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ADOLESCENT BRAIN ON COCAINE: SHORT- AND LONG-TERM MOLECULAR CHANGES FOLLOWING REPEATED PSYCHOSTIMULANT EXPOSURE

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1. Introduction

1.1. Cocaine Addiction

Over the past decades, there was an overall increase in the consumption of illicit drugs. Over 246 million of people around the world tried illegal drugs at some point in their lives (UNODC, 2015). In Europe, almost 80 million of people, a quarter of the adult population, experienced drugs of abuse (EMCDDA, 2015). Among the broad and growing market of illicit drugs, cocaine represents the most commonly used psychostimulant. A recent study report that 15.6 million of Europeans from 15 to 64 years old have experienced cocaine at least once in their lifetime and about 3.4 million of them have used illicit drugs in the last year, almost 2.3 million of people from 15 to 34 years old. In Italy, individuals who experienced at least once cocaine are estimated around 4.2% of the total population and 1% of them used cocaine in the last 12 months (EMCDDA, 2015).

These alarming estimates demonstrate that the use of cocaine represents a broad and still unsolved problem, especially in the young population. Among the regular drug users, it is possible to identify two groups: the first is composed of the so-called "socially integrated" drug users that use drugs sporadically over their life, mainly during the weekends, parties or other special occasions. Many of socially integrated users report to control their drug intake depending on the context (Edwards, 2001). However, it has been recently reported that people who initially use drugs only on the weekends often start using them over the week, thus becoming frequently drug users (Bernstein et al., 2015). The second group includes the so-called "marginalized users", in particular those who smoke crack-cocaine mainly because of the low price on the illegal market. Besides the socioeconomic point of view, advances in neuroscience identified drug addiction as a chronic brain disorder that relies on the contribution of genetic, neurodevelopmental, and sociocultural factors. While initial and voluntary drug use activates in the brain the so-called

"reward neurocircuits", repeated drug use alters the physiological brain functions by interfering with self-control on drug-taking behavior leading to a substantial increase over the time of drug intake (Volkow and Morales, 2015) despite negative consequences (Holden, 2001).

Most of the psychostimulants, and in particular cocaine, activate dopaminergic neurons within the reward neurocircuits (Nestler, 2001). In detail, activation of mesolimbic pathway, i.e. dopaminergic neurons projecting from ventral tegmental area (VTA) to the nucleus accumbens (NAc), promotes and sustains goal-directed and reward-mediated behaviors (Nestler, 2005; Koob and Volkow, 2010). Activation of the mesocortical pathway, i.e. dopaminergic neurons projecting from VTA to the medial prefrontal cortex (mPFC), underlies drug-seeking behaviors and relapse even after long period of abstinence (Nestler, 2005; Koob and Volkow, 2010). The vicious "addiction cycle" starts when individual experience for the first time cocaine. In fact, the first pleasure gives rise, in most of the cases, to a desire to take the drug again. From the molecular point of view, acute effects of cocaine rely on the blockade of the presynaptic dopamine transporter (DAT), thus reducing the clearance of dopamine from the synaptic cleft (Nestler, 2005). This results in enhanced synaptic levels of dopamine (Nestler, 2005) which exerts its activity binding postsynaptic dopamine receptors, that are G-protein coupled receptors, that in turn activates intracellular signaling pathway, thus increasing the neuronal activity underlying the short-term effects of cocaine (Volkow et al., 1999). Although those neuronal circuits are physiologically activated by natural reward stimuli, repeated drug use produces a substantial shift from a transient neuroplasticity to a stable and long-lasting neuroadaptation leading to addiction (Kalivas and O'Brien, 2008).

Besides the drug-taking behavior, one of the major clinical issues of drug addiction is the relapse even after long period of abstinence. Among the factors that trigger reinstatement of drug-seeking behavior, stress, drug itself and the drug-associated environmental cues are known to induce relapse in both rodents and humans (Anderson and Pierce, 2005; Crombag et al., 2008; Beardsley and Shelton, 2012; Bossert et al., 2013; Moran-Santa Maria et al., 2014; Mantsch et al., 2015). Among factors mentioned above, stress is the major cause of relapse during the early phases of withdrawal. In fact at this stage, addicts display a "negative emotional state", i.e. loneliness or depressive state that, perhaps, could drive the re-use of drugs of abuse as a means of

"self-medication" (Koob, 2015). Moreover, re-exposure to drugs of abuse appear to drive relapse based on the strong release of dopamine in the reward neurocircuitry thus recalling the molecular memories of drug use, a common property of rewarding drugs (Beardsley and Shelton, 2012). Last, environmental cues, such as places, people and objects paired with cocaine-use produce strong craving, presumably trough the classic conditioning (Crombag et al., 2008). Since repeated use of cocaine generates profound alterations in the brain's reward neurocircuitry, cognitive and behavioral approaches to treat cocaine addiction are often insufficient. Thus, understanding the biological basis and the short- and long-term molecular changes induced by the psychostimulant are necessary to develop new pharmacological interventions.

1.2. Adolescence Brain and Drug Use

Adolescence in humans has been suggested as a vulnerable period of life with a unique sensitivity to experience illicit drugs. Data from European Drug Report of 2015 (EMCDDA, 2015) show that 4.1% of the Italian population, aged 15 or older, experienced cocaine, 1% among the school population (EMCDDA, 2015). Such alarming trend has been reported to increase susceptibility to develop cocaine dependence in adulthood (Laviola et al., 1999). Although in the last decade drug dependence has been considered an adult brain disorder, it is important to point up that drug use peaks during adolescence (EMCDDA, 2015). This alarming trend relies, perhaps, on the fact that during adolescence the brain is still developing and undergoes profound structural and neurochemical changes making the brain particularly sensitive to the pharmacological effects of drugs of abuse. Accordingly, is clear that interfering with the proper maturation of the brain through the early-onset drug use might set the stage to develop drug addiction later on in life.

The brain maturation during adolescence tends to occur from the back to the front with a specific temporal profile (Gogtay et al., 2004). In particular, nucleus accumbens and prefrontal cortex follow a specific pattern of changes leading to a characteristic bottom up and back to front maturation (Gogtay et al., 2004). Accordingly, the nucleus accumbens, involved in reward

properties of drugs of abuse reaches its mature state earlier than the prefrontal cortex, more involved in decision-making behavior (Gogtay et al., 2004). Based on these evidence, adolescents fail to modulate intense emotions and inappropriate behaviors and they are likely to take risk and feel more the reward effects rather than adverse effects of drugs of abuse (Spear, 2002). Overall, these features may render adolescents more vulnerable to experience drugs of abuse and, perhaps, set the stage to develop drug addiction later on in life. Accordingly, more studies are needed to understand the drug-induced molecular changes in the adolescent brain that may contribute to develop addiction in adulthood. From the preclinical point of view, adolescent rats have a good face-validity regarding physiological and molecular changes if compared to those associated with humans (Laviola et al., 1999). In rodents, adolescence is considered a transitional period usually between postnatal days (PND) 28 to 60 (Sengupta, 2013). In addition to sexual maturation, adolescence in rodents is characterized by high level of social interaction, propensity to play as well as high levels of risk-taking, sensation seeking and novelty-seeking (Adriani et al., 1998; Spear, 2000). Accordingly, adolescence in rodents can be considered a reliable translational model to investigate the drug addiction in humans. Based on this line of evidence, Wong and colleagues (2013) nicely demonstrated that adolescent rats are more susceptible to the reward properties of cocaine compared to adults over different doses of cocaine and procedures of drug self-administration (Wong et al., 2013), making them more sensible to stress-induced reinstatement of cocaine seeking behavior (Wong and Marinelli, 2015). Even though there is a growing preclinical interest in the field of early-onset drug use, how repeated use of cocaine results in loss of control over drug-intake and the underlying molecular changes are still unknown.

1.3. Prefrontal cortex in drug addiction

In the last decades, evidence accumulated indicates the leading role of the prefrontal cortex in modulating drug-seeking behaviors. The rodent's prefrontal cortex can be grossly divided into the orbitofrontal cortex (oPFC) and the medial prefrontal cortex (mPFC), which contains the anterior cingulate cortex, infralimbic cortex (IL), prelimbic cortex (PL), and the medial oPFC

(Moorman et al., 2014). The medial prefrontal cortex (mPFC) structurally includes distinct sub-regions that differently regulate cognitive, emotional, and motivational processes. The mPFC receives from and projects to multiple areas of the limbic and sensory systems involved in encoding reward motivation including the hippocampus and amygdala (Gabbott et al., 2006), as well as different subregions of the nucleus accumbens (Moorman et al., 2014). Many parallel and independent circuits comprise the mesocorticolimbic system, with distinct regions of the mPFC innervating specific subcortical structures. However, most of clinical and preclinical studies aimed at identifying drug-induced activation of neuronal pathways, focused on the midbrain dopaminergic areas, such as VTA and substantia nigra (SN) and the related brain regions to which they project, i.e. ventral and dorsal striatum. Recently both preclinical and clinical studies emphasized the central role of prefrontal cortex in addictive behaviors and the dysregulation of the cortical balance between executive and inhibitory functions has emerged as the core motive driving drug addiction (Goldstein and Volkow, 2011).

In particular, the PL cortex projects preferentially to the NAc core and the basolateral and lateral nuclei of the amygdala. Recently it has been demonstrated that this subregion of the prefrontal cortex modulates different aspects of drug-seeking behaviors (Moorman et al., 2014). In fact, pharmacological inactivation of the PL region reduces drug-seeking during cocaine self-administration as well as the prime-, stress- and cue-induced reinstatement of cocaine-seeking behavior (McFarland and Kalivas, 2001; Capriles et al., 2003; McLaughlin and See, 2003) that relies, perhaps, on the reduced release of glutamate in the NAc core. (McFarland and Kalivas, 2001). In fact, injection of cocaine in the PL cortex potentiate reinstatement of drug-seeking behavior (Park et al., 2002) and increase the release of glutamate in NAc core (McFarland and Kalivas, 2001; Park et al., 2002; McFarland et al., 2003). On the other hand, the IL subregion of the prefrontal cortex plays an active role in the suppression of drug-seeking behavior and preferentially innervates the NAc shell, the basal, central (cAM) and medial nuclei of amygdala (Moorman et al., 2014). Although pharmacological inhibition of IL cortex does not result in reduced drug seeking behavior (Pierce et al., 1998) nor in the modulation of stress-, cue- and prime-induced reinstatement (McFarland and Kalivas, 2001; McLaughlin and See, 2003), its pharmacological activation decreases the reinstatement of cocaine-seeking behavior (Peters et al.,

2008). Moreover, optogenetic activation of the IL cortex facilitates extinction from cocaine self-administration (Van Eden and Buijs, 2000). Accordingly, taking into account that during adolescence the prefrontal cortex is still developing, it is possible to speculate that this brain region could be more sensitive to the pharmacologic effects of cocaine and interfering with the proper development of the cortical subregions through the early-onset drug use may alter the homeostasis within the corticolimbic pathway.

1.4. Cocaine-induced neuroplastic changes in reward circuits

Although addiction is a chronic brain disorder characterized by compulsive drug seeking and use, associated with high vulnerability to relapse even after long period of abstinence, the underlying drug-induced molecular changes are still elusive. However, this phenomenon could be at least in part explained by long-lasting changes in the brain functions and structure mediating this pathological behavior (Kalivas and O'Brien, 2008). Recently, it has been shown that cocaine as well as other drugs of abuse, induce functional changes in mesolimbic and mesocortical pathway that physiologically mediates goal-directed behavior and ensure survival needs. Accordingly, it is possible to identify several stages in the progression of the pathology starting from acute effects, mainly mediated by dopamine neurotransmission in the reward neurocircuits. Then, repeated use of the drugs induces the transition from recreational use of the drug to actual addiction. Last, during the end stage of addiction individual lose the control over the drug intake, mainly related to a diminished pleasure leading to increase the intake of the drug over the time (Kalivas et al., 2005). At the stage of acute effects, the cocaine-induced increase of dopamine in the reward pathways activates postsynaptic receptors: activation of D1 receptors induce activation of intracellular signaling pathway, i.e. cAMP-dependent protein kinase (PKA). Activated PKA phosphorylates transcription factors such as cAMP response element binding protein (CREB), which in turn induces the transcription of immediate early genes, such as c-fos and the activity-regulated cytoskeleton-associated protein (Arc/Arg3.1) (Konradi et al., 1994), thus promoting short-term neuroplastic changes. Moreover, chronic cocaine-induced activation of D2 receptor

increase RGS9-2 in nucleus accumbens, thus modulating the rewarding properties of cocaine and different drugs of abuse (Rahman et al., 2003).

Repeated use of cocaine promotes the transition to addiction through long-lasting structural and functional changes. Among the factors driving the transition, ΔFosB has been demonstrated to play a crucial role in the process by which drugs of abuse cause the addiction state (Nestler, 2001). At the "end stage" of addiction to drugs of abuse, short-term neuroadaptations becomes stable promoting profound functional and structural changes (Kalivas and O'Brien, 2008), most of them underlying the vulnerability to relapse, even after long period of abstinence. Recently, among the cocaine-induced neuroadaptation, the glutamatergic neurotransmission has emerged as one of the systems whose alteration contributes, at least in part, to relapse even after long period of abstinence (Kalivas, 2004).

1.5. Glutamate system in cocaine addiction

Although in the last decades the concept of drug addiction has been widely associated with alteration of dopamine neurotransmission in the reward pathways, dysregulation of mesocorticolimbic glutamate pathways has a leading role in developing and maintenance of addictive behaviors and, in particular, in the reinstatement of drug-seeking behavior (Kalivas, 2009). It is well established that acute effects of cocaine are associated with the enhancement of the dopaminergic tone from VTA to NAc and mPFC, underlying the reward properties of the drug and the locomotor hyperactivity reported in both rodents and humans. However, the reasons why such behavior lasts for years is not completely clear and recently has been proposed that long-term maladaptive neuroplasticity in the glutamatergic neurotransmission might sustain the loss of control over drug intake (Kalivas, 2004). Such hypothesis is supported by several electrophysiological studies demonstrating that acute injection of cocaine does not alter glutamate levels or release in nucleus accumbens (Bell et al., 2000). However, a challenge of reinforcing drug after chronic cocaine self-administration increase extracellular levels of glutamate in NAc core (Miguens et al., 2008), further supporting the idea that it is not cocaine or the reinforcing

drug *per se* to induce addictive behaviors but, rather, a coordinated series of neuroadaptations in the reward neurocircuits set in motion by repeated drug use.

Glutamate is the most abundant excitatory neurotransmitter in the brain (Martinez-Lozada and Ortega, 2015) and it acts via two class of membrane receptors, i.e. ionotropic (iGluRs) and metabotropic (mGluRs) glutamate receptors (Kumar and Mayer, 2013; Karakas et al., 2015). The ionotropic glutamate receptors are subdivided in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (also known as AMPA receptors), N-Methyl-D-Aspartate receptor (also known as NMDA receptors) and Kainate receptors (Bowie, 2012; Bouvier et al., 2015). All of the ionotropic glutamate receptors are nonselective permeable to cations such as Na^+, K^+ and Ca^{2+}, thus producing a post-synaptic excitatory response.

NMDA subfamily of glutamate receptors are heterodimers with a different subunits composition. Up to date, results from several studies identified seven different subunits belonging to three major families: GluN1, GluN2 (GluN2A, GluN2B, GluN2C, GluN2D) and GluN3 (GluN3A and GluN3B) (Paoletti and Neyton, 2007; Paoletti et al., 2013). Functional NMDA receptors display a tetrameric structure composed of two GluN1 subunits and often two GluN2 subunits or GluN2/GluN3 subunits (Paoletti and Neyton, 2007; Paoletti et al., 2013).

AMPA receptors are permeable to cations such as Na^+ and K^+ and depending on the subunit composition, also to Ca^{2+}. Functional AMPA receptors have a tetrameric composition and to date four major subunits have been identified, i.e. from GluR1 to GluR4, either in monomers or heterodimers composition (Gan et al., 2015). Besides the receptors composition and their location, the functional organization of glutamatergic synapse appears to be more complex and its homeostasis depends on the coordinated functions of several neuronal structures, organized in the so-called "tripartite synapse" (Bridges et al., 2012; Smith et al., 2015). Such organization comprises pre- and post-synaptic neurons as well as the glia. In the ventral striatum, and in particular in NAc core, almost 60% of the synaptic glutamate derives from the glia via the action of cysteine-glutamate exchanger (xCT) (Baker et al., 2002; Bridges et al., 2012), whose function is to uptake cysteine and release glutamate that, in turn, interacts with the pre-extrasynaptic mGluRs, thus finely modulating the glutamatergic neurotransmission (Bridges et al., 2012). Recently has been shown that release of glutamate from xCT activates pre-

extrasynaptic mGluR2 receptors, whose physiological function is to attenuates glutamate release from presynaptic neurons (Moran et al., 2005). The release of glutamate is counterbalanced by the action of glutamate transporters, which uptake glutamate from the synaptic cleft to the glial cells (Danbolt, 2001). Among the glutamate transporters, the glial transporter 1 (GLT-1) is the most abundant and is responsible for >90% of glutamate clearance. Recently has been demonstrated that repeated administration of cocaine and other addictive drugs downregulates xCT, thus reducing the basal levels of glutamate derived from glial cells (Kalivas, 2009) and increasing the presynaptic release of glutamate by reducing the inhibitory tone on mGluR2 receptors (Kalivas, 2009). Moreover, repeated exposure to drugs of abuse such as cocaine, nicotine and heroin reduce GLT-1 expression in NAc (Gipson et al., 2013; Shen et al., 2014; Reissner et al., 2015). Such impairments in the homeostasis of glutamate neurotransmission have been proposed as the molecular background capable of generating relapse (Kalivas, 2009). It is important to note that treatment with ceftriaxone, a beta-lactam antibiotic, restores GLT-1 expression and attenuates cue- and prime-induced reinstatement of drug seeking behavior (Sari et al., 2009; Knackstedt et al., 2010; Trantham-Davidson et al., 2012; Fischer et al., 2013). Recently, it has been also demonstrated that treatment with N-Acetylcysteine increases the activity of xCT and restores GLT-1 expression, thus resulting effective in modulating relapse in humans (Knackstedt et al., 2009; Schmaal et al., 2011; Froeliger et al., 2015) and reinstatement of cocaine- and nicotine-seeking behavior in rodents (Kupchik et al., 2012; Reichel and See, 2012; Ramirez-Nino et al., 2013; Frankowska et al., 2014). Moreover, mGluR2 positive allosteric modulators reduce the cue-induced reinstatement of cocaine-seeking behavior in monkeys (Justinova et al., 2015) and rodents (Cannella et al., 2013).

Altogether, these preclinical findings point to the corticoaccumbal glutamatergic system as a promising target to treat addiction-related behaviors (Roberts-Wolfe and Kalivas, 2015). Although pharmacologic modulation of mGluR2 receptors has been widely associated with modulation of reinstatement of drug-seeking behavior, it is important to note also that postsynaptic mGluR5 receptors play a significant role in the behavioral effects of psychostimulants (Swanson and Kalivas, 2000; Pomierny-Chamiolo et al., 2015). In fact, mice

lacking mGluR5 receptor do not self-administer cocaine and do not show the increased locomotor activity after cocaine treatment (Chiamulera et al., 2001).

A growing body of evidence suggest that long-term exposure to cocaine might induce corticolimbic neuroadaptation of AMPA receptors in terms of composition and function underlying the cue-induced relapse even after long period of abstinence (Pierce and Wolf, 2013; Loweth et al., 2014; Ma et al., 2014). Such phenomena it is known as "incubation" of cue-induced craving (Pickens et al., 2011; Ma et al., 2014). As previously discussed, AMPA receptors are tetrameric structure, comprising GluA1-GluA4 subunits (Gan et al., 2015). Depending on the presence or not of GluA2 subunit, its function is different, i.e. AMPA GluA2 lacking receptors are Ca^{2+} permeable (CP-AMPARs), presenting a higher single channel conductance compared to those presenting the GluA2 subunits (Liu and Cull-Candy, 2000; Plant et al., 2006). Emerging evidence suggests that reduced synaptic expression of GluA2 subunit of AMPARs in NAc is associated with incubation of cocaine-seeking behavior (Conrad et al., 2008), thus increasing the responsiveness to excitatory inputs capable of triggering reinstatement to drugs of abuse (Conrad et al., 2008). Also, prolonged withdrawal (45 days) from Long-Access (LgA) cocaine self-administration (6h/day for 10 days) "incubate" cocaine craving (Grimm et al., 2001; Grimm et al., 2003; Ma et al., 2014) reducing the synaptic expression of GluA2 subunits (Conrad et al., 2008) that in turn might increase the reactivity of nucleus accumbens to the cue-induced reinstatement of drug-seeking behavior (Briand et al., 2014). Besides the presence or not in the receptor, the ability of GluA2 subunits to modulate Ca^{2+} influx also depends on mRNA editing state (Wright and Vissel, 2012). In particular, repeated use of cocaine produces the activation of Adenosine deaminase acting on RNA 2 (ADAR2), the key enzyme that catalyzes the editing of GluA2 pre-mRNA at Q/R site 607 (Rueter et al., 1995). Unedited GluA2(Q) subunit generate Ca^{2+} permeable AMPARs, while the edited GluA2(R) subunit generate Ca^{2+} impermeable AMPARs (Wright and Vissel, 2012). Forced abstinence (7 days) from chronic cocaine self-administration (21 days) increases GluA2 Q/R ratio, associated with reduced expression of ADAR2 in NAc shell, but not NAc Core (Schmidt et al., 2015). Interestingly, viral overexpression of ADAR2 in NAc Shell attenuates the cue-induced reinstatement of cocaine seeking behavior (Schmidt et al., 2015), further supporting the idea of glutamate

neurotransmission in NAc shell as a potential target to modulate the cue-induced reinstatement after withdrawal from drugs of abuse (Marchant et al., 2014).

1.6. Brain-Derived Neurotrophic Factor in cocaine addiction and withdrawal

BDNF belongs to the family of Neurotrophic factors, involved in synaptic plasticity, memory processes, cell survival (Lu et al., 2005; Hempstead, 2015). BDNF gene structure is well conserved among species, from fish to mammals (Aid et al., 2007; Pruunsild et al., 2007) and consists of multiple exons (eleven in humans, nine in rodents) that are regulated in a developmental, tissue-specific, and activity-dependent manner (Aid et al., 2007). Moreover, the *BDNF gene* structure shows two alternative 3' polyadenylation sites that result in the synthesis of two different pool of mRNAs: transcripts with a short 3' untranslated region (UTR), and transcripts with a long 3'UTR (Timmusk et al., 1993); those with a short 3'UTR are mainly localized in the soma, while those with a long 3'UTR are targeted to the dendrites for local translation (An et al., 2008; Vicario et al., 2015) in response to different stimuli (Greer and Greenberg, 2008). The single coding sequence generate a 32KDa precursor form of BDNF (proBDNF) whose processing by cleavage either by intracellular or extracellular enzymes, generates a mature form (14KDa) of the neurotrophin (Lu et al., 2005; Hempstead, 2015). Neuronal activity triggers the release of BDNF that exerts its biological action binding its high-affinity tropomyosin receptor kinase B (TrkB). This results in receptor dimerization and auto-phosphorylation of tyrosine residues in the catalytic domain (Tyr706/707) thus activating three different intracellular downstream pathways, i.e. the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3-K), and phospholipase C-γ (PLC-γ) that in turn modulate gene expression (Numakawa et al., 2010).

The contribution of BDNF in drug addiction, and in particular in cocaine addiction behaviors, has been widely investigated in the mesocorticolimbic system (Corominas et al., 2007; McGinty et al., 2010; McCarthy et al., 2012; Li and Wolf, 2015). Horger et al. (Horger et al., 1999) provided the first demonstration that accumbal BDNF mediates cocaine-induced

behaviors. In fact, chronic intra-NAc infusion of BDNF increased cocaine-induced responding for a cue previously paired with water in thirsty rats (Horger et al., 1999). This effect lasted up to 5 weeks after cessation of BDNF infusion. This study was the inspiration to investigate further the role of BDNF in drug self-administration, reinstatement, and incubation of drug craving. Graham et al. (Graham et al., 2007; Graham et al., 2009) demonstrate the key role of accumbal BDNF and its high-affinity receptor TrkB in the rewarding properties of cocaine, using the self-administration protocol. Infusion of the neurotrophin in NAc shell for five days immediately after the self-administration session increases the rewarding properties of cocaine. In fact, rats self-administer more cocaine over a different range of doses (Graham et al., 2007). Moreover, infusion of BDNF in the NAc increase the rat's motivation to get cocaine under a progressive ratio (PR) schedule of reinforcement (Graham et al., 2007) and display a reduction of extinction responding and prime-, cue- and stress-induced reinstatement of cocaine-seeking behavior (Graham et al., 2007). Moreover, injection of Anti-TrkB Viral Vector (AVV) in the NAc shell decreases cocaine self-administration under fixed ratio 1 (FR1) schedule of reinforcement, over the different range of doses used (Graham et al., 2009), an effect similar to that observed with intra-NAc infusion of BDNF antiserum (Graham et al., 2007). Also, acute injection of BDNF in the VTA after the last cocaine self-administration session induces long-lasting enhancement of cue-induced reinstatement of drug-seeking behavior for up to 30 days (Lu et al., 2004) further supporting the idea of subcortical contribution of BDNF modulation as the driving force of the pro-seeking behaviors observed in preclinical studies.

However, an opposite effect has been shown when BDNF was infused in cortical regions. In fact, bilateral infusion of BDNF into the prelimbic cortex immediately after the last of ten cocaine self-administration sessions reduce extinction responding as well as the cue- and prime-induced reinstatement of cocaine-seeking behavior (Berglind et al., 2007), an effect mediated by TrkB-induced activation of ERK and reversed by U0126, which blocks ERK activity (Whitfield et al., 2011). In addition to attenuating cocaine-seeking behavior, infusion of BDNF in mPFC rescues the cocaine-induced reduction of extracellular glutamate levels in NAc as well as prevents the strong release of glutamate during reinstatement test (Berglind et al., 2009), suggesting that the anti-craving effect of cortical infusion of BDNF relies, at least in part, on the modulation of

cocaine-induced glutamatergic neuroadaptations within the corticolimbic pathway. However, how BDNF acts in the mPFC to modulate cocaine seeking and glutamatergic activity is not fully understood. In fact, it has been demonstrated that exogenous BDNF can be internalized in neurons, thus becoming available for activity-dependent release (Santi et al., 2006) or it can be anterogradely transported to the NAc and dorsal striatum (dS) (Altar and DiStefano, 1998). Accordingly, it is possible to speculate that BDNF can functionally act on site or exert its action on distal projection regions. Based on this consideration, McGinty and colleagues (2010) demonstrated that infusion of the scavenger TrkB-fc in the mPFC 20 minutes before BDNF infusion completely blunts the anti-craving effects of the neurotrophin (McGinty et al., 2010) suggesting, at least in part, that the anti-craving effect of cortical infusion of BDNF are mediated by the activation of its high-affinity receptor in the mPFC.

However, less is known about the modulation of BDNF after long-term withdrawal from the last drug exposure. The pioneering study in this field was conducted by Grimm and colleagues (2003), showing that long-term withdrawal from ten long-access (6h/d) sessions of cocaine self-administration increase BDNF protein levels in a time-dependent manner from the last drug exposure as a function of abstinence duration and the brain region investigated. In particular, the authors show a time-dependent increase of the neurotrophin up to 90 days after the last cocaine self-administration session, but not sucrose, in VTA, NAc and amygdala. Such increase was paralleled by a concomitant time-dependent potentiation of the cue-induced reinstatement of cocaine-seeking behavior, suggesting a relationship between BDNF and the mounting desire over the time to get cocaine, the so-called "incubation" of cocaine craving. More recently, the role of BDNF has been also associated with the concept of "drug expectation". In particular, Geoffroy and colleagues (2015) showed that non-contingent repeated exposure to cocaine increase the plasma levels of BDNF after 1 or 14 days of withdrawal compared to control animals, when measured at the usual time point at which animals had been exposed to the drug. Beside the drug-induced modulation of BDNF and its withdrawal-induced up-regulation, the role of neurotrophin has been also investigated in trans-generational studies (Vassoler et al., 2013; Sadri-Vakili, 2014; Vassoler and Sadri-Vakili, 2014). The elegant work of Vassoler and colleagues (2013) show a heritable phenotype resulting from chronic cocaine self-administration in rats. In

particular, ten days of cocaine self-administration produce a delay in the acquisition and reduced maintenance of cocaine self-administration in male, but not female, offspring (Vassoler et al., 2013). Interestingly, in the mPFC of the offspring that self-administered less cocaine, there was an increase of BDNF protein as well as a selective up-regulation of BDNF exonIV associated with hyperacetylation of the promoter region (Vassoler et al., 2013). Moreover, male offspring showed less motivation to get cocaine under progressive ratio (PR) schedule of reinforcement, a behavior reversed by systemic administration of TrkB antagonist ANA-12 (Vassoler et al., 2013), thus paradoxically suggesting that a father's addiction protect his son from drug addiction.

Altogether, preclinical studies dissect the contribution of BDNF in modulating addictive behaviors depending on the brain region investigated, suggesting the potential role of the cortical neurotrophin in modulating cocaine craving. Accordingly, drug-induced modulation of the neurotrophin might be of clinical interest in order to follow addiction habits and the probability to relapse (McGinty and Mendelson, 2011). Taking into account that blood levels of BDNF reflect those in the brain (Klein et al., 2011), Carrol D'Sa and colleagues (2011) reported the first evidence that higher BDNF serum levels in abstinent cocaine users correlates with a shorter time to relapse (D'Sa et al., 2011), and inversely correlates with the severity of crack-cocaine use (Sordi et al., 2014; von Diemen et al., 2014). Moreover, early withdrawal from crack-cocaine use increases plasma levels of BDNF (Corominas-Roso et al., 2013; von Diemen et al., 2014), suggesting the neurotrophin as a good candidate biomarker to predict relapse, the outcome of the inpatient treatment and a promising system to modulate drug addiction behaviors.

1.7. Activity-Regulated Cytoskeletal-associate protein in cocaine addiction

Arc (Activity-regulated cytoskeleton-associated protein), also known as activity-regulated gene 3.1 (arg3.1) (Lyford et al., 1995b), belongs to the family of immediate early genes (IEGs) whose induction activity-mediated is critical for learning and memory (Greer and Greenberg, 2008; Shepherd and Bear, 2011) and can be considered a validated marker of neuronal activity (Kawashima et al., 2014). For example, inhibition of Arc expression in hippocampus using

antisense oligonucleotides impairs consolidation of long-term memory for a spatial water maze task (Guzowski et al., 2000). Besides its well-established role in mediating cognitive functions, its modulation has been associated with drug-induced neuroplasticity (Korpi et al., 2015). A single injection of cocaine induces Arc mRNA expression 2h later with a dose- and temporal-dependent profile in striatum, prefrontal cortex and hippocampus while its expression returns to controls 24h later (Fumagalli et al., 2006b). Moreover, subchronic (5 daily injections) or chronic (14 daily injections) non-contingent treatment with cocaine produces a long-lasting up-regulation of Arc, depending on the length of exposure to the drug and the brain region investigated (Fumagalli et al., 2006b), an effect that last up to 3 days after the last injection (Fumagalli et al., 2006b). Based on this lines of evidence, Fumagalli and colleagues (2009b) showed that a single 2h session of cocaine self-administration up-regulates Arc expression in the mPFC immediately after the behavioral task, suggesting that a single session is sufficient to induce profound changes shaping a goal-oriented behavior in rats, sustained by up-regulation of Arc (Fumagalli et al., 2009b). Caffino and colleagues (2011) investigate whether stress (acute or repeated) could modulate Arc expression after a single injection of cocaine (10mg/kg). Interestingly, injection of cocaine immediately after acute stress potentiate Arc expression, while the chronic stress attenuates the response to cocaine in prefrontal cortex (Caffino et al., 2011), suggesting Arc as a good candidate whose modulation by stress can influence the response to cocaine (Caffino et al., 2011). Moreover, re-exposure to environmental stimuli previously associated with cocaine self-administration, increase Arc expression in dorsolateral striatum (dlS) after 15 days of forced abstinence (Hearing et al., 2008), while the selective inactivation of Arc in dlS 3h before the context-induced reinstatement of drug seeking, significantly attenuates Arc expression but failed to alter the response during the test (Hearing et al., 2011). However, inactivation of Arc slows down the responses to subsequent extinction tests 24h and 48h later (Hearing et al., 2011). Take together, these results demonstrate the key role of Arc in modulating addiction-related behaviors. Given that cocaine addiction induces profound neuroadaptation in mesolimbic and mesocortical pathways, Arc could participate, at least in part, to cocaine-induced long-term changes as well as in incubation of cocaine craving.

