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# The cerebellum on cocaine: plasticity and metaplasticity

**Vazquez-Sanroman D[1], Leto K[2,3], Cerezo-Garcia M[4], Carbo-Gas M[1], Sanchis-Segura C[1], Carulli D[2,3], Rossi F[2,3,†], Miquel M[1,*]**

[1]Área de Psicobiología, Universitat Jaume I, Spain
[2]Department of Neuroscience, Neuroscience Institute of Turin (NIT), University of Turin, Italy
[3]Neuroscience Institute of the Cavalieri-Ottolenghi Foundation (NICO), University of Turin, Italy
[4]Plant Physiology Section, Department of CAMN, Universitat Jaume I, Spain

[†]In memoriam

*Correspondence to: Marta Miquel, Área de Psicobiología, Universitat Jaume I, Avenida Sos Baynat, 12071 Castellon de la Plana, Spain. E-mail: miquel@psb.uji.es

## 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 1-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 mature BDNF 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. Significant pruning in Purkinje dendrite arborization and reduction in the size and density of Purkinje boutons contacting deep cerebellar projection neurons accompanied cocaine-dependent increase in proBDNF. Cocaine-associated effects point to the inhibitory Purkinje function impairment, as was evidenced by lower activity in these cells. Moreover, the probability of any remodelling in Purkinje synapses appears to be decreased due to an upregulation of extracellular matrix components in the PNNs surrounding the medial nuclear neurons.

**Keywords:** BDNF; cerebellum; cocaine; mice; sensitization; 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 drug-taking (Hyman, Malenka & Nestler 2006). Cocaine-dependent neuronal plasticity includes molecular and structural modifications in the cortical–striatal–limbic networks (Hyman *et al*. 2006; Noori, Spanagel & Hansson 2012) with a ventral to dorsal gradient (Everitt & Robbins 2005). In addition, addictive drugs induce restrictive metaplasticity 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 & Maiti 1976; Perciavalle, Berretta & Raffaele 1989; Ikai *et al*. 1992; Bostan, Dum & Strick 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 (Ikai *et al*. 1992; Schweighofer, Doya & Kuroda 2004). Earlier studies found a D1-dependent increase in cFOS immunoreactivity (cFOS-IR) levels in the granule cell layer of the rat vermis chronically treated with cocaine or amphetamine (Klitenick, Tham & Fibiger 1995). Furthermore, recordings of extracellular activity in the cerebellar cortex showed that cocaine is able to suppress spontaneous firing and glutamate-induced activation of Purkinje (PK) cells (Jiménez-Rivera *et al*. 2000).

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; Volkow *et al*. 2003; Anderson *et al*. 2006).

In the present study, we tested the effects of a chronic cocaine treatment on short- and long-lasting plasticity mechanisms in the cerebellum. We also addressed an unexplored way of approaching drug-induced cerebellar metaplasticity, namely the analysis of perineuronal nets (PNNs) in the projection neurons of 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 overexpressing 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 the Supporting Information. Additionally, each legend accounts for a brief methodological description.

### Subjects

Male Balb/c AnNHsd mice (Harlan, Barcelona, Spain) were housed in groups of five with 12 hours light–dark cycle and free access to food (Panlab, Barcelona, Spain) and tap water. They were handled for 3 weeks before the 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 life-span curve provided by Harlan, all experiments took place in the adult stage.

From the total sample of mice tested for behaviour, different control and experimental groups were used for molecular and cellular experiments.

### Cocaine treatment

Cocaine hydrochloride (Alcaliber, Madrid, Spain), dissolved in 0.9 percent saline (2 mg/ml or 1 mg/ml), was intraperitoneally (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 designed with only two groups (repeated saline and repeated cocaine).

### RNA extraction, reverse transcription and real-time polymerase chain reaction (RT-PCR) analysis

Briefly, total RNA was extracted from cerebellar vermis tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen Inc., Hilden, Germany) 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 Ultra-Turrax T25 basic (Ika Labortechnik, Staufen, Germany). Quantification of RNA was carried out with a Nanodrop 1000 spectrophotometer (Fisher Scientific, Madrid, Spain). Total RNA extracted was used to synthesize complementary DNA (cDNA) with the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Madrid, Spain). Primer and probe sequences for BDNF variants were designed using the splice variants (see Supporting Information Table S1). Real-time PCR was conducted using the SYBR Green PCR Kit (Thermo Scientific, Madrid, Spain) and the SmartCycler II instrument (Cepheid, Sunnyvale, CA, USA).

### Western immunoblotting

proBDNF, mature BDNF, p75R, TrkBR, tPA (tissue plasminogen activator), dopamine receptor $D_3$and ΔFosB protein levels were quantified in the cerebellar vermis by Western blotting (WB) (see Supporting Information Table S2 and the text in the Supporting Information for more details). The film signals were scanned at 600 dpi (EPSON 11344, Suwa, NGN, Japan), and levels of the band density were blind processed and quantified by densitometry with FIJI software (Schindelin *et al*. 2012). Every sample was replicated at least twice to ensure the reproducibility of the method.

### Immunohistochemistry and immunofluorescence

A whole description of the antibodies, reagents and procedures used may be seen in the Supporting Information, including Supporting Information Tables S3 and S4. The vermis 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 (Turin, Italy). Confocal images were taken in a first plane 1 μm thick in single planes at a resolution of 1024 × 1024 and 100 Hz speed. Laser intensity, gain and offset were maintained constant in each analysis. Quantitative evaluations were made using the FIJI software (Schindelin *et al*. 2012).

### Morphometric analysis in PK and quantification of PNNs

A full description and explanation of antibodies and procedures for the morphometric analyses may be found in the Supporting Information (see also Supporting Information Table S4). Due to variability in size of PK terminals between saline and cocaine-treated animals, we corrected the raw data by applying the Abercrombie formula (Abercrombie 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's HSD (honest significant difference) tests were performed as parametric *post hoc* tests, when required. The level of significance was set at $P < 0.05$. We applied $\chi^2$ tests to compare the distribution of frequencies relative to staining intensity categories of *Wisteria floribunda* agglutinin (WFA).

## Results

### Cocaine-induced stimulating motor behaviour

As expected, after seven alternate IP cocaine administrations (20 mg/kg), Balb mice ($n = 18$) developed sniffing sensitization {one-way repeated measures ANOVA: the cocaine effect [degrees of freedom (d.f.) = 1,20; $F = 461.22$; $P < 0.001$]; day of treatment effect (d.f. = 6120; $F = 16.21$; $P < 0.001$); the interaction effect (d.f. = 6120; $F = 22.15$; $P < 0.001$)}. Tukey's *post hoc* tests demonstrated sensitization after four cocaine administrations ($P < 0.001$). After the sixth cocaine injection, mice remained undisturbed in the animal facilities for 1 week until they were tested again under a lower cocaine dose (10 mg/kg) (Fig. 1). Sniffing sensitization was maintained after a 1-week washout period ($P < 0.001$ for comparisons between the first and seventh cocaine administration).

