Full-length Article

Title

Short-term withdrawal from repeated exposure to cocaine during adolescence modulates dynorphin mRNA levels and BDNF signaling in the rat nucleus accumbens

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Abstract

BACKGROUND: Early-life stressful events affect the neurobiological maturation of cerebral circuitries including the endogenous opioid system and the effects elicited by adolescent cocaine exposure on this system have been poorly investigated. Here, we evaluated whether cocaine exposure during adolescence causes short- or long-term alterations in mRNAs codifying for selected elements belonging to the opioid system. Moreover, since brain-derived neurotrophic factor (BDNF) may undergo simultaneous alterations with the opioid peptide dynorphin, we also evaluated its signaling pathway as well.

METHODS: Adolescent male rats were exposed to cocaine (20 mg/kg/day) from post-natal day (PND) 28 to PND42, approximately corresponding to human adolescence. After short- (PND45) or long-term (PND90) abstinence, prodynorphin-κ-opioid receptor (pDYN-KOP) and pronociceptin-nociceptin receptor (pN/OFQ-NOP) gene expression were evaluated in the nucleus accumbens (NAc) and hippocampus (Hip) together with the analysis of BDNF signaling pathways.

RESULTS: In the NAc of PND45 rats, pDYN mRNA levels were up-regulated, an effect paralled by increased BDNF signaling. Differently from NAc, pDYN mRNA levels were down-regulated in the Hip of PND45 rats without significant changes of BDNF pathway. At variance from PND45 rats, we did not find any significant alteration of the investigated parameters either in NAc and Hip of PND90 rats.

CONCLUSIONS: Our results indicate that the short-term withdrawal from adolescent cocaine exposure is characterized by a parallel pDYN mRNA and BDNF signaling increase in the NAc. Given the depressive-like state experienced during short abstinence in humans, we hypothesize that such changes may contribute to promote the risk of cocaine abuse escalation and relapse.

Keywords: cocaine, adolescence, dynorphin; opioid receptor, nociceptin, BDNF

1. Introduction

Adolescents represent the population at highest risk of abuse-related behaviors because of their impulsivity and higher vulnerability to the reinforcing properties of drugs of abuse; in addition, they appear to suffer the severe effects of abstinence to a lesser extent (Acheson et al., 1999; Doherty et al., 2009; Li and Frantz, 2009). In this regard, relevant evidence showed that the cue-induced reinstatement of cocaine seeking after withdrawal was attenuated in rats exposed to cocaine during adolescence compared with rats exposed during adulthood (Li and Frantz, 2009).

Adolescence is a crucial stage of brain development (Casey et al., 2008; Ernst and Korelitz, 2009), during which cortical and limbic brain regions mature following a specific temporal profile (Spear 2000; Caballero et al., 2016) providing a critical neurochemical network responsible for the maturation of cognitive processes.

Our group recently demonstrated that exposure to cocaine during adolescence may impact the developing brain. Indeed, a single cocaine exposure is sufficient to alter neuroplasticity in the adolescent brain (Giannotti et al., 2015; Caffino et al., 2017; Caffino et al., 2018) whereas repeated exposure to the psychostimulant during adolescence causes short- as well as long-term alterations at behavioral, molecular and structural level (Giannotti et al., 2013, 2014; 2016; Caffino et al., 2014, 2015a, 2018). These lines of evidence have been corroborated by structural and functional evidence from Gourley's group showing that exposure to cocaine during adolescence impairs cognitive tasks and brain plasticity (Gourley et al., 2012; DePoy et al., 2014, 2017; Hinton et al., 2014).

Interestingly, early-life manipulations have also been shown to involve changes in the endogenous opioid system (Ploj and Nylander, 2003; Ploj et al., 2002), prompting us to hypothesize that interfering with the developing brain, i.e. exposing adolescent rats to cocaine, may impact such system. Actually, several lines of evidence indicate that different addictive substances such as cocaine, alcohol and heroin affect the endogenous opioid system (D'Addario et al. 2007a, 2013a, 2013b; Sillivan et al., 2013; Caputi et al., 2014, 2016) and corroborate the hypothesis that this system might represent a promising target for addiction therapy (Lutz and Kieffer, 2013; Belzeaux

et al., 2018). In details, the dynorphinergic system has long been proposed to mediate negative emotional states and, indeed, it is strongly involved in stress-induced reinstatement of cocaine seeking (Valdez et al., 2007; Mantsch et al., 2016). Another endogenous peptide, known as nociceptin/orphanin FQ (N/OFQ), acts as critical mediator in the hedonic dysregulation (Devine et al., 2001; Sakoori and Murphy, 2008) mainly attenuating the stress-like responses (Ciccocioppo et al., 2003; Martin-Fardon et al., 2010). In this regard, we recently reported that chronic cocaine infusion induces significant neurochemical alterations in prodynorphin - κ-opioid receptor (pDYN-KOP) and in the proN/OFQ - nociceptin receptor (pN/OFQ-NOP) systems in limbic regions, an effect paralleled by consistent histone modifications (Caputi et al., 2014).