1.8. basic Fibroblast Growth Factor-2 in cocaine Addiction

Basic fibroblast growth factor-2 (bFGF-2) is the prototype member of a family of heparin-binding growth factors with a broad spectrum of actions on different cell types in the central nervous system and widely distributed within the brain (Ford-Perriss et al., 2001). Recently it has been demonstrated the link between FGF-2 and the dopaminergic system (Flores and Stewart, 2000; Fumagalli et al., 2004; Forget et al., 2006). In particular, Fumagalli and colleagues (2004) demonstrated that a single dose of the atypical antipsychotic quetiapine (10mg/kg) up-regulates FGF-2 under reduced NMDA receptor activity induced by a single injection of MK-801 (Fumagalli et al., 2004). However, whether FGF-2 is involved in the long-lasting drug-induced neuroplastic changes in cocaine addiction remain elusive. Accordingly, Fumagalli and colleagues (2006a) demonstrated that a single intraperitoneal (IP) injection of cocaine is sufficient to up-regulate FGF-2 mRNA levels in a dose-dependent manner (1-5-10-20mg/kg) in dlS and mPFC, while its expression was up-regulated in hippocampus only at the dose of 20mg/kg (Fumagalli et al., 2006a). Interestingly, acute treatment with cocaine (5mg/kg) up-regulates FGF-2 expression in a time-dependent manner in dlS and mPFC and its expression returns to controls 24h after the acute injection of cocaine (Fumagalli et al., 2006a). Moreover, repeated non-contingent injection of cocaine (5 or 14 days) produce a long-lasting up-regulation of FGF-2 that was evident after 72h in the mPFC and persist up to 14 days in dlS (Fumagalli et al., 2006a), suggesting that its modulation might participate, at least in part, to the drug-induced neuroadaptations set in motion by repeated exposure to the psychostimulant.

Although the modulation of FGF-2 by stress has been widely investigated in rodents (Molteni et al., 2001), its modulation produced by the combination of cocaine and stress remains elusive. It is important to note that stress is a strong environmental factor capable of triggering relapse in both humans and rodents (Beardsley and Shelton, 2012). Accordingly, it seems to be crucial to investigate the contribution of cocaine exposure in modulating the physiological response of the neurotrophic system. Accordingly, Fumagalli and colleagues (2008) showed that stress and cocaine interact to modulate FGF-2 expression in mPFC and dlS. In particular, acute

stress potentiates FGF-2 the response to an acute injection of cocaine in the mPFC, whereas chronic stress prevents its up-regulation (Fumagalli et al., 2008). However, in the dlS, the expression of FGF-2 was potentiated after repeated stress in response to acute injection of cocaine, while no interaction was observed when acute stress and single exposure to cocaine were combined (Fumagalli et al., 2008), suggesting that FGF-2 is modulated by stress and cocaine depending on the modality of stress and the brain region investigated. However, the role of FGF-2 in drug addiction behavior is not completely understood.

Recently it has been demonstrated the role of cortical FGF-2 in modulating drug-seeking behavior in animal models of drug addiction. In particular, Hafenbreidel and colleagues (2015) demonstrated that inhibition of FGF-2 in infralimbic subregion of the mPFC, facilitates drug extinction after cocaine self-administration (Hafenbreidel et al., 2015). In particular, when rats reached stable self-administration (16-20 sessions), they received intra infralimbic injection of the antibody against bFGF2 before the first four days of 30 min of extinction session (Hafenbreidel et al., 2015) to minimize extinction learning during the first four sessions and better manipulate initial learning. Then, rats underwent 90 min of extinction sessions to investigate the effect of neutralized bFGF-2 on extinction of cocaine seeking-behavior. Interestingly, rats infused with anti-FGF-2 showed a robust reduction of the active lever presses in the first of 90 min sessions of extinction, compared to those infused with vehicle (Hafenbreidel et al., 2015). Moreover, bFGF-2 protein was up-regulated in IL cortex after cocaine self-administration but reduced after extinction (Hafenbreidel et al., 2015), suggesting that extinction of drug-seeking behavior reduce bFGF-2 levels in IL cortex and neutralizing its activity facilitate this process. Altogether these results point to FGF-2 as a promising marker whose modulation could underlie the different sensitivity to drugs of abuse after stress as well as the extinction of cocaine-seeking behavior and might be involved in long-term neuroplastic changes following repeated exposure to psychostimulants.

1.9. Stress-related system in cocaine addiction

Adolescence can be considered, almost by definition, as a period of enhanced sensitivity to stress (Spear, 2000), characterized by a concomitant series of changes including physical maturation, hormonal changes and brain development (Blakemore, 2008b, a, 2012; Mills et al., 2014). Moreover, adolescence is known as the most common period of life for the onset and manifestation of psychiatric disorders (Kessler et al., 2005), characterized by exacerbated stress and anxiety manifestations (McCauley Ohannessian, 2014; Gale et al., 2015; Haller et al., 2015): of note, during adolescence, suicide is the fourth leading cause of death (Goldsztein et al., 2008; Ortin et al., 2012). During adolescence hormonal changes drive a substantial shift in stress reactivity. Physiologically, when an individual experiences a stressful situation, two systems are activated to cope with the event: immediately there is a release in the blood flow of epinephrine and norepinephrine that mediates the so-called "fight-or-flight" reaction; then a slower, but more protracted, hormonal response comes in and involves the activation of hypothalamic-pituitary-adrenal (HPA) axis. Such hormonal response is initiated by the activation of neurons in the paraventricular nucleus of the hypothalamus (PVN) that, in turn, release corticotropin-releasing hormone (CRH) which acts on pituitary gland by inducing the release of adrenocorticotropic hormone (ACTH) in the blood stream. ACTH acts on adrenal glands, inducing both synthesis and release of glucocorticoids (cortisol in humans and corticosterone in rodents). Once the stress is over, the signal is terminated by a negative feedback exerted by glucocorticoids on the pituitary gland, thus reducing the production and release of CRH and ACTH. Notably, even if during adolescence the levels of stress hormones remain stable, the amount and duration of stress response undergoes substantial changes. In particular, adolescents show a stronger as well as protracted ACTH and corticosterone response compared to adults after different stressor (Vazquez and Akil, 1993), in both males and females. Those observations point to adolescence as a critical period of high sensitivity to stressful situations and it is possible to imply its contribution in the development of addiction and vulnerability to relapse since the stress is a well-known risk factor to initiate drug use and precipitate relapse (Sinha, 2008). Accordingly, there is

growing literature in humans reporting the effect of stress on drug use initiation and escalation in adolescents and young adults (Fishbein et al., 2006; Caldeira et al., 2012). From the preclinical point of view, stress in rodents acts by potentiating the acquisition and the escalation of drug self-administration of different drugs of abuse, including cocaine (Piazza and Le Moal, 1996; Piazza and Le Moal, 1998), probably increasing the reinforcing efficacy of drugs itself. In fact, it has been shown that stress-induced increase of glucocorticoids enhance dopamine levels in NAc (Thierry et al., 1976) and adrenalectomy reduces the dopamine levels under basal conditions and following exposure to psychostimulants (Thierry et al., 1976), an effect that is reversed by corticosterone replacement. Glucocorticoids act in the brain by activating two different receptors: Type I (mineralcorticoid) receptors (MR also known as Nr3c2) and Type II (glucocorticoid) receptors (GR also known as Nr3c1) (de Kloet, 2000), which both modulate gene expression. Recently, it has been shown that pharmacological modulation of the GR receptor induces changes in cocaine self-administration: in particular, acute injection of mifepristone (30mg/kg I.P.), a GR antagonist, before the last self-administration session in mice with a history of chronic cocaine self-administration, reduces the reinforcing properties of the drug (Fiancette et al., 2010) (Deroche-Gamonet et al., 2003). Moreover, inactivation of GR in the whole brain reduces the motivation to self-administer cocaine (Tronche et al., 1999; Deroche-Gamonet et al., 2003) and, recently, it has been shown that selective inactivation of GR in mesocorticolimbic pathway attenuates sensitizing, rewarding and reinforcing properties of cocaine (Ambroggi et al., 2009; Barik et al., 2013). In particular, Ambroggi and colleagues (2009) to get further insight into the contribution of GR in cocaine addiction behaviors, employed a mice model with a selective inactivation of Nr3c1 gene expression obtained by mating mice $Nr3c1^{loxp}$ with those expressing Cre recombinase under the promoter of D1a receptor, thus inactivating Nr3c1 in all the dopaminergic projections including NAc, dS and mPFC. Interestingly, GR-$D1a^{Cre}$ mice display a reduction of reinforcing properties of cocaine and showed less motivation to get cocaine under progressive ratio schedule of reinforcement (Ambroggi et al., 2009). Moreover, Gourley and colleagues (2012b) demonstrated that repeated exposure to corticosterone as well as the acute treatment with the GR antagonist RU38486, impairs the decision-making of the animals through the desensitization of GRs leading to an increased vulnerability to develop habits (Gourley et al.,

2012b), one of the features of addictive-like behaviors (Everitt and Robbins, 2005; Everitt, 2014). However, despite the growing interest on how the GR system modulates addiction-like behaviors at adulthood, less is known about the contribution of GR system in the development of addiction-related behaviors during adolescence.

2. Aim

Addiction to drugs of abuse, and in particular cocaine addiction, is a chronic relapsing brain disorder characterized by loss of control over drug intake that relies on the transition from recreational and controlled drug intake to compulsive use (Kalivas and O'Brien, 2008) despite negative consequences (Koob, 2003). The reasons why people get addicted and crave for drugs are still unknown, and so are the molecular mechanisms underlying the addictive state. However, there is a growing body of evidence suggesting that repeated drug use generates enduring and stable neuroplastic changes (Kalivas and O'Brien, 2008) that contribute to develop maladaptive behaviors (Ersche et al., 2011) driving the transition from recreational use to uncontrolled drug intake (Kalivas and O'Brien, 2008) and therefore increasing the propensity to relapse after withdrawal (Buchta and Riegel, 2015). In fact, cocaine addiction can also be considered as a form of maladaptive learning or drug-induced habit (Robbins and Everitt, 1999; Everitt and Robbins, 2005, 2015) that relies, over the time, on the stabilization of cocaine-induced transient neuroplasticity (Kalivas and O'Brien, 2008). Such maladaptive neuroplasticity generates impulsivity and high risk-taking behaviors (Zhu et al., 2015) that underlie to the initiation of drug use and contribute to maintain the drug-taking behavior (Gould, 2010).

However, the overall picture is much more complex and the evidence accumulated so far indicates that the development of addictive behaviors relies on the intricate interplay between genetic, developmental and sociological vulnerabilities (Kreek et al., 2005). Considering the aforementioned risk factors, adolescence well represents the combination of all these components. In fact, adolescence is a vulnerable period of development during which the brain undergoes a coordinated series of functional and structural changes under the influence of changes in gene expression and environmental factors (Leather, 2009). During adolescence the brain is still developing, and the last brain region that reaches its mature form is the prefrontal cortex, i.e. the brain region modulating decision-making processes and emotional states (Fellows and Farah,

2007). Based on this evidence, it is possible to state that adolescents fail to modulate their impulsive behaviors. In fact, adolescents display high levels of risk-taking behaviors (Leather, 2009; Jacobus et al., 2013) and seek intense feelings (Wong and Marinelli, 2015) due to the earlier development of the limbic system compared to the cortical regions (Gogtay et al., 2004). Accordingly, it is clear that adolescence represents a critical period of vulnerability to initiate drug use (Fuhrmann et al., 2015), perhaps setting the stage to develop addictive behaviors later in life.

In the last decade, our laboratory has contributed to clarify the pharmacological effects of cocaine in the adult brain on different molecular systems (Fumagalli et al., 2006b; Fumagalli et al., 2006a; Fumagalli et al., 2007, 2008; Fumagalli et al., 2009a; Fumagalli et al., 2009b; Fumagalli et al., 2013), in different paradigms of psychostimulant exposure (Fumagalli et al., 2006a; Fumagalli et al., 2009b; Caffino et al., 2014) and challenging situations (Fumagalli et al., 2008; Fumagalli et al., 2009a; Caffino et al., 2011). Although drug addiction can be considered an adult brain disorder, the onset of drug use peaks during adolescence (Munno et al., 2015). Accordingly, in order to understand the molecular mechanisms underlying the drug-induced maladaptive behaviors in adults it is necessary to take a step backwards and investigate the cocaine-induced molecular alterations in adolescents.

Therefore, during my Ph.D., I focused on analyzing the short- and long-term molecular changes following repeated exposure to cocaine during adolescence, dissecting its contribution in modulating different molecular systems. Moreover, I decided to explore the contribution of the medial prefrontal cortex that is still developing during adolescence (Gogtay et al., 2004) and it might be more vulnerable to the action of psychostimulants. The results of these studies are presented in the form in which they have been published (Giannotti et al., 2013; Giannotti et al., 2014; Caffino et al., 2015b; Caffino et al., 2015a; Giannotti et al., 2015).

In particular, first, I investigated whether the rapid response of the glutamatergic system to an acute stress was influenced by previous cocaine history during adolescence. In fact, evidence exists that stress induces changes in glutamate neurotransmission in the prefrontal cortex (mPFC) and the hippocampus (Hip), thereby influencing some aspects of cognitive processes (Gould, 2010). Moreover, it is known that stressful experiences increase the vulnerability of an individual

to relapse to drug use, even after prolonged abstinence (Wingo et al., 2015). However, how stress acts on the adolescent brain to trigger relapse is complicated and still not fully understood.

Second, I investigated whether chronic exposure to cocaine during adolescence may have altered the stress-related system, thus producing a vulnerable molecular background. In fact, it is known that short-term abstinence from drugs of abuse leads to a negative emotional state that might be responsible of the high risk to relapse during early phases of withdrawal (Koob, 2015). Moreover, it has been demonstrated that adolescents are more sensitive to stressful situations (Fuhrmann et al., 2015) and the modulation of the stress system contributes to different features of addictive behaviors in adults (Koob, 2008).

Then, I investigated whether long-term withdrawal from developmental exposure to cocaine could modulate the expression of the Brain-Derived Neurotrophic Factor (BDNF) and its associated signaling pathway, since it has been demonstrated in adult animals a time-dependent increase of BDNF expression as a function of abstinence duration, the so-called "incubation" of cocaine craving (Grimm et al., 2001; Grimm et al., 2003), which can be considered as a mounting desire to get cocaine over the time. Accordingly, it might be possible that abstinence-induced modulation of BDNF could drive not only the drug use later in life, but also it might be functionally relevant in modulating relapse to drugs of abuse.

Finally, I investigated whether the short- and long-term abstinence from developmental exposure to cocaine could alter the expression of Fibroblast Growth Factor 2 (FGF-2) and how the duration of withdrawal could modulate the expression of the neurotrophin following an acute stress. In fact, evidence exists that cocaine in adulthood alters the expression of FGF-2 in a time-dependent profile from the last drug exposure depending on the brain region investigated (Fumagalli et al., 2006a). However, understanding how developmental exposure to cocaine modulates FGF-2 and how the system copes to a stressful situation could help to get further insight into the complexity of neurobiology on drug use during adolescence. Further, I investigated if the first exposure to cocaine during development might alter the modulation of FGF-2 expression to a second injection of cocaine to investigate whether early cocaine priming alters the mechanisms regulating the trophic response in a brain region-specific fashion.

3. Materials and Methods

3.1. Experimental procedures

The adolescent rats used in these studies were generated by mating Sprague-Dawley rats weighing 250g (Charles River, Calco, Italy) and housed under standard conditions of temperature and humidity under artificial light (from 07:00 to 19:00 hours). A maximum of two male siblings was taken from each litter to reduce "litter effects" (Chapman and Stern, 1978).

Experiment A (Section 4.1.): to investigate whether the developmental exposure to cocaine could modulate the response of the glutamatergic system in a challenge situation, male rats were treated subcutaneously with cocaine (20 mg/kg/day) (MacFarlan-Smith, Edinburgh, UK) or saline from postnatal day 28 (PND 28) to PND 42, a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004). A group of rats, treated either with saline and cocaine was sacrificed three days after the end of treatment (PND 45) while another group of rats with the same treatment regimen and withdrawal time underwent to a single session of forced swim test (FST) for 5 minutes and scarified 15 minutes after the end of the stress procedure.

Experiment B (section 4.2.): to investigate whether repeated exposure to cocaine during adolescence could alter the stress related system, male rats were treated subcutaneously with cocaine (20 mg/kg/day) (MacFarlan-Smith, Edinburgh, UK) or saline from postnatal day 28 (PND 28) to PND 42, a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004). A group of rats, treated either with saline and cocaine with no further manipulations was sacrificed three days after the end of treatment (PND 45).

Experiment C and D (Sections 4.3. and 4.4.): to investigate the long-term molecular changes set in motion by repeated exposure to cocaine during adolescence, male rats were treated subcutaneously with cocaine (20 mg/kg/day) (MacFarlan-Smith, Edinburgh, UK) or saline from

postnatal day 28 (PND 28) to PND 42, a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004). Thus, rats treated either with saline and cocaine, were left undisturbed in their home cages till adulthood and sacrificed at PND 90.

Experiment E (section 4.5.): to investigate the short- and long-term molecular changes set in motion by developmental exposure to cocaine on the FGF-2 system, and how exposure to cocaine could modulate the response to an acute stress, male rats were treated subcutaneously with cocaine (20 mg/kg/day) (MacFarlan-Smith, Edinburgh, UK) or saline from postnatal day 28 (PND 28) to PND 42, a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004). A group of rats, treated either with saline and cocaine, was sacrificed three days and 48 days after the end of treatment (PND 45 and PND 90 respectively). Another group of rats with the same treatment exposure and withdrawal time from the last drug exposure underwent to a single session of forced swim test (FST) for 5 minutes and scarified 15 minutes after the end of the stress procedure.

Experiment F (section 4.6.): to investigate whether a priming of cocaine could alter the trophic response to a second injection, male rats were exposed to the first intraperitoneal injection (i.p.) of cocaine (20 mg/kg) or saline at postnatal day 35 (PND 35) and then challenged with a second i.p. injection of cocaine (10 mg/kg) or saline at PND 36, i.e. 24 hours after the first one, or 7 days later at PND 42, during a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004; Maldonado and Kirstein, 2005). Rats were sacrificed 2 hours after the second injection.

Following the sacrifice, the Frontal cortex (Approximately from Bregma +3.70 to Bregma +2.20), mPFC (Approximately from Bregma +3.70 to Bregma +2.20), Striatum (Approximately from Bregma +2.20 to Bregma +0.48), Nucleus Accumbens (Approximately from Bregma +2.20 to Bregma +0.48) and Hippocampus (Approximately from Bregma -1.80 to Bregma -6.80) (Paxinos and Watson, 2005) were immediately dissected from 2-mm thick slices, frozen on dry ice and stored at -80°C.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., supplement 40, 18 Febbraio, 1992, Circolare No. 8, G.U., 14 Luglio, 1994) and international laws and policies

(EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care & Use of Laboratory Animals, U.S. National Research Council, 1996).

3.2. 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 using the NanoDrop spectrophotometer (Thermo Fisher Scientific Inc). Following total RNA extraction, the samples were processed for real-time reverse transcription polymerase chain reaction (real-time RT-PCR) to assess mRNA levels. Briefly, an aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed by TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iScript™ one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 well formats in triplicate as multiplexed reactions. Data were analyzed with the comparative threshold cycle (ΔΔCt) method using 36B4, β-actin and 18S as reference genes. The primer efficiencies were experimentally set up for each couple of primers.

Probes and primers were purchased from Eurofins MWG-Operon. 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. The complete set of primers and probes used in these studies are listed in Tab. 2, section 6. (appendix)

3.3. microRNA real-time qPCR.

According to the manufacturer's instructions, two microlitres (μl) of RNA solution 5ng/μl were reverse transcribed in 10μl of reaction mix using the miRCURY LNA™ Universal-RT microRNA PCR kit (#203300, Exiqon). cDNA was diluted 80X and assayed by real-time PCR according to the protocol for miRCURY LNA™ Universal-RT microRNA PCR. SYBR Green (SYBR® Green master mix, Exiqon, #203450) based RT-PCR was performed in CFX384 real-time system (Bio-Rad Laboratories) and LNA-enhanced miRNA specific primers sets were used to quantify hsa-let-7-d (#204124, Exiqon), hsa-miR-124 (#204319, Exiqon), hsa-miR-132 (#204129, Exiqon). Each reaction was performed in triplicate and data were analyzed with the comparative threshold cycle (ΔΔCt) method using U6 snRNA (#203907, Exiqon) and RNU5G (#203908, Exiqon) as stably expressed reference genes.

3.4. Protein Extraction and Western Blot Analyses

The brain tissues were homogenized in a glass-glass Potter using a cold buffer containing 0.32M sucrose, 1mM Hepes solution, 0.1mM EGTA, 0.1mM PMSF, pH=7.4, in a presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. The crude synaptosomal fraction was prepared as previously described. The homogenized tissues were centrifuged at 1000 g for 10 min; The resulting pellet (P1), corresponding to the nuclear fraction, was re-suspended in a buffer containing 20mM Hepes, 0.1mM DTT, 0.1mM EGTA, with protease and phosphatase inhibitors. The supernatant (S1) was centrifuged at 9000 g for 15 min to obtain a pellet (P2) corresponding to the crude synaptosomal fraction, which was re-suspended in a buffer containing 20mM Hepes, 0.1mM DTT, 0.1mM EGTA, with protease and phosphatase inhibitors, and the resulting supernatant (S2) corresponds to a clarified fraction of cytosolic proteins. Total amount of proteins was measured by the Bio-Rad Protein Assay, using bovine serum albumin (BSA) as the calibration standard (Bio-Rad Laboratories, Milan, Italy).

For the molecular analysis of BDNF, its high-affinity receptor TrkB as well as its associated signaling pathway, 10μg of proteins for each line were run under reducing conditions on the Criterion TGX precast gels (Bio-Rad Laboratories, Milan, Italy) and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Milan, Italy). For the other molecular analysis, 10μg of proteins for each sample were run on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel under reducing conditions, and proteins were then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, 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. Antibodies used for protein analysis are listed in Tab. 1, section 6. (appendix).

3.5. Dendritic spine labeling and morphological classification

Neuronal labeling and morphological classification of dendritic spines in layer V of medial prefrontal cortex were carried out using a lipophilic membrane tracer as previously reported with minor modifications (Malinverno et al., 2010). Three days after the end of the chronic cocaine treatment (PND45), rats (3 rats/group) were deeply anesthetized and perfused with 0.1M phosphate buffer (PB) followed by 1.5% paraformaldehyde (PFA) in PB. Brains were removed from the skulls and were postfixed at 4°C in 4% PFA in PB for 40 min. 2-mm thick slices corresponding to plates 5-9 of the atlas of Paxinos and Watson and containing the mPFC have been dissected from the postfixed brains and stained with a lipophilic dye, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DilC18(3)) (Life Technologies). Brain sections were left overnight at room temperature in PB to allow the Dil to completely diffuse through labeled neurons. Sections were, then, postfixed in 4% PFA for 40 min at 4°C, washed three times in PB and 150-μm thick coronal slices were prepared using a vibratome. Slices were then mounted, covered in fluoromount (Sigma-Aldrich), and analyzed on a Zeiss LSM-510 laser confocal microscope with 63X objective. Individual dendrites were selected randomly and their

spines were traced manually. The number of neurons used for quantification is at least 15 for each experimental group. Analysis of dendritic spine morphology was performed with ImageJ software; for each dendritic spine length, the head and neck width were measured, which was used to classify dendritic spines into three categories (thin, stubby and mushroom) (Harris et al., 1992). In particular, the length and the ratio between the width of head and the width of neck (Wh/Wn) were used as parameters for the classification as follows: protrusions having a length of more than 3 μm were considered as filopodia, the others as spines; spines with a Wh/Wn ratio bigger than 1.7 were considered mushrooms; spines with a Wh/Wn ratio smaller than 1.7 were divided in stubby, if shorter than 1 μm, and thin if longer than 1 μm (Gardoni et al., 2012). An operator who was 'blind' to the experimental conditions performed both image acquisition and quantification.

3.6. Analysis of plasma corticosterone levels

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

3.7. Statistical analysis

Data were collected in individual animals (independent determinations) and are presented as means and standard errors.

Experiment A (Section 4.1.): Changes produced by cocaine treatment and acute stress alone as well as by their combination were analyzed using a two-way analysis of variance (ANOVA), with adolescent cocaine treatment and acute stress as independent variables. When dictated by relevant interaction terms, Single Contrast Post-Hoc Test (SCPHT) was used to

characterize differences among individual groups of rats. However, when no interaction between cocaine treatment and stress was observed, only the main effects were reported. The immobility time, measured during the swim stress, was analyzed by an unpaired Student's t test. Statistical significance was assumed at $p<0.05$.

Experiment B (section 4.2.): The effects produced by repeated cocaine treatment were analyzed by an unpaired Student's t-test. Statistical significance was assumed at $p<0.05$. Statistical evaluation of all confocal experiments was performed by an unpaired Student's t-test. Statistical significance was assumed at $p<0.05$.

Experiment C and D (Sections 4.3. and 4.4.): The long-term effects produced by repeated cocaine treatment were analyzed by an unpaired Student's t-test. Statistical significance was assumed at $p<0.05$.

Experiment E (Section 4.5.): Changes produced by cocaine treatment and acute stress alone as well as by their combination were analyzed using a two-way analysis of variance (ANOVA), with adolescent cocaine treatment and acute stress as independent variables. When dictated by relevant interaction terms, Single Contrast Post-Hoc Test (SCPHT) was used to characterize differences among individual groups of rats. However, when no interaction between cocaine treatment and stress was observed, only the main effects were reported. The immobility time, measured during the swim stress, was analyzed by an unpaired Student's t test. Statistical significance was assumed at $p<0.05$

Experiment F (section 4.6.): To determine treatment-related differences, we first subjected the values to a three-way (analysis of variance) ANOVA, incorporating the following variables: first injection (saline vs. cocaine), second injection (saline vs cocaine) and time between injections (24 hours vs. 7 days). As dictated by the relevant interaction terms, low-order ANOVAs were used to determine treatment effects and interactions followed by Fisher's protected least significant difference (Fisher's LSD test) to characterize differences among individual values. Significance for all tests was assumed at $p<0.05$.

4. Results and discussion

4.1. Stress rapidly reorganizes the glutamate synapse in the prefrontal cortex of cocaine-withdrawn adolescent rats

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4.1.1. Introduction

Drug addiction is a chronic relapsing disorder hypothesized to be produced by drug-induced neuroplasticity that renders individuals vulnerable to craving-inducing stimuli. Besides alterations in central dopamine homeostasis (Berridge and Robinson, 1998), addiction liability has also been associated to changes in prefrontal-accumbens glutamate transmission (Kalivas et al., 2005; Gipson et al., 2014). Several studies have investigated the role of glutamate in the nucleus accumbens (NAc) (Ghasemzadeh et al., 2011; Huang et al., 2011; Xie et al., 2012; Purgianto et al., 2013) while we know less about cocaine-induced glutamate plasticity in the medial prefrontal cortex (mPFC), although the mPFC has been described as a key site for compulsive drug-seeking (Everitt and Robbins, 2005). In fact, evidence exists showing that long-term exposure to cocaine does not alter glutamatergic receptor protein expression in mPFC (Tang et al., 2004; Hemby et al., 2005; Freeman et al., 2008) whereas withdrawal from cocaine leads to robust changes in the redistribution of these cortical receptors (Ghasemzadeh et al., 2009; Ghasemzadeh et al., 2011; Ben-Shahar et al., 2013).

Among the factors that cause relapse to drug-seeking, stress plays an important role. In fact, cocaine users are extremely sensitive to stressful events (Sinha et al., 2003; Fox et al., 2008; Chaplin et al., 2010). Studies in animals have shown that acute stress can induce relapse to drug seeking following chronic exposure to cocaine (Erb et al., 1996; Ahmed and Koob, 2005), but the contribution of glutamate to stress-induced cocaine reinstatement is still elusive. In the current study, we incorporated a paradigm of acute swim stress to investigate, for the first time in detail, the functional responsiveness of the mPFC glutamate system in animals with a prior history of cocaine. We have used adolescent rats because these animals are more vulnerable to cocaine when compared to adult rats (Wong et al., 2013).

Glutamate is the major excitatory neurotransmitter in the brain. It is stored within intracellular secretory vesicles via the action of vesicular glutamate transporters (vGLUTs) that do not only serve storage functions, but are also involved in the regulation of glutamate release (Bellocchio et al., 2000). Once released, glutamate activates post-synaptic ionotropic and metabotropic receptors before removal from the extracellular space into glial cells through the

action of excitatory amino acid transporters (EAAT1 and EAAT2) (Bridges et al., 2012). Once inside the glial cells, glutamine synthetase (GS) converts glutamate into glutamine, which is then released by glial cells and taken up by glutamate neurons (Broer and Brookes, 2001) thereby replenishing the pre-synaptic vesicular stores of glutamate. In addition, glutamate homeostasis is modulated also by an antiporter that exchanges extracellular cysteine for intracellular glutamate and is also responsible of the extra-synaptic release of glutamate (Bridges et al., 2012).

We show that swim stress rapidly reorganizes the expression of the above-mentioned components of the glutamatergic synapse in the mPFC of cocaine-withdrawn adolescent rats. The observed cocaine-induced sensitization of the glutamatergic synapse to stress may contribute to the increased sensitivity to stress observed in cocaine users as well as to stress-induced reinstatement of cocaine seeking.

4.1.2. Results

Figure 1A illustrates the experimental paradigm that was designed to investigate whether the rapid stress-induced response of the glutamatergic synapse was influenced by a previous history of cocaine. Medial prefrontal cortex (mPFC) was dissected as depicted in Figure 1B. Figure 1C shows the behavioral response to the 5 min stress in both saline- and cocaine-treated rats. We measured the time that the animals were immobile during the 5 min of forced swimming, an index of pro-depressive symptoms that are known to be related, at least in part, to increased glutamate transmission (Sanacora et al., 2012). Interestingly, cocaine-treated rats showed higher immobility when compared to saline-treated rats (+81%, $p< 0.01$, Student's t-test) (Fig. 1C). We also measured the plasma levels of corticosterone and found a significant effect of stress ($F_{1,33}= 57.74$; $p= .22E-07$; two-way ANOVA), but no cocaine x stress interaction ($F_{1,33}= 0.86$; $p= 0.358$; two-way ANOVA). Notably, cocaine alone did not alter the plasma levels of corticosterone (Fig. 1D).

