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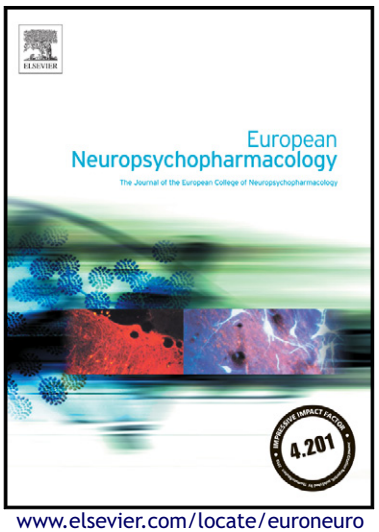

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Short-term withdrawal from developmental exposure to cocaine activates the glucocorticoid receptor and alters spine dynamics

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Abstract

Although glucocorticoid receptors (GRs) contribute to the action of cocaine, their role following developmental exposure to the psychostimulant is still unknown. To address this issue, we exposed adolescent male rats to cocaine (20mg/kg/day) from post-natal day (PND) 28 to PND 42 and sacrificed them at PND 45 or 90. We studied the medial prefrontal cortex (mPFC), a brain region that is still developing during adolescence. In PND 45 rats we found enhanced GR transcription and translation as well as increased trafficking toward the nucleus of the receptor, with no alteration in plasma corticosterone levels. We also showed reduced expression of the GR co-chaperone FKBP51, that normally keeps the receptor in the cytoplasm, and increased expression of Src1, which cooperates in the activation of GR transcriptional activity, revealing that short withdrawal alter the finely tuned mechanisms regulating GR action. Since activation of GRs regulate dendritic spine morphology, we next investigated spine dynamics in cocaine-withdrawn rats. We found that PSD95, cofilin and F-actin, molecules regulating spine actin network, are reduced in the mPFC of PND 45 rats suggesting reduced spine density, confirmed by confocal imaging. Further, formation of filopodia, i.e. the inactive spines, is enhanced suggesting the formation of non-functional spines. Of note, no changes were found in molecules related to GR machinery or spine dynamics following long-term abstinence, i.e. in adult rats (PND 90). These findings demonstrate that short withdrawal

promotes plastic changes in the developing brain via the dysregulation of the GR system and alterations in the spine network.

Keywords

Glucocorticoid receptor, dendritic spine morphology, cocaine, prefrontal cortex, adolescence

1. Introduction

Several studies have established a critical role for glucocorticoids in cocaine-induced responses. In fact, corticosterone facilitates the acquisition of cocaine self-administration in rats (Mantsch et al., 1998) and potentiates the reinstatement of cocaine-seeking (Graf et al., 2013). Further, evidence exists showing that alterations of the glucocorticoid receptor (GR) expression affect the action of cocaine; in fact the constitutive knockout of the glucocorticoid receptor (GR) prevents, while its forebrain overexpression facilitates, cocaine-induced behavioral sensitization and attenuates its reinforcing effects (Deroche-Gamonet et al., 2003; Wei et al., 2004). Moreover, mifepristone, a GR antagonist, reduces the reinforcing properties of cocaine (Fiancette et al., 2010), confirming that the modulation of GRs influences the action of the psycostimulant. Taken together, these results suggest that cocaine may act not only by altering glucocorticoid release but also via changes in GR expression and/or activity.

Thus, based on the cited literature, it is possible to hypotesize an hyperactivation of GRs following exposure to cocaine. This would be interesting in view of the evidence that activation of GRs, as shown following treatment with corticosterone or GR agonists, causes dendritic atrophy and spine loss in the rat medial prefrontal cortex (mPFC) (Cerqueira et al., 2007; Wellman, 2001) while pretreatment with a GR antagonist prevented the cortical decrease in spine density caused by stress (Liu and Aghajanian, 2008) suggesting that hyperactivation of cortical GRs may affect spine dynamics.

Accordingly, we decided to investigate the effects of repeated cocaine exposure during adolescence on GR trafficking and spine dynamics in the mPFC, a brain region that is still developing during adolescence, a period of life characterized by high vulnerability to drugs of abuse (Chambers et al., 2003; Crews et al., 2007).

We thus exposed male adolescent rats to cocaine (20mg/kg/day) from postnatal day (PND) 28 to PND 42 and sacrificed them after 3 (PND 45) or 48 (PND 90) days of drug withdrawal in order to draw a dynamic picture of the effects produced by short- and long-term drug withdrawal following developmental exposure to the psychostimulant.

2. Experimental procedures

The adolescent rats used in this study were generated by mating Sprague Dawley rats weighting 250g (Charles River, Calco, Italy) and housed under standard conditions of temperature and humidity under artificial light (from 07:00 to 19:00 hours). A maximum of two male siblings was taken from each litter in order to reduce "litter effects" (Chapman and 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 and Izenwasser, 2004). **Injections were made into the home cage under the loose skin between the shoulder blades.**

Following the end of the treatment, one group of animals was sacrificed after three days of withdrawal (PND 45) and compared with PND 45 rats treated with saline whereas another group of animals was left undisturbed in their home cages until adulthood and sacrificed at PND 90 and compared with PND 90 rats exposed, during adolescence, to daily saline injections. 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 and Use of Laboratory Animals, National Academies Press, 8th Edition, 2011).

RNA Preparation and Real-Time Polymerase Chain Reaction

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 (Giannotti et al., 2014). 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 iScript™ one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 well formats in triplicate as multiplexed reactions. Data were analyzed with the comparative threshold cycle (ΔΔCt) method using 36B4 as reference gene (Fumagalli et al., 2012). 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 min (RNA retrotranscription) and then at 95°C for 5 min (Retrotranscriptase inactivation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reaction.

