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Cocaine-induced plasticity in the cerebellum of sensitised mice.

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Conflict of Interest Statement

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

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Abstract

Rationale: **Prior research has accumulated a substantial amount of evidence on the ability of cocaine to produce short- and long-lasting molecular and structural plasticity in the corticostriatal-limbic circuitry. However, traditionally, the cerebellum has not been included in the addiction circuitry, even though growing evidence supports its involvement in the behavioural changes observed after repeated drug experiences.** *Objectives:* **In the present study, we explored the ability of seven cocaine administrations to alter plasticity in the cerebellar vermis.** *Methods***: In this study, after six cocaine injections, one injection every 48 h, mice remained undisturbed for one month in their home cages. Following this withdrawal period, they received a new cocaine injection of a lower dose. Locomotion, behavioural stereotypes and several molecular and structural cerebellar parameters were evaluated**. *Results*: Cerebellar proBDNF and mature BDNF levels were both enhanced by cocaine. The high BDNF expression was associated with dendritic sprouting and increased terminal size in Purkinje neurons. Additionally, we found a reduction in extracellular matrix components that might facilitate the subsequent remodelling of Purkinje-nuclear neuron synapses. *Conclusions*: **Although speculative, it is possible that these cocaine-dependent cerebellar changes were incubated during withdrawal and manifested by the last drug injection. Importantly, the present findings indicate that cocaine is able to promote plasticity modifications in the cerebellum of sensitised animals similar to those in the basal ganglia.**

Key words: cerebellum, cocaine, sensitisation, withdrawal, BDNF, GluR2

Introduction

In the last three decades, research on addiction has found significant evidence regarding the ability of cocaine to induce short- and long-lasting molecular and structural plasticity in the corticostriatal-limbic circuitry (Corbit et al. 2012; Everitt and Robins 2005; Murray et al. 2013; Willuhn et al. 2012). **Despite increasing evidence for the involvement of the cerebellum in drug-related behavioural alterations, however, this structure has been traditionally overlooked in addiction research (Carbo-Gas et al. 2014ab; Moulton et al. 2014; Vazquez-Sanroman et al. 2015). This is striking because experimental data have shown that the cerebellum mediates the consolidation of emotional memories, the persistence of behavioural repertories and the development of reward-induced learning (Strata et al. 2011; Yalachkov et al. 2010).**

Several of the cocaine-dependent modifications in neural plasticity appear to be incubated during withdrawal periods following a repeated experience with the drug. Indeed, both BDNF concentrations and GluR1/GluR2 trafficking progressively increased long after the cessation of exposure to cocaine (Boudreau et al. 2007; Boudreau and Wolf 2005; Ghasemzadeh et al. 2009; Grimm et al. 2003), thus mediating the incubation of the craving (Li et al. 2013; Loweth et al. 2014). Furthermore, striatal GluR1/GluR2 trafficking correlated with behavioural sensitisation after 21 days but not after a shorter period following repeated cocaine administrations (Boudreau and Wolf 2005).

The present investigation aimed to analyse molecular and structural plasticity in the cerebellum of cocaine-sensitised mice. In this study, a withdrawal period of one month preceded the last cocaine injection. Cocaine-induced cerebellar plasticity

was dramatically different from that which was observed when the withdrawal period was as short as one week (Vazquez-Sanroman et al. 2015).

Methods

Subjects and treatments

Four-week-old male Balb /c AnNHsd mice (Harlan, Barcelona, Spain) (N=32) were housed for four weeks in our animal facilities before the experiment was initiated. They remained in a 12-h light-dark cycle and had free access to food (Panlab S.L, Barcelona, Spain) and tap water. All experimental protocols were performed during the light phase. Daily handling and habituation to experimental procedures were addressed during the last two weeks preceding behavioural testing. All animal procedures were approved by the Jaume I University Ethical Committee for Animal Welfare and performed in accordance with the European Community Council Directive (86/609/ECC) and the Spanish directive BOE 34/ 11370/2013.

Cocaine administration

Mice received six saline (n=15) or cocaine (n=16) injections (20 mg/kg of cocaine hydrochloride, 2 mg/ml diluted in 0.9% saline) (Alcaliber, Madrid, Spain), one injection every 48 h, followed by a one-month withdrawal period. During this time, animals remained undisturbed in their home cages. On the 30^{th} day, the mice received either a final (7^{th}) saline or cocaine injection (10 mg/kg; 1 mg/ml). Animals were exposed to an open-field chamber immediately after each cocaine injection. This intermittent cocaine treatment has been shown to induce robust motor sensitisation (Miquel et al., 2003; Vazquez-Sanroman et al., 2015). All sessions were videotaped and analysed by a blind observer (see supplementary material (S1) and Vazquez-Sanroman et al. (2015) for task details).

Brain sampling

With respect to RT-PCR experiments (n=4) and western blot analyses (n=5), mice were sacrificed by cervical dislocation 2 or 24 h after the last cocaine injection, respectively. For immunofluorescence analysis (n=5), subjects were deeply anaesthetised with pentobarbital (Pfizer) (60 mg/kg) and perfused transcardially 24 h after the last cocaine or saline administration. The cerebellar vermis was sliced at 40 μm and used for sequential immunofluorescence labelling on free-floating sections (see S1 for additional information).

RNA extraction and real-time PCR analysis

Primer and probe sequences for BDNF variants were designed using the splice variants previously characterised and reported (Ng et al. 2012) (see Table 1 and S1). The complete procedure has been published previously elsewhere (Vazquez-Sanroman et al. 2015).

Western Immunoblotting

Pro-BDNF, mature BDNF, p75R, TrkB and tPA protein levels in the cerebellar vermis were quantified by western blotting, following the procedure as previously published (Vazquez-Sanroman et al. 2015) (see S1 and Table 2).

Immunofluorescence

Cerebellar sections were incubated with primary antibodies at 4ºC either overnight or for 48 h in PBS 0.1 M Triton X-100 and 1.5% serum. Cerebellar samples were then exposed to secondary antibodies conjugated with fluorochromes for 1 h at room temperature (Table 3 and S1).

Imaging analysis and morphometric estimations

Confocal images were acquired using a Nikon Eclipse-C1 confocal microscope (Nikon

Europe). Images were taken at the 1 μm-thick plane of acquisition in single planes at a resolution of 1024x1024. Quantitative and morphometric evaluations were made using the ImageJ free software (NIH sponsored image analysis software, USA) (Vazquez-Sanroman et al. 2015).

Statistical analysis

For all statistical analyses, we used the STATISTICA 7 software package (Statsoft, Inc., Tulsa, OK, USA). When data fulfilled normality requirements, they were analysed by means of parametric statistical tests (ANOVA) and expressed as the mean and standard error of the mean (SEM). Behavioural data were tested by means of two-way ANOVA of repeated measures. Posthoc mean comparisons were accomplished using Tukey tests that protect against Type 1 errors. The level of significance was set at $p < 0.05$. To compare proportions, Mann-Whitney U and X^2-tests were used.

Results

Motor sensitisation after chronic cocaine administration

As expected, mice that received repeated cocaine injections developed progressive orofacial stereotype sensitisation [two-way repeated measures ANOVA: the cocaine effect (df=1, 28; F=76.21; p<0.0001), number of injections effect (df=6, 168; F=25.22; p<0.0001) and interaction (df=6,168; F=24.12; p<0.0001)]. A Tukey LSD test demonstrated significant differences (p<0.001) from the fourth day of cocaine treatment. The persistence of sensitisation was revealed by a new cocaine challenge (p< 0.001) administered after a one-month withdrawal period (Figure 1). In addition, we observed an increase in cocaine-dependent locomotor stimulation that sensitised from the 3rd cocaine administration (p<0.0001) and which was retained after a one month period of withdrawal (Figure 1) [two-way repeated measures ANOVA: the cocaine

effect (df= 1, 28; F= 121.26 p<0.0001), number of injections effect (df=6, 168; F=29.00; p<0.01)] and interaction (df=6, 168; F=17.18; p<0.01)].

