Anhedonic-like behavior and BDNF dysregulation following a single injection of cocaine during adolescence

Lucia Caffino[1], Francesca Mottarlini[1], Jessica Mingardi[2], Gian Maria Zita[3], Alessandro Barbon[2] and Fabio Fumagalli[1]

[1]Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Via Balzaretti 9, 20133 Milano, Italy; [2]Biology and Genetic Division; Department of Molecular and Translational Medicine; University of Brescia; Brescia, Italy; [3] Dipartimento di Salute Mentale e Dipendenze, ASST Fatebenefratelli-Sacco, Milan, Italy.

*Corresponding author: Fabio Fumagalli, Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Via Balzaretti 9, 20133 Milano, Italy Phone 39-2-50318298; E-mail: Fabio.Fumagalli@unimi.it

Running title: Adolescent cocaine exposure causes anhedonia and alters BDNF signaling

Conflict of interest

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

Abstract

We have previously demonstrated that a single exposure to cocaine during adolescence causes several behavioural and neurobiological changes, highlighting the unique vulnerability of this period of life. The purpose of our work was to investigate whether a single exposure to cocaine during brain development is sufficient to shape a negative emotional state in adolescent rats.

A single injection of cocaine during adolescence followed by measurement of sucrose consumption, a measure of anhedonia, identifies two separate groups of rats, i.e. anhedonic (AN) and non anhedonic (NON-AN) rats. AN rats show reduced ability to synthesize, traffic and translate the neurotrophin at synaptic level, reduced activation of hippocampal BDNF signaling, reduced BDNF plasma levels and a steep rise of corticosterone secretion. Conversely, NON-AN rats exhibit reduced trafficking of BDNF while up-regulating hippocampal BDNF synthesis and stabilizing its downstream signaling with no changes of BDNF and corticosterone plasma levels. Adult rats showed no signs of anhedonia, an increase of BDNF both in hippocampus and plasma and decreased levels of corticosterone.

In conclusion, our findings reveal a complex central and peripheral dysregulation of BDNF-related mechanisms that instead are preserved in NON-AN rats, suggesting that BDNF modulation dictates behavioural vulnerability vs. resiliency to cocaine-induced anhedonia, a profile uniquely restricted to adolescent rats.

Key words: BDNF, cocaine, hippocampus, anhedonia, corticosterone.

1. Introduction

It is well established that adolescence is a period of unique susceptibility to drug abuse (Andersen 2003; Ernst *et al.* 2009; Spear 2000; Kelley 2004; Casey *et al.* 2008). We and others have contributed to demonstrate that interfering with such vulnerable period through exposure to cocaine may alter the correct trajectories of brain development causing functional consequence through behavioural, molecular and structural changes (Giannotti *et al.* 2014; Caffino *et al.* 2015a; Caffino *et al.* 2015b; Caffino *et al.* 2017b; Caffino *et al.* 2018; Caputi *et al.* 2019; Caffino *et al.* 2017a; DePoy *et al.* 2014). In addition, we have shown that also a single exposure to cocaine during this vulnerable period may be functionally relevant. In fact, we have recently demonstrated that a single injection of the psychostimulant during brain development is sufficient to disrupt actin dynamics (Caffino *et al.* 2017b) and to alter dendritic spine morphology (Caffino *et al.* 2018). In antoher manuscript, we found that a single exposure to cocaine reduced the expression of the trophic factor FGF-2 when measured 7 days after the single injection (Giannotti *et al.* 2015), suggesting a long-lasting effect on a molecule implicated in depression (Evans *et al.* 2004; Riva *et al.* 2005) and whose expression has been shown to be up-regulated by antidepressant treatments in rodents (Maragnoli *et al.* 2004; Bachis *et al.* 2008). The latter finding is indeed interesting since it raises the intriguing possibility, yet unexplored, that a single injection of cocaine during a sensitive period of brain development may be sufficient to cause a negative emotional state in rodents.

Thus, our major aims were 1) to investigate whether a single injection of cocaine during adolescence is sufficient to cause negative emotional state through the evaluation of one of its core symptoms, i.e. anhedonia, and 2) to find a putative, underlying molecular mechanism. To this end, we have focused our attention on Brain Derived Neurotrophic Factor (BDNF), since this neurotrophin has been implicated in the dysregulation of emotional states (Fumagalli *et al.* 2007; Bjorkholm & Monteggia 2016), cognition (Kesslak *et al.* 1998; Bekinschtein *et al.* 2014; Leo *et al.* 2018) as well as in the action of psychostimulants (Fumagalli *et al.* 2013; Li & Wolf 2015; Verheij *et al.* 2016; Leo *et al.* 2018). Further, evidence exists showing that the modulation of hippocampal BDNF directly controls anhedonia (Taliaz *et al.* 2010; Dong *et al.* 2018). The hippocampus is an important part of the limbic system involved in the regulation of emotion and cognition. Although it has not been extensively studied with respect to drug abuse, it is integrated anatomically and functionally into the addiction network and it interacts with addiction-related areas (Castilla-Ortega *et al.* 2016): for these reasons, neuroplastic changes in this brain region may be critical for the effects of drugs of abuse.