List of the primers and probes used in our experiments:

Nr3c1: Forward primer 5'-GAAAAGCCATCGTCAAAAGGG-3', Reverse Primer 5'-TGGAAGCAGTAGGTAAGGAGA-3', Probe 5'-AGCTTTGTCAGTTGGTAAAACCGTTGC-3';

Src1: Forward primer 5'-AAGTGATGACTCGTGGCACT-3', Reverse Primer 5'-TCCCATGATGAAAGGCTGCA-3', Probe 5'-AAAGCACAAGGATGGCAAGG-3';

CaD: Forward primer 5'-AGGAGGAGGCTGATCGAAAA-3', Reverse Primer 5'-TCTTCTGGCGTTTCTCAGCA-3', Probe 5'-AGAGAGGAGGAAGAGAAGAGGA-3';

36B4: Forward primer 5'-TTCCCACTGGCTGAAAAGGT-3', Reverse Primer 5'-CGCAGCCGCAAATGC-3', Probe 5'-AAGGCCTTCCTGGCCGATCCATC-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, 1mM Hepes solution, 0.1 mM EGTA, 0.1 mM PMSF, pH=7.4, in presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. Nuclear fraction, cytosolic fraction and crude synaptosomal fraction were prepared as previously described (Fumagalli et al., 2010). The homogenized tissues were centrifuged at 1000 g for 10 min. The resulting pellet P1, corresponding to the nuclear fraction, was resuspended in a buffer containing 20 mM Hepes, 0.1 mM DTT, 0.1 mM EGTA, with protease and phosphatase inhibitors. The supernatant (S1) was centrifuged at 9000 g for 15 min to obtain the pellet corresponding to the crude synaptosomal fraction, which was resuspended in a buffer containing 20 mM Hepes, 0.1 mM DTT, 0.1 mM EGTA, with protease and phosphatase inhibitors, and the resulting supernatant S2 corresponds to a clarified fraction of cytosolic proteins. Total proteins have been measured in the nuclear fraction, in the crude synaptosomal fraction and in the cytosolic fraction by the Bio-Rad Protein Assay, using bovine serum albumin as the calibration standard (Bio-Rad Laboratories, Segrate, Milan, Italy). pGR S232 and FKBP51 were measured in the cytosolic fraction, GR levels were evaluated in both nuclear and cytosolic fractions, whereas PSD95, F-

Actin, pCofilin S3 and Cofilin analyses were performed in the crude synaptosomal fraction. Ten µg of proteins for each sample were run on an SDS-10% polyacrylamide gel under reducing conditions and then electrophoretically transferred onto PVDF membranes (GE Healthcare, Milan, Italy) for pGR S232 and GR or onto nitrocellulose membranes (Bio-Rad Laboratories, Segrate, Milan, Italy) for FKBP51, PSD95, F-Actin, pCofilin S3 and Cofilin. Blots were blocked one hour at room temperature with 10% non-fat dry milk in TBS + 0,1% Tween-20 buffer, incubated with antibodies against the phosphorylated forms of the proteins and then stripped and reprobed with the antibodies against corresponding total proteins.

The conditions of the primary antibodies were the following: anti phosphoGR Ser232 (1:1000, Abcam, UK), anti GR (1:500, Thermo Scientific, USA), anti FKBP51 (1:2000, Abcam, UK), anti PSD95 (1:4000, Cell Signaling, USA), anti F-Actin (1:1000, Abcam, UK), anti phospho-Cofilin (Ser3) (1:1000, Cell Signaling, USA), anti Cofilin (1:2000, Cell Signaling, USA), and anti β-actin (1:10000, Sigma-Aldrich, Milan, Italy). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories) and analyzed using the Image Lab software from Bio-Rad.

Analysis of plasma corticosterone levels

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

Dendritic spine labeling and morphological classification

Neuronal labeling and morphological classification of dendritic spines in layer V of medial prefrontal cortex were carried out using a lipophilic membrane tracer as previously reported with

minor modifications (Malinverno et al., 2010). Three days after the end of the chronic cocaine treatment (PND45), rats (3 rats/group) were deeply anesthetized and perfused with 0.1 M phosphate buffer (PB) followed by 1.5% paraformaldehyde (PFA) in PB. Brains were removed from the skulls and were postfixed at 4°C in 4% PFA in PB for 40 min. 2-mm thick slices corresponding to plates 5-9 of the atlas of Paxinos and Watson and containing the mPFC have been dissected from the postfixed brains and stained with a lipophilic dye, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DilC18(3)) (Life Technologies). Brain sections were left overnight at room temperature in PB to allow the Dil to completely diffuse through labeled neurons. Sections were, then, postfixed in 4% PFA for 40 min at 4°C, washed three times in PB and 150-µm thick coronal slices were prepared using a vibratome. Slices were then mounted, covered in fluoromount (Sigma-Aldrich), and analyzed on a Zeiss LSM-510 laser confocal microscope with 63X objective. Individual dendrites were selected randomly and their spines were traced manually. The number of neurons used for quantification is at least 15 for each experimental group. Analysis of dendritic spine morphology was performed with ImageJ software; for each dendritic spine length, the head and neck width were measured, which was used to classify dendritic spines into three categories (thin, stubby and mushroom) (Harris et al., 1992). In particular, the length and the ratio between the width of head and the width of neck (Wh/Wn) were used as parameters for the classification as follows: protrusions having a length of more than 3 µm were considered as filopodia, the others as spines; spines with a Wh/Wn ratio bigger than 1.7 were considered mushrooms; spines with a Wh/Wn ratio smaller than 1.7 were divided in stubby, if shorter than 1 µm, and thin if longer than 1 µm (Gardoni et al., 2012). An operator who was 'blind' to the experimental conditions performed both image acquisition and quantification.

