

Stress rapidly dysregulates the glutamatergic synapse in the prefrontal cortex of cocaine-withdrawn adolescent rats

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ABSTRACT

Although several lines of evidence have shown that chronic cocaine use is associated with stress system dysregulation, the underlying neurochemical mechanisms are still elusive. To investigate whether the rapid stress-induced response of the glutamatergic synapse was influenced by a previous history of cocaine, rats were exposed to repeated cocaine injections during adolescence [from postnatal day (PND) 28–42], subjected to a single swim stress (5 minutes) three days later (PND 45) and sacrificed 15 minutes after the end of this stressor. Critical determinants of glutamatergic homeostasis were measured in the medial prefrontal cortex (mPFC) whereas circulating corticosterone levels were measured in the plasma.

Exposure to stress in saline-treated animals did not show changes in the crucial determinants of the glutamatergic synapse. Conversely, in cocaine-treated animals, stress dynamically altered the glutamatergic synapse by: (1) enhancing the presynaptic vesicular mediators of glutamate release; (2) reducing the transporters responsible for glutamate clearance; (3) increasing the postsynaptic responsiveness of the N-methyl-D-aspartate subunit GluN1; and (4) causing hyperresponsive spines as evidenced by increased activation of the postsynaptic cdc42-Pak pathway.

These findings indicate that exposure to cocaine during adolescence sensitizes mPFC glutamatergic synapses to stress. It is suggested that changes in glutamatergic signaling may contribute to the increased sensitivity to stress observed in cocaine users. Moreover, glutamatergic processes may play an important role in stress-induced reinstatement of cocaine seeking.

Keywords: Adolescence, cocaine, glia, glutamate, prefrontal cortex, stress.

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INTRODUCTION

Drug addiction is a chronic relapsing disorder hypothesized to be produced by drug-induced neuroplasticity that renders individuals vulnerable to craving-inducing stimuli. Besides alterations in central dopamine homeostasis (Berridge & Robinson 1998), addiction liability has also been associated to changes in prefrontal-accumbens glutamate transmission (Kalivas, Volkow & Seamans 2005; Gipson, Kupchik & Kalivas 2013). Several studies

have investigated the role of glutamate in the nucleus accumbens (Huang *et al.* 2011; Xie *et al.* 2012; Purgianto *et al.* 2013) while we know less about cocaine-induced glutamate plasticity in the medial prefrontal cortex (mPFC), although the mPFC has been described as a key site for compulsive drug-seeking (Everitt & Robbins 2005). In fact, long-term exposure to cocaine leads to contrasting results with respect to glutamate receptor modulation (Hemby, Horman & Tang 2005; Freeman *et al.* 2008; Kasanetz *et al.* 2013) whereas withdrawal

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from cocaine leads to robust changes in the redistribution of these cortical receptors (Ghasemzadeh, Mueller & Vasudevan 2009; Ben-Shahar *et al.* 2013).

Among the factors that cause relapse to drug-seeking, stress plays an important role. In fact, cocaine users are extremely sensitive to stressful events (Sinha *et al.* 2003; Fox *et al.* 2008). Studies in animals have shown that acute stress can induce relapse to drug seeking following chronic exposure to cocaine (Erb, Shaham & Stewart 1996; Ahmed & Koob 2005), but the contribution of glutamate to stress-induced cocaine reinstatement is still elusive. In the current study, we incorporated a paradigm of acute swim stress to investigate, for the first time in detail, the functional responsiveness of the mPFC glutamate system in animals with a prior history of cocaine. We have used adolescent rats because these animals are more vulnerable to cocaine when compared with adult rats (Wong *et al.* 2013).

Glutamate is the major excitatory neurotransmitter in the brain. It is stored within intracellular secretory vesicles via the action of vesicular glutamate transporters (vGLUTs) that do not only serve storage functions, but are also involved in the regulation of glutamate release (Bellocchio *et al.* 2000). Once released, glutamate activates postsynaptic ionotropic and metabotropic receptors before removal from the extracellular space into glial cells through the action of excitatory amino acid transporters (EAAT1 and EAAT2; Bridges *et al.* 2012). Once inside the glial cells, glutamine synthetase (GS) converts glutamate into glutamine, which is then released by glial cells and taken up by glutamate neurons (Broer & Brookes 2001) thereby replenishing the presynaptic vesicular stores of glutamate. In addition, glutamate homeostasis is modulated also by an antiporter that exchanges extracellular cystine for intracellular glutamate and is also responsible of the extra-synaptic release of glutamate (Bridges *et al.* 2012).

We show that swim stress rapidly dysregulates the expression of the components of the glutamatergic synapse in the mPFC of cocaine-withdrawn adolescent rats mentioned earlier. The observed cocaine-induced sensitization of the glutamatergic synapse to stress may contribute to the increased sensitivity to stress observed in cocaine users as well as to stress-induced reinstatement of cocaine seeking.

MATERIAL AND METHODS

Experimental procedures

The adolescent rats used in this study were generated by mating Sprague Dawley rats weighting 250 g (Charles River, Calco, Italy) and housed under standard conditions of temperature and humidity under artificial light (from

7:00 AM to 7:00 PM). A maximum of two male siblings was taken from each litter in order to reduce 'litter effects' (Chapman & Stern 1978). Male rats were treated subcutaneously with cocaine (20 mg/kg/day; MacFarlan-Smith, Edinburgh, UK) or saline from postnatal day 28 (PND 28) to PND 42, a period that roughly approximates adolescence in humans (Collins & Izenwasser 2004).

Following the end of this treatment, animals were left undisturbed in their home cages until PND 45 when they were randomly divided into four experimental groups. Group 1 was formed by rats exposed to saline from PND 28 to 42 and sacrificed on PND 45 without any further manipulation; group 2 was formed by rats exposed to saline from PND 28 to 42 and sacrificed on PND 45 following acute stress; group 3 was formed by rats exposed to cocaine from PND 28 to 42 and sacrificed on PND 45 without any further manipulation; group 4 was formed by rats exposed to cocaine from PND 28 to 42 and sacrificed on PND 45 following acute stress. A schematic picture is shown in Fig. 1a. Swim stress was chosen since we have previously shown that it rapidly modulates the phosphorylation of glutamate postsynaptic receptors in the brain of adult rats, a measure of activation of the glutamate synapse (Fumagalli *et al.* 2009b). Also, this stress allows us to have behavioral measures of depressive symptoms, which are known to occur during the initial phase of cocaine withdrawal in humans and animal models (Gawin 1991; Perrine *et al.* 2008). Rats were individually placed in glass cylinders containing water (24°C) at a depth of 30 cm. Depth of the water was adequate to prevent rats from touching the bottom of the cylinder with their tails. Water was replaced between each test and temperature was monitored constantly. Three independent investigators, blind to the experimental design, measured the time the animals were immobile during the 5 minutes test. Immobility was defined as lack of movement of three paws and only minimal movement of the fourth or lack of movement of all four paws.