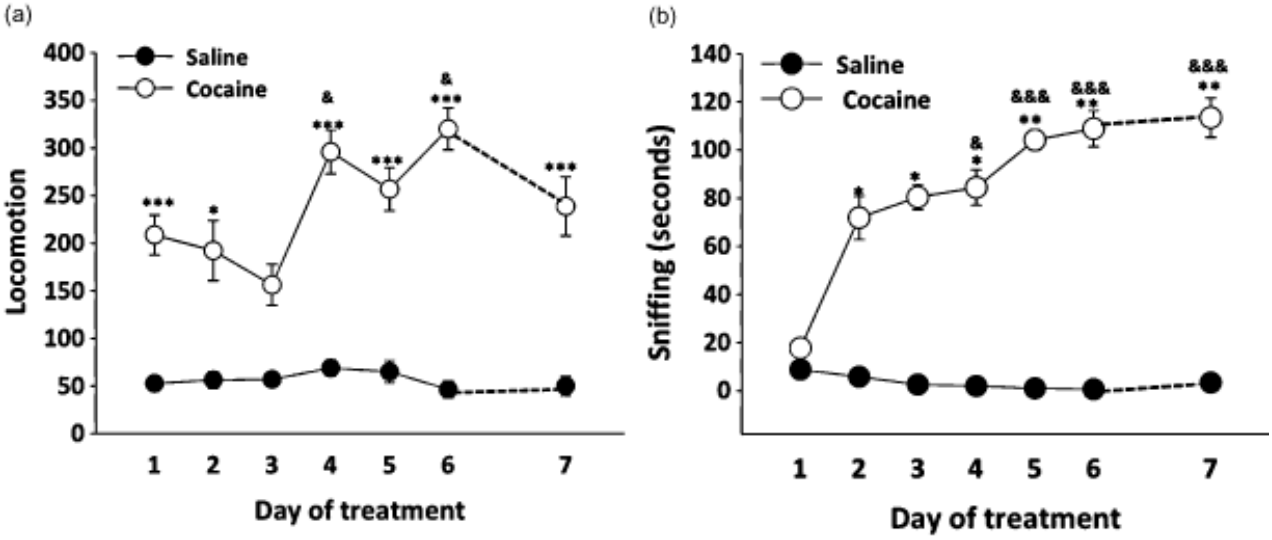


**Figure 1.** Effects of repeated cocaine administration on motor behaviour. The treatment protocol consisted of two phases. During the first phase, six saline or cocaine injections (20 mg/kg) every 48 hours were administered to two different groups of mice (saline $n = 17$; cocaine $n = 18$). Immediately after each of these injections, mice were placed in the open-field chambers for 15 minutes. The second phase of this protocol took place 7 days after the sixth testing session and it was identical to the previous one, except for the fact that cocaine-treated mice were challenged with a lower cocaine dose (10 mg/kg). Each one of the testing sessions was videotaped and scored by a researcher blinded to the treatment conditions. Locomotion was measured as the number of counts in 15 minutes, considering as a count each time that the mice crossed with all four legs one of the lines dividing the open field into four equal quadrants. For sniffing behaviour, we considered three representative minutes throughout the 15-minute period (3′–4′; 7′–8′; 13′–14′). During the minute recorded, we registered the amount of seconds spent doing sniffing and head bobbing. Mean ± SEM of (a) counts in 15 minutes and (b) the total amount of time spent doing sniffing comparing across the sessions. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for comparison between treatments; &$P < 0.05$, &&$P < 0.01$, &&&$P < 0.001$ for comparison with the first cocaine injection).

In addition, we evaluated locomotion in these mice. After the first cocaine injection, there was an acute enhancement of locomotion, which increased during the last days of treatment before withdrawal. Nevertheless, locomotor sensitization could not be maintained after a 1-week drug-free period [one-way repeated measures ANOVA: the cocaine effect (d.f. = 1,20; $F = 24.37$; $P < 0.001$); days of treatment effect (d.f. = 6120; $F = 2.81$; $P < 0.01$); the interaction effect (d.f. = 6120; $F = 1.86$; $P = 0.09$)] (Fig. 1).

## Cocaine-induced BDNF mechanisms in the cerebellum

In cocaine-treated mice, we observed a significant increase in proBDNF protein levels (d.f. = 1,8; $F = 7.22$; $P < 0.05$), but no change in the mature isoform (d.f. = 1,8; $F = 0.21$; $P > 0.05$) (Fig. 2). Therefore, we asked whether such elevation in proBDNF was the result of either an enhancement of transcriptional activity or an increase in cleavage mechanisms.

