Repeated cocaine exposure dysregulates BDNF expression and signaling in the mesocorticolimbic pathway of the adolescent rat

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Abstract

Objectives Long-term abstinence following cocaine exposure up-regulates BDNF expression in the mesocorticolimbic pathway. Given the increased vulnerability to drug abuse typical of adolescence, we hypothesized that changes in BDNF expression may become manifest early after the end of cocaine treatment in the adolescent brain.

Methods Rats received cocaine injections from postnatal day 28 (PND28) to PND42 and the mesocorticolimbic expression of BDNF was measured by real-time PCR and Western blotting at PND43.

Results In the ventral tegmental area (VTA), BDNF-trkB expression and phosphorylation are enhanced while the intracellular signaling is unaltered. In the nucleus accumbens (NAc) shell and core, BDNF and its signaling were down-regulated. In the prelimbic (PL) cortex, we found reduced BDNF expression and increased phosphoprylation of trkB, ERK and AKT. In the infralimbic (IL) cortex, increased BDNF expression was coupled with reduced activity and expression of its downstream targets. To evaluate the role of glutamate on BDNF-independent changes, we investigated the expression of the transporter GLT-1 and the activation of the NMDA receptor subunit GluN2, which were both increased in the PL cortex while reduced in the IL cortex.

Conclusions Our results show that adolescent cocaine exposure modulates BDNF system early after treatment in the mesocorticolimbic pathway, identifying a complex but specific set of changes that could provide clues for treatment.

1. Introduction

Brain Derived Neurotrophic Factor (BDNF) belongs to the neurotrophin family of growth factors highly expressed in the brain. Accumulating evidence has shown that changes in BDNF expression accompany various disorders including psychiatric disorders such as depression and schizophrenia (Autry and Monteggia, 2012), but also neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease and multiple sclerosis (Fumagalli et al., 2006a; Fumagalli et al., 2006b; KhorshidAhmad et al., 2016). Recently, attention has been focused on the critical role played by BDNF in the action of drugs of abuse, primarily cocaine. Evidence has been accumulated showing that acute or chronic exposure to cocaine, either contingent or non-contingent, modulates BDNF expression in the rat brain (Filip et al., 2006; Fumagalli et al., 2009; Fumagalli et al., 2007; Fumagalli et al., 2013; McGinty et al., 2010; Sadri-Vakili et al., 2010) highlighting a critical role of the neurotrophin in the action of the psychostimulant. This evidence has been further corroborated by a recent work showing that systemic administration of an antagonist of trkB, the high-affinity receptor of BDNF, reduces cocaine-taking behavior in a long-access self-administration model (Verheij et al., 2016). Interestingly, its accumulation in the mesocorticolimbic network occurs following long-term abstinence, an effect that was suggested to contribute to the incubation of cocaine craving (Grimm et al., 2003). The role of BDNF in the mesocorticolimbic pathway is further strengthened by its exogenous administration that, depending on the area of infusion after cocaine exposure, causes different effects. In fact, infusing BDNF levels in the medial prefrontal cortex (mPFC) leads to suppression of cocaine seeking (Berglind et al., 2007; Berglind et al., 2009; Whitfield et al., 2011) whereas BDNF infusion in the ventral tegmental area (VTA) and nucleus accumbens (NAc) shell enhances cocaine seeking behavior (Graham et al., 2007; Lu et al., 2004).

However, most of the studies focus on the contribution of BDNF in the action of cocaine in the adult brain and much less is known about the role of the neurotrophin in a vulnerable period of life such as adolescence. Adolescence may be considered a peculiar period for brain development

(Shapiro et al., 2017) due to its unique sensitivity to risky behaviors such as, for instance, drug abuse (Kelley et al., 2004) since key brain regions, such as mPFC, which govern decision making, are still maturing (Caballero et al., 2016).

We have previously shown that repeated administration of cocaine during adolescence causes a significant up-regulation of BDNF measured several weeks after the treatment cessation (Giannotti et al., 2014), an information limited to long-term abstinence in the mPFC, i.e. the final station of the mesocorticolimbic pathway. However, no evidence exists showing whether changes in BDNF expression occur early after the end of developmental exposure to cocaine in the mesocorticolimbic pathway. Taking into account that the increased vulnerability to drug abuse typical of adolescence may be due, at least in part, to the fact that dopamine neurons in the VTA display increased firing during adolescence (McCutcheon et al., 2012), we hypothesized that cocaine-induced changes in BDNF expression and signaling in the mesocorticolimbic pathway may become manifest early after the end of treatment in the adolescent brain.

