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

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ABSTRACT

Cocaine is a psychostimulant whose abuse causes a social and economic burden for our society. Most of the published literature deals with acute effects of cocaine or short-term abstinence in adult animals whereas much less information exists on neuroplastic changes following long-term abstinence. We have recently shown that long-term abstinence following developmental exposure to cocaine results in increased Activity Regulated Cytoskeletal-associated protein (Arc/Arg3.1) expression in the crude synaptosomal fraction (Giannotti et al., 2013). Given that Arc/Arg3.1 localizes not only at active synapse but also in the nucleus (Ozuno et al., 2012; Korb et al., 2013; Bloomer et al., 2007), we investigated Arc/Arg3.1 protein levels in the whole homogenate and the nuclear fraction of animals exposed to cocaine during adolescence.

We observed increased expression of Arc/Arg3.1 in both fractions suggesting that up-regulation of Arc/Arg3.1 protein may be partly due to increased nuclear expression of Arc/Arg3.1 in the rat medial prefrontal cortex (mPFC) of rats sacrificed at postnatal day 90, i.e. following 48 days of abstinence. This effect seems to cause reduced Gria1 transcription. We also found reduced expression of fragile X mental retardation gene (FMR1), which normally inhibits Arc/Arg3.1 translation together with reduced expression of Ubiquitin-protein ligase E3A (Ube3a) that normally causes Arc/Arg3.1 protein degradation via ubiquitination. Further, we found increased expression of metabotropic glutamate receptor 5 (GRM5), which is also involved in the regulation of Arc/Arg3.1 expression. Taken together, our findings show that abstinence from developmental exposure to cocaine is associated with alterations in the finely tuned mechanisms that regulate Arc/Arg3.1 expression.

Keywords: cocaine, adolescence, medial prefrontal cortex, Arc/Arg3.1, FMR1, Ube3a, GRM5

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., 1995). 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., 2006). 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 (Fumagalli et al., 2009; Hearing et al., 2008; 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., 2006) 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 in the crude synaptosomal fraction are elevated as a result of long-term abstinence following exposure to cocaine during adolescence (Giannotti et al., 2013b). However, while we have dissected in details the mechanisms of BDNF up-regulation (Giannotti et al., 2013b), we have not investigated whether 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 the nuclear expression of Arc and 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.

Experimental procedures

The adolescent rats used in this study were generated by mating Sprague Dawley rats weighting 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 were taken from each litter in order to reduce "litter effects" (Chapman and Stern, 1978). Male rats were treated subcutaneously with cocaine (20 mg/kg/day) (MacFarlan-Smith, Edinburgh, UK) or saline from postnatal day 28 (PND 28) to PND 42, a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004).

Following the end of this treatment, animals were left undisturbed in their home cages until PND 45 or PND 90. At the scheduled time of sacrifice, the mPFC (defined as Cg1, Cg3, and IL subregions) was dissected from a 2mm section extending from approximately bregma +5.16 to +3.24 (Paxinos and Watson, 2005), 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 and Use of Laboratory Animals, National Academies Press, 8th Edition, 2011).

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 (Giannotti et al., 2013a). Following total RNA extraction, the samples were processed for real-time reverse transcription polymerase chain reaction (real time RT-PCR) to assess mRNA levels, as previously described (Fumagalli et al., 2012). 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^{TM} one-step RT-PCR kit for probes (Bio-Rad Laboratories). Each experimental group was composed of at least 6 rats. Samples were run in 384 well formats in triplicate as multiplexed reactions with a normalizing internal control (36B4).

Probes and primers were purchased from Eurofins MWG-Operon. Their sequences are shown in Table 1. The primer efficiencies were experimentally set up for each couple of primers. 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. A comparative cycle threshold ($\Delta\Delta$Ct) method was used to calculate the relative target gene expression.

Preparation of Protein Extracts and Western Blot Analysis

Preparation of protein extracts was described previously (Fumagalli et al., 2010). mPFC was homogenized in a glass-glass potter using a cold buffer containing 0.32 M sucrose, 1mM Hepes solution, 0.1 mM EGTA, 0.1 mM PMSF, pH=7.4, in presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. The homogenized tissues were centrifuged at 1000 g for 10 min; the resulting pellet corresponding to the nuclear fraction was resuspended in a buffer containing 20 mM Hepes, 0.1 mM DTT, 0.1 mM EGTA, in presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. Total proteins have been measured in the whole homogenate and in the nuclear fraction by the Bio-Rad Protein Assay, using bovine serum albumin as the calibration standard (Bio-Rad Laboratories, Milan, Italy).