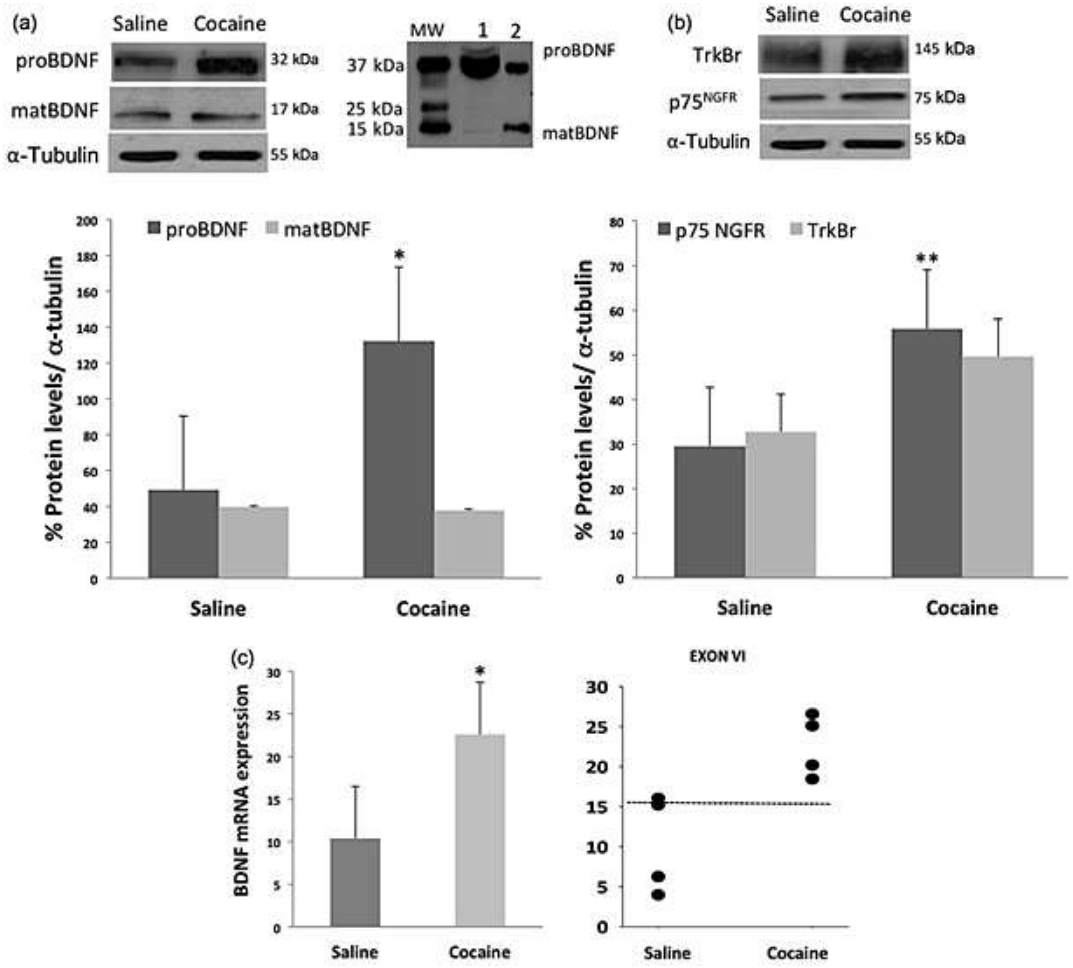


**Figure 2.** Effects of chronic cocaine administration on BDNF-related transcripts and proteins. The effects of repeated cocaine administrations on protein levels were quantified and expressed as the percentage of arbitrary units of densitometry normalized by the α-tubulin level in the same blot. Cerebellar samples were obtained 24 hours after the last cocaine injection. Two replicas were performed per subject ($n = 5$). (a) Top panel depicts representative immunoblottings for the two bands detected by the antibody rabbit anti-BDNF. For detection of proBDNF (32 kDa) and mature BDNF (matBDNF) (14 kDa), we used an antibody that recognizes both isoforms. On the right-hand side, a representative Western blot for a positive control of proBDNF is shown. Sample 1 corresponds to 37-kDa biologically active BDNF protein (50 µg) produced in *Escherichia coli* incubated with anti-BDNF (Santa Cruz Biotechnology, CA, USA; sc-546). Sample 2 represents the band identified in the cerebellum tissue. Cocaine raised the precursor isoform of BDNF in the cerebellum, without affecting the mature isoform. As can be seen, we were able to detect the proBDNF band at 37 kDa in the cerebellar samples. Data represent the average of percentages of pro- and mature BDNF expression (mean + SE; $*P < 0.05$). (b) $p75^{NGFR}$ and TrkB receptor levels in the cerebellar vermis. Cocaine selectively increased proBDNF receptor levels ($p75^{NGFR}$) ($**P < 0.01$). (c) The right panel depicts the scatterplot of mRNA BDNF levels for exon VI. Expression of exon VI was significantly higher in the cerebellum of cocaine-treated mice ($n = 4$) ($*P < 0.05$). The dotted line represents the median value. Cerebellar samples were obtained 2 hours after the last saline or cocaine injection.

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 (d.f.= 1,6; $U = 3$; $P < 0.05$), without inducing any significant change in exons IV (d.f. = 1,6; $U = 0$; $P > 0.05$) and I (d.f. = 1,6; $U = 5$; $P > 0.05$). No changes in the β-tubulin expression were identified (d.f. = 1,6; $U = 1$; $P > 0.05$) (Fig. 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 (d.f. = 1,6; $U = 8$; $P > 0.05$) or protein expression (d.f. = 1,6; $F = 0.40$; $P > 0.05$) in the mouse cerebellar vermis.

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 (d.f. = 1,8; $F = 13.36$; $P < 0.01$), but TrkBR levels remained unchanged (d.f. = 1,8; $F = 6.68$; $P > 0.05$) (Fig. 2).

To further describe the effects of chronically administered cocaine, we analysed BDNF expression in different cellular lineages of the cerebellar cortex ($n = 5$) (Fig. 3). To encompass a comprehensive sampling of the vermis, we selected four 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 PK 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 PK and other cell types corresponded to proBDNF.

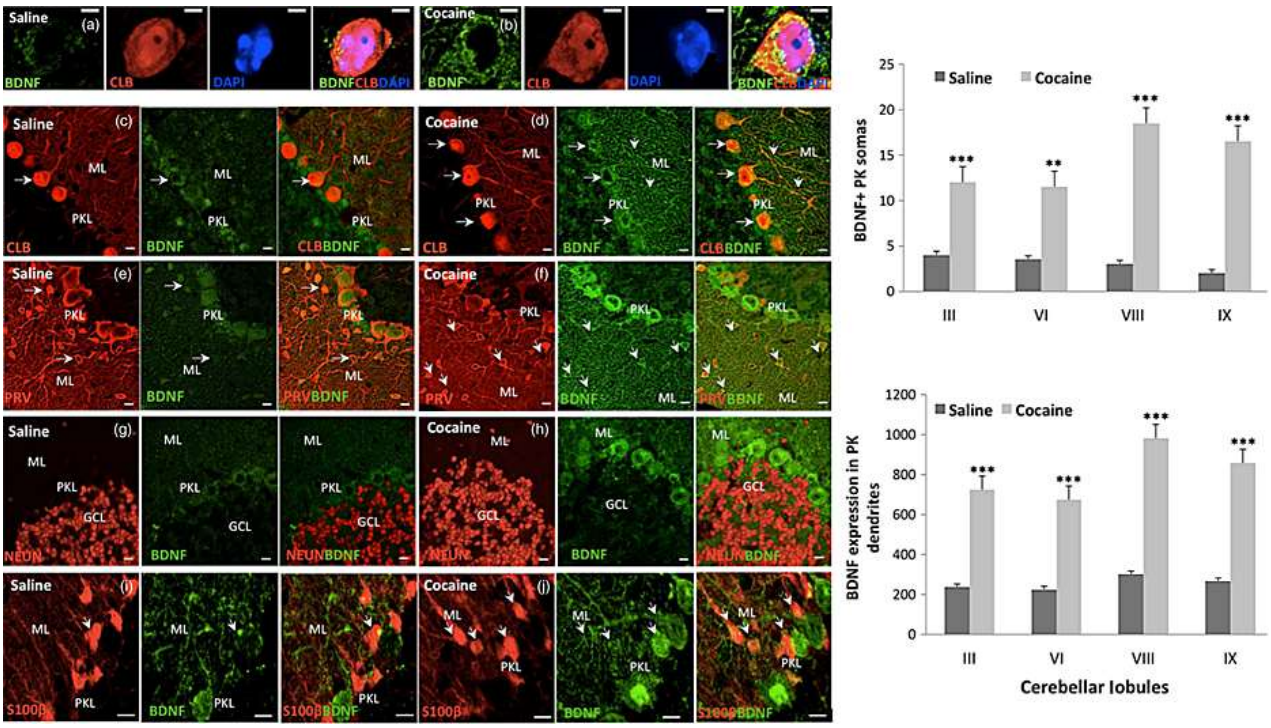


**Figure 3.** BDNF expression in different cellular types in the cerebellum. On the left side panel, representative confocal images of BDNF (green) expression in different cerebellar cell types (labelled by specific markers, in red) after saline or cocaine administration are included. (a–d) Purkinje cells (marked by calbindin); (e and f) interneurons (marked by parvalbumin) at the molecular cell layer; (g and h) granule cells (marked by NeuN). For all photographs, scale bar: 10 μm. GCL = granule cell layer; ML = molecular cell layer; PKL = Purkinje cell layer. White arrows show BDNF specific co-localizations. The right side panels display the number (mean ± SEM) of somas and dendrites showing full co-localization between a specific Purkinje neuron marker (calbindin) and the proBDNF signal. This quantification was carried out by an experimenter blinded to the experimental conditions using a cerebellar region of interest of 90 000 $\mu m^2$. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for differences between groups). See the main text and the Supporting Information for further details.