Statistical analysis

Data were collected in individual animals (independent determinations) and are presented as means and standard errors. The molecular changes produced by cocaine treatment was analyzed by an unpaired Student's *t* test. Statistical evaluation of all confocal experiments was performed by using an unpaired Student's *t* test. Statistical significance was assumed at $p<0.05$.

3. Results

GRs are members of the nuclear receptor superfamily of ligand-dependent transcription factors, which translocate into the nucleus to regulate the expression of downstream genes through direct binding to DNA response elements, i.e. GRE (de Kloet et al., 2005). Hence, glucocorticoids are powerful regulators of brain homeostasis and can produce structural and functional changes mediating emotion and stress response in selected brain regions (McKlveen et al., 2013). We first investigated the trascriptional levels of the gene encoding for GR, Nr3c1. We found an increase in Nr3c1 mRNA levels in the mPFC of cocaine-withdrawn rats at PND 45 (+25%, $p<0.05$, **Student's t-test**; Fig. 1a) whereas no changes were observed in the mineralcorticoid receptor (Nr3c2) mRNA levels (data not shown). We next examined the protein levels of GR in the cytoplasm and nucleus. We found that GR expression was increased in both cellular fractions (cytoplasm: +36%, $p<0.05$, **Student's t-test**; nucleus: +34%, $p<0.01$, **Student's t-test**) (Fig. 1b). The analysis of translocation of GR from the cytoplasm to the nucleus, an index of GR function, revealed an increased nucleus/cytosol ratio of GR protein (+17%, $p<0.05$, **Student's t-test**) in the mPFC of PND 45 cocaine-withdrawn rats.

It is known that the activity of nuclear GRs on gene expression is sustained by co-activators (Kurihara et al., 2002; Meijer et al., 2006). Accordingly, we next evaluated whether the increased translocation of the receptor is correlated to increased activity by analyzing the expression levels of the nuclear receptor coativator-1 (Src1). We found that, three days after the last adolescent cocaine

exposure, Src1 mRNA levels are increased (+30%, $p<0.05$; Fig. 1d, **Student's t-test**), suggesting that increased GR nuclear trafficking is coupled with an enhanced activity of the GR.

GR translocation to the nucleus also depends on a specific phosphorylation in its serine residue 232 (Galliher-Beckley et al., 2008; Wang et al., 2002) and on a physical interaction with a large chaperone protein complex consisting mainly of FK506 binding protein 51 (FKBP51) (Tatro et al., 2009). Interestingly, we found increased GR phosphorylation in Ser232 (Fig. 2a) and reduced FKBP51 expression (Fig. 2b) in the cortical cytosolic fraction of cocaine-withdrawn rats (+27% $p<0.05$ and -26%, $p<0.05$, respectively, **Student's t-test**).

In order to verify if the observed dysregulation of the GR system might be due to altered levels of circulating glucocorticoids, we measured corticosterone levels in the plasma of PND 45 cocaine-withdrawn rats. Three days after the end of the cocaine treatment plasma levels of corticosterone were not different from control animals (Fig. 3).

Recently, GR has been proposed as a mediator of the plastic changes in the architecture of different brain areas contributing to alter dendritic spine density and morphology as well as spine actin cytoskeleton (Jafari et al., 2012; Liston and Gan, 2011; Liu and Aghajanian, 2008). Moreover, GR is critical for cognitive and emotional processes (de Kloet et al., 1999; Gass et al., 2001; Holsboer, 2000; Liston et al., 2006; Lupien et al., 2007). Since drug abuse impacts on the brain by usurping the neuronal mechanisms that contribute to learning and memory increasing the risk of drug dependence (Gould, 2010; Hyman et al., 2006; O'Brien and Anthony, 2005), we hypothesized that the herein found cocaine-induced alterations of GR-dependent mechanisms might result in dendritic atrophy and spine loss, an effect that may contribute to drug-induced neuroadaptations set in motion by early cocaine withdrawal. We then investigated some crucial effectors of the signaling pathways that regulate spine actin network. Figure 4a shows the reduced expression of Caldesmon (CaD) (-21%, $p<0.05$, **Student's t-test**, Fig. 3a), an actin-linked regulatory protein that is negatively regulated when GR is activated (Tanokashira et al., 2012), presumably contributing to cocaine-induced abnormal spine development. Interestingly, as shown in figure 4b, we found

reduced levels of PSD95, a well-established marker of postsynaptic density, in the cortical crude synaptosomal fraction of PND 45 cocaine-withdrawn rats (-21%, $p<0.05$, **Student's t-test**). We also observed a reduced expression of the filamentous actin (F-Actin) (-41%, $p<0.01$, **Student's t-test**) (Fig. 4c), the major cytoskeletal element in dendritic spines, and, accordingly, reduced phosphorylation levels of cofilin in Serine 3 (-46%, $p<0.05$, **Student's t-test**) (Fig. 4d), an actin-depolimerizing factor linked to destabilization of actin polymers and spine loss in its dephosphorylated form (Gu et al., 2010).

Since these data point to an alteration in the regulatory steps leading to formation, stabilization and elimination of the dendritic spines, we analyzed cocaine-induced spine remodeling using a fluorescent dyolistic labeling tecnique. Accordingly, we observed a reduction in cortical dendritic spine density in the mPFC of cocaine-withdrawn rats (-0.91 spine/μm, $p<0.05$; Fig 5, **Student's t-test**) with no effects on their lenght and head size (data not shown). Further, morphological analyses using a highly validated classification method (see Materials and Methods section) and evaluating the shape of all protrusions (mushroom, thin, stubby and filopodia) were performed and revealed an increased formation of filopodia in cocaine-withdrawn rats (+4.76%, $p<0.05$, **Student's t-test**) (Fig. 5b), i.e the immature protrusions that may contribute to maladaptive learning with no significant changes in the percentage of mushroom-, thin- and stubby-shaped spines.