Western blot analysis was performed on the whole homogenate and on the nuclear fraction. Total protein concentrations were adjusted to the same amount for all samples (10 mg per lane). All the samples were run on a sodium dodecyl sulfate (SDS)-10% polyacrilamide gel under reducing conditions, and proteins were then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad). Blots were blocked with 10% nonfat dry milk then incubated with primary antibody. The conditions of the primary antibodies were the following: anti Arc/Arg3.1 (1:500, BD Transduction Laboratories, USA), anti GluA1 (1:2000, Santa Cruz Biotechnology, USA) and anti β-Actin (1:10000, Sigma-Aldrich, Italy). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories).

Statistical analysis

Data were collected in individual animals (independent determinations) and are presented as means and standard errors. The effects produced by repeated cocaine treatment were analyzed by an unpaired Student's t test. Statistical significance was assumed at $p<0.05$.

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). 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). 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.

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). 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). Indeed, we found reduced Gria1 mRNA levels (-21%, $p<0.05$) (Fig. 5A). Also, we evaluated the levels of AMPA subunit GluA1 protein land found them reduced too (-29%; $p<0.01$) (Fig 5B), presumably as a consequence of reduced transcription.

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., 2006), 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., 2013b) 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, which in turn may lead to reduced GluA1 synthesis, adding novel evidence to the inverse relationships existing between Arc/Arg3.1 and AMPA receptors following long-term cocaine withdrawal (Giannotti et al., 2013b; Korb et al., 2013).

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., 2013b). 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., 2013b). 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).

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Disclosure/Conflict of Interest

The authors declare that they have no conflict of interest.

Table 1 Sequences of forward and reverse primers and probes used in Real-Time PCR analysis.

Gene	Forward primer	Reverse Primer	Probe
Arc/Arg3.1	5'- ACTGTCTCTGTAGGTGTGGG -3'	5'- GGGCTAACAGTGTAGTCGTAG -3'	5'- ATCAGCTTCCTGGCAGTAGGGC -3'
Ube3a	5'- GTCCTGGGTCTGGCTATTTAC -3'	5'- AGTCTCCCAAGTCACAAAACG -3'	5'- TCCCCATTAGCTTCCTGTACACAACC-3'
FMR1	5'- ATGGTCAAGGAATGGGTCG -3'	5'- TCTCCCTCTCTTCCTCTGTTG -3'	5'- CTGCCGTGCCCCCTATTTCTGTAA -3'
GRM5	5'- AGCGCCTGTGCTCAGTTAGT -3'	5'- AGACTTCTCGGATGCTTGGA -3'	5'- GCTTTCATTCTCATCTGTATTCAGC -3'
SENP1	5'- CTCTACACCGAGCTTTCACG -3'	5'- AGTTTCTCCATTGTCCATTTGC -3'	5'- ACCCTTCCTCAGACAGTTTCCTTGG -3'
Gria1	5'-CCTCGAAGATCCTTACGTGATG-3'	5'-TCGCTGACAATCTCAAGTCG-3'	5'-ATAGCGGTCATTGCCCTCAAACTGG-3'
36B4	5'-TTCCCACTGGCTGAAAAGGT-3'	5'-CGCAGCCGCAAATGC-3'	5'-AAGGCCTTCCTGGCCGATCCATC-3'

FIGURE LEGENDS

Figure 1. Abstinence from developmental exposure to cocaine alters the transcriptional regulation of Arc/Arg3.1.

Panel A and B show 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.

* $p<0.05$ and *** $p<0.001$ vs. control rats.

Figure 2. Abstinence from developmental exposure to cocaine alters the translational regulation of Arc/Arg3.1.

Panel A and B show 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.

Figure 3. Abstinence from developmental exposure to cocaine alters Arc/Arg3.1 protein levels in the nuclear fraction.

Panel A and B show 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.

Figure 4. Abstinence from developmental exposure to cocaine alters the mechanisms responsible of Arc/Arg3.1 translation: effects at PND 90.

Panel A shows FMR1 mRNA levels, panel B shows Ube3a mRNA levels, panel C shows GRM5 mRNA levels, while panel D shows 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.

Figure 5. Abstinence from developmental exposure to cocaine alters Gria1 transcription and translation: effects at PND 90.

Panel A shows Gria1 mRNA levels, whereas panel B shows 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.

Figure 6. Abstinence from repeated cocaine administration during adolescence up-regulates Arc/Arg3.1 expression in the rat mPFC (panel B): comparison with control rats (panel 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 details, we have previously shown that 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 reduction of these receptors at the membrane level (Giannotti et al., 2013b). In the present manuscript 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 withdrawal 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.

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