Accordingly, male adolescent rats were exposed to repeated cocaine treatment from postnatal day (PND) 28 to PND 42, a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004). Rats were sacrificed at PND 43, i.e. 24 hours after the last drug exposure, which can be considered a time point free of cocaine and therefore useful to investigate the effects set in motion by the repeated exposure to the psychostimulant, with no abstinence involved. The detailed analysis of the mesocorticolimbic network took advantage of the punching technique (Giannotti et al., 2016), which allows the microdissection of small brain regions such as the VTA, the prelimbic (PL) and infralimbic (IL) portions of the mPFC and the shell and core portions of the NAc (NAc shell and NAc core, respectively).

2. Experimental procedures

2.1. Animals and 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 to 19:00 hours). A maximum of two male siblings was taken from six different litters in order to reduce "litter effects" (Chapman and Stern, 1978). Twelve male rats were treated subcutaneously with cocaine (20 mg/kg/day; n=6) (Space Import-Export Srl, Milan, Italy) or saline (n=6) from postnatal day 28 (PND 28) to PND 42, a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004). Animals were always treated in the morning at the same time, in order to avoid any stress due to unpredictability of the treatment. Following the end of treatment, animals were sacrificed 24 hours (PND 43) after the last exposure to cocaine. All the brain subregions were bilaterally microdissected according to the rat brain atlas (Paxinos and Watson, 2005). In detail, Infralimbic (IL) and Prelimbic (PL) subregions of the prefrontal cortex (approximately from Bregma +4.20 to Bregma +2.52 mm), Nucleus accumbens core (cNAc) and shell (sNAc) (approximately from Bregma +2.76 to Bregma +0.84 mm) and ventral tegmental area (VTA) (approximately from Bregma −4.80 to Bregma −5.40 mm) were collected from freshly dissected brain sections of 220 μm using a sterile 1-mm-diameter needle (Giannotti et al., 2016) and stored at −80 °C until being processed for molecular analysis. 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. The experiments have been reported in compliance with the ARRIVE guidelines.

2.2.RNA Preparation and Real-Time Polymerase Chain Reaction

Sample for RNA analysis were taken from the same animals used for protein measurements. 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. 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 (Giannotti et al., 2014). Briefly, an aliquot of each samples was treated with DNase to avoid DNA contamination. RNA was analyzed by TaqMan qRT-PCR instrument (CFX384 real time system, Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 wells formats in triplicate as multiplexed reactions. Data were analyzed with the comparative threshold cycle (ΔΔCt) method using 36B4 as the reference gene. The primer efficiencies were experimentally set up for each couple of primers.

Primers and probe for the BDNF's exons were purchased from Applied Biosystem (BDNF exon IV: ID Rn01484927_m1 and BDNF exon VI: ID Rn01484928_m1). Primers and probe for total BDNF and 36B4 were purchased from Eurofins MWG-Operon. Their sequences are shown below:

- total BDNF: forward primer 5'-AAGTCTGCATTACATTCCTCGA-3', reverse primer 5' GTTTTCTGAAAGAGGGACAGTTTAT-3', probe 5'-TGTGGTTTGTTGCCGTTGCCAAG-3';
- 36B4: forward primer 5'-TTCCCACTGGCTGAAAAGGT-3', reverse primer 5'-CGCAGCCGCAAATGC-3', probe 5'-AAGGCCTTCCTGGCCGATCCATC-3'.

Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reaction.

