Involving the cerebellum in cocaine-induced memory: Pattern of cFos expression in mice trained to acquire conditioned preference for cocaine.

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Conflict of Interest (COI) Statement

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

Abstract

Due to its capital role in drug seeking, consumption and addictive behaviour there is a growing interest in identifying the neural circuits and molecular mechanisms underlying the formation, maintenance and retrieval of drug related memories. Human studies focused on neuronal systems that store and control drug-conditioned memories have found cerebellar activations during the retrieval of drug-associated cues memory. However, at the preclinical level, almost no attention has been paid to a possible role of the cerebellum in drug-related memories. In the present study, we ought to fill this gap by aiming to investigate the pattern of neuronal activation (as revealed by cFos expression) in different regions of the prefrontal cortex and cerebellum of mice trained to develop conditioned preference for an olfactory stimulus (CS+) paired with cocaine. Our results indicate that CS+ preference was directly associated with cFos expression in cells at the apical region of the granule cell layer of the cerebellar vermis, this relationship being more prominent in some specific lobules. Conversely, cFos+ immunostaining in other cerebellar regions seems unrelated to CS+ preference but to other aspects of the conditioning procedure. At the prefrontal cortex, cFos expression seemed to be related to cocaine administration rather than to its ability to establish conditioned preference. The present results suggest that as it has been observed in some clinical studies, the cerebellum might be an important and largely overlooked part of the neural circuits involved in generating, maintaining and/or retrieving drug memories.

Key words: cocaine, preference, conditioning, cerebellum, vermis, mice.

Introduction

Several processes underlie motivational alterations in drug seeking and taking behaviour. Indeed, conditioned reinforcement, incentive motivation, behavioural sensitization and maladaptive stimulus–response learning, all contribute to orienting the response toward drug-related stimuli (Kalivas & Volkow 2005; Hyman, Malenka & Nestler 2006; Everitt et al. 2008; Robinson & Berridge 2008; Koob & Volkow 2010). Specifically, Pavlovian conditioning tunes the motivational impact of drug-associated stimuli by strengthening memory of drug-related cues and thus, boosting the importance of stimuli and contexts that enclose drug seeking and taking (Everitt & Robbins 2005). Drug associated cues and contexts guide drug-seeking and have an important effect on drug intake, gaining progressively more control over an individual's behaviour as some of them transit through successive behavioural stages towards habitual consumption and ultimately reaching the addicted state.

Due to the relevance for drug seeking and taking, there has been a growing interest in identifying the neural circuits and molecular mechanisms underlying the formation, maintenance and retrieval of drug related memories. It has been argued that Pavlovian and instrumental conditioned memories are controlled and stored by dopamine-glutamate interactions into the nucleus accumbens, basolateral amygdala, hippocampus and prefrontal cortex (Bower & Parson 2003). Chronic drug abuse produces a re-organization of these prefronto-striatal-limbic networks via their effects on neurotransmitter systems (Nestler 2005), neuronal morphology (Nestler 2005) and functional interactions within and between neuronal assemblies that belong to this circuitry (Belin & Everitt 2008; Noorin et al. 2012).

Over the past decades, it has become clear that the cerebellum constitutes functional loop circuits with different brain areas previously involved in drug effects

