A single cocaine administration alters dendritic spine morphology and impairs glutamate receptor synaptic retention in the medial prefrontal cortex of adolescent rats

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Declaration of Interest

None

Abstract

The brain is still maturing during adolescence and interfering with such a vulnerable period may lead to structural and functional consequences. We investigated the effect of a single cocaine exposure on dendritic spine structure and glutamate dynamics in the medial prefrontal cortex (mPFC) of adolescent rats 7 days after a single cocaine administration.

We found a reduced number of dendritic spines, suggesting that cocaine lowers the density of dendritic spines in the mPFC of adolescent rats. Since dendritic spines are postsynaptic glutamatergic protrusions, we investigated the main determinants of glutamate postsynaptic responsiveness. In the postsynaptic density, cocaine reduced the expression of the NMDA receptor subunits GluN1, GluN2A and GluN2B as well as of the AMPA GluA1 and GluA2 subunits. Cocaine also impaired their synaptic stability since the expression of the scaffolding proteins SAP102 and SAP97, critical for the anchoring of such receptors at the postsynaptic membrane, was reduced as well. The expression of PSD-95 and Arc/Arg3.1, which play structural and functional roles in glutamate neurons, was also similarly reduced. Such changes were not found in the whole homogenate, ruling out a translational effect of cocaine and implying, rather, an impaired synaptic retention at the active zones of the synapse. Notably, neither these critical glutamate determinants nor the density and morphology of the dendritic spines were altered in the mPFC of adult animals, suggesting that a single cocaine exposure selectively impairs the developmental trajectory of the glutamate synapse.

These results indicate a dynamic impairment of mPFC glutamate homeostasis during a critical developmental window that persists for at least one week after a single cocaine administration. Our results identify dysfunctional glutamate synapse as a major contributor to the mechanisms that distinguish adolescent vs. adult outcomes of a single cocaine exposure.

1. Introduction

Dendritic spines are defined as small protrusions arising from dendrites, used by excitatory glutamatergic synapses of the central nervous system (CNS) to communicate. They are remarkably dynamic structures that may undergo adaptive changes following different stimuli, either physiological (learning tasks, environmental factors) (Swanson et al., 2017) or pathological (i.e. drugs of abuse) (DePoy et al., 2017). Such changes often reflect modifications in the number and shape of dendritic spines, events that are closely linked to alterations in the postsynaptic density composition and synaptic strength.

While the primary target of cocaine is the dopamine transporter (Rocha et al., 1998), the effects of cocaine exposure may set in motion different mechanisms including, among the others, morphological changes. To this end, several lines of evidence have shown that changes in dendritic spines are peculiar of exposure to the psychostimulant cocaine. Most of the available data involve repeated exposure to cocaine in adult animals and show increased spine density in several brain regions, primarily nucleus accumbens and also ventral tegmental area (Lee et al., 2006; Norrholm et al., 2003; Robinson et al., 2001; Robinson and Kolb, 1999; Sarti et al., 2007). Conversely, in the orbitofrontal cortex, evidence exists showing dendritic spine loss (DePoy et al., 2017; Gourley et al., 2012), whereas in the medial prefrontal cortex (mPFC), results are contrasting with work reporting increased spine density (Robinson and Kolb, 1999) and other showing spine density reduction (Radley et al., 2015; Rasakham et al., 2014);. Notably, in the same brain region, changes in the expression of critical neuroplastic proteins have been shown following repeated exposure to cocaine (Caffino et al., 2015a; Caffino et al., 2018; Chen et al., 2011; Fumagalli et al., 2013; Giannotti et al., 2014; Sun and Rebec, 2006; Verheij et al., 2016) and cocaine-induced cortical dysregulation has been observed in humans (Bolla et al., 2004; Goldstein and Volkow, 2002). The importance of investigating morphological changes in the mPFC is highly enhanced when considering adolescence, a critical period for brain development (Yohn and Blendy, 2017) characterized by synaptic reorganization and pruning of dendritic spines. During this vulnerable period of life, dendritic spine refinement, which defines the overall structural stability, is still occurring and, therefore, cocaine interference during such a crucial period may have functional consequences. Interestingly, repeated exposure to cocaine during the sensitive period of adolescence reduced spine density in the rat mPFC (Caffino et al., 2015b; Gourley et al., 2012; Zhu et al., 2018). Additionally, we have recently shown that repeated exposure to cocaine during adolescence alters the expression of cytoskeletal proteins such as Arc/Arg3.1 in the mPFC (Caffino et al., 2017a). Taken together, these results call for a unifying hypothesis, which implies cortical dendritic spine regulation and cytoskeletal

rearrangements as critical markers of repeated exposure to cocaine during adolescence.