We thus have investigated the effect of a single injection of cocaine during adolescence in rats exposed to the sucrose preference test. Accordingly, adolescent rats at post-natal day (PND) 35 were first exposed to a single cocaine injection or saline and, a week later (i.e. at PND42), the same rats were subjected to the sucrose preference test to measure anhedonia. Behavioural measurements were accompanied by in-depth evaluation of BDNF levels both in the brain (hippocampus) and plasma. Further, levels of corticosterone in the plasma and hippocampal glucocorticoid receptor expression were measured, as activation of hypothalamic-pituitary adrenal (HPA) axis is strictly correlated to negative emotional states. In order to verify that changes set in motion by a single exposure to cocaine during brain development could be specific of adolescence or, rather, common to adulthood, we exposed adult rats to the same experimental paradigm, performing the same behavioural and molecular evaluations in these rats.

2. Material and Methods

2.1 Experimental procedures

Adolescent and adult 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 & Stern 1978).

Rats were shipped to us at post-natal day 28. They were habituated in the facility before starting the cocaine exposure for one week. In details, rats were left undisturbed for two days and then they were handled for 5 days before the injection: such manipulation was performed in order to avoid any potential bias due to stress-related effects.

Experiment 1. Forty rats were exposed to a single intraperitoneal injection (i.p.) of saline (n = 12) or cocaine (n = 28) (20 mg/kg) at postnatal day 35 (PND 35) and 7 days later, i.e. at PND 42, during a period that roughly approximates adolescence in humans (Collins & Izenwasser 2004; Maldonado & Kirstein 2005), animals were exposed at the sucrose preference test and three hours later they were sacrificed. A total of 2 cocaine- and 2 saline-exposed animals were removed from the study because they did not respond properly to the sucrose preference test. At the scheduled time of sacrifice, the whole hippocampus (Hip) from 7 saline- and 20 cocaine-exposed rats was grossly dissected from 2-mm thick slices, frozen on dry ice and stored at -80°C to perform the molecular analysis; whereas the whole brain from 3 saline- and 6 cocaine-exposed rats was removed and post-fixed in PFA 4% for 24 hours to perform in situ hybridization.

Experiment 2. To investigate whether the effects of a single cocaine administration were different between adolescent and adult animals, we incorporated 22 adult rats that were treated with saline or cocaine i.p. (20 mg/kg) at postnatal day 70 and then, at PND 77, they were exposed at the sucrose preference test and three hours later sacrificed. A total of 3 cocaine- and 1 saline-treated rats were removed from the study because they did not respond properly to the sucrose preference test. At the scheduled time of sacrifice, the whole hippocampus (Hip) was grossly dissected from 2-mm thick slices, frozen on dry ice and stored at -80°C.

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 Sucrose preference test

The sucrose preference test (SPT) was used to assess anhedonia at PND 42 or at PND 77 in rats exposed to a single injection of cocaine at PND 35 or at PND70, respectively. Rats were habituated to 2 water bottles for 5 days and exposed to water deprivation overnight (19:00-9:00 hours) the night before the test day. During a 1-hour session (9:00-10:00 hours), rats were housed individually and given access to two bottles, positioned side-by-side at the rear of the cage, one containing regular water and the other containing 1% sucrose solution diluted in tap water. At the end of the test, all animals are returned to group housing with food and water *ad libitum*.

The solution has been allowed to warm to room temperature (22 °C ± 1 °C) 1 h before introducing to animals. Before and after consumption, fluid was measured by weighing each bottle. Sucrose preference was calculated as sucrose intake(g)/total fluid (sucrose + water) intake (g) X 100. Rats showing a sucrose/(total fluid intake) ratio above 0.5 were defined as NON-anehdonic, and those showing a lower sucrose/(total fluid intake) ratio were defined as anhedonic. This criterion was based on the fact that the averaged ratio of the control animals group showed a sucrose/(total fluid intake) ratio above 0.5 (Strekalova *et al.* 2006).

2.3 RNA Preparation and Real-Time Polymerase Chain Reaction

RNA measures were taken in the same animals as the protein measures. Total RNA was isolated by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Segrate, Milan, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. 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 (Caffino *et al.* 2019).

The primer efficiencies were experimentally set up for each couple of primers. Probe and primers for and *Bdnf exon VI* were purchased from Life Technologies (*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';

- *Bdnf exon I*: forward primer 5'-GGGAGACGAGATTTTAAGACACTG-3', reverse primer 5'-GTCATCACTCTTCTCACCTGG-3', probe 5'-TTGTGGCTTTGCTGTCCTGGAGA-3';

- *36B4*: forward primer 5'-TTCCCACTGGCTGAAAAGGT-3', reverse primer 5'- CGCAGCCGCAAATGC-3', probe 5'-AAGGCCTTCCTGGCCGATCCATC-3'.