Animals were sacrificed 15 minutes after the end of the swim stress. Following the sacrifice, the mPFC (defined as Cg1, Cg3 and IL subregions) corresponding to plates 5–9 of the atlas of Paxinos and Watson (Paxinos and Watson, 2005) has been immediately dissected from 2-mm thick slices, frozen on dry ice and stored at –80°C. Trunk blood from each rat was collected in heparinized tubes for quantification of corticosterone plasma levels. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., supplement 40, 18 Febbraio, 1992, Circolare no. 8, G.U., 14 Luglio, 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care & Use of Laboratory Animals, U.S. National Research Council, 1996).

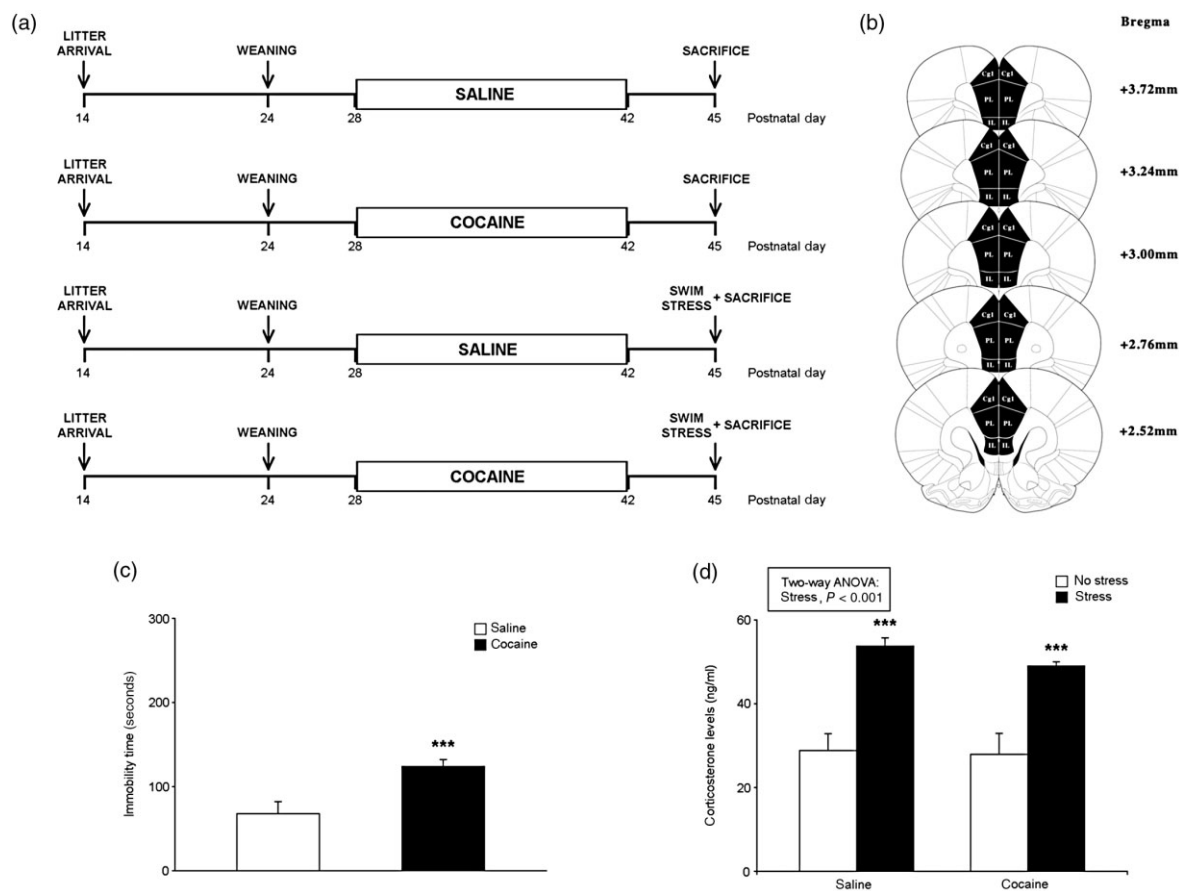


Figure 1 Schematic representation of the experimental procedure, specific coordinates of medial prefrontal cortex dissection and general analyses characterizing the stress experiment.

Panel (a) shows the experimental paradigm. Animals were treated with cocaine (20 mg/kg) or saline during early adolescence [postnatal day (PND) 28–PND 42]. After the end of the adolescent treatment, animals were left undisturbed in their home cages. On PND 45, half of the animals exposed to cocaine or to saline, were subjected to 5 minutes of swim stress and sacrificed 15 minutes after the end of this stressor. The other half of the animals was left in their home cages and sacrificed at the same time as their swim stress-exposed counterparts.

Panel (b) shows the dissection of the medial prefrontal cortex. This procedure was undertaken according to the coordinates indicated by the atlas of Paxinos and Watson, 2005 (please see the Material and Methods section). The medial prefrontal cortex was then stored at -80°C until processing.

Panel (c) shows the time (seconds) the animals spent immobile during the 5 minutes of swim stress. Measurements were taken by three independent investigators who were blind to the experimental design (** $P < 0.001$, unpaired Student's *t*-test).

Panel (d) shows the levels of circulating corticosterone (expressed in ng/ml). The main effects of analysis of variance appear at the top of the panel (** $P < 0.001$)

RNA preparation and gene expression analysis by quantitative real-time PCR

RNA measures were taken in the same animals as the protein measures. Total RNA was isolated by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Segrate, Milan, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis (Caffino, Racagni & Fumagalli 2011). Following total RNA extraction, the samples were processed for real-time reverse transcription polymerase chain reaction (real-time RT-PCR) to assess mRNA levels, as previously

described (Fumagalli *et al.* 2012). Briefly, an aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed by TaqMan qRT-PCR instrument (CFX384 real time system, Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Each experimental group was composed of at least six rats. Samples were run in 384 well formats in triplicate as multiplexed reactions. Data were analyzed with the comparative threshold cycle ($\Delta\Delta\text{Ct}$) method using *36B4*, β -actin and *18S* as reference genes (Barbon *et al.* 2010). The primer efficiencies were experimentally set up for each couple of primers.

Probes and primers were purchased from Eurofins MWG-Operon. Their sequences are shown below. Thermal cycling was initiated with an incubation at 50°C for 10 minutes (RNA retrotranscription) and then at 95°C for 5 minutes (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 seconds to enable the melting process and then for 30 seconds at 60°C for the annealing and extension reaction.

