K-type GA isoforms. The presence of KGA is much higher than LGA in the whole mouse brain measured by qPCR using poly(A+)mRNA (authors' unpublished results, manuscript in preparation). In general terms, KGA biosynthetic enzyme expression is much more abundant than LGA in prefrontal cortex, hippocampus, striatum and cerebellum. These data are consistent with our previous results at the protein level, showing that KGA immunolabeling was more pronounced in the mammalian brain than LGA (Olalla et al. 2002; Olalla et al. 2008). This differential pattern of expression could be due to the fact that KGA was located in the mitochondrial compartment while LGA was mostly found in the neuronal nuclei (Olalla et al. 2002). Glutaminase activity was not affected by acute cocaine challenge neither in vehicle-exposed nor in cocaine-exposed animals. However, two-way ANOVA revealed that repeated cocaine exposure elicited a decrease in GA activity in both, the dorsal striatum and the prefrontal cortex. Neither a vehicle injection nor a challenge with a cocaine injection was able to recover the GA activity values to those shown by acute-control or acute-cocaine treated animals. Cocaine-induced sensitization is associated to enhanced glutamate transmission in both brain areas (Nasif et al., 2005a and 2005b). This action of cocaine is produced by dynamic changes in both glutamate release (Pierce et al., 1996; McFarland et al, 2003) and glutamate receptor signalling ((Engblom et al. 2008; Ghasemzadeh et al., 2009a; Kim et al,, 2009; Xi et al., 2002; Xi and steketee, 2008). In this context, the decrease in GA activity may be either a counter-regulatory inhibition to compensate the enhanced

glutamate output or be related to the fact that withdrawal from repeated exposure to cocaine reduces basal extracellular Glu levels in the basal ganglia (Schmidt and Pierce, 2010). Since we observed enhanced GA activity 5 days after cessation of repeated cocaine exposure, this explanation seems to be plausible.

The second relevant finding is the confirmation of the modification of the expression GA isoforms expression as result of cocaine administration. Cocaine has been shown to alter Glu transmission in ventral tegmental area (VTA) and its projection areas, both directly to the accumbens nucleus (NAc) or indirectly (substantia nigra, medial prefrontal cortex, septo-hippocampal area, cerebellum) contributing in the psychostimulant behavioral effects (Kalivas 2004; Kalivas et al. 2009). But the role of GA enzymes and EAAC1 transport on the mediation of cocaine-induced sensitization was unknown in these brain regions. This is a relevant issue since the prefrontal-cortex/dorsal striatum circuit is the key anatomical target for chronic cocaine actions. Understanding glutamate dynamics in this circuit may help us to understand the vulnerability to develop compulsive drug-seeking habits in cocaine addiction (Everitt et al. 2008). In the dorsal striatum, we observed an increased expression of the mRNAS coding for KGA and LGA after acute cocaine exposure in cocaine sensitized animals, which contrasts with the decrease in the activity observed in these areas. This indicates that cocaine exposure dissociates the up-regulation of GA mRNA isoforms from protein activity. This finding stresses the importance of monitoring not only mRNA expression but protein levels and activity, since cell phenotype is derived from the later (Pradet-Balade et al., 2001). Similar dissociations have been described for glutamate receptors in VTA of cocaine exposed-animals (Choi et al., 2011).

The decrease in the expression of the EAAC1 carrier after acute cocaine, in both control and cocaine-sensitized animals, suggest that this Glu transporter plays also a role in the neuroadaptions to cocaine. Although we do not have functional evaluation of its activity, previous results support this idea. Thus, the decrease in basal accumbal Glu during withdrawal from chronic cocaine exposure has been attributed to a cocaine-modulation of the activity of the cysteine-Glu antiporter (Baker DA et al., 2002 and 2003).

Finally, major changes were observed in the prefrontal cortex-dorsal striatum circuit, in agreement with the described role for these brain areas in psychostimulant-induced sensitization. Outside from this circuit, our data indicated that neither GA activity nor LGA and EAAC1 mRNA levels were unaffected in mouse hippocampus or cerebellum, with the exception of a small increase in cerebellar expression of the transporter as

result of cocaine challenge. The only robust effect observed after cocaine exposure outside the cortico-striatal circuit was a decrease in KGA expression in the hippocampus after acute cocaine in both control and cocaine-sensitized animals. This decrease in KGA expression has an impact neither in protein levels nor in total GA activity. This pattern of restricted regional effects of cocaine has been found also in other members of the Glutamate signalling pathway such as the mGluR8 whose protein expression levels in the hippocampus and prefrontal cortex also remained constant after acute cocaine injection while they were down-regulated in the striatum (Zhang et al. 2009).