2.4 Riboprobes preparation for in situ hybridization

Digoxigenin (DIG)-labeled riboprobes detecting *total Bdnf* and *Bdnf exon VI* transcripts were generated from PCR templates adapted with SP6 and T7 RNA polymerase sites (Tornese *et al.* 2019). Riboprobes were transcribed using a DIG RNA Labeling Kit (Thermo Scientific, Monza, MB, Italy), according to the manufacturer's instructions. Specifically, the 20 µl transcription mixture included 200 ng of template purified cDNA, 1x Transcription Buffer, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.17 mM UTP, 0.33 mM DIG-UTP (Life Technologies, Monza, MB, Italy), and 40 U of either T7 or SP6 RNA polymerase (Russo *et al.* 2013). The reaction was incubated for 2 h at 37° C and stopped by adding 0.5 M EDTA. The riboprobes were then purified in NucAwayTM spin columns (Ambion, Monza, MB, Italy) and quantified with Nanodrop 1000 (Thermo Scientific, Monza, MB, Italy).

2.5 In situ hybridization

After sacrifice, brains were fixed in the 4% PFA solution for 24 h and subsequently placed in 30% sucrose before sectioning. Coronal slices (40 µm) were prepared and stored in cryo-protectant sectioning buffer (30% ethylene glycol, 30% glycerol and 0.05M phosphate buffer) at -20°C until further processing.

In situ hybridization experiments on brain slices were carried out under RNAse-free conditions. In situ hybridization was developed using the Vectastain® Elite ABC-Peroxidase Staining Kit (Vector Laboratories, Segrate, MI, Italy) (La Via *et al.* 2013; Russo *et al.* 2013). Free-floating sections were post-fixated over-night in 4% PFA at 4°C. Following 3 washes in 0.1 Tween 20 in 1x PBS (PBST) at RT for 5 min the slices were quickly washed in H2O, permeabilized with 2.3% sodium meta-periodate (Sigma-Aldrich, Milano, Italy) in H_2O at RT for 5 min, and then quickly washed in H2O. After these steps, the sections were incubated in 1% sodium borohydride (Sigma-Aldrich, Milano, Italy) in 0.1 M Tris-HCl buffer pH 7.5 at RT for 10 min, and then washed twice in PBST at RT for 3 min. The slices were digested with 8 µg/ml proteinase K (Sigma-Aldrich, Milano, Italy) in PBST at RT for 20 min and washed twice in PBST at RT for 5 min. After digestion, the tissue slices were fixed in 4% PFA at RT for 5 min and washed three times in PBST at RT for 10 min. Slices were then incubated ON at 55° C in the hybridization solution containing 20 mM Tris-HCl (pH7.5) (Sigma-Aldrich, Milano, Italy), 1 mM EDTA (Gibco, Monza, MB, Italy),

1x Denhardt's solution (Invitrogen, Monza, MB, Italy), 300 mM NaCl, 100 mM DTT (Fluka, Milano, Italy), 0.5 mg/ml salmon sperm DNA (Gibco, Monza, MB, Italy), 0.5 mg/ml polyadenylic acid (Sigma-Aldrich, Milano, Italy), and 50% formamide (Sigma-Aldrich, Milano, Italy), supplemented with 10% dextransulfate and 100 ng/ml DIG-labeled riboprobes. The next day, the slices were washed twice in 2x saline sodium citrate, 0.1 % Tween 20 (SSCT), and 50% deionized formamide at 55° C for 30 min; for 20 min in 2x SSCT at 55° C; and twice in 0.2 x SSCT at 60° C for 30 min. Subsequently, the slices were detected using the peroxidase method with biotinylated donkey anti-mouse IgG antibodies and diaminobenzidine as chromogen (Vector Laboratories, Segrate, MI, Italy). The images of in situ hybridization were acquired using LSM510 Meta (Carl Zeiss, Germany) confocal microscope, and the maximal distance of hybridization signal in dendrites (maximal distance of dendritic labeling) was determined by AxionVision LE64 (Zeiss, Milano, Italy) using the function Measure Length. Dendrites of pyramidal neurons in CA1 and CA3 hippocampal regions have been analyzed; 3 rats for group and 2-3 slices for rat were analyzed. All the experiments were coded and analyzed in a blinded manner.

2.6 Preparation of Protein Extracts and Western Blot Analyses

Proteins were extracted as previously described (Caffino *et al.* 2018) with minor modifications. Briefly, Hip was homogenized in a teflon-glass potter in cold 0.32M sucrose buffer pH 7.4 containing 1 mM HEPES, 1 mM MgCl2 1 mM NaHCO3 and 0.1 mM PMSF, in presence of commercial cocktail 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 1000 g for 10 min obtaining a pellet (P1) corresponding to the nuclear fraction. The supernatant (S1) was then centrifuged at 9000g for 15 min to obtain a clarified fraction of cytosolic proteins (S2) and a pellet (P2). The pellet (P2), corresponding to a crude membrane fraction, was resuspended in a buffer containing 75mM KCl and 1% Triton X-100 and centrifuged at 100,000 x g for 1h. 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 20mM HEPES, protease and phosphatase inhibitors and stored at −20°C in presence of glycerol 30%. Total proteins have been measured in the total homogenate, in P1, S2 and TIF fractions according to the Bio-Rad Protein Assay, using bovine serum albumin as the calibration standard (Bio-Rad Laboratories, Segrate, Milan, Italy). Equal amount of proteins (10 ug) were run on criterion TGX precast gels (Bio-Rad Laboratories) under reducing conditions and electrophoretically transferred onto nitrocellulose membrane (Bio-Rad Laboratories). Blots were blocked 1 h at room temperature with I-Block solution (Life Technologies Italia,

Italy) 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 phospho-Akt S473 (1:1000, Cell Signaling Technology, USA); anti total Akt (1:1000, Cell Signaling Technology, USA); anti GR (1:500, ThermoScientific, USA). 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) and analyzed using the Image Lab software from Bio-Rad. Gels were run 3 times each and the results represent the average from 3 different western blots.