Based on these experimental lines of evidence, the present study aimed at evaluating whether rats exposed to cocaine during adolescence exhibit short- or long-lasting alterations in pDYN-KOP and pN/OFQ-NOP systems. Further, since dynorphin has been proposed as a downstream effector of the brain-derived neurotrophic factor (BDNF) and given that the pDYN expression pattern might parallel that of BDNF (Croll et al., 1994; Kim et al., 2000; Logrip et al., 2008), we also evaluated whether repeated cocaine exposure during adolescence may dysregulate BDNF signaling after short or long-term abstinence.

To address these questions, we exposed male rats to prolonged cocaine administration from postnatal day (PND) 28 to PND 42, a period of development that roughly approximates adolescence in humans (Collins and Izenwasser, 2004). Animals were sacrificed at PND45 or PND90 to evaluate the alteration pattern of endogenous opioids (pDYN-KOP and pN/OFQ-NOP systems) and BDNF signaling following short- and long-term withdrawal. We focused our analysis on the nucleus accumbens (NAc), which plays a critical role in the reinforcing properties of cocaine (Di Chiara et al., 2004). Further, we analyzed the hippocampus (Hip) that is crucial for the negative emotional state occurring during drug abstinence (Koob, 2012) and also because of its feature to undergo periodic structural and functional changes from adolescence to adulthood (Koehl, 2015; Risher et al., 2015; Zhu et al., 2016).

To pursue this issue, we decided to take advantage of a previously conducted experiment (Giannotti et al., 2014) with a dual purpose: 1) to use tissues from rats that already showed neuroplastic changes as a consequence of developmental exposure to cocaine and 2) to adhere with the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines, which ask to maximize information published and to minimize unnecessary studies reducing the numbers of animals whenever possible.

2. Materials and methods

2.1 Experimental procedures

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 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 PND28 to PND42, a period that include periadolescence (PND 28-35) and the beginning of mid adolescent rat phases, and that roughly approximates adolescence in humans (Collins and Izenwasser, 2004). In order to avoid any stress due to unpredictability of the treatment, animals were always treated in the morning at the same time. Following the end of this treatment, animals were left undisturbed in their home cages until the time of sacrifice, PND45 or PND90.

The NAc (approximately + 2.76 to + 0.84 mm from Bregma) and the Hip (grossly dissected) were collected from freshly dissected brain (Paxinos and Watson, 2005), immediately frozen on dry ice and stored at -80°C. All animal procedures were carried out in accordance with 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. Experiments have been reported in compliance with the ARRIVE guidelines.

2.1 RNA Preparation and Real-Time Quantitative RT-PCR

Total RNA was isolated from one single hemisphere of NAc and Hip by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Segrate, Milan, Italy) according to the manufacturer's instructions. Following total RNA extraction, the samples were processed for quantitative real-time reverse transcription polymerase chain reaction (real time qRT-PCR) to assess mRNA levels.

In brief, RNA integrity was checked by 1% agarose gel electrophoresis and RNA concentrations were measured by spectrophotometry (OD260/OD280 1.8 > ratio >2). Total RNA was reverse transcribed with the GeneAmp RNA PCR kit (Life Technologies) and the relative abundance of each mRNA of interest was assessed by real-time qRT-PCR using the Syber Green gene expression Master Mix (Life Technologies) in a Step One Real-Time PCR System (Life Technologies). All data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous reference gene. Relative expression of different gene transcripts was calculated by the Delta-Delta Ct (ΔΔCt) method and converted to relative expression ratio ($2^{-\Delta\Delta Ct}$) for statistical analysis (Livak and Schmittgen, 2001). Results are represented as fold change in mRNA levels. The primers used for PCR amplification were designed using Primer 3 and are reported in Table 1.