Since adolescence is considered a window of vulnerability to drugs of abuse, we have recently investigated whether a single exposure to cocaine during this exquisitely sensitive maturational period would be sufficient to alter brain plasticity. We have found that a single cocaine exposure alters expression of neurotrophic factors such as FGF-2 (Giannotti et al., 2015) and disrupts actin dynamics (Caffino et al., 2017b). Based on these lines of evidence, we hypothesized that a single injection of cocaine during adolescence would be sufficient to trigger enduring morphological changes in the developing brain, altering the glutamate synapse. To this end, we investigated spine morphology in the mPFC of rats exposed to a single injection of cocaine during adolescence, completing our analysis by examining the expression of the main NMDA and AMPA receptor subunits, their main scaffolding proteins SAP102 and SAP97, which cluster glutamate receptors at the postsynaptic membrane of excitatory synapses, as well as proteins involved into structure and function of glutamate neurons, such as PSD-95 and Arc/Arg3.1 (Chowdhury et al., 2006; Peebles et al., 2010; Vickers et al., 2006). Accordingly, male adolescent rats were exposed to a single injection of cocaine at postnatal day (PND) 35, a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004). Rats were sacrificed at PND 42, i.e. 7 days after the drug exposure: the choice of this time point derives from previous evidence from our laboratory showing molecular changes indicative of cortical reorganization 7 days after a single administration of cocaine (Caffino et al., 2017b; Giannotti et al., 2015). Moreover, in order to investigate whether a single injection of cocaine during adolescence might impact the glutamate synapse differently from adulthood, we exposed adult rats to the same treatment schedule and sacrificed them 7 days after treatment, i.e. on PND 77, to evaluate both spine morphology and the expression of markers of the glutamate synapse in the mPFC.

2. Material and Methods

2.1 Experimental procedures

The adolescent Sprague-Dawley male rats used in this study were obtained from Charles River (Calco, Italy) and housed under standard conditions of temperature and humidity under artificial light (from 07:00 am to 7.00 pm). A maximum of two male siblings was taken from each litter in order to reduce "litter effects" (Chapman and Stern, 1978). Twelve rats were exposed to a single intraperitoneal injection (i.p.) of saline or cocaine (20 mg/kg) at postnatal day 35 (PND 35) and sacrificed 7 days later, i.e. at PND 42, during a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004) (Maldonado and Kirstein, 2005) To investigate whether the effects of a single cocaine administration were different between adolescent and adult animals, we incorporated twelve adult rats (6 x group) that were treated with saline or cocaine (20 mg/kg) at postnatal day 70 and then sacrificed 7 days later, at PND 77. At the scheduled time of sacrifice, the medial prefrontal cortex (defined as Cg1, Cg3, and PL and IL sub-regions) corresponding to plates 5-9 of the atlas of Paxinos and Watson, from both adolescent and adult rats, was dissected from 2-mm thick slices (Paxinos and Watson, 2005), 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.

In order to have a more comprehensive picture of dendritic spine dynamics in the adolescent and adult brain, twelve more animals (6 adolescents and 6 adults), randomly divided in two groups (6 rats treated with cocaine, 3 at PND 35 and 3 at PND 70, and 6 rats treated with saline, 3 at PND 35 and 3 at PND70), were deeply anesthetized (at PND 42 or at PND 77) and perfused with 0.1 M phosphate buffer (PB) followed by 1.5% paraformaldehyde (PFA) in PB for morphological analyses.