Cocaine produced a more than twofold enhancement of BDNF expression in the soma [Lob III: (d.f. = 1,8; $F = 14.40$; $P < 0.01$); Lob VI: (d.f. = 1,8; $F = 10.0$; $P < 0.01$); Lob VIII: (d.f. = 1,8; $F = 59.66$; $P < 0.001$); and Lob IX: (d.f. = 1,8; $F = 34.50$; $P < 0.001$)] and dendrites of PK neurons [Lob III: (d.f. = 1,8; $F = 23.29$; $P < 0.001$); Lob VI: (d.f. = 1,8; $F = 19.92$; $P < 0.01$); Lob VIII: (d.f. = 1,8; $F = 100.20$; $P < 0.001$); and Lob IX (d.f. = 1,8; $F = 108.13$; $P < 0.001$)].

In addition, we also noticed BDNF expression in the granule cell layer, although it was not expressed in granule cells as co-labelling with NeuN could not be demonstrated (Fig. 3). The presence of BDNF surrounding granule cells could be due to synaptic-associated mechanisms related to mossy fibres, 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 (d.f. = 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 being linked to dopamine $D_3$ receptor expression (Payer, Balasubramaniam & Boileau 2013), we next evaluated the effects of our experimental cocaine treatment conditions on $D_3$ levels. As expected, 24 hours following the cocaine challenge, we observed up to fourfold $D_3$ receptor levels in the vermis (d.f. = 1,8; $F = 15.13$; $P < 0.01$) (Supporting Information Fig. S1).

### Cocaine effects on structural plasticity in PK neurons

As BDNF has been involved in dendritic spine remodelling (Schratt *et al*. 2006), we next evaluated in the same mice the cocaine effects on the density of dendritic spines in the PK dendritic tree (Fig. 4). Worthy of mention, in animals treated with cocaine, PK dendritic spines were clearly and significantly pruned. This reduction was a global effect affecting all lobules assessed [Lob III (d.f. = 1,8; $F = 12.66$; $P < 0.01$); Lob VI (d.f. = 1,8; $F = 80.65$; $P < 0.001$); Lob VIII (d.f. = 1,8; $F = 7.99$; $P < 0.05$); and Lob IX (d.f. = 1,8; $F = 49.13$; $P < 0.001$)]. No cocaine effects were observed on the total number of PK cells labelled by calbindin.

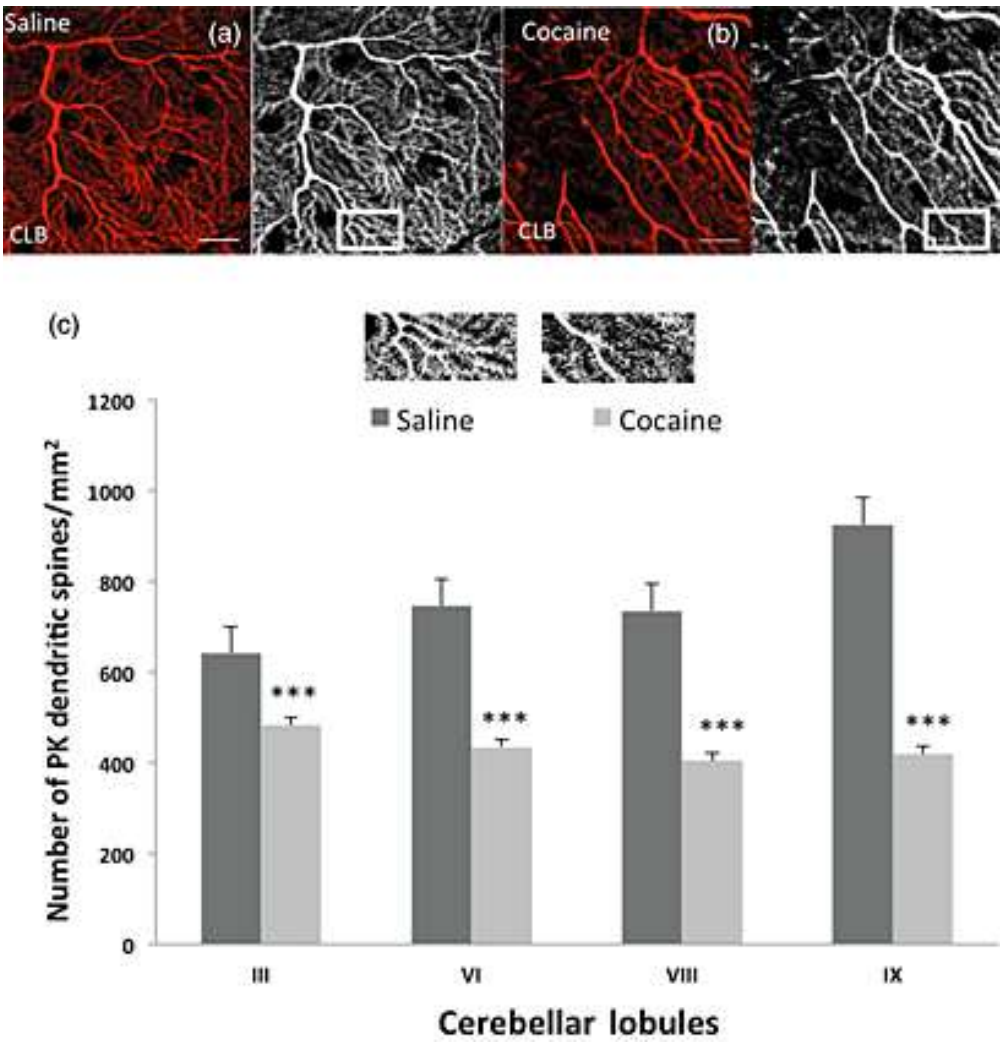


**Figure 4.** Cocaine effects on Purkinje (PK) dendritic arbour in several cerebellar lobules. The dendritic tree of PK neurons of saline- (a) or cocaine- (b) treated mice was visualized using an anti-calbindin antibody (red). Fluorescence microphotographs were taken using a 40× objective and a 4× zoom for a total magnification of 160×. In a second step, microphotographs were converted to grey-RGB pictures. We traced two different regions of interest (ROIs) of 10 000 μm$^2$ in the PK dendritic tree: one near the soma and another in the most distal part of the arborization tree. White squares indicate the ROIs for quantification. (c) Quantification of the density of dendritic spines of PK neurons. Data are depicted as mean ± SEM number o neurons per square millimetre (***$P < 0.001$, **$P < 0.01$). Cocaine induced a significant dendritic shrinkage in PK cells. Scale bar: 20 μm.