In summary, our data suggest that cocaine sensitization is associated to prefrontal cortex-dorsal striatum decrease in the activity of Glu synthetic enzymes, and to a modulation of the expression of GA isoforms and the EAAC1 Glu transporter. Minor changes were observed in the hippocampus and cerebellum indicating than in this model of cocaine actions the main relevant target is the dorsal striatum and functionally associated PFC. These observed changes in the levels of Glu biosynthetic activity and transporter may alter excitatory neurotransmission in the mesocorticolimbic dopamine system, which could play a significant role in the enduring biochemical and behavioural effects of cocaine. Further research is needed to understand the temporal dynamics of GA contribution to cocaine-induced behavioural sensitization.

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References

Aledo JC, Gomez-Fabre PM, Olalla L, Marquez J (2000) Identification of two human glutaminase loci and tissue-specific expression of the two related genes. Mamm Genome 11: 1107-10

Aoyama K, Suh SW, Hamby AM, Liu J, Chan WY, Chen Y, Swanson RA (2006) Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse. Nat Neurosci 9: 119-26

Baker DA, McFarland K, Lake RW, Shen H, Tang XC, Toda S, Kalivas PW. (2003) Neuroadaptations in cystine-glutamate exchange underlie cocaine relapse. Nat Neurosci 67:743-9.

Baker DA, Shen H, Kalivas PW. (2002) Cystine/glutamate exchange serves as the source for extracellular glutamate: modifications by repeated cocaine administration. Amino Acids. 23:161-2.

Choi KH, Edwards S, Graham DL, Larson EB, Whisler KN, Simmons D, Friedman AK, Walsh JJ, Rahman Z, Monteggia LM, Eisch AJ, Neve RL, Nestler EJ, Han MH, Self DW. (2011) Reinforcement-related regulation of AMPA glutamate receptor subunits in the ventral tegmental area enhances motivation for cocaine. J Neurosci. 2011 May 25;31(21):7927-37.

Collingridge GL, Lester RA (1989) Excitatory amino acid receptors in the vertebrate central nervous system. Pharmacol Rev 41: 143-210

Conti F, Weinberg RJ (1999) Shaping excitation at glutamatergic synapses. Trends Neurosci 22: 451-8

Curthoys NP, Watford M (1995) Regulation of glutaminase activity and glutamine metabolism. Annu Rev Nutr 15: 133-59

Dong Y, Nasif FJ, Tsui JJ, Ju WY, Cooper DC, Hu XT, Malenka RC, White FJ (2005) Cocaine-induced plasticity of intrinsic membrane properties in prefrontal cortex pyramidal neurons: adaptations in potassium currents. J Neurosci 25: 936-40

Engblom D, Bilbao A, Sanchis-Segura C, Dahan L, Perreau-Lenz S, Balland B, Parkitna JR, Lujan R, Halbout B, Mameli M, Parlato R, Sprengel R, Luscher C, Schutz G, Spanagel R (2008) Glutamate receptors on dopamine neurons control the persistence of cocaine seeking. Neuron 59: 497-508

Erecinska M, Silver IA (1990) Metabolism and role of glutamate in mammalian brain. Prog Neurobiol 35: 245-96

Everitt BJ, Belin D, Economidou D, Pelloux Y, Dalley JW, Robbins TW (2008) Review. Neural mechanisms underlying the vulnerability to develop compulsive drug-seeking habits and addiction. Philos Trans R Soc Lond B Biol Sci 363: 3125-35

Everitt BJ, Wolf ME (2002) Psychomotor stimulant addiction: a neural systems perspective. J Neurosci 22: 3312-20

Ferrario CR, Li X, Wang X, Reimers JM, Uejima JL, Wolf ME. (2010) The role of glutamate receptor redistribution in locomotor sensitization to cocaine. Neuropsychopharmacology. 35:818-33.