2.3. Preparation of Protein Extracts and Western Blot Analyses

As previously described (Caffino et al., 2017), bilateral punches of the sub-regions of interest were homogenized in a teflon-glass potter in cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 0.1 mM EGTA and 0.1 mM PMSF, in the presence of commercial cocktails of protease

(Roche, Monza, Italy), RNAse (Euroclone, Milan, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy) inhibitors and then sonicated. For infralimbic and prelimbic cortices, an aliquot of the remaining homogenate was 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 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%. Equal amounts of proteins (10µg) of the total homogenate were run under reducing conditions on the criterion TGX precast gels (Bio-Rad Laboratories, Milan, Italy) whereas equal amounts of proteins (8µg) of the TIF fraction 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, 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 mBDNF (1:500, Icosagen, Estonia); anti phospho-trkB Y706 (1:1000, Santa Cruz Biotechnology, USA); anti total trkB (1:750, Santa Cruz Biotechnology, USA); anti phospho-ERK2 T185/187 (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); anti GLT-1 (1:5000, Abcam, UK); anti phospho-GluN2B S1303 (1:1000, Upstate, Milan, Italy); anti GluN2B (1:1000, Santa Cruz Biotechnology, 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). Each sample was run three times and the average of the three different Western blots is shown in the histograms.

2.4. Statistical analysis

Data were collected in individual animals (independent determinations) and are presented as means ± standard errors. The effects produced by repeated cocaine treatment were analyzed by an unpaired Student's t test (GraphPad Prism v7.0, La Jolla, California, USA) assuming that both populations have the same standard deviation. Statistical significance was assumed at $p<0.05$.

3. Results

3.1 Effect of developmental exposure to cocaine on BDNF expression and signaling in different reward-related brain regions.

3.1.1 Ventral tegmental area (VTA)

Figure 1 shows a detailed analysis of the BDNF system in the VTA (panel G), region in which originates cell bodies of mesolimbic dopamine (DA) neurons. Repeated exposure to cocaine during adolescence has led to reduced mRNA levels of total BDNF (-37%, $t_{(10)}$=2.98, p=0.014), effect that should be ascribed to the reduced levels of BDNF exon IV (-45%, $t_{(10)}$=2.87, p=0.017), the most abundant isoform, sensitive to neuronal activity, and BDNF exon VI (-32%, $t_{(10)}$=2.63, p=0.025; panel A), the isoform dendritically targeted, transcripts. The transcriptional reduction of the neurotrophin was counteracted by increased expression of the mature form of BDNF (mBDNF) (+64%, $t_{(10)}$=3.19, p=0.0097; panel B); increased phosphorylation (+43%, $t_{(10)}$=4.09, p=0.002) and expression (+31%, $t_{(10)}$=2.54, p=0.029) of the high affinity BDNF receptor trkB in Y706 (panel C); increased protein levels of ERK2 (+57%, $t_{(10)}$=5.12, p=0.0005) with no change in ERK 2 T185/187 phosphorylation (+2%, $t_{(10)}$=0.16, p=0.87; panel D) and increased protein levels of Akt (+32%, $t_{(8)}$=4.82, p=0.0013) with no change in Akt phosphorylation in S473 (+13%, $t_{(10)}$=1.66, p=0.13; panel E).

3.1.2 Infralimbic cortex (IL)

Prefrontal cortical BDNF has been shown to play a role in the neuroplasticity mediated by early in life exposure to cocaine (Hinton et al., 2014): we here investigated its sub-regional expression profile. Figure 2 shows a detailed analysis of the BDNF system in the infralimbic cortex (IL) (panel G). Repeated exposure to cocaine during adolescence has led to reduced mRNA levels of total BDNF (-15%, $t_{(10)}$=2.30, p=0.045), BDNF exon IV (-19%, $t_{(10)}$=2.51, p=0.031) with no changes on BDNF exon VI (+8%, $t_{(10)}$=1.08, p=0.304; panel A); increased protein levels of the mBDNF (+28%, $t_{(9)}$=4.57, p=0.0013; panel B); reduced phosphorylation in Y706 (-34%,

$t_{(10)}$=2.34, p=0.041) and expression (+26%, $t_{(11)}$=3.30, p=0.007) of the high affinity BDNF receptor trkB (panel C); reduced ERK 2 T185/187 phosphorylation (-22%, $t_{(10)}$=2.45, p=0.034) and expression (-22%, $t_{(10)}$=2.49, p=0.032; panel D) and reduced phosphorylation of Akt in S473(-16%, $t_{(11)}$=2.54, p=0.028) with no change in Akt expression (-1%, $t_{(11)}$=0.09, p=0.929; panel E).