Procedures involving animals were conducted at the Department of Pharmacological and Biomolecular Sciences, which adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). All efforts were made to minimize animal suffering and to keep the lowest number of animals used. The experiments have been reported in compliance with the ARRIVE guidelines.

2.2 Dendritic spine labeling and morphological classification

Neuronal labeling and morphological classification of dendritic spines in layer V of medial prefrontal cortex, primarily formed by glutamatergic pyramidal neurons, were carried out using a

lipophilic membrane tracer as previously published (Malinverno et al., 2010). Seven days after the single cocaine injection (PND 42 or PND 77), 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. Two-mm thick slices corresponding to plates 5-9 of the atlas of Paxinos and Watson and containing the mPFC have been dissected from the post-fixed 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, post-fixed 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 randomly selected and their protrusions manually traced. The number of neurons used for quantification is at least 20 for each experimental group (from each neuron, a different number of dendritic segments was analyzed), all neurons analyzed were belonging to 6 hemispheres per group. The average dendritic length analyzed is 150 μm for the adolescent mPFC (total dendrites length analyzed: 12000 μm) and 137 μm for the adult mPFC (total dendrites length analyzed: 10000 μm). We analysed around 12 dendritic segments for each hemisphere. Analysis of dendritic spine morphology was performed with ImageJ software; for each protrusion we measured spine length, head and neck width, 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 dendritic spines; dendritic spines with a Wh/Wn ratio bigger than 1.7 were considered mushrooms; dendritic 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.

2.3 Preparation of Protein Extracts and Western Blot Analyses

Proteins were extracted as previously described (Caffino et al., 2017b) with minor modifications. Briefly, mPFC was homogenized in a teflon-glass potter in cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 1 mM $MgCl_2$, 1 mM $NaHCO_3$ and 0.1 mM PMSF, in presence of commercial cocktails of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy) inhibitors and an aliquot of

each homogenate was then sonicated. The remaining homogenate was centrifuged at 800 g for 5 min; the obtained supernatant was then centrifuged at 13000 g for 15 min obtaining a pellet. This pellet was resuspended in buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100,000 x g for 1 h. The resulting supernatant, referred as Triton X-100 soluble fraction (TSF), was stored at -20°C; the pellet, referred as postsynaptic density (PSD) or Triton X-100 insoluble fraction (TIF), was homogenized in a glass–glass potter in 20 mM HEPES, protease and phosphatase inhibitors and stored at -20°C in presence of glycerol 30%. Total proteins have been measured in the total homogenate and in the TIF fraction according to the Bradford Protein Assay procedure (Bio-Rad, Milan, Italy), using bovine serum albumin as calibration standard.

Equal amounts of proteins of the homogenate (10µg) and of TIF fraction (8µg) were run on a sodium dodecyl sulfate-8% polyacrylamide gel under reducing conditions and then electrophoretically transferred onto nitrocellulose membranes (GE Healthcare, Milan, Italy). Blots were blocked one hour at room temperature with 10% non-fat dry milk in TBS + 0,1% Tween-20 buffer and incubated with antibodies against the proteins of interest.

The conditions of the primary antibodies were the following: anti GluN1 (1:1000, Invitrogen, Carlsbad, CA, USA, RRID: AB_2533060), anti GluN2B (1:1000, Santa Cruz Biotechonology, Santa Cruz, CA, USA, RRID: AB_670229), anti GluN2A (1:1000, Invitrogen, RRID: AB_2536209), anti GluA1 (1:2000, Santa Cruz Biotechonology, RRID: AB_641040), anti GluA2 (1:2000, Cell Signaling Technology Inc., RRID: AB_10622024), anti PSD-95 (1:4000, Cell Signaling Technology Inc., RRID: AB_2292883), anti SAP102 (1:1000, Cell Signaling Technology Inc. RRID: AB_2092180), anti SAP97 (1:1000, AbCam, Cambridge, UK, RRID: AB_2091910), anti Arc/Arg3.1 (1:500, BD Transduction Laboratories, San Jose, CA, USA, RRID: AB_399886) and anti □-Actin (1:10000, Sigma-Aldrich, RRID: AB_476697).

Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories). Gels were run 3 times each and the results represent the average from 3 different runs.

