The cerebellum on cocaine: plasticity and metaplasticity.

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Abstract

Despite the fact that several data have supported the involvement of the cerebellum in the functional alterations observed after prolonged cocaine use, this brain structure has been traditionally ignored and excluded from the circuitry affected by addictive drugs. In the present study, we investigated the effects of a chronic cocaine treatment on molecular and structural plasticity in the cerebellum, including BDNF, D3 dopamine receptors, ΔFosB, the Glu2 AMPA receptor subunit, structural modifications in Purkinje neurons, and finally, the evaluation of perineuronal nets (PNNs) in the projection neurons of the medial nucleus, the output of the cerebellar vermis. In the current experimental conditions in which repeated cocaine treatment was followed by a one-week withdrawal period and a new cocaine challenge, our results showed that cocaine induced a large increase in cerebellar proBDNF levels and its expression in Purkinje neurons, with the matureBDNF expression remaining unchanged. Together with this, cocaine-treated mice exhibited a substantial enhancement of D3 receptor levels. Both ΔFosB and AMPA receptor Glu2 subunit expressions were enhanced in cocaine-treated animals, but only GluR2 cerebellar levels were related to the propensity to exhibit sniffing sensitization. Moreover, cocaine-dependent increase in proBDNF was associated with pruning in Purkinje dendrite arborisation and a reduction in the size and density of Purkinje boutons contacting deep cerebellar projection neurons. This reduced neurite complexity in Purkinje was accompanied by an up-regulation of extracellular matrix components in the perineuronal nets surrounding medial nuclear neurons, thereby decreasing the probability of any remodelling in these synapses.

Key words: cerebellum, mice, cocaine, sensitization, BDNF, ΔFosB

Introduction

It is widely accepted that addictive drugs promote long-lasting changes in brain plasticity mechanisms, which could underlie the transition from a recreational use of drugs to a compulsive drug seeking and taking (Hyman et al., 2006). Cocaine-dependent neuronal plasticity includes molecular and structural modifications in the cortical-striatal-limbic networks (Hyman et al., 2006; Noori et al., 2012) with a ventral to dorsal gradient (Everitt and Robins, 2005). In addition, addictive drugs induce restrictive metaplasticiy in such a way so that the probability of any new change is decreased (Moussawi et al., 2009; Kasanetz et al., 2010).

Despite scattered data having supported the involvement of the cerebellum in the functional alterations observed after repeated cocaine use, this brain structure has been traditionally ignored and excluded from the circuitry affected by addictive drugs (Miquel et al., 2009; Moulton et al., 2014). Increasing evidence has demonstrated that the cerebellum is anatomically and functionally related to the basal ganglia-prefrontal networks (Snider and Maiti, 1976; Perciavalle et al., 1989; Panagopoulos et al., 1991; Ikai, et al., 1992; Bostan et al., 2013; Herrera-Meza et al., 2014). Moreover, dopamine-glutamate interactions, which sustain an important part of the molecular pathophysiology of cocaine addiction, have also been described in the cerebellum (Snider et al., 1976; Panagopoulos et al., 1991; Ikai et al., 1992; Schweighofer et al., 2004). Earlier studies found a D1-dependent increase in cFOS-IR levels in the granule cell layer of the rat vermis chronically treated with cocaine or amphetamine (Klitenick et al., 1995). Furthermore, recordings of extracellular activity in the cerebelar cortex showed that cocaine is able to suppress spontaneous firing and glutamate-induced activation of Purkinje cells (Jimenez-Rivera et al., 2000).

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Very recently, we showed that the expression of conditioned preference towards an odour associated with cocaine was positively correlated with cFOS expression in cells at the dorsal region of the granule cell layer of the cerebellar vermis (Carbo-Gas et al., 2014). These findings are clearly coincident with those of some clinical reports showing cerebellar activations during exposure to drug-associated cues in human cocaine addicted individuals (Grant et al., 1996; Bonson et al., 2002; Volkow et al., 2003; Anderson et al., 2006).

In the present study, we tested the effects of a chronic cocaine treatment on molecular and structural plasticity in the cerebellum, including BDNF, D3 dopamine receptors, ΔFosB, the Glu2 AMPA receptor subunit and structural modifications in Purkinje neurons. We also tackled the analysis of perineuronal nets (PNNs) in the large projection neurons of the medial nucleus. These neurons received strong innervation from GABAergic Purkinje axons, and they operated as the output from the cerebellar vermis. PNNs are formed by extracellular matrix (ECM) components, which restrict neuronal plasticity in order to stabilize specific connection patterns (Foscarin et al., 2011; Carulli et al., 2013). Therefore, by reducing or over-expressing these signalling regulatory molecules, cocaine might be able to change conditions for synaptic change in the outputs from the cerebellum.

Methods

A full and comprehensive explanation of protocols can be found in supplemental materials. Additionaly, each legend accounts for a brief methological description.

Subjects

Male Balb /c AnNHsd mice (Harlan) were housed in groups of five with 12hr light-dark cycle and free access to food (Panlab) and tap water. They were handled for three weeks

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before experiments started. The experimental protocols were performed during the light

phase. All animal procedures were approved by the Ethical Committee for Animal

Welfare of Jaume I University and performed in accordance with the European

Community Council Directive of 24 November 1986 (86/609/EEC), Spanish directives

1201/2005, 13/2007 and the NIH Guide for the care and use of laboratory animals. All

experiments were addressed in housing and experimental conditions in order to avoid

animal suffering and to reduce the number of animals used. According to the lifespan

curve provided by Harlan (Spain), all experiments took place in the adult stage.

Cocaine treatment

Cocaine hydrochloride (Alcaliber, Spain), dissolved in 0.9% saline (2mg/ml or 1mg/ml)

was IP injected starting on postnatal day 77. It is important to remark that the present

research was mainly aimed at investigating the effects of a chronic cocaine

administration on cerebellar plasticity. Therefore, the present experiments were

desingned with only two groups (repeated saline and repeated cocaine).

Brain sampling

From the total sample of mice tested for behaviour, different control and experimental

groups were used for molecular and cellular experiments.