2.2 Preparation of protein extracts and Western blot analyses

The other hemisphere of NAc and Hip 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, and then sonicated. Homogenate was prepared as previously described (Fumagalli et al., 2009). Total proteins were measured in the whole homogenate by the Bio-Rad Protein Assay, using bovine serum albumin as the calibration standard (Bio-Rad Laboratories, Milan, Italy). Equal amounts of

protein were run under reducing conditions on the criterion TGX precast gels (Bio-Rad Laboratories, Milan, Italy) and then electrophoretically transferred onto polyvinylidene difluoride 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, 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: proBDNF (1:2000, GeneTex, USA); mBDNF (1:500, Icosagen, Estonia); anti total trkB (1:750, Santa Cruz Biotechnology, USA); anti phopsho-ERK2 T202/204 (1:1000, Cell Signaling Technology, USA); anti total ERK2 (1:5000, Cell Signaling Technology, USA); anti phopsho-Akt S473 (1:1000, Cell Signaling Technology, USA); anti total Akt (1:1000, Cell Signaling Technology, USA) and anti β-Actin (1:10000, Sigma-Aldrich, Italy). 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).

2.4 Statistical Analysis

Data were collected from individual animals and are reported as the mean of values ± standard error of the mean (SEM) (n/assay = 6). qPCR results, produced by short- (PND45) and long-term (PND90) abstinence from chronic cocaine exposure, were statistically analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Western blotting results were analyzed by unpaired Student's t test. Subjects were eliminated from the final dataset if their data deviated from the mean by 2 SDs (standard deviations). Statistical analysis was performed using GraphPad Prism software package (version 5.00 for Windows, GraphPad Software, San Diego CA, USA, www.graphpad.com) and statistical significance was set at $p < 0.05$.

3. Results

3.1 Gene expression analysis

Rats were sacrificed at PND45 and PND90, i.e. 3 and 48 days after the last exposure to cocaine or saline. Gene expression analysis was performed via qRT-PCR in the NAc and Hip of saline- and cocaine-treated rats.

In the NAc, adolescent cocaine exposure promoted a significant increase of pDYN mRNA levels at PND45 (1.45 ± 0.11 vs saline 1.00 ± 0.04, $F_{(1,20)} = 4.04$, $p < 0.05$) (Fig. 1a) with no significant changes detected for the other investigated genes (KOP at PND45: 1.09 ± 0.11 vs saline 1.00 ± 0.09, n.s.; pN/OFQ at PND45: 1.03 ± 0.04 vs saline 1.00 ± 0.05, n.s.; NOP at PND45: 1.14 ± 0.09 vs saline 1.00 ± 0.06, n.s.) (Fig.1). At PND90, no changes were observed in the cocaine-treated group compared with controls (pDYN at PND90: 1.01 ± 0.10 vs saline 1.00 ± 0.16, n.s.; KOP at PND90: 0.92 ± 0.09 vs saline 1.00 ± 0.07, n.s.; pN/OFQ at PND90: 1.01 ± 0.05 vs saline 1.00 ± 0.07, n.s.; NOP at PND90: 1.03 ± 0.09 vs saline 1.00 ± 0.06, n.s.) (Fig. 1). Bonferroni post hoc test revealed a significant age x treatment interaction in the analysis of pDYN mRNA levels ($F_{(1, 20)} = 3.77$, $p < 0.05$) (Fig.1a).

In the Hip, a significant decrease of pDYN mRNA levels was detected at PND45 (0.77 ± 0.02 vs saline 1.00 ± 0.05, $F_{(1,20)} = 17.03$, $p < 0.01$) (Fig. 2a) with no other changes at the same time point for the other investigated genes (KOP at PND45: 1.15 ± 0.04 vs saline 1.00 ± 0.12, n.s.; pN/OFQ at PND45: 1.05 ± 0.03 vs saline 1.00 ± 0.07, n.s.; NOP at PND45: 1.02 ± 0.06 vs saline 1.00 ± 0.07, n.s.) (Fig.2). No significant alterations of investigated genes were observed at PND90 (pDYN at PND90: 0.84 ± 0.06 vs saline 1.00 ± 0.05, n.s.; KOP at PND90: 0.87 ± 0.07 vs saline 1.00 ± 0.04, n.s.; pN/OFQ at PND90: 1.03 ± 0.05 vs saline 1.00 ± 0.04, n.s.; NOP at PND90: 1.03 ± 0.04 vs saline 1.00 ± 0.04, n.s.) (Fig. 2). Tabular result of KOP gene expression analysis in the Hip indicated that no significant effect was referred to treatment and interaction parameters overall.