and addictive behaviour such as prefrontal and associative non-motor cortices, the basal ganglia (Bostan, Dum & Strick 2010) and limbic system (Heath et al. 1978). Remarkably, several cerebellar regions have bidirectional connections with the prefrontal and sensorimotor cortices (Dum & Strick 2003; Kelly & Strick 2003), and the striatum (Hoshi et al. 2005; Bostan et al. 2010). Additionally, the medial part of the cerebellum (vermis) connects to dopamine neurons in the ventral Tegmental Area (VTA) and substantia nigra (Snider, Maiti & Snider 1976; Middleton & Strick 2000) and the VTA sends dopaminergic projections to the vermis (Snider & Maiti 1976; Ikai et al. 1992; Schweighofer, Doya & Koroda 2004), forming a reciprocal midbrain-cerebellar circuit. Moreover, activation of the prelimbic subdivision of the medial prefrontal cortex produces electrophysiological responses in the contralateral vermis (Watson, Jones & Apps 2009) and electrical stimulation of the fastigial nucleus, which receives projections from the vermis, evoking neuronal activity in the amygdala and hippocampus (Heath et al. 1978). All of these anatomical findings challenge the traditional view of the cerebellum as a subcortical isolated motor structure and support its involvement in functional networks affected by addictive drugs (Miquel et al. 2009). Indeed, psychostimulant administration increases cFos-like immunoreactivity in the rat granule cell layer of the vermis at a wide range of doses (Klitenick, Tham & Fibiger 1995). Also, sensitization of cFos and jun-B mRNA has been demonstrated in the cerebellar cortex of cocaine sensitized rats (Couceyro et al. 1994). After cocaine administration, Purkinje soma and dendrites augment the expression of Homer 1b/c and 3a/b (Jimenez-Rivera et al. 2000). These long homer isoforms are a crucial link between mGluR and IP_3-dependent intracellular Ca^{2+} signalling, and they are considered as an important step of synaptic remodelling and spine morphogenesis (Skumlinski, Kalivas & Worley 2006). Furthermore, elevations in the relative cerebral blood volume in the

cerebellar dentate nucleus have been demonstrated in nonhuman primate studies mapping DA function with amphetamine (Jenkins et al. 2004). From these findings, it is clear that molecular and cellular actions of addictive drugs in the cerebellum involve long-term adaptive changes in receptors, neurotransmitters and intracellular signalling transduction pathways.

At the clinical level, human studies have found cerebellar activations during the exposure to drug-associated cues (Grant et al. 1996; Schneider et al. 2001; Bonson et al. 2002; Volkow et al. 2003). Furthermore, Anderson and co-workers (2006) have suggested that the relevance of the cerebellum in modulating incentive drug-related stimuli would be increased when the prefrontal lobule is compromised by disease or chronic drug use. However, probably because there are no experimental animal studies aimed at the involvement of the cerebellum in drug-associated memories, almost no attention has been paid to these findings and so, to date, the cerebellum has not been considered as part of the circuitry that sustains addictive behaviour.

Therefore, by trying to fill this gap, the main objective of the present study was to investigate the pattern of neuronal activation as revealed by cFos immunoreactivity in the cerebellum and prefrontal cortex in mice trained to develop conditioned preference to an olfactory stimulus paired with cocaine. We proposed that repeated experience with cocaine would produce a different pattern of cFos expression in the vermis to that observed in the prefrontal cortex. Also, we expected the pattern of cFos expression to be related to cocaine-induced conditioned preference.

Methods

Subjects

Three-week-old Swiss male mice were purchased from Janvier S.A. and maintained in our colony room (Jaume I University. Spain) for 30 days prior to experiments (N=55). Handling was carried out daily for 5 minutes for 21 days before the experiments began. The colony room was kept at a temperature of 22 $\pm$ 2 ºC with lights on from 08:00 to 20:00 hours. Animals were housed in standard-conditions with laboratory rodent chow and tap water ad libitum. At the age of seven-weeks experimental procedures began. Behavioural tests were conducted within the first five hours of the light cycle. All animal procedures were performed in accordance with the European Community Council directive (86/609/ECC), Real Decreto 1201/2005 and the local directive DOGV 13/2007.

Pharmacological agents

All drugs were administered intraperitoneally (IP). Cocaine hydrochloride (2mg/ml) (Alcaliber S.A., Spain) was dissolved in 0.9% w/v saline and injected immediately before each conditioning trial. Saline solution 0.9% w/v was used as the vehicle control.