Cocaine-induced proBDNF and mature BDNF mechanisms in the cerebellum

We found that both proBDNF [one-way ANOVA (df= 1, 8; F=10.99 p<0.01)] and mature BDNF levels [one-way ANOVA (df= 1, 8; F=21.58 p<0.001)] were enhanced (Figure 2ab). The increased levels of both BDNF isoforms could have derived from a gradual enhancement of BDNF transcriptional activity during the long-term drug-free period. However, we did not observe significant differences in any of the BDNF exons evaluated (I, IV and VI). Instead, cocaine administration elevated the levels of tPA, the tissue plasminogen activator responsible for proBDNF cleavage [one-way ANOVA (df= 1, 8; F=35.51 p<0.001)] (Figure 2cd). As expected, both $P75^{NGFR}$ [one-way ANOVA (df= 1, 8; F=32.40 p<0.001)] and TrkB receptor levels [one-way ANOVA (df= 1, 8; F=88.06 p<0.001)] were also increased (Figure 2ef).

We focused the analysis on two anterior (III and VI) and two posterior (VIII and IX) cerebellar lobules to obtain a comprehensive sampling of the vermis. Cocaine only increased BDNF expression in the Purkinje somata of the posterior lobules VIII [one-way ANOVA (df= 1, 8; F=9.03 p<0.01)] and IX [one-way ANOVA (df= 1, 8; F=13.12 p<0.001)] (Figure 3ab). We also observed higher BDNF expression in the Purkinje dendritic tree in all of the lobules examined [one-way ANOVAs: Lobule III (df= 1, 8; F=22.73 p<0.001), Lobule VI (df= 1, 8; F=86.95 p<0.001), Lobule VIII (df= 1, 8; F=10.54 p<0.001) and Lobule IX (df= 1, 8; F=48.29 p<0.001)] (Figure 3ac).

Morphometric analysis of Purkinje neurons

Cocaine-treated mice showed a significant increase in the density of Purkinje dendritic spines. Cocaine effects were selectively seen in lobule VIII [one-way ANOVA (df= 1,8; F=7.96 p<0.05)] and lobule IX [one-way ANOVA (df= 1,8; F=21.33 p<0.01)] (Figure

4). Next, we addressed whether a longer cocaine drug-free period might also remodel Purkinje terminals and found an increase in the size of Purkinje terminals (Figure 5ac) [one-way ANOVA (df= 1,8; F=7.83 $p<0.05$)] but a decrease in the number of terminals per mm^2 (Figure 5bc) [one-way ANOVA (df= 1,8; F=9.29 $p<0.01$)]. This decrease in density was because the increased Purkinje terminal size was not accompanied by an expansion of neuron perikaryon in the deep medial nucleus [one-way ANOVA (df= 1,98; F=1.18 $p>0.05$)] (Figure 5).

Cocaine-dependent AMPA receptor-2 subunit (GluR2) expression in Purkinje neurons

GluR2 expression was selectively increased in the soma and the dendrites of Purkinje cells of lobule VIII [one-way ANOVA for somatic expression (df= 1, 8; F=11.07 $p<0.01$) and for dendritic expression (df= 1, 8; F=19.47 $p<0.001$)] and lobule IX [soma: (df= 1, 8; F=154.35 $p<0.001$), dendrites (df= 1, 8; F=22.67 $p<0.001$)] (Figure 6). **After preventing membrane permeabilisation of the GluR2 antibody, the signal was only maintained in the Purkinje dendrites of lobule IX, as indicated by Mann Whitney tests ($p<0.05$) (n=3) (Figure 7). This finding suggests an external position of GluR2 subunits, which occurred selectively in a posterior cerebellar region.**

GABA vesicular transporter immunolabelling

To estimate whether cocaine-induced changes could have impacted Purkinje inhibitory control on the deep medial nucleus neurons, we addressed a fluorescence immunostaining of the GABA vesicular transporter (vGAT) in Purkinje axon terminals (Figure 8). Previously, we observed that repeated experience with cocaine followed by a short withdrawal period reduced Purkinje activity (cFOS-IR) (Vazquez-Sanroman et al. 2015). Accordingly, we used cerebellar samples from mice exposed to such a condition as a positive control for the accuracy of procedural issues (Figure 8b). As expected, the number of Purkinje axon terminals surrounded by vGAT was reduced ($p<0.01$).

Therefore, we replicated the previously observed cocaine-dependent reduction in Purkinje activity. However, we failed to find any significant change in vGAT expression after a one-month period of withdrawal (Figure 8acd).

Changes in perineuronal nets (PNNs) after cocaine treatment

We observed that 76% of the medial nucleus neurons from cocaine-treated mice expressed faint WFA intensity [X^2 (1)=7.37 p<0.01)] compared to 44% from the saline group. Furthermore, cocaine treatment reduced the proportion of medial neurons exhibiting WFA medium intensity [X^2 (1)=10.28 p<0.01)].

Discussion

Behavioural abnormalities in cocaine addiction develop gradually and progressively during the course of repeated exposure to the drug and can last for months or years after the cessation of drug use (Nestler 2004). It has been suggested that the development of sensitisation after a repeated drug experience could promote the transition from recreational sporadic drug use to an escalated pattern of consumption in subjects with vulnerability (Piazza and Deroche-Gamonet 2013).

In the present study, cocaine-induced motor sensitisation was long lasting and persisted after a one-month withdrawal period. The expression of behavioural sensitisation was accompanied by changes in the cerebellum that were similar to those previously demonstrated in the striatumcortico-limbic circuitry (Grimm et al. 2003; Robinson et al. 2001). Cocaine-sensitised mice showed increased cerebellar BDNF levels, changes in the expression of Glu2 AMPA subunits and permissive conditions for neurite outgrowth in Purkinje neurons. Remarkably, these cocaine-induced cerebellar modifications are substantially different from the observed modifications when a withdrawal period of one week preceded the last cocaine challenge (Vazquez-Sanroman et al. 2015). Under such conditions, cocaine

promoted a cerebellar accumulation of proBDNF and higher levels of its receptor p75^{NGFR} to the detriment of mature BDNF mechanisms. These changes were associated with pruning in the dendritic spines, a reduction in size and density of the Purkinje synaptic terminals, and an increase in the proportion of deep nucleus neurons expressing strong perineuronal nets.

Cocaine raised both proBDNF and mature BDNF mechanisms, promoting dendritic spine growth and remodelling of axon terminals in Purkinje neurons

Several studies have found that repeated non-contingent exposure to cocaine leads to an increase in endogenous BDNF (for recent reviews see Li and Wolf 2014; McGinty et al. 2010). **The present findings indicate that both proBDNF and mature BDNF mechanisms were enhanced in the cerebellum of cocaine-sensitised animals. Nevertheless, we did not find cocaine-associated changes in mRNA BDNF levels. Supporting our results, endogenous protein levels but not mRNA levels have been found to be enhanced in the NAc of cocaine-treated animals after long periods of withdrawal (Li et al. 2013). As an explanation for this dissociation, it has been reported that BDNF might increase if the translation of pre-existing mRNA is induced (Lau et al. 2010). Indeed, cocaine effects on cerebellar BDNF appear to rely on post-transcriptional mechanisms. In the present conditions, the concentration of the tissue plasminogen activator tPA was enhanced in cocaine-treated cerebella. Thus, it appears that proBDNF levels were raised by cocaine,** but because the cleavage was also stimulated, the precursor and mature stages of the protein remained balanced.

Previous research demonstrated increases in mRNA tPA levels in the NAc, striatum, VTA and hippocampus after chronic cocaine administration (Bahi and Dreyer 2008). Strikingly, mice lacking tPA exhibited enhanced locomotor sensitisation after a repeated

experience with cocaine (Ripley et al., 1999). However, in another study, cocaine-induced sensitisation was attenuated in tPA knockout mice (Maiya et al., 2009). Moreover, wild-type animals overexpressing tPA in the NAc also demonstrated enhanced sensitivity to chronic amphetamine and morphine administration (Bahi and Dreyer 2008). Overall, the results were contradictory and difficult to explain. On the one hand, opposite tPA manipulations led to an enhancement of sensitivity to cocaine effects. On the other hand, by deleting tPA, conflicting results were also observed.