Cerebellar neurons in the medial nucleus projecting to other areas in the brain receive strong GABAergic innervation on the perikaryon from PK axons. To ask whether cocaine might affect structural plasticity at the level of synaptic PK 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 PK terminals were labelled with calbindin 28K (Fig. 5). The soma size of the deep medial nuclear neurons was unknot affected by cocaine (d.f. = 1,97; $F = 0.30$; $P > 0.05$). However, density of PK terminals (d.f. = 1,8; $F = 5.36$; $P < 0.05$) and their size were reduced (d.f. = 1,8; $F = 4.56$; $P < 0.05$).

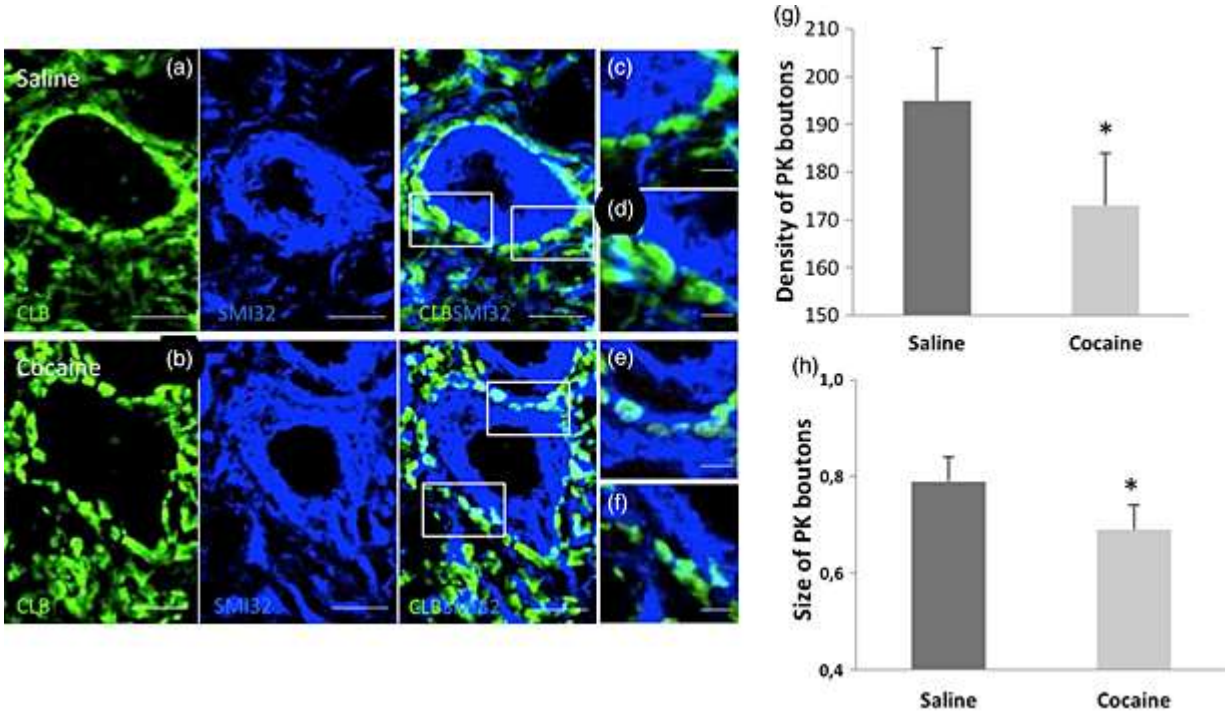


**Figure 5.** Purkinje (PK) synaptic terminals contacting medial nuclear projection neurons. (a and b) Deep cerebellar neurons were identified with anti-SMI32 antibody (blue) and PK synaptic boutons were stained by calbindin (green). Scale bar: 20 μm. (c–f) White squares display a digital amplification of the axosomatic contacts. We analysed approximately 100 PK terminals per subject. The amplification bar represents 10 μm. Data are depicted as mean ± SEM. (g and h) Cocaine reduced the density (g) and size (h) of PK terminals contacting medial neurons (*$P < 0.05$).

## 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 (Fig. 6). 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 [d.f. (1); $\chi^2 = 0.19$; $P > 0.05$]. 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 [d.f. (1); $\chi^2 = 80.31$; $P < 0.01$].

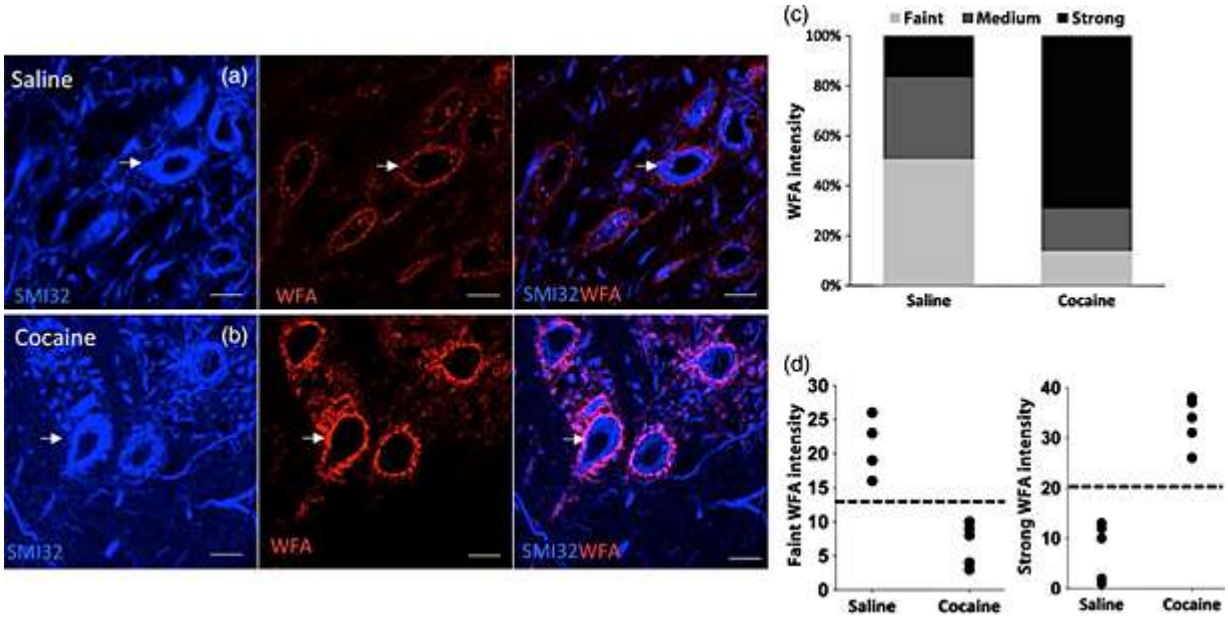


**Figure 6.** Perineuronal nets (PNNs) in the medial nucleus. (a and b) Confocal microphotographs of medial nuclear projection neurons (identified by SMI32; blue). Those neurons bearing a PNN (white arrows in the photographs) were identified by an antibody against *Wisteria floribunda* agglutinin (WFA; red). Staining intensity was assessed from confocal images (40× objective, 2.0× zoom; final magnification of 80×) by randomly selecting 15 pixels of each net and calculating their average (50 PNNs + neurons per animal). Each PNN was assigned to one of three categories (faint = 0–33 percent, medium = 34–66 percent, strong = 67–100 percent of the maximum staining intensity). Scale bar represents 20 μm. (c) Cocaine significantly increased the proportion of neurons that expressed strong PNNs. (d) Scatterplots for WFA intensity distribution in cocaine and saline-treated mice.