The following is the list of the primers and probe used in our experiments:

- vGLUT1: Forward primer 5'-ACTGCCTCACCTTGTC ATG-3', Reverse Primer 5'-GTAGCTTCCATCCCGAA ACC-3', Probe 5'-CTTTCGCACATTGGTCGTGGACAT T-3';
- vGAT: Forward primer 5'-ACGACAAACCCAAGAT CACG-3', Reverse Primer 5'-GTAGACCCAGCAGCAA CATG-3', Probe 5'-TTCCAGCCCGCTTCCCACG-3';
- GAD67: Forward primer 5'-ATACTTGGTGTGGCGT AGC-3', Reverse Primer 5'-AGGAAAGCAGTTCTT GGAG-3', Probe 5'-AAAACCTGGGCCTGAAGATCTGT GGT-3';
- EAAT1: Forward primer 5'-GGGTTTTTCATTGGAGGG TTG-3', Reverse Primer 5'-ACGGGAAGCACAAATC TGG-3', Probe 5'-TGATGGGCAGGGTGGCAGAA-3';
- EAAT2: Forward primer 5'-TTGCTGGCATTTCCAA GC-3', Reverse Primer 5'-TTAATGGTTGCTCCGACTG G-3', Probe 5'-CAAGCGTGTGACCAGATTCGTCT-3';
- X_c⁻: Forward primer 5'-TCTTCGATACAAACGCCAG-3', Reverse Primer 5'-CGAGTAAAGGGAGAGGACA AC-3', Probe 5'-CCATGAAGAGGCAGGTGAAGGAA AA-3';
- GS: Forward primer 5'-ATGGTCTGAGGTGCATTGAG-3', Reverse Primer 5'-TGATGTTGGAGGTTTCGTGG-3', Probe 5'-ATGTGGTACTGGTGCCTCTTGCTC-3';
- Cdc42: Forward primer 5'-AAGGCTGTCAAGTATGT GGAG-3', Reverse Primer 5'-GCTCTGGAGATGCGTTC ATAG-3', Probe 5'-CCTGCGGCTCTTCTTCGGTTCT-3';
- β-actin: Forward primer 5'-CACTTCTACAATGAG CTGCG-3', Reverse Primer 5'-CTGGATGGCTACGTA CATGG-3', Probe 5'-TCTGGGTCATCTTTTCACGGTT GGC-3';
- 18S: Forward primer 5'-GTAACCCGTTGAACCCCA TT-3', Reverse Primer 5'-CCATCCAATCGGTAGTAGC G-3', Probe 5'-TGCAATTAATCCCATGAACGAGG-3';
- 36B4: Forward primer 5'-TTCCCACTGGCTGAAAAG GT-3', Reverse Primer 5'-CGCAGCCGCAAATGC-3', Probe 5'-AAGGCCTTCTGGCCGATCCATC-3'.

Preparation of protein extracts and Western blot analyses

mPFC was homogenized in a glass-glass potter using a cold buffer containing 0.32 M sucrose, 1 mM

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) solution, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM phenylmethanesulfonyl fluoride, pH = 7.4, in presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. Crude synaptosomal fraction was prepared as previously described (Fumagalli *et al.* 2009a). The homogenized tissues were centrifuged at 1000 g for 10 minutes; the resulting supernatant was centrifuged at 9000 g for 15 minutes to obtain the pellet corresponding to the crude synaptosomal fraction, which was resuspended in a buffer containing 20 mM HEPES, 0.1 mM dithiothreitol, 0.1 mM EGTA, in presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. Total proteins have been measured in the crude synaptosomal fraction by the Bio-Rad Protein Assay (Bio-Rad Laboratories). Ten micrograms of proteins for each sample were run on an sodium dodecyl sulfate-10% polyacrylamide gel under reducing conditions and then electrophoretically transferred onto nitrocellulose membranes (GE Healthcare, Milan, Italy). Blots were blocked 1 hour at room temperature with 10% non-fat dry milk in tris buffered saline + 0.1% Tween-20 buffer, incubated with antibodies against the phosphorylated forms of the proteins and then stripped and reprobated with the antibodies against corresponding total proteins.

The conditions of the primary antibodies were the following: anti phospho-GluN1 (Ser896; 1:1000, Affinity Bioreagents, Golden, CO, USA), anti phospho-αCaMKII (T286; 1:2000, Thermo Scientific, Waltham, MA, USA), anti phospho-GluN2B (Ser1303; 1:1000, Upstate, Lake Placid, NY, USA), anti phospho-GluA1 (Ser831; 1:500, Affinity Bioreagents), anti phospho-GluA2 (Ser880; 1:1000, Abcam, Cambridge, UK), anti phospho-Pak1 (Thr423; 1:1000, Cell Signaling Technology Inc., Beverly, MA, USA), anti total GluN1 (1:1000, Zymed Laboratories, San Francisco, CA, USA), anti total αCaMKII (1:5000, Chemicon, Temecula, CA, USA), anti total GluN2B (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti total GluN2A (1:1000, Zymed Laboratories), anti total GluA1 (1:2000, Upstate), anti total GluA2 (1:2000, Alomone, Jerusalem, Israel), anti total Pak1 (1:1000, Cell Signaling), anti-Arc (activity-regulated cytoskeletal-associated protein; 1:500, BD Transduction Laboratories, San Jose, CA, USA) and anti β-actin (1:10 000, Sigma-Aldrich). Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories). The activation of the proteins investigated were expressed as a ratio between the phosphorylated and the respective total forms and analyzed using the Image Lab software from Bio-Rad Laboratories.

Analysis of plasma corticosterone levels

Plasma was separated by centrifugation (6500 g for 10 minutes) and corticosterone levels were determined by an enzyme-linked immunosorbent assay using a commercial kit, according to the manufacturers' instructions (IBL, Hamburg, Germany).

STATISTICAL ANALYSIS

Data were collected in individual animals (independent determinations) and are presented as means and standard errors. Changes produced by cocaine treatment and acute stress alone as well as by their combination were analyzed using a two-way analysis of variance (ANOVA), with adolescent cocaine treatment and acute stress as independent variables. When dictated by relevant interaction terms, single contrast post-hoc test (SCPHT) was used to characterize differences among individual groups of rats. However, when no interaction between cocaine treatment and stress was observed, only the main effects were reported. The immobility time, measured during the swim stress, was analyzed by an unpaired Student's *t* test. Statistical significance was assumed at $P < 0.05$.

RESULTS

Figure 1a illustrates the experimental paradigm that was designed to investigate whether the rapid stress-induced response of the glutamatergic synapse was influenced by a previous history of cocaine. mPFC was dissected as depicted in Fig. 1b. Figure 1c shows the behavioral response to the 5 minutes stress in both saline- and cocaine-treated rats. We measured the time that the animals were immobile during the 5 minutes of forced swimming, an index of pro-depressive symptoms that are known to be related, at least in part, to increased glutamate transmission (Sanacora, Treccani & Popoli 2012). Interestingly, cocaine-treated rats showed higher immobility when compared with saline-treated rats (+81%, $P < 0.01$, Student's *t*-test; Fig. 1c). We also measured the plasma levels of corticosterone and found a significant effect of stress ($F_{1,33} = 57.74$; $P = 0.22E-07$; two-way ANOVA), but no cocaine \times stress interaction ($F_{1,33} = 0.86$; $P = 0.358$; two-way ANOVA). Notably, cocaine alone did not alter the plasma levels of corticosterone (Fig. 1d).