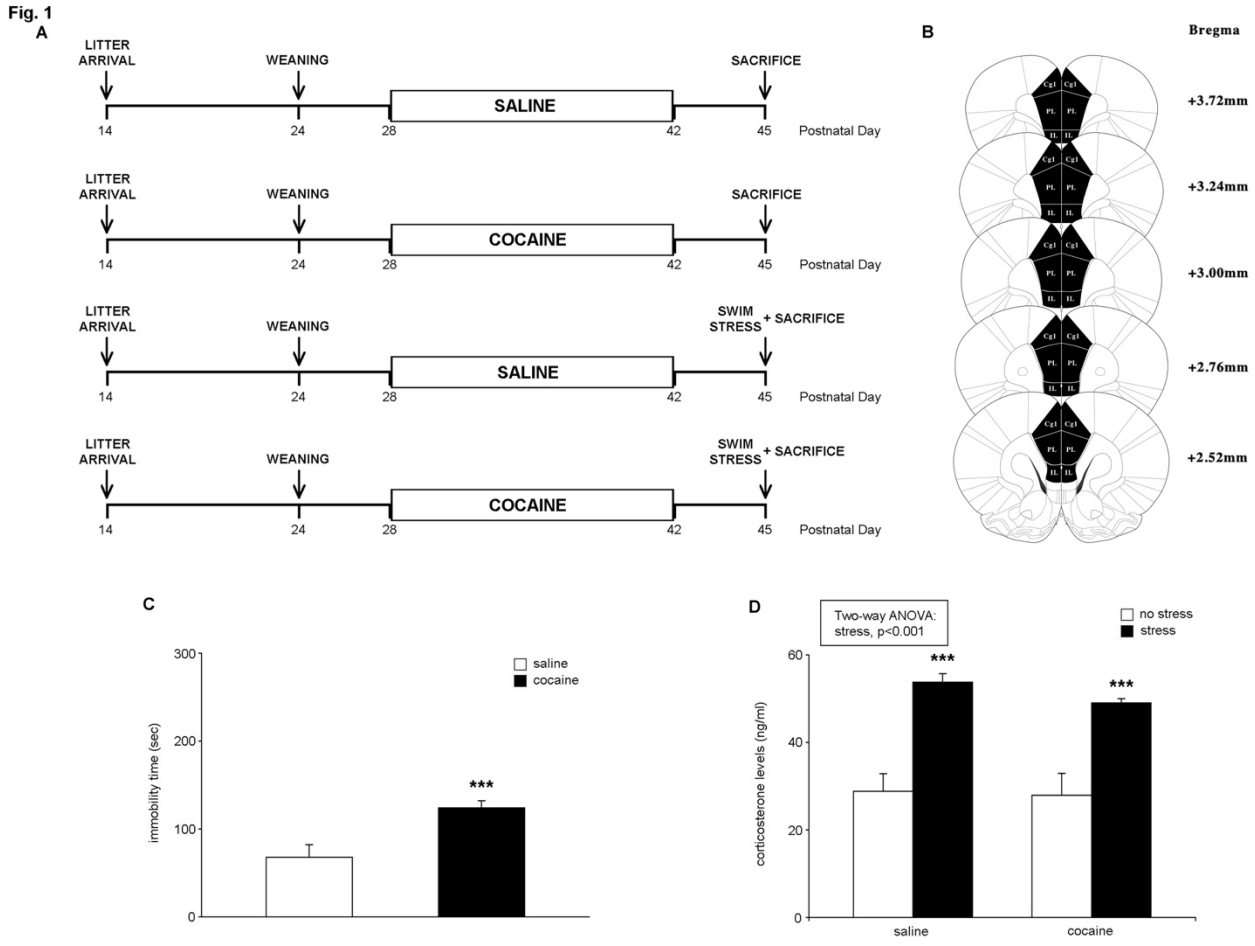

Figure 1: Schematic representation of the experimental procedure, specific coordinates of medial prefrontal cortex dissection and general analyses characterizing the stress experiment. Panel (A) shows the experimental paradigm. Animals were treated with cocaine (20 mg/kg) or saline during early adolescence [postnatal day (PND) 28-PND 42]. After the end of the adolescent treatment, animals were left undisturbed in their home cages. On PND 45, half of the animals exposed to cocaine or to saline, were subjected to 5 minutes of swim stress and sacrificed 15 minutes after the end of this stressor. The other half of the animals was left in their home cages and sacrificed at the same time as their swim stress-exposed counterparts. Panel (B) shows the dissection of the medial prefrontal cortex. This procedure was undertaken according to the coordinates indicated by the atlas of Paxinos and Watson, 2005 (please see the Material and Methods section). The medial prefrontal cortex was then stored at –80°C until processing. Panel (C) shows the time (seconds) the animals spent immobile during the 5 minutes of swim stress. Measurements were taken by three independent investigators who were blind to the experimental design (***P < 0.001, unpaired Student's t-test). Panel (D) shows the levels of circulating corticosterone (expressed in ng/ml). The main effects of analysis of variance appear at the top of the panel (***P < 0. 001).

We next measured the expression of several molecular determinants of the glutamate synapse in response to the combination of adolescent exposure to cocaine and acute stress. Given that the short interval between the end of stress and sacrifice (15 min) is likely to favor changes that occur rapidly, we focused on measuring gene expression and/or phosphorylation of the

different glutamate determinants, since these represent the initial responses, whereas changes in corresponding protein levels take much longer.

Figure 2 shows the contribution of cortical glial cells to the effects produced by the combination of cocaine and stress. Considering the glutamate transporters EAAT1 and EAAT2, their response to stress was influenced by prior cocaine history. Two-way ANOVA showed a cocaine x stress interaction for EAAT1 ($F_{1,35}$= 5.48; p= 0.026) and EAAT2 ($F_{1,30}$= 6.77; p= 0.015) (Fig. 2A and 2B). The single intergroup comparisons revealed that stress decreased the levels of these transporters in cocaine-treated animals (EAAT1: -14% vs. cocaine-no stress, $F_{1,17}$= 6.15, p= 0.038; EAAT2: -23% vs. cocaine-no stress, $F_{1,15}$= 12.07, p= 0.0036; SCPHT), but not in saline-treated rats (EAAT1: +4% vs. saline-no stress, $F_{1,18}$= 0.65, p= 0.854; EAAT2: +2% vs. saline-no stress, $F_{1,15}$= 0.06, p= 1.632; SCPHT) (Fig. 2A and 2B). We also measured the expression of another transporter expressed by glial cells, such as the glucose transporter 1 (Glut-1). No changes in Glut-1 mRNA levels were found suggesting that the combination of cocaine and stress selectively targets glial glutamate transport (data not shown).

Besides glutamate transporters, extracellular glutamate levels may also be modulated via the regulation of the glial cysteine/glutamate antiporter (system Xc-) that exchanges extracellular cysteine for intracellular glutamate (Bridges et al., 2012). Two-way ANOVA indicated cocaine x stress interaction ($F_{1,33}$= 6.78; p= 0.015) for system Xc- mRNA levels (Fig. 2C). The analysis of the single treatment effects revealed a significant reduction of system Xc- in the mPFC of animals exposed to cocaine and stress (-19% vs. cocaine-no stress, $F_{1,17}$= 6.16, p= 0,038; SCPHT) with no effects in saline-treated rats (+9% vs. saline-no stress, $F_{1,16}$= 1.42, p= 0.486; SCPHT) (Fig. 2C). To further characterize the contribution of glial cells, we analyzed the effect of the combination of cocaine and stress on the enzyme that converts glutamate into glutamine in the glia, i.e. glutamine synthetase (GS). Two-way ANOVA indicated cocaine x stress interaction ($F_{1,33}$= 5.71; p= 0.024) for GS mRNA levels (Fig. 2D). We subdivided the data for individual intergroup comparisons and found that stress evoked a significant elevation of GS mRNA levels only in rats that had received cocaine during adolescence (+26% vs. cocaine-no stress, $F_{1,16}$= 16.61, p= 0.0006; SCPHT) with no effects in saline-treated rats (+7% vs. saline-no stress; $F_{1,17}$= 0.62, p= 0.878; SCPHT) (Fig. 2D).

Fig. 2

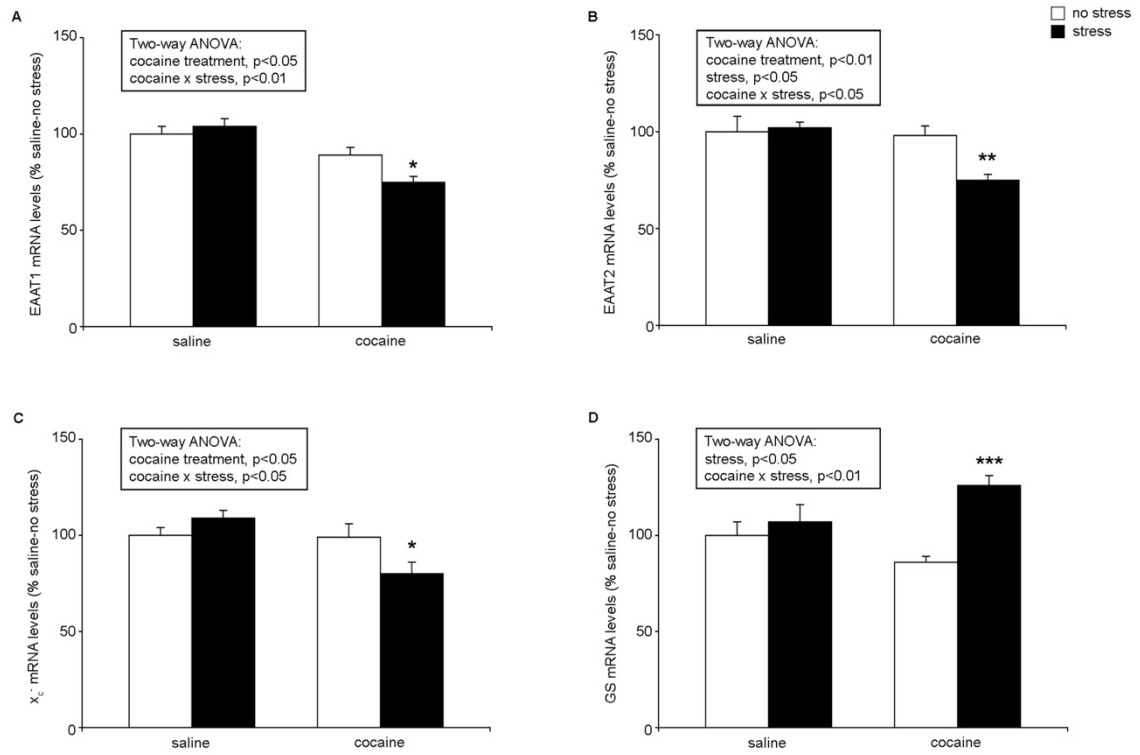

Figure 2: Effect of repeated cocaine treatment during adolescence [postnatal day (PND) 28 to PND 42] on glial determinants of glutamate neurotransmission and on the subsequent response to acute stress assessed at PND 45 in the medial prefrontal cortex. Panel (A) and (B) show the glial glutamate transporter mRNA levels, EAAT1 and EAAT2; panel C shows the glial glutamate exchanger mRNA levels, Xc^-, while panel D shows the glutamine synthetase mRNA levels, GS. Asterisks denote the significant effect of the acute stress in cocaine-treated rats versus cocaine-no stress (*P < 0.05; **P < 0.01 and ***P < 0.001)

Next we investigated the pre-synaptic terminal (Fig. 3). Two-way ANOVA indicated a cocaine x stress interaction ($F_{1,30}$= 27.78; p= 0.000016) for the vesicular glutamate transporter 1 (vGLUT1) mRNA levels (Fig. 3A). Thus, we subdivided the data for individual intergroup comparisons. Stress evoked a marked up-regulation of vGLUT1 mRNA levels only in rats that had received cocaine during adolescence (+106% vs. cocaine-no stress, $F_{1,12}$= 36.08, p= 0.000004; SCPHT) with no effects in saline-treated rats (-14% vs. saline-no stress, $F_{1,18}$= 0.95, p= 0.676; SCPHT) (Fig. 3A). The analysis of the vesicular GABA transporter (vGAT) mRNA levels revealed only a main effect of cocaine treatment ($F_{1,33}$= 22.07; p= 0.00006; two-way ANOVA) (Fig. 3B) with no significant interaction between cocaine and stress ($F_{1,33}$= 0.125; p= 0.727; two-way ANOVA) (Fig. 3B). We next analyzed GAD 67 mRNA levels, the GABA synthesizing enzyme isoform highly expressed in the central nervous system (Soghomonian and Martin, 1998). Two-way ANOVA indicated a significant cocaine x stress interaction ($F_{1,32}$=

4.54; p= 0.041). The analysis of the single treatment effects revealed a significant reduction of GAD67 in the mPFC of animals exposed to cocaine and stress (-28% vs. cocaine-no stress, F1,15= 12.74, p= 0.003; SCPHT) with no effects in saline-treated rats (-5% vs. saline-no stress, F1,17= 0.472, p= 0.996; SCPHT) (Fig. 3C).

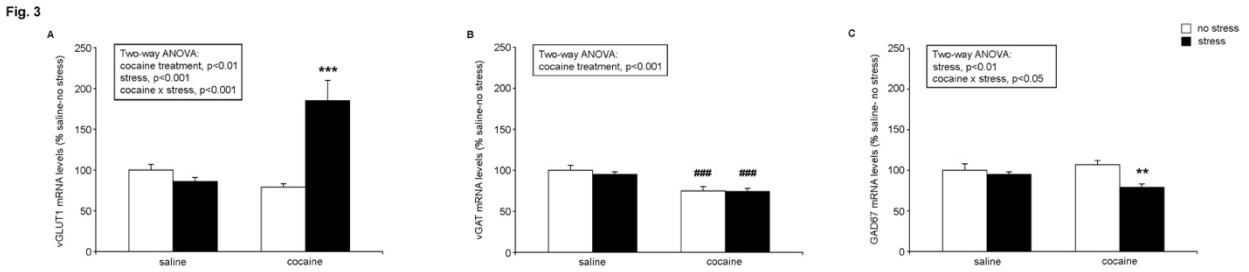

Figure 3: Effect of repeated cocaine treatment during adolescence (PND28 to PND 42) on presynaptic determinants of glutamate transmission. Panel (A) shows the vesicular glutamate transporter (vGLUT1) mRNA levels; panel (B) shows the vesicular gamma-aminobutyric acid transporter (vGAT) mRNA levels; panel (C) shows glutamate decarboxylase 67 (GAD 67) mRNA levels. The main effects of analysis of variance appear at the top of the panel ($^{###}$P<0.001). Asterisks denote the significant effect of the acute stress in cocaine-treated rats versus cocaine-no stress (**P < 0.01; ***P < 0.001).

Next, we investigated the post-synaptic terminal and analyzed the activation of the obligatory NMDA receptor subunit, GluN1, in the crude synaptosomal fraction, expressed as a ratio between the phosphorylated and the total levels of protein. As shown in figure 4A, two-way ANOVA indicated a significant cocaine x stress interaction (F1,22= 5.46; p= 0.031). Examining the individual treatment effects, we found that swim stress enhanced the phosphorylation levels of GluN1 in cocaine-treated rats (+66% vs. cocaine-no stress; F1,11= 18.60, p= 0.0008; SCPHT) with no effects in saline-treated rats (+15% vs. saline-no stress, F1,11= 1.02, p= 0.652; SCPHT) (Fig. 4A). No changes in the total levels of NMDA GluN1 (data not shown). We also measured the phosphorylation and total levels of the other NMDA (GluN2A, GluN2B) as well as AMPA receptor subunits (GluA1 and GluA2) and found no significant effects in any of these receptors (Table 1), suggesting that the combination of cocaine and stress specifically activates the obligatory NMDA receptor subunit, GluN1.

	Sal-sham	*Sal-stress*	*Coc-sham*	*Coc-stress*
GluN2A	100 ± 14	104 ± 10	110 ± 9	118 ± 9
pGluN2B/GluN2B	100 ± 10	95 ± 15	98 ± 12	112 ± 11
pGluA1/GluA1	100 ± 11	152 ± 13**	101 ± 9	140 ± 19**
pGluA2/GluA2	100 ± 4	100 ± 6	96 ± 4	107 ± 7

The results represent the mean ± standard error of measurement of at least six independent determinations for each experimental group (**$P < 0.004$).

Table 1: Levels of glutamate NMDA receptor subunits GluN2A and GluN2B as well as of the AMPA receptor subunits GluA1 and GluA2 following the exposure to saline or cocaine during adolescence and subsequent exposure to stress in rat mPFC.

Given that the acute activation of the NMDA receptor may lead to long-term structural and functional changes in spine plasticity through the modulation of CaMKII-Cdc42-Pak1 transduction pathway (Murakoshi et al., 2011), we also investigated these crucial effectors of such action. As shown in figure 4B, two-way ANOVA indicated only a significant stress effect on the activation of αCaMKII, expressed as a ratio between the phosphorylated and its total levels ($F_{1,19}$= 10.52; p= 0.005; two-way ANOVA). Post-hoc testing indicated that acute stress enhanced the phosphorylation of αCaMKII in cocaine-withdrawn rats (+73% vs. cocaine-no stress, $F_{1,10}$= 6.38, p= 0.044; SCPHT) but not in saline-treated rats (+40% vs. saline-no stress, $F_{1,9}$= 4.23, p= 0.112; SCPHT) (Fig. 4B).

Two-way ANOVA indicated a cocaine x stress interaction ($F_{1,35}$= 27.61; p= 0.00001; two-way ANOVA) for Cdc42 mRNA levels. Examining the individual treatment effects, we found that acute stress caused a significant elevation of Cdc42 mRNA levels in the mPFC of cocaine-treated rats (+43% vs. cocaine-no stress, $F_{1,17}$= 31.12, p= 0.000004; SCPHT), but not in saline-treated rats (-14% vs. cocaine-no stress, $F_{1,18}$= 27.61, p= 0.164; SCPHT) (Fig. 4C). The analysis of the activation of Pak1 kinase (expressed as ratio between the phosphorylated and total levels of the protein) also revealed a significant interaction between cocaine and stress ($F_{1,20}$= 5.44; p= 0.033; two-way ANOVA) with stress increasing Pak1 phosphorylation levels in cocaine-treated rats (+49% vs. cocaine-no stress, $F_{1,10}$= 11.27, p= 0.008; SCPHT), but not in saline-treated rats (+1% vs. saline-no stress, $F_{1,10}$= 0.003, p= 1.91; SCPHT) (Fig. 4D). No changes were observed when analyzing the total levels of Pak1 (data not shown).

Fig. 4

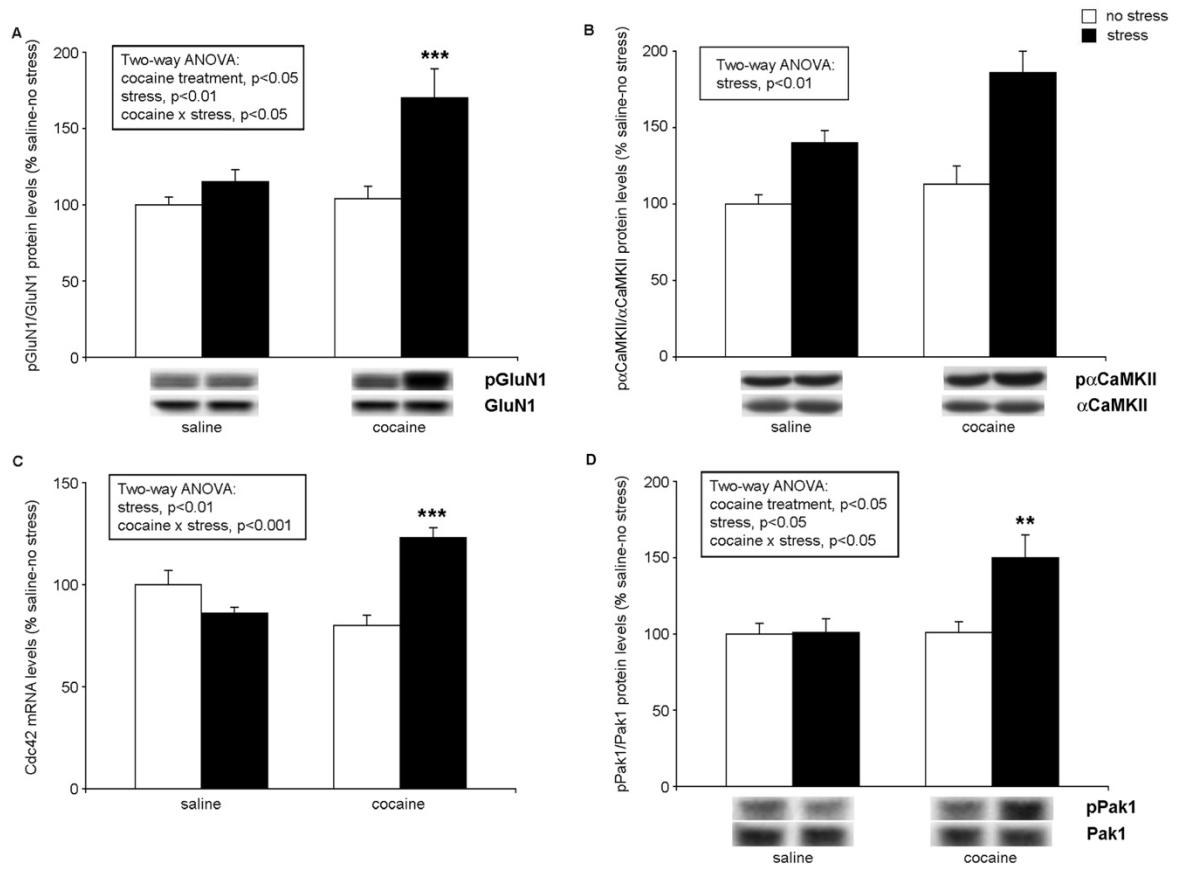

Figure 4: Effect of repeated cocaine treatment during adolescence (PND28 to PND 42) on postsynaptic determinants of glutamate transmission. Panel (A) shows the activation of the NMDA GluN1 receptor subunit whereas panel (B) shows the activation of the αCaMKII, both expressed as the ratio between the phosphorylated and the total form of the protein; panel (C) shows Cdc42 mRNA levels whereas panel (D) shows the phosphorylation of Pak1, expressed as the ratio between the phosphorylated and the total form of the protein. Asterisks denote the significant effect of the acute stress in cocaine-treated rats versus cocaine-no stress (**P < 0.01; ***P < 0.001). Below the graphs in panel (A), (B) and (D) are shown representative bands of the respective proteins from Western blots.

It has been postulated that long-term cocaine administration produces a reduction in the baseline neuronal activity of the mPFC, but that exposure to a stimulus capable of generating drug-seeking (i.e. cocaine priming, cues or stress) would produce a hyperactive state (Goldstein and Volkow, 2011; Jentsch and Taylor, 1999). This may explain the higher responsivity to stress observed in the glutamate synapse of cocaine-treated rats. Accordingly, we decided to measure protein expression of the immediate early gene Arc (Activity Regulated Cytoskeletal-associated protein), a well-established marker of neuronal activity. As shown in figure 5, two-way ANOVA indicated a significant cocaine x stress interaction ($F_{1,30}$= 12.54; p= 0.002; two-way ANOVA).

Examining the individual treatment effects, repeated exposure to cocaine reduced Arc expression (-20% vs. saline-no stress, F1,14= 5.90, p= 0.035; SCPHT); interestingly, Arc expression was significantly increased only in the mPFC of cocaine-withdrawn rats exposed to stress (+39% vs. cocaine-no stress, F1,15= 20.96, p= 0.0002; SCPHT) but not to saline (-4% vs. saline-no stress, F1,15= 0.16, p= 0.692; SCPHT) (Fig. 5).

Fig. 5

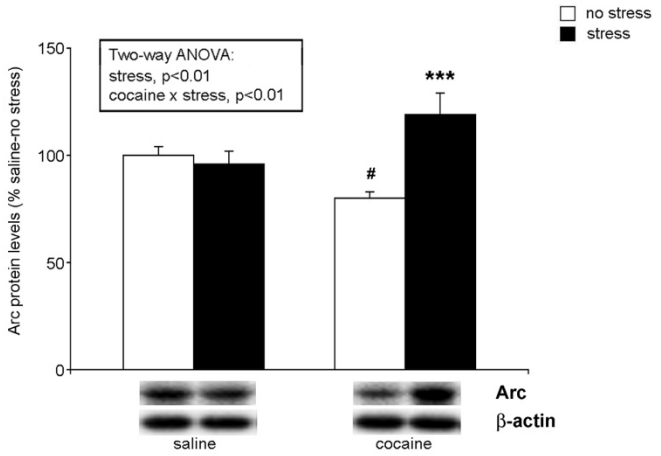

Figure 5: Effect of repeated cocaine treatment during adolescence (PND28 to PND 42) on Arc protein levels. Dagger denotes the significant effect of repeated cocaine treatment during adolescence versus saline-no stress ($^{\#}P<0.05$). Asterisks denote the significant effect of the acute stress in cocaine-treated rats versus cocaine-no stress (***P < 0.001). Below the graph are shown representative bands of Arc protein from Western blots.

4.1.3. Discussion

The present results show that adolescent cocaine exposure sensitizes glutamatergic responses of the mPFC to acute stress, presumably through cocaine-induced reduction of baseline mPFC neuronal activity that may generate a hyperactive state in response to a stimulus capable of generating drug seeking (i.e. acute stress) (Jentsch and Taylor, 1999; Goldstein and Volkow, 2011). Our findings indicate that, in cocaine-withdrawn adolescent rats, the response to a brief stressor produces a reorganization of the mPFC within a time-frame of minutes. These findings may help to explain, at least in part, the hypersensitivity to stress observed in cocaine users during early abstinence (Sinha, 2001; Fox et al., 2008; Chaplin et al., 2010).

4.1.4. Effects of acute stress on glutamate-related glial elements in the mPFC of cocaine-treated rats.

Glutamate signaling requires a dynamic and coordinated interplay among neurons and glial cells (Bridges et al., 2012). We found that the glial glutamate transporters EAAT1 and EAAT2 mRNA levels are reduced following the combination of cocaine and stress. This suggests that the clearance of the extracellular glutamate from the synaptic space is impaired after stress in the mPFC of cocaine-treated rats, pointing to increased excitatory neurotransmission in these animals. Interestingly, exposure to the combination of cocaine and stress also reduced the mRNA levels of the glutamate antiporter system Xc. Since this antiporter modulates the efflux of glutamate at extra-synaptic sites, which stimulates pre-synaptic mGluR 2/3 receptors to inhibit glutamate release (Baker et al., 2002), reduced expression of this antiporter by the combination of cocaine and stress may potentiate synaptic excitatory neurotransmission. The reduction of EAAT1 and EAAT2 mRNA levels may also be seen as an adaptive response to maintain physiological levels of glutamate in the extra-synaptic space to avoid excessive clearance.

We also observed a significant elevation of GS, the enzyme that converts glutamate into glutamine, in glial cells. Since glutamine is then released by glial cells and taken up by glutamate neurons (Broer and Brookes, 2001), increased expression of GS by the combination of cocaine and stress may lead to higher availability of glutamate inside the pre-synaptic glutamate neurons.

Our data suggest that glial cells strongly contribute to the observed dysregulation of glutamate homeostasis in animals exposed to the combination of cocaine and stress through at least two different mechanisms, i.e. by reducing glutamate reuptake and by increasing glutamate availability at the pre-synaptic level.

4.1.5. Effects of acute stress on pre- and post-synaptic elements of the glutamatergic synapse in the mPFC of cocaine-treated rats.

Interestingly, vGLUT1 mRNA levels were markedly increased in the mPFC of only rats exposed to the combination of cocaine and stress. Based on previously published evidence showing that vGLUT1 expression directly regulates glutamate release and the efficacy of glutamate neurotransmission (Wojcik et al., 2004; Wilson et al., 2005), our data suggest that more glutamate might be available for activity-dependent glutamate release, presumably as a consequence of the above-mentioned increased glutamine synthesis in glial cells.

We then shifted our attention to the GABA network, since it has recently been shown that non-contingent cocaine exposure during adolescence disrupts GABA functions in the mPFC (Cass et al., 2013). In contrast to the mRNA levels of vGLUT1, the mRNA levels of vGAT, the protein responsible of GABA storage and release, were reduced in the mPFC of animals exposed to the combination of cocaine and stress. Since GABA is an inhibitory neurotransmitter, a reduction of vGAT may further contribute to the potentiation of the excitatory neurotransmission in cocaine-withdrawn rats exposed to stress. This is further strengthened by the evidence that the mRNA levels of GAD 67, the enzyme responsible of converting glutamate into GABA, is reduced in the mPFC of rats exposed to the combination of cocaine and stress suggesting a reduced conversion of glutamate into GABA and, therefore, an accumulation of the excitatory neurotransmitter.

The analysis of post-synaptic glutamatergic elements also revealed changes indicative of hyper-responsive glutamatergic synapse. In fact, we found a selective activation of the obligatory subunit GluN1 in the mPFC of rats exposed to the combination of cocaine and stress. Such activation of the NMDA receptor may increase calcium influx that, in turn, activates downstream

kinases. To this end, we measured the phosphorylation of αCaMKII, a sensor of intracellular calcium levels (Coultrap and Bayer, 2012) and found it increased in the mPFC of cocaine-withdrawn rats. It is known that the activation of the NMDA receptor leads to changes in spine structural plasticity, an effect that may contribute to reinstatement of cocaine seeking (Toda et al., 2006), via the activation of the αCaMKII-cdc42-Pak signal transduction pathway (Murakoshi et al., 2011). Accordingly, we measured both cdc42 and Pak and found them enhanced by stress only in the mPFC of cocaine-treated rats. These data show increased responsiveness of cortical spines to stress only in animals with a previous history of cocaine exposure.

A potential limitation of our results may derive from the nature of cocaine exposure employed in the present study (i.e. non contingent) which is known to be more stressful relative to self-administration (Mantsch and Goeders, 2000); however, no differences in corticosterone levels were observed in the plasma of cocaine-treated rats when compared to saline-treated animals suggesting that the modality of cocaine treatment can not be considered a potential confound under our experimental conditions. Also, one may argue that we primarily rely on mRNA/phosphorylation measures to infer changes in function. However, the evaluation of the rapid coping to the acute stress needs to rely on measuring changes in gene expression and/or phosphorylation since these represent the initial responses, whereas changes in corresponding protein levels take much longer.

4.1.6. Conclusions

Exposure to acute stress in adolescent cocaine-treated rats leads to widespread changes in both glial and synaptic regulators of glutamate neurotransmission leading to reduced glutamate clearance, increased packaging of glutamate in the pre-synaptic vesicular stores, increased conversion of glutamate to glutamine, all of which would be combined with data consistent with increased activation of NMDA receptors (Figure 6). These results suggest that hyper-reactive glutamatergic synapses in the mPFC may contribute to the hypersensitivity to stress observed in abstinent cocaine users (Sinha, 2001; Fox et al., 2008; Chaplin et al., 2010).