2.4 Analysis of Plasma Corticosterone Levels

Plasma was separated by centrifugation (3000g for 20 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).

2.5 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 and was calculated by hemisphere (6 hemispheres/group). Samples were eliminated from the final dataset if their data deviated from the mean by 2 SDs. Statistical significance was assumed at $p<0.05$.

3. Results

Figure 1 shows the effect of a single administration of cocaine during adolescence on spine remodeling measured 7 days later using a fluorescent dyolistic labeling technique. As shown in figure 1A, we observed a reduction in the density of dendritic spines in the mPFC of cocaine-treated rats (-0.88 dendritic spines/10μm, $t_{(10)}$=2.71, p=0.022). Further, morphological analyses were performed using a highly validated method of dendritic spines classification (please see Materials and Methods for details) to evaluate the shape of all protrusions (mushroom, thin, stubby and filopodia). No significant changes in the percentage of mushroom-, thin- and stubby-shaped dendritic spines (-0.43% mushroom/spines, $t_{(10)}$=0.08, p=0.94; -0.48% stubby/spines, $t_{(10)}$=0.51, p=0.62; -5.63% thin/spines, $t_{(10)}$=0.44, p=0.67); notably, we found an increased formation of filopodia, i.e the immature protrusions, in cocaine-treated rats (+10.64%, $t_{(10)}$=2.29, p=0.045) (Fig. 1B). We furthered the morphological analysis by examining spine length and head width size of cortical dendritic spines. While no changes were found in dendritic spine length (+0.02, $t_{(10)}$=0.63, p=0.54), head width size was increased in cocaine-treated rats (+0.10, $t_{(10)}$=2.48, p=0.033; Fig. 1C), an effect that appeared to be localized in the mushroom-type dendritic spines (length: +0.11, $t_{(10)}$=0.85, p=0.41; head width: +0.18%, $t_{(10)}$=2.56, p=0.028; Fig. 1D).

To determine whether changes in structural plasticity paralleled changes in the organization of dendritic proteins at the postsynaptic level 7 days after a single psychostimulant administration, we examined the expression of the main glutamate receptors in the mPFC homogenate and postsynaptic density (PSD) of cocaine-treated rats in comparison with saline-treated rats. Akin to spine morphology, we found reduced expression of the obligatory NMDA subunit GluN1 (-35%, $t_{(9)}$=2.47, p=0.036; Fig. 2A), the NMDA accessory subunits GluN2A and GluN2B (GluN2A: -24%, $t_{(8)}$=2.31, p=0.049, Fig. 2B; GluN2B: -24%, $t_{(9)}$=2.60, p=0.029, Fig. 2C) and the AMPA glutamate receptor subunits, GluA1 and GluA2 (GluA1= -52%, $t_{(8)}$=3.36, p=0.0099, Fig. 2D; GluA2= -42%, $t_{(9)}$=3.11, p=0.013, Fig. 2E) in the PSD of rats exposed to a single cocaine administration whereas no changes were observed in the whole homogenate (GluN1: +19%, $t_{(10)}$=1.93, p=0.083, Fig. 2A; GluN2A: +12%, $t_{(10)}$=0.69, p=0.51, Fig. 2B; GluN2B: +22%, $t_{(10)}$=1.02, p=0.33, Fig. 2A; GluA1: +10%, $t_{(10)}$=0.03, p=0.97, Fig. 2D; GluA2: 0%, $t_{(8)}$=0.0006, p=0.9996, Fig. 2E).

We then analyzed the expression of the main scaffolding proteins of glutamate receptors, i.e. those proteins that anchor the receptors to the membrane but that are, also, critical for the synaptic localization of newly synthesized receptor towards dendritic spines (Naisbitt et al., 2000). Figure 3 shows that a single exposure to cocaine during development did not alter the expression of PSD-95, SAP102 and SAP97 in the whole homogenate (PSD-95: +19%, $t_{(11)}$=0.209, p=0.838, Fig. 3A; SAP102: -8%,

$t_{(10)}$=0.654, p=0.528, Fig. 3B and SAP97: -4%, $t_{(9)}$=0.166, p=0.872, Fig. 3C) while it significantly reduced the expression of these scaffolds in the postsynaptic density (PSD-95: -43%, $t_{(9)}$=2.842, p=0.019, Fig. 3A; SAP102: -41%, $t_{(8)}$=3.421, p=0.009, Fig. 3B and SAP97: -31%, $t_{(9)}$=2.426, p=0.038, Fig. 3C).