RNA extraction, reverse transcription and real-time PCR analysis

Briefly, total RNA was extracted from cerebellar vermis tissue using the RNeasy Lipid

Tissue Mini Kit (Qiagen Inc.) To avoid contaminating DNA, the samples were treated

with DNAse I. The tissue was ground to a fine powder in liquid nitrogen, and

homogenized using a Polytron Ultraturrax T25 basic (Ika Labortechnik). Quantification

of RNA was carried out with a Nanodrop 1000 spectrophotometer (Fisher Scientific).

Total RNA extracted was used to synthesize cDNA with the High Capacity RNA-to-

cDNA Master Mix (Applied Biosystems). Primer and probe sequences for BDNF

variants were designed using the splice variants (see table S1). Real-time PCR was

conducted using the SYBR Green PCR Kit (Thermo Scientific) and the SmartCycler II

instrument (Cepheid).

Western Immunoblotting

Pro-BDNF, mature-BDNF, p75R, TrkBR, tPA (tissue plasminogen activator tPA),

dopamine receptor D_3 and $\Delta FosB$ protein levels were quantified in the cerebellar vermis

by Western Blotting (WB) (see table S2 and text in supplemental materials for more

details). The film signals were scanned at 600 dpi (EPSON 11344) and levels of the

band density were blind processed and quantified by densitometry with ImageJ

software. Every sample was replicated at least twice to ensure the reproducibility of the

method.

Immunohistochemistrty and immunofluorescence

A whole description of the antibodies, reagents and procedures used may be seen in

supplemental materials, including tables S3 and S4. The vermal tissue for

immunofluorescent labelling was obtained 24 hours after the last cocaine injection

following perfusion and fixation. Fluorescent-labelled sections were examined in

confocal microscope Nikon Eclipse-1C. Confocal images were taken in a first plane

1μm thick in single planes at a resolution of 1024x1024 and 100 Hz speed. Laser

intensity, gain and offset were maintained constant in each analysis. Quantitative

evaluations were made using the Image J software (NIH sponsored image analysis

software).

Morphometric analysis in Purkinje and quantification of perineuronal nets (PNNs)

A full description and explanation of antibodies and procedures for the morphometric

analyses may be found in supplemental materials (see also table S4) and in legends. Due

to variability in size of PK terminals between saline and cocaine-treated animals, we

corrected the raw data by applying the Abercrombie formula (Abercromie, 1946).

Statistical analysis

All behavioural and biochemical experiments were performed blind. For all statistical

analyses we used the STATISTICA 7 software package (Statsoft, Inc., Tulsa, OK,

USA). Data were analysed by means of parametric and non-parametric statistics,

including repeated measures ANOVA, one-way ANOVAs as well as Mann-Whitney U

test. Tukey HSD tests were performed as parametric *post-hoc* tests, when required. The

level of significance was set at $p < 0.05$. We applied X^2-tests to compare the distribution

of frequencies relative to staining intensity categories of WFA. Spearman Rank Order

Correlations (RHO index) was reckoned to ascertain the degree of correlation between

variables analysed.

Results

Cocaine-induced stimulating motor behaviour

As expected, after seven alternate IP cocaine administrations (20mg/kg), Balb mice

(n=18) developed sniffing sensitization [One way repeated measures ANOVA: the

cocaine effect (df=1,20; F=461.22; p<0.001), day of treatment effect (df= 6,120; F=

16.21; p<0.001), the interaction effect (df= 6,120; F= 22.15; p<0.001)]. Tukey posthoc

tests demonstrated sensitization after five cocaine administrations (p<0.001). After the

sixth cocaine injection, mice remained undisturbed in the animal facilities for one week

until they were tested again under a lower cocaine dose (10mg/kg) (Figure 1). Sniffing

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sensitization was maintained after a one-week washout period (p< 0.001 for

comparisons between the first and seventh cocaine administration). We then calculated

an individual sniffing sensitization index by subtracting the sniffing score showed after

the first cocaine injection from the score we recorded following the seventh one. This

sensitization index was used to estimate which of the cocaine-dependent plasticity

modifications in the cerebellum were related to sensitization.

Cocaine-induced BDNF mechanisms in the cerebellum

In cocaine treated mice, we observed a significant increase in proBDNF protein levels

[df=1,8; F=7.22; p<0.05], but no change in the mature isoform [df=1,8; F=0.21; p>0.05]

(Figure 2). Therefore, we asked whether such elevation in proBDNF was either the

result of an enhancement of transcriptional activity or an increase in cleavage

mechanisms.

We first analysed bdnf mRNA expression by RT-PCR (n=4). In the vermis tissue,

measurable levels of mRNA were observed for bdnf exons I, IV and VI. We analysed

each exon levels by means of Mann-Whitney U tests. Chronic cocaine administration

increased levels of exon VI [df=1,6; U=3; p<0.05], without inducing any significant

change in exons IV [df=1,6; U=0; p>0.05] and I [df=1,6; U=5; p>0.05]. No changes in

β-tubulin expression were identified [df=1,6; U=1; p>0.05] (Figure 2).

We then evaluated mRNA and protein levels of tPA the protease that converts

proBDNF into mature BDNF. We found that chronic cocaine administration did not

modify either levels of the tPA transcript [df=1,6; U=8; p>0.05] or protein expression

[df=1,6; F=0.40; p>0.05] in the mouse cerebellar vermis.

In the present experimental conditions, BDNF concentrations did not appear to be

related to cocaine-induced sniffing sensitization, as no significant correlation could be

demonstrated.

We next explored whether chronic cocaine administration would differentially affect any of the BDNF receptor subtypes. We determined $p75^{NGRF}$ and TrkB receptor levels by WB analysis. After seven cocaine injections, levels of the $p75^{NGRF}$ receptor subtype were increased in the cerebellar vermis [df=1,8; F=13.36; p<0.01]; but TrkBR levels remained unchanged [df=1,8; F=6.68; p>0.05] (Figure 2).