2.6 Analysis of plasma corticosterone and BDNF levels

Samples of blood from each rat were collected in tubes containing EDTA (250 μL x 2 mL of blood collected) as anticoagulant agent. Plasma was separated by centrifugation (6500 g for 10 min). Corticosterone and BDNF levels were determined by an enzyme-linked immunosorbent assay (ELISA) using a commercial kit according to the manufacturers' instructions (Tecan, Italy; AbCam, UK, respectively).

2.7 Statistical analysis

Data were collected in individual animals (independent determinations) and are presented as means and standard errors. Behavioral and molecular changes produced by cocaine treatment during adolescence were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Molecular changes of in situ hybridization produced by cocaine treatment during adolescence were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Behavioral and molecular changes produced by cocaine treatment during adulthood were analyzed by unpaired Student's t-test. Pearson's product-moment coefficients were calculated to study potential correlations between molecular and behavioral measurements in the pooled group of control, AN and NON-AN rats. Subjects were eliminated from the final dataset if their data deviated from the mean by 2 SDs. Prism 8.0 (GraphPad) was used to analyze all the data. Statistical significance was assumed at $p<0.05$.

3. Results

Seven days after a single injection of cocaine or saline during adolescence, rats were exposed to the sucrose preference test. Sucrose preference ($F_{(2,33)}$=31.50, p<0.0001), shown in Fig.1a, allowed the identification of two separated populations of cocaine-exposed rats: those preferring water over sucrose (-46%, p=0.0013 vs saline) named anhedonic rats (from now on called AN rats) and those preferring sucrose over water (+44%, p=0.0035 vs saline; +90%, p<0.0001 vs AN), named non anhedonic rats (from now on called NON-AN rats). Importantly, the test was not influenced by the amount of water drunk by rats as both AN and NON-AN rats drank a similar amount of liquid (measured as the amount of water sucrose-free + water added with sucrose; $F_{(2,33)}$=1.078, p=0.3521) (Fig. 1b), suggesting that the reduced sucrose intake in the AN group of rats is not linked to thirsty-related problems.

In an attempt to find a potential mechanism to explain, at least partially, the difference between AN and NON-AN rats, we focused our attention on the neurotrophin BDNF, i.e. a molecule whose hippocampal regulation has been tightly linked to negative emotional states (Smith *et al.* 1995; Bjorkholm & Monteggia 2016). One-way ANOVA revealed that total *Bdnf* mRNA levels ($F_{(2,22)}$=14.74, p<0.0001) were reduced in the hippocampus of AN rats (-31% vs saline, p=0.0494; -64% vs NON-AN, p<0.0001) while increased in the same brain region of NON-AN rats (+33% vs saline, p=0.0438) (Fig. 2a). We next measured the expression of specific BDNF isoforms, *Bdnf exon I, Bdnf exon IV*, known to be activity-dependent and localized in the soma, and *Bdnf exon VI*, known to be targeted to dendrites (Chiaruttini *et al.* 2008). One-way ANOVA revealed a reduction of *Bdnf exon I* ($F_{(2,24)}$=6.219, p=0.0067) *and Bdnf exon IV* mRNA levels ($F_{(2,24)}$=8.279, p=0.0018) in the hippocampus of only AN rats (*Bdnf exon I:* -18% vs saline, p=0.0187; -18% vs NON-AN, p=0.0165; *Bdnf exon IV:* -16% vs saline, p=0.0017; -11% vs NON-AN, p=0.0402). One-way ANOVA indicated a reduction of *Bdnf exon VI* mRNA levels ($F_{(2,24)}$=4.579, p=0.0207; Fig 2d) both in AN (-14% vs saline, p=0.0362) and NON-AN rats (-15% vs saline, p=0.0297). As a further step of BDNF transcripts characterization, we performed *in situ* hybridization experiments (Figure 2g) to assess changes in *Bdnf* mRNA dendritic localization, since local translation and release of BDNF at synapses is likely dependent on dendritic transcripts. A moderate but significant decrease in the trafficking of *total Bdnf* mRNA was found in the CA3 region of hippocampus ($F_{(2,631)}$=11.65, p<0.0001; Fig 2e) of both AN (-5.95μm vs saline, p=0.0003) and NON-AN (-5.9μm vs saline, p<0.0001). A similar pattern was found for *Bdnf exon VI* trafficking ($F_{(2,788)}$=13.06, p<0.0001; AN: -5.18μm vs saline, p=0.0001; NON-AN: -5.81μm vs saline, p<0.001 Fig 2f), while *Bdnf* dendritic trafficking was only marginally affected in CA1 region of Hippocampus (total *Bdnf*: $F_{(2,696)}$=3.022, p=0.05, Fig 2e; *Bdnf exon VI*: $F_{(2,761)}$=3.568, p=0.0287, NON-AN: -2.1318μm vs saline, p=0.0328, Fig 2d).