3.1.3 Prelimbic cortex (PL)

Figure 3 shows a detailed analysis of the BDNF system in the PL (panel G). Repeated exposure to cocaine during adolescence has led to no significant changes of total as well as exon IV and exon VI mRNA levels (total BDNF: +4%, $t_{(10)}$=1.12, p=0.291; BDNF exon IV: +8%, $t_{(10)}$=1.55, p=0.153; BDNF exon VI: -1%, $t_{(10)}$=0.181, p=0.86; panel A); reduced protein levels of the mBDNF (-29%, $t_{(10)}$=2.66, p=0.024; panel B); increased phosphorylation of trkB receptor in Y706 (+81%, $t_{(9)}$=3.46, p=0.007; panel C), ERK2 T185/187 (+31%, $t_{(11)}$=3.84, p=0.003; panel D) and Akt S473 (+19%, $t_{(11)}$=2.82, p=0.017; panel E) with no changes in the expression levels of trkB (-7%, $t_{(11)}$=0.54, p=0.599; panel C), ERK2 (-10%, $t_{(11)}$=0.98, p=0.35; panel D) and Akt (-10%, $t_{(11)}$=1.31, p=0.217; panel E).

3.1.4 Nucleus accumbens shell (sNAc)

The NAc, endpoint of the dopaminergic innervation of the mesolimbic pathway, has been shown to be composed by two anatomically and functionally distinct regions, the core and the shell, differentially involved in the addictive process. Since BDNF mRNA levels in the NAc are below detection thresholds (Altar et al. 1997; Conner et al. 1997), in this brain area we focused our analysis on BDNF protein levels and its signaling pathway. Figure 4 shows a detailed analysis of the BDNF system in the NAc shell (panel F). Repeated exposure to cocaine during adolescence has led to reduced protein levels of the mBDNF (-15%, $t_{(10)}$=2.56, p=0.029; panel A); reduced

trkB expression levels (-15% $t_{(10)}$=3.42, p=0.007) with no changes of trkB phosphorylation in Y706 (-3%, $t_{(10)}$=0.19, p=0.851; panel B); reduced phosphorylation of T185/187 ERK2 (-15%, $t_{(10)}$=2.33, p=0.042) with no change in ERK 2 expression (+6%, $t_{(10)}$=0.72, p=0.489; panel C) and reduced phosphorylation of Akt in S473 (-16%, $t_{(10)}$=2.66, p=0.024) with no change in Akt expression (+4%, $t_{(10)}$=1.34, p=0.209; panel D).

3.1.5 Nucleus accumbens core (cNAc)

Figure 5 shows a detailed analysis of the BDNF system in the nucleus accumbens core (cNAc) (panel F). Repeated exposure to cocaine during adolescence has led to reduced protein levels of the mBDNF (-19%, $t_{(10)}$=2.32, p=0.043; panel A); reduced trkB Y706 phosphorylation (-33%, $t_{(10)}$=2.66, p=0.024) and expression (-26%, $t_{(10)}$=2.64, p=0.025; panel C); no changes in ERK2 T185/187 phosphorylation and expression (pERK2: -15%, $t_{(10)}$=1.04, p=0.324; ERK2: -8%, $t_{(10)}$=0.96, p=0.36; panel D) and reduced phosphorylation of Akt in S473 (-22%, $t_{(10)}$=2.58, p=0.027) with no change in Akt expression (+1%, $t_{(10)}$=0.07, p=0.947; panel E).

3.2. Effect of developmental exposure to cocaine on GLT-1 expression in different reward-related brain regions.

Since cocaine-induced changes in glutamate transmission contribute to drug seeking behavior and since glutamatergic projections from the mPFC to the NAc and VTA make functional contacts with DA neurons, we hypothesized that, in parallel with the herein shown alteration of BDNF, adolescent cocaine exposure may have altered glutamate signaling in the mesocorticolimbic pathway. To this end, we evaluated the expression levels of the main glial glutamate transporter GLT-1 in the different brain regions investigated: this transporter removes most of removes the glutamate from the synaptic cleft and can be considered an indirect measure of synaptic glutamate levels.We found that developmental exposure to cocaine enhanced GLT-1 expression in VTA (+43%, $t_{(9)}$=2.36, p=0.043; panel A), PL cortex (+42%, $t_{(10)}$=3.06, p=0.012;

panel C) and NAc shell (+43%, $t_{(9)}$=2.55, p=0.031; panel D) whereas the transporter expression was reduced in IL cortex (-24%, $t_{(9)}$=2.38, p=0.041; panel B) and NAc core (-25%, $t_{(9)}$=3.42, p=0.008; panel E).