Further, we decided to investigate the expression of the activity-driven protein Arc/Arg3.1, known to have structural and functional roles in glutamate neurons (Bramham et al., 2008). Again, the levels of Arc/Arg3.1 were significantly reduced in the PSD fraction (Arc/Arg3.1: -30%, $t_{(10)}$=3.59, p=0.005, Fig. 3D) but not in the homogenate (Arc/Arg3.1: +13%, $t_{(10)}$= 1.55, p=0.15, Fig. 3D).

In order to investigate whether a single exposure to cocaine alters the homeostasis of the glutamate synapse also at adulthood. We examined spine remodeling in the mPFC of adult animals sacrificed 7 days after the single injection, i.e. at PND 77. Table 1 shows no changes in the density of dendritic spines and in the percentage of mushroom, thin and stubby-shaped spines. We also did not find any change in filopodia as well as in spine length and head width size (table 1). In addition, we examined the expression of NMDA and AMPA receptors, their main scaffolding proteins as well as the expression of PSD-95 and Arc/Arg3.1. Table 2 shows no changes in the expression of these critical determinants of the glutamate synapse in both homogenate and PSD fractions (statistical analyses are shown in legend of Table 2).

Since Radley and coworkers have hypothesized a link between increased corticosterone levels and mPFC structural impairment following repeated cocaine exposure (Radley et al., 2015), we decided to evaluate whether a single injection of cocaine was sufficient to alter corticosterone levels 7 days after the administration. Notably, we found that corticosterone levels were markedly and significantly increased in the plasma of adolescent animals that were exposed to cocaine (+196.6 ng/ml, $t_{(10)}$=3.23, p=0.009, Fig. 4a) while unaltered in the plasma of adult rats that were administered the psychostimulant (-0.77 ng/ml, $t_{(10)}$=0.03, p=0.98, Fig. 4b).

4. Discussion

We here provide evidence that a single exposure to cocaine during adolescence is sufficient to cause dendritic spine alterations associated with impaired postsynaptic glutamate signaling in the rat medial prefrontal cortex (mPFC) indicating structural and functional deficit in mPFC neurons (Fig. 5).

A single injection of cocaine during adolescence uniquely alters distinct features of dendritic spine morphology in the mPFC. In fact, we found a reduction in the density of dendritic spines coupled with an increase of filopodia, i.e. the immature dendritic protrusions. These results suggest that cocaine interfered with dendritic spine formation by influencing their maturation in a critical period of brain development. In addition, the evidence that a single exposure to cocaine enhanced immature dendritic protrusions may be indicative of an increased turnover of immature structures that display less potential for synaptic strengthening. In addition, morphological analyses revealed that cocaine exposure during development did not alter dendritic spine length but, rather, it significantly enlarged the head of the active protrusions, an effect that appears to be primarily located in the mushroom-shaped dendritic spines, which represent the most active type of dendritic spines (Bourne and Harris, 2007). Such effect may reflect a protective response (DePoy et al., 2014) or an attempt of the mature synapses, which are reduced in number, to restore a physiological synaptic communication, a possibility that may be, however, frustrated by the reduced expression of glutamate receptor subunits (Fig. 5).