To determine whether changes in transcription paralleled changes in translation, we examined

the expression of BDNF and its high affinity receptor trkB in the Hip homogenate and postsynaptic density (PSD), where synaptic transmission takes place, of cocaine-treated rats in comparison with saline-treated rats. These analyses are crucial to dissect the effect of adolescent cocaine exposure on BDNF protein translation, i.e. whole homogenate, from the availability of the neurotrophin at synaptic sites, i.e. PSD fraction. Figure 3a shows that the expression of the mature form of BDNF (mBDNF) was reduced in the homogenate of hippocampus ($F_{(2,24)}$=18.24, p<0.0001) of AN rats (-14% vs saline, p=0.0496; -32% vs NON-AN, p<0.0001) while increased in the same brain region of NON-AN rats (+18%, p=0.0169). In the post-synaptic density, mBDNF levels ($F_{(2,23)}$=6.091, p=0.0075; Fig 3b) were downregulated in the hippocampus of AN rats (-21% vs saline, p=0.0171; -19% vs NON-AN, p=0.0237) while unaltered in the same brain region of NON-AN rats (-2% vs saline, p=0.9689). We next measured the expression and activation of the BDNF high affinity receptor trkB (Fig. 3c, d). In the homogenate (Fig 3c), we did not find any significant difference for both the phosphorylated form of trkB in Y706 ($F_{(2,22)}$=0.3075, p=0.7384) and trkB total levels ($F_{(2,24)}$=1.142, p=0.3360). Conversely, in the postsynaptic density (Fig 3d), we found that the phosphorylation of trkB in Y706 ($F_{(2,23)}$=10.51, p=0.0006) was significantly reduced in the hippocampus of AN (-29% vs saline, p=0.0054; -33% vs NON-AN, p=0.0009) whereas no changes were observed in the same brain region of NON-AN rats (+4% vs saline, p=0.9033). Similarly, trkB protein levels ($F_{(2,24)}$=6.582, p=0.0053) were reduced in the hippocampus of AN (-26% vs. saline, p=0.0073; -19% vs. NON-AN, p=0.0346) but not in the same brain region of NON-AN rats (-7% vs. saline, p=0.6864).

Next, we examined the main BDNF-dependent intracellular pathways, i.e. Akt and Erk2, in the homogenate of AN and NON-AN rats. The analysis of the ratio pAkt S_{473}/Akt ($F_{(2,23)}$=11.37, p=0.0004) revealed a reduction in AN rats (-20% vs. saline, p=0.0357; -33% vs. NON-AN, p=0.0003) with no changes in NON-AN rats (+13% vs. saline, p=0.2254) (Fig. 4a). Similarly, the analysis of the ratio pErk2 $T_{185/187}$/Erk2 ($F_{(2,24)}$=9.947, p=0.0007) showed a reduction in AN rats (-29% vs. saline, p=0.0023; -26% vs. NON-AN, p=0.0031) with no changes in NON-AN rats (-3% vs. saline, p=0.9311) (Fig. 4b).

To investigate the potential relationship between sucrose preference and mBDNF levels we performed a Pearson's product–moment correlation analysis. As shown in Figure 5, Pearson's correlation analysis revealed that sucrose preference correlates positively with mBDNF levels in both homogenate (r=0.5953, p=0.0011, Fig. 5a) and post-synaptic density (r=0.4340, p=0.0267, Fig. 5b).

We next measured the levels of the neurotrophin in the plasma as it has been previously observed that plasma BDNF levels are reduced during negative emotional states in humans (Lee *et al.* 2007). One-way ANOVA revealed that BDNF levels ($F_{(2,24)}$=5.468, p=0.011) are significantly reduced in the plasma of AN (-345 pg/μl vs. saline, p=0.008), but not NON-AN (-216 pg/μl vs. saline, p=0.1387),

rats (Fig. 6).

The following step was to measure glucocorticoid release in the two subpopulations of rats, as the hypothalamic-pituitary adrenal (HPA) axis has been shown to be dysfunctional during negative emotional states (Zorn *et al.* 2017). We found increased corticosterone levels in the plasma of AN rats ($F_{(2,23)}$=9.551, p=0.001; +3.89 ng/ml vs saline, p=0.0267; +5.62 ng/ml vs NON-AN, p=0.001) with no significant variations from control levels in NON-AN rats (-1.73 ng/ml vs saline, p=0.4869) (Fig. 7a). Of note, Pearson's correlation analysis revealed that sucrose preference negatively correlates with circulating corticosterone levels (r=-0.6311, p=0.0013, Fig. 7b). It is widely established that corticosterone binds to glucocorticoid receptors (GR) that, by moving into the nucleus, regulate the expression of downstream genes through the binding to DNA response elements (de Kloet *et al.* 2005). Thus, we measured the ratio between GR expression in the nucleus and cytoplasm, an index of GR translocation into the nucleus. We found a significant reduction of such ratio in both AN ($F_{(2,24)}$=23.89, p<0.0001; -46% vs saline, p<0.0001) and NON-AN (-21% vs saline, p=0.0193) rats when compared to saline-treated rats; however, the reduction was significantly lower in AN rats when compared to NON-N rats (-25% vs NON-AN, p=0.0014, Fig. 7c).