Behavioural procedures and experimental design

In a first step, the effect of the number of pairing sessions (2, 4 or 8) between an odour (lavender or papaya) and cocaine (20 mg/kg) was evaluated in three separate groups of mice (n=12, 16 and 15, respectively). These daily-pairing sessions took place in a specific conditioning environment (a rectangular Plexiglass box of 30x15x20 cm) and the odours used as CS+ and CS- were counterbalanced between animals and

sessions following an ABAB schedule. Thus, one of the odours acted as CS+ and was associated with IP cocaine (20 mg/kg). On alternate days, mice were exposed to a different odour (CS-) associated with saline administration. Cocaine-induced odour preference was assessed in a 30 minutes drug-free test using a T-maze in which CS+ and CS- were presented simultaneously but in opposite arms. The preference test took place 24 hours after the last cocaine administration. All test sessions were videotaped and the time spent (TS) in each arm of the maze was registered manually from the recorded test sessions during the last 20 minutes by a blind observer. Preference score was calculated as TS in CS+ / (TS in CS^+ + TS in CS^-).

In a second step, regardless of their number of pairings at the training phase, tissue samples from individuals having CS+ preference scores higher or lower than the arbitrary cut off point of 60% were randomly picked out to conform the thereafter-called "conditioned" (n=7) and "non-conditioned" (n=6) groups, respectively. In these subjects appropriate samples (see following sections) were collected to evaluate cFos staining on cerebellar and prefrontal areas. For identical purpose, two additional groups of mice were generated. First, the "saline" group members (n=6) received saline injections associated with both odours. Second, the "unpaired" group members (n=7) received cocaine (20 mg/kg) injections randomly associated with any of those odours. Both groups were designed to match the number of pairings of those received by the members of the "conditioned group".

Perfusion and dissection protocol

Animals were deeply anesthetized with sodium pentobarbital (30mg/kg) 70 minutes after the preference test and perfused transcardially, first with 0.9% saline solution and then with 4% paraformaldehyde. After perfusion, the frontal cortex and the

vermis cerebellum were quickly dissected and placed in a container with 4% paraformaldehyde for 24 hours. After this time, tissue was cryoprotected in 30% sucrose solution until complete immersion.

Tissue sections

Brain tissue was rapidly frozen by immersion in liquid nitrogen and sections were performed at 40 µm with a cryostat microtome (Microm HM560. ThermoFisher Scientific). Six series of tissue sections were collected and stored at -80^{o}C in cryoprotectant solution. Sagittal sections of the cerebellum were selected according to the lateral coordinates -0.04 mm and 0.72 mm, comprising the vermis cerebellum (Paxinos and Franklin, 2008). Coronal sections from bregma 2.22 mm to 1.94 mm (Paxinos and Franklin, 2008) were considered as the prefrontal cortex.

cFos Immunohistochemistry

Immunohistochemistry was performed on free-floating sections. For peroxidative immunostaining, tissue peroxidases were eliminated with 0.3% of H2O2 and methanol 20%, during a period of 30 minutes**. Tissue was incubated for 48 h with a polyclonal primary antibody, rabbit anti-cFos (1:500, Santa Cruz Biotechnology) or overnight with rabbit anti-DAT (dopamine transporter) (Abcam) in smooth agitation at 4°C.** In a second step, sections were exposed to an affinity-purified secondary biotinylated antibody, donkey anti-rabbit (1:400) (Vector Labs, BA-2000) for 120 minutes at room temperature**.** For magnification, we used preassembled biotin-avidin peroxidase complex according to the Vector Labs recommendations (ABC Elite, Vector Labs). Sections were exposed to DAB solution free of nickel component until the tissue developed an intense brown staining. Then, the tissue was rinsed and mounted.

To obtain a clear view of cFos cellular expression, some additional tissue obtained from the same mice was rinsed and pre-blocked with 5% donkey serum and 0.3% triton X-100 for one hour. Cerebellar sections were incubated at 4°C for 48 hours with primary antibody rabbit anti-cFos (1:500 Santa Cruz Biothechnology). Thereafter, samples were exposed in the dark to AlexaFluor 647 dye anti-rabbit (1:500; Vector Lab) for two hours. To stain Purkinje neurons, sections reacted with rabbit anti-calbindin (1:500; Chemicon, Millipore) for 48 hours, and then with AlexaFluor 488 donkey anti-rabbit (1:500; Invitrogen) for two hours. Tissues were rinsed with PBS and mounted with fluorsave reagent (Calbiochem).