Last, we decided to analyze the mPFC of adult animals (PND 90) to investigate whether the herein shown changes in GR machinery persist until adulthood, i.e. after a long period of abstinence. No changes were found in Nr3c1 mRNA levels (-3%, $p>0.05$; Fig. 6, **Student's t-test**) and GR protein levels both in the cytosol (-8%, $p>0.05$, **Student's t-test**) and nucleus (-1%, $p>0.05$, **Student's t-test**) (Fig. 6b) as well as in the nucleus/cytosol ratio (-6%, $p>0.05$; Fig. 6c, **Student's t-test**) of cocaine-withdrawn rats at PND 90. Next, we measured also the expression and activation of the molecular determinants of spine dynamics in the mPFC of PND 90 rats. No changes were

observed in the expression of PSD95 (Fig. 6d) and F-actin (Fig. 6e) as well as in the expression and phosphorylation of cofilin (Fig. 6f) in the mPFC of adult rats.

4. Discussion

Our study indicates that short-term withdrawal from developmental exposure to cocaine dysregulates the glucocorticoid receptor (GR) system through a coordinated series of changes all converging into determining hyperactivation of the GR. Further, we found reduced density and altered morphology of spines suggesting impaired reorganization of the cortical network in the mPFC of cocaine-withdrawn rats.

Besides increased transcription and translation of GR, witnessed by increased mRNA and protein levels of the receptor itself, we found augmented trafficking of GR toward the nucleus in cocaine-withdrawn rats. This occurs not only via changes in GR protein expression in the different cell compartments (i.e. cytosol and nucleus) but also through the modulation of the molecules that contribute to the shuttling of the GR toward the nucleus. In fact, short-term withdrawal from developmental cocaine exposure reduced the expression of FK506 binding protein 51 (FKBP51), which normally retains the receptor in the cytoplasm. Concomitantly, short-term withdrawal activates the phosphorylation of the GR, which contributes to the translocation of the receptor to the nucleus. The activation of GR is further confirmed by the increased expression of Src1, which co-activates GRs after nuclear import to induce gene expression (Meijer et al., 2005). Taken together, these results are indicative of an overall dysregulation of the machinery governing GR trafficking set in motion by the short-term withdrawal from developmental cocaine exposure providing mechanistic insights for withdrawal-induced GR activation. The importance of the alteration of such mechanisms is reinforced by the evidence that all these alterations occurred without changes in the circulating levels of glucocorticoids, although we cannot exclude that the hormonal levels might have been altered at different time points. Of note, the effects produced by short withdrawal wane in animals abstinent for 48 days, indicating that they are peculiar of the short-term withdrawal, an

observation that might be of functional relevance given the altered response to stress observed in cocaine users during early abstinence (Fox et al., 2008; Sinha et al., 2003). Further, since we have previously shown that short-term withdrawal from developmental cocaine exposure causes an abnormal response to a stress challenge leading to pro-depressive symptoms (Caffino et al., 2015), we suggest that the herein shown dysregulation of the GR system may contribute to the negative emotional state observed in humans during early periods of abstinence (Gould, 2010; Koob, 2013). Although the non contingent exposure of cocaine herein employed might represent a limitation of this manuscript, we decided to focus on the pharmacologic properties of the psychostimulant administration and the short abstinence from it.

Interestingly, short-term withdrawal from adolescent cocaine exposure also altered the dynamics of dendritic spines. In fact, we found a reduction of F-actin stability through changes in the expression and activation of caldesmon and cofilin, an effect which results in reduced spine density, as shown by reduced levels of PSD95 and confirmed by confocal imaging. In addition, we found a higher number of immature protrusions (filopodia), which are unable to make functional contacts, thus indicating the formation of inactive spines in the mPFC of cocaine-withdrawn rats.

Our data are in contrast with previous works, showing increased cortical spine density after long-term withdrawal (Robinson et al., 2001; Robinson and Kolb, 1999): however, besides the different duration of abstinence, these data were obtained in adult animals whereas our findings are in line with a reduction in cortical spine density observed following cocaine treatment during adolescence (Gourley et al., 2012a).

Since drugs of abuse appear to engage the same pathways that allow normal learning and memory (Kelley, 2004; Robinson and Kolb, 2004), we speculate that cocaine-induced increase of GR functions, associated with altered structural remodeling, may compromise the physiological functioning of cortical synaptic networks. In fact, evidence exists that the activation of GRs results in cognitive impairments via alterations in dendritic spine morphology (Finsterwald and Alberini, 2014; Gourley et al., 2012b; Swanson et al., 2013). In this view, our data may contribute to explain

the loss of inhibitory control occuring during early withdrawal: in fact, the hyperactivation of the GR system and the reduced structural remodeling of the mPFC may contribute to confer greater vulnerability to the addictive properties of cocaine (Chambers et al., 2003). Future studies employing mutant mice for glucocorticoid receptors (Reichardt et al., 1998) may allow to investigate the mechanistic link between cocaine-induced changes in GR expression and spine density observed in mPFC.

In conclusion, our findings suggest that short-term withdrawal from developmental exposure to cocaine causes an overall dysregulation of the finely tuned mechanisms governing GR trafficking, reinforcing the possibility that GR is a potential target to reduce cocaine abuse (Deroche-Gamonet et al., 2003). Further, changes in GR activity may contribute to alterations in spine density and morphology, which participate to the overall synaptic dysregulation caused by the developmental exposure to cocaine.

Contributors

Lucia Caffino, Giorgio Racagni and Fabio Fumagalli designed the study and wrote the protocol.

Lucia Caffino performed western blot and dendritic spine analyses.