To further describe the effects of chronically administered cocaine, we analysed BDNF expression in different cellular lineages of the cerebellar cortex (n=5) (Figure 3). To encompass a comprehensive sampling of the vermis, we selected 4 cerebellar lobules for the analysis (III, VI, VIII, IX), each of them selectively interconnected with different networks of the basal ganglia and cerebral cortices. Using double fluorescent immunostaining, we observed BDNF expression in many of the cerebellar cellular types, including Purkinje neurons (soma and dendritic arbor), inhibitory interneurons and Bergman glia. Due to the fact that in the WB analysis we were only able to demonstrate cocaine effects on proBDNF levels, it is very plausible that most of the BDNF detected in Purkinje and other cell types corresponded to proBDNF.

Cocaine produced a more than two-fold enhancement of BDNF expression in the soma [Lob III: (df=1,8; F=14.40; p<0.01); Lob VI: (df=1,8; F=10.0; p<0.01); Lob VIII: (df=1,8; F=59.66; p<0.001); Lob IX: (df=1,8; F=34.50; p<0.001)] and dendrites of Purkinje neurons [Lob III: (df=1,8; F=23.29; p<0.001); Lob VI: (df=1,8; F=19.92; p<0.01); Lob VIII: (df=1,8; F=100.20; p<0.001); Lob IX (df=1,8; F=108.13; p<0.001)]. As expected, BDNF expression in the soma directly correlates with the dendritic expression [Lob III: RHO=0.67, p<0.05; Lob VI: RHO=0.71, p<0.05; Lob VIII RHO=0.82, p<0.01; Lob IX RHO=0.78, p<0.05].

In addition, we also noticed BDNF expression in the granule cell layer, though it was not expressed in granule cells as co-labelling with NeuN could not be demonstrated

(Figure 3). The presence of BDNF surrounding granule cells could be due to synaptic-associated mechanisms related to mossy fibers, as we detected higher levels of glutamate transporter (vGlut) in some of the tissue preparations (labelling not shown). Cocaine effects on BDNF expression in the inhibitory interneurons of the molecular layer were weak and limited to lobule VI, where we observed higher levels in mice treated chronically with cocaine [df=1,8; F=6.08; p<0.05]. However, we did not find a clear expression of BDNF in Bergmann glia.

Dopamine D_3 receptor levels

Due to BDNF enhancement has been linked to dopamine D_3 receptor expression (Payer et al., 2013), we next evaluated the effects of our experimental cocaine treatment conditions on D_3 levels. Twenty-four hours following the cocaine challenge we observed up to four-fold D_3 receptor levels in the vermis [df=1,8; F=15.13; p<0.01] (Figure S1).

ΔFosB protein levels and cellular expression of AMPA receptor-2 subunit (GluR2) in the cerebellum

ΔFosB binds AP-1 DNA sequences found in promoters of many genes and thereby can both repress or activate gene transcription (Renthal et al., 2009). One of these target genes is the AMPA glutamate receptor GluR2 subunit. Therefore, we next explored cocaine effects on cerebellar ΔFosB levels and addressed the estimation of GluR2 subunit expression in the cerebellar cortex. Under our experimental conditions, we found a five-fold increase in ΔFosB levels in the vermis [df=1,8; F=12.28; p<0.01] (Figure 4). Then, we analysed the number of positive Purkinje somas for GluR2, and by densitometry, GluR2 expression in the Purkinje dendritic tree (Figure 4).

Higher GluR2 levels were observed in Purkinje soma [df=1,8; F=60.77; p<0.001] and dendrites of lobule III [df=1,8; F=14.60; p<0.01], as well as in dendrites of lobule VIII [df=1,8; F=262; p<0.001]. Lobule VI [df=1,8; F=23.83; p<0.01] and IX [df=1,8; F=4.99; p<0.05] of cocaine-treated animals only showed differences in GluR2 expression in the soma (Figure 4). Additionally, we accomplished an immunoanalysis against GluR2 without any membrane permeabilisation in order to identify the internal or external position of the AMPA subunit in Purkinje somas and dendrites. As seen in Figure 4, only in the cocaine group the GluR2 signal was prevented in dendrites but maintained in Purkinje somas and surrounding interneurons when membrane permeabilisation was not accomplished, suggesting an endocytosis of the AMPA GluR2 subunits in Purkinje dendrites after cocaine treatment.

Remarkably, sniffing sensitization did not correlate with the cerebellar expression of ΔFosB but it did with GluR2 expression in soma and dendrites of lobule III [soma; RHO= 0.73, p<0.05; dendrites; RHO= 0.70, p<0.05].

cFOS expression in the cerebellum

To estimate cerebellar neuronal activity, we evaluated cFOS expression in Purkinje neurons, the granule cell layer and in the medial deep nucleus.

Ninety minutes following the priming cocaine injection, we observed higher cFOS immunoreactivity (cFOS-IR) in granule cells [Lob III: (df 1,8; F=152.01; p<0.01), Lob VIII (df 1,8; F=31.28; p<0.01) and Lob IX (df 1,8; F=137.27; p<0.001)]. We also found that Purkinje neurons of lobules VIII [(df 1,8) F=66.85; p<0.001] and IX [(df 1,8) F=49.68, p<0.001] expressed lower levels of cFOS-IR (Figure 5). We next analysed cFos expression in the deep medial neurons (Figure 5). As expected from the reduced Purkinje activity, the large projection neurons of the medial nucleus showed higher activity [df=1,37; F=12.53; p<0.01].

Cocaine effects on structural plasticity in Purkinje neurons

As BDNF has been involved in dendritic spine formation (Schratt et al., 2006), we next evaluate in the same mice the cocaine effects on the number of dendritic spines in the Purkinje dendritic tree (Figure 6). Worthy of mention, in animals treated with cocaine Purkinje dendritic spines were clearly and significantly pruned. This reduction was a global effect affecting all lobules assessed [Lob III (df=1,8; F=12.66; p<0.01); Lob VI (df=1,8; F=80.65; p<0.001); Lob VIII (df=1,8; F=7.99; p<0.05) and Lob IX (df=1,8; F=49.13; p<0.001)]. No cocaine effects were observed on the total number of Purkinje cells labelled by calbindine.

Meaningfully, the higher BDNF levels in Purkinje somas the lower the density of dendritic spines. This relationship was found in the anterior and posterior vermis [Lob III: RHO=-0.69, p<0.05; Lob VIII: RHO=-0.64, p<0.05; Lob IX: RHO=-0.73, p<0.05], but not in lobule VI, where there was not a significant correlation between BDNF and structural changes.