Fonnum F (1984) Glutamate: a neurotransmitter in mammalian brain. J Neurochem 42: 1-11

Ghasemzadeh MB, Mueller C, Vasudevan P (2009a) Behavioral sensitization to cocaine is associated with increased glutamate receptor trafficking to the postsynaptic density after extended withdrawal period. Neuroscience 159: 414-26

Ghasemzadeh MB, Vasudevan P, Mueller C, Seubert C, Mantsch JR (2009b) Neuroadaptations in the cellular and postsynaptic group 1 metabotropic glutamate receptor mGluR5 and Homer proteins following extinction of cocaine self-administration. Neurosci Lett 452: 167-71

Gomez-Fabre PM, Aledo JC, Del Castillo-Olivares A, Alonso FJ, Nunez De Castro I, Campos JA, Marquez J (2000) Molecular cloning, sequencing and expression studies of the human breast cancer cell glutaminase. Biochem J 345 Pt 2: 365-75

Heini HG, Gebhardt R, Brecht A, Mecke D (1987) Purification and characterization of rat liver glutaminase. Eur J Biochem 162: 541-6

Ito R, Dalley JW, Robbins TW, Everitt BJ (2002) Dopamine release in the dorsal striatum during cocaine-seeking behavior under the control of a drug-associated cue. J Neurosci 22: 6247-53

Kalivas PW (2004) Glutamate systems in cocaine addiction. Curr Opin Pharmacol 4: 23-9

Kalivas PW, Lalumiere RT, Knackstedt L, Shen H (2009) Glutamate transmission in addiction. Neuropharmacology 56 Suppl 1: 169-73

Kanai Y, Bhide PG, DiFiglia M, Hediger MA (1995) Neuronal high-affinity glutamate transport in the rat central nervous system. Neuroreport 6: 2357-62

Kanai Y, Hediger MA (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 360: 467-71

Kauer JA, Malenka RC (2007) Synaptic plasticity and addiction. Nat Rev Neurosci 8: 844-58

Kim M, Au E, Neve R, Yoon BJ. (2009)AMPA receptor trafficking in the dorsal striatum is critical for behavioral sensitization to cocaine in juvenile mice. Biochem Biophys Res Commun.379:65-9.

Kovacevic Z, McGivan JD (1983) Mitochondrial metabolism of glutamine and glutamate and its physiological significance. Physiol Rev 63: 547-605

Kvamme K (1984) Glutamine Metabolism in Mammalian Tissues. In: Häussinger D, Sies H (eds) Enzymes of cerebral glutamine metabolism. Springer Verlag, Berlin, pp 32-48

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J Neurosci 15: 1835-53

Marquez J, de la Oliva AR, Mates JM, Segura JA, Alonso FJ (2006) Glutaminase: a multifaceted protein not only involved in generating glutamate. Neurochem Int 48: 465-71

McFarland K, Lapish CC, Kalivas PW. (2003) Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior. J Neurosci. 23:3531-7.

Miguens M, Crespo JA, Del Olmo N, Higuera-Matas A, Montoya GL, Garcia-Lecumberri C, Ambrosio E (2008) Differential cocaine-induced modulation of glutamate and dopamine transporters after contingent and non-contingent administration. Neuropharmacology 55: 771-9

Mohn AR, Yao WD, Caron MG (2004) Genetic and genomic approaches to reward and addiction. Neuropharmacology 47 Suppl 1: 101-10

Nasif FJ, Hu XT, White FJ (2005a) Repeated cocaine administration increases voltage-sensitive calcium currents in response to membrane depolarization in medial prefrontal cortex pyramidal neurons. J Neurosci 25: 3674-9

Nasif FJ, Sidiropoulou K, Hu XT, White FJ (2005b) Repeated cocaine administration increases membrane excitability of pyramidal neurons in the rat medial prefrontal cortex. J Pharmacol Exp Ther 312: 1305-13

Nicklas WJ, Zeevalk G, Hyndman A (1987) Interactions between neurons and glia in glutamate/glutamine compartmentation. Biochem Soc Trans 15: 208-10

Nieoullon A, Canolle B, Masmejean F, Guillet B, Pisano P, Lortet S (2006) The neuronal excitatory amino acid transporter EAAC1/EAAT3: does it represent a major actor at the brain excitatory synapse? J Neurochem 98: 1007-18