We are aware that most of the available data in the literature have shown increased dendritic spine density in the nucleus accumbens, orbitofrontal cortex and ventral tegmental area following repeated cocaine exposure in the adult animal (Lee et al., 2006; Norrholm et al., 2003; Robinson et al., 2001; Robinson and Kolb, 1999; Sarti et al., 2007). Of note, Robinson and Kolb (1999) found increased spine density in the mPFC following repeated exposure to cocaine in adult animals whereas, in a recent elegant paper using 3D imaging and analysis of dendritic spine morphometry, Radley and associates (2015) have shown that, in adult rats, chronic cocaine self-administration reduced spine density in the pre-limbic sub-region of the mPFC (Radley et al., 2015), an effect that was not observed in yoked-cocaine animals. Interestingly, Zhu and colleagues (2017) found that chronic cocaine administration during adolescence reduced dendritic spine density in the mPFC, an effect that persists until adulthood (Zhu et al., 2018). Our findings, showing reduced spine density after a single administration of cocaine, suggest that a single injection of the psychostimulant during this exquisitely vulnerable period of brain development recapitulates structural changes of the glutamate synapse that are usually seen only after chronic psychostimulant exposure. This notion is reinforced by the evidence that we found increased corticosterone plasma levels 7 days after the single non-contingent exposure to cocaine during

adolescence, an effect that is similar to that seen by Radley and colleagues after repeated contingent exposure to the psychostimulant. This evidence may suggest a common mechanism for the regressive prefrontal structural plasticity that may link acute exposure to cocaine during adolescence and repeated exposure to cocaine in adult animals (Radley et al., 2015). The evidence that, in adolescent animals, morphological changes are observed also in animals exposed to non-contingent administration of cocaine raises the intriguing possibility that, during adolescence, the structural changes caused by cocaine may not depend on how cocaine is encountered.

Based on these results, we hypothesized an alteration in the composition of the glutamate synapse caused by adolescent exposure to cocaine that might account for the reduced number of dendritic spines along dendrites. To this end, we investigated the expression of PSD-95, a structural protein of the glutamate synapse and a critical regulator of dendritic spines in vivo (Vickers et al., 2006). The reduced expression of PSD-95 in the postsynaptic density (PSD) fraction supports the morphological observation. Besides such a structural deficit, we also demonstrated an impaired synaptic retention of the main glutamate NMDA and AMPA receptor subunits since their expression was significantly reduced in the PSD fraction, but not in the whole cortical homogenate. These results imply that cocaine has not influenced the translation of these receptors but, rather, their synaptic retention (Fig. 5).

Such impairment is strengthened by two different, but tightly linked, events i.e. the reduced expression of 1) the main scaffolding proteins of NMDA (SAP102) and AMPA (SAP97) receptors and 2) the cytoskeletal protein Arc/Arg3.1: notably, alterations in both proteins occurred, again, in the PSD fraction only, corroborating the dynamic impairment of the PSD itself. SAP97 and SAP102 are versatile proteins pivotal for the synaptic localization of AMPA and NMDA receptors (Kim et al., 2005; Vickers et al., 2006) and also critical for the anchoring of such receptors at the postsynaptic membrane: their reduction indicates impaired delivery and reduced synaptic stability of such receptors at the postsynaptic membrane, thus providing a mechanism for the glutamate deficit herein shown. Arc/Arg3.1 is an activity-driven protein localized at active synapses, with structural and functional roles in glutamate neurons, such as regulation of excitatory synapse strength (Chowdhury et al., 2006) and dendritic spine morphology (Peebles et al., 2010); notably, Arc/Arg3.1 also interacts with components of the actin cytoskeleton, a feature known to be altered by adolescent cocaine exposure (Caffino et al., 2017b).

Taken together, the complex set of molecular analyses herein shown point to (mal)adaptive cocaine-induced glutamatergic rearrangements occurring in the adolescent brain following a single administration of cocaine during adolescence. Notably, such changes were specific of adolescent rats since they were not observed in the mPFC of adult rats, suggesting that adolescents differ substantially

from adults with respect to the consequences brought about by single exposure to cocaine, as a single administration of the psychostimulant selectively impairs the trajectory of the developing glutamate synapse only.

4.1 Conclusion

In closing, a single exposure to cocaine during a critical developmental window is sufficient to lower the number of dendritic spines and impair glutamate receptor retention at the post-synaptic density (Fig. 4). Our results point to dysfunctional glutamate synapse as a major contributor to the mechanisms that distinguish developmental vs. adult outcomes of a single cocaine exposure. These events are functionally relevant since the failure in recruiting critical glutamate determinants at synaptic level might reduce the transition to active dendritic spines, leading to a functional destabilization of the mPFC. The dichotomy between the effects of cocaine on glutamate dynamics in the adolescent and adult brains is likely to play a critical role in the greater vulnerability of developing neurons to cocaine. The inability of the developing glutamate system to adapt to the single cocaine administration may represent one of the multiple mechanisms that confer sensitivity to adolescents following exposure to drugs of abuse.