We next measured the expression of several molecular determinants of the glutamate synapse in response to the combination of adolescent exposure to cocaine and acute stress. Given that the short interval between the end of stress and sacrifice (15 minutes) is likely to favor changes that occur rapidly, we focused on measuring gene expression and/or phosphorylation of the different glutamate

determinants, since these represent the initial responses, whereas changes in corresponding protein levels take much longer.

Figure 2 shows the contribution of cortical glial cells to the effects produced by the combination of cocaine and stress. Considering the glutamate transporters EAAT1 and EAAT2, their response to stress was influenced by prior cocaine history. Two-way ANOVA showed a cocaine \times stress interaction for EAAT1 ($F_{1,35} = 5.48$; $P = 0.026$) and EAAT2 ($F_{1,30} = 6.77$; $P = 0.015$; Fig. 2a & 2b). The single intergroup comparisons revealed that stress decreased the levels of these transporters in cocaine-treated animals (EAAT1: -14% versus cocaine-no stress, $F_{1,17} = 6.15$, $P = 0.038$; EAAT2: -23% versus cocaine-no stress, $F_{1,15} = 12.07$, $P = 0.0036$; SCPHT), but not in saline-treated rats (EAAT1: +4% versus saline-no stress, $F_{1,18} = 0.65$, $P = 0.854$; EAAT2: +2% versus saline-no stress, $F_{1,15} = 0.06$, $P = 1.632$; SCPHT; Fig. 2a & 2b). We also measured the expression of another transporter expressed by glial cells, such as the glucose transporter 1 (Glut-1). No changes in Glut-1 mRNA levels were found suggesting that the combination of cocaine and stress selectively targets glial glutamate transport (data not shown).

Besides glutamate transporters, extracellular glutamate levels may also be modulated via the regulation of the glial cystine/glutamate antiporter (system X_c^-) that exchanges extracellular cystine for intracellular glutamate (Bridges *et al.* 2012). Two-way ANOVA indicated cocaine \times stress interaction ($F_{1,33} = 6.78$; $P = 0.015$) for system X_c^- mRNA levels (Fig. 2c). The analysis of the single treatment effects revealed a significant reduction of system X_c^- in the mPFC of animals exposed to cocaine and stress (-19% versus cocaine-no stress, $F_{1,17} = 6.16$, $P = 0.038$; SCPHT) with no effects in saline-treated rats (+9% versus saline-no stress, $F_{1,16} = 1.42$, $P = 0.486$; SCPHT; Fig. 2c). To further characterize the contribution of glial cells, we analyzed the effect of the combination of cocaine and stress on the enzyme that converts glutamate into glutamine in the glia, i.e. GS. Two-way ANOVA indicated cocaine \times stress interaction ($F_{1,33} = 5.71$; $P = 0.024$) for GS mRNA levels (Fig. 2d). We subdivided the data for individual intergroup comparisons and found that stress evoked a significant elevation of GS mRNA levels only in rats that had received cocaine during adolescence (+26% versus cocaine-no stress, $F_{1,16} = 16.61$, $P = 0.0006$; SCPHT) with no effects in saline-treated rats (+7% versus saline-no stress; $F_{1,17} = 0.62$, $P = 0.878$; SCPHT; Fig. 2d).

Next we investigated the presynaptic terminal (Fig. 3). Two way ANOVA indicated a cocaine \times stress interaction ($F_{1,30} = 27.78$; $P = 0.000016$) for vGLUT1 mRNA levels (Fig. 3a). Thus, we subdivided the data for individual intergroup comparisons. Stress evoked a marked

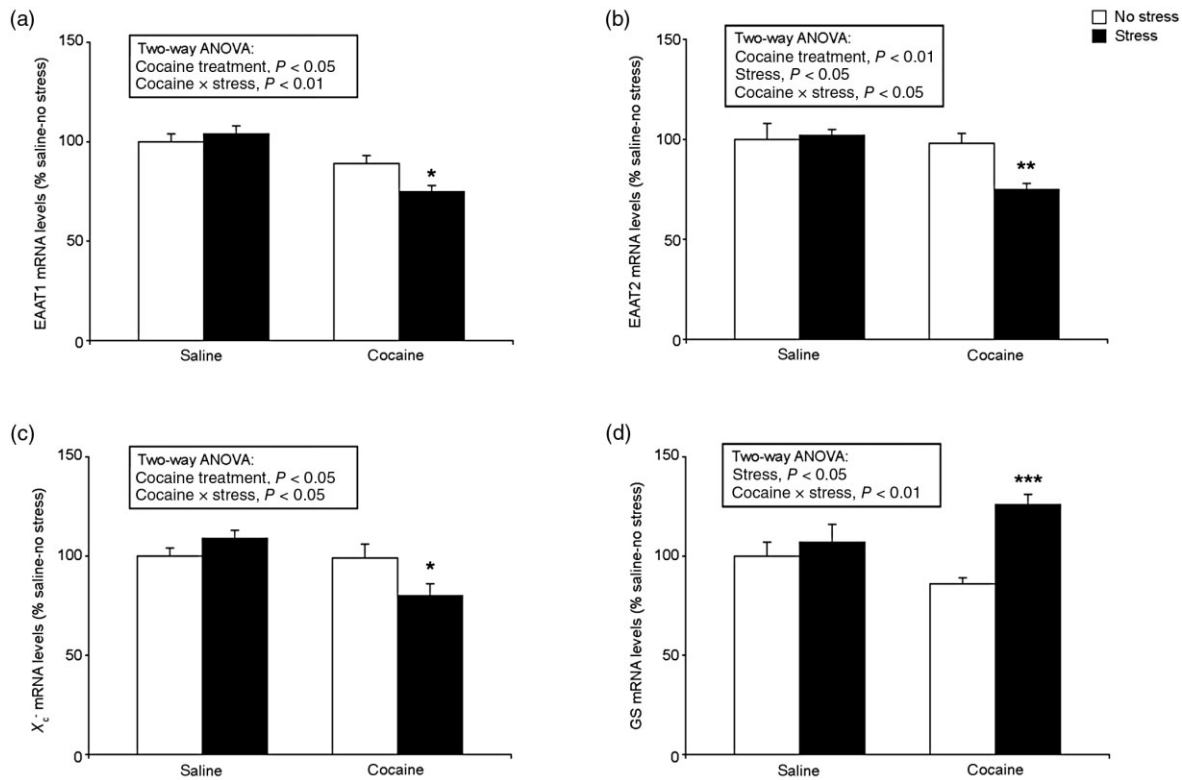


Figure 2 Effect of repeated cocaine treatment during adolescence [postnatal day (PND) 28 to PND 42] on glial determinants of glutamate neurotransmission and on the subsequent response to acute stress assessed at PND 45 in the medial prefrontal cortex. Panel (a) and (b) show the glial glutamate transporter mRNA levels, EAAT1 and EAAT2; panel C shows the glial glutamate exchanger mRNA levels, X_c⁻, while panel D shows the glutamine synthetase mRNA levels, GS. Asterisks denote the significant effect of the acute stress in cocaine-treated rats versus cocaine-no stress (* $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$)