Olalla L, Gutierrez A, Campos JA, Khan ZU, Alonso FJ, Segura JA, Marquez J, Aledo JC (2002) Nuclear localization of L-type glutaminase in mammalian brain. J Biol Chem 277: 38939-44

Olalla L, Gutierrez A, Jimenez AJ, Lopez-Tellez JF, Khan ZU, Perez J, Alonso FJ, de la Rosa V, Campos-Sandoval JA, Segura JA, Aledo JC, Marquez J (2008) Expression of the scaffolding PDZ protein glutaminase-interacting protein in mammalian brain. J Neurosci Res 86: 281-92

Paxinos G, Franklin KBJ (2001) The mouse brain in stereotaxic coordinates, 2nd edn. Academic, Academic

Pierce RC, Bell K, Duffy P, Kalivas PW. (1996) Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization. J Neurosci. 16:1550-60.

Pradet-Balade B, Boulmé F, Beug H, Müllner EW, Garcia-Sanz JA. (2001) Translation control: bridging the gap between genomics and proteomics? Trends Biochem Sci. 26:225-9.

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. Neuron 13: 713-25

Schmidt HD, Pierce RC. (2010) Cocaine-induced neuroadaptations in glutamate transmission: potential therapeutic targets for craving and addiction. Ann N Y Acad Sci. 1187:35-75.

Shashidharan P, Huntley GW, Meyer T, Morrison JH, Plaitakis A (1994) Neuron-specific human glutamate transporter: molecular cloning, characterization and expression in human brain. Brain Res 662: 245-50

Velaz-Faircloth M, McGraw TS, alandro MS, Fremeau RT, Jr., Kilberg MS, Anderson KJ (1996) Characterization and distribution of the neuronal glutamate transporter EAAC1 in rat brain. Am J Physiol 270: C67-75

Xi ZX, Ramamoorthy S, Baker DA, Shen H, Samuvel DJ, Kalivas PW (2002) Modulation of group II metabotropic glutamate receptor signaling by chronic cocaine. J Pharmacol Exp Ther 303: 608-15

Xie X, Steketee JD (2008) Repeated exposure to cocaine alters the modulation of mesocorticolimbic glutamate transmission by medial prefrontal cortex Group II metabotropic glutamate receptors. J Neurochem 107: 186-96

Zhang GC, Vu K, Parelkar NK, Mao LM, Stanford IM, Fibuch EE, Wang JQ (2009) Acute administration of cocaine reduces metabotropic glutamate receptor 8 protein expression in the rat striatum in vivo. Neurosci Lett 449: 224-7

Figure Legends

Fig. 1: A. Acute cocaine dose-response curve in C57BL/6J mice. Different doses of acute cocaine (0, 5, 10 and 20 mg/kg) showed an increase in total distance travelled in the open-field. The 10-20 mg/Kg doses significantly increased distance travelled compared to other doses. **p<0.01 and *** p<0.001 compared to vehicle group, or ## p<0.01 and ### p<0.001 compared to cocaine 20 mg/kg group. **B.** Chronic cocaine conditioning. Acquisition of the cocaine conditioned response increased during 5 treatment's days. Cocaine-treated group showed a significant increase in locomotor activity compared to control group by chronic drug-injections. *** p<0.001 compared to vehicle group each treatment day. **C.** Conditioned Locomotion (CL) response induced by chronic cocaine administration after a vehicle injection. ** p<0.01 compared to vehicle group. **D.** Behavioral Sensitization (BS) response was measured with animals conditioned with cocaine/vehicle (20mg/kg) and administered with vehicle/cocaine (10 mg/kg) in the probe. Acute-cocaine treated groups (10 mg/kg) showed increased locomotor activity compared with control groups. Between previously cocaine-treated groups, the enhancement was significantly higher in cocaine-conditioned pretreated mice (20mg/kg) than non-conditioned pretreated mice as consequence of cocaine-induced sensitization. *** p<0.001 compared to vehicle groups and ### p<0.001 compared to cocaine 10 mg/kg group. All results are presented as means ± SEM (n = at least 8 per group).