In order to investigate whether the behavioural and molecular changes above shown are peculiar of adolescence or, instead, common to adulthood, we exposed adult rats to the same treatment and analysed the same behavioural and molecular parameters. The sucrose preference test revealed that all rats preferred the sucrose solution (Fig. 8a). Adult rats exposed to a single injection of cocaine exhibited increased levels of mBDNF (+21%, $t_{(16)}$=2.787, p=0.0132, Fig. 8b), enhanced phosphorylation and expression of trkB (ptrkB: +22%, $t_{(16)}$=2.299, p=0.0353; trkB: +19%, $t_{(16)}$=3.203, p=0.0055; Fig. 8c) whereas no changes were observed for the ratio pAkt S_{473}/Akt and pErk2 $T_{185/187}$/Erk2 (data not shown). Peripheral analyses revealed that a single injection of cocaine up-regulated BDNF expression in the plasma of adult rats (+504 pg/μl, $t_{(16)}$=2.841, p=0.0118; Fig. 8d) while reducing corticosterone plasma levels (-7.35 ng/ml, $t_{(16)}$=2.245, p=0.0392; Fig. 8e).

4. Discussion

Our findings show that a single injection of cocaine during adolescence identifies two separated, emotionally distinct subpopulations of rats in response to the sucrose preference test, i.e. anhedonic (AN) and non-anhedonic (NON-AN) rats. Such clustering is accompanied by a different modulation of the neurotrophin BDNF at both central and peripheral level.

Anhedonia has been critically associated to abstinence in humans (Garfield *et al.* 2017; Gawin & Kleber 1986) and withdrawal in rodents (Markou & Koob 1991; Scheggi *et al.* 2011) following repeated exposure to drugs of abuse. Our results represent the first evidence that a single exposure to cocaine during adolescence is sufficient to shape an anhedonic phenotype in rats, rewriting the standard view that prolonged exposure to drugs of abuse is necessary to cause depressive-like symptoms. Indeed, albeit occurring after a single cocaine injection, such effects are far from being simply acute as they last, at least, for a week. Of note, the lack of an anhedonic-like behaviour in adult rats exposed to the same treatment bolsters the notion that the adolescent brain is uniquely receptive to the emotional effects caused by a single cocaine exposure.

Vulnerability or resiliency to anhedonia following a single cocaine exposure during adolescence seems to depend upon the modulation of BDNF, both at central and peripheral level. In the homogenate of hippocampus of adolescent AN rats, we found a significant reduction of *Bdnf* mRNA and protein levels, of the high affinity receptor trkB as well as of major BDNF-mediate intracellular pathways, such as Akt and ERK2. Notably, in adolescent AN rats, BDNF expression was reduced also at the synaptic level. Intriguingly, adolescent AN rats show reduced BDNF plasma levels as well. The evidence that we found a significant association between reduced BDNF protein levels (both in the homogenate and in the post-synaptic density) and the anhedonic behaviour fosters the possibility that a decrease of hippocampal BDNF signalling may contribute to the anhedonic phenotype, an effect that may be strengthened by the reduced peripheral levels of the neurotrophin. Of note, the contribution of reduced BDNF levels to an anhedonic phenotype has been recently suggested by Martin and associates in an elegant paper employing BDNF heterozygous mice (Martis *et al.* 2019). Additionally, we also found reduced mRNA trafficking at dendrites in AN rats suggesting both reduced trafficking and local protein synthesis of the neurotrophin. Thus, it appears that vulnerability to AN critically depends upon reduced BDNF synaptic levels, perhaps lowering the threshold to develop anhedonic behaviour. Conversely, in NON-AN rats, BDNF expression is increased in the whole homogenate, leading to a stabilization of its downstream signalling, unaltered in the synaptic fraction and reduced as mRNA transport raising the possibility that NON-AN rats compensate the reduced trafficking of *Bdnf* mRNA by up-regulating the protein levels of the

neurotrophin: it is suggested that their resiliency to AN relies on their ability to create an hippocampal reservoir of the neurotrophin to be used in case of demand (Taliaz *et al.* 2011). Strikingly, this is in line with recent evidence showing higher mBDNF levels in the hippocampus of rats resilient to chronic mild stress (Tornese *et al.* 2019) suggesting that the elevation of the hippocampal expression of this neurotrophin may be considered a reliable marker of resiliency that may be independent from the experimental context. Under our experimental conditions, such resiliency may be corroborated by the evidence that BDNF levels in the plasma of NON-AN adolescent rats are maintained at control levels.