Giuseppe Giannotti and Chiara Malpighi performed real time PCR experiments.

Lucia Caffino, Giuseppe Giannotti and Chiara Malpighi managed the literature searches.

Lucia Caffino and Giuseppe Giannotti undertook the statistical analysis.

Lucia Caffino and Fabio Fumagalli wrote the first draft of the manuscript.

All authors contributed to and have approved the final manuscript.

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The Foundation Zardi-Gori had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the paper for publication.

Conflict of interest

The authors declare no conflict of interest in relation to the work herein described.

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Figure legends

Fig. 1 Glucocorticoid receptor mRNA and protein and co-activator Src1 mRNA levels are altered in the mPFC of cocaine-withdrawn PND 45 rats.

Panel a shows Nr3c1 mRNA levels in the mPFC of PND 45 rats. Panel b shows the GR protein levels in the cytosolic and nuclear fraction of the mPFC of PND 45 rats. Panel c shows the ratio between nuclear and cytosolic GR protein levels in the mPFC of PND 45 rats. Panel d shows Src1 mRNA levels in the mPFC of PND 45 rats. Representative Western blot bands of GR are shown below the graphs shown in panel b.

The results, expressed as % of saline-treated rats, represent the mean ± S.E.M. of at least 5 samples. * $p<0.05$, ** $p<0.01$ vs. saline-treated rats.

Fig. 2 Effects of short-term abstinence on pGR S232 and FKBP51 protein levels in the mPFC of cocaine-withdrawn PND 45 rats.

Panel a shows the GR phosphorylation levels in Ser232 measured in the cytosolic fraction of the mPFC of PND 45 rats. Panel b shows FKBP51 protein levels measured in the cytosolic fraction.

Representative Western blot bands of pGR S232 and FKBP51 are shown below the graph shown in panel a and b.

The results, expressed as % of saline-treated rats, represent the mean ± S.E.M. of at least 5 samples.

* $p<0.05$ vs. saline-treated rats.

Fig. 3 Effects of short-term withdrawal from developmental cocaine exposure on hypotalamus-pituitary-adrenal axis (HPA) activity.

The levels of plasma circulating glucocorticoids were expressed in nanogram per milliliter and represent the average of at least 7 animals for each experimental group.

Fig. 4 Effects of short-term abstinence on spine-related proteins in the mPFC of cocaine-withdrawn PND 45 rats.

Panel a shows Caldesmon mRNA levels in the mPFC of PND 45 rats. PSD95 protein levels (panel b), F-Actin protein levels (panel c) and pCofilin Ser3 and total Cofilin (panel d) were measured in the crude synaptosomal fraction.

The results, expressed as % of saline-treated rats, represent the mean ± S.E.M. of at least 6 samples.

* $p<0.05$ vs. saline-treated rats.

Fig. 5 Effects of short-term abstinence on dendritic spine morphology in the layer V of the mPFC of cocaine-withdrawn PND 45 rats.

Panel a shows representative images of dendrites segment from the mPFC of saline (top) and cocaine-treated animals (bottom). Panel b shows the total spine density in the mPFC. Panel c shows the percentage of total protrusions belonging to different categories depending on their morphology (mushroom, thin, stubby and filopodia).

n>700 spines from 15 different neurons for each group, 3 animals/group

* $p<0.05$ vs. saline-treated rats.

Fig. 6 Effects of long-term abstinence on GR mRNA and protein levels and on spine-related proteins in the mPFC of cocaine-withdrawn PND 90 rats.

Panel a shows Nr3c1 mRNA levels in the mPFC of PND 90 rats. Panel b shows the GR protein levels in the cytosolic and nuclear fraction of the mPFC of PND 90 rats. Panel c shows the ratio between nuclear and cytosolic GR protein levels in the mPFC of PND 90 rats. PSD95 protein levels (panel d), F-Actin protein levels (panel e) and pCofilin Ser3 and total Cofilin (panel f) were measured in the crude synaptosomal fraction.of the mPFC of PND 90 rats.

The results, expressed as % of saline-treated rats, represent the mean ± S.E.M. of at least 6 samples.