Immunostaining Analysis

Images were captured in an optical microscope (Nikon E-800) with 40x lens for the cerebellum and 20x lens for the prefrontal cortex. We considered cFos positive (cFos+) peroxidase staining those cells showing a brown labelling in the nucleus (see figure 1A).

We counted the first plane of 3 sagittal sections at the granule cell layer of the vermis cerebellum (L -0.04 to 0.72mm) (Paxinos and Franklin, 2008) in selected ROIs of 20,000 μm^2 at the apical (external surface of the internal granular layer) and medial zone (deep portions of lobule) of each cerebellar lobule, for a total area of 40,000 μm^2 per lobule and section. Purkinje neurons were counted in an area of 20,000 μm^2 in the apical and medial regions and they were considered cFos+ when exhibiting a uniform and constant staining in the soma (see Figure 1A). For the prefrontal cortex, we counted cFos+ neurons in ROIs of 20,000 μm^2 of the cingulate, prelimbic, infralimbic and orbitofrontal medial cortex (from bregma 2.22 mm to bregma 1.94 mm) (Figure 7). Cell count was performed automatically with ImageJ (now FIJI) software. Fluorescent

microphotographs were taken with an Olympus FV1000 confocal microscope with 60x oil lens (Figure 1B).

Statistics

All statistical analyses were conducted using the Statistica 6.0 software package (Statsoft, Inc). Behavioural data were analysed by means of one-way ANOVA followed by Tukey HSD post-hoc tests and by means of Kruskal-Wallis ANOVA by ranks and chi-squared tests for dyadic comparisons. Differences between groups on cFos staining at different brain regions were analysed using separate one-way (group) MANOVAs followed up by univariate ANOVAs and Tukey HSD tests, when possible. In all these analyses, the number of pairings at the training phase was used as a covariate. Finally, Pearson's r correlation index was used to ascertain the degree of correlation between preference for the CS+ preference and cFos staining in particular brain regions. The level of significance was set at $p<0.05$.

Results

A one-way ANOVA revealed that the number of pairings during the training phase had a significant effect on the group-averaged preference scores on the test day [$F_{2,36}=3,97$, $p<0.05$]. Tukey HSD based comparisons revealed that a training protocol consisting of 8 cocaine-odour pairings produced a statistically significant higher group preference than that observed at the 2 pairings group ($p<0.05$). These results are displayed in figure 2A. On the other hand, Figure 2B depicts individual preference scores subjected to 2, 4 and 8 conditioning trials. From these data it is readily observable that almost half of the individuals treated with 2 pairings during the training phase showed preference scores below the theoretical indifference critical point (50%)

whereas this only occurred in one subject (out of 13) of the 8 pairings group. Furthermore, a larger number of cocaine-odour pairings seems to increase the minimum, but less clearly maximal, preference scores within each group. Thus, it seems that the number of pairings at the training phase displaced the preference scores distribution upwards rather than changing the highest preference values reached by a subset of individuals of each group. Accordingly, a Kruskal-Wallis ANOVA by ranks comparing the proportion of individuals above and below the overall median revealed a significant effect of the number of pairings [$H(2, n=39)=7.31$, $p<0.05$]. Subsequent dyadic Chi-square based comparisons revealed that on the 8 pairings group the proportion of subjects displaying preference scores higher than the overall median value was higher than expected [Chi-square= 11.39, $p<0.01$]. Taken as a whole, these results seem to indicate that the higher the number of pairings, the higher the proportion of subjects surpassing the indifference scores range and, therefore, the higher the group-averaged preference.