Intriguingly, 7 days after a single injection of cocaine, AN rats exhibited significantly higher levels of circulating glucocorticoids compared to controls at variance from NON AN rats whose glucocorticoid levels were unaltered. Our results recapitulate the elevation of glucocorticoid release previously found in cocaine-withdrawn rats (Zhou *et al.* 2011; Georgiou *et al.* 2016) and abstinent cocaine users (Gawin & Kleber 1985; Kalechstein *et al.* 2002; Contoreggi *et al.* 2003; Morie *et al.* 2014) thus supporting the translational validity of these findings and further pointing to adolescence as a critical period of high vulnerability since it exacerbates the effect of a single exposure to cocaine. We hypothesize that the markedly reduced nuclear translocation of GR in AN rats, at variance from the attenuated decline observed in NON-AN rats, tones down the natural feedback on the HPA axis, thus representing a mechanism to explain the elevation of corticosterone levels observed in AN rats.

In order to investigate whether the emotional response to a single injection of cocaine was restricted to adolescence or, instead, common to adulthood, we exposed adult rats to the same experimental paradigm. We found that adult rats showed preference to sucrose. Further, we found increased expression of mBDNF and trkB in the hippocampus of adult rats as well as higher BDNF levels and reduced corticosterone levels in their plasma. These results indicate that adult rats are not vulnerable to the effects of the single dose of cocaine, presumably via their ability to keep stable levels of the neurotrophin both in hippocampus and plasma, stressing the notion that resilience cannot be merely interpreted as reversal of vulnerability.

We are aware that our study holds some limitations. For instance, we analyzed the whole hippocampus without differentiating between dorsal and ventral sub-regions: however, to the best of our knowledge, there is no evidence about a different role played by these two sub-regions in the modulation of anhedonic-like behaviors following cocaine exposure. In addition, given the within-subject design type of study, we could measure brain and plasma BDNF concentration only at the end of the study, i.e. 7 days after the single injection: this does not inform us about the time of onset of both the behavioural and molecular changes while instructing, however, on the long-lasting nature of such alterations.

5. Conclusions

In conclusion, AN rats exhibit reduced hippocampal BDNF signalling together with a decline of plasma BDNF expression and increased corticosterone plasma levels thus recapitulating a clinical, depression-like endophenotype. Conversely, the resilience to AN shown by NON-AN rats appears to be due, at least in part, to increased hippocampal BDNF signalling together with stabilized BDNF downstream signaling and corticosterone plasma levels. This resilient feature is shared by adult rats that do not exhibit signs of anhedonia, up-regulate BDNF and trkB expression and stabilize the neurotrophin downstream signalling. These data also reveal BDNF as a versatile and finely-tuned molecule with unique properties in the effects caused by different drugs of abuse, in line with our previous data showing that an opposite modulation of BDNF can dissect the antidepressant from the reinforcing properties of ketamine (Caffino *et al.* 2016).

Since anhedonia represents a core feature of addiction (Tang et al., 2015), further studies are needed to evaluate whether AN/NON-AN adolescent rats show different proneness to substance use later in life in order to identify the underlying mechanisms. This issue is of critical importance since an association between anhedonia and marijuana use escalation across mid-adolescence in humans has been demonstrated (Leventhal *et al.* 2017). Since symptoms of anhedonia have been found to be associated with relatively poor outcomes of treatment response (Crits-Christoph *et al.* 2018), our findings suggest that first exposure to cocaine during adolescence might have profound consequences, however often overlooked.

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Declaration of Interest

None.

Author's contribution

LC and FM performed the animal studies. LC and FM performed the molecular analyses. JM and AB performed the *in situ* hybridization experiments. LC and FM did the statistical analyses. LC, FM and JM managed the literature searches. LC, GZ, AB and FF designed the study and wrote the protocol. LC, GZ,

AB and FF interpreted the data and wrote the manuscript. All authors contributed to and have approved the final manuscript.

Figure legends

Fig 1 Effect of a single injection of cocaine during adolescence on sucrose preference test. Male rats were exposed to a single injection of saline or cocaine (20 mg/kg) at postnatal day 35 (PND 35) and 7 days later, i.e. at PND 42, sucrose preference test was performed. Sucrose preference was calculated as sucrose intake(g)/total fluid (sucrose + water) intake (g) X 100 and data are presented in panel (a). In panel (b), total liquid intake, calculated as sucrose + water intake (g), is shown.

Bar graphs represent the mean ± SEM from at least seven independent determinations. $^{*}p<0.05$ vs saline-treated rats; $^{\$\$\$}p<0.001$ vs NON-Anhedonic rats (one-way ANOVA followed by Tukey's multiple comparisons test).

Fig 2 Effect of a single injection of cocaine during adolescence on *Bdnf* mRNA levels in the hippocampus (Hip).

The data represent the mRNA levels for *total Bdnf* (a), *Bdnf exon I* (b), *Bdnf exon IV* (c) and *Bdnf exon VI* (d) in the whole hippocampus expressed as a percentage of saline-treated rats. In panel (e) and (f), total *Bdnf* and *Bdnf exon VI* mRNA trafficking are shown in the CA1 (left) and CA3 (right) subregions of the hippocampus. Panel (g) shows representative images of in situ hybridization of total *Bdnf* mRNA levels in CA1 and CA3 regions of hippocampus.