However, the narrative report showed the presence of a borderline result for interaction parameter. Indeed, Bonferroni post hoc test revealed a significant age x treatment interaction in the analysis of KOP mRNA levels ($F_{(1, 20)} = 3.45$, $p < 0.05$) (Fig.2b).

3.2 Protein analysis

Fig 3 shows that both the precursor (proBDNF) and the mature form (mBDNF) of BDNF are significantly increased in the NAc of cocaine-treated PND45 rats (proBDNF: 141 ± 14 vs saline 100 ± 11, $t_{(9)}= 2.338$, $p = 0.044$; mBDNF: 135 ± 11 vs saline 100 ± 9, $t_{(9)}= 2.49$, $p = 0.034$; unpaired two-tailed t test; panel a). Panel b shows that the expression of the high affinity BDNF receptor, trkB, is up-regulated as well (136 ± 11 vs saline 100 ± 11, $t_{(10)} = 2.314$, $p = 0.043$, unpaired two-tailed t test). In line with the enhancement of BDNF-trkB mediated signalling, we found increased phosphorylation of the BDNF-dependent Akt pathway (pAkt S473: 133 ± 5 vs saline 100 ± 9, $t_{(9)} = 3.02$, $p = 0.015$; Akt: 119 ± 7 vs saline 100 ± 9, $t_{(10)} = 1.666$, $p = 0.127$, unpaired two-tailed t test; panel c) with no changes in ERK2 pathway (pERK2 T202/204: 104 ± 13 vs saline 100 ± 18, $t_{(10)} = 0.18$, $p = 0.861$; ERK2: 109 ± 10 vs saline 100 ± 11, $t_{(10)} = 0.605$, $p = 0.56$, unpaired two-tailed t test; panel d).

Fig 4 shows no changes in the hippocampal expression of both proBDNF (99 ± 4 vs saline 100 ± 5, $t_{(10)} = 0.158$, $p = 0.877$; unpaired two-tailed t test; panel a) and mBDNF (94 ± 5 vs saline 100 ± 4, $t_{(10)} = 0.914$, $p = 0.38$; unpaired two-tailed t test; panel a) as well as in the BDNF receptor trkB (110 ± 7 vs saline 100 ± 5, $t_{(10)} = 1.125$, $p = 0.285$; unpaired two-tailed t test; panel b). In line with the lack of changes in the neurotrophin, no alterations were observed in both phosphorylation and expression of Akt (pAkt S473: 101 ± 6 vs saline 100 ± 8, $t_{(10)} = 0.102$, $p = 0.921$; Akt: 104 ± 7 vs saline 100 ± 4, $t_{(10)} = 0.473$, $p = 0.645$, unpaired two-tailed t test; panel c) and ERK2 pathway (pERK2 T202/204: 104 ± 7 vs saline 100 ± 5, $t_{(10)} = 0.465$, $p = 0.652$; ERK2: 114 ± 7 vs saline 100 ± 3, $t_{(10)} = 1.730$, $p = 0.112$, unpaired two-tailed t test; panel d).

4. Discussion

The major finding of our study is that repeated exposure to cocaine during adolescence evokes dynorphinergic alterations that heavily depend on the brain regions investigated. In particular, pDYN mRNA levels increased in the NAc while decreasing in the Hip after short-term withdrawal. Conversely, no changes were observed for the nociceptin system either after short- or long-term withdrawal in both investigated regions.

The dynorphinergic system is widely distributed within the mesocorticolimbic regions (Meng et al., 1993; Simonin et al., 1995) where it is known to mediate stress responses and to play a significant role in regulating the reward circuitry. In fact, pDYN-KOP system mediates negative emotional responses such as dysphoria, anhedonia, anxiety and depression (McLaughlin et al., 2006; Land et al., 2008; Bruchas et al., 2008).