Fig. 1

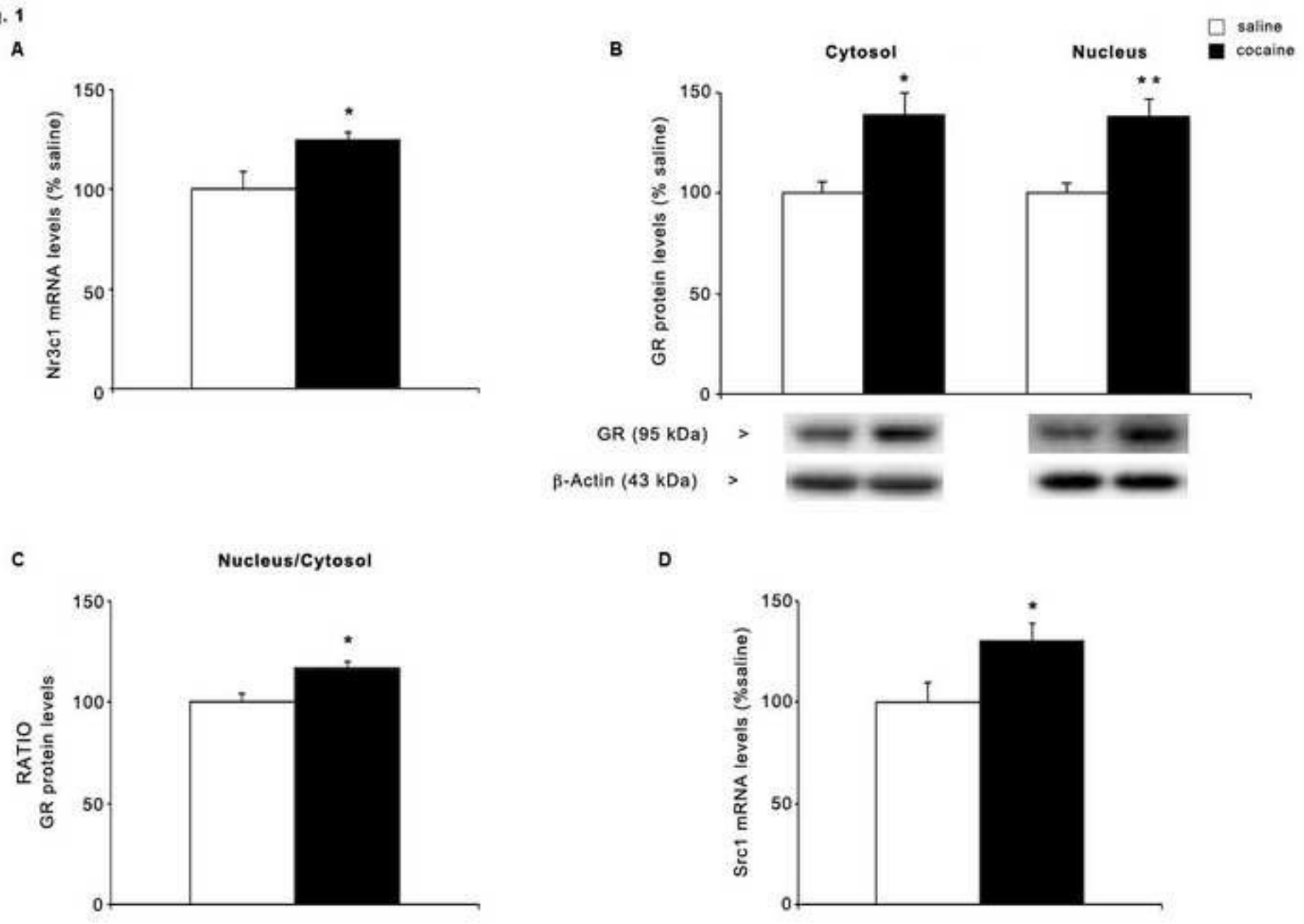
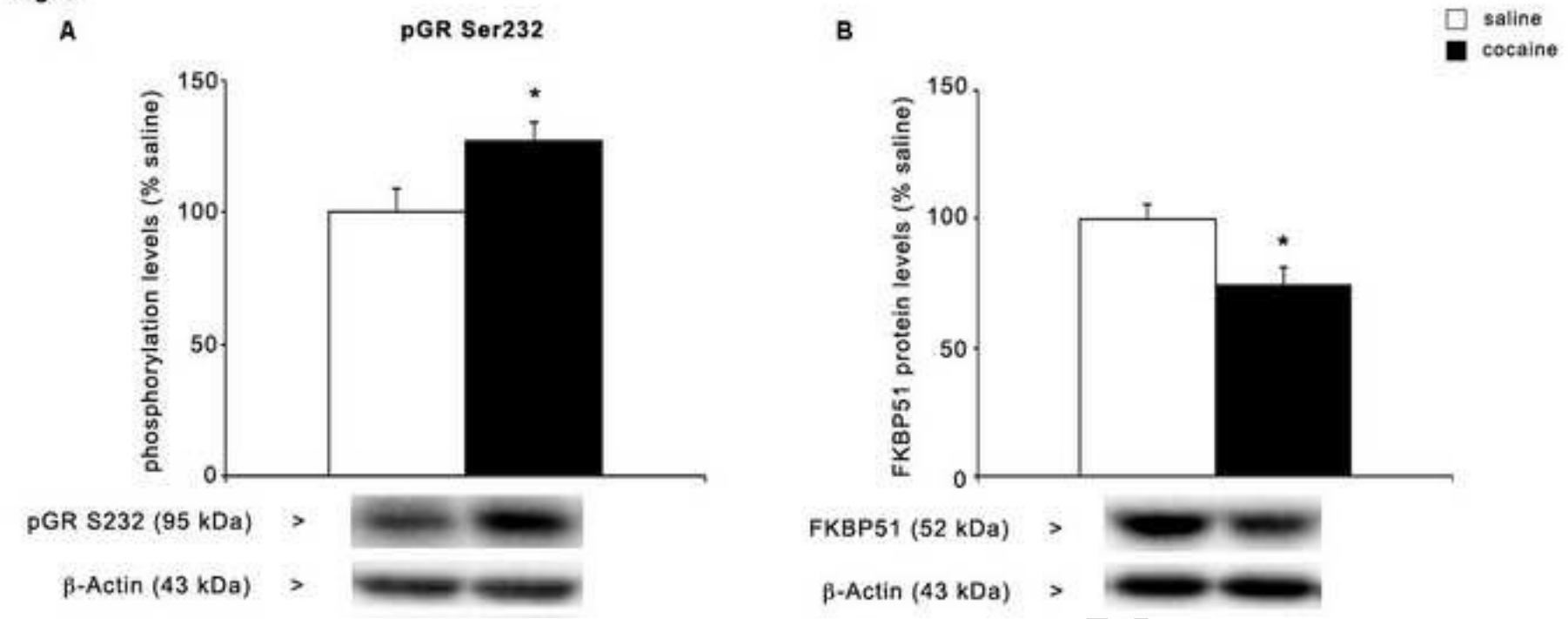