3.3. Effect of developmental exposure to cocaine on GluN2B expression and phosphorylation in different reward-related brain regions.

Finally, to evaluate if the altered glutamate transmission may interfere with the activation of BDNF-mediated intracellular signaling pathway in a BDNF-indipendent manner we investigated the effect of repeated exposure to cocaine during adolescence on the expression and phosphorylation of the glutamate NMDA receptor GluN2B in the IL cortex (panel A) and PL cortex (panel B). We found that developmental exposure to cocaine decreased GluN2B(S1303) phosphorylation (-27%, $t_{(10)}$=3.65, p=0.005) with no changes in GluNB total levels (+17%, $t_{(11)}$=1.56, p=0.147) whereas, in the PL cortex, GluN2B(S1303) phosphorylation was up-regulated (+65%, $t_{(8)}$=2.44, p=0.041) with no changes in GluNB total levels (+17%, $t_{(10)}$=0.73, p=0.485).

4. Discussion

It is clearly established that BDNF expression is elevated after long-term cocaine abstinence in reward-related brain regions, an effect that contributes to cocaine craving (Grimm et al., 2003). We here provide evidence that exposure to cocaine during adolescence modulates BDNF expression and signaling as early as 24h after the end of repeated psychostimulant exposure in reward-related brain regions. Although we cannot rule out that part of the effects herein shown might be due to the last injection of cocaine, we tend to exclude this possibility as 24 hours after the end of treatment rats are in a cocaine-free state.

4.1. *Adolescent exposure to cocaine alters BDNF and its associated signaling in the ventral tegmental area (VTA)*.

In the VTA, where dopaminergic neurons of the mesocorticolimbic pathway originate, cocaine promoted BDNF-trkB expression and phosphorylation, an effect that is not reflected in the activation of its intracellular signaling. In the adult animal, elevation of BDNF in the VTA either due to long-term abstinence (Grimm et al., 2003) or exogenous infusion (Lu et al., 2004) mediates incubation of cocaine-craving or potentiates relapse (Lu et al., 2004). We here show that, in the adolescent rat, 24 hours are sufficient to trigger a significant BDNF increase (although we cannot exclude that such increase is due to the treatment itself) perhaps because dopamine neurons in the VTA fire faster in adolescent than in adult rats (McCutcheon et al., 2012): accordingly, the lack of activation of the intracellular BDNF-mediated signaling in the VTA may tone down the activation of these neurons. Further, the reduced mRNA levels of total BDNF and the highly abundant activity-dependent exon IV may represent an attempt to reduce the increased BDNF expression. We hypothesize that up-regulation of BDNF levels may occur in the post-synaptic dopamine neurons, rather than representing an increased presynaptic pool ready to be released, since the reduced expression of the dendritically localized BDNF exon VI (Chiaruttini et al., 2008) might contribute to reduce BDNF release from the presynaptic cortical glutamate projections into the VTA. Since it

has been shown that reinstatement to drug-seeking is driven by disturbances in the glutamatergic transmission (Kalivas, 2009), we investigated the expression of GLT-1, i.e. the glial glutamate transporter that removes most of glutamate from the synaptic cleft (Baker et al., 2002), known to be regulated by different drugs of abuse (Roberts-Wolfe and Kalivas, 2015). In addition, BDNF modulates astrocytic GLT-1 expression via different signaling pathways (Rodriguez-Kern et al., 2003) implying a bidirectional modulation of glutamatergic homeostasis via BDNF activity (Gulyaeva, 2017): as such, GLT-1 modulation may represent a common drug-induced neuroadaptation contributing to relapse vulnerability. Interestingly, we found increased GLT-1 expression, suggesting a reduced glutamate tone that may mitigate dopamine activity, since the activation of glutamate receptors on VTA dopamine neurons enhances the *in vivo* firing rate (Georges and Aston-Jones, 2002; Zweifel et al., 2009).