up-regulation of vGLUT1 mRNA levels only in rats that had received cocaine during adolescence (+106% versus cocaine-no stress, $F_{1,12} = 36.08$, $P = 0.000004$; SCPHT) with no effects in saline-treated rats (-14% versus saline-no stress, $F_{1,18} = 0.95$, $P = 0.676$; SCPHT; Fig. 3a). The analysis of the vesicular gamma-aminobutyric acid (GABA) transporter (vGAT) mRNA levels revealed only a main effect of cocaine treatment ($F_{1,33} = 22.07$; $P = 0.00006$; two-way ANOVA; Fig. 3b) with no significant interaction between cocaine and stress ($F_{1,33} = 0.125$; $P = 0.727$; two-way ANOVA; Fig. 3b). We next analyzed GAD67 mRNA levels, the GABA synthesizing enzyme isoform highly expressed in the central nervous system (Soghomonian & Martin 1998). Two-way ANOVA indicated a significant cocaine \times stress interaction ($F_{1,32} = 4.54$; $P = 0.041$). The analysis of the single treatment effects revealed a significant reduction of GAD67 in the mPFC of animals exposed to cocaine and stress (-28% versus cocaine-no stress, $F_{1,15} = 12.74$, $P = 0.003$; SCPHT) with no effects in saline-treated rats (-5% versus saline-no stress, $F_{1,17} = 0.472$, $P = 0.996$; SCPHT; Fig. 3c).

Next, we investigated the postsynaptic terminal and analyzed the activation of the obligatory N-methyl-D-

aspartate (NMDA) receptor subunit, GluN1, in the crude synaptosomal fraction, expressed as a ratio between the phosphorylated and the total levels of protein. As shown in Fig. 4a, two-way ANOVA indicated a significant cocaine \times stress interaction ($F_{1,22} = 5.46$; $P = 0.031$). Examining the individual treatment effects, we found that swim stress enhanced the phosphorylation levels of GluN1 in cocaine-treated rats (+66% versus cocaine-no stress; $F_{1,11} = 18.60$, $P = 0.0008$; SCPHT) with no effects in saline-treated rats (+15% versus saline-no stress, $F_{1,11} = 1.02$, $P = 0.652$; SCPHT; Fig. 4a). No changes in the total levels of NMDA GluN1 were found (data not shown). We also measured the phosphorylation and total levels of the other NMDA (GluN2A, GluN2B) as well as AMPA receptor subunits (GluA1 and GluA2) and found no significant effects in any of these receptors (Table 1), suggesting that the combination of cocaine and stress specifically activates the obligatory NMDA receptor subunit, GluN1.

Given that the acute activation of the NMDA receptor may lead to long-term structural and functional changes in spine plasticity through the modulation of CaMKII-Cdc42-Pak1 transduction pathway (Murakoshi, Wang & Yasuda 2011), we also investigated these crucial effectors of such action. As shown in Fig. 4b, two-way ANOVA

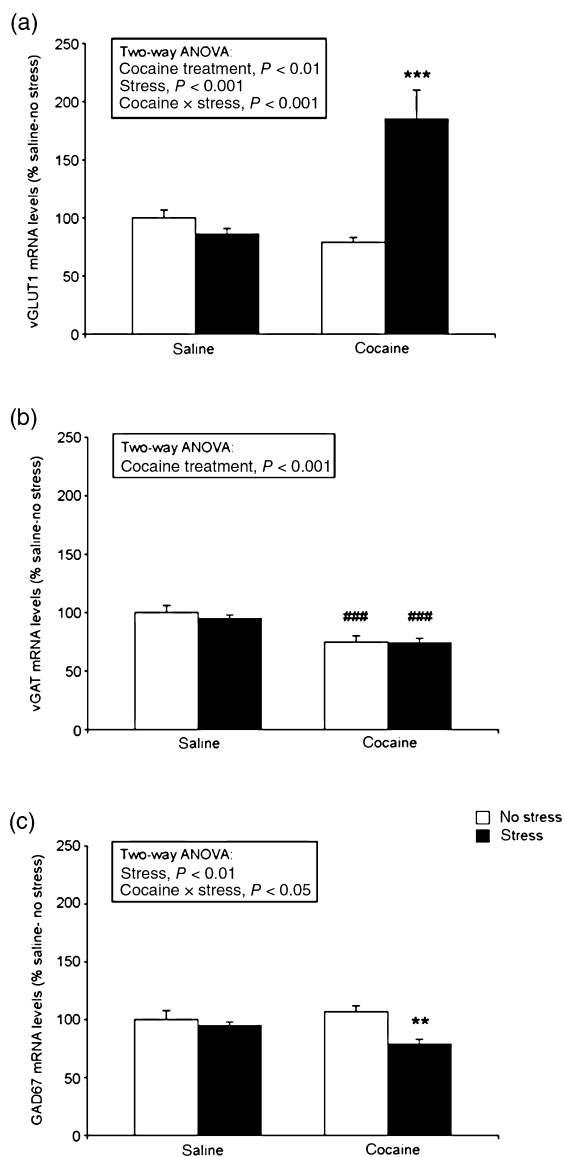


Figure 3 Effect of repeated cocaine treatment during adolescence (PND28 to PND 42) on presynaptic determinants of glutamate transmission.

Panel (a) shows the vesicular glutamate transporter (vGLUT1) mRNA levels; panel (b) shows the vesicular gamma-aminobutyric acid transporter (vGAT) mRNA levels; panel (c) shows glutamate decarboxylase 67 (GAD 67) mRNA levels. The main effects of analysis of variance appear at the top of the panel (### $P < 0.001$). Asterisks denote the significant effect of the acute stress in cocaine-treated rats versus cocaine-no stress (** $P < 0.01$; *** $P < 0.001$)

indicated a significant stress effect on the activation of α CaMKII ($F_{1,19} = 10.52$; $P = 0.005$). *Post hoc* testing indicated that acute stress enhanced the phosphorylation of α CaMKII in cocaine-withdrawn rats (+73% versus cocaine-no stress, $F_{1,10} = 6.38$, $P = 0.044$; SCPHT), but not in saline-treated rats (+40% versus saline-no stress, $F_{1,9} = 4.23$, $P = 0.112$; SCPHT; Fig. 4b).