The anhedonic state, primarily mediated by the dynorphinergic system activation, is also the most typical sign of withdrawal and may promote the risk to escalate drug abuse and to relapse (Bruchas et al, 2010; Wee and Koob, 2010; Butelman et al., 2012). For this reason, KOP antagonists have been proposed to block the aversive effects of withdrawal reducing anxiety-like behavior and to counteract stress-induced reinstatement of drug seeking (Wee and Koob, 2010; Schlosburg et al., 2013). It is worth noting that the increase of dynorphinergic tone in addiction-relevant brain areas such as the NAc, amygdala and bed nucleus of the stria terminalis has been postulated as a counter-regulatory mechanism toward drug-induced dopamine increase (Koob, 2008). In other words, the dynorphinergic activation in the NAc works to decrease dopaminergic function (Werling et al, 1998; Margolis et al., 2003; Koob, 2008) and, indeed, different patterns of cocaine administration promote the rapid increase of dynorphin in this brain area (Turchan et al., 1998; Wee and Koob, 2010; Caputi et al., 2014). In this frame, our data show that repeated developmental exposure to cocaine causes a significant pDYN gene expression increase in cocaine-withdrawn rats, thus

indicating that the high dynorphinergic tone persists in the absence of cocaine in the NAc. With respect to the functional relevance, such increase of pDYN may favour relapse since the KOP/dynorphinergic tone inhibition may oppose to the dysphoric state occurring during abstinence (Butelman et al., 2012).

In addition, the increase in pDYN gene expression may also contribute to the depressive-like state experienced during short abstinence (Barr et al., 2002; Shirayama and Chaki, 2006). This observation is relevant in the light of our previous data revealing that cocaine-withdrawn rats at PND45 exhibited a depressive-like behavior in a swim stress paradigm (Caffino et al., 2015b). Therefore, the here reported up-regulation of pDYN mRNA levels in the NAc might also participate to the pro-depressive state caused by early withdrawal from cocaine.

According with the hypothesis of a linkage between pDYN and BDNF (Croll et al., 1994; Kim et al., 2000; Logrip et al., 2008; Palmisano et al., 2018) and since an increase of BDNF expression in the NAc has been shown to mediate pro-depressive behavior (Eisch et al., 2003), we decided to investigate BDNF expression and its signaling pathway only after short-term withdrawal, at the time point that cocaine abstinence altered pDYN gene expression. We indeed found increased expression of both BDNF precursor (proBDNF) and mature BDNF (mBDNF), coupled with the up-regulation of its high affinity receptor trkB and of Akt, with no effect on ERK2 activation. These results suggest that the pro-depressive phenotype we have previously observed in rats exposed to cocaine during adolescence (Caffino et al., 2015b) may be, at least in part, due to the up-regulation of the BDNF-trkB-Akt pathway in the NAc.

A different picture can be drawn for the Hip. In this brain region, we observed a significant decrease of pDYN gene expression after short abstinence without changes in late withdrawal. We previously observed that, after seven days of cocaine infusion, pDYN mRNA levels were unaltered in rat Hip (D'Addario et al., 2007b). These data suggest that the herein shown changes in pDYN mRNA levels may be due to the withdrawal condition and/or to the period of life of exposure (adolescence vs. adulthood).

The reduction of pDYN gene expression here reported, after a short abstinence from developmental chronic cocaine exposure, might facilitate the increase of glutamate release since, at hippocampal level, the dynorphinergic system modulates transmission at all excitatory synapses (Wagner et al., 1993; Jeub et al., 1999; Terman et al., 2000; Drake et al., 2007). Actually, a recent report analyzed the consequences of chronic cocaine intoxication identifying seizures as one of major negative effects (Rolland et al., 2011). Therefore, since different studies associate seizures and cocaine (Choy-Kwong and Lipton, 1989; Pascual-Leone et al., 1990), it is tempting to speculate that the prolonged cocaine exposure during adolescence might induce neurological disorders including seizures manifestation likely related to the decrease of the dynorphinergic tone (Simonato and Romualdi, 1996).

Differently from what observed in the NAc, no significant changes of proBDNF, mBDNF and trkB protein levels were observed in the Hip of cocaine-withdrawn rats at PND45, indicating that, in this brain region, changes in pDYN mRNA levels are not paralleled by changes in BDNF expression. Such discrepancy is not surprising since previous studies have shown that the BDNF infusion into the Hip of rats decreased dynorphin peptide and mRNA levels (Croll et al., 1994), suggesting that BDNF may play a regionally specific role in modulating neuropeptide expression.

Interestingly, the changes of pDYN expression observed at PND45 vanished when the neuropeptide expression was measured at PND90 suggesting that this system is mainly modified during the early withdrawal, which is characterized by the occurrence of severe negative emotional states. In addition, it is also likely that the lack of pDYN mRNA alterations at PND90 after adolescence cocaine exposure could be related to a low sensitivity to cue-induced reinstatement of cocaine seeking behavior (Li and Frantz, 2009).