Fig. 2



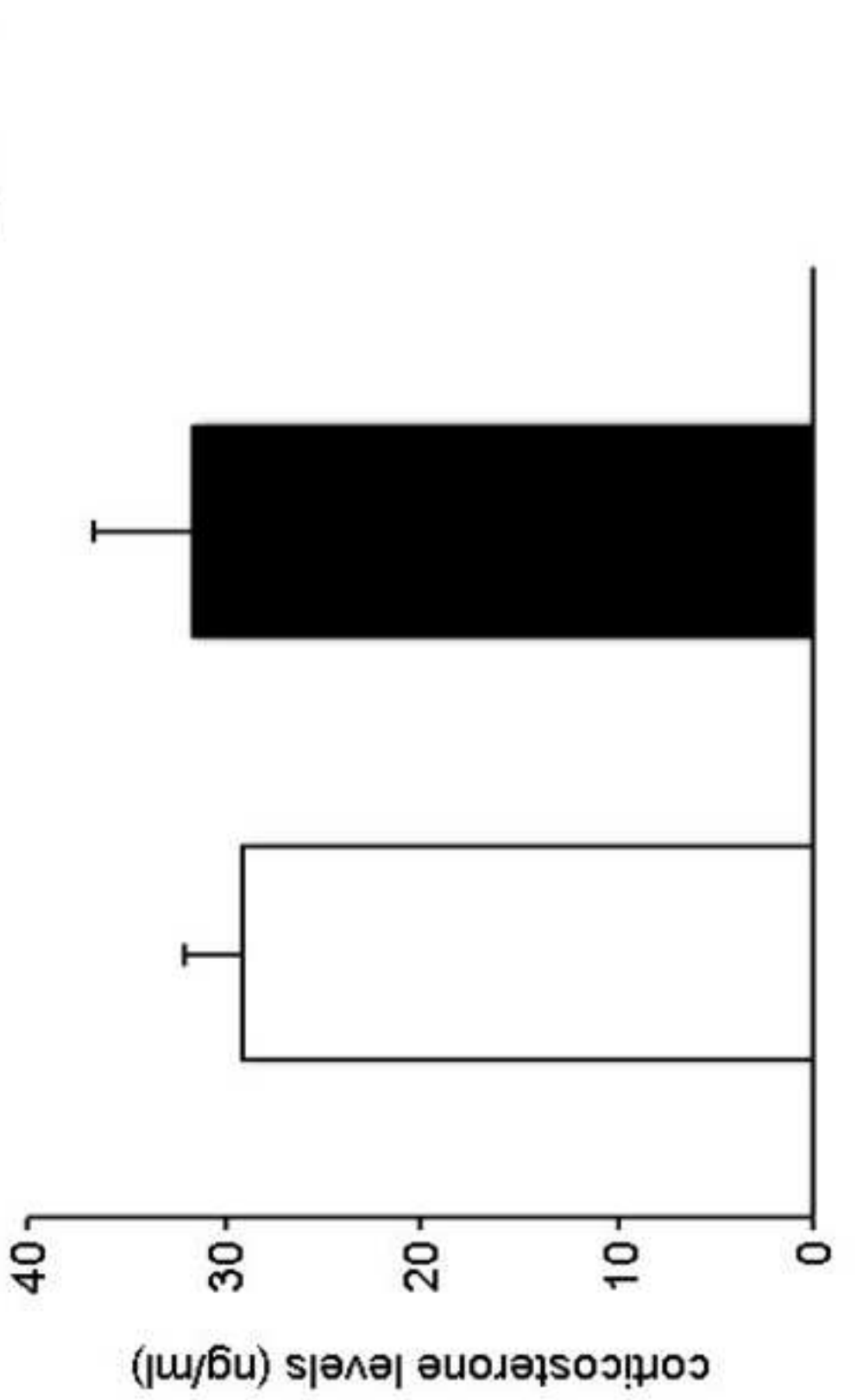


Fig. 3

Fig. 4