Two-way ANOVA indicated a cocaine \times stress interaction ($F_{1,35} = 27.61$; $P = 0.00001$) for Cdc42 mRNA levels. Examining the individual treatment effects, we found that acute stress caused a significant elevation of Cdc42 mRNA levels in the mPFC of cocaine-treated rats (+43% versus cocaine-no stress, $F_{1,17} = 31.12$, $P = 0.000004$; SCPHT), but not in saline-treated rats (−14% versus cocaine-no stress, $F_{1,18} = 27.61$, $P = 0.164$; SCPHT; Fig. 4c). The analysis of the activation of Pak1 kinase (expressed as ratio between the phosphorylated and total levels of the protein) also revealed a significant interaction between cocaine and stress ($F_{1,20} = 5.44$; $P = 0.033$; two-way ANOVA) with stress increasing Pak1 phosphorylation levels in cocaine-treated rats (+49% versus cocaine-no stress, $F_{1,10} = 11.27$, $P = 0.008$; SCPHT), but not in saline-treated rats (+1% versus saline-no stress, $F_{1,10} = 0.003$, $P = 1.91$; SCPHT; Fig. 4d). No changes were observed when analyzing the total levels of Pak1 (data not shown).

It has been postulated that long-term cocaine administration produces a reduction in the baseline neuronal activity of the mPFC, but that exposure to a stimulus capable of generating drug-seeking (i.e. cocaine priming, cues or stress) would produce a hyperactive state (Jentsch & Taylor 1999; Goldstein & Volkow 2011). This may explain the higher responsivity to stress observed in the glutamate synapse of cocaine-treated rats. Accordingly, we decided to measure protein expression of the immediate early gene Arc, a well-established marker of neuronal activity. As shown in Fig. 5, two-way ANOVA indicated a significant cocaine \times stress interaction ($F_{1,30} = 12.54$; $P = 0.002$). Examining the individual treatment effects, repeated exposure to cocaine reduced Arc expression (−20% versus saline-no stress, $F_{1,14} = 5.90$, $P = 0.035$; SCPHT); interestingly, Arc expression was significantly increased only in the mPFC of cocaine-withdrawn rats exposed to stress (+39% versus cocaine-no stress, $F_{1,15} = 20.96$, $P = 0.0002$; SCPHT), but not in saline-treated rats (−4% versus saline-no stress, $F_{1,15} = 0.16$, $P = 0.692$; SCPHT; Fig. 5).

DISCUSSION

The present results show that adolescent cocaine exposure sensitizes glutamatergic responses of the mPFC to acute stress, presumably through cocaine-induced reduction of baseline mPFC neuronal activity that may generate a hyperactive state in response to a stimulus capable of generating drug seeking (i.e. acute stress; Jentsch & Taylor 1999; Goldstein & Volkow 2011). Our findings indicate that, in cocaine-withdrawn adolescent rats, the glutamate response to a brief stressor is dysregulated in the mPFC within a time frame of minutes. These findings may help to explain, at least in

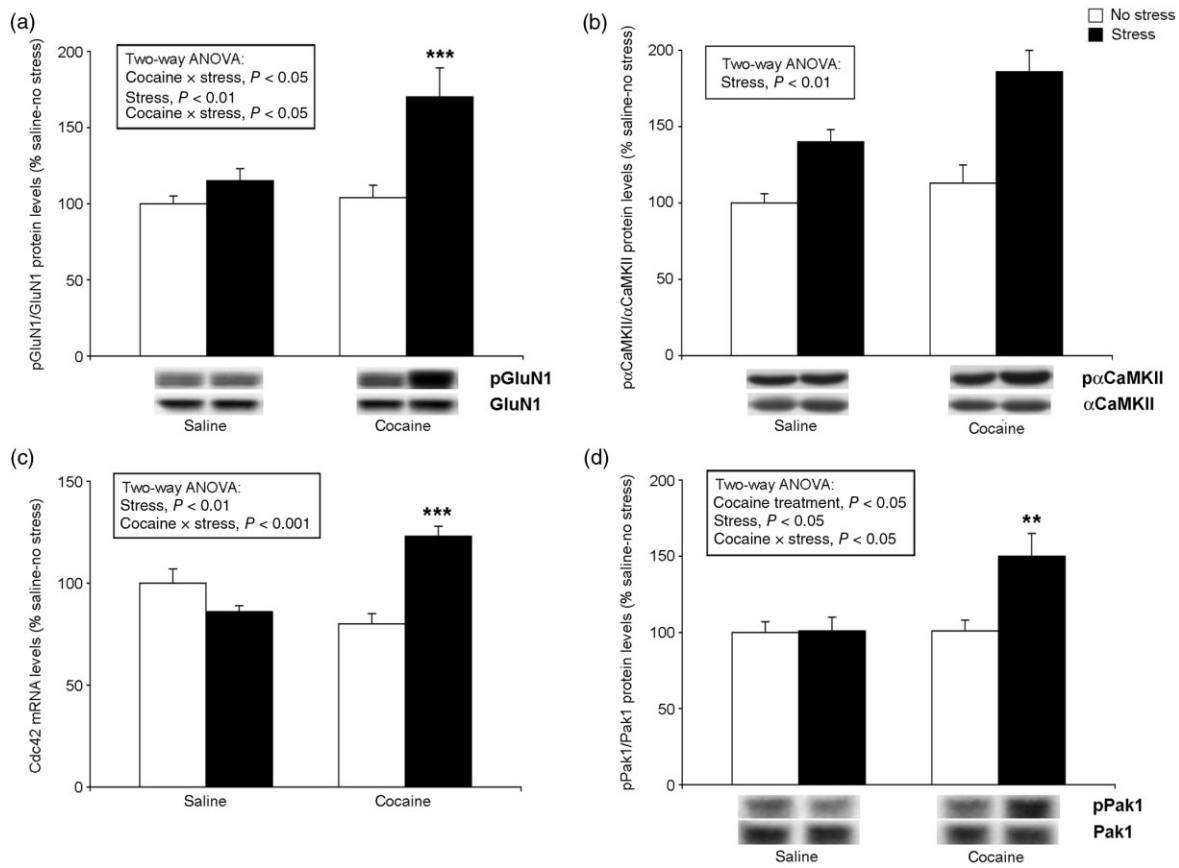


Figure 4 Effect of repeated cocaine treatment during adolescence (PND28 to PND 42) on postsynaptic determinants of glutamate transmission.

Panel (a) shows the activation of the NMDA GluN1 receptor subunit whereas panel (b) shows the activation of the α CaMKII, both expressed as the ratio between the phosphorylated and the total form of the protein; panel (c) shows Cdc42 mRNA levels whereas panel (d) shows the phosphorylation of Pak1, expressed as the ratio between the phosphorylated and the total form of the protein. Asterisks denote the significant effect of the acute stress in cocaine-treated rats versus cocaine-no stress (** $P < 0.01$; *** $P < 0.001$). Below the graphs in panel (a), (b) and (d) are shown representative bands of the respective proteins from Western blots

Table 1 Levels of glutamate NMDA receptor subunits GluN2A and GluN2B as well as of the AMPA receptor subunits GluA1 and GluA2 following the exposure to saline or cocaine during adolescence and subsequent exposure to stress in rat mPFC.