The functional role of mature BDNF in parallel fibres/Purkinje synapses was mediated by TrkB receptors (Lu and Figurov 1997). The activation of TrkBR by stimulating currents of sodium conductance enhances $Ca2^{+}$ influx into dendritic spines, thereby fostering dendritic plasticity (Kafitz et al. 1999; Kovalchuk et al. 2002). Thus, BDNF might stimulate activity-dependent dendritic sprouting and axonal remodelling (Jeanneteau et al. 2010; Tanaka et al. 2008). Accordingly, repeated exposure to cocaine followed by an extended abstinence increased dendritic branching in the NAc (Robinson et al. 2001). In the present study, BDNF expression in Purkinje cells was accompanied by a high density of dendritic spines and a larger size of presynaptic terminals contacting medial nuclear projection neurons. Nevertheless, we failed to find higher Purkinje activity after these cocaine-induced changes, likely because Purkinje neurons exhibit high spontaneous activity (De Zeeuw et al. 1994).

A few studies have strongly suggested that a causal relationship between cocaine-dependent enhancement of endogenous BDNF levels and the development of drug-induced sensitisation exists. Either forebrain over-expression of a dominant negative TrkB receptor or a selective suppression of BDNF expression by a conditional knockout indicates that the development of cocaine-induced motor sensitisation relies on endogenous BDNF mechanisms (Crooks et al. 2010; Huang et al. 2011). Moreover,

increasing both BDNF synthesis and release in striatalcortico-limbic neurons is critical to promoting lasting changes in synaptic strength, which underlies psychostimulant-induced sensitisation (Bahi et al. 2008; Grimm et al. 2003; Robinson et al. 2001). Nonetheless, further research is needed to clarify whether a cocaine-dependent increase in cerebellar BDNF is functionally linked to the development of motor sensitisation.

Cocaine enhances the expression of GluR2 AMPA subunits on the cell surface of Purkinje neurons

In the cerebellum, unlike other brain areas, plasticity of Purkinje-parallel fibre synapses depends almost completely on GluR2 subunit trafficking (Hansel et al. 2005; Kakegawa and Yuzaki 2005; Petralia et al. 1997). When delivering to the Purkinje cell surface in an activity-dependent manner, GluR2 subunits promote long-term potentiation in these synapses. On the contrary, if these receptors are internalised, Purkinje neurons develop long-term depression (Kakegawa and Yuzaki 2005). Our recent results indicate that Glu2R expression in the cerebellar vermis was increased in cocaine-sensitised mice (Vazquez-Sanroman et al. 2015). Moreover, by preventing membrane permeabilisation, GluR2 expression was precluded selectively in dendrites, suggesting Glu2R subunit endocytosis.

Now, we also find an upregulation of GluR2 expression in cocaine-treated cerebella. However, after preventing the penetration of the antibody, the GluR2 signal was still clearly observed in the Purkinje dendritic tree of lobule IX. These results suggest GluR2 insertion and maintenance on the cell surface in this lobule. **Therefore, it seems that GluR2 trafficking toward synapses was stimulated during long withdrawal in selective cerebellar regions of cocaine-sensitised animals.** Interestingly, a bidirectional relationship between BDNF and AMPARs subunit expression has been suggested. In hippocampal and neocortical cell cultures, BDNF can regulate GluR2

trafficking, thus promoting their expression on the cell surface (Caldeira et al. 2007; Narisawa-Saito et al. 2002). Moreover, it has been hypothesised that AMPARs surface expression in the NAc could be responsible for the cocaine-induced increase in endogenous BDNF (Li and Wolf 2014).

Under the present conditions, there was a regionalisation of Glu2R expression. **The effect was selectively observed in the posterior cerebellum, lobules VIII and IX. It has been further determined that the expression of a cocaine-induced preference towards odour cues is correlated with activity in these cerebellar regions (Carbo-Gas et al. 2014ab). In humans, these lobules have also been found to be activated by cocaine-related cues (Anderson et al. 2006; Grant et al. 1996)**. **Additionally, Lobule VIII is a part of the sensorimotor network connected to motor and premotor areas and to the somatosensory cortex (Bostan et al. 2013; Suzuki et al. 2012), and importantly, it is involved in automating behavioural repertoires towards drug-related cues (Miquel et al. 2009; Moulton et al. 2014; Yalachkov et al. 2010).**

However, a causal link between cocaine-induced cerebellar plasticity and the development of sensitisation has not been demonstrated thus far. Nonetheless, while similar changes in the NAc have been associated with cocaine-induced sensitisation following a 14-day withdrawal period, such changes have not been found after a shorter period of 24 h (Boudreau et al. 2007; Boudreau and Wolf 2005; Ghasemzadeh et al. 2009).

Cocaine facilitates conditions for structural remodelling in the deep medial nucleus neurons

At the end of brain development, several neuronal phenotypes express perineuronal nets (PNNs), a specialised extracellular matrix composed of chondroitin sulfate

proteoglycans surrounding the soma and restricting neuronal plasticity to stabilise circuits (Carulli et al. 2006; Foscarin et al. 2011). The large glutamatergic projection neurons in the deep medial nucleus of the cerebellum are enveloped by PNNs. These PNNs are under the dynamic regulation of environmental factors (Foscarin et al. 2011). Previously, we demonstrated that restrictive structural plasticity in Purkinje neurons of cocaine-treated mice was accompanied by an upregulation of PNNs in these large glutamatergic medial nuclear neurons that project out from the cerebellum (Vazquez-Sanroman et al. 2015). Now, we again obtained contrasting results. When a long withdrawal period was included, cocaine decreased the expression of PNNs in the medial neurons and facilitated further synapsis remodelling.

The maintenance and restructuring of the extracellular matrix components are enzymatic-dependent. The matrix metalloproteases (MMPs) are a family of proteolytic enzymes that participate in the remodelling of the ECM (Stamenkovic 2003) and require serine proteinases such as plasmin or other MMPs for activation. Indeed, tPA plasminogen protease contributes to the conversion of pro-MMPs to active MMP forms (Wright and Harding 2009). Therefore, one can expect increasing levels of tPA to produce higher MMP activity and thereby a down-regulation in PNNs. In agreement with this hypothesis, when tPA was not affected by cocaine, as was the case in our earlier study, PNN structure was maintained. However, when tPA was enhanced, extracellular matrix expression decreased. Restructuring the extracellular matrix in the whole brain after the inhibition of the MMPs could reduce sensitivity to drug-related cues, thereby preventing reinstatement and relapse (Brown et al. 2007; Van den Oever et al. 2010). **Furthermore, two recently published studies have demonstrated anatomical and functional specificity of the effects of PNN disruption (Slaker et al., 2015; Xue et al. 2014), while RECK, a membrane-anchored MMP inhibitor, has**

been found to be overexpressed in the hippocampus of cocaine addicts (Mash et al. 2007). Thus, strategies that target the regulatory molecules of the extracellular matrix may restore or restrict the neuronal plasticity potential.

Concluding remarks

Overall, our findings have again demonstrated the ability of cocaine to modify molecular and structural plasticity in the cerebellum. **In the present investigation, we used the same cocaine dose and same number of injections as previously published (Vazquez-Sanroman et al. 2015). However, we observed contrasting cocaine-induced effects on cerebellar plasticity. It is noteworthy that the only difference between our previous investigation and the present one was the length of the withdrawal period included before the last cocaine challenge. Our current findings indicate that following a prolonged withdrawal, a new cocaine challenge revealed a different trend in dendritic and axonal Purkinje remodelling. In this case, Purkinje neurons appeared to increase their input and output strength as a result of the cocaine treatment. Similar plastic modifications have been described in the striatum and linked to the incubation of craving after long periods of withdrawal (Li et al. 2013; Loweth et al. 2014).**

Remarkably, the cerebellar changes shown here do not seem to be exclusively due to the repeated experience with cocaine. Rather, to be induced, the changes seem to require a washout time. Nevertheless, an important matter for future consideration is to know the extent to which this cerebellar plasticity contributes to the observed long-lasting motor sensitisation effect.