Cerebellar neurons in the medial nucleus projecting to other areas in the brain receive strong GABAergic innervation on the perikaryon from Purkinje axons. To ask whether cocaine might affect structural plasticity at the level of synaptic Purkinje terminals and thus change the probability of releasing information out of the cerebellum, we accomplished a morphometric analysis in the deep medial nucleus. Somas of the large deep cerebellar nuclear neurons were stained by SMI32 and Purkinje terminals were labelled with calbindin 28K (Figure 7). The soma size of the deep medial nuclear neurons was unaffected by cocaine [df=1,97; F=0.30; p>0.05]. However, density of Purkinje terminals [df=1,8; F=5.36; p<0.05], and their size was reduced [df=1,8; F=4.56; p<0.05]. Of note, density of Purkinje terminals inversely correlated with proBDNF expression in the soma of lobule VIII [RHO=-0.68, p<0.05].

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Perineuronal nets (PNNs) in the deep cerebellar medial nucleus

Adult deep cerebellar nuclear neurons develop PNNs that envelop the soma of the large projection neurons, thereby restricting neuronal remodelling. Therefore, in the present study, we explored cocaine effects on PNNs of the large projection neurons in the medial nucleus. To properly identify PNNs, we immunolabelled nuclear neurons by SMI32 and WFA (Figure 8). Then, we analysed cocaine effects on the number of deep nuclear neurons that sustained a PNN. We did not observe any effect of cocaine on the total number of deep nuclear neurons expressing a PNN [df (1); $X^2 = 0.19$; p>0.05]. We next addressed an analysis of WFA intensity by dividing neurons expressing PNNs into three categories: faint, medium and strong. The analysis of distribution of the nets in these categories demonstrated that cocaine treated mice exhibited a larger proportion of deep nuclear neurons with strong WFA intensity than that observed in saline treated animals [df (1); $X^2 = 80.31$; p<0,01]. Furthermore, proBDNF expression in soma and dendrites significantly correlates with the proportion of neurons displaying strong WFA staining in all of the lobules evaluated. The higher the proBDNF expression in Purkinje somas the higher the proportion of deep cerebellar neurons expressing strong WFA intensity [Lob III: RHO=0.72, p<0.05; Lob VI: RHO=0.64, p<0.05; Lob VIII: RHO=0.78, p<0.05; Lob IX: r^2= RHO=0.81, p<0.01]. The same relationship was found for the dendritic expression of proBDNF [Lob III: RHO=0.76, p<0.05; Lob VI: RHO=0.71, p<0.05; Lob VIII: RHO=0.76, p<0.05; Lob IX: RHO=0.76, p<0.05]. Moreover, dendritic expression of GluR2 in lobules III, VI and VIII positively correlates with the proportion of medial neurons expressing strong WFA intensity [Lobule III: RHO=0.87, p<0.01; Lobule VI: RHO=0.72; p<0.05; Lobule VIII: RHO=0.67, p<0.05].

Finally, a correlational analysis between the number of Purkinje dendritic spines and the proportion of neurons expressing strong WFA intensity showed an inverse relationship. Thus, the lower the density of dendritic spines in the Purkinje cell tree the higher the proportion of PNNs expressing strong WFA intensity [Lob III: RHO=-0.70, p<0.05; Lob VIII: RHO=-0.63, p<0.05; Lob IX: RHO=-0.76, p<0.05].

Discussion

BDNF and ΔFosB are both pivotal mechanisms driving cocaine-dependent plasticity in the striatum-cortico-limbic circuits as well as the persistent behavioural changes associated (Nestler, 2001; McGinty et al., 2010). Here, we showed that the cerebellar vermis of cocaine-treated mice exhibited a dramatic increase in proBDNF and ΔFosB levels that were accompanied by structural modifications in Purkinje cells and in the deep medial nucleus. Thus, the present findings demonstrate the substantial ability of cocaine to induce molecular and structural changes in the cerebellum. The cocaine-dependent changes we describe here might be mediated by a direct cocaine effect on cerebellar dopamine transporters. Nevertheless, it is also possible that any cocaine-induced striatal DAT blockade has contributed to the cerebellar changes through either the VTA-cerebellar (Ikai et al., 1992) or striatal-cerebellar projections (Bostan et al., 2013).

proBDNF accumulates in the cerebellar cortex of cocaine-treated animals

It has been described that acute and repeated cocaine treatments, as well as cocaine self-administration, increase the expression of endogenous BDNF in the striatal-cortico-limbic circuitry (Graham et al., 2007; Fumagalli et al., 2007; Li et al., 2013). The levels of BDNF are progressively and selectively elevated during cocaine withdrawal in different regions of this circuit (Graham et al., 2007; Li et al., 2013).

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It is worth mentioning that our cocaine treatment protocol involved six alternant cocaine injections followed by a one-week drug free period, after which mice received a priming injection with a lower cocaine dose. Twenty-four hours later, in cocaine-treated mice we were able to demonstrate a dramatic increase in the expression of proBDNF within the soma and dendritic tree of Purkinje neurons and the surrounding Bergmann glia that was especially prominent in the posterior cerebellum (lobules VIII and IX). In contrast, mature BDNF levels remained unchanged. Interestingly and paralleling our findings, earlier results showed that 72 hours following five daily consecutive cocaine injections proBDNF levels were enhanced in the striatum with striatal matureBDNF levels remaining unaltered (Fumagalli et al., 2007).

The precursor and mature forms of BDNF have been shown to exert very different roles (Greenberg et al., 2009) though both forms are biologically active (Yang et al., 2009). proBDNF is an active precursor of BDNF that binds preferentially to p75 ^{NGRF} (Teng et al., 2005). Accordingly, we also described selective elevations of *p75R* levels in the vermis of cocaine-treated animals. This neurotrophin could be released in an activity-dependent manner as proBDNF and converted extracellularly into a mature protein by the tissue plasminogen activator tpA (Yang et al., 2009). In our study, cocaine treatment did not affect cerebellar tpA levels.