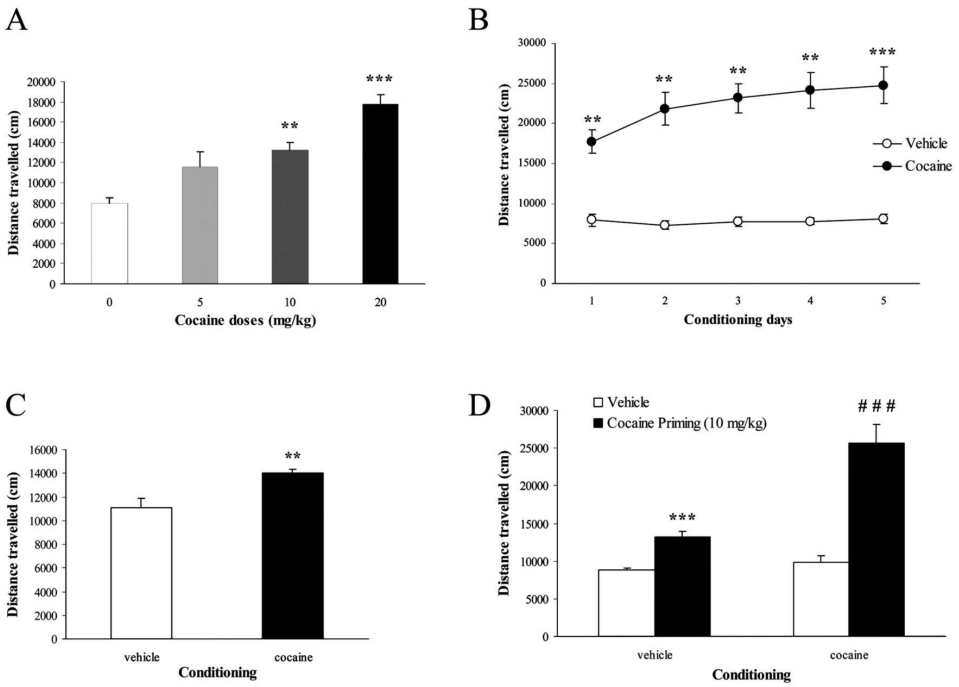
Fig. 2: Quantitative real-time PCR analysis of liver-type glutaminase (LGA) mRNA expression normalized to the levels of β-actin mRNA in different brain regions. Each value corresponds to mRNA levels in C57BL/6J mice after acute treatment (sensitization with vehicle or cocaine for 1 day) and chronic pretreatment (conditioning with vehicle or cocaine for 5 days). **A.** Comparison of the expression of LGA in the striatum of cocaine acute treated and chronic pretreated mice. **B.** Comparison of the expression of LGA in the hippocampus of cocaine acute treated and chronic pretreated mice. **C.** Comparison of the expression of LGA in the prefrontal cortex of cocaine acute treated and chronic pretreated mice. **D.** Comparison of the expression of LGA in the cerebellum of cocaine acute treated and chronic pretreated mice. Bars indicate the mean value ± SEM. Two-way ANOVA followed by Bonferroni post hoc test, *p<0.05; **p<0.01; ***p<0.001 (n = 7-8 per group)..

Fig. 3: Quantitative real-time PCR analysis of kidney-type glutaminase (KGA) mRNA expression normalized to the levels of β-actin mRNA in different brain regions. Each value corresponds to mRNA levels in C57BL/6J mice after acute treatment (sensitization with vehicle or cocaine for 1 day) and chronic pretreatment (conditioning with vehicle or cocaine for 5 days). **A.** Comparison of the expression of KGA in the striatum of cocaine acute treated and chronic pretreated mice. **B.** Comparison of the expression of KGA in the hippocampus of cocaine acute treated and chronic pretreated mice. **C.** Comparison of the expression of KGA in the prefrontal cortex of cocaine acute treated and chronic pretreated mice. **D.** Comparison of the expression of KGA in the cerebellum of cocaine acute treated and chronic pretreated mice. Bars indicate the mean value ± SEM. Two-way ANOVA followed by Bonferroni post hoc test, *p<0.05; **p<0.01; ***p<0.001 (n = 7-8 per group).