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Table 1. Sequences of primers used in RT-PCR protocol

Gene	Forward	Reverse
mBDNF I	5′ttaccttcctgatctgttgg3′	5′gtcatcactcttctcacctgg3
mBDNF IIC	5′ggctggaatagactcttggc3′	5′gtcatcactcttctcacctgg3′
mDNFB IV	5′agctgccttgatgtttactttg3′	5′cgtttacttctttcatgggcg3′
mBDNF VI	5′ggaccagaagcgtgacaac3′	5′atgcaaccgaagtatgaaataacc3′
tPA	5'tgtctttaaggcagggaagt3'	5'gtcacacctttcccaacata3'

Table 2. Western blot antibodies and conditions

Protein	**SDS-PAGE**	**□g**	**Transfer Parameters**	**Primary Ab**	**Goat anti-rabbit peroxidase-conjugated (Bio-Rad, USA)**
pro-BDNF	15%	60	300 mA/2 h	1:100; 32 kDa band (sc-546; Santa Cruz Biotechnology, Santa Cruz, CA, USA)	1:25,000
mature-BDNF	15%	60	300 mA/2 h	1:100; 17 kDa band (sc-546, Santa Cruz, Biotechnology, Santa Cruz, CA, USA)	1:25,000
$p75^{NGFR}$	10%	30	90 volts/1 h	1:500; 75 kDa band (ab8874, Abcam, Cambridge, UK)	1:50,000
TrkB	10%	30	90 volts/1 h	1:500; 145 kDa band (07-225, Millipore, Billerica, Massachusetts, USA)	1:50,000
tPA	10%	60	90 volts/1 h	1:100; 70 kDA band (sc-15346, Santa Cruz Biotechnology, Santa Cruz, CA, USA)	1:50,000

Table 3. Primary and secondary antibodies

Primary antibody	**Secondary antibody**
Rabbit anti-BDNF (1:100; sc-20981, Santa Cruz Biotechnology, Santa Cruz, CA, USA)	Donkey anti-rabbit FITC (1:200; FI1000, VectorLabs, Peterborough, UK)
Mouse anti-Calbindine D28K (1:1500, CB300, Swant, Switzerland) Rabbit anti-Calbindine D28K (1:1500, CB38, Swant, Switzerland)	Cy3 conjugated donkey anti-mouse (1:250; 715-167-003, Jackson ImmunoResearch labs, Pennsylvania, USA)
Mouse anti-SMI32 (1:500, SMI3212, Sterbenger, Covance, USA).	Donkey anti-mouse Alexa Fluor 647 (1:500; 715-605-150, Jackson ImmunoResearch labs, Pennsylvania, USA)
Wisteria floribunda agglutinin (WFA) (1:200, L1516-2MG, Sigma Aldrich, Madrid, Spain)	Donkey anti-rabbit Alexa Fluor 488 (1:500; A-2106, Invitrogen Life Technologies, New York, USA)
Mouse anti-glutamate receptor 2 (GluR2) monoclonal antibody (1:75, 32-0300, Invitrogen, California, USA)	Biotinylated goat anti-rabbit SA-506 (1:250,VectorLabs, Peterborough, UK) Streptavidin Texas red (1:500, VectorLabs, Peterborough, UK)
Guinea pig polyclonal anti-vesicular GABA transporter (vGAT), (1:100, 131004, Synaptic Systems, Göttingen, Germany)	

Legends

Fig. 1 Cocaine-induced motor behaviour a) Mice received six cocaine injections, one injection every 48 h. They then remained undisturbed in their home cages for one month. On the 30th day, animals were injected with a lower cocaine dose 24 h before perfusion**.** b**)** Subjects were tested in an open field after six alternant saline (n=15) or cocaine (n=16) injections. Mean ± SEM of stereotypes (time spent sniffing and head bobbing) were compared throughout the period. Mean ± SEM of locomotion counts were compared throughout the period (*p<0.05, **p<0.01, p<0.001 between-group comparisons; ## p<0.01; ###p<0.001 within-group comparisons).

Fig. 2 Protein levels of BDNF mechanisms in the cerebellum In all cases, protein levels were expressed as the percentage of α-tubuline expression**.** a) Data represent the average BDNF levels (Mean + SE; ** p< 0.01, ***p<0.001). b) Representative immunoblottings for the two bands were detected by the antibody rabbit anti-BDNF. The proBDNF and mature BDNF bands detected corresponded to 32 and 17 kDa MW, respectively. BDNF antibody specificity had been tested previously elsewhere (Vazquez-Sanroman et al. 2015). c) Data represent the average of the tpA levels (Mean + SE; ***p<0.001). d) Representative immunoblottings for tPA band 40 kDa. e) Data represent the average receptor levels (Mean + SE; ***p<0.001). f) Representative immunoblottings for P75^{NGFR} band 32 kDa and TrkBR band 95 kDa. In all cases, two replicas were performed per subject (n = 5). Precursor and mature BDNF protein mechanisms as well as tPA protein levels increased in cocaine-treated mice.

Fig. 3 BDNF expression in Purkinje neurons a) Confocal images of BDNF expression (green) in soma and dendrites of Purkinje neurons (red) of lobule IX. The confocal images were taken at 80x. Scale bar 20 μm. b) Percentage of BDNF+ Purkinje somas from the total number of somas labelled with calbindine (CBL). c) Densitometry of BDNF expression in the Purkinje dendritic tree (Mean + SE; **p<0.01). Purkinje neurons of the posterior cerebellum increased BDNF expression in cocaine-treated subjects.

Fig. 4 Cocaine effects on Purkinje dendritic spine density a) The dendritic tree was visualised using calbindine (CBL) (red). Microphotographs were then converted to a grey-RGB scale. Pictures were acquired at 40x with a 4x zoom for a final amplification of 160x. Scale bar 10 μm. b) Number of dendritic spines per mm^2 throughout the lobules analysed (Mean + SEM; *p<0.05, ***p<0.001) (***p<0.001, *p<0.05).

Dendritic spine density selectively increased in Purkinje neurons of lobules VIII and IX in cocaine-treated mice.

Fig. 5 Cocaine effects on synaptic Purkinje boutons contacting medial nuclear projection neurons Cocaine effects on the perimeter (a) and density (b) of Purkinje terminals contacting medial neurons (Mean + SEM *p<0.05; **p<0.01). Purkinje-medial neuronal contacts increased the size and reduced the density in cocaine-treated mice. c) Confocal images were taken with 40x objective and a 2.0x zoom for a final magnification of 80x. We used anti-SMI32 antibody (blue) to identify medial nuclear neurons and calbindine (green) to visualise Purkinje synaptic terminals. Scale bar 20 μm; amplification bar represents 10 μm.

Fig. 6 GluR2 expression in Purkinje neurons a) Data represent the average positive Purkinje somas for GluR2 throughout the cerebellar lobules assessed. b) Densitometry of GluR2 expression at the Purkinje dendritic tree. The analysis was performed in a ROI of 90,000 μm^2 (**p<0.01, ***p<0.001). c) Confocal images of GluR2 expression (red) in Purkinje neurons stained by calbindine (green). Scale bar of 20 μm.

Fig. 7 GluR2 expression in Purkinje somas and dendrites after preventing membrane permeabilisation of the GluR2 antibody a) Confocal images of GluR2 expression. Scale bar of 20 μm. We conducted an immunofluorescence labelling of Glu2R expression, but this time prevented membrane permeabilisation to determine the internal or external position of GluR2 subunits (n=3). GluR2 expression was retained in cocaine-treated animals, and it was selectively enhanced in the Purkinje dendritic tree of lobule IX.