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

ΔFosB is one of the transcription factors that have been shown to accumulate after chronic psychostimulant administration (Larson *et al*. 2010). It binds AP-1 DNA sequences found in promoters of many genes and thereby can both repress and 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 fivefold increase in the ΔFosB levels in the vermis (d.f. = 1,8; $F = 12.28$; $P < 0.01$) (Fig. 7). Then, we analysed the number of positive PK somas for GluR2, and by densitometry, GluR2 expression in the PK dendritic tree (Fig. 7).

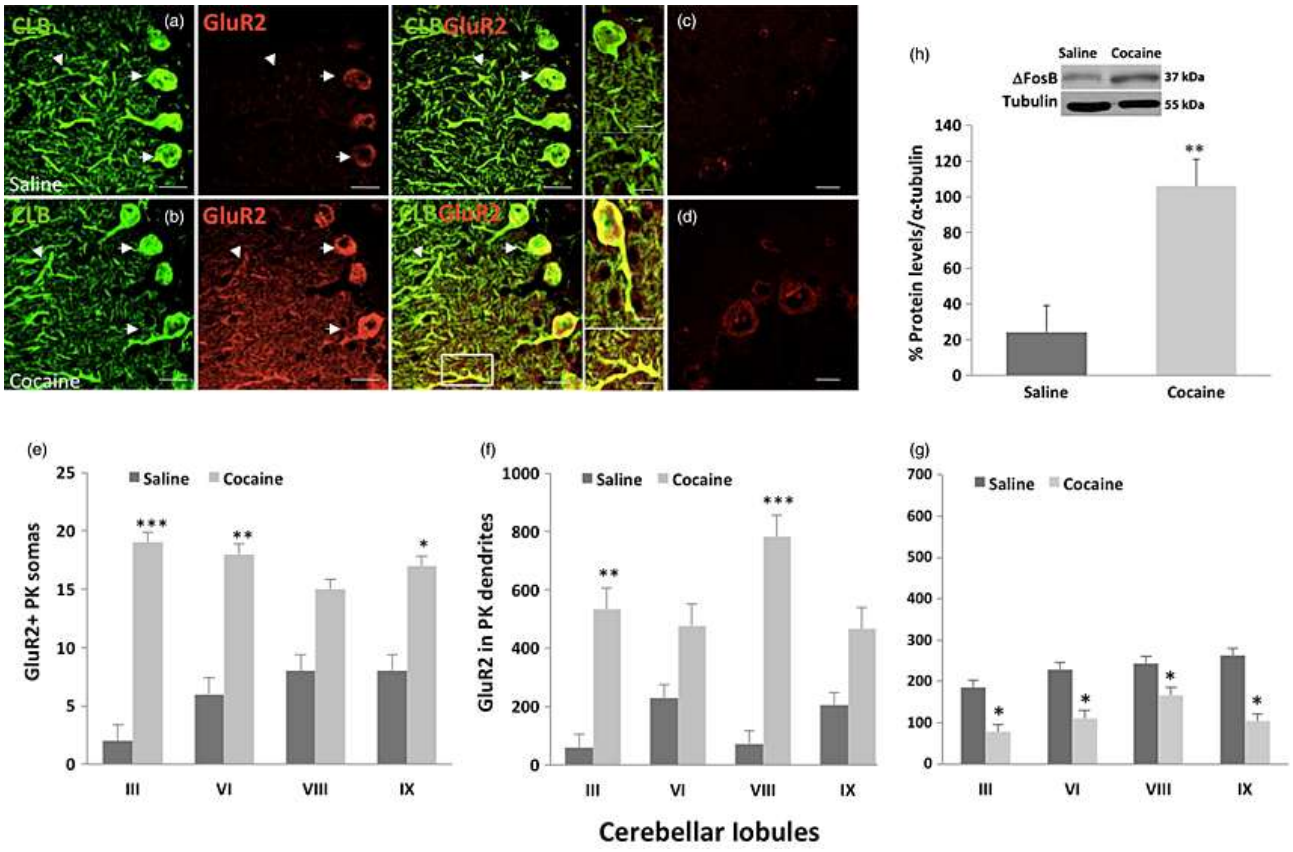


**Figure 7.** ΔFosB levels and GluR2 expression in the cerebellar vermis. Photographs (a)–(d) illustrate the expression of GluR2 AMPA subunit (red) in Purkinje (PK) neurons (identified by calbindin expression in green). White arrows indicate positive GluR2/calbindin co-labelling. White squares depict a digital amplification of a co-localized soma and dendrites. Panel (e) depicts the number of GluR2-positive PK somas, whereas panel (f) depicts GluR2 levels on the dendrites of PK cells as measured by densitometry (data are presented as mean ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Cocaine significantly increased the number of cells displaying GluR2/calbindin co-expression (a and b; e and f), but this ability was lost when membrane permeabilization was prevented (d–b; g). Panel (h) shows the 350 percent increase of ΔFosB levels observed in the cerebellum after repeated cocaine administration (data are shown as mean ± SEM of the percentage abundance over the α-tubulin levels in the same blot; $P < 0.001$). See the main text and the Supporting Information for further details.

Higher GluR2 levels were observed in PK soma (d.f. = 1,8; $F = 60.77$; $P < 0.001$) and dendrites of lobule III (d.f .= 1,8; $F = 14.60$; $P < 0.01$), as well as in dendrites of lobule VIII (d.f. = 1,8; $F = 262$; $P < 0.001$). Lobules VI (d.f. = 1,8; $F = 23.83$; $P < 0.01$) and IX (d.f. = 1,8; $F = 4.99$; $P < 0.05$) of cocaine-treated animals only showed differences in GluR2 expression in the soma (Fig. 7). Additionally, we accomplished an immunoanalysis against GluR2 without any membrane permeabilization in order to identify the internal or external position of the AMPA subunit in PK somas and dendrites. As seen in Fig. 7, only in the cocaine group the GluR2 signal was prevented in dendrites but maintained in PK somas when membrane permeabilization was not accomplished, suggesting an endocytosis of the AMPA GluR2 subunits in PK dendrites after cocaine treatment (for all lobules: $\chi^2 = 3.85$; d.f. = 1; $P < 0.05$).