Furthermore, these hypersensitive glutamatergic synapses in the mPFC may contribute to the negative emotional state and stress-induced reinstatement generally observed in animal models of cocaine abuse (Baker et al., 2003; McFarland et al., 2003; Koob, 2008). Notably, when compared to saline-treated rats exposed to swim stress, cocaine-treated rats exposed to swim stress showed increased immobility, a measure of depressive like-behavior. The evidence that, in cocaine-treated rats, acute stress recapitulates a depressive-like behavior that is usually only seen after chronic stress (Willner, 1997; Kompagne et al., 2008) suggests that withdrawal from our cocaine paradigm may have led to a latent negative emotional state (Koob and Le Moal, 1997), which is precipitated by an acute stressor. Interestingly, most of the markers that were altered following the combination of cocaine and stress are, indeed, changed in animal models of depression (Banasr et al., 2010; Eriksson et al., 2012). The changes in glutamate homeostasis herein observed may therefore contribute, at least in part, to the pro-depressive symptoms occurring during the initial phase of cocaine withdrawal (Gawin, 1991; Markou et al., 1992; Perrine et al., 2008).

Fig. 6

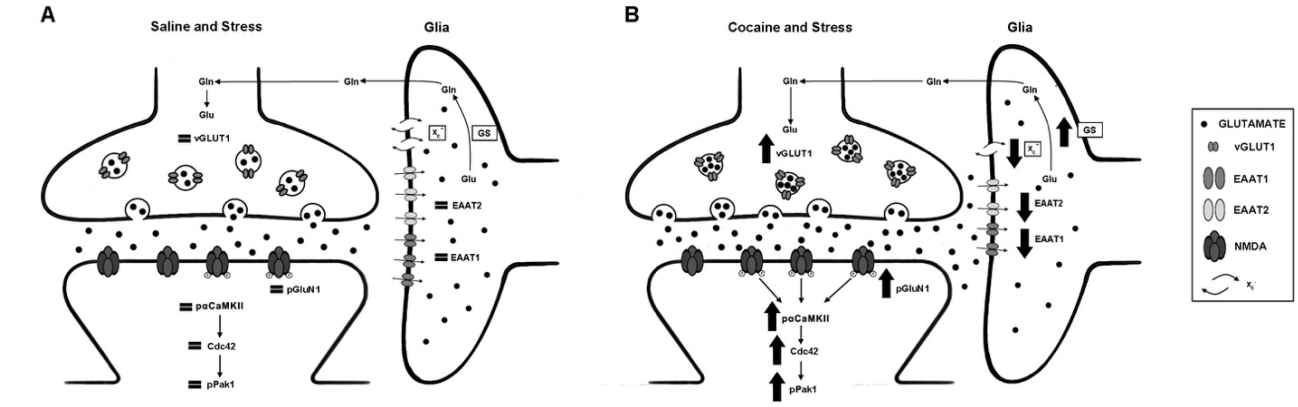

Figure 6: Exposure to repeated cocaine administration during adolescence alters the response of the cortical glutamate synapse to an acute stressor (panel b): comparison with rats exposed to saline and stress (panel a). The homeostasis of the glutamate synapse is altered in the mPFC of rats exposed to the combination of cocaine and stress. In the cocaine + stress group, glial function is altered as evidenced by reduced expression of the main glial glutamate transporters EAAT1 and EAAT2 that leads to diminished clearance of extracellular glutamate. Changes in glial homeostasis are further revealed by a reduced expression of the cystine/glutamate antiporter, system Xc^-, which modulates non-vesicular glutamate release. Further, increased expression of GS leads to higher availability of glutamine that is released by astrocytes and taken up by presynaptic neurons. This may contribute to the increased expression of vGLUT1 implying that more glutamate is available for activity-dependent glutamate release. The potentiation of the excitatory transmission results in increased activation of the postsynaptic NMDA receptor subunit GluN1 and CaMKII and increased responsiveness of cortical spines as measured by increased Cdc42 expression and Pak1 phosphorylation. αCaMKII = αCa^{++}/calmodulin-dependent protein kinase; Cdc42, cell division cycle 42, GTP binding protein; EAAT1, excitatory amino acid transporter 1; EAAT2, excitatory amino acid transporter 2; Gln, glutamine; Glu, glutamate; GluN1, glutamate NMDA receptor subunit 1; GS, glutamine synthetase; Pak1, p21-Activated Kinase 1; vGLUT1, vesicular glutamate transporter 1; Xc^-, cysteine/glutamate antiporter.

4.2. Short-term abstinence from developmental exposure to cocaine activates the glucocorticoid receptor and reduces spine density

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4.2.1. Introduction

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

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

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

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

4.2.2. Results

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

It is known that the activity of nuclear GRs on gene expression is sustained by co-activators (Kurihara et al., 2002; Meijer et al., 2006). Accordingly, we next evaluated whether the increased translocation of the receptor is correlated to increased activity by analyzing the expression levels of the nuclear receptor coativator-1 (Src1). We found that, three days after the last adolescent cocaine exposure, Src1 mRNA levels are increased (+30%, $p<0.05$; Fig. 1d), suggesting that increased GR nuclear trafficking is coupled with an enhanced activity of the GR.

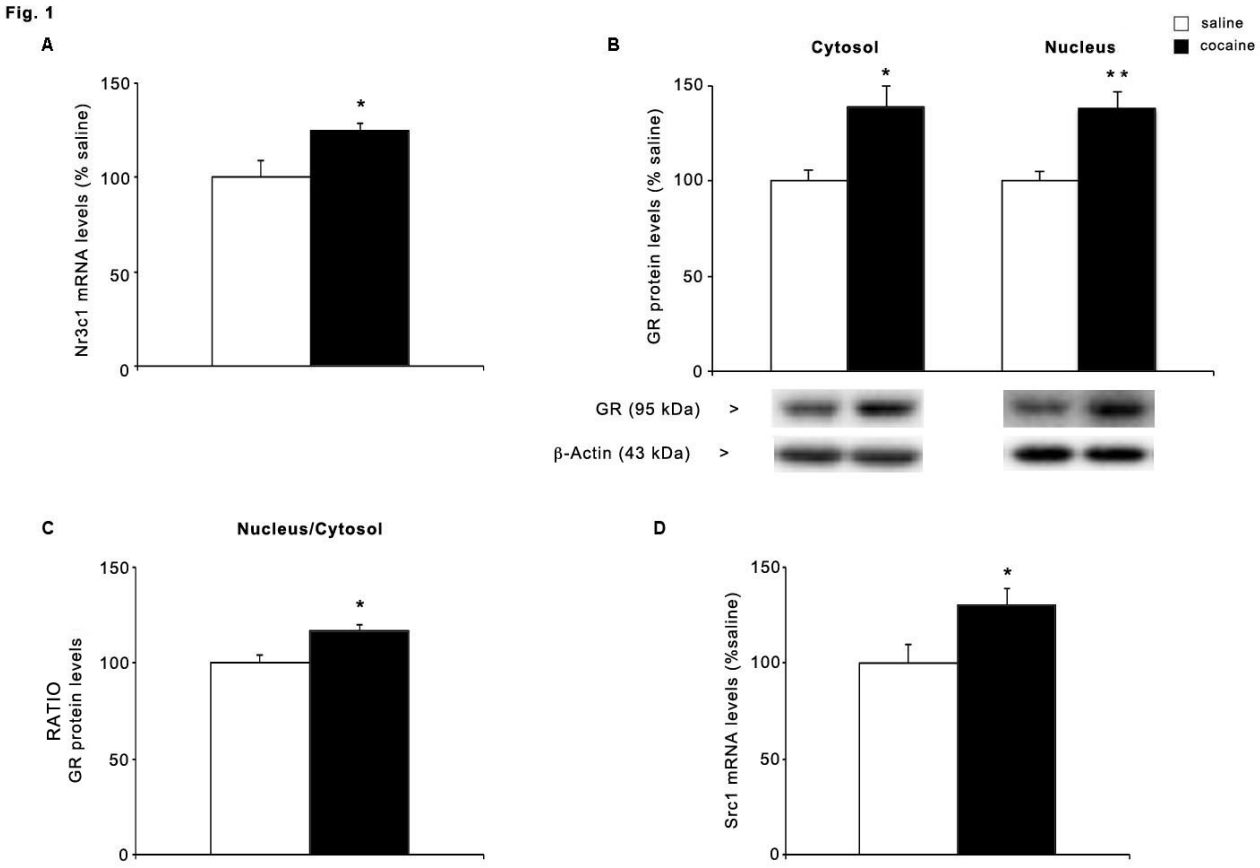

Figure 1: Glucocorticoid receptor mRNA and protein and co-activator Src1 mRNA levels are altered in the mPFC of cocaine- withdrawn PND 45 rats. Panel A shows Nr3c1 mRNA levels in the mPFC of PND 45 rats. Panel B shows the GR protein levels in the cytosolic and nuclear fraction of the mPFC of PND 45 rats. Panel C shows the ratio between nuclear and cytosolic GR protein levels in the mPFC of PND 45 rats. Panel D shows Src1 mRNA levels in the mPFC of PND 45 rats. Representative Western blot bands of GR are shown below the graphs shown in panel B. The results, expressed as % of saline-treated rats, represent the mean7±S.E.M. of at least 5 samples. *p<0.05, *p<0.01 vs. saline- treated rats.

GR translocation to the nucleus also depends on a specific phosphorylation in its serine residue 232 (Wang et al., 2002; Galliher-Beckley et al., 2008) and on a physical interaction with a large chaperone protein complex consisting mainly of FK506 binding protein 51 (FKBP51) (Tatro et al., 2009). Interestingly, we found increased GR phosphorylation in Ser232 (Fig. 2a) and reduced FKBP51 expression (Fig. 2b) in the cortical cytosolic fraction of cocaine-withdrawn rats (+27% p<0.05 and -26%, p<0.05, respectively).

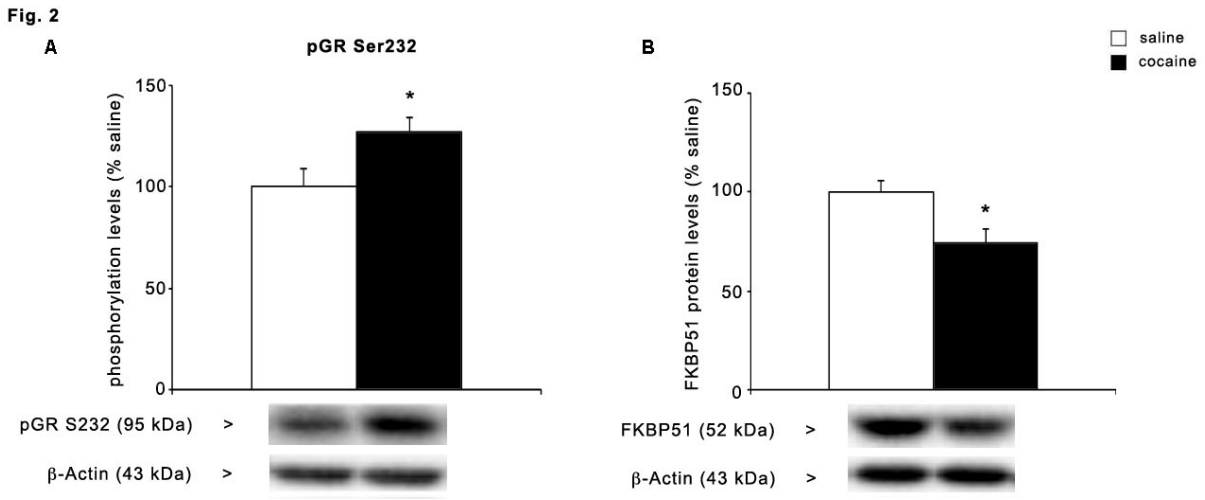

Figure 2: Effects of short-term abstinence on pGR S232 and FKBP51 protein levels in the mPFC of cocaine-withdrawn PND 45 rats. Panel A shows the GR phosphorylation levels in Ser232 measured in the cytosolic fraction of the mPFC of PND 45 rats. Panel b shows FKBP51 protein levels measured in the cytosolic fraction. Representative Western blot bands of pGR S232 and FKBP51 are shown below the graph shown in panel A and B. The results, expressed as % of saline-treated rats, represent the mean±S.E.M. of at least 5 samples. * $p<0.05$ vs. saline- treated rats.

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

Fig. 3

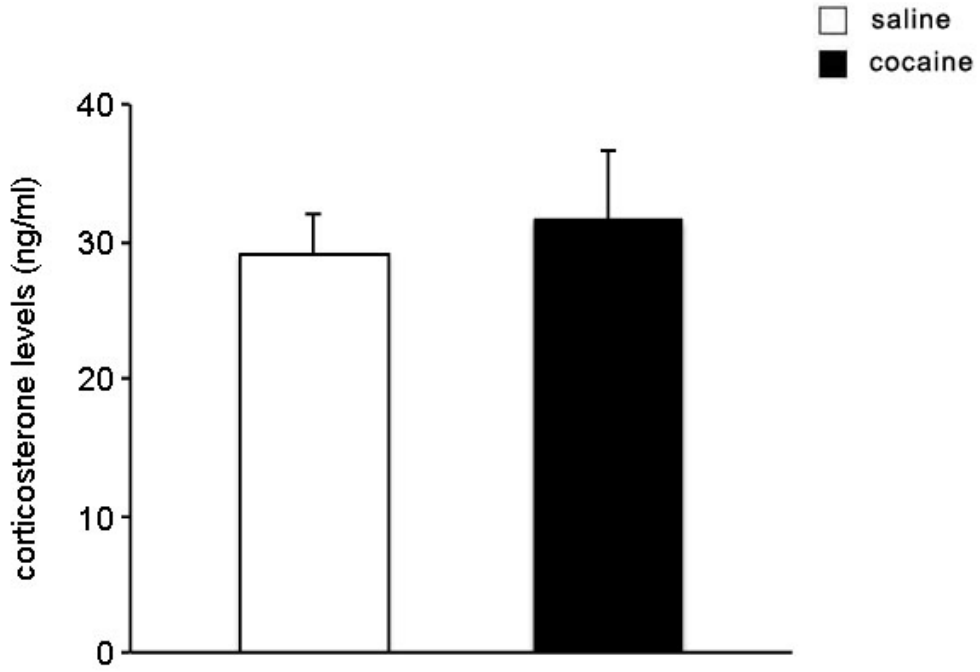

Figure 3: Effects of short-term withdrawal from developmental cocaine exposure on hypothalamus-pituitary-adrenal axis (HPA) activity. The levels of plasma glucocorticoids were expressed in nanogram per milliliter and represent the average of at least 7 animals for each experimental group.

Recently, GR has been proposed as a mediator of the plastic changes in the architecture of different brain areas contributing to alter dendritic spine density and morphology as well as spine actin cytoskeleton (Liu and Aghajanian, 2008; Liston and Gan, 2011; Jafari et al., 2012). Moreover, GR is critical for cognitive and emotional processes (de Kloet et al., 1999; Holsboer, 2000; Gass et al., 2001; Liston et al., 2006; Lupien et al., 2007). Since drug abuse impacts on the brain by usurping the neuronal mechanisms that contribute to learning and memory increasing the risk of drug dependence (O'Brien and Anthony, 2005; Hyman et al., 2006; Gould, 2010), we hypothesized that the herein found cocaine-induced alterations of GR-dependent mechanisms might result in dendritic atrophy and spine loss, an effect that may contribute to drug-induced neuroadaptations set in motion by early cocaine withdrawal. We then investigated some crucial effectors of the signaling pathways that regulate spine actin network. Figure 4a shows the reduced expression of Caldesmon (CaD) (-21%, $p<0.05$, Fig. 3a), an actin-linked regulatory protein that is negatively regulated when GR is activated (Tanokashira et al., 2012), presumably contributing to cocaine-induced abnormal spine development. Interestingly, as shown in figure

4b, we found reduced levels of PSD95, a well-established marker of postsynaptic density, in the cortical crude synaptosomal fraction of PND 45 cocaine-withdrawn rats (-21%, $p<0.05$). We also observed a reduced expression of the filamentous actin (F-Actin) (-41%, $p<0.01$) (Fig. 4c), the major cytoskeletal element in dendritic spines, and, accordingly, reduced phosphorylation levels of cofilin in Serine 3 (-46%, $p<0.05$) (Fig. 4d), an actin-depolimerizing factor linked to destabilization of actin polymers and spine loss in its dephosphorylated form (Gu et al., 2010).

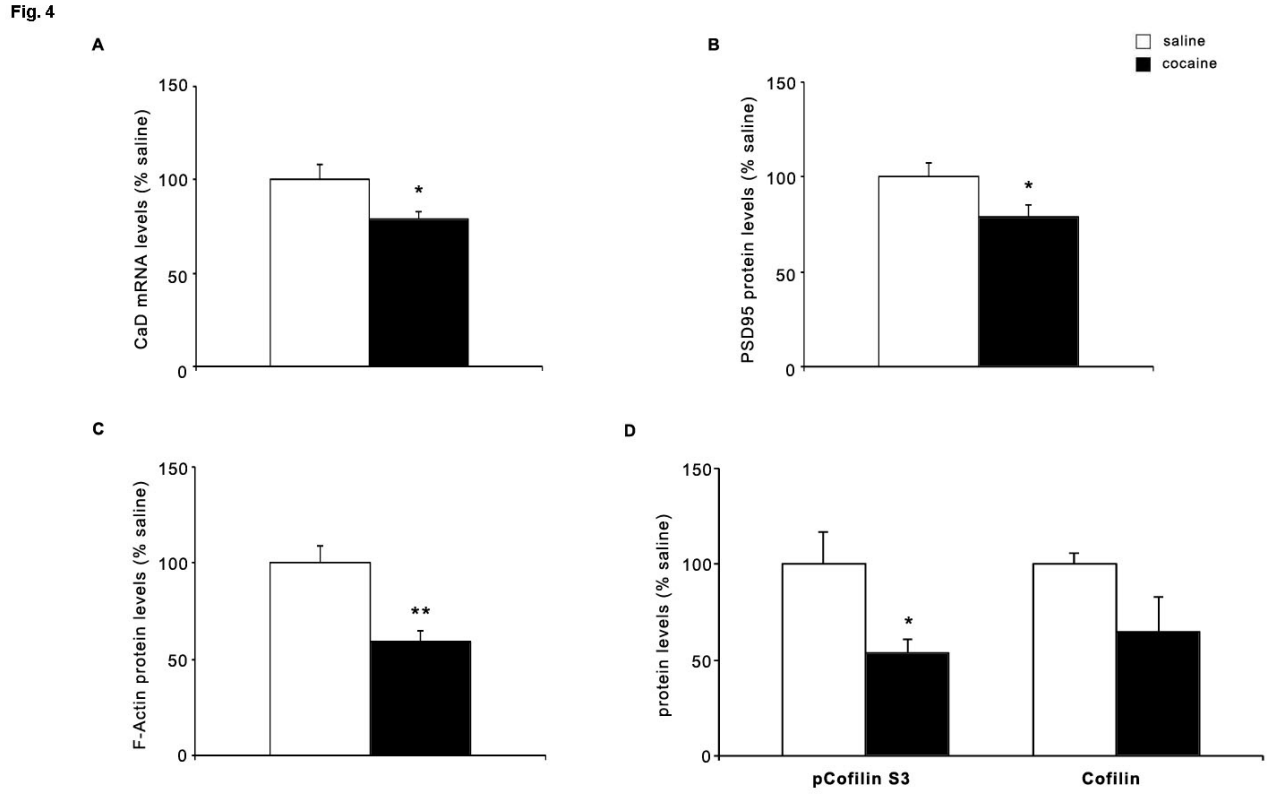

Figure 4: Effects of short-term abstinence on spine-related proteins in the mPFC of cocaine-withdrawn PND 45 rats. panel A shows Caldesmon (CaD) mRNA levels in the mPFC of PND 45 rats. PSD95 protein levels (panel B), F-Actin protein levels (panel C) and pCofilin Ser3 and total Cofilin (panel D) were measured in the crude synaptosomal fraction. The results, expressed as % of saline-treated rats, represent the mean±S.E.M. of at least 6 samples. *$p<0.05$ vs. saline- treated rats.

Since these data point to an alteration in the regulatory steps leading to formation, stabilization and elimination of the dendritic spines, we analyzed cocaine-induced spine remodeling using a fluorescent dyolistic labeling tecnique. Accordingly, we observed a reduction in cortical dendritic spine density in the mPFC of cocaine-withdrawn rats (-0.91 spine/μm, $p<0.05$; Fig 5a) with no effects on their lenght and head size (data not shown). Further, morphological analyses using a highly validated classification method (see Materials and Methods

section) and evaluating the shape of all protrusions (mushroom, thin, stubby and filopodia) were performed and revealed an increased formation of filopodia in cocaine-withdrawn rats (+4.76%, $p<0.05$) (Fig. 5b), i.e the immature protrusions that may contribute to maladaptive learning with no significant changes in the percentage of mushroom-, thin- and stubby-shaped spines.

Fig. 5

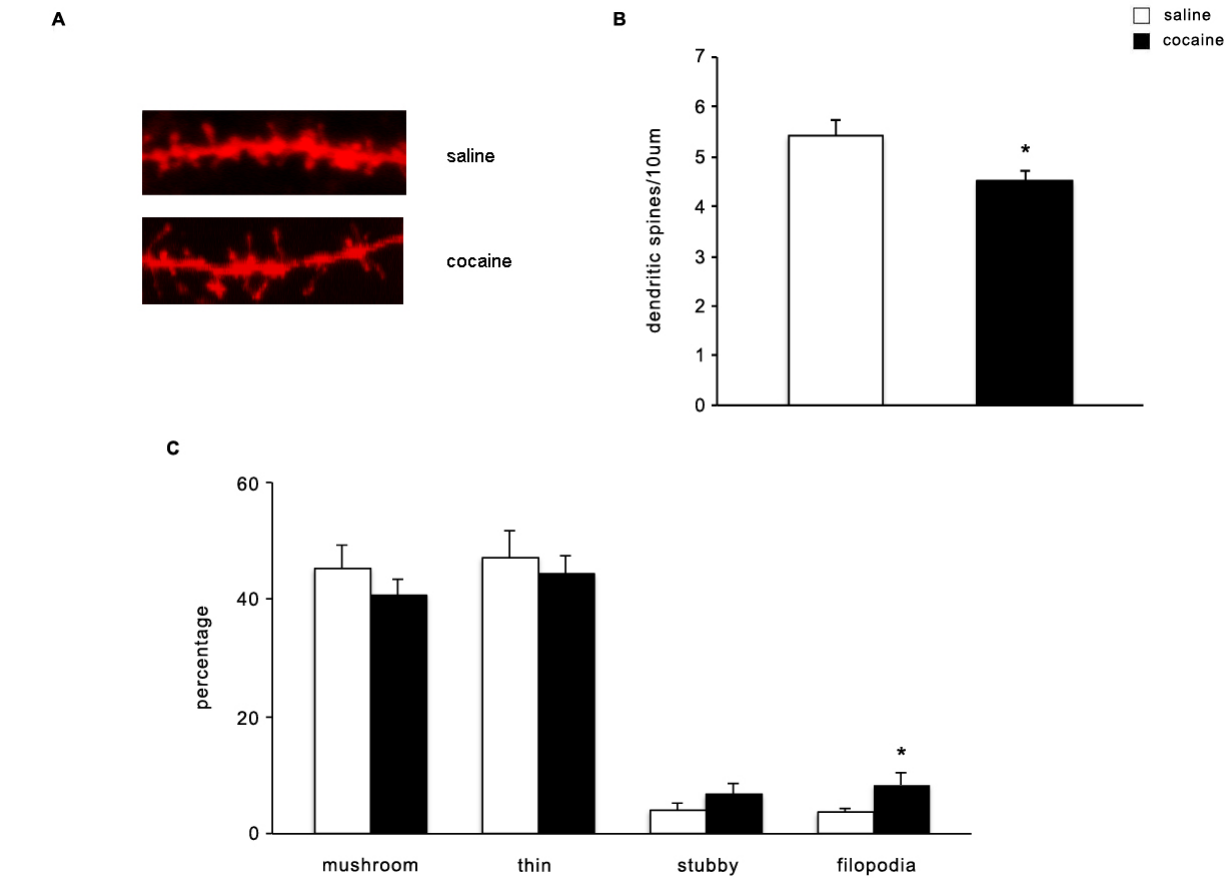

Figure 5: Effects of short-term abstinence on dendritic spine morphology in the layer V of the mPFC of cocaine-withdrawn PND 45 rats. Panel A shows representative images of dendrite segments from the mPFC of saline (top) and cocaine-treated animals (bottom). Panel B shows the total spine density in the mPFC. Panel C shows the percentage of total protrusions belonging to different categories depending on their morphology (mushroom, thin, stubby and filopodia). N>700 spines from 15 different neurons for each group, 3 animals/group. *$p<0.05$ vs. saline-treated rats.

Last, we decided to analyze the mPFC of adult animals (PND 90) to investigate whether the herein shown changes in GR machinery persist until adulthood, i.e. after a long period of abstinence. No changes were found in Nr3c1 mRNA levels (-3%, $p>0.05$; Fig. 6a) and GR protein levels both in the cytosol (-8%, $p>0.05$) and nucleus (-1%, $p>0.05$) (Fig. 6b) as well as in the nucleus/cytosol ratio (-6%, $p>0.05$; Fig. 6c) of cocaine-withdrawn rats at PND 90. Next,

we measured also the expression and activation of the molecular determinants of spine dynamics in the mPFC of PND 90 rats. No changes were observed in the expression of PSD95 (Fig. 6d) and F-actin (Fig. 6e) as well as in the expression and phosphorylation of cofilin (Fig. 6f) in the mPFC of adult rats.

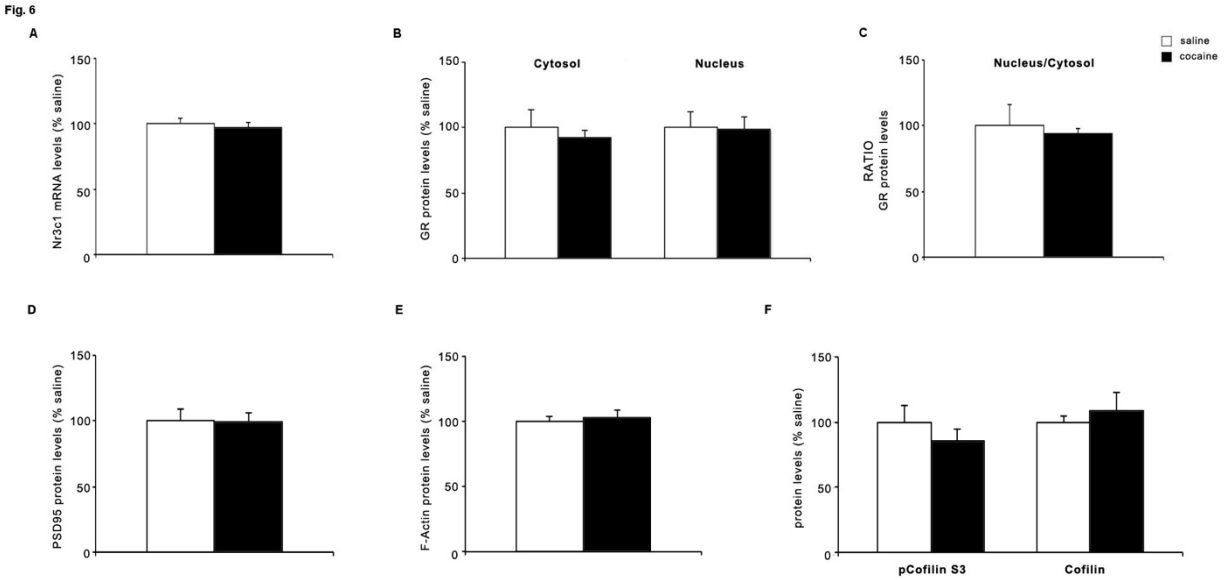

Figure 6: Effects of long-term abstinence on GR mRNA and protein levels and on spine-related proteins in the mPFC of cocaine- withdrawn PND 90 rats. Panel A shows Nr3c1 mRNA levels in the mPFC of PND 90 rats. Panel B shows GR protein levels in the cytosolic and nuclear fraction of the mPFC of PND 90 rats. Panel C shows the ratio between nuclear and cytosolic GR protein levels in the mPFC of PND 90 rats. PSD95 protein levels (panel D), F-Actin protein levels (panel E) and pCofilin Ser3 and total Cofilin (panel F) were measured in the crude synaptosomal fraction of the mPFC of PND 90 rats. The results, expressed as % of saline-treated rats, represent the mean±S.E.M. of at least 6 samples.

4.2.3. Discussion

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

Besides increased transcription and translation of GR, witnessed by increased mRNA and protein levels of the receptor itself, we found augmented trafficking of GR toward the nucleus in cocaine-withdrawn rats. This occurs not only via changes in GR protein expression in the different cell compartments (i.e. cytosol and nucleus) but also through the modulation of the molecules that contribute to the shuttling of the GR toward the nucleus. In fact, short-term withdrawal from developmental cocaine exposure reduced the expression of FK506 binding protein 51 (FKBP51), which normally retains the receptor in the cytoplasm. Concomitantly, short-term withdrawal activates the phosphorylation of the GR, which contributes to the translocation of the receptor to the nucleus. The activation of GR is further confirmed by the increased expression of Src1, which co-activates GRs after nuclear import to induce gene expression (Meijer et al., 2005). Taken together, these results are indicative of an overall dysregulation of the machinery governing GR trafficking set in motion by the short-term withdrawal from developmental cocaine exposure providing mechanistic insights for withdrawal-induced GR activation. The importance of the alteration of such mechanisms is reinforced by the evidence that all these alterations occurred without changes in the circulating levels of glucocorticoids, although we cannot exclude that the hormonal levels might have been altered at different time points. Of note, the effects produced by short withdrawal wane in animals abstinent for 48 days, indicating that they are peculiar of the short-term withdrawal, an observation that might be of functional relevance given the altered response to stress observed in cocaine users during early abstinence (Sinha et al., 2003; Fox et al., 2008). Further, since we have previously shown that short-term withdrawal from developmental cocaine exposure causes an abnormal response to a stress challenge leading to pro-depressive symptoms (Caffino et al.,

2015b), we suggest that the herein shown dysregulation of the GR system may contribute to the negative emotional state observed in humans during early periods of abstinence (Gould, 2010; Koob, 2013). Although the non contingent exposure to cocaine herein employed might represent a limitation of this manuscript, we decided to focus on the pharmacologic properties of the psychostimulant administration and the short abstinence from it.

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

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

Since drugs of abuse appear to engage the same pathways that allow normal learning and memory (Kelley, 2004; Robinson and Kolb, 2004), we speculate that cocaine-induced increase of GR functions, associated with altered structural remodeling, may compromise the physiological functioning of cortical synaptic networks. In fact, evidence exists that the activation of GRs results in cognitive impairments via alterations in dendritic spine morphology (Gourley et al., 2012b; Swanson et al., 2013; Finsterwald and Alberini, 2014). In this view, our data may contribute to explain the loss of inhibitory control occuring during early withdrawal: in fact, the hyperactivation of the GR system and the reduced structural remodeling of the mPFC may contribute to confer greater vulnerability to the addictive properties of cocaine (Chambers et al., 2003). Future studies employing mutant mice for glucocorticoid receptors (Reichardt et al., 1998) may allow to investigate the mechanistic link between cocaine-induced changes in GR expression and spine density observed in mPFC. Future studies employing mutant mice for

glucocorticoid receptors (Reichardt et al., 1998) may allow to investigate the mechanistic link between cocaine-induced changes in GR expression and spine density observed in mPFC.