Fig. 8 Vesicular GABA transporter (vGAT) expression in the deep medial neurons surrounded by Purkinje axon projections Confocal images of vGAT were taken with 40x objective and a 2.0x zoom for a final magnification of 80x. We used CBL (green) to visualise Purkinje synaptic terminals and anti-vGAT antibody (red) to identify vGAT expression. White arrows point to an example of a double labelled deep medial neuron. Scale bar 20 μm. a) After a one-month withdrawal period. b) After a one week withdrawal period. Intensity of vGAT staining was not significantly affected by cocaine treatment.

Fig. 9 Perineuronal nets (PNN) in the medial nucleus a) Confocal images (80x) of medial nuclear projection neurons (SMI32 blue) bearing a perineuronal net identified by

a *Wisteria floribunda agglutinin* (WFA) (red). Scale bar represents 20 μm. b) Proportion of SMI32-WFA positive deep medial neurons for each WFA intensity condition. Cocaine reduced the proportion of neurons expressing strong and medium intensities.

Supplementary material

Behavioural testing

The sensitisation protocol involved six cocaine injections given on alternate days (20 mg/kg). After a one month withdrawal period, a new cocaine challenge with a lower dose (10 mg/kg) was administered. In previous studies, it was found that intermittent cocaine administration leads to increased motor sensitisation (see Robinson and Berridge 2003 for a review). This finding seems to be because neuroadaptations are promoted through intermittent drug treatment (Heidbreder et al., 1996; Robinson and Berridge 2003). Thus, when a lower drug dose is administered, motor sensitisation is easily revealed. In fact, this procedure has been used in previous studies, thereby revealing consistent and robust cocaine-induced behavioural sensitisation (Miquel et al., 2003, Vazquez-Sanroman et al., 2015). The open field chamber consisted of a clear glass cylinder 25 cm in diameter and 30 cm in height in a test room illuminated with soft white light. The base of the cylinder was divided into four equal quadrants by two intersecting lines drawn on the floor. For the evaluation of locomotion, the whole 15-min period was considered. A locomotion score was assigned every time an animal crossed from one quadrant to another on all four legs. For behavioural stereotypes, we considered the number of seconds spent sniffing and head bobbing during each of the three representative minutes throughout the 15-min period (3' to 4'; 7' to 8'; 13' to 14').

Brain sampling

Different control and experimental groups were used for molecular and cellular experiments. For RT-PCR experiments (n=4) and western blot analysis (n=5), mice were sacrificed by cervical dislocation 2 h or 24 h after the last cocaine injection, respectively. For both protocols the cerebellar vermis was immediately removed,

dissected and frozen in liquid nitrogen and stored at -80ºC. For immunofluorescence analysis (n=5), subjects were deeply anaesthetised with pentobarbital (Pfizer) (60 mg/kg) and perfused transcardially with a saline solution at 0.9% followed by 4% paraformaldehyde at room temperature 24 h after the cocaine administration. The brains were cryoprotected in 30% sucrose solution for a 72-h period. The cerebellar vermis was sliced at 40 μm and used for sequential immunofluorescense labelling on free-floating sections.

RNA extraction and real-time PCR analysis

To prevent contaminating DNA, the samples were treated with DNAse I. The tissue was ground to a fine powder in liquid nitrogen and homogenised using a Polytron Ultraturrax T25 basic (Ika Labortechnik). Quantification of RNA was performed with a Nanodrop 1000 spectrophotometer (Fisher Scientific). Total RNA extracted was used to synthesise cDNA with the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Total RNA was extracted from the cerebellar vermis using the RNeasy Lipid Tissue Mini Kit (Qiagen Inc.) according to the manufacturer's instructions. Reactions were conducted at 25ºC for 10 min, 37ºC for 120 min and 85ºC for 5 min in a Mastercycler (Eppendorf). Real-time PCR was conducted using the SYBR Green PCR Kit (Thermo Scientific) and the SmartCycler II instrument (Cepheid). The parameters were set as follows: 95ºC for 10 min, followed by 45 cycles of 95ºC for 15 sec and 60ºC for 1 min.

Western immunoblotting

Cerebellar tissue samples were homogenised in an ice-cold lysis buffer [137 mM NaCl, 20 mM Tris–HCl (pH 8.8), 1% NP40, 10 □g/ml of aprotinin, leupetin, 0.5 mM orto sodium vanadate and 0.1 mM PMSF, protease inhibitors] and quantified for a final protein concentration as required for each experiment (different amounts of protein

were used in each experiment). Homogenates were centrifuged at 14,000 rpm for 15 min at 4°C. Aliquots of supernatants were collected and used for Bradfort quantification of total protein, and the remaining aliquots were stored at −80°C until analysis. Every sample was boiled for 5 min. Equal amounts for each protein were separated by SDS-PAGE during a period of 1 h at 90 volts, and then transferred to a nitrocellulose membrane. Membranes were blocked overnight with 5% non-fat dry milk, filters were then reacted with goat anti-rabbit peroxidase-conjugated antibody and developed by enhanced chemiluminescence. Antibodies and concentrations are presented in Table 2. Filters were probed with anti-αtubulin monoclonal antibody (1:1000; Chemicon, Millipore) or GAPDH (1:150,000; Sigma Aldrich) as internal standards for protein quantification. The film signals were scanned at 600 dpi (EPSON 11344) and the levels of the band density were processed with FIJI software (Schindelin et al. 2012).

Immunofluorescence

Following several rinses with PBS 0.1 M 0.25% triton X-100 and followed by a pre-block in 15% donkey serum, cerebellar sections were incubated either with primary antibodies at 4ºC overnight or for 48 h in PBS 0.1 M Triton X-100 and 1.5% serum (see Table 3 for further details about antibodies and concentrations used). After several rinses, tissue was incubated for 1 h at room temperature with secondary antibodies conjugated to fluorochromes (see Table 3). Once fluorescence reaction occurred, the sections were mounted in Mowiol (Calbiochem).

Imaging analysis and morphometric estimations

The number of BDNF and GluR2 positive Purkinje somas was quantified by selecting an ROI of 90,000 μm^2. We also evaluated BDNF and GluR2 signals in the Purkinje dendrites by densitometry in an ROI of 90,000 μm^2 at the molecular layer. The densitometry analysis was performed with ImageJ software by subtracting the

background acquired in an area where the BDNF signal was not present from the brightness obtained in the ROI placed in the dendritic tree at the molecular layer. Densitometry data for the analysis were the average of the signals obtained from three cerebellar slices per animal.

To estimate the number of dendritic spines in Purkinje neurons, we stained neurons with immunofluorescent Calbindine 28K (Vazquez-Sanroman et al. 2015). The confocal images were taken with a 40x objective and a 4x zoom for a total magnification of 160x. Pictures were then converted with split channel plugging into a grey scale. Two different ROIs of 10,000μm were traced in the Purkinje dendritic tree, one proximal to the soma and the other in the distal region of the tree.

We also assessed the density and size of Purkinje axon (PC) terminals contacting the soma of the large projecting neurons in the medial nucleus (DCN) by labelling nuclear neurons with *neurofilament-H non-phosphorylated* (SMI32). For each animal, we measured the perimeter of 50 DCN neurons in which the soma was visible. Only those DCN neurons in which the soma was clearly visible by SMI32 were included in the analysis. The size of Purkinje axon terminals was measured by drawing a line around the perimeter of the terminal visualised by calbindine staining. One hundred Purkinje terminals were analysed per animal. Due to variability in the size of PC terminals, we corrected the raw data by applying the Abercrombie formula (Abercromie 1946). We also traced the perimeter of DCN neurons using ImageJ in the same confocal images with a 40x objective and a 2.0x zoom for a final magnification of 80x.