## cFOS expression in the cerebellum

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

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

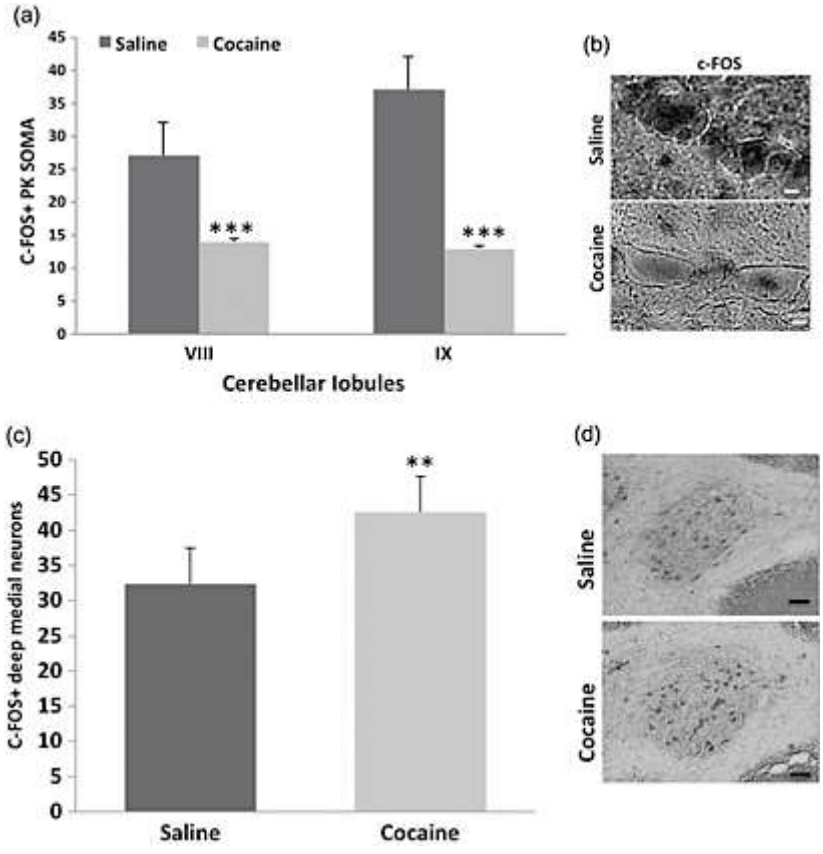


**Figure 8.** cFOS immunolabelling in the cerebellum. cFOS expression was reduced in Purkinje (PK) neurons but increased in medial nuclear neurons of cocaine-treated mice. (a) Number (mean ± SEM) of cFOS immunoreactivity (cFOS-IR) positive somas of PK neurons in saline- and cocaine-treated mice (***$P < 0.001$). (b) An example of cFOS + PK somas (magnification of 80×; scale bar of 10 μ). (c) Number (mean ± SEM) of cFOS-IR positive somas of medial nucleus neurons in saline- and cocaine-treated mice (**$P < 0.01$). (d) Representative microphotographs of cFOS-IR in the medial nucleus (magnification of 20×). See the main text and the Supporting Information for further details.

## 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, Whitfield & Berglind 2010). Here, we showed that the cerebellar vermis of cocaine-treated mice exhibited a dramatic increase in proBDNF and ΔFosB levels, which were accompanied by structural modifications in PK 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 have been mediated by a direct cocaine effect on cerebellar dopamine transporters (DATs). Nevertheless, it is also possible that any cocaine-induced striatal DAT blockade has contributed to the cerebellar changes through either the ventral tegmental area–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 (Fumagalli *et al*. 2007; Graham *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).

It is worth mentioning that our cocaine treatment protocol involved six alternant cocaine injections followed by a 1-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 PK neurons 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 mature BDNF 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), although 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 tPA (Yang *et al*. 2009). In our study, cocaine treatment did not affect cerebellar tPA levels, although from the present data, it is not possible to discard a cocaine-dependent reduction in the enzymatic activity. Definitely, further investigation is needed to test this issue.

It has been described that BDNF synthesis is produced from nine exons resulting in an identical protein (Liu & 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, Abel & Lynch 2014). Under the present conditions, we observed an upward trend in exon IV of cocaine-treated animals, although it did not reach statistical significance.

Adult PK cells constitutively express BDNF (Kawamoto *et al*. 1996). Cocaine-dependent proBDNF enhancement was accompanied in our study 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* gene (Zagrebelsky *et al*. 2005). The functional role of the proBDNF accumulation in PK neurons is far

from being clear. The possibility has been suggested that cocaine-dependent proBDNF 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 role of proBDNF in PK cells, but it is clear that under the present experimental conditions, proBDNF accumulation accompanied restrictive remodelling in the same 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 proteolytic 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.

**PK synaptic contacts and metaplasticity in the deep medial nucleus are both affected by cocaine**

The large cerebellar neurons in the medial nucleus projecting to other brain regions receive strong GABAergic innervation on the soma from PK axons. Here, we showed that PK axons contacting medial nuclear neurons reduced their size and density after the cocaine regimen in the same animals expressing an accumulation of proBDNF. It is plausible therefore that somatic proBDNF accumulation influenced the structural remodelling of PK 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 PK 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 as we also detected cFOS-IR expressed in small inhibitory nuclear neurons that could influence the final output from the medial nucleus.

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 PNN consisting of molecules (versican, aggrecan, neurocan, brevican, hyaluronan, tenascin-R and semaphorin 3A) of ECM 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, 2013). Genetic, pharmacological and environmental strategies to inhibit the regulatory molecules of the ECM may restore neuronal plasticity potential (Köppe *et al*. 1997; 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 deep medial nucleus (DMN) neurons and the axons of PK cells (Foscarin *et al*. 2011). One of the most remarkable and totally new finding we present here is that the reduction in PK neurite complexity in cocaine-treated mice was accompanied by upregulation of PNNs in the large glutamatergic medial nuclear neurons that project out of the cerebellum. This result indicates that repeated cocaine treatment creates metaplastic restrictive conditions for cerebellar plasticity to be induced.

A few earlier 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 prefrontal 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 that opens new avenues of research regarding not only drug-dependent mechanisms controlling PNN formation but also therapeutic approaches and protective environmental strategies.

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. 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 PK-parallel fibre (PF) synapses relies almost entirely on GluR2 subunit trafficking (Petralia *et al*. 1997; Hansel 2005; Kakegawa & Yuzaki 2005). During cerebellar plasticity, GluR2 subunits are delivered to the PK cell surface in an activity-dependent process promoting long-term potentiation in these synapses. In contrast, GluR2 endocytosis causes long-term depression (Kakegawa & Yuzaki 2005). Evidence derived from the present findings showed that PK 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 permeabilization was prevented, the GluR2 signal was lost in PK dendrites, indicating that cocaine promoted GluR2 subunit internalization in PK arbour. On the contrary, GluR2 expression was preserved in the cell surface of PK somas and interneurons. Based upon the present findings, one could expect PK activity to be depressed. Indeed, following cocaine administration, we found a significant decrease in the activity of PK cells that was accompanied by higher activation of granule cells, the source of the PFs. Speculatively, GluR2 could be overexpressed in PK cells of cocaine-treated animals to compensate for the reduction in spine density and thus the likely decrease in PF input strength.