Adult Purkinje cells constitutively express BDNF (Kawamoto et al., 1996). It has been described that BDNF synthesis is produced from 9 exons resulting in an identical protein (Liu and Xu, 2006). An analysis of the BDNF transcripts by RT-PCR 2 hours following the cocaine challenge suggested that cocaine-associated cerebellar proBDNF elevation might derive from the recruitment of exon VI transcription. Previous data showed higher exon IV levels in the prefrontal cortex and striatum of rodents treated chronically with cocaine (Peterson et al., 2014). Under the present conditions, we

observed an upward trend in exon IV of cocaine-treated animals, though it did not reach statistical significance.

Cocaine-dependent proBDNF enhancement was accompanied by dendritic spine shrinkage. Supporting our results, it has been described that *p75R* activation by proBDNF negatively regulated dendritic morphology because hippocampal neurons showed higher dendritic spine density after knocking-down *p75R* gen (Zagrebelsky et al., 2005).

The functional role of the proBDNF accumulation in Purkinje neurons is far from being clear. The possibility has been suggested that cocaine-dependent pro-BDNF increase in the striatum may represent a 'reservoir' of neurotrophin to be used in case of demand (Fumagalli et al., 2007) and released in an activity-dependent manner (Yang et al., 2009). Further investigation is required to ascertain the proBDNF role in Purkinje cells, but it is clear that under the present experimental conditions proBDNF accumulation was associated with a restrictive remodelling in these neurons.

Considerable evidence related BDNF mechanisms with dopamine D_3 receptor expression (Payer et al., 2013). Interestingly, D3 receptor inactivation using knockout D3 -/- mice has been associated with an increase in the conversion ratio of proBDNF into the mature protein by inducing tpA proteolitic activity in the hippocampus and prefrontal cortex (Castorina et al., 2013). Supporting these results under the present conditions, higher proBDNF levels accompanied greater D3 expression in the cerebellum of animals with cocaine experience.

Repeated-cocaine administration increases ΔFosB levels and affects GluR2 AMPA subunit trafficking in the cerebellar vermis.

ΔFosB levels gradually rise and persist within nucleus accumbens and dorsal striatum neurons after repeated cocaine administration, mediating long-lasting neuronal and

behavioural effects in response to chronic cocaine use (Larson et al., 2010). ΔFosB

stability allows it to regulate long-term transcriptional activity in the basal ganglia

(Renthal et al., 2009). Constitutive ΔFosB expression in the cerebellum was described

two decades ago (Chen et al., 1995). However, to our best knowledge the present work

is the first report demonstrating an increase in ΔFosB levels in the cerebellum after a

repeated experience with cocaine. Cocaine-treated cerebella exhibited a large

accumulation of ΔFosB 24 hours following the priming cocaine injection. However,

such accumulation did not correlate with cocaine-induced sniffing sensitization

expression. In contrast to our results, mice overexpressing ΔFosB in the striatum

exhibited increased motor responses to cocaine administration (Kelz et al., 1999).

Therefore, it appears that under the present treatment conditions cocaine-induced

cerebellar ΔFosB enhancement is the result of the pharmacological properties of the

drug affecting cerebellar transcription, but it is not related to motor neuroadaptations.

ΔFosB effects are partially mediated by the transcription and trafficking of calcium

impermeable AMPAR (GluR2-containing) (Kelz et al., 1999). GluR2 trafficking is one

of the most common mechanisms for AMPAR remodelling. In the cerebellum, in

contrast to other brain areas, plasticity of Purkinje-parallel fiber synapses relies almost

entirely on GluR2 subunit trafficking (Petralia et al., 1997; Hansel, 2005; Kakegawa

and Yuzaki, 2005). During cerebellar plasticity, GluR2 subunits are delivered to the

Purkinje cell surface in an activity-dependent process promoting long-term potentiation

(LTP) in these synapses. In contrast, GluR2 endocytosis causes long-term depression

(LTD) (Kakegawa and Yuzaki, 2005). Evidence derived from the present findings

showed that Purkinje neurons, and some interneurons of the inner molecular layer

(probably basket cells) from cocaine-treated mice, expressed high levels of Glu2

AMPAR subunit. Importantly, when membrane permeabilisation was prevented the

GluR2 signal was lost in Purkinje dendrites, indicating that cocaine promoted GluR2 subunit internalization in Purkinje arbor. On the contrary, GluR2 expression was preserved in the cell surface of Purkinje somas and interneurons. Based on the present findings, one could expect Purkinje activity to be depressed. Indeed, following cocaine administration we found a significant decrease in the activity of Purkinje cells that was accompanied by higher activation of granule cells, the source of the parallel fibers (PF). Speculatively, GluR2 could be overexpressed in Purkinje cells of cocaine-treated animals to compensate for the reduction in spine density, and thus the likely decrease in PF input strength.

GluR2 cell surface trafficking in the Nacc has been associated with cocaine-induced sensitization after prolonged but not short-lasting withdrawal (24 hours) (Boudreau et al., 2007; Ghasemzadeh et al., 2009). In the present study this association was found for lobule III, where GluR2 expression directly correlated with the propensity to sensitize. In the nucleus accumbens GluR2 subunits increase after 14 days of cocaine withdrawal in sensitized mice, but it decayed following a cocaine or saline challenge (Boudreau et al., 2007). It is conceivable, but unlikely, that GluR2 endocytosis observed in Purkinje dendrites was a transient effect derived from the challenge with cocaine. In the study of Boudreau and co-workers, GluR2 expression after cocaine challenge was not different to that observed in either saline-challenged animals or those that never received cocaine. Rather, the present findings have shown clear differences regarding cerebellar GluR2 expression between cocaine- and saline-treated groups.

Cocaine effects on structural plasticity and metaplasticity in the deep medial nucleus: The Perineuronal nets.