To address whether Purkinje inhibitory control onto DCN medial neurons changed as a result of cocaine-dependent molecular and structural modifications, we analysed the vesicular GABA transporter signal (vGAT) surrounding Purkinje terminals. We co-labelled the DCN slices with anti-calbindine and anti-vGAT antibodies (see Table 3). In

each microphotograph, all CLB+/vGAT+ terminals were identified and a vGAT staining intensity analysis was performed on confocal images taken under a 40x objective and a 2.5x zoom for a final magnification of 100x. We measured the brightness intensity (range 0-255) of 50 vGAT+ terminals by randomly selecting 15 pixels around the net formed by the synaptic Purkinje terminals and calculated their average intensity. The background brightness, taken from a non-stained region of the cortical molecular layer, was subtracted from the brightness measurements. For each vGAT+ neuron, intensity was arranged in three categories from the lowest (faint) to the highest (strong) intensity.

Perineural nets (PNNs) in the medial projection neurons

To evaluate the proportion of DCN neurons supporting a PNN, thecerebellar sections where the medial nucleus was clearly visible by *Wisteria floribunda agglutinin* (WFA) immunochemistry were labelled. In each section, we sampled all of the SMI32+ DCN neurons stained and counted how many of them were surrounded by WFA. Additionally, we performed an analysis of WFA staining intensity on 80x confocal images. We measured the brightness intensity (range 0-255) of 50 PNNs+ neurons per animal by randomly selecting 15 pixels in the PNN and calculating their average (as previously explained). Each net was assigned to one of three categories of staining intensity that ranged from the lowest to the highest value of WFA intensity: faint= 0 to 33%, medium= 34 to 66% and strong= 67 to100% of the maximum staining intensity (Foscarin et al. 2011; Vazquez-Sanroman et al. 2015).

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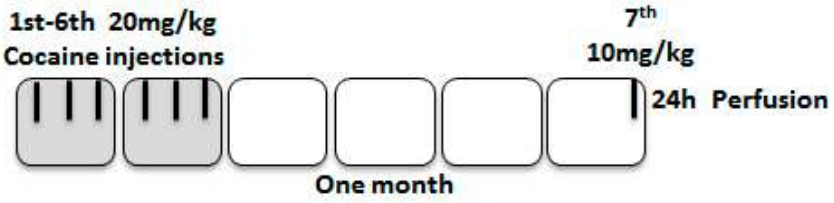
a



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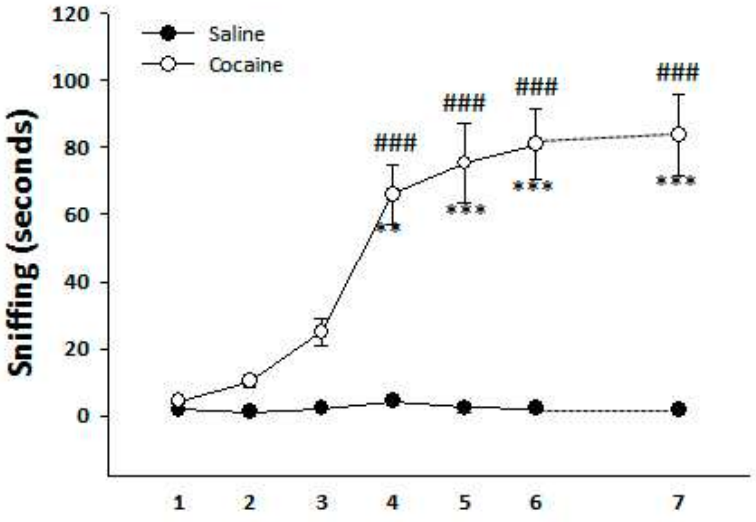
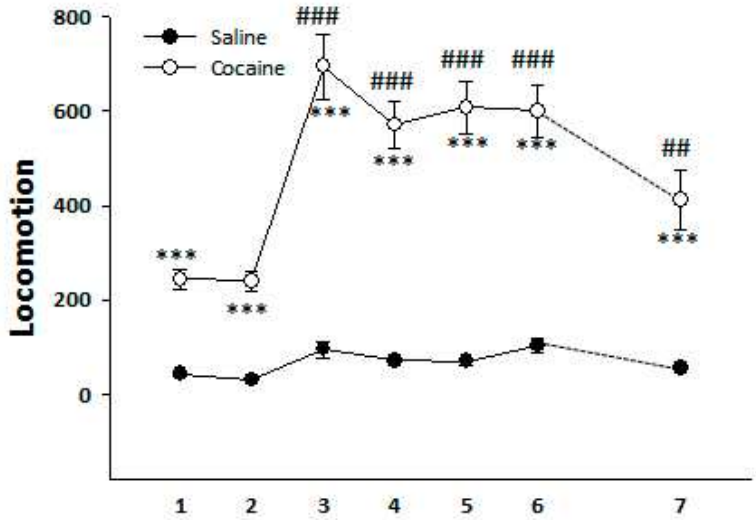
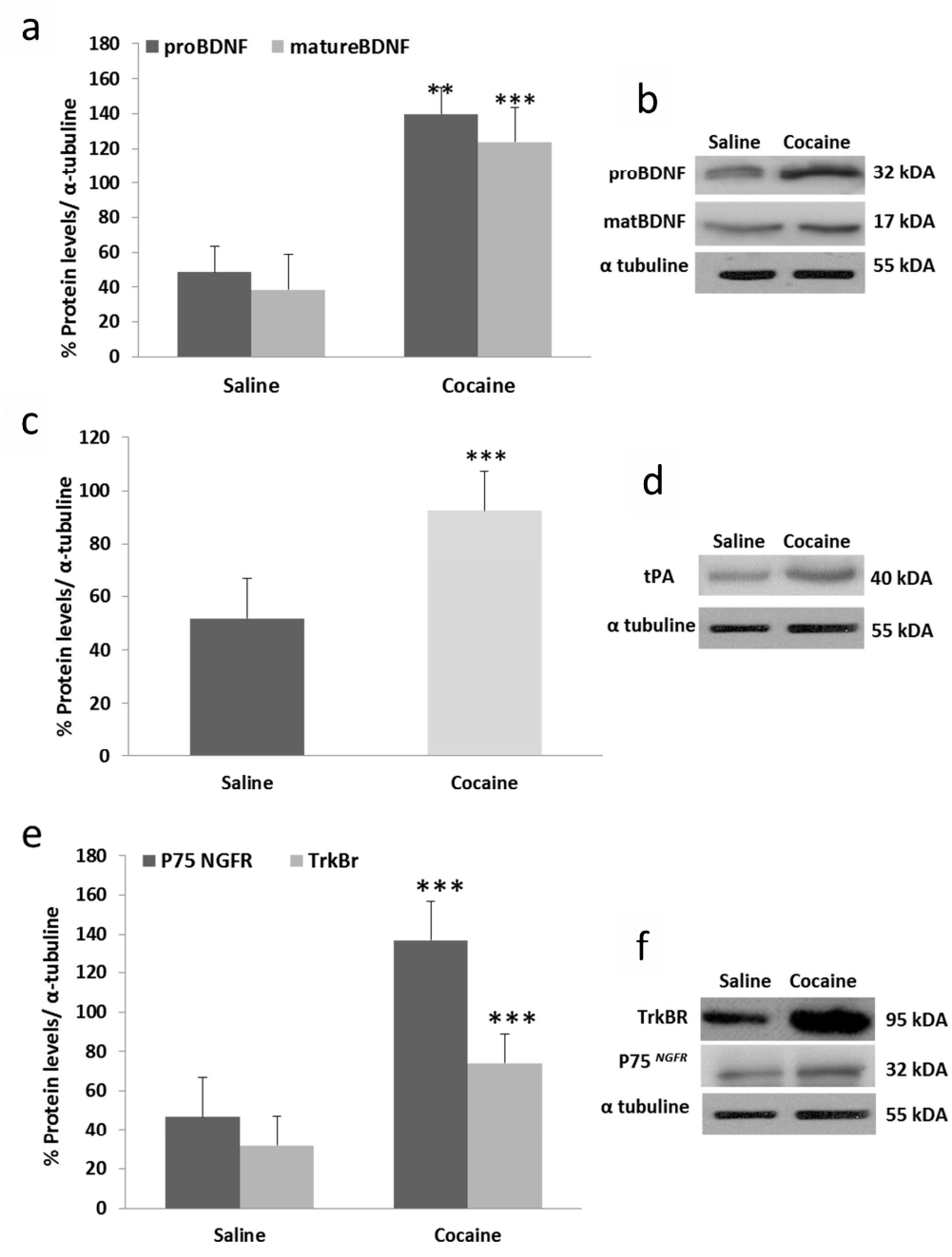