	Sal-sham	Sal-stress	Coc-sham	Coc-stress
GluN2A	100 ± 14	104 ± 10	110 ± 9	118 ± 9
pGluN2B/GluN2B	100 ± 10	95 ± 15	98 ± 12	112 ± 11
pGluA1/GluA1	100 ± 11	152 ± 13**	101 ± 9	140 ± 19**
pGluA2/GluA2	100 ± 4	100 ± 6	96 ± 4	107 ± 7

The results represent the mean ± standard error of measurement of at least six independent determinations for each experimental group (** $P < 0.004$).

part, the hypersensitivity to stress observed in cocaine users during early abstinence (Sinha *et al.* 2003; Fox *et al.* 2008).

Effects of acute stress on glutamate-related glial elements in the mPFC of cocaine-treated rats

Glutamate signaling requires a dynamic and coordinated interplay among neurons and glial cells (Bridges *et al.*

2012). We found that the glial glutamate transporters EAAT1 and EAAT2 mRNA levels are reduced following the combination of cocaine and stress. This suggests that the clearance of the extracellular glutamate from the synaptic space is impaired after stress in the mPFC of cocaine-treated rats, pointing to increased excitatory neurotransmission in these animals. Interestingly, exposure to the combination of cocaine and stress also reduced the mRNA levels of the glutamate antiporter

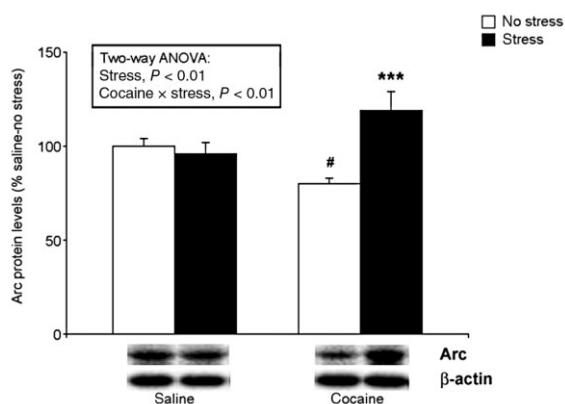


Figure 5 Effect of repeated cocaine treatment during adolescence (PND28 to PND 42) on Arc protein levels.

Dagger denotes the significant effect of repeated cocaine treatment during adolescence versus saline-no stress ($^{\#}P < 0.05$). Asterisks denote the significant effect of the acute stress in cocaine-treated rats versus cocaine-no stress ($***P < 0.001$). Below the graph are shown representative bands of Arc protein from Western blots

system X_c^- . Because this antiporter modulates the efflux of glutamate at extra-synaptic sites, which stimulates presynaptic mGluR 2/3 receptors to inhibit glutamate release (Baker *et al.* 2002), reduced expression of this antiporter by the combination of cocaine and stress may potentiate synaptic excitatory neurotransmission. The reduction of EAAT1 and EAAT2 mRNA levels may also be seen as an adaptive response to maintain physiological levels of glutamate in the extra-synaptic space to avoid excessive clearance.

We also observed a significant elevation of GS, the enzyme that converts glutamate into glutamine, in glial cells. Because glutamine is then released by glial cells and taken up by glutamate neurons (Broer & Brookes 2001), increased expression of GS by the combination of cocaine and stress may lead to higher availability of glutamate inside the presynaptic glutamate neurons. Our data suggest that glial cells strongly contribute to the observed dysregulation of glutamate homeostasis in animals exposed to the combination of cocaine and stress through at least two different mechanisms, i.e. by reducing glutamate reuptake and by increasing glutamate availability at the presynaptic level.

Effects of acute stress on pre- and postsynaptic elements of the glutamatergic synapse in the mPFC of cocaine-treated rats

Interestingly, vGLUT1 mRNA levels were markedly increased in the mPFC of only rats exposed to the combination of cocaine and stress. Based on previously published evidence showing that vGLUT1 expression directly regulates glutamate release and the efficacy of glutamate neurotransmission (Wojcik *et al.* 2004; Wilson *et al.*

2005), our data suggest that more glutamate might be available for activity-dependent glutamate release, presumably as a consequence of the increased glutamine synthesis in glial cells mentioned earlier.

We then shifted our attention to the GABA network, as it has recently been shown that non-contingent cocaine exposure during adolescence disrupts GABA functions in the mPFC (Cass *et al.* 2013). In contrast to the mRNA levels of vGLUT1, the mRNA levels of vGAT, the protein responsible of GABA storage and release, were reduced in the mPFC of animals exposed to the combination of cocaine and stress. Because GABA is an inhibitory neurotransmitter, a reduction of vGAT may further contribute to the potentiation of the excitatory neurotransmission in cocaine-withdrawn rats exposed to stress. This is further strengthened by the evidence that the mRNA levels of GAD 67, the enzyme responsible of converting glutamate into GABA, is reduced in the mPFC of rats exposed to the combination of cocaine and stress suggesting a reduced conversion of glutamate into GABA and, therefore, an accumulation of the excitatory neurotransmitter.

The analysis of postsynaptic glutamatergic elements also revealed changes indicative of hyperresponsive glutamatergic synapse. In fact, we found a selective activation of the obligatory subunit GluN1 in the mPFC of rats exposed to the combination of cocaine and stress. Such activation of the NMDA receptor may increase calcium influx that, in turn, activates downstream kinases. To this end, we measured the phosphorylation of α CaMKII, a sensor of intracellular calcium levels (Coultrap & Bayer 2012) and found it increased in the mPFC of cocaine-withdrawn rats. It is known that the activation of the NMDA receptor leads to changes in spine structural plasticity, an effect that may contribute to reinstatement of cocaine seeking (Toda *et al.* 2006), via the activation of the CaMKII-Cdc42-Pak signal transduction pathway (Murakoshi *et al.* 2011). Accordingly we measured both Cdc42 and Pak and found them enhanced by stress only in the mPFC of cocaine-treated rats. These data show increased responsiveness of cortical spines to stress only in animals with a previous history of cocaine exposure.