4.2. *Adolescent exposure to cocaine alters BDNF and its associated signaling in the nucleus accumbens shell and core as well as in infralimbic (IL) and prelimbic (PL) cortices: modulation by glutamate*.

We next investigated the brain regions of the reward pathway receiving dopamine projections from the VTA. i.e. medial prefrontal cortex and NAc. The mPFC receives dopamine inputs from the VTA (mesocortical pathway) and sends glutamate projections back to the VTA and NAc; the NAc receives dopamine projections from VTA (mesolimbic pathway) and glutamate afferents from mPFC. In the PL cortex, in contrast to reduced mBDNF levels, we found a BDNF-independent activation of the downstream pathway. Interestingly, it has been demonstrated that infusion of BDNF in the PL cortex immediately after the last cocaine SA session suppresses cocaine seeking (Berglind et al., 2007), preventing the cocaine-induced ERK shut-off (Whitfield et al., 2011) and restoring the corticostriatal glutamate tone (Berglind et al., 2009), acting on a specific subpopulation of pyramidal neurons projecting to the NAc core. Besides trkB-induced activation, regulation of ERK activity is indeed mediated by the activation of GluN2B-containig NMDARs

(Ivanov et al., 2006). In the context of cocaine addiction, it has been shown that selective inhibition of GluN2B-containing NMDARs in PL cortex prevents the BDNF-mediated up-regulation of pERK thus blocking the neurotrophin-mediated attenuation of cocaine-seeking (Go et al., 2016). Accordingly, we decided to investigate the BDNF-independent activation of pERK by evaluating the contribution of NMDARs. Interestingly, we found an increased phosphorylation of GluN2B(S1303) in PL cortex, which increases channel conductance (Hall and Soderling, 1997), activating downstream targets such as ERK (Ivanov et al., 2006). Thus, the BDNF-independent activation of the downstream pathway may represent an early defensive strategy of PL neurons to cope with the cocaine-induced neuroadaptation in the corticolimbic pathway. Of note, we found reduced GLT-1 expression in the NAc core, as previously shown in adult rats (Scofield et al., 2016), an effect that may contribute to increase glutamate levels in the synaptic cleft via activation of cortical ERK2. Moreover, the reduced BDNF levels coupled with reduced expression and activity of BDNF and trkB in the NAc core may reflect reduced cortico-accumbal trafficking of the neurotrophin from the PL cortex, as an early attempt to oppose cocaine-seeking since infusion of BDNF in the NAc core potentiates drug-seeking behavior (Lu et al., 2004).

In the IL cortex we found a different subregional profile since mBDNF protein levels are increased whereas activity and expression of the trkB receptor as well as its downstream pathway are reduced. Infusion of BDNF in the IL cortex potentiates extinction memories through the IL cortex–hippocampus pathway (Peters et al., 2010) pointing to activation of the IL cortex as critical to modulate relapse (Augur et al., 2016). Accordingly, it is possible to speculate that the increase of BDNF in the IL may represent an early attempt to counteract seeking behaviors by potentiating the glutamate neuronal firing of pyramidal neurons projecting to the hippocampus, rather than NAc shell where BDNF levels were reduced. In fact, attenuation of BDNF-trkB activity in the NAc shell suppresses relapse (Li et al., 2013) further supporting the idea that the cocaine-mediated dampening of the BDNF system through the modulation of glutamate activity in the IL cortex-NAc shell pathway may represent an early attempt to oppose to relapse: such hypothesis is strengthened by

reduced levels of pGluN2B (S1303) in IL cortex and increased protein levels of GLT-1 in NAc shell.

A potential limitation of our results may derive from the non-contingent nature of cocaine exposure employed in the present study; however, we and others have previously demonstrated that BDNF expression is enhanced following both contingent and non-contingent drug administration (Fumagalli et al., 2013; Giannotti et al., 2014; Verheji et al., 2016; Grimm et al., 2003), suggesting that the neurotrophin up-regulation is due to the pharmacologic, rather than motivational, action of cocaine. Further, at first sight, our results may suffer from the single time-point of sacrifice following the repeated exposure to cocaine during development. However, our first aim was to investigate the changes in the BDNF system early after the end of psychostimulant exposure since the majority of manuscripts have, so far, primarily evaluated the expression of BDNF following long-term abstinence.