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

4.3. Prolonged abstinence from developmental cocaine exposure dysregulates BDNF and its signaling network in the medial prefrontal cortex of adult rats

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4.3.1. Introduction

It is now well established that adolescence is a vulnerable period of development characterized by a unique sensitivity to drug abuse (Kelley et al., 2004). In fact, during this period, maturational changes are still occurring in the adolescent brain (Spear, 2000; Casey et al., 2008; Ernst et al., 2009) which make it more sensitive to the effects of abused drugs (Andersen, 2003). However, the mechanisms underlying such higher sensitivity are still obscure.

Cocaine is a highly addictive psychostimulant, whose abuse causes a great social and economic burden worldwide. Identifying the molecular mechanisms that might contribute to the craving and relapse after cocaine withdrawal represents, so far, an unmet need. Recent research has pointed to the neurotrophin BDNF as an important player in the action of cocaine. In fact, acute or repeated cocaine exposure increased BDNF expression in different brain regions (Filip et al., 2006; Fumagalli et al., 2007; Fumagalli et al., 2009a; Sadri-Vakili et al., 2010; Fumagalli et al., 2013) suggesting that the modulation of neurotrophin expression may participate to the adaptations set in motion by the exposure to the psychostimulant. The expression of BDNF is also increased as a function of abstinence; in fact Grimm and associates (2003) have shown that neurotrophin protein levels progressively increased after long-term withdrawal in the nucleus accumbens (NAc) ventral tegmental area (VTA) and amygdala (Grimm et al., 2003), suggesting that BDNF plays an important role in cocaine craving.

In particular, it has been recently reported that BDNF holds a protective role not only toward cocaine-mediated behaviors but also biochemical and molecular alterations in the rat medial prefrontal cortex (mPFC) (Berglind et al., 2009; McGinty et al., 2010). In fact, infusion of the neurotrophin in the mPFC suppressed cocaine seeking (Berglind et al., 2007), whereas BDNF infusion in the NAc or VTA increased cocaine seeking (Horger et al., 1999; Lu et al., 2004; Graham et al., 2007) suggesting that BDNF might be a pro- or anti-addictive factor depending on the brain region of the infusion. Interestingly, mPFC and NAc are strictly connected in the regulation of BDNF since Berglind and associates (2009) have shown that elevating BDNF levels in the mPFC normalizes cocaine-induced alterations in extracellular

glutamate within the nucleus accumbens (Berglind et al., 2009) providing a potential molecular mechanism for the BDNF-induced protection against cocaine seeking.

Based on these lines of evidence, we hypothesized that since adolescence is characterized by higher vulnerability to drugs of abuse and given that protection toward such sensitivity is afforded by increased BDNF expression in the mPFC, repeated exposure to cocaine during development would lead to reduced expression of the neurotrophin and its associated signaling network in this brain region, thus explaining the higher vulnerability of adolescents to cocaine. To this end, we exposed male rats to repeated 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 then sacrificed after 3 (PND 45) or 48 (PND 90) days of drug withdrawal to draw a dynamic picture of the effects produced by cocaine early and long after the end of adolescence.

4.3.2. Results

Figure 1A shows that repeated exposure to cocaine during adolescence increases BDNF mRNA levels in the mPFC of PND 90 (+71%, $p<0.05$), but not PND 45 rats (+11%, $p>0.05$). The analysis of the different BDNF isoforms at the 5' UTR revealed that such increase could be ascribed to BDNF exon IV (+68%, $p<0.01$) (Fig. 1B), i.e. the most abundant isoform, sensitive to neuronal activity (Pruunsild et al., 2011). No changes were instead observed on the mRNA levels of other major transcripts expressed in the prefrontal cortex (exon I, exon II, exon VI) (data not shown). The analysis of the transcription factors involved in the modulation of BDNF exon IV revealed a selective activation of nuclear factor B (NF-kB) (+70%, $p<0.01$) and calcium responsive factor (CaRF) (+25%, $p<0.05$) in the mPFC of PND 90 rats treated with cocaine during adolescence (Fig. 1C). No changes were instead observed for cAMP responsive element binding protein (CREB) (-8%, $p>0.05$) and neuronal Per-Arnt-Sim domain protein 4 (NPAS 4) (-3%, $p>0.05$), two other transcription factors regulating exon IV (Fig. 1C).

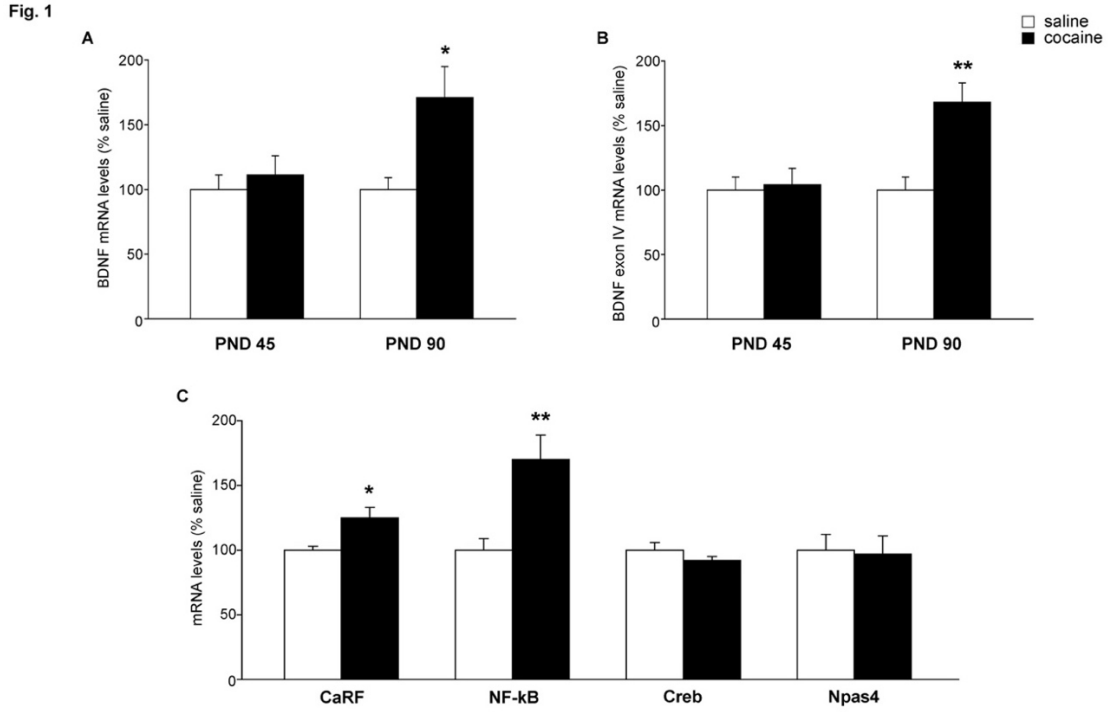

Figure 1: Developmental exposure to cocaine alters the transcriptional regulation of BDNF: molecular mechanisms. Panel (A) and (B) show total and exon IV BDNF mRNA levels, respectively, in the mPFC of PD 45 and PD 90 rats following repeated exposure to cocaine during adolescence. Panel (C) shows the mRNA levels of several transcription factors involved in the regulation of exon IV mRNA levels in the mPFC of PD 90 rats. The results, expressed as % of saline-treated rats, represent the mean±S.E.M. of six samples; *$p<0.05$ and **$p<0.01$ vs. saline-treated rats.

The transcriptional changes of BDNF after repeated exposure to cocaine were paralleled by significant alterations in its protein levels, as shown in Figure 2. Developmental exposure to cocaine increased both proBDNF and mBDNF levels in the homogenate of PND 90 rats (proBDNF: +20%, $p<0.05$; mBDNF: +27%, $p<0.01$) (Fig. 2B) but not in PND 45 rats (proBDNF: -7%, $p>0.05$; mBDNF: -7%, $p>0.05$) (Fig. 2A) suggesting an increased synthesis and processing of BDNF. Given that a significant effect on BDNF was observed only at PND 90, from now on we focused our attention on this time point.

The increased expression of mBDNF may be sustained by alterations in the expression of some miRNAs regulating BDNF expression. In particular, we focused our attention on let7d, miR124 and miR132, three different microRNAs that regulate, among the others, cocaine-induced BDNF expression (Chandrasekar and Dreyer, 2009). Indeed, we observed a profound decrease of these miRNAs that may very well contribute to the increased expression of proBDNF (let7-d: -81%, $p<0.001$; miR124: -66%, $p<0.01$ and miR132: -84%, $p<0.001$) (Fig. 2C). Another option relies on the possibility that repeated cocaine treatment has altered the activity of tissue plasminogen activator (tPA), one of the extracellular proteases converting proBDNF into mBDNF (Pang et al., 2004). Indeed, tPA mRNA levels were increased in the mPFC of cocaine-treated rats at PND 90 (+22% vs. saline-treated rats, $p<0.05$) (Fig. 2D).

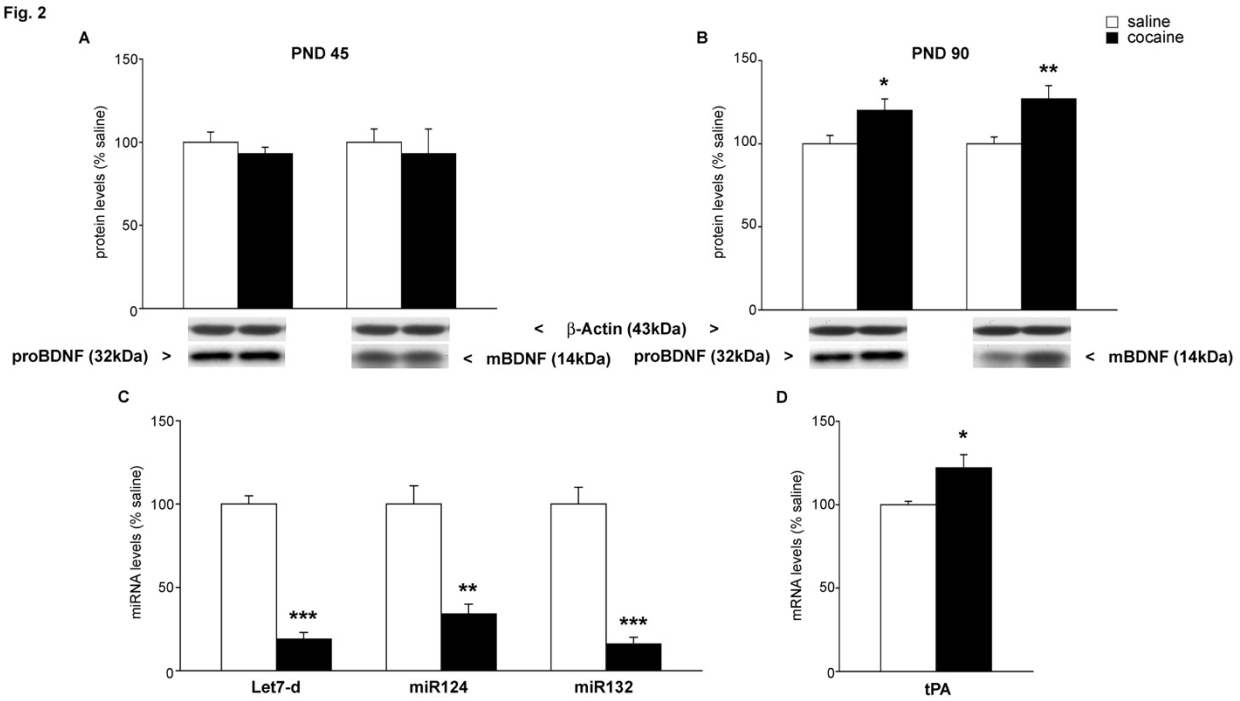

Figure 2: Developmental exposure to cocaine alters the translational regulation of BDNF: molecular mechanisms. Panel (A) and (B) show the levels of precursor BDNF (proBDNF) and mature BDNF (mBDNF), respectively, in the mPFC from PD 45 and PD 90 rats following repeated exposure to cocaine during adolescence. Representative Western blot bands of pro- and mBDNF are shown below the graphs shown in panel (A) and (B). Panel (C) shows the modulation of several microRNA (miRNAs) implicated in the regulation of BDNF following exposure to cocaine. Panel (D) shows the mRNA levels of tissue plasminogen activator (tPA), one of the extracellular proteases that govern the proteolytic conversion of proBDNF into mBDNF. The results, expressed as % of saline-treated rats, represent the mean±S.E.M. of six samples; *p<0.05, **p<0.01 and ***p<0.001 vs. saline-treated rats.

Indeed, we found an increased trkB expression in the crude synaptosomal fraction of the mPFC from cocaine-withdrawn rats (+35%, $p<0.05$) with no changes in the homogenate (+9%, $p>0.05$) (Fig. 3A), suggesting an increased trafficking of the receptor toward the membrane. Moreover, we found that prolonged abstinence from developmental cocaine induced a selective activation of Akt (pAkt S473: +39%, $p<0.05$; Akt: +12%, $p>0.05$) but not ERK2 (pERK2 T185/187: -10%, $p>0.05$; ERK2: -3%, $p>0.05$) (Fig. 3B). Akt activation is linked to the pathway of mTOR and S6 that, accordingly, were examined under our experimental conditions. We found that, in the mPFC of cocaine-withdrawn rats, the phosphorylation of mTOR is enhanced (pmTOR S2448: +30%, $p<0.05$; mTOR: -9%, $p>0.05$) and so is the phosphorylation of S6 (pS6K S240/244: +96%, $p<0.001$; S6K: +22%, $p>0.05$) (Fig. 3C) suggesting an overall activation of the translational machinery as a consequence of prolonged abstinence.

Among the different targets of S6, we decided to investigate the expression of the immediate early gene Arc, an effector early gene with a multifaceted role in brain plasticity. In fact, changes in Arc expression may represent changes in neuronal activity but also may impact synaptic plasticity, by, for example, altering the surface expression of glutamate AMPA receptors (Chowdhury et al., 2006; Rial Verde et al., 2006). Interestingly, abstinence-induced S6 activation resulted in increased expression of Arc in the cortical crude synaptosomal fraction of cocaine-withdrawn rats (+24%, $p<0.05$) (Fig. 3D). Such increase resulted in reduced membrane AMPA receptor expression (-22%, $p<0.05$) (Fig. 3E).

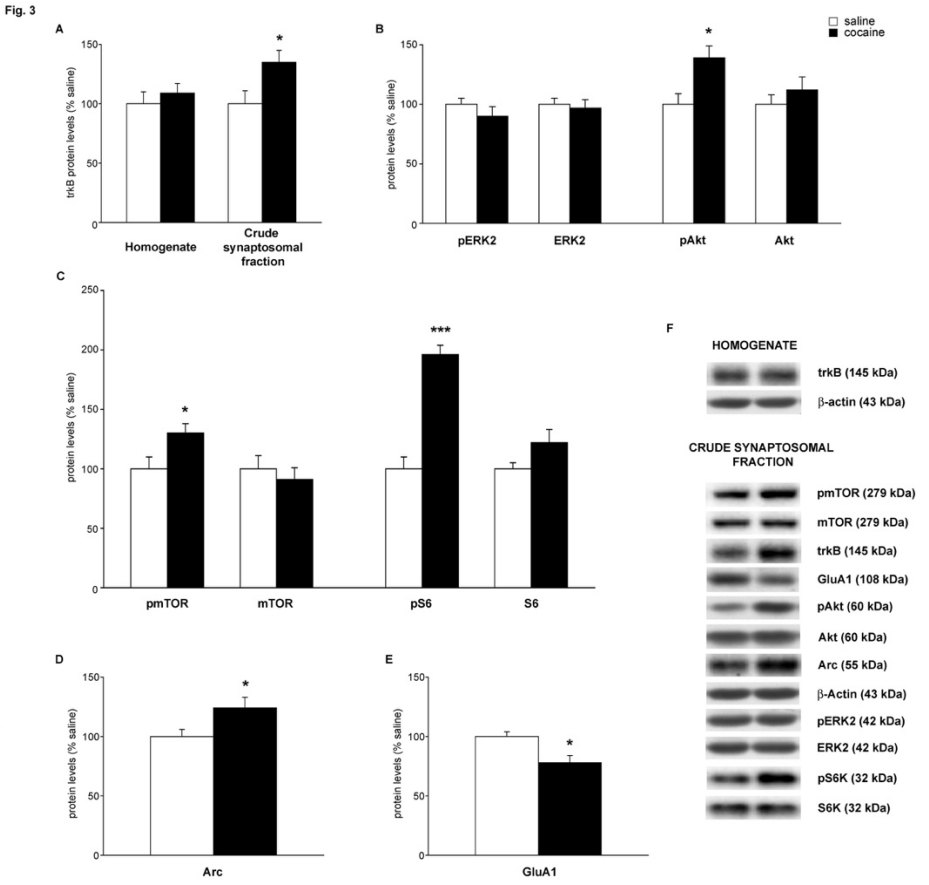

Figure 3: Developmental exposure to cocaine alters BDNF-induced intracellular pathways and neuroplastic markers. Panel (A) shows the expression of the high affinity BDNF receptor trkB following repeated exposure to cocaine in the mPFC of PD 90 rats in the whole homogenate and crude synaptosomal fraction. Panel (B) shows the phosphorylation and expression of ERK 2 and Akt in the mPFC of PD 90 rats in the crude synaptosomal fraction. Panel (C) shows the activation of the Akt-dependent pathway (mTOR and S6K) in the crude synaptosomal fraction. Panel (D) shows the expression of the immediate early gene Arc whereas panel (E) shows the expression of the main AMPA subunit of glutamate receptors, i.e. GluA1, in the crude synaptosomal fraction. Representative Western blot bands are shown in panel (F). The results, expressed as % of saline-treated rats, represent the mean±S.E.M. of six samples; *$p<0.05$ and ***$p<0.001$ vs. saline-treated rats.

4.3.3. Discussion

Our data are the first to show that abstinence from adolescent exposure to cocaine increases BDNF and its signaling network in the medial prefrontal cortex (mPFC) of adult rats. These results provide novel mechanisms governing abstinence-induced changes in BDNF and its related signaling, suggesting that withdrawal from adolescence may impact adult brain homeostasis.

BDNF expression is increased as a function of abstinence duration since its expression is not altered 3 days after the end of treatment, but only 48 days later. Notably, prolonged abstinence from developmental cocaine exposure has selectively up-regulated BDNF exon IV, which represents the major activity-dependent transcript of the neurotrophin. This presumably occurs through the activation of transcription factors such as NF-kB and CaRF that are known to regulate BDNF exon IV.

Changes in BDNF mRNA levels were accompanied by up-regulation of BDNF protein levels suggesting that abstinence from adolescent cocaine exposure regulates also the translation of the neurotrophin. Abstinence-induced increase of BDNF protein levels occurs through, at least, two distinct mechanisms. First, repeated exposure to cocaine during adolescence caused a profound down-regulation of some miRNAs that are known to control BDNF expression (Chandrasekar and Dreyer, 2009), an effect that may very well contribute to the long-lasting proBDNF up-regulation herein observed. Second, the mRNA levels of tPA, the enzyme responsible of the cleavage of proBDNF into mBDNF, is increased in the mPFC of PND 90 rats exposed to cocaine during adolescence. The up-regulation of the extracellular proteases tPA is indicative of increased processing leading to higher levels of released mBDNF.

The increase of BDNF expression represents the first step of a series of dynamic changes that lead to an increase of BDNF-dependent pathways. In fact, we found a parallel up-regulation of its high affinity receptor trkB, that might be considered an index of activation upon neurotrophin release (Saarelainen et al., 2003). TrkB enhancement resulted in a selective activation of the Akt-dependent pathway that, in turn, activated both mTOR and S6 phosphorylation (Gong et al., 2006), a canonical pathway known to be activated by BDNF

(Swiech et al., 2008) thereby promoting mRNA translation (Hoeffer and Klann, 2010). These results suggest that prolonged abstinence from developmental cocaine has permanently altered the physiological regulation of the translational machinery. Interestingly, dysregulation of this pathway has been observed in the brain of cocaine addicts (Alvaro-Bartolome et al., 2011).

The widespread impact on neuroplasticity exerted by the developmental exposure to cocaine is further highlighted by the increased expression of Arc. Arc is a critical crossroad of various signals that converge into the stabilization of synaptic inputs (Bramham et al., 2010) and it has been implicated in the acute or chronic action of cocaine (Fumagalli et al., 2006b; Fumagalli et al., 2009b; Caffino et al., 2011; Hearing et al., 2011) as well as in the relapse to cocaine seeking (Hearing et al., 2008). Arc is a target of mTOR-S6 kinase pathway (Panja and Bramham, 2014) and, accordingly, we observed increased Arc expression in the mPFC of PND 90 rats thus identifying Arc as a marker of developmental abstinence-induced long-term adaptations. Interestingly, recent evidence has shown a crucial role for Arc in regulating AMPA-type glutamate receptor endocytosis (Chowdhury et al., 2006; Rial Verde et al., 2006). Accordingly, the levels of GluA1, the main AMPA subunit, are reduced in the crude synaptosomal fraction of mPFC from PND 90 cocaine-withdrawn rats, suggesting that long-term abstinence from developmental cocaine exposure indirectly influences glutamate neurotransmission, presumably through changes in the BDNF-Arc pathway.

To the best of our knowledge, this is the first evidence showing that long-term abstinence from exposure to cocaine during adolescence alters the expression of BDNF and its associated network at adulthood thus ruling out the possibility that the increased vulnerability of adolescents to drugs of abuse might rely upon the reduction of BDNF levels. Our data are in conflict with recent published evidence showing reduced BDNF expression following early, but not long, abstinence from repeated cocaine treatment at adulthood (McGinty et al., 2010), an effect that seems to occur via alteration of the MAP kinase pathway (Whitfield et al., 2011). The most parsimonious explanation for this discrepancy is that the timing of cocaine exposure (development vs. adulthood) dictates the profile of BDNF expression. Intriguingly, the cocaine–induced developmental effect seems to depend upon the activation of a pathway different from that activated by the adult treatment (i.e. PI3 kinase instead of MAP kinase) since we found

activation of Akt but not ERK 1/2. Our results are in line with the hypothesis that enhanced BDNF expression might represent an adaptive, defensive strategy to oppose cocaine-seeking and might be interpreted as a compensatory reaction in an attempt, perhaps, to normalize drug-induced alterations in glutamate transmission in the NAc (Berglind et al., 2009). Interestingly, although there is no evidence of cocaine-induced changes in mTOR pathway in the mPFC, it has been demonstrated that, in the Nac, such pathway regulates cocaine seeking (Wang et al., 2010). In analogy with the protective effect evoked by BDNF infusion in the mPFC (Berglind et al., 2007), we may speculate that the herein shown increase of the mTOR pathway may prevent cocaine seeking.

4.3.4. Conclusion

Repeated exposure to cocaine during adolescence causes a significant up-regulation of the neurotrophin BDNF which occurs through different mechanisms both at the transcriptional and translational level. Such increase promotes increased phosphorylation of Akt, through the high affinity BDNF receptor trkB, that culminates into the activation of the mTOR-S6 kinase pathway, indicating that mRNA translation and long-lasting synaptic plasticity is altered in the mPFC of cocaine-withdrawn adolescent rats. The effects of the developmental exposure to cocaine, however, go beyond the alteration of the BDNF system since it results in translational changes of Arc and GluA1 expression (Fig. 4) revealing a global effect on mPFC neuroplasticity.

Taken together, these data indicate that abstinence from repeated exposure to cocaine during adolescence has set in motion disparate mechanisms, that affect a wide variety of targets involved in brain plasticity ranging from the neurotrophin BDNF to the immediate early gene Arc and the AMPA glutamate receptor GluA1. These findings indicate that interfering with the correct development of the mPFC by repeated exposure to cocaine dysregulates the BDNF system and its associated network and reveal novel mechanisms that underlie the prolonged abstinence from early in life exposure to cocaine. This previously unappreciated, and highly dynamic, way of regulating cortical neuroplasticity by early delivery of, and long-term abstinence from,

adolescent cocaine may provide novel potential targets for the treatment of psychostimulant abuse.

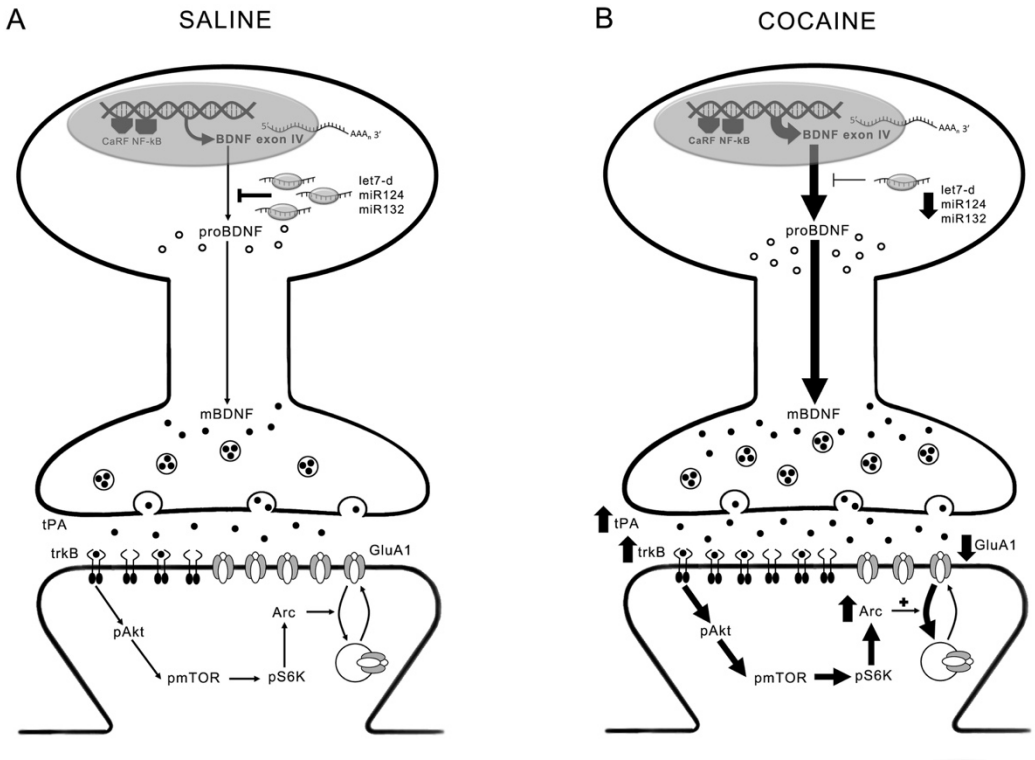

Figure 4: Schematic representation of the changes of BDNF and its associated network set in motion by the developmental exposure to cocaine (panel b): comparison with rats exposed to saline (panel a).

4.4. Long-term abstinence from developmental cocaine exposure alters Arc/Arg3.1 modulation in the rat medial prefrontal cortex.

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4.4.1. Introduction

Understanding the reason(s) why people get addicted and crave for drugs represents a critical, although still unmet, need. Repeated exposure to cocaine brings about a constellation of changes in brain homeostasis ranging from altered release of neurotransmitters and changes in the expression of neuroplastic molecules to structural modifications, with recent results casting fresh light on epigenetic and microRNA mechanisms (Jonkman and Kenny, 2013). Taken together, these findings show that cocaine exerts its deleterious effects through a series of coordinated changes that occur in specific brain areas.

Among the molecules that participate in the action of cocaine, attention has been recently focused on the effector immediate early gene (IEG) Activity Regulated Cytoskeletal-associated protein (Arc/Arg3.1) (Lyford et al., 1995a). While initial studies showed a prominent localization of Arc/Arg3.1 at active synapses (Dynes and Steward, 2007) where it can be locally synthesized, recent lines of evidence have challenged this observation showing that Arc/Arg3.1 protein is also localized in inactive synapses where it can be accumulated (Okuno et al., 2012). Interestingly, Arc/Arg3.1 is present not only in dendrites (Korb et al., 2013; Bloomer et al., 2007) but also in the cell nucleus adding complexity, but perhaps also specificity, to its modulation, although the role of nuclear Arc/Arg3.1 is largely unknown.

Single or repeated cocaine exposure increase Arc/Arg3.1 expression (Fosnaugh et al., 1995; Freeman et al., 2002), an effect that is strictly related to the activation of dopamine D1 receptors (Fumagalli et al., 2006b). A role for Arc/Arg3.1 in rat medial prefrontal cortex (mPFC) has been suggested in the associative processing of drug-associated contextual stimuli, in the extinction of cocaine-seeking as well as in cue-elicited reinstatement of cocaine seeking (Hearing et al., 2008; Fumagalli et al., 2009b; Hearing et al., 2011; Ziolkowska et al., 2011) pointing to Arc/Arg3.1 as a critical mediator of cocaine's action.

We have previously shown that changes in Arc/Arg3.1 mRNA and protein levels vanished within two weeks after the end of treatment in the adult animals (Fumagalli et al., 2006b) indicating that adult exposure to cocaine does not cause long-lasting changes in Arc/Arg3.1 protein levels. Interestingly, we have recently shown that the expression of the neurotrophin

BDNF, a molecule strictly connected to Arc/Arg3.1 (Yin et al., 2002; Ying et al., 2002), and Arc/Arg3.1 itself are elevated as a result of long-term abstinence following exposure to cocaine during adolescence (Giannotti et al., 2014). However, while we have dissected in details the mechanisms of BDNF up-regulation (Giannotti et al., 2014), we have not investigated whether the long-term abstinence from developmental cocaine exposure alters nuclear Arc/Arg3.1 expression and whether it alters the inhibitory and degradative cellular mechanisms that may contribute to Arc/Arg3.1 up-regulation. To this end, we decided to focus our attention on several proteins such as FMR1, Ube3a and GRM5 (Park et al., 2008; Shepherd and Bear, 2011) that are known to regulate Arc/Arg3.1 protein levels and analyzed the mPFC of rats exposed to cocaine from postnatal day (PND) 28 to PND 42, a period of life that approximates adolescence in humans. Animals were sacrificed early after the end of treatment (PND 45) or at adulthood (PND 90) in an attempt to draw a dynamic picture of the effects produced by short- and long-term abstinence from developmental cocaine exposure.

4.4.2. Results and Discussion

Figure 1 shows the effect of repeated exposure to cocaine during adolescence on Arc/Arg3.1 mRNA and protein levels at PND 45 and PND 90. Arc/Arg3.1 mRNA levels were markedly increased at PND 45 (+111%, $p<0.001$) (Fig. 1A) with no effects at PND 90 (-14%, $p>0.05$) (Fig. 1B).

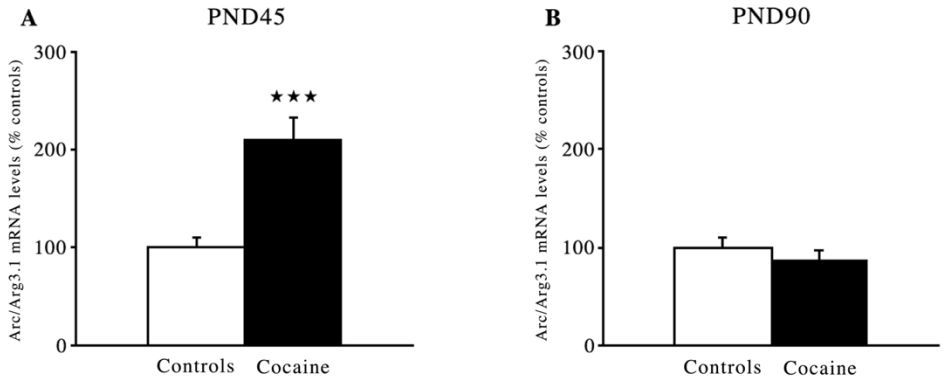

Figure 1: Abstinence from developmental exposure to cocaine alters the transcriptional regulation of Arc/Arg3.1. Arc/ Arg3.1 mRNA levels in the mPFC of PND 45 (A) and PND 90 (B) rats following repeated exposure to cocaine during adolescence. The results, expressed as % of control rats, represent the mean ± S.E.M. of, at least, 8 samples. ***p<0.001 vs. control rats.