The large cerebellar neurons in the medial nucleus projecting to other brain regions receive strong GABAergic innervation on the soma from Purkinje axons. Here, we

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showed that Purkinje axons contacting medial nuclear neurons reduced their size and density after cocaine regimen. Of note, proBDNF expression in Purkinje somas of lobule VIII inversely correlates with the size of Purkinje terminals. Therefore, it appears that somatic proBDNF accumulation in lobule VIII selectively influenced the structural remodelling of Purkinje synaptic contacts with medial nuclear neurons. One can speculate that by reducing size and density of GABA synaptic terminals, as well as the activity of Purkinje cells, nuclear projecting neurons could remain disinhibited. In support of this speculation, we found higher neuronal activity in the medial nucleus 90 minutes after the last cocaine challenge. Notwithstanding, this result has to be taken with caution since we also detected cFOS-IR expressed in small inhibitory nuclear neurons that could influence the final output from the medial nucleus. Maybe so there was no relationship between cocaine-induced nuclear neuronal activity and sniffing sensitization.

External factors might promote structural remodelling of brain circuitry by modulating the activity of regulatory molecules that restricted neuronal plasticity in order to stabilize circuits (Foscarin et al., 2011). These plasticity inhibitory mechanisms take place in a cartilage-like structure called Perineuronal net (PNN) consisting of molecules (versican, aggrecan, neurocan, brevican, hyaluronan, tenascin-R and semaphorin 3A) of extracellular matrix that enwraps the perikaryon of several neurons and which may create restrictive conditions for the emergence of new synaptic contacts and neuronal plasticity modifications (Brückner et al., 1993; Carulli et al., 2006; Carulli et al., 2013). Genetic, pharmacological and environmental strategies to inhibit the regulatory molecules of the extracellular matrix may restore neuronal plasticity potential (Köppe et al., 1997; Corvetti and Rossi, 2005; Foscarin et al., 2011; Carulli et al., 2013). Previous observations indicate that in the deep cerebellar nuclei the PNN structure is maintained

through a dynamic interaction between DCN neurons and the axons of Purkinje cells (Foscarin el al., 2011). One of the most remarkable and totally new finding we present here is that the reduction in Purkinje neurite complexity in cocaine-treated mice was accompanied by up-regulation of PNNs in the large glutamatergic medial nuclear neurons that project out of the cerebellum. Moreover, proBDNF and GluR2 expression were positively associated with the proportion of the nuclear neurons enwrapped by a strong PNN. These resuls indicate that repeated cocaine treatment creates metaplastic restrictive conditions for cerebellar plasticity to be induced.

A few erlier studies have approached the analysis of PNNs in animals treated with addictive drugs (Brown et al., 2007; Van den Oever et al., 2010). In these papers, the restoration of the PNNs in the hippocampus (Brown et al., 2007) and the medial prefrotal cortex (Van den Oever et al., 2010) by inhibiting the metalloproteinase 9 decreased sensitivity to drug-related cues, preventing reinstatement.

The functional consequences of cocaine-induced modulation of PNNs in the cerebellum required further exploration, but in our opinion, the drug-induced regulation of PNNs is a very significant finding which opens new avenues of research regarding, not only drug-dependent mechanisms controlling PNNs formation, but also therapeutic approaches and protective environmental strategies.

Concluding remarks

Only in two synapses do Purkinje cells transform all excitatory information into inhibitory signals that adjust nuclear neuron output (Grüsser-Cornehls and Bäurle, 2001), therefore affecting spatiotemporal patterns of Purkinje activity would allow different subsets of inhibitory neurons to control cerebellar output (Person and Raman, 2012). Under the present conditions of cocaine administration, the inhibitory function of Purkinje neurons appears to be decreased leading to a higher activity in deep medial

neurons (Figure 9). Importantly, the only cocaine-dependent molecular change that correlates with a propensity for developing sniffing sensitization was detected in lobule III. This lobule receives dopaminergic afferents from the VTA and expresses dopamine transporter (Ikai, et al., 1992). Moreover, lobule III is a component of the sensorimotor network (Bostan et al., 2013), this being the network that supports the final control of the transition to addictive behaviour (Belin et al., 2013). Of note, lobule III has been involved in automatizing behavioural repertories toward drug-related cues (Miquel et al., 2009; Yalachkov et al., 2010). Therefore, cocaine-induced modifications in this cerebellar region might contribute to promoting the re-wiring of cortico-limbic-cerebellar circuitry and the behavioural stable effects featured in drug addiction.

Author's contribution

DV-S accomplished all drug treatments and behavioural experiments as well as immunohistochemistry, immunofluorescence, western blot, and RT-PCR techniques, also confocal images acquisition, data and image analysis. MC-G collaborated in the obtention of the tissue samples and in processing the tissue for histological procedures, as well as in the images analysis. MIC-G supervised and was involved in RT-PCR analysis. KL and DC closely supervised all immunofluorescence techniques and analysis of confocal images. CS took part in the statistical analyses and the discussion of the results. FR† and MM were the supervisors of this research and they were reponsibles for the hypothesis, desing and data elaboration. MM was in charge of the final manuscript.