4.3 *Conclusion*

In conclusion, we found that repeated exposure to cocaine during adolescence modulates the BDNF system in the mesocorticolimbic pathway as early as 24h after psychostimulant exposure. Notably, while long-term abstinence appears to uniform BDNF responses in reward-related brain regions toward a general elevation of the neurotrophin (Grimm et a., 2003; Giannotti et al., 2014), the sacrifice early after the last drug exposure draws a different picture allowing the delineation of a peculiar region-dependent profile of cocaine-induced BDNF dynamics.

Based on these findings, we propose a cohesive hypothesis linking BDNF expression and signaling in the mesocorticolimbic pathways of the developing brain (Fig. 8). Prolonged exposure to cocaine during development induces BDNF expression and trkB phosphorylation in the VTA as early as 24 hours after the last drug exposure, an effect that may influence both NAc shell and core response as shown by reduced BDNF levels, which may compensate for the VTA dopamine

activation (Ren et al., 2015). The activation of BDNF signaling in the PL cortex may contribute to re-establish physiologic extracellular levels of NAc glutamate, via glutamate afferent from the PL cortex, thus inhibiting cocaine-seeking; concomitantly, the dampened activation of the IL cortex may oppose to relapse via glutamate activity modulation in the IL cortex-sNAc pathway. Perhaps, as abstinence prolongs, such mechanisms become exhausted and are no longer working thus contributing to the increased expression of BDNF in the NAc that contributes to drive the incubation of cocaine craving (Grimm et al., 2003). These results, identifying a complex but specific set of changes of the BDNF system in the mesolimbic pathway, may serve to pave the way for future functional studies that could manipulate this pathway by chemogenetic approaches or miRNAs.

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Financial Disclosures/Conflicts of Interest

The authors report no conflicts of interest.

Figure legends

Figure 1. Effect of developmental exposure to cocaine on BDNF expression and signaling in the ventral tegmental area (VTA). Rats were treated with cocaine from PND 28 to PND 42. Twenty-four hours after the end of treatment, i.e. on PND 43, rats were sacrificed.

In saline- and cocaine-treated rats, we examined: total, exon IV and exon VI BDNF mRNA levels (panel A); mBDNF protein levels (panel B); trkB phosphorylation and expression (panel C); ERK2 phosphorylation and expression (panel D) and Akt phosphorylation and expression (panel E). Representative immunoblots are shown for ptrkBY706 and trkB (145 kDa), pAktS473 and Akt (60 kDa), pERK2T185-187 and ERK2 (42 kDa), mBDNF (14 kDa) and -actin (43 kDa) proteins in the VTA homogenates (panel F). Panel G shows a representative brain section with VTA region punched highlighted in black.

The results, expressed as % of saline-treated rats, represent the mean ± S.E.M. of at least 5 samples; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. saline-treated rats.

S = saline; C = cocaine

Figure 2. Effect of developmental exposure to cocaine on BDNF expression and signaling in the infralimbic (IL) cortex. Rats were treated with cocaine from PND 28 to PND 42. Twenty-four hours after the end of treatment, i.e. on PND 43, rats were sacrificed.

In saline- and cocaine-treated rats, we examined: total, exon IV and exon VI BDNF mRNA levels (panel A); mBDNF protein levels (panel B); trkB phosphorylation and expression (panel C); ERK2 phosphorylation and expression (panel D) and Akt phosphorylation and expression (panel E). Representative immunoblots are shown for ptrkBY706 and trkB (145 kDa), pAktS473 and Akt (60 kDa), pERK2T185-187 and ERK2 (42 kDa), mBDNF (14 kDa) and -actin (43 kDa) proteins in the IL homogenates (panel F). Panel G shows a representative brain section with IL region punched highlighted in black.

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

S = saline; C = cocaine

Figure 3. Effect of developmental exposure to cocaine on BDNF expression and signaling in the infralimbic (PL) cortex. Rats were treated with cocaine from PND 28 to PND 42. Twenty-four hours after the end of treatment, i.e. on PND 43, rats were sacrificed.