Conversely, Arc/Arg3.1 protein levels were not affected at PND 45 (-15%, $p>0.05$) (Fig. 2A) while significantly increased in the mPFC homogenate of PND 90 rats (+63%, $p<0.05$) (Fig. 2B).

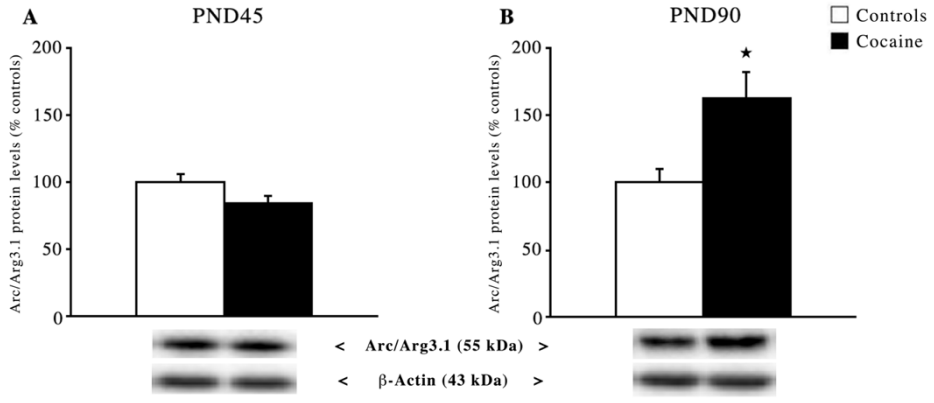

Figure 2: Abstinence from developmental exposure to cocaine alters the translational regulation of Arc/Arg3.1. Arc/Arg3.1 protein levels in the whole homogenate of the mPFC of PND 45 (A) and PND 90 (B) rats. The results, expressed as % of control rats, represent the mean ± S.E.M. of, at least, 6 samples. *p<0.05 vs. control rats.

Such increase may be ascribed to enhanced Arc/Arg3.1 protein levels in the nucleus (+41%, $p<0.05$), as shown in Fig. 3b. No changes were instead observed in the nuclear expression of Arc/Arg3.1 at PND 45 (-6%%, $p>0.05$) (Fig. 3a). Based on these data, from now on we decided to focus our attention to the mPFC of PND 90 rats, in an attempt to find a potential molecular explanation of the increased levels of Arc/Arg3.1 protein observed as a result of long-term abstinence.

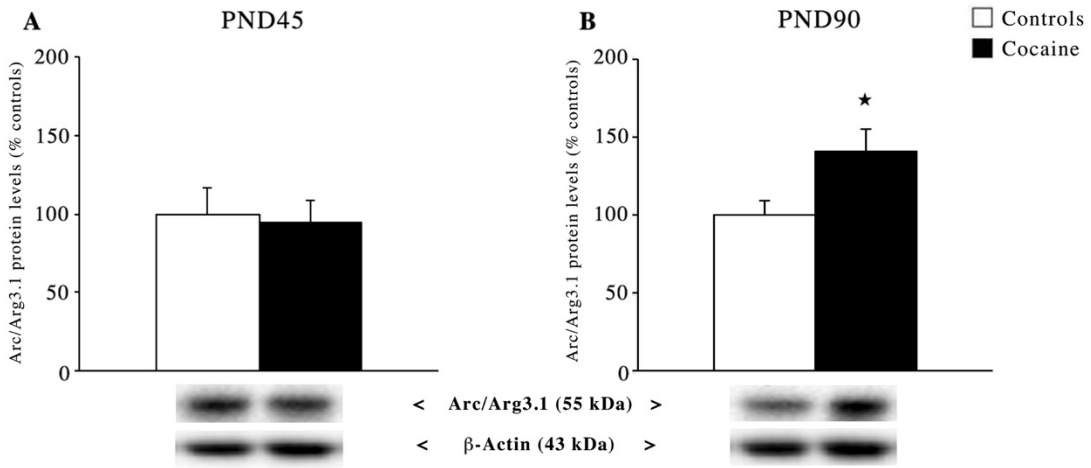

Figure 3: Abstinence from developmental exposure to cocaine alters Arc/Arg3.1 protein levels in the nuclear fraction. Arc/Arg3.1 protein levels in the nuclear fraction of the mPFC of PND 45 (A) and PND 90 (B) rats. The results, expressed as % of control rats, represent the mean ± S.E.M. of, at least, 6 samples. *$p<0.05$ vs. control rats

Besides neurotransmitters, other mechanisms come into play in the physiological regulation of Arc/Arg3.1 thus providing other potential sites of regulation by cocaine. Arc/Arg3.1 translation at dendrites is inhibited by FMRP (fragile X mental retardation protein) (Shepherd and Bear, 2011). Interestingly, its mRNA, FMR1, is significantly reduced in the mPFC of PND 90 rats (-19%, $p<0.05$) (Fig. 4A). Also, the turn-over of Arc/Arg3.1 protein is modulated at different levels, i.e. via ubiquitination and sumoylation, both leading to Arc/Arg3.1 degradation through the proteasome system (Shepherd and Bear, 2011). Arc/Arg3.1 protein is degraded via ubiquitination, primarily via the action of Ubiquitin-protein ligase E3A (Ube3a) (Shepherd and Bear, 2011). Intriguingly, the analysis of Ube3a reveals a reduction (-26%, $p<0.01$) (Fig. 4B). No changes in the expression levels of Sentrin/SUMO-specific protease 1 (SENP1) (+2%,

p>0.05), that controls Arc/Arg3.1 SUMOylation, were observed (Fig. 4D). A further level of regulation of Arc/Arg3.1 protein is through the activation of the metabotropic glutamate receptor 5 (GRM5) (Shepherd and Bear, 2011). Interestingly, long-term abstinence from cocaine enhanced the expression of GRM5 (+37%, p<0.05) (Fig. 4C).

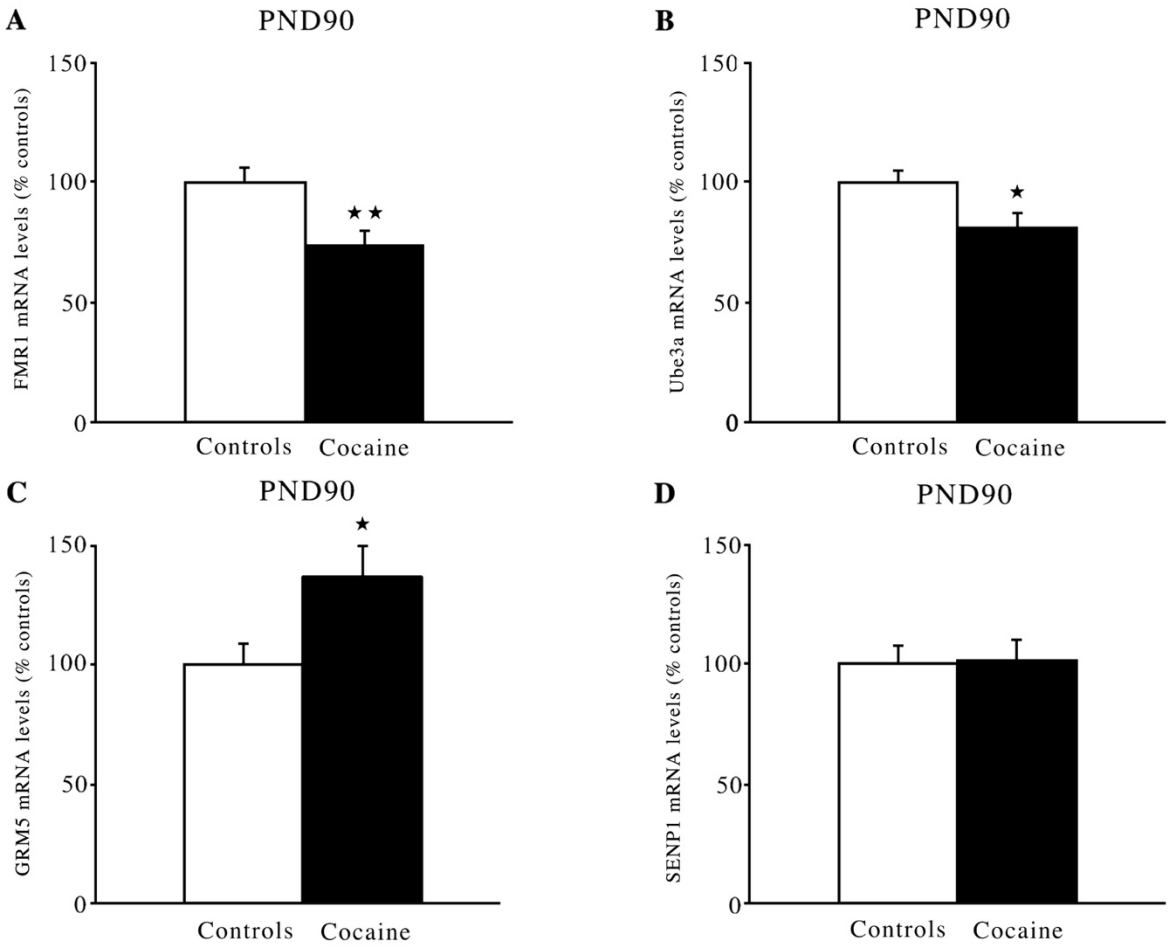

Figure 4: Abstinence from developmental exposure to cocaine alters the mechanisms responsible of Arc/Arg3.1 translation: effects at PND 90. a FMR1 mRNA levels, b Ube3a mRNA levels, c GRM5 mRNA levels, d SENP1 mRNA levels. These measures were undertaken in the mPFC of PND 90 cocaine-withdrawn rats. The results, expressed as % of control rats, represent the mean ± S.E.M. of, at least, 6 samples. *p<0.05 and **p<0.01 vs. control rats

While these changes may contribute to explain Arc/Arg3.1 up-regulation, we next measured the transcription of Gria1, the main glutamate AMPA receptor subunit, a target of increased nuclear Arc/Arg3.1 expression (Korb et al., 2013) and its translation in the whole

homogenate. Indeed, we found reduced Gria1 mRNA levels (-21%, $p<0.05$) (Fig. 5A) and reduced GluA1 protein levels (-29%; $p<0.01$) (Fig 5B).

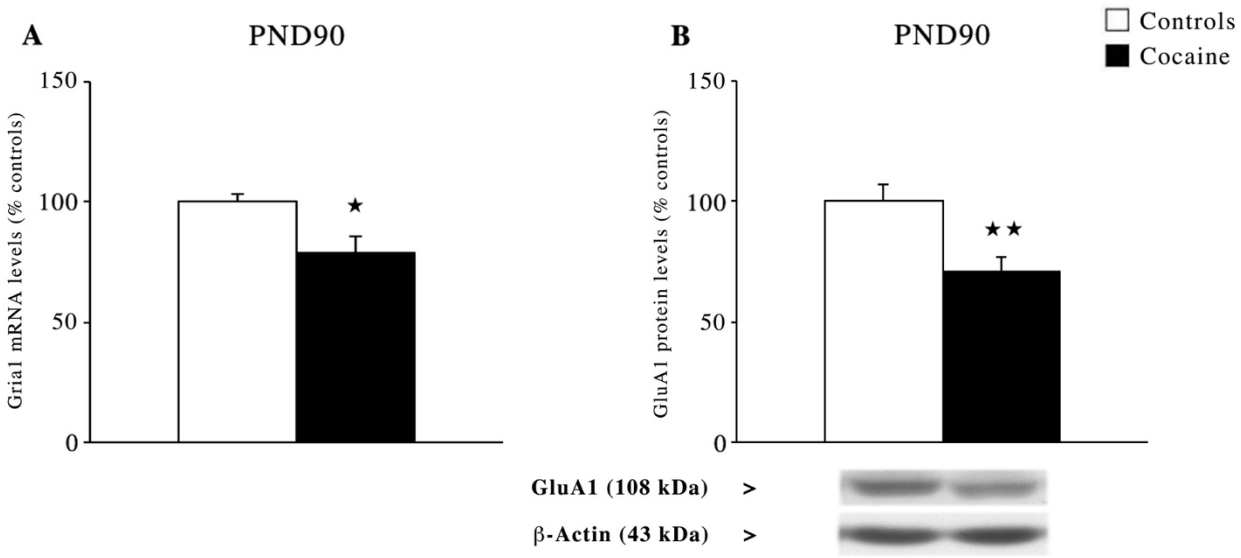

Figure 5: Abstinence from developmental exposure to cocaine alters Gria1 transcription and translation: effects at PND 90. a Gria1 mRNA levels; b GluA1 protein levels in the whole homogenate. These measures were undertaken in the mPFC of PND 90 cocaine-withdrawn rats. The results are expressed as % of control rats and represent the mean ± S.E.M. of, at least, 6 samples. *$p<0.05$ and **$p<0.01$ vs. control rats

Interesting differences were observed when comparing short (3 days) and long (48 days) abstinence from developmental exposure to cocaine. Arc/Arg3.1 mRNA levels were markedly increased 3 days after the end of the treatment (PND 45) but declined back to control levels at PND 90; conversely, Arc/Arg3.1 protein levels were markedly enhanced at PND 90 while unchanged at PND 45. Whereas evidence exists that increased Arc/Arg3.1 protein levels dissipate within two weeks if cocaine is administered at adulthood (Fumagalli et al., 2006b), we here show that long-term abstinence from developmental cocaine exposure causes an enduring Arc/Arg3.1 up-regulation, which persists until, at least, PND 90. Of note, this is in line with the evidence that Arc/Arg3.1 protein levels are increased in the mPFC of mice that were withdrawn for one month from repeated amphetamine administered at adolescence (Calabrese et al., 2013), suggesting that the enhancement of Arc/Arg3.1 expression might be a sign of abstinence-triggered adaptations following long-term withdrawal of psychostimulants. The most parsimonious explanation for such long-lasting increase is that the timing of cocaine exposure (adolescence or adulthood) dictates the profile and duration of Arc/Arg3.1 expression.

While there is no doubt that Arc/Arg3.1 mRNA is locally translated in dendrites and plays an important role in synaptic changes underlying plastic modifications necessary for long-term memory formation (Bramham et al., 2010; Shepherd and Bear, 2011), it must be taken into account that Arc/Arg3.1 protein is found not only in dendrites, but also at high levels in the nucleus (Bloomer et al., 2007; Korb et al., 2013). Thus, we hypothesize that the increased expression of Arc/Arg3.1 in the whole homogenate may depend not only on its enhancement in the crude synaptosomal fraction (Giannotti et al., 2014) but also on the up-regulation herein observed in the nuclear fraction. Also, the increase of nuclear Arc/Arg3.1 might participate to the regulation of glutamate AMPA receptors since we observed reduced Gria1 transcription, adding novel evidence to the inverse relationships existing between Arc/Arg3.1 and AMPA receptors following long-term cocaine withdrawal (Korb et al., 2013; Giannotti et al., 2014)

It is acknowledged that FMR1 and Ube3a contribute to the physiological Arc/Arg3.1 modulation (Shepherd and Bear, 2011); our data suggest their implication in the modulation of the effects on Arc/Arg3.1 expression in the long-term abstinence from cocaine. We found that abstinence from developmental exposure to cocaine reduced the expression of FMR1, which physiologically represses Arc/Arg3.1 translation (Shepherd and Bear, 2011), and Ube3a, that targets Arc/Arg3.1 to proteasome for degradation (Greer et al., 2010), suggesting that both mechanisms might contribute to the observed increase in Arc/Arg3.1 expression. Concomitantly, we found that abstinence increased the expression of GRM5 that, under normal conditions, promotes Arc/Arg3.1 translation and synthesis (Park et al., 2008).

Taken together, it appears that long-term abstinence from repeated exposure to cocaine during adolescence alters the machinery responsible of the regulation of Arc/Arg3.1 expression through the concomitant alteration of various, independent mechanisms involved in its physiological regulation and that were not previously associated with cocaine-induced abstinence (Fig. 6). These mechanisms involve both the nucleus (present manuscript) and the crude synaptosomal fraction (Giannotti et al., 2014). We have recently shown that long-term abstinence after exposure to cocaine during adolescence up-regulates BDNF and its transduction pathways in the mPFC of adult rats (Giannotti et al., 2014). These results, together with the data of the current manuscript, reveal novel mechanisms associated with prolonged abstinence from

cocaine that cause enduring changes in brain homeostasis, via Arc/Arg3.1 modulation. It might seem premature, although suggested by these data, to propose a cohesive, mechanistic hypothesis that links changes in BDNF and Arc/Arg3.1 as molecular signatures of long-term abstinence from cocaine. However, given that Arc/Arg3.1 may be a partner of BDNF in mediating the adaptive changes caused by psychostimulants (Calabrese et al., 2013), we may speculate that an abstinence-induced alteration in the pathway of BDNF signaling together with changes in the inhibitory and degradation pathways that regulate Arc/Arg3.1 synthesis may contribute to the incubation of cocaine craving (Grimm et al., 2003).

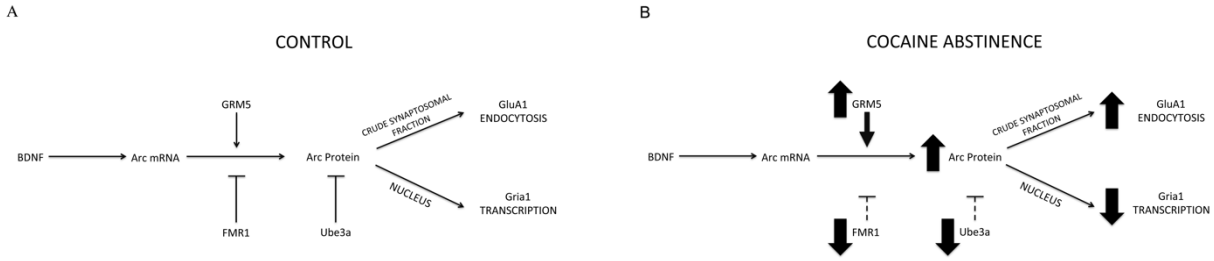

Figure 6: Abstinence from repeated cocaine administration during adolescence up-regulates Arc/Arg3.1 expression in the rat mPFC (b) in comparison with control rats (a). Under physiological conditions, Arc/Arg3.1 protein levels are regulated by several, different but converging and finely tuned, mechanisms. Following long-term abstinence from developmental exposure to cocaine, these mechanisms are altered. In detail, we have previously shown that the long-term abstinence from early exposure to cocaine up-regulates BDNF levels and its signaling pathways leading to increased levels of Arc/Arg3.1 in the crude synaptosomal fraction, an effect that promoted GluA1 AMPA receptor endocytosis and subsequent reduc- tion of these receptors at the membrane level (Giannotti et al., 2014). In the present study, we show that abstinence from developmental exposure to cocaine up-regulates Arc/Arg3.1 protein levels in the whole homogenate, an effect presumably due to increased nuclear expression of Arc/Arg3.1 that, in turn, leads to reduced Gria1 mRNA levels: this reduction might contribute to Arc/Arg3.1-induced down-regulation of glutamate AMPA receptors. Further, long-term with- drawal from developmental cocaine has also altered the inhibitory and degradative molecules that contribute to the regulation of Arc/Arg3.1 protein levels. In fact, we found reduced expression of FMR1, which physiologically represses Arc/Arg3.1 translation and Ube3a that normally targets Arc/Arg3.1 to proteasome for degradation together with enhanced expression of GRM5 that, under normal conditions, promotes Arc/Arg3.1 translation and synthesis

4.5. Dynamic modulation of basic Fibroblast Growth Factor (FGF-2) expression in the rat brain following repeated exposure to cocaine during adolescence

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http://www.ncbi.nlm.nih.gov/pubmed/22895673

4.5.1. Introduction

Adolescent exposure to drugs of abuse can produce long-lasting changes in brain structure and function that endure into adulthood (Laviola et al., 1999; Chambers et al., 2003; Carlezon and Konradi, 2004). Evidence exists that it predisposes to drug abuse later in life (Merline et al., 2004; Casey and Jones, 2010; Stone et al., 2012) presumably because the brain is still developing (Spear, 2000; Casey et al., 2008; Ernst et al., 2009) and it is more vulnerable to the effects of the psychostimulant (Andersen, 2003).

Emerging evidence suggests that dysregulation of neurotrophic factors participate to drug abuse vulnerability (Thomas et al., 2008; McGinty et al., 2010). We hypothesized that a role might be played by basic fibroblast growth factor (FGF-2), the prototype member of a family of heparin-binding growth factors with a broad spectrum of actions on different cell types in the central nervous system (Reuss and von Bohlen und Halbach, 2003; Itoh and Ornitz, 2004). FGF-2 fulfills several criteria that need to be met to accomplish such hypothesis. First, FGF-2 is expressed in the developing brain (Gomez-Pinilla et al., 1994; Monfils et al., 2006). Second, dopamine, i.e. the neurotransmitter primarily responsible of the action of cocaine (Wise and Bozarth, 1987), modulates FGF-2 expression (Roceri et al., 2001; Fumagalli et al., 2003). Third, FGF-2 expression is modulated by acute or repeated exposure to psychostimulants (amphetamine, cocaine) (Flores et al., 2000; Flores and Stewart, 2000; Fumagalli et al., 2006a; Mueller et al., 2006; Turner et al., 2009) as well as by acute or chronic stress (Fumagalli et al., 2005; Bland et al., 2007; Fumagalli et al., 2008). Fourth, exogenous administration of FGF-2 early in life enhanced the acquisition of cocaine self-administration in adulthood as elegantly shown by Turner and associates (Turner et al., 2009), suggesting that FGF-2 may contribute to the initial vulnerability to cocaine addiction. Taken together, these different, but connected, lines of evidence point to FGF-2 as a potential target of the action of cocaine during development.

In this manuscript, we focused our attention on the effects of repeated exposure to cocaine during adolescence [from post-natal day (PND) 28 to PND 42] on FGF-2 mRNA levels, sacrificing the animals at different time-points, i.e. PND 45 and PND 90, in order to evaluate the short- and long-term effect of delivery of cocaine during adolescence on brain plasticity.

Further, we decided to add a degree of complexity to our experimental design by exposing PND 45 and PND 90 animals to a single stress. The choice to investigate the effect of adolescent exposure to cocaine on the response to a challenge derives from the need to couple, in a single animal, different adverse events that may occur throughout life. We believe that this approach is uniquely suited to investigate how drug abuse, in a vulnerable period for brain development, may dynamically influence brain homeostasis, perhaps via the modulation of trophic factors such as FGF-2.

4.5.2. Results

In the prefrontal cortex of PND 45 rats we found no effect of cocaine ($F_{1,34} = 3.13$, $p = 0.086$, two-way ANOVA) or stress ($F_{1,34} = 0.86$, $p = 0.36$, two-way ANOVA) but a significant cocaine x stress interaction ($F_{1,34} = 8.19$, $p = 0.0076$, two-way ANOVA). Indeed, stress significantly decreased the FGF-2 mRNA levels in cocaine-pretreated rats (-27% vs Cocaine/Sham; $F_{1,16} = 6.73$, $p = 0.028$, two-way ANOVA with SCPH test) (Fig 1a), whereas no effects were observed in animals that received saline injections (+15% vs Saline/Sham; $F_{1,18} = 2.00$, $p > 0.05$, two-way ANOVA with SCPH test). Conversely, in adult rats we found a main effect of cocaine ($F_{1,39} = 12.59$, $p = 0.0011$, two-way ANOVA), no effect of stress ($F_{1,39} = 0.094$, $p = 0.76$, two-way ANOVA) and a significant stress x cocaine interaction ($F_{1,39} = 4.24$, $p = 0.046$, two-way ANOVA) (Figure 1b). In details, repeated cocaine administration during adolescence induced an increase in FGF-2 mRNA levels at PND 90 (+53% vs Saline/Sham; $F_{1,19} = 15.32$, $p = 0.0008$, two-way ANOVA with SCPH test). The response to stress in PND 90 rats was, however, different since we observed a tendency to an increase after acute swim stress in saline-pretreated rats, whereas a slight decrease was found in animals injected with cocaine during adolescence.

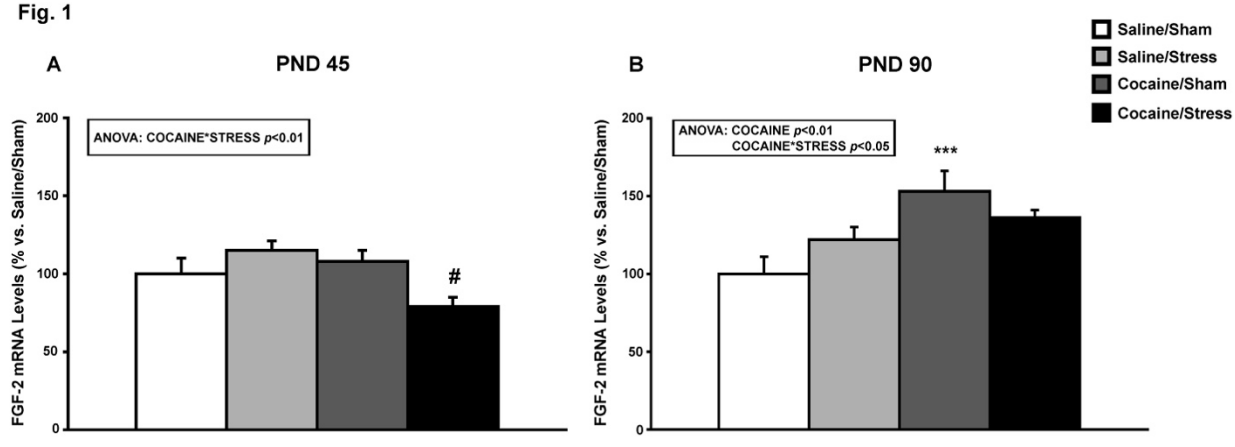

Fig. 1: Response to a single stress exposure (5-min swim stress and sacrificed 15 min later) in animals exposed to cocaine (20 mg/kg) or saline during adolescence: effects on FGF-2 mRNA levels in rat prefrontal cortex of PND 45 (a) and PND 90 (b) rats. The results, expressed as percentage of saline/sham rats, represent the mean ± SEM; triple asterisks $p < 0.001$ vs. saline/sham rats; number sign $p < 0.05$ vs. cocaine/sham rats (two-way ANOVA followed by SCPHT). Global ANOVA analysis appears in the upper box.

The analysis of hippocampal FGF-2 mRNA levels revealed a different pattern of FGF-2 expression. In fact, in PND 45 animals, we observed a main effect of cocaine treatment ($F_{1,34}$ = 26.61, p= 0.000015, two-way ANOVA) and of stress ($F_{1,34}$ = 6.31, p= 0.017, two-way ANOVA) (Fig. 2a). When animals that had received cocaine treatment during adolescence were then exposed to acute swim stress, they showed a higher increase of hippocampal FGF-2 mRNA levels with respect to saline-treated animals, that was however not significant statistically and therefore we did not proceed with further sub testing [cocaine x stress interaction ($F_{1,34}$ = 1.33, p= 0.25, two-way ANOVA)] (Fig. 2a).

These effects observed at PND 45, however, were not long-lasting since no effect of cocaine ($F_{1,39}$ = 0.33, p= 0.56, two-way ANOVA), stress ($F_{1,39}$ = 2.02, p= 0.16, two-way ANOVA) and no interaction as well ($F_{1,39}$ = 0.0094, p= 0.92, two-way ANOVA) (Fig. 2b) on FGF-2 gene expression were detected in the hippocampus of adult animals.

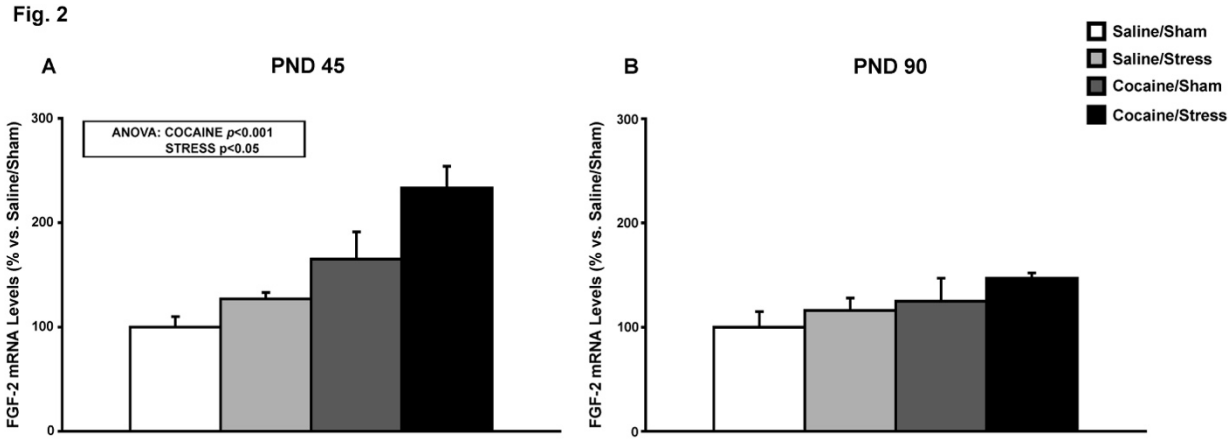

Fig. 2: Response to a single stress exposure (5-min swim stress and sacrificed 15 min later) in animals exposed to cocaine (20 mg/kg) or saline during adolescence: effects on FGF-2 mRNA levels in rat hippocampus of PND 45 (a) and PND 90 (b) rats. The results, expressed as percentage of saline/sham rats, represent the mean ± SEM. Global ANOVA analysis appears in the upper box.

At variance from what we observed in prefrontal cortex and hippocampus, no effects of cocaine treatment and/or of stress were observed in PND45 and PND90 rats both in striatum (Fig. 3a and 3b) as well as nucleus accumbens (Fig. 3c and 3d) [Striatum: PND45 (cocaine, $F_{1,35}$ = 0.0049, p= 0.94; stress: $F_{1,35}$ = 0.33, p= 0.57; cocaine x stress interaction: $F_{1,35}$ = 0.22, p=

0.64) PND90 (cocaine, F1,39 = 0.72, p= 0.40; stress: F1,39 = 0.025, p= 0.87; cocaine x stress interaction: F1,39 = 0.00027, p= 0.98); Nucleus accumbens: PND45 (cocaine, F1,34 = 3.49, p= 0.071; stress: F1,34 = 0.92, p= 0.34; cocaine x stress interaction: F1,34 = 3.47, p= 0.072) PND90 (cocaine, F1,34 = 2.76, p= 0.10; stress: F1,34 = 0.62, p= 0.43; cocaine x stress interaction: F1,34 = 0.38, p= 0.54)].