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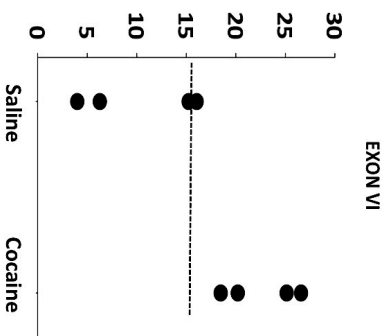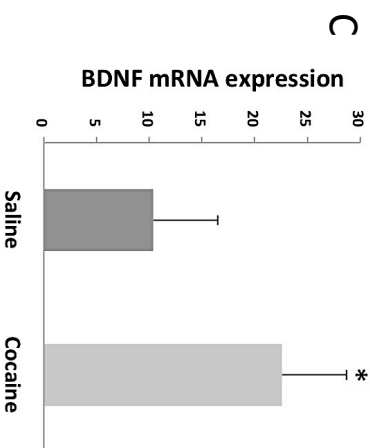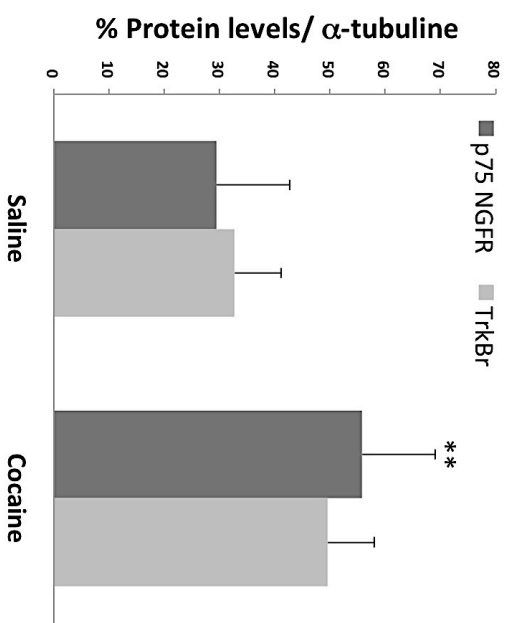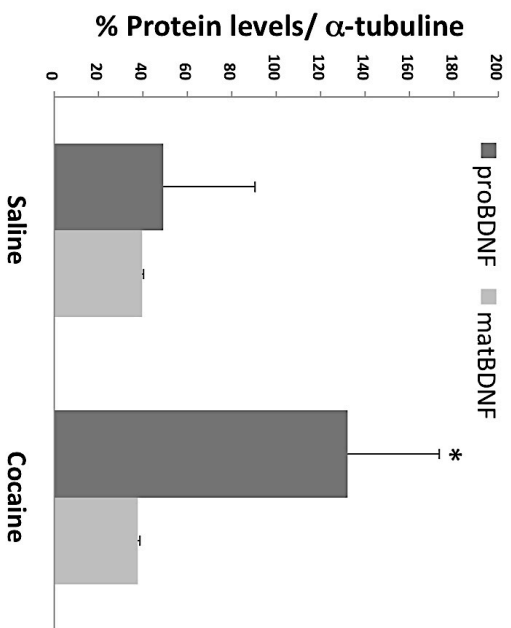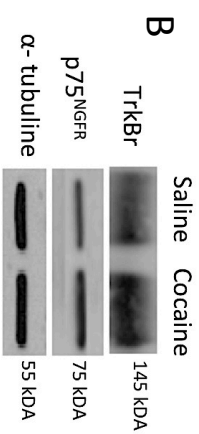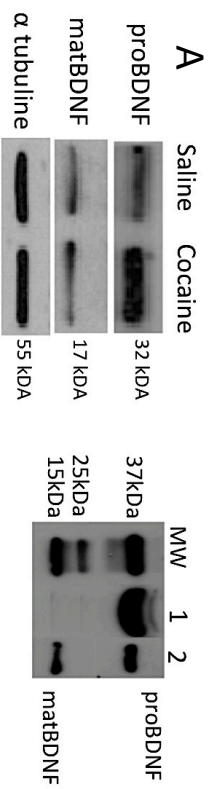
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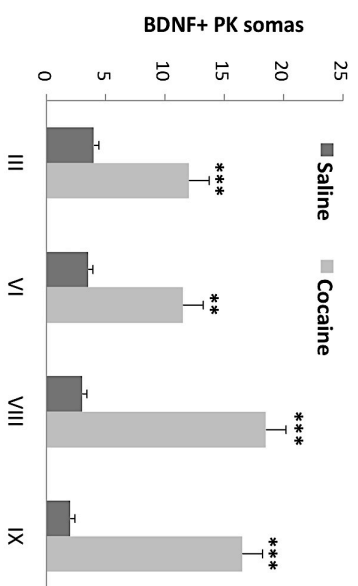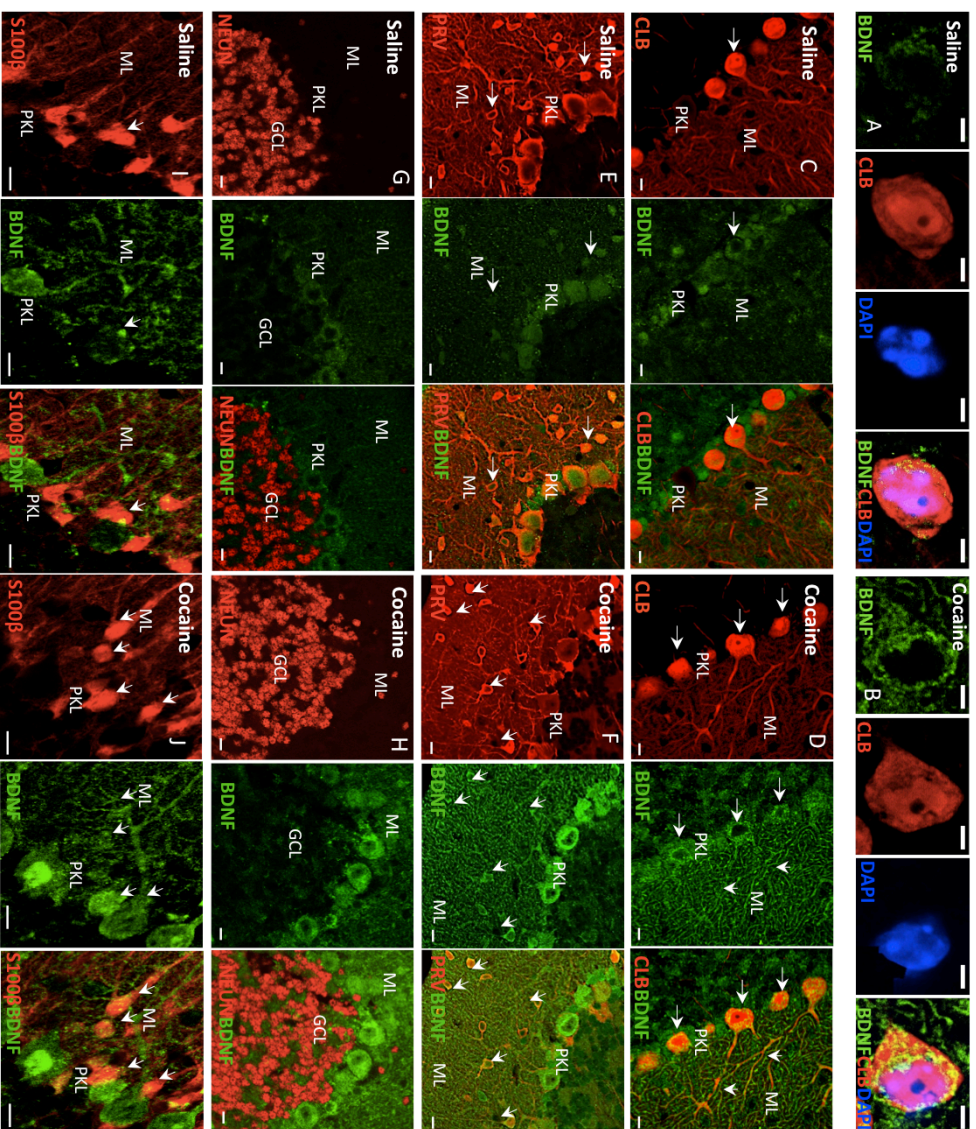