The N/OFQ system, being widely distributed in brain areas involved in the motivational effects of addictive drugs (Neal et al., 1999; Mollereau and Mouledous, 2000), plays a relevant role in drug addiction (Witkin et al., 2014) working as a powerful anti-stress system to buffer the activation of brain stress systems (Koob, 2015).

As mentioned above, the nociceptin system did not show any significant gene expression alteration either after short- or long-term withdrawal, in both investigated regions. We previously reported that chronic cocaine infusion using osmotic mini-pumps induces significant pN/OFQ-NOP down-regulation in the NAc (Caputi et al., 2014). However, in that study, gene expression was measured immediately after the end of treatment, whereas in the present manuscript analyses have been undertaken 3 and 48 days after the last cocaine injection. Hence, the different result here observed highlights for the first time that the nociceptinergic response is no longer present in the absence of cocaine, i.e. following short- or long-term drug withdrawal, according with the anti-reward activity of the N/OFQ-NOP system that is not required during the abstinence phases.

5. Conclusions

In conclusion, our data show that repeated cocaine exposure during adolescence interferes with the dynorphinergic system likely leading to the development of depressive-like phenotype observed during early abstinence (Caffino et al., 2015b), through a mechanism that appears to involve up-regulation of the neurotrophin BDNF and its intracellular signaling. Notwithstanding these results, additional research will be necessary to fully elucidate our hypothesis also taking into account that the alterations here reported are peculiar of early withdrawal phase, which therefore confirms to be a feasible therapeutic window (Butelman et al., 2012). Hence, these results provide further support to the notion that the modulation of the dynorphinergic system can be used as therapeutic strategy in cocaine addiction (Helal et al., 2017; Valenza et al., 2017).

Acknowledgement

This work was supported by grants from RFO2016 (to PR), RFO2015 (to SC) and MIUR Progetto Eccellenza (to FF).

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Figure Legends

Figure 1. Effects of short- (PND45) and long-term (PND90) abstinence from chronic cocaine (20 mg/kg) or saline exposure during adolescence on a) pDYN, b) KOP, c) pN/OFQ and d) NOP mRNA levels in the rat nucleus accumbens (NAc). Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± standard error of the mean (SEM) of six rats per group (* $p < 0.05$ vs. PND45 saline; # $p < 0.05$ vs. PND45 cocaine; data were analyzed by two-way ANOVA followed by Bonferroni post hoc test).

Figure 2. Effects of short- (PND45) and long-term (PND90) abstinence from chronic cocaine (20 mg/kg) or saline exposure during adolescence on a) pDYN, b) KOP, c) pN/OFQ and d) NOP mRNA levels in the rat hippocampus (Hip). Data represent 2^{-DDCt} values calculated by DDCt

method and are expressed as mean ± standard error of the mean (SEM) of six rats per group (** $p < 0.01$ vs. PND45 saline; # $p < 0.05$ vs. PND45 cocaine; data were analyzed by two-way ANOVA followed by Bonferroni post hoc test).

Figure 3. Effects of short- term (PND45) abstinence from chronic cocaine (20 mg/kg) or saline exposure during adolescence on a) proBDNF and mBDNF, b) trkB, c) pAkt S473 and Akt and d) pERK2 T202/204 and ERK2 protein levels in the rat nucleus accumbens (NAc). Below each graph, representative immunoblots are shown for proBDNF, mBDNF, trkB, pAkt S473, Akt, pERK2 T202/204 and ERK2 proteins in the homogenate of NAc. Data, expressed as % of saline-treated rats, represent the mean ± standard error of the mean (SEM) of five-six rats per group (* $p < 0.05$ vs. saline; data were analyzed by unpaired two-tailed Student's t test).

Figure 4. Effects of short- term (PND45) abstinence from chronic cocaine (20 mg/kg) or saline exposure during adolescence on a) proBDNF and mBDNF, b) trkB, c) pAkt S473 and Akt and d) pERK2 T202/204 and ERK2 protein levels in the rat hippocampus (Hip). Below each graph, representative immunoblots are shown for proBDNF, mBDNF, trkB, pAkt S473, Akt, pERK2 T202/204 and ERK2 proteins in the homogenate of Hip. Data, expressed as % of saline-treated rats, represent the mean ± standard error of the mean (SEM) of six rats per group.