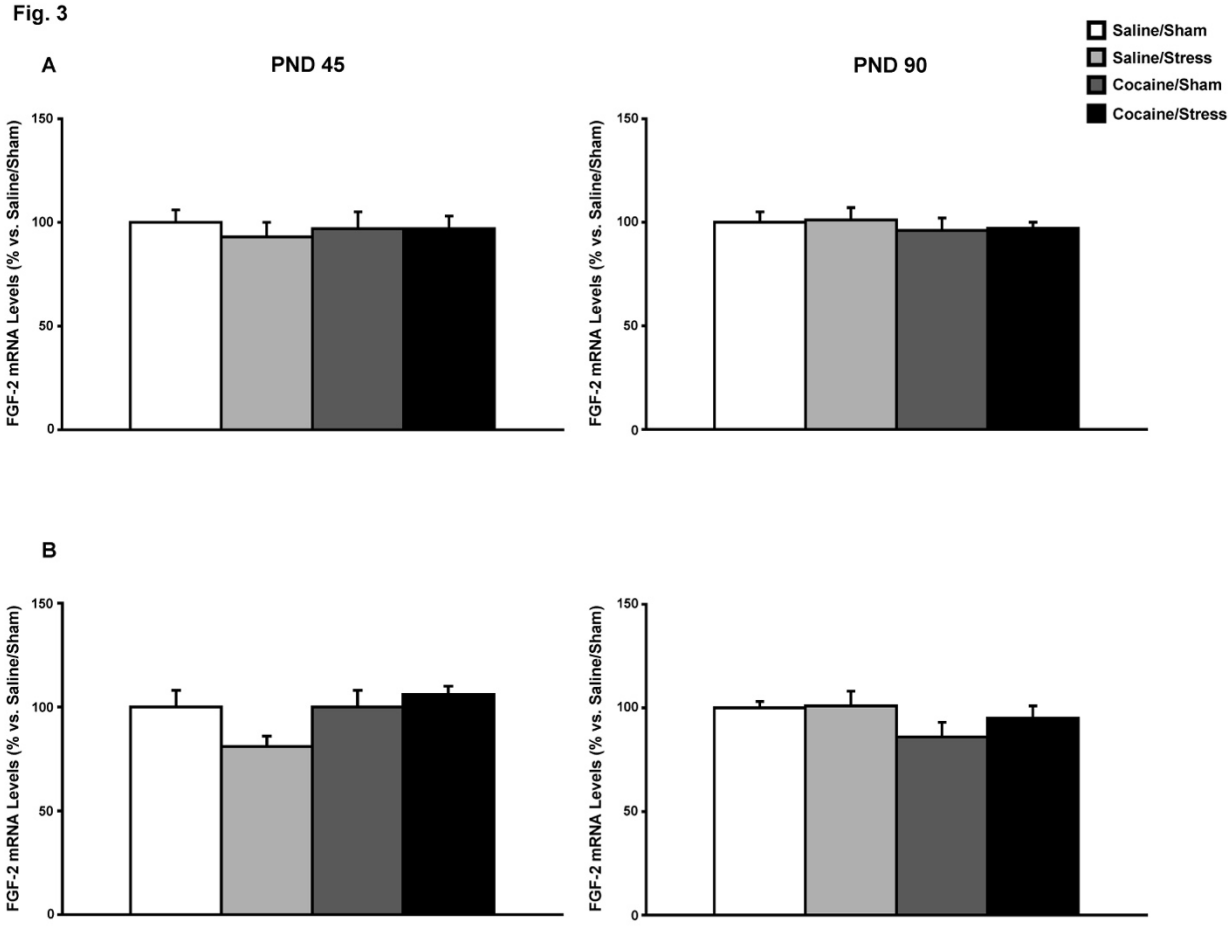

Fig. 3: Response to a single stress exposure (5-min swim stress and sacrificed 15 min later) in animals exposed to cocaine (20 mg/kg) or saline during adolescence: effects on FGF-2 mRNA levels in the striatum of PND 45 (a) and PND 90 (b) rats and in the nucleus accumbens of PND 45 (c) and PND 90 (d) rats. The results, expressed as percentage of saline/sham rats, represent the mean ± SEM

It is well established that FGF-2 is primarily localized in astrocytes (Gonzalez et al., 1995) and changes in astroglial expression of the trophic factor have been observed after a repeated regimen of amphetamine administration (Flores and Stewart, 2000). To this end, we decided to investigate the expression of a known astrocyte marker, i.e. glial fibrillary acidic protein (GFAP), in the prefrontal cortex and hippocampus, to verify whether changes in FGF-2 expression could be ascribed to glial FGF-2. In the prefrontal cortex of PND 45 rats we found a significant effect of cocaine ($F_{1,29} = 4.41$, $p = 0.046$), no effect of stress ($F_{1,29} = 2.71$, $p = 0.11$) and cocaine x stress interaction ($F_{1,29} = 4.63$, $p = 0.041$) (Figure 4a). In details, GFAP mRNA levels were significantly reduced in rats exposed to cocaine at adolescence and then subjected to stress (-38% vs Cocaine/Sham; $F_{1,14} = 6.99$, $p = 0.027$, two-way ANOVA with SCPH test) whereas no changes were detected in saline-treated rats (+5% vs Saline/Sham; $F_{1,15} = 0.13$, $p > 0.05$, two-way ANOVA with SCPH test). In PND 90 rats, we found an effect of cocaine ($F_{1,38} = 4.17$, $p = 0.048$), an effect of stress ($F_{1,38} = 4.69$, $p = 0.037$) but no cocaine x stress interaction ($F_{1,38} = 1.68$, $p = 0.20$) (Figure 4b). In the hippocampus of PND 45 rats we found no effect of stress ($F_{1,34} = 0.30$ $p = 0.58$), no effect of cocaine ($F_{1,34} = 0.041$ $p = 0.84$) and no cocaine x stress interaction ($F_{1,34} = 0.81$ $p = 0.37$) on GFAP mRNA levels (Figure 4c) and so we did in the hippocampus of PND 90 rats [stress: $F_{1,40} = 0.58$ $p = 0.44$; cocaine: $F_{1,40} = 2.44$ $p = 0.12$; cocaine x stress: $F_{1,40} = 3.77$ $p = 0.059$] (Figure 4d).

Fig. 4

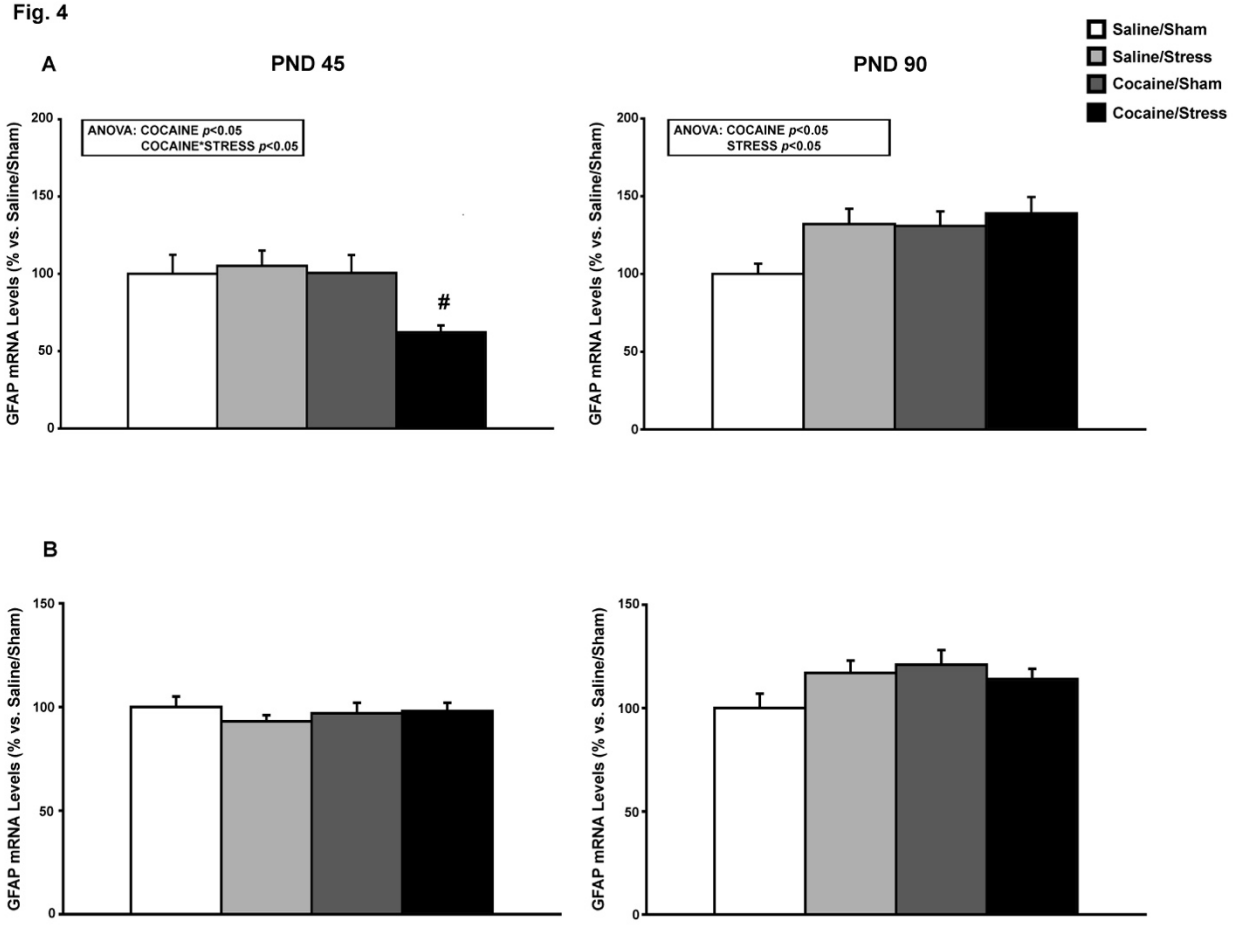

Fig. 4: Response to a single stress exposure (5-min swim stress and sacrificed 15 min later) in animals exposed to cocaine (20 mg/kg) or saline during adolescence: effects on GFAP mRNA levels in the rat prefrontal cortex of PND 45 (a) and PND 90 (b) rats and in the hippocampus of PND 45 (c) and PND 90 (d) rats. The results, expressed as percentage of saline/sham rats, represent the mean ± SEM. Number sign $p < 0.05$ vs. cocaine/sham rats (two-way ANOVA followed by SCPHT). Global ANOVA analysis appears in the upper box

4.5.3. Discussion

The analysis of FGF-2 mRNA levels following repeated exposure to cocaine during adolescence revealed two major effects. First, baseline FGF-2 mRNA levels are increased in the prefrontal cortex of adult rats, suggesting a long-lasting imprinting of cocaine treatment delivered during adolescence; second, adolescent exposure to cocaine altered the subsequent trophic response to stress in the prefrontal cortex, revealing a dynamic modulation of FGF-2 mRNA levels under adverse conditions. These data point to this trophic factor as a uniquely sensitive target of adolescent exposure to cocaine and highlight the critical role of prefrontal cortex in mediating such changes.

FGF-2 mRNA levels were increased in the prefrontal cortex of PND 90 rats exposed to cocaine during adolescence (from PND 28 to PND 42) revealing a long-lasting effect promoted by the psychostimulant. Such increase was not observed at PND 45 suggesting that FGF-2 mRNA levels progressively increase after cocaine withdrawal, perhaps as a result of the so-called 'incubation of craving' (Lu et al., 2004; Pickens et al., 2011), a process which occurs via adaptations in the corticolimbic reward system that develop over time. The potential relevance of increased FGF-2 mRNA levels at adulthood following exposure to cocaine during adolescence is obscure but different possibilities can be put forward. Turner and associates (2009) have elegantly shown that early in life administration to FGF-2 leads to increased drug taking behavior at adulthood. This evidence suggests that the increase in FGF-2 mRNA levels in the prefrontal cortex of adult rats exposed to cocaine during adolescence might be related to increased propensity to take cocaine, perhaps representing an index of vulnerability to drug abuse. The higher levels of FGF-2 observed at adulthood may also contribute to the morphological changes, critical for drug seeking, observed following psychostimulant addiction (Diaz Heijtz et al., 2003; Robinson and Kolb, 2004): to this end, Mueller and colleagues (2006) have shown that enhanced dendritic growth following repeated, early in life exposure to amphetamine contributes to drug seeking and it is dependent on FGF-2 (Mueller et al., 2006). It is important to point out that increased expression of FGF-2 in the prefrontal cortex may also have antidepressant effect (Turner et al., 2008; Perez et al., 2009; Turner et al., 2011; Elsayed et al., 2012); although these lines of

evidence involved exogenous administration of FGF-2, rather than the modulation of the trophic factor levels, we can not rule out the possibility that such increase may also positively influence brain homeostasis. To this end, the herein reported, long-lasting changes in FGF-2 expression add complexity as well as specificity to the modulation of neuroplasticity by drugs of abuse.

In addition to determining changes in the cortical baseline FGF-2 mRNA levels, we found that developmental exposure to cocaine altered the FGF-2 response to stress. In fact, whereas acute stress tended to increase FGF-2 mRNA levels in animals exposed to saline at adolescence, it reduced FGF-2 gene expression in the prefrontal cortex of PND 45 animals with a similar trend also in PND 90 rats. This suggests that cocaine exposure at adolescence might reduce neuronal responsiveness to external stimuli. Interestingly, cocaine and stress interacted to down-regulate GFAP mRNA levels at PND 45; this effect closely parallels the reduction of FGF-2 mRNA levels and points to glial FGF-2 as the primary source of the effects produced by cocaine and stress. The possibility exists that GFAP down-regulation at PND 45 may represent a mechanism set in motion by cocaine to avoid the recruitment of rapid, neuroprotective mechanisms, mediated by FGF-2, thus rendering the cell more sensitive to further insults.

A different effect was observed in the hippocampus where, although dopamine terminals are scarce, cocaine seems to modulate FGF-2 mRNA levels, perhaps implying the contribution of mechanisms independent of dopamine. Interestingly, FGF-2 gene expression appears to be regulated at PND 45, i.e. soon after the end of adolescence, whereas such effect wanes at adulthood. Indeed, at PND 45, cocaine enhances FGF-2 mRNA levels, an effect that seems to be potentiated by stress. At adulthood, although the trend appears to be the same, the magnitude of the effect is reduced and not statistically significant, suggesting the transient nature of FGF-2 mRNA elevation in hippocampus, although we do not know when such increase dissipates since we have no intermediate time-points between PND 45 and PND 90.

Prefrontal cortex appears to be the brain region mostly affected by adolescent exposure to cocaine whereas other brain regions, traditionally considered to be the major targets of the action of the psychostimulant, such as striatum and nucleus accumbens, did not show any change. Although this finding appears surprising at first sight since these brain regions govern dopamine transmission following repeated exposure to cocaine (Rocha et al., 1998), however, evidence exists

that presynaptic dopamine function is largely unaltered in rats exposed to cocaine early in life (Phillips et al., 2003) and that dopamine uptake rate is increased in response to escalated cocaine self-administration (Oleson et al., 2009), underlying that the effects of the long-term exposure to cocaine are not strictly related to the primary mechanism of action of the psychostimulant. In addition, we had previously demonstrated that acute or repeated exposure to cocaine modulates the expression of the trophic factor in adult rat striatum (Fumagalli et al., 2006a). Therefore, the lack of effect in the striatum and in the nucleus accumbens might indicate that perturbation of dopamine transmission during adolescence is not sufficient to produce changes in FGF-2 mRNA levels in these brain regions or that such changes wane earlier than PND 45.

In conclusion, our results demonstrate that exposure to cocaine during adolescence causes a long-lasting elevation of FGF-2 mRNA levels in the rat prefrontal cortex. Since FGF- mRNA levels are not altered at PND 45, these data indicate that modulation of FGF-2 expression depends on the withdrawal time and point to a role of FGF-2 in the incubation of cocaine craving after drug withdrawal (Grimm et al., 2001). Further, cocaine treatment during brain development alters the FGF-2 response to adverse events such as an acute stress, revealing the influence of the developmental psychostimulant treatment on the dynamic modulation of this trophic factor. Taken together, these data suggest that exposure to cocaine when the brain is still maturing may have long-lasting, dynamic and, perhaps, functional consequences on prefrontal cortex homeostasis.

4.6. A single exposure to cocaine during development elicits regionally-selective changes in basal FGF-2 gene expression and alters the trophic response to a second injection.

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4.6.1. Introduction

Brain buildup is extremely sensitive to the influence of interfering events that may hamper the physiological developmental trajectory (Spear, 2000; Casey et al., 2008; Ernst et al., 2009). Among the factors that may promote brain vulnerability, exposure to drugs of abuse during adolescence, and/or abstinence from it, is indeed of great importance, perhaps through changes in the expression of neuroplastic molecules. Since neurotrophic factors participate to brain development by displaying various actions on different cell populations in the central nervous system (Reuss and von Bohlen und Halbach, 2003; Itoh and Ornitz, 2004), dysregulation of their synthesis may contribute to the above mentioned vulnerability. Among the others, basic fibroblast growth factor (FGF-2) is the prototype member of a family of heparin-binding growth factors whose contribution to brain development has been extensively documented (Riva et al., 2005). Indeed, FGF-2 might represent a target of the early exposure to drugs of abuse since it is expressed in the developing brain (Gomez-Pinilla et al., 1994; Monfils et al., 2006) and, when administered early in life, it potentiated the acquisition of cocaine self-administration (Turner et al., 2009) and enhanced cocaine sensitization at adulthood (Clinton et al., 2012). To this end, we have recently shown that abstinence following adolescent exposure to cocaine leads to short- and long-term changes in basic Fibroblast growth factor (FGF-2) gene expression in the rat brain (Giannotti et al., 2013) further pointing to brain development as a sensitive period for external stimuli.

Additionally, we have recently shown that exposure to cocaine during adolescence leads to changes in FGF-2 mRNA levels that last into adulthood (Giannotti et al., 2013) confirming that FGF-2 is a putative target of the action of cocaine during development suggesting the possibility that modulation of FGF-2 mRNA levels may participate to the vulnerability of the adolescent brain to drug seeking.

In this manuscript, we decided to address two questions. First, to evaluate if a single injection of the psychostimulant during development, i.e. postnatal day (PND) 35, is sufficient to alter the trophic response, short (24h) or long (one week) after the injection. Second, to investigate whether and how the exposure to a second administration of cocaine 24 hours (PND

36) or a week later (PND 42) might influence the trophic factor transcription. Taken together, the findings deriving from this two-hit approach will increase our knowledge on how drug abuse at adolescence might dynamically influence brain homeostasis, via changes in FGF-2 mediated trophic response.

4.6.2. Results

In the mPFC, three-way ANOVA revealed an effect of the first injection ($F_{1,45}$=8.48, p=0.006) and a first injection × second injection interaction ($F_{1,45}$=7.03, p=0.012) on FGF-2 mRNA levels. No significant effect of the time between injections was instead detected ($F_{1,45}$=2.18, p=0.149). Thus, we made intergroup comparisons by two-way ANOVAs (first injection × second injection) for each group (i.e. animals challenged with cocaine 24h and 7 days after the first injection). In the 24 hour-challenged group, two-way ANOVA did not detect significant differences (first injection: $F_{1,25}$=1.23, p=0.280; second injection: $F_{1,25}$=0.181, p=0.68; first injection x second injection: $F_{1,25}$=2.30, p=0.145). Conversely, in the 7 day-challenged group, we found effects of the first injection ($F_{1,20}$=7.64, p=0.014) and a significant first injection x second injection interaction ($F_{1,20}$=4.51, p=0.049). The post hoc analysis revealed that FGF-2 mRNA levels were increased 7 days after the first cocaine administration compared with the control group (+57%, p=0.003, Fisher's LSD test). In animals that were exposed to the first injection of cocaine, the second injection with the psychostimulant significantly decreased FGF-2 mRNA levels (-35%, p=0.047, Fisher's LSD test), an effect that was not observed in saline-pretreated animals (+14%, p=0.429).

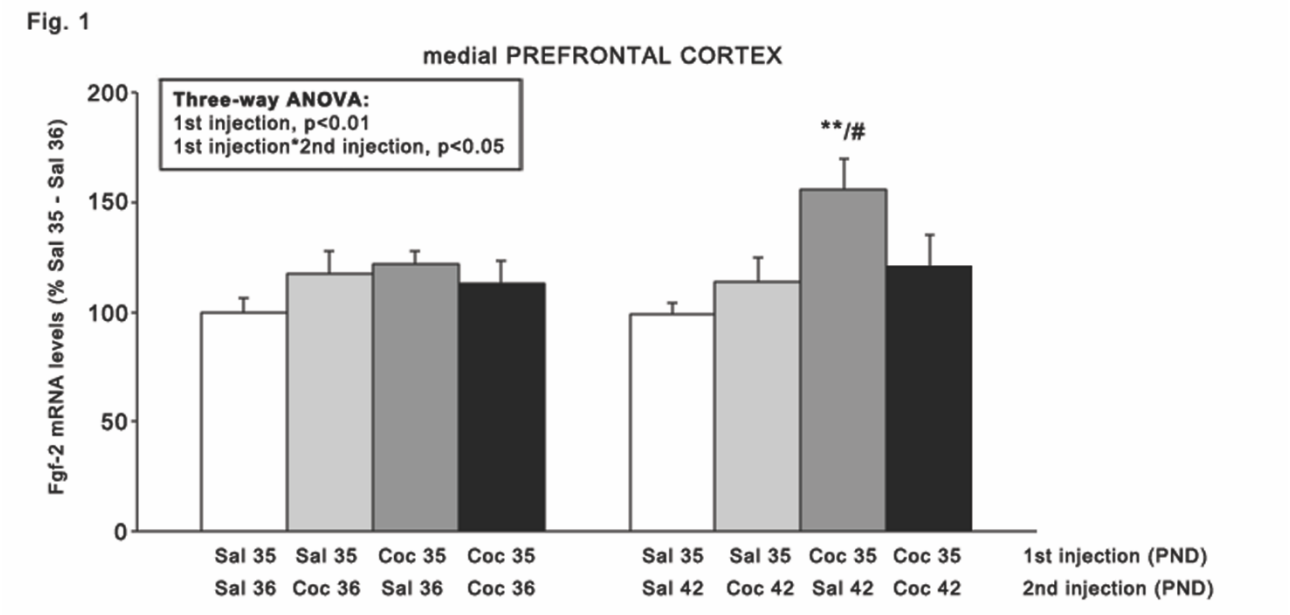

Figure 1: Effects of a single injection of cocaine during brain development on FGF-2 mRNA levels in the rat medial prefrontal cortex: modulation by a second cocaine injection. Adolescent male rats (PND 35) were exposed to a first injection of cocaine (20 mg/kg) or saline at PND 35; then, each group was divided in four different groups receiving, respectively, cocaine (10 mg/kg) or saline at PND36 or PND 42. Rats were sacrificed 2 hours after the second injection with cocaine or saline. The results, expressed as % of SalineP35/SalineP36 rats, represent the mean ± S.E.M. of at least 5 independent determinations; $^{**}p<0.01$ vs. SalineP35/SalineP42 rats; $^{\#}p<0.05$ vs. CocaineP35/CocaineP42 rats (two-way ANOVA followed by Fisher LSD test). Global ANOVA analysis appears in the upper box.

In the frontal cortex, three-way ANOVA of FGF-2 found only an effect of the time between injections ($F_{1,48}$=6.89, p=0.012) (first injection: $F_{1,48}$=0.556, p=0.460; second injection: $F_{1,48}$=0.177, p=0.676) but the first injection of cocaine did not influence the trophic effects of the second injection (interaction between first injection x second injection x time between injections: $F_{1,48}$=0.014, p=0.905) (Fig. 2). Accordingly, we did not subdivide the statistical analysis in this region.

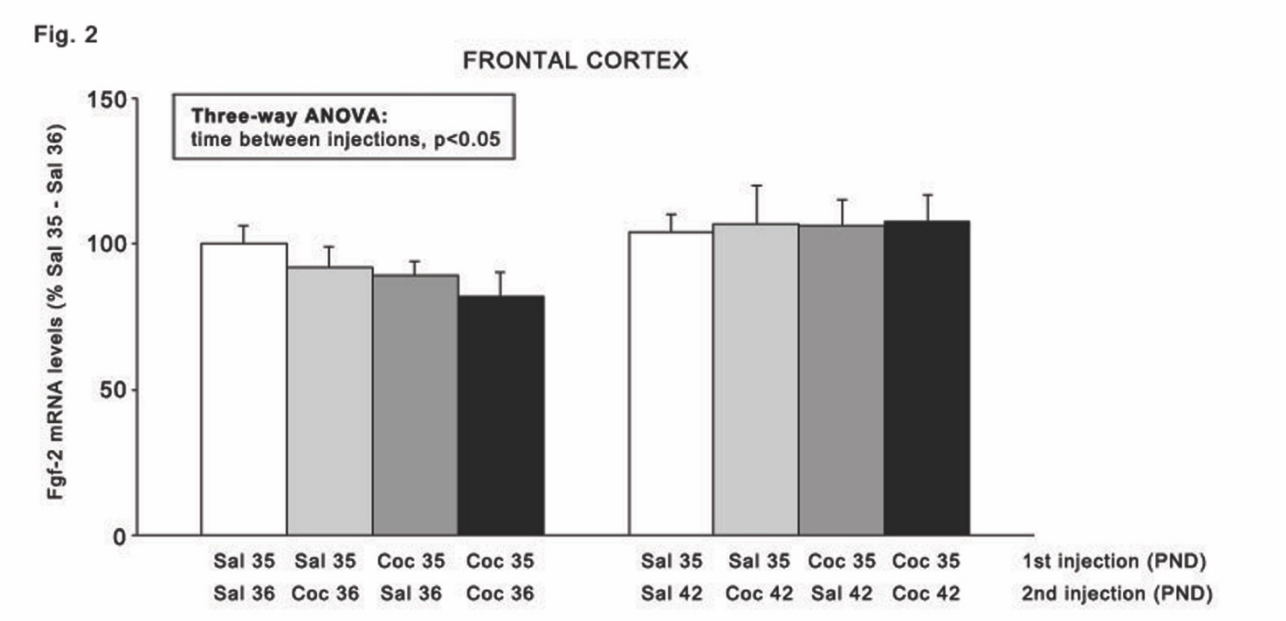

Figure 2: Effects of a single injection of cocaine during brain development on FGF-2 mRNA levels in the rat frontal cortex: modulation by a second cocaine injection. Adolescent male rats (PND 35) were exposed to a first injection of cocaine (20 mg/kg) or saline at PND 35; then, each group was divided in four different groups receiving, respectively, cocaine (10 mg/kg) or saline at PND 36 or PND 42. Rats were sacrificed 2 hours after the second injection with cocaine or saline. The results, expressed as % of SalineP35/SalineP36 rats, represent the mean ± S.E.M. of at least 5 independent determinations. Global ANOVA analysis appears in the upper box.

Unlike the findings for the frontal cortex, in the NAc three-way ANOVA revealed effects of the second injection ($F_{1,47}$=13.59, p=0.001), first injection x second injection interaction ($F_{1,47}$=5.68, p=0.022) and first injection x second injection x time between injections interaction ($F_{1,47}$=4.58, p=0.039) (Fig. 3). Examining the individual treatment effects, we found that a single cocaine injection increased FGF-2 mRNA levels 24 hours later (+28% vs. Sal35-Sal36, p=0.010, Fisher's LSD test), an effect that returned to controls level 7 days after the single cocaine injection (+5% vs. Sal35-Sal42, p=0.682, Fisher's LSD test). The response to the second cocaine injection was different in saline- and cocaine-pretreated animals when challenged 24h later. In fact, the cocaine-induced increase in FGF-2 mRNA levels observed in saline-pretreated rats (+36% vs.

Sal35-Sal36, p=0.001, Fisher's LSD test) was prevented in cocaine-pretreated animals (-15% vs. Coc35-Sal36, p=0.203, Fisher's LSD test). On the other hand, when measured 7 days later, the second injection of cocaine induced an increase independently of the pretreatment. In fact, FGF-2 mRNA levels were increased in both the saline- (+32% vs. Sal35-Sal42, p=0.004, Fisher's LSD test) and cocaine-pretreated rats (+29% vs. Coc35-Sal42, p=0.025, Fisher's LSD test).

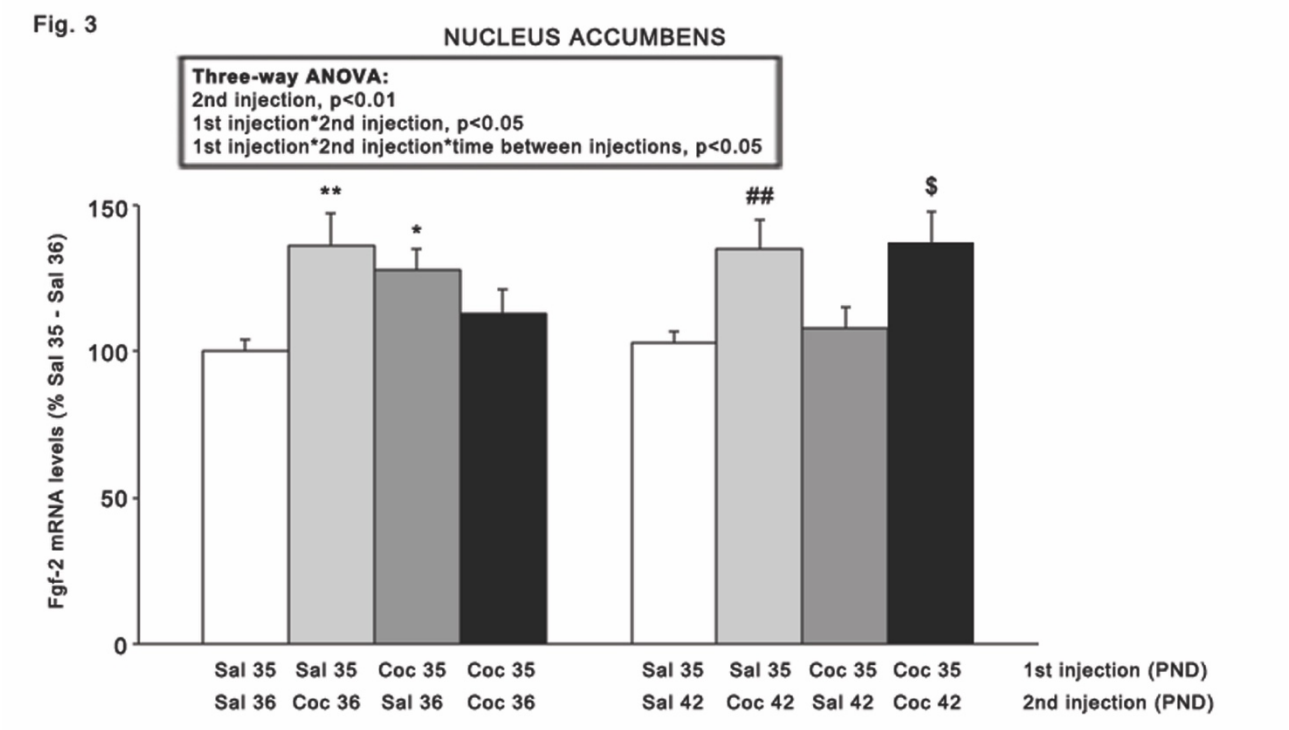

Figure 3: Effects of a single injection of cocaine during brain development on FGF-2 mRNA levels in the rat nucleus accumbens: modulation by a second cocaine injection. Adolescent male rats (PND 35) were exposed to a first injection of cocaine (20 mg/kg) or saline at PND 35; then, each group was divided in four different groups receiving, respectively, cocaine (10 mg/kg) or saline at PND 36 or PND 42. Rats were sacrificed 2 hours after the second injection with cocaine or saline. The results, expressed as % of SalineP35/SalineP36 rats, represent the mean ± S.E.M. of at least 5 independent determinations; $^{*}p<0.05$, $^{**}p<0.01$ vs. SalineP35/SalineP36 rats; $^{\#\#}p<0.01$ vs. SalineP35/SalineP42 rats; $^{\$}p<0.05$ vs. CocaineP35/SalineP42 rats (three-way ANOVA followed by Fisher's LSD test). Global ANOVA analysis appears in the upper box.