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 PK 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 from 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.

**Cerebellar plasticity and cocaine-induced sensitization**

Moderate to high doses of cocaine induce an increase in locomotor activity as well as in a group of behaviours, including sniffing, rearing, grooming and ‘head bobbing’ (Blanchard *et al*. 2000). As long as the cocaine administration is prolonged, some of these behaviours become sensitized (Robinson & Berridge 2008). Of note, sniffing sensitization has been the focus of particular experimental attention as a key stereotypy associated with cocaine (Blanchard *et al*. 2000). As expected, after seven cocaine administrations, we observed a fivefold increase in sniffing and head bobbing behaviours in most (60 percent), but not all, of the drug-treated mice. However, we did not observe cocaine-dependent locomotor sensitization, likely because sniffing and locomotion are displayed as incompatible behavioural phenotypes.

Motor sensitization is relevant to addiction-related changes because it reflects the engagement of the incentive motivational systems activated by addictive drugs (Robinson & Berridge 2008). Nevertheless, psychostimulant-induced sniffing sensitization has also been used as an experimental model of the stimulant psychosis and vulnerability in schizophrenia (Vanderschuren & Kalivas 2000). In any case, these three different conditions—psychostimulant-induced behavioural sensitization,

psychostimulant-induced psychoses and chronic schizophrenia—share a common pathophysiology linked to an upregulation of mesolimbic dopaminergic system activity (Ujike 2002).

Data drawn from the present study have indicated that a repeated regimen with cocaine that was able to induce motor sensitization in 60 percent of the cocaine-treated mice also produced dramatic modifications in cerebellar neuroplasticity. Even so, our findings do not support a causal link between cocaine-dependent cerebellar changes and the development of cocaine-induced motor sensitization, as has been demonstrated repeatedly for the striatum (Kelz *et al*. 1999). Therefore, further research should be focused on unravelling whether cerebellar changes could mediate behavioural sensitization.

## Concluding Remarks and Future Developments

Only in two synapses do PK cells transform all excitatory information into inhibitory signals that adjust nuclear neuron output (Grüsser-Cornehls & Bäurle 2001), therefore affecting spatiotemporal patterns of PK activity would allow different subsets of inhibitory neurons to control cerebellar output (Person & Raman 2012). Under the present conditions of cocaine administration, the inhibitory function of PK neurons appears to be decreased, leading to a higher activity in deep medial neurons (Fig. 9). An important matter for future consideration is the question of whether these cerebellar changes are due to the repeated cocaine effects or whether they are a consequence of cocaine abstinence due to the time elapsed since the last cocaine administration.

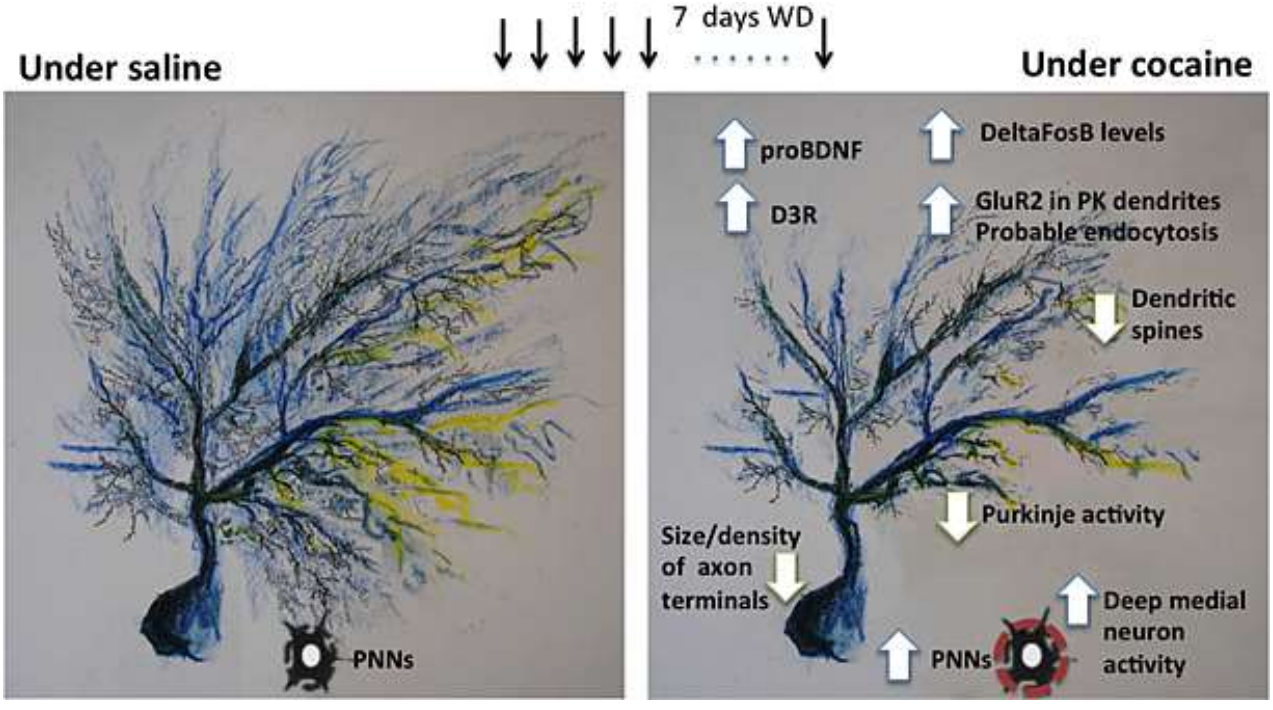


**Figure 9.** Molecular and structural distinctive features of cocaine-induced plasticity in the cerebellum after seven cocaine administrations including a 1-week withdrawal period (WD). Cocaine-treated mice showed an accumulation of proBDNF, an increase in GluR2 expression in Purkinje cells that was selectively prevented in dendrites when no membrane permeabilization was allowed, and a downregulation of the complexity in Purkinje neurites. Therefore, our results point to a decrease in the Purkinje inhibitory function over the deep medial neurons. As expected, the reduced activity of Purkinje cells resulted in an upregulation of deep cerebellar nuclear neuron activity. Moreover, cocaine triggered the higher expression of perineuronal nets (PNNs) in the medial nucleus, leading to a restrictive metaplastic state that would result in a reduced likelihood of further changes on cortico-nuclear synapses.

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**Conflict of Interest**

The authors of the present manuscript declare no conflict of interest.

**Authors 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 obtaining the tissue samples and in processing the tissue for histological procedures, as well as in the image 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 responsible for the hypothesis, design and data elaboration. MM was in charge of the final manuscript. All authors critically reviewed content and approved final version for publication.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher's web-site. Filename (153K): adb12223-sup-0001-si.docx

**Description**

**Figure S1** Cocaine effects on cerebellar dopamine D3 receptor levels
**Table S1** Sequence of primers used in RT-PCR protocol
**Table S2** Western-blot conditions for the different proteins
**Table S3** Immunoperoxidase antibodies
**Table S4** Combination of immune fluorescence antibodies

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