Fig. 4: Quantitative real-time PCR analysis of neuronal Glu transporter (EAAC1) mRNA expression normalized to the levels of β-actin mRNA in different brain regions. Each value corresponds to mRNA levels in C57BL/6J mice after acute treatment (sensitization with vehicle or cocaine for 1 day) and chronic pretreatment (conditioning with vehicle or cocaine for 5 days). **A.** Comparison of the expression of EAAC1 in the striatum of cocaine acute treated and chronic pretreated mice. **B.** Comparison of the expression of EAAC1 in the hippocampus of cocaine acute treated and chronic pretreated mice. **C.** Comparison of the expression of EAAC1 in the prefrontal cortex of cocaine acute treated and chronic pretreated mice. **D.** Comparison of the expression of EAAC1 in the cerebellum of cocaine acute treated and chronic pretreated mice. Bars indicate the mean value ± SEM. Two-way ANOVA followed by Bonferroni post hoc test, *p<0.05; **p<0.01; ***p<0.001 (n = 7-8 per group).

Fig. 5: Quantitative analysis of LGA protein levels in Striatum (A) (n = 5) and Prefrontal Cortex (B) (n = 6), and KGA in Striatum (C) (n = 6) and Hippocampus (D) (n = 6) after acute treatment vs. chronic pretreatment with vehicle and cocaine in mice by using western blot analysis. Protein levels were normalized to β-actin as arbitrary units of optical density (mean ± SEM) as arbitrary units of optical density (mean ± SEM). **p<0.01

Fig. 6: Quantification of the enzymatic activity to glutaminase in Striatum (A) (n = 6), Hippocampus (B) (n = 6) and Prefrontal Cortex (C) (n = 6) after acute treatment vs. chronic pretreatment with vehicle and cocaine in mice. *p<0.05; ***p<0.001.



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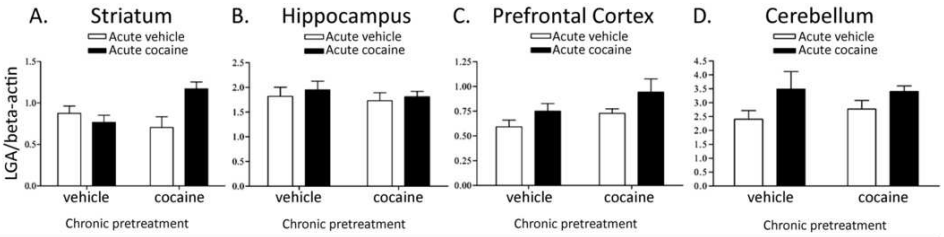

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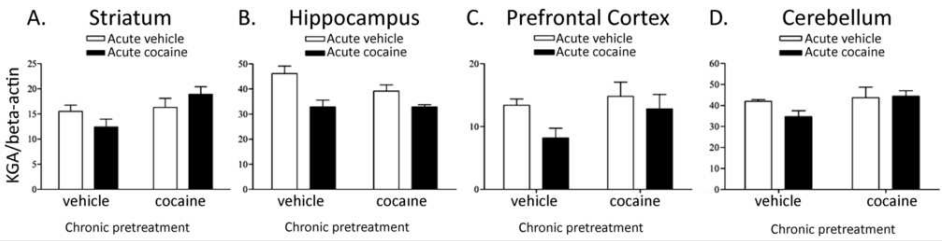

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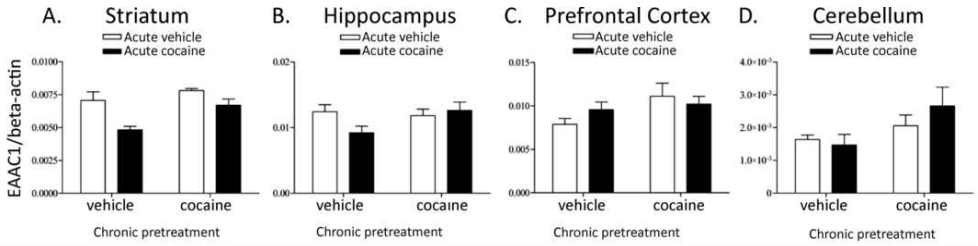