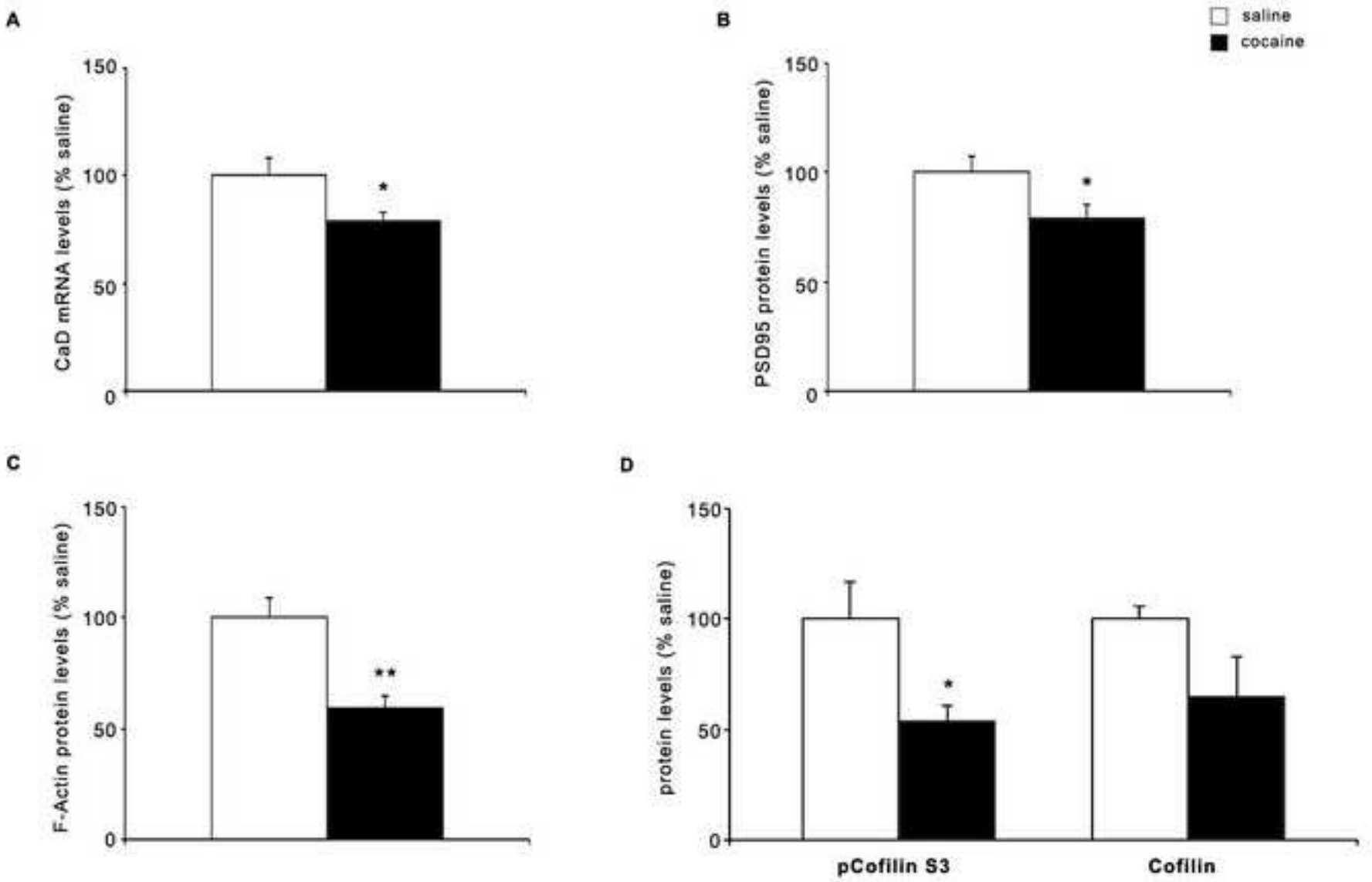


Fig. 5

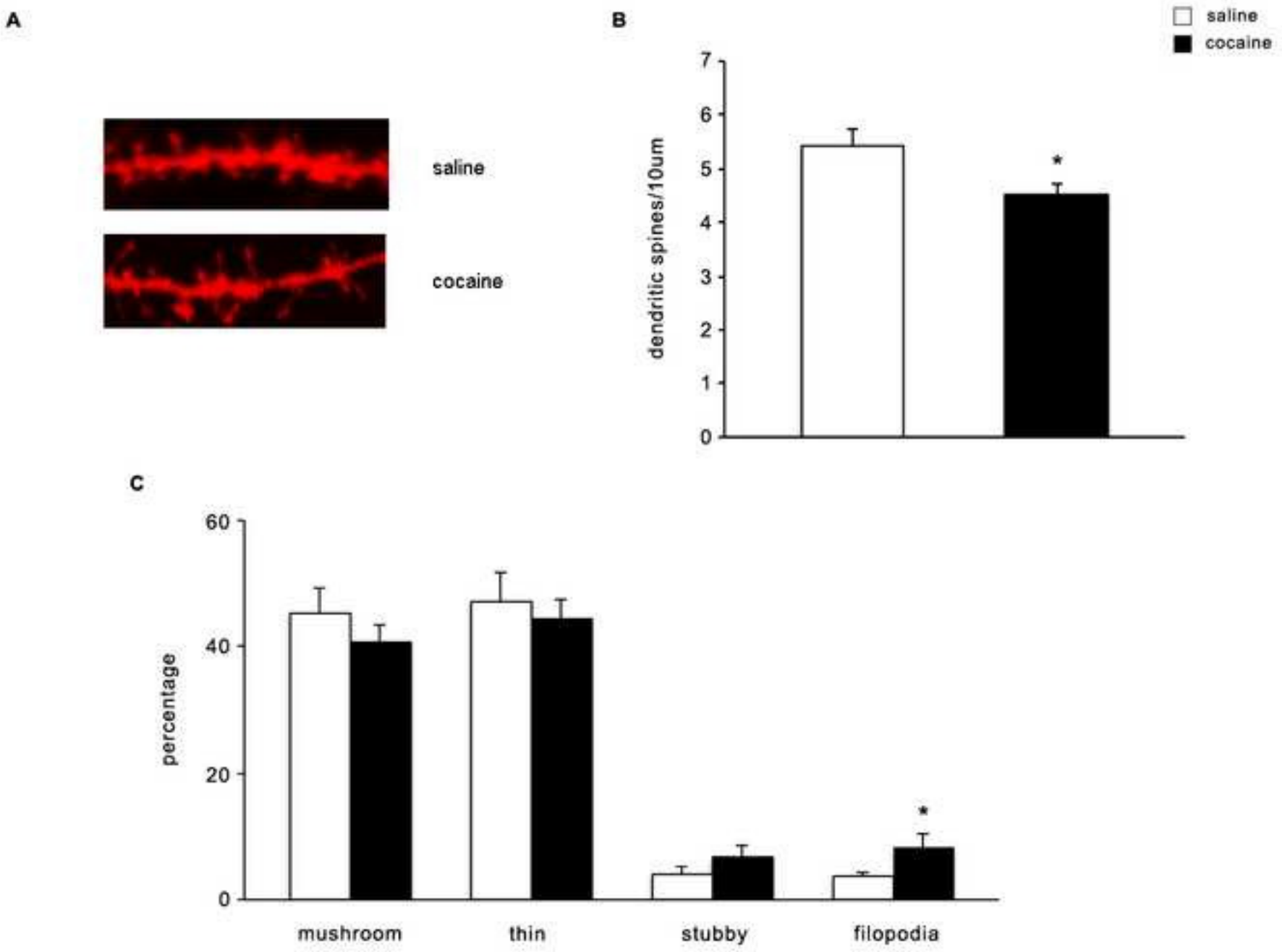


Fig. 6