Bar graphs represent the mean ± SEM from at least seven independent determinations. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs saline-treated rats; $^{\$}p<0.05$ and $^{\$\$\$}p<0.001$ vs NON-Anhedonic rats (one-way ANOVA followed by Tukey's multiple comparisons test).

Fig 3 Effect of a single injection of cocaine during adolescence on mBDNF and trkB protein levels in the whole homogenate (Homo) and in the post-synaptic density (PSD) of rat Hip.

Panel (a) and (b) show mBDNF protein levels in the Homo and PSD of Hip (respectively). Panel c and d show the phosphorylation in Tyr706 (left side) and the total form (right side) of the trkB receptor in the Homo and PSD of Hip (respectively). In the upper panel, representative immunoblots are shown for mBDNF (14 kDa), ptrkB Y706 (145 kDa), trkB (145 kDa) and β-Actin (43 kDa) proteins in the homogenate and post-synaptic density of hippocampus.

Histograms, expressed as percentages of saline-treated rats, represent the mean ± SEM of at least seven rats per group. $^{*}p<0.05$ and $^{**}p<0.01$ vs saline-treated rats; $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs NON-Anhedonic rats (one-way ANOVA followed by Tukey's multiple comparisons test).

S=saline; A=Anhedonic; N-An=NON-Anhedonic

Fig 4 Effect of a single injection of cocaine during adolescence on BDNF-downstream signalling in the Homo of rat Hip.

Panel (a) and panel (b) show the activation of Akt and ERK2, respectively, both expressed as the ratio between the phosphorylated and the total form of the protein. Below the graphs, representative immunoblots are shown for pAkt Ser473 (60 kDa), Akt (60 kDa), pERK2 Thr185-Tyr187 (42 kDa) and ERK2 (42 kDa) proteins in the whole homogenate of hippocampus.

Histograms, expressed as percentages of saline-treated rats, represent the mean ± SEM of at least seven rats per group. $^{*}p<0.05$ and $^{**}p<0.01$ vs saline-treated rats; $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs NON-Anhedonic rats (one-way ANOVA followed by Tukey's multiple comparisons test).

S=saline; A=Anhedonic; N-An=NON-Anhedonic

Fig 5 Pearson's product–moment correlation(r) analyses between mBDNF protein levels and sucrose preference in homogenate (panel a) and post-synaptic density (panel b) of hippocampus of rats exposed to a single injection of cocaine during adolescence.

Fig 6 Effect of a single injection of cocaine during adolescence on BDNF plasma levels.

Histograms, expressed as picogram per milliliter, represent the mean ± SEM of at least seven rats per group. $^{*}p<0.05$ vs saline-treated rats (one-way ANOVA followed by Tukey's multiple comparisons test).

Fig 7 Effect of a single injection of cocaine during adolescence on hypotalamus-pituitary-adrenal axis (HPA) activity.

Panel (a) shows the levels of corticosterone, expressed as nanogram per milliliter, in the plasma of AN and NON-AN rats. Panel (b) shows Pearson's product–moment correlation (r) analyses between corticosterone plasma levels and sucrose preference. Panel (c) shows the ratio between nuclear and cytosolic glucocorticoid receptor (GR) protein levels in the hippocampus of rats exposed to a single injection of cocaine during adolescence. Below the graph, representative immunoblots are shown for GR (95 kDa) protein in the nucleus and cytosol of hippocampus.

Histograms, expressed as percentages of saline-treated rats in panel C, represent the mean ± SEM of at least seven rats per group. $^{*}p<0.05$ and $^{***}p<0.001$ vs saline-treated rats; $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs NON-Anhedonic rats (one-way ANOVA followed by Tukey's multiple comparisons test).

S=saline; A=Anhedonic; N-An=NON-Anhedonic

Fig 8 Effect of a single injection of cocaine during adulthood on sucrose preference and on BDNF-downstream signalling in the rat hippocampus.

Panel (a) shows sucrose preference measured seven days after a single cocaine injection during adulthood. Panel (b) and (c) show mBDNF protein levels and the phosphorylation in Tyr706 (left side) and the total form (right side) of the trkB receptor in the whole homogenate of hippocampus (respectively). In panel (f), representative immunoblots are shown for mBDNF (14 kDa), ptrkB Y706 (145 kDa), trkB (145 kDa) and β-Actin (43 kDa) proteins in the whole homogenate of hippocampus. Panel (d) and (e) shows the levels of BDNF, expressed as picogram per milliliter, and corticosterone, expressed as nanogram per milliliter, in the plasma of AN and NON-AN rats (respectively).

Histograms, expressed as percentages of saline-treated rats in panel (b) and (c), represent the mean ± SEM of at least seven rats per group. $^{*}p<0.05$ and $^{**}p<0.01$ vs saline-treated rats (unpaired Student's t test).

S=saline; C=cocaine

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