Figure 4a shows the mRNA levels of FGF-2 in the hippocampus. In this brain region, three-way ANOVA revealed only a significant effect of the first injection x second injection interaction ($F_{1,45}$=11.89, p=0.001) (first injection: $F_{1,45}$=3.83, p=0.058; second injection: $F_{1,45}$=0.216, p=0.645; time between injections: $F_{1,45}$=2.39, p=0.131; interaction between first injection x second injection x time between injections: $F_{1,45}$=0.657, p=0.423). Examining the individual treatment effects, lower order ANOVA (first injection × second injection) were performed for each group (24h- and 7 days-challenged). Rats that received the second injection 24h later showed a significant effect of the second injection ($F_{1,23}$=8.66, p=0.008) and a first injection × second injection interaction ($F_{1,23}$=4.48, p=0.048). Twentyfour hours after the first cocaine injection, FGF-2 was significantly reduced (-22% vs. Sal35-Sal36, p=0.038, Fisher's LSD test). The second injection of cocaine caused a decrease in FGF-2 mRNA levels in saline-pretreated rat (-37% vs. Sal35-Sal36, p=0.001, Fisher's LSD test), but did not further reduce the FGF-2 levels in cocaine-pretreated rats (-6% vs. Coc35-Sal36, p=0.591, Fisher's LSD test). Two-way ANOVA performed on the 7 day-challenged group showed an effect of the second injection ($F_{1,22}$=8.51, p=0.009) and a first injection × second injection interaction ($F_{1,22}$=7.30, p=0.015). FGF-2 mRNA levels were reduced 7 days after the first cocaine injection (-42% vs. Sal35-Sal42, p=0.007, Fisher's LSD test). Cocaine challenge did not alter FGF-2 levels in saline-pretreated animals (+2% vs. Sal35-Sal42, p=0.873, Fisher's LSD test), but the second cocaine injection significantly increased FGF-2 levels in cocaine–pretreated rats (+52% vs. Coc35-Sal42, p=0.001, Fisher's LSD test).

Since this latter effect was intriguing because of the magnitude of FGF-2 enhancement, we hypothesized that the first injection of cocaine reduced the hippocampal baseline activity such that the response to a second stimulus could determine hippocampal hyperactivity. Therefore, we decided to measure the mRNA levels of Activity Regulated Cytoskeletal associated protein (Arc), a well established index of neuronal activity (Lyford et al., 1995a). Three-way ANOVA showed effects of the first injection ($F_{1,46}$=10.94, p=0.002), time between injections ($F_{1,46}$=13.65, p=0.0007), a first injection x second injection interaction ($F_{1,46}$=17.29, p=0.0002) and a first injection x second injection x time between injections interaction ($F_{1,46}$=6.09, p=0.018). As observed for FGF-2 mRNA, the post-hoc analysis revealed a reduction in Arc mRNA levels 7

days after the first cocaine injection (-50% vs. Sal35-Sal42, p=0.000001, Fisher's LSD test). The second injection of cocaine 7 days later altered differently Arc mRNA based on the pretreatment. In fact, the second cocaine injection reduced Arc levels in saline-pretreated animals (-23% vs. Sal35-Sal42, p=0.007, Fisher's LSD test), whereas it increased Arc mRNA in cocaine-pretreated rats (+33% vs. Coc35-Sal42, p=0.0005, Fisher's LSD test).

Fig. 4

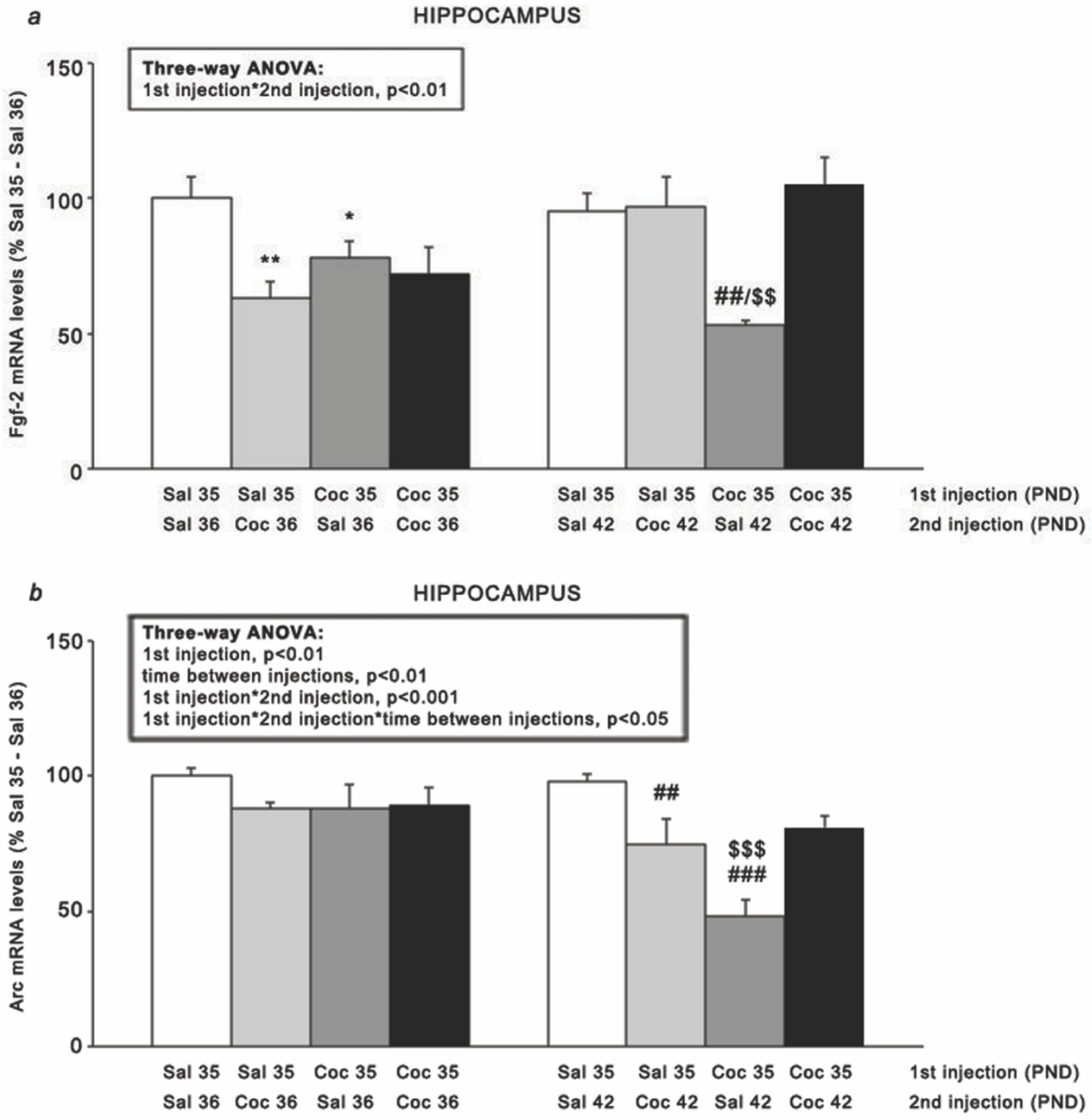

Figure 4. Effects of a single injection of cocaine during brain development on FGF-2 (panel a) and Arc (panel B) mRNA levels in the rat hippocampus: modulation by a second cocaine injection. Adolescent male rats (PND 35) were exposed to a first injection of cocaine (20 mg/kg) or saline at PND 35; then, each group was divided in four different groups receiving, respectively, cocaine (10 mg/kg) or saline at PND 36 or PND 42. Rats were sacrificed 2 hours after the second injection with cocaine or saline. In panel A the results, expressed as % of SalineP35/SalineP36 rats, represent the mean ± S.E.M. of at least 5 independent determinations; $^{*}p<0.05$, $^{**}p<0.01$ vs. SalineP35/SalineP36 rats; $^{\#\#}p<0.01$ vs. SalineP35/SalineP42 rats; $^{\$\$}p<0.01$ vs. CocaineP35/CocaineP42 rats (two-way ANOVA followed by Fisher's LSD test). In panel B, the results, expressed as % of SalineP35/SalineP36 rats, represent the mean ± S.E.M. of at least 5 independent determinations; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. SalineP35/SalineP42 rats; $^{\$\$\$}p<0.001$ vs. CocaineP35/CocaineP42 rats (two-way ANOVA followed by Fisher's LSD test). Global ANOVA analysis appears in the upper box.

4.6.3. Discussion

Our data reveal that a single exposure to cocaine during brain development is sufficient to alter FGF-2 mRNA levels in the rat brain. Additionally, our results show that a single exposure to cocaine, during the sensitive period of adolescence, influences the response to a second dose of the psychostimulant suggesting that cocaine priming may have altered the mechanisms regulating the trophic response.

Indeed, we found regional differences in the FGF-2 response as either the first or the second injection of cocaine by themselves up-regulated FGF-2 mRNA in the mPFC and Nac while down-regulating it in the hippocampus.

In the NAc, baseline FGF-2 mRNA levels were increased 24 hours after a single injection of cocaine while returning back to control levels 7 days later. However, when the second injection was given 24 hours after the first, it increased FGF-2 mRNA levels in saline- but not in cocaine-pretreated animals. At variance, 7 days later, the FGF-2 increase was independent from the type of pretreatment. This evidence suggests that a previous exposure to cocaine has altered the responsiveness of the cell to a subsequent exposure to the psychostimulant within a short (24 hour), but not long (1 week), time-frame thereby indicating a significant, although transient, influence of cocaine priming. These data show that changes in the modulation of FGF-2 mRNA levels depend on how early cocaine is encountered during development suggesting that brain vulnerability during adolescence may be partially related to altered FGF-2 mediated trophic response. Of note, the inability to further elevate FGF-2 mRNA levels in the NAc at PND 36 might reduce neuroplasticity in animals previously exposed to cocaine, as observed in adult animals (Kolb et al., 2003).

In the mPFC, FGF-2 mRNA levels were increased 7 days after a single injection of cocaine while no effects were observed 24 hours later, revealing a quite lasting effect promoted by the acute psychostimulant administration. Since it has been previously demonstrated that a single, exogenous administration of FGF-2 early in life causes increased drug taking behavior at adulthood (Turner et al., 2009; Clinton et al., 2012), the herein shown increase in FGF-2 mRNA levels might be suggestive of vulnerability to take cocaine, perhaps representing a previously

unappreciated contribution to initial incubation of cocaine-seeking. However, the opposite may also be true given that FGF2 knockout mice display augmented responsiveness to psychostimulants (Fadda et al., 2007), thereby suggesting that increased FGF-2 mRNA levels may represent a defensive, protective strategy to oppose to the behavioral effects of cocaine. However, it this holds true, the neuroprotection afforded by FGF-2 increase may be compromised by the evidence that the trophic response to a second injection is significantly reduced in cocaine-pretreated rats.

The analysis of FGF-2 regulation in the hippocampus led to interesting and unexpected results. First, a single injection of cocaine reduced FGF-2 mRNA levels, when measured 24 hours later, an effect that became more consistent 7 days later. This suggests that cocaine markedly reduces the hippocampal trophic response during adolescence, an effect that we did not observe at adulthood (Fumagalli et al., 2006a). Such reduction might be functionally relevant. In fact, a deficit of FGF-2 expression causes defects in hippocampal neurogenesis, an effect that seems to be critical for relapse (Deschaux et al., 2014). Further, the reduction observed 7 days after the first exposure to cocaine might impair working memory, as shown by Sudai and colleagues following a similarly high dosage of cocaine (Sudai et al., 2011). Notably, since a single injection of cocaine is sufficient to induce anxiety in rats (Kohtz et al., 2010), an effect that has been linked to lower levels of hippocampal FGF-2 (Turner et al., 2011), our data suggest that reduced hippocampal levels of FGF-2 may represent a sign of vulnerability for cocaine-induced anxiety. saSecond, the second injection of cocaine reduced FGF-2 mRNA levels in saline-, but not cocaine-, pretreated animals 24 hours after the first drug administration; conversely, when the second challenge with cocaine was given a week later, a marked increase was observed in cocaine-primed animals with no effect in animals previously exposed to saline. This suggests that a single injection of cocaine during adolescence has altered the hippocampal trophic response in a way that depends on the lag phase from the first injection. The possibility exists that, in the hippocampus, a single injection of cocaine is sufficient to produce a reduction in the baseline activity of this brain region that, following a discrete stimulus capable of generating drug seeking (i.e. cocaine injection), it results in the transition to hippocampal hyperactivity. This possibility is corroborated by the evidence that the gene expression of Activity Regulated Cytoskeletal

associated protein (Arc), a well established index of neuronal activity (Bramham et al., 2008), follows exactly the same expression profile of FGF-2.

Taken together, our findings highlight a dynamic modulation of FGF-2 mRNA levels following a single injection of cocaine during development: in fact, while eliciting baseline changes both 24 hours and 7 days later, it also alters the response to a second injection of the psychostimulant, in a way that depends on the brain region investigated. Our results show the impact that a single injection with cocaine may cause when administered during a highly sensitive period for brain maturation such as adolescence. Given that FGF-2 expression has been shown to be long-lastingly altered by other types of early in life manipulations such as prenatal stress (Fumagalli et al., 2005), the present findings further point to FGF-2 as a critical modulator of brain development under various experimental conditions.

5. Overall summary and conclusions

Altogether, the results obtained during my Ph.D. add important preclinical evidence to the understanding of the short- and long-term molecular changes set in motion by repeated exposure to cocaine during adolescence, dissecting its contribution in the modulation of different molecular systems and processes.

Indeed, we found that three days of withdrawal from adolescent exposure to cocaine alters the ability of the glutamatergic system to cope with a stressful event. Recently, it has been highlighted the role of the cortical glutamatergic neurotransmission in response to a challenging situation (Moghaddam and Jackson, 2004; Popoli et al., 2012). Although stress is an environmental factor capable of generating relapse in adults (Mantsch et al., 2015) via the increase of the corticoaccumbal glutamatergic activity (McFarland et al., 2003), we reported the first evidence of the hyperactive glutamatergic synapse in response to acute stress after short-term withdrawal from developmental exposure to cocaine. In particular, we went on to dissect the contribution of the different components of the so-called "tripartite" glutamatergic synapse. At the presynaptic level, we found an enhanced expression of the vesicular glutamate transporter-1 (vGlut1) together with a reduced expression of the vesicular GABA transporter (vGAT), suggesting an imbalance between the excitatory and inhibitory system, toward the excitatory neurotransmission. Further, we investigated the contribution of the glial components and found a reduction in all the glial glutamate transporters herein investigated, suggesting a reduced clearance of glutamate from the synaptic cleft. Such potentiation of the glutamatergic neurotransmission results in the postsynaptic increased activation of the GluN1 subunit of NMDA receptors, which in turn activates an intracellular cascade, i.e. αCaMKII, Cdc42 and PAK-1. Notably, three days of withdrawal are sufficient to induce a pro-depressive like phenotype. In fact, those rats display an enhanced immobility time when compared to their saline

counterparts during the forced swim test, in line with the "negative emotional state" observed in the early phases of withdrawal in adults (Koob, 2015).

To get deeper insights into the molecular changes underlying the hypersensitivity to stress and the pro-depressive behavior, we decided to investigate whether chronic exposure to cocaine during adolescence may have altered the stress-related system, thus producing a vulnerable molecular background. Interestingly, after short-term withdrawal from adolescent exposure to cocaine, we found increased GR transcription and translation as well as increased nuclear translocation of the receptor, an effect that was associated with a reduced expression of the GR co-chaperone FKBP5 that, under physiological conditions, keeps the receptor in the cytoplasm (Tatro et al., 2009). We also found reduced spine density, in agreement with dendritic spine loss observed in animal models of depression (Sato, 2013). These findings suggest a dysregulation of GR-dependent cortical signaling that may contribute to the depressive-like behavior in the early phases of cocaine withdrawal and might represent the molecular mechanism to explain, at least in part, the hypersensitivity to stress observed in cocaine users in the early stages of abstinence (Kreek and Koob, 1998; Koob, 2015).

One of the major clinical issues of drug addiction is relapse even after long period of abstinence. Accordingly, besides the short-term molecular changes, we investigated also the long-term pharmacological effects brought about by repeated exposure to cocaine during adolescence. Although it is well-known that cocaine-induced modulation of molecules regulating neuroplastic processes such as Activity-regulated cytoskeleton-associated protein (Arc) and brain-derived neurotrophic factor (BDNF) play a crucial role in modulating addictive behaviors in adulthood (Fosnaugh et al., 1995; Schoenbaum et al., 2007; McGinty et al., 2010; Ziolkowska et al., 2011; Li and Wolf, 2015), the cocaine-induced neuroplastic changes in the adolescent brain are still unknown. Accordingly, we investigated whether repeated exposure to cocaine during adolescence might have altered the BDNF expression in mPFC, since it has been shown by Grimm and colleagues (2001; 2003) a time-dependent increase of the neurotrophin as a consequences of abstinence duration, the so-called "incubation" of cocaine craving.

Interestingly, we found that long-term withdrawal from developmental exposure to cocaine has altered the transcriptional and translational mechanisms governing the neurotrophin

expression up to 48 days of withdrawal. In particular, we found a withdrawal-induced up-regulation of total BDNF mRNA levels in the mPFC of PND90 rats, an effect sustained by increased expression of BDNF exon IV through the transcription factors CaRF and NF-kB. Enhanced BDNF mRNA levels result in an increased expression of both precursor and mature forms of BDNF, suggesting not only an increased translation but also an enhanced processing of the neurotrophin. Moreover, we found a reduction of several miRNAs governing the translation of BDNF and known to be modulated by repeated exposure to cocaine, an effect that might, perhaps, contribute to the increased expression of proBDNF. Further, the withdrawal-induced expression of BDNF results in the activation of the trkB/AKT pathway, which, through the increased phosphorylation of S6 kinase, may drive the increase of Arc protein levels in the nucleus and crude synaptosomal fraction. We also investigated the inhibitory and degradative pathways regulating Arc expression and found reduced FMR1 and Ube3a mRNA levels as well as increased GRM5 mRNA levels. The up-regulation of Arc protein might lead, in turn, to a reduction of AMPA GluA1 mRNA and protein levels, indicating that long-term withdrawal alters markers of synaptic plasticity through different, but converging mechanisms. These findings point to BDNF and Arc/Arg3.1 as molecular signatures of the long-term withdrawal from repeated exposure to cocaine during adolescence. Given that Arc/Arg3.1 may be a partner of BDNF in mediating such adaptive changes, we may speculate that withdrawal-induced alteration in the pathway of BDNF together with the changes of molecules regulating Arc/Arg3.1 synthesis and degradation may contribute to the incubation of cocaine craving.

Lastly, since it has been shown that cocaine modulates FGF-2 in a region- and time-specific manner from the last drug exposure in adulthood (Fumagalli et al., 2006a; Fumagalli et al., 2008), and taking into account that it has been recently shown that infusion of FGF-2 in the prefrontal cortex modulates addictive behaviors (Hafenbreidel et al., 2015), we investigated whether developmental exposure to cocaine could modulate its expression in a time- and region-specific manner. Interestingly we found that short-term withdrawal from developmental exposure to cocaine selectively increase FGF-2 expression in the hippocampus while its expression was increased in the mPFC after long-term withdrawal, a time-dependent increase similar to that

observed for BDNF, suggesting an overall drug-induced modulation of the neurotrophins system that relies on a withdrawal-induced increase from the last drug exposure.

Since adolescence could be considered a period of high vulnerability to initiate drug use, next we investigated whether a single exposure to cocaine could modulate the trophic response to a second injection. Interestingly, we found that cocaine priming during adolescence is sufficient to alter the mechanisms underlying the trophic response to a second injection in a region- and time-dependent manner. In particular, we found that a single injection is sufficient to enhance FGF-2 levels in mPFC seven days after, while its expression, as well as ARC expression, was reduced in hippocampus. Interestingly, the challenge of cocaine seven days later induces a reduction of the neurotrophin in the mPFC while its expression returns to controls in hippocampus in cocaine pretreated animals. Taken together, these results suggest that a single exposure to cocaine during adolescence *per sè* is sufficient to modulate FGF-2 expression and it modulates the trophic response to a second injection. Moreover, the baseline reduction of FGF-2 in hippocampus, accompanied by reduction in ARC expression, might suggest that a single injection of cocaine during adolescence is sufficient to induce, seven days later, a reduction of the baseline neuronal activity. Taken together, these results point to adolescence as a period of high vulnerability, suggesting that a single injection of cocaine, rather than the repeated exposure, is sufficient to alter the molecular mechanisms underlying the physiological brain functions, perhaps setting the molecular stage to develop addiction to drugs of abuse later on in life.

In conclusion, the results obtained during my Ph.D. add further weight to the notion of adolescence as a critical period of life during which interfering with the proper development of the brain might produce a vulnerable molecular background and, perhaps, may set the stage to initiate drug use or relapse to drugs of abuse later on in life. Moreover, it is clear from our preclinical data that the medial prefrontal cortex has a leading role in mediating the pharmacological effects of developmental exposure to cocaine, and that this brain region appears to be more vulnerable to either short- and long-term effects brought about by the psychostimulant. Such increased vulnerability perhaps relies on the fact that cortical regions are still developing during adolescence (Gogtay et al., 2004) and, therefore, they appear to be more sensitive to the pharmacological effects of cocaine. Even though we are aware of the fact that our

studies do not take into account the reinforcing properties of cocaine underlying the active drug-taking behavior, our main issue was to investigate whether the pure pharmacological properties of cocaine could induce maladaptive neuroplasticity in a brain region that is still developing during adolescence, i.e. the prefrontal cortex. However, further studies are in progress to address this issue.

Therefore, our preclinical data provide new insights into the neural mechanisms underlying developmental exposure to cocaine by highlighting the complex interaction between different brain systems, thus making the picture even more puzzling. Accordingly, more studies are needed to clarify the contribution of each of those systems in modulating addictive behaviors in adolescents as well as the contribution in modulating the long-term consequences of developmental exposure to cocaine. Thus, I believe that the results presented in my Ph.D. dissertation represent the starting point to explore further the long-standing problem of drug use during adolescence.

6. Appendix

Table 1: complete list of antibodies and experimental conditions employed in the studies

Primary Antibody	Dilution	Company
proBDNF	1:2000	GeneTex, USA
mBDNF	1:500	Santa Cruz Biotechnology, USA
anti-total trkB	1:750	Santa Cruz Biotechnology, USA
anti phopsho-ERK2 T185/187	1:1000	Cell Signaling Technology, USA
anti-total ERK2	1:5000	Santa Cruz Biotechnology, USA
anti-phopsho Akt S473	1:1000	Cell Signaling Technology, USA
anti-total Akt	1:1000	Cell Signaling Technology, USA
anti-phopsho mTOR S2448	1:1000	Cell Signaling Technology, USA
anti-phopsho S6 S240/244	1:1000	Cell Signaling Technology, USA
anti-total S6	1:1000	Cell Signaling Technology, USA
anti-total GluA1	1:2000	Upstate, USA
anti-Arc	1:500	BD Transduction Laboratories, USA
Anti-phospho GluN1 S896	1:1000	Affinity Bioreagents, USA
anti-phospho αCaMKII T286	1:2000	Thermo Scientific, USA
anti-phospho GluN2B S1303	1:1000	Upstate, USA
anti-phospho GluA1 S831	1:500	Affinity Bioreagents, USA
anti-phospho GluA2 S880	1:1000	Abcam, UK
anti-phospho Pak1 T423	1:1000	Cell Signaling, USA
anti-total GluN1	1:1000	Zymed Laboratories, USA
anti-total αCaMKII	1:5000	Chemicon, USA
anti-total GluN2B	1:1000	Santa Cruz Biotechonology, USA
anti-total GluN2A	1:1000	Zymed Laboratories, USA
anti-total GluA2	1:2000	Alomone, Israel
anti-total Pak1	1:1000	Cell Signaling, USA
anti-phospho GR S232	1:1000	Abcam, UK
anti-total GR	1:500	Thermo Scientific, USA
Anti-FKBP51	1:2000	Abcam, UK
Anti-PSD95	1:4000	Cell Signaling, USA
anti F-Actin	1:1000	Abcam, UK
anti-phospho Cofilin S3	1:1000	Cell Signaling, USA
anti-total Cofilin	1:2000	Cell Signaling, USA
β-Actin	1:10000	Sigma-Aldrich, Italy

Table 2: complete list of primers and probes employed in the studies

Gene	Primers	Probe
Arc/Arg3.1	FWD: 5'-ACTGTCTCTGTAGGTGTGGG-3' REV: 5'-GGGCTAACAGTGTAGTCGTAG-3'	5'- ATCAGCTTCCTGGCAGTAGGGC -3'
Ube3a	FWD: 5'-GTCCTGGGTCTGGCTATTTAC-3' REV: 5'-AGTCTCCCAAGTCACAAAACG-3'	5'- TCCCCATTAGCTTCCTGTACACAACC-3'
FMR1	FWD: 5'-ATGGTCAAGGAATGGGTCG-3' REV: 5'-TCTCCCTCTCTTCCTCTGTTG-3'	5'- CTGCCGTGCCCCCTATTTCTGTAA -3'
GRM5	FWD: 5'-AGCGCCTGTGCTCAGTTAGT-3' REV: 5'-AGACTTCTCGGATGCTTGGA-3'	5'- GCTTTCATTCTCATCTGTATTCAGC -3'
SENP1	FWD: 5'-CTCTACACCGAGCTTTCACG-3' REV: 5'-AGTTTCTCCATTGTCCATTTGC-3'	5'- ACCCTTCCTCAGACAGTTTCCTTGG -3'
Gria1	FWD: 5'-CCTCGAAGATCCTTACGTGATG-3' REV: 5'-TCGCTGACAATCTCAAGTCG-3'	5'-ATAGCGGTCATTGCCCTCAAACTGG-3'
BDNF	FWD: 5'-AAGTCTGCATTACATTCCTCGA-3' REV: 5'-GTTTTCTGAAAGAGGGACAGTTTAT-3'	5'-TGTGGTTTGTTGCCGTTGCCAAG-3'
BDNF exon IV	FWD: 5'-CATATCGGCCACCAAAGACT-3' REV: 5'-GTCATCACTCTTCTCACCTGG-3'	5'-TCTAGAACCTTGGGGACCGGTCTT-3'
BDNF exon I	FWD: 5'-GGGAGACGAGATTTTAAGACACT-3' REV: 5'-GTCATCACTCTTCTCACCTGG-3'	5'-TTGTGGCTTTGCTGTCCTGGAGA-3'
BDNF exon VI	FWD: 5'-CTGGCAGGCTTTGATGAGAC-3' REV: 5'-GTCATCACTCTTCTCACCTGG-3'	5'-AGCTTTGTGTGGACCCTGAGTTCC-3'
BDNF exon IIb	FWD: 5'-AGTTGGCTTCCTAGCGGTGTA-3' REV: 5'-GTCATCACTCTTCTCACCTGG-3'	5'-AATAGACTCTTGGCAAGCTCCGGTT-3'
CaRF	FWD: 5'-GAGATGCACACACCATTCCA-3' REV: 5'-GTGTTGGCTCATTGGGTTCT-3'	5'-CAGCCATCCAGCTCTTGTTGAAGA-3'
Npas4	FWD: 5'-GGAAGTTGCTATACCTGTCGG-3' REV: 5'-GTCGTAAATACTGTCACCCTGG-3'	5'-CATAGAATGGCCCAGATGCTCGCT-3'
NF-kB	FWD: 5'-CTACGAGACCTTCAAGAGCATC-3' REV: 5'-GATGTTGAAAAGGCATAGGGC-3'	5'-AATGGACCAACTGAACCCCGGC-3'
Creb1a/b	FWD: 5'-AGATTCTAGTGCCCAGCAAC-3' REV: 5'-CTGTGCGAATCTGGTATGTTTG-3'	5'-TGTTCAAGCTGCCTCTGGTGATGT-3'
tPA	FWD: 5'-CAAAATGAAGGGAGAGCTGTTG-3' REV: 5'-TGTTGGTAAGTTGTCTGAGTCTG-3'	5'-CTGCTTTGTGGAGTGGCGTTCA-3'
vGLUT1	FWD: 5'-ACTGCCTCACCTTGTCATG-3' REV: 5'-GTAGCTTCCATCCCGAAACC-3'	5'-CTTTCGCACATTGGTCGTGGACATT-3'
vGAT	FWD: 5'-ACGACAAACCCAAGATCACG-3' REV: 5'-GTAGACCCAGCACGAACATG-3'	5'-TTCCAGCCCGCTTCCCACG-3'
GAD67	FWD: 5'-ATACTTGGTGTGGCGTAGC-3' REV: 5'-AGGAAAGCAGGTTCTTGGAG-3'	5'-AAAACTGGGCCTGAAGATCTGTGGT-3'
EAAT1	FWD: 5'-GGGTTTTCATTGGAGGGTTG-3' REV: 5'-ACGGGAAGCACAAATCTGG-3'	5'-TGATGGGCAGGGTGGCAGAA-3'
EAAT2	FWD: 5'-TTGCTGGCATTTTCCAAGC-3' REV: 5'-TTAATGGTTGCTCCGACTGG-3'	5'-CAAGCGTGTGACCAGATTCGTCCT-3'
X_C^-	FWD: 5'-TCTTCGATACAAACGCCCAG-3' REV: 5'-CGAGTAAAGGGAGAGGACAAC-3'	5'-CCATGAAGAGGCAGGTGAAGGAGAA-3'
GS	FWD: 5'-ATGGTCTGAGGTGCATTGAG-3' REV: 5'-TGATGTTGGAGGTTTCGTGG-3'	5'-ATGTGGTACTGGTGCCTCTTGCTC-3'
Cdc42	FWD: 5'-AAGGCTGTCAAGTATGTGGAG-3' REV: 5'-GCTCTGGAGATGCGTTCATAG-3'	5'-CCTGCGGCTCTTCTTCGGTTCT-3'
Nr3c1	FWD: 5'-GAAAAGCCATCGTCAAAAGGG-3' REV: 5'-TGGAAGCAGTAGGTAAGGAGA-3'	5'-AGCTTTGTCAGTTGGTAAAACCGTTGC-3'
Src1	FWD: 5'-AAGTGATGACTCGTGGCACT-3' REV: 5'-TCCCATGATGAAAGGCTGCA-3'	5'-AAAGCACAAGGATGGCAAGG-3'
CaD	FWD: 5'-AGGAGGAGGCTGATCGAAAA-3' REV: 5'-TCTTCTGGCGTTTCTCAGCA-3'	5'-AGAGAGGAGGAAGAGAAGAGGA-3'
GFAP	FWD: 5'-GACTTTCTCCAACCTCCAGATC-3' REV: 5'-CTCCTGCTTCGACTCCTTAATG-3'	5'-CCGCATCTCCACCGTCTTTACCA-3'

FGF-2	ID Rn00570809_m1	
actin	FWD: 5'-CACTTTCTACAATGAGCTGCG-3' REV: 5'-CTGGATGGCTACGTACATGG-3'	5'-TCTGGGTCATCTTTTCACGGTTGGC-3'
18S	FWD: 5'-GTAACCCGTTGAACCCCATT-3' REV: 5'-CCATCCAATCGGTAGTAGCG-3'	5'-TGCAATTATTCCCCATGAACGAGG-3'
36B4	FWD: 5'-TTCCCACTGGCTGAAAAGGT-3' REV: 5'-CGCAGCCGCAAATGC-3'	5'-AAGGCCTTCCTGGCCGATCCATC-3'

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