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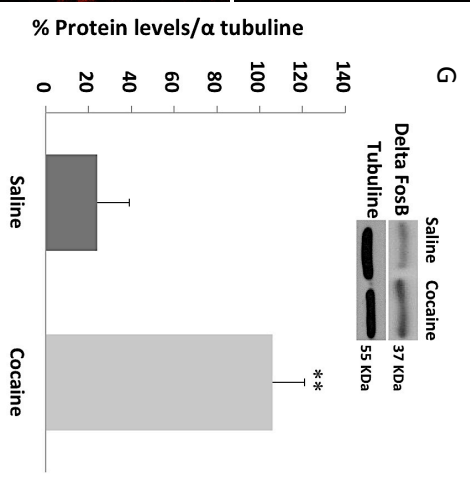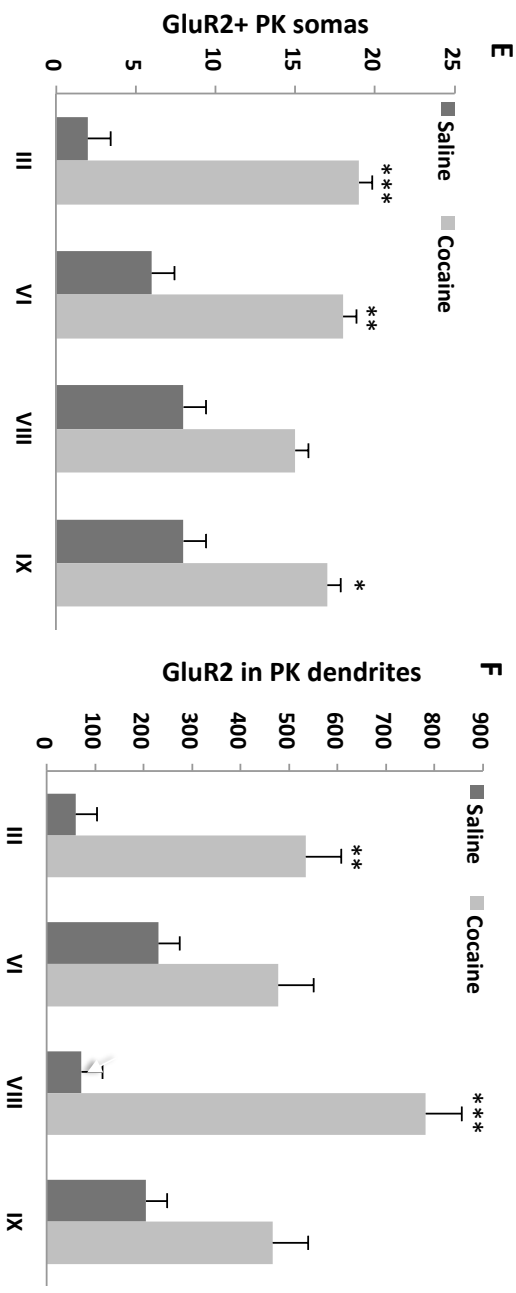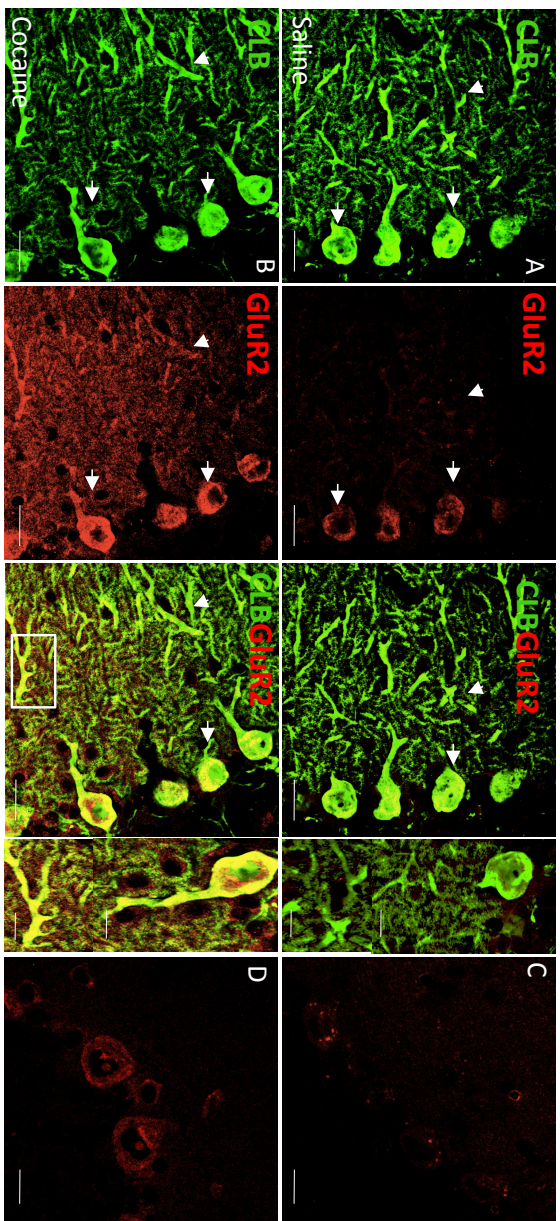
Cerebellar Lobules

Addiction Biology

Repeated Cocaine
7WD

proBDNF expression

GluR2 in PK dendrites
possible endocytosis of
GluR2

Dendritic spines

Size/density
of axon
terminals

Purkinje activity

Deep medial neurons activity

PNNs

DeltaFosB levels

D3R

proBDNF levels