A potential limitation of our results may derive from the nature of cocaine exposure employed in the present study (i.e. non contingent) which is known to be more stressful relative to self-administration (Mantsch & Goeders 2000); however, no differences in corticosterone levels were observed in the plasma of cocaine-treated rats when compared with saline-treated animals suggesting that the modality of cocaine treatment can not be considered a potential confound under our experimental conditions. Also, one may argue that we primarily rely on mRNA/phosphorylation measures to infer changes in

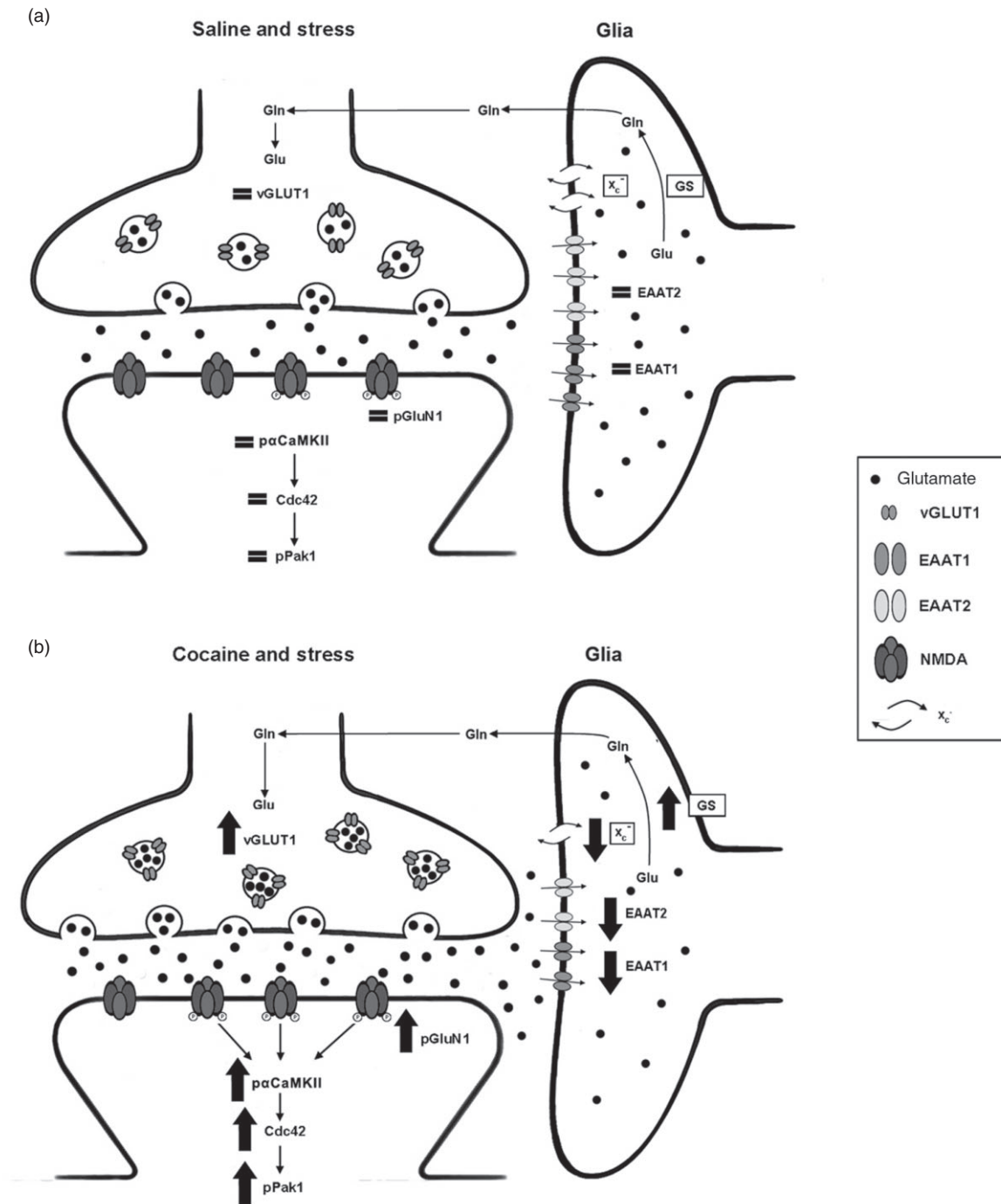


Figure 6 Exposure to repeated cocaine administration during adolescence alters the response of the cortical glutamate synapse to an acute stressor (panel b); comparison with rats exposed to saline and stress (panel a).

The homeostasis of the glutamate synapse is altered in the mPFC of rats exposed to the combination of cocaine and stress. In the cocaine + stress group, glial function is altered as evidenced by reduced expression of the main glial glutamate transporters EAAT1 and EAAT2 that leads to diminished clearance of extracellular glutamate. Changes in glial homeostasis are further revealed by a reduced expression of the cystine/glutamate antiporter, system X_c^- , which modulates non-vesicular glutamate release. Further, increased expression of GS leads to higher availability of glutamine that is released by astrocytes and taken up by presynaptic neurons. This may contribute to the increased expression of vGLUT1 implying that more glutamate is available for activity-dependent glutamate release. The potentiation of the excitatory transmission results in increased activation of the postsynaptic NMDA receptor subunit GluN1 and CaMKII and increased responsiveness of cortical spines as measured by increased Cdc42 expression and Pak1 phosphorylation. α CaMKII = α Ca²⁺/calmodulin-dependent protein kinase; Cdc42, cell division cycle 42, GTP binding protein; EAAT1, excitatory amino acid transporter 1; EAAT2, excitatory amino acid transporter 2; Gln, glutamine; Glu, glutamate; GluN1, glutamate NMDA receptor subunit 1; GS, glutamine synthetase; Pak1, p21-Activated Kinase 1; vGLUT1, vesicular glutamate transporter 1; X_c^- , cystine/glutamate antiporter

function. However, the evaluation of the rapid coping to the acute stress needs to rely on measuring changes in gene expression and/or phosphorylation as these represent the initial responses, whereas changes in corresponding protein levels take much longer.

CONCLUSIONS

Exposure to acute stress in adolescent cocaine-treated rats leads to widespread changes in both glial and synaptic regulators of glutamate neurotransmission leading to reduced glutamate clearance, increased packaging of glutamate in the presynaptic vesicular stores, increased conversion of glutamate to glutamine, all of which would be combined with data consistent with increased activation of NMDA receptors (Fig. 6). These results suggest that hyperreactive glutamatergic synapses in the mPFC may contribute to the hypersensitivity to stress observed in abstinent cocaine users (Sinha *et al.* 2003; Fox *et al.* 2008).

Furthermore, these hypersensitive glutamatergic synapses in the mPFC may contribute to the negative emotional state and stress-induced reinstatement generally observed in animal models of cocaine abuse (Baker *et al.* 2003; McFarland, Lapish & Kalivas 2003; Koob 2008). Notably, when compared with saline-treated rats exposed to swim stress, cocaine-treated rats exposed to swim stress showed increased immobility, a measure of depressive-like behavior. The evidence that, in cocaine-treated rats, acute stress recapitulates a depressive-like behavior that is usually only seen after chronic stress (Willner 1997; Kompagne *et al.* 2008) suggests that withdrawal from our cocaine paradigm may have led to a latent negative emotional state (Koob & Le Moal 1997), which is precipitated by an acute stressor. Interestingly, most of the markers that were altered following the combination of cocaine and stress are, indeed, changed in animal models of depression (Banar *et al.* 2010; Eriksson *et al.* 2012). The changes in glutamate homeostasis herein observed may therefore contribute, at least in part, to the pro-depressive symptoms occurring during the initial phase of cocaine withdrawal (Gawin 1991; Perrine *et al.* 2008).

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Disclosure/Conflict of Interest

The authors report no biomedical financial interests or potential conflicts of interest.

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