Administrations of cocaine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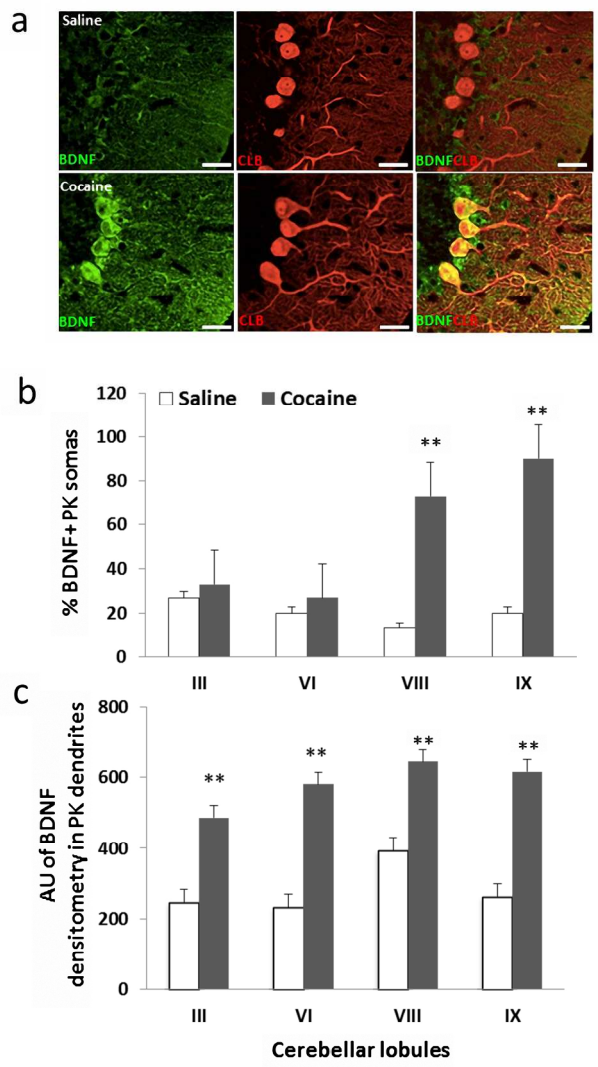

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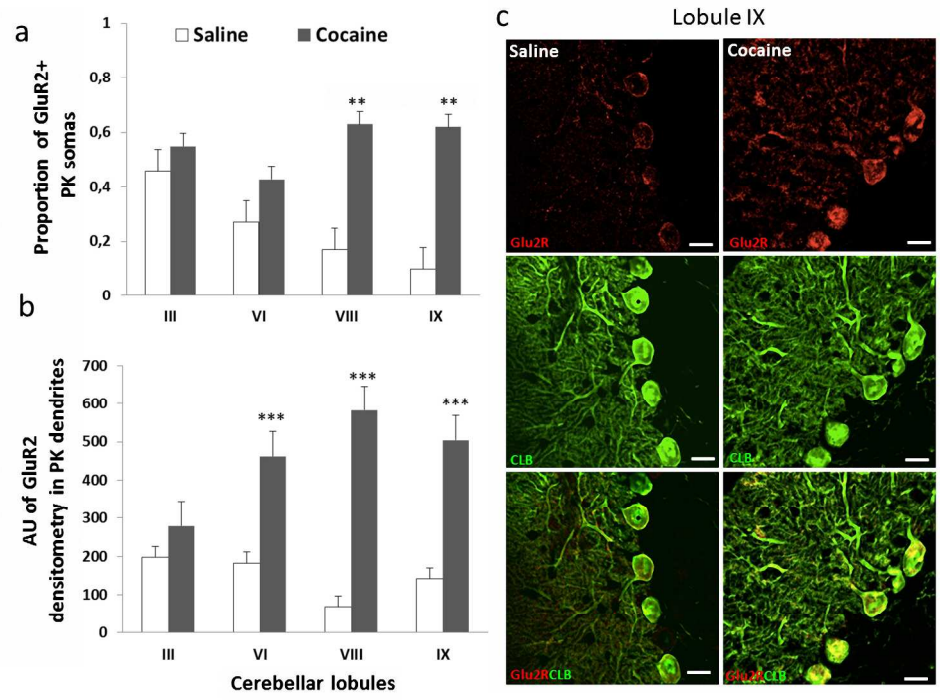

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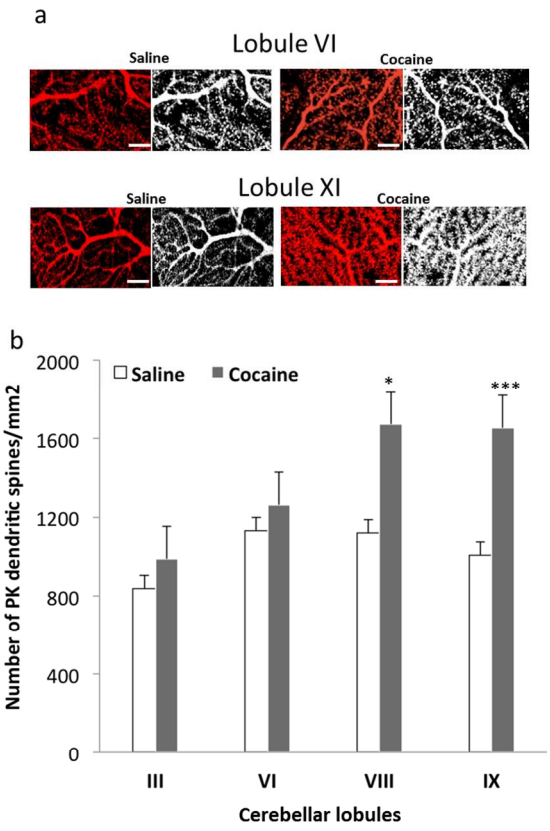

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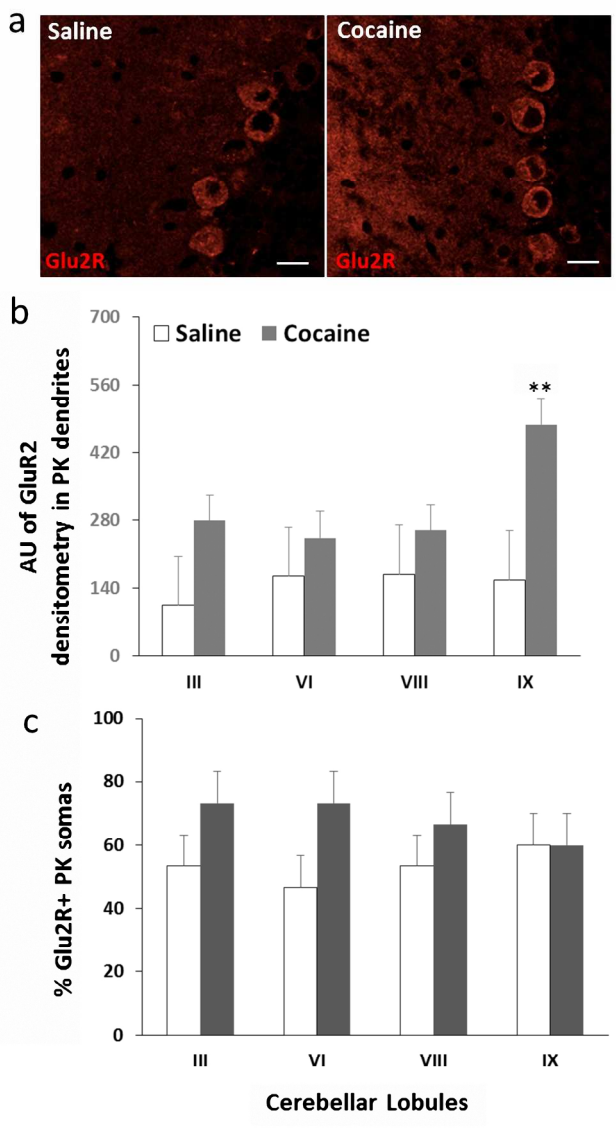