In saline- and cocaine-treated rats, we examined: total, exon IV and exon VI BDNF mRNA levels (panel A); mBDNF protein levels (panel B); trkB phosphorylation and expression (panel C); ERK2 phosphorylation and expression (panel D) and Akt phosphorylation and expression (panel E). Representative immunoblots are shown for ptrkBY706 and trkB (145 kDa), pAktS473 and Akt (60 kDa), pERK2T185-187 and ERK2 (42 kDa), mBDNF (14 kDa) and -actin (43 kDa) proteins in the PL homogenates (panel F). Panel G shows a representative brain section with PL region punched highlighted in black.

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

S = saline; C = cocaine

Figure 4. Effect of developmental exposure to cocaine on BDNF expression and signaling in the nucleus accumbens shell (NAc shell). Rats were treated with cocaine from PND 28 to PND 42. Twenty-four hours after the end of treatment, i.e. on PND 43, rats were sacrificed.

In saline- and cocaine-treated rats, we examined: mBDNF protein levels (panel A); trkB phosphorylation and expression (panel B); ERK2 phosphorylation and expression (panel C) and Akt phosphorylation and expression (panel D). Representative immunoblots are shown for ptrkBY706 and trkB (145 kDa), pAktS473 and Akt (60 kDa), pERK2T185-187 and ERK2 (42 kDa), mBDNF (14 kDa) and β-actin (43 kDa) proteins in the sNAc homogenates (panel E). Panel F

shows a representative brain section with NAc shell region punched highlighted in black.

The results, expressed as % of saline-treated rats, represent the mean ± S.E.M. of 6 samples; * $p<0.05$ and ** $p<0.01$ vs. saline-treated rats.

S = saline; C = cocaine

Figure 5. Effect of developmental exposure to cocaine on BDNF expression and signaling in the nucleus accumbens core (NAc core). Rats were treated with cocaine from PND 28 to PND 42. Twenty-four hours after the end of treatment, i.e. on PND 43, rats were sacrificed.

In saline- and cocaine-treated rats, we examined: mBDNF protein levels (panel A); trkB phosphorylation and expression (panel B); ERK2 phosphorylation and expression (panel C) and Akt phosphorylation and expression (panel E). Representative immunoblots are shown for ptrkBY706 and trkB (145 kDa), pAktS473 and Akt (60 kDa), pERK2T185-187 and ERK2 (42 kDa), mBDNF (14 kDa) and β-actin (43 kDa) proteins in the NAc core homogenates (panel E). Panel F shows a representative brain section with NAc core region punched highlighted in black.

The results, expressed as % of saline-treated rats, represent the mean ± S.E.M. of 6 samples; * $p<0.05$, vs. saline-treated rats.

S = saline; C = cocaine

Figure 6. Effect of developmental exposure to cocaine on GLT-1 expression in different brain subregions. Rats were treated with cocaine from PND 28 to PND 42. Twenty-four hours after the end of treatment, i.e. on PND 43, rats were sacrificed. GLT-1 protein levels were measured in VTA (panel A), IL cortex (panel B), PL cortex (panel C), NAc shell (panel D) and NAc core (panel E). Representative immunoblots are shown below the respective graphs.

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

Figure 7. Effect of developmental exposure to cocaine on GluN2B expression and phosphorylation in different brain regions. Rats were treated with cocaine from PND 28 to PND 42. Twenty-four hours after the end of treatment, i.e. on PND 43, rats were sacrificed. GluN2B phosphorylation in S1303 and expression were measured in IL cortex (panel A) and PL cortex (panel B). Representative immunoblots are shown below the respective graphs.

The results, expressed as % of saline-treated rats, represent the mean ± S.E.M. of at least 4 samples; * $p<0.05$ and ** $p<0.01$ vs. saline-treated rats.

Figure 8. Schematic representation of the changes of BDNF and its associated network caused by exposure to cocaine during development.

The solid line shows the dopaminergic projections from VTA to NAc and from VTA to PFC. The dotted line shows the glutamatergic projections from PFC to NAc and to VTA. The single brain (sub)regions are enlarged to show the summary of BDNF-related changes.

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