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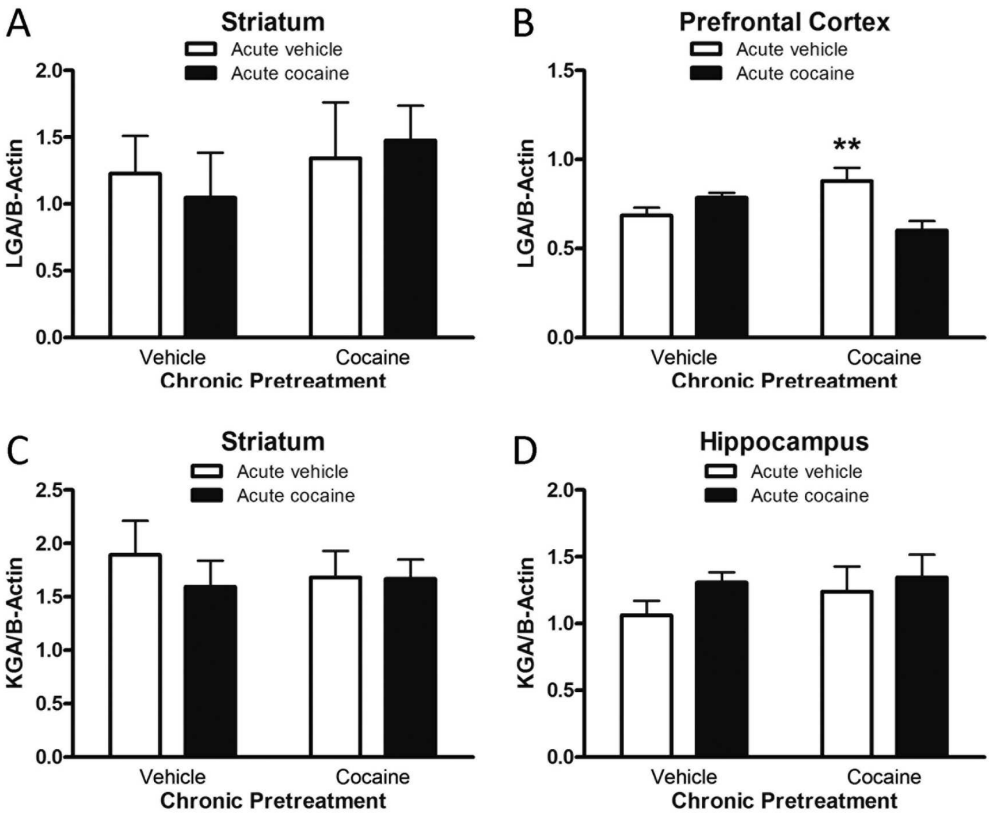

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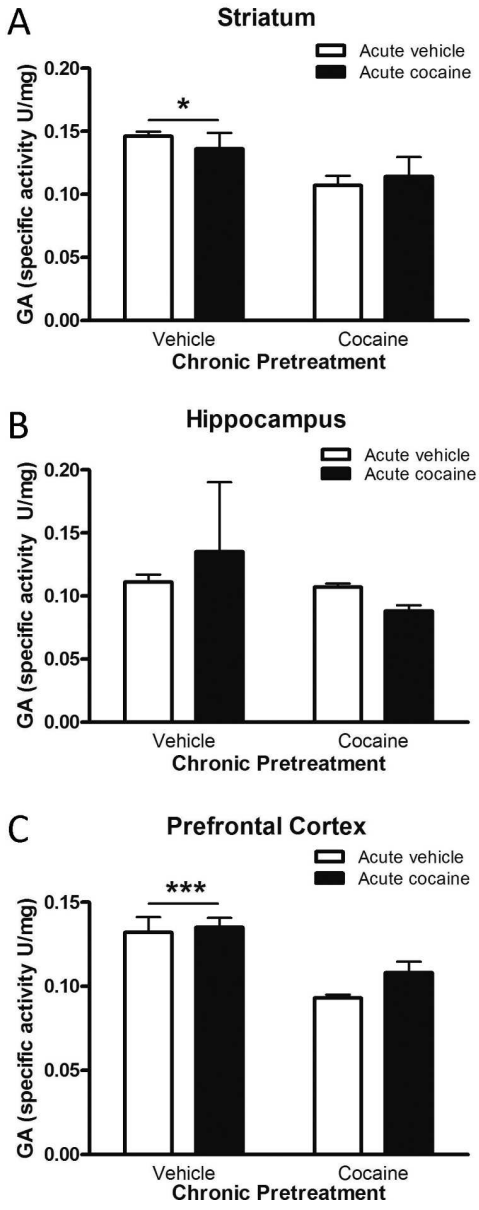

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