These findings add complexity as well as specificity to our previous data and reinforce the notion that a single cocaine exposure during adolescence is sufficient to trigger functional and structural changes in the rat brain. We have previously shown that a single injection of cocaine during adolescence influences the response to a second challenge of the psychostimulant, altering trophic response (Giannotti et al., 2015) as well as actin dynamics (Caffino et al., 2017b) thus suggesting that early cocaine priming might affect plasticity in the developing brain. Further studies will evaluate whether a single exposure to cocaine during adolescence influences addiction liability at adulthood.

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Declaration of Interest

None.

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Figure legends

Figure 1. Effects of a single cocaine exposure at post-natal (PND) 35 on dendritic spine morphology in the layer V of the mPFC measured 7 days later (PND 42).

Panel a shows total spine density in the mPFC. Panel b shows the percentage of total protrusions belonging to different categories depending on their morphology (mushroom, thin, stubby and filopodia). Panel c shows the average spines length (left) and the average spines head width (right) of the total spines measured. Panel d shows the average spines length (left) and the average spines head width (right) of the mushroom-shaped spines. Panel e shows representative images of dendrite segments from the mPFC of saline (top) and cocaine-treated animals (bottom).

n>2500 spines from at least 20 different neurons for each group, around 12 dendritic segments for each hemisphere, 6 hemispheres/group

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

Figure 2. Effects of a single cocaine exposure at PND 35 on glutamate receptor subunit expression in the whole homogenate (left column of each panel) and postsynaptic density (PSD, right column of each panel) measured 7 days later (PND 42). Panels A through C show the expression of the main NMDA receptor subunits GluN1, GluN2A and GluN2B; panels D and E show the expression of the main AMPA receptor subunits GluA1 and GluA2. Panel F shows representative immunoblots for GluN2A, GluN2B, GluN1, GluA1, GluA2 proteins in the homogenate and PSD of mPFC.

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.

Figure 3. Effects of a single cocaine exposure at PND 35 on PSD-95, SAP102, SAP97 and Arc/Arg3.1 expression in the whole homogenate (left column of each panel) and postsynaptic density (PSD, right column of each panel) measured 7 days later (PND 42). Panel A shows the protein levels of PSD-95; panel B shows the protein levels of SAP102, panel C shows the protein levels of SAP97 and panel D shows the protein levels of Arc/Arg3.1. Below each graph, representative immunoblots are shown for PSD-95, SAP102, SAP97 and Arc/Arg3.1 proteins in the homogenate and PSD of mPFC.

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.

Figure 4. Effect of a single exposure to cocaine on the levels of circulating corticosterone in adolescent (panel a) and adult (panel b) animals. The levels of plasma glucocorticoids were expressed in ng/ml and represent the mean ± S.E.M. of 6 animals for each experimental group ** $p<0.01$ vs. saline-treated rats

Figure 5. Schematic representation of the changes observed 7 days after a single exposure to cocaine during adolescence in the mPFC.

A single administration of cocaine during adolescence has reduced the number of dendritic spines. Among the different types of dendritic spines, filopodia are instead increased. The analysis of several determinants of the glutamate synapse has revealed an overall impairment in the expression receptors, scaffolding proteins as well as cytoskeletal proteins pointing to a dysfunctional glutamate synapse.

GluA1, glutamate AMPA receptor subunit 1; *GluA2,* glutamate AMPA receptor subunit 1; *GluN1,* glutamate NMDA receptor subunit 1; *GluN2A,* glutamate NMDA receptor subunit 2A; *GluN2B,* glutamate NMDA receptor subunit 2B; *PSD-95,* postsynaptic density protein 95*; SAP102,* synapse-associated protein 102*; SAP97,* postsynaptic density protein 97*; Arc/Arg3.1,* activity-regulated cytoskeleton associated protein.