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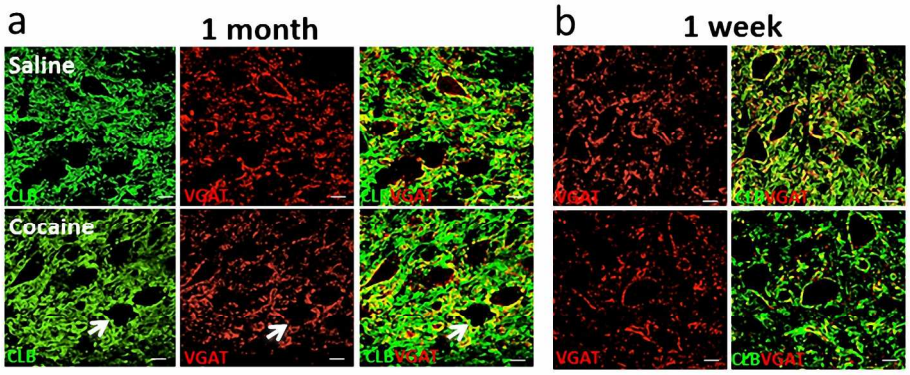

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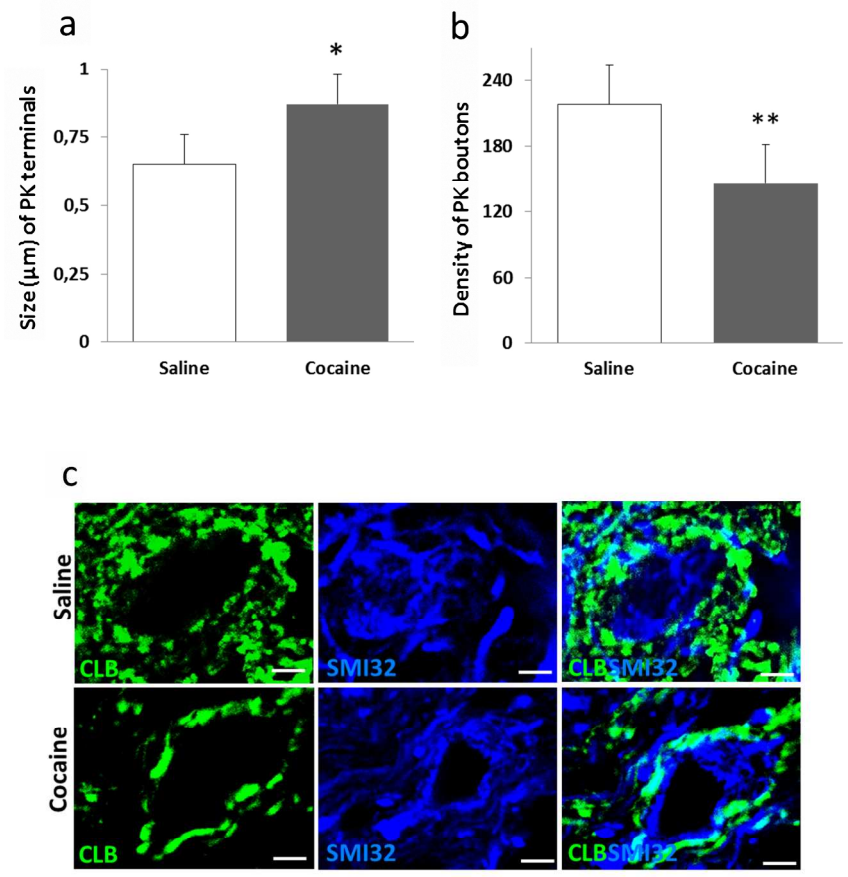
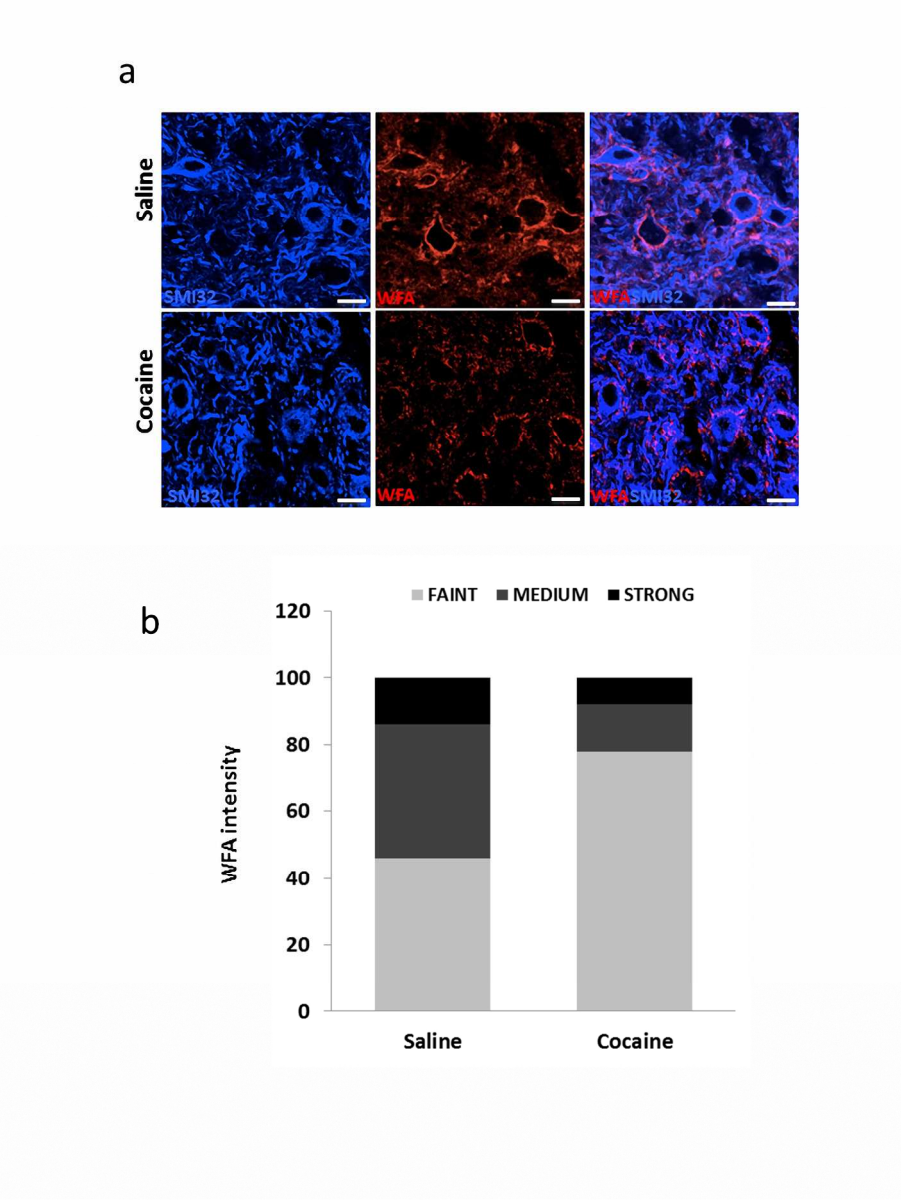


190x254mm (300 x 300 DPI)



190x254mm (300 x 300 DPI)