In a second step, regardless of their number of pairings at the training phase, these individuals' samples were divided into two groups having CS+ preference scores higher or lower than the arbitrary cut off point of 60%. From each one of these two new groups, mice were randomly picked out to conform the thereafter-called "conditioned" (n=7) and "non-conditioned" (n=6) groups, respectively. For subsequent analysis, these two groups were compared against the "saline" and the "unpaired" groups (see methods section for further details). As expected, an ANCOVA comparing the preference scores of all four treatment groups revealed a significant effect of the treatment factor ($F_{3,33}$= 21.53; $p<0.001$) whereas the number of pairings, which had been used as covariate, did not affect those scores ($F_{2,33}$= 1.39; $p=0.24$). Post-hoc mean comparisons were performed using the Tukey HSD test, which showed that the "conditioned" group was

different from all the other treatment groups ($p<0.01$ in all cases) and that the preference scores of the "saline", "unpaired" and "non-conditioned" groups had no difference among them ($p>0.05$ in all cases). These results are depicted in Figure 3.

When comparing locomotor activity recorded during the preference test, no significant differences were seen among any of the four groups ($F_{2,19}$= 1,76; p=0.18). Means and standard error of the mean were as follow: the saline group = 8352.74±966; the unpaired group =13935.11±2735; the non-conditioned group = 9266.08±1465; the conditioned group = 8277.34±2717.67.

Trying to identify evidence for a differential involvement of fronto-cerebellar networks on subjects exhibiting CS+ preference, we examined cFos expression on several cortical and cerebellar regions in each one of these four experimental groups. Regarding the cerebellum, we first analysed cFos expression in the granule cell layer of different vermal lobules. As revealed by a one-way MANCOVA, the treatment group produced an effect that approached, but did not reach, statistical significance [Wilk's= 0.14; $F_{24,41}$=1.61, p=0.08], whereas the number of pairings did not even have a trend towards producing any relevant effect [Wilk's=0.59; $F_{8,14}$=1.20, p=0.36].

These results prompted us to analyse cFos expression in further detail, then separating the functionally distinct apical and medial regions of the granule cell layer of different cerebellar lobules (Fig1, 4, 5). A one-way MANCOVA revealed a significant effect of the group [Wilk's= 0.11; $F_{24,41}$=1.93, $p<0.05$] but not of the number of pairings, which was used as covariate [Wilk's= 0.56; $F_{8,14}$=1.35, p=0.29]. Subsequent univariate analyses showed a significant effect of the group in all cerebellar vermis lobules ($p<0.01$ in all cases, see Table 1 for further details). Interestingly, as revealed by Tukey HSD post-hoc comparisons, in all cases the "conditioned" group displayed a significantly higher ($p<0.01$) number of cFos positive neurons than the "saline", the

"unpaired" and the "non-conditioned" groups, which did not differ among themselves regarding cFos staining. These results are depicted on the different panels of Figure 4. Furthermore, as summarized in Table 2, individual levels of cFos staining were significantly and positively correlated to their corresponding CS+ preference scores at lobules, being the correlation indexes highest at lobules VIII, IX and X. Taken together, these results seem to indicate that CS+ preference is related to the activity of cells in the apical region of the granule cell layer of the cerebellar vermis and that this relationship might be more prominent in some specific lobules.

On the other hand, a separate one-way MANCOVA comparing cFos expression in the medial region of the granule cell layer also revealed an effect of the group [Wilk's= 0.11; $F_{24,41}$=1.94, $p<0.05$] but not of the number of pairings [Wilk's= 0.63; $F_{8,14}$=1.00, p=0.47], which was used as a covariate. Follow-up univariate analyses yielded a significant group effect at all cerebellar vermis lobules ($p<0.01$ in all cases, see Table 4 for further details). However, when post-hoc mean comparisons for each dependent variable were performed, statistically significant differences were focused on the "unpaired" group, which exhibit significantly lower ($p<0.01$) cFos staining levels than the other groups in most of these comparisons. These results are presented in detail on the different panels of Figure 5 and, conversely to what was observed for the apical region, they seem to suggest that cellular activity in the medial region of the granular layer of the cerebellar vermis is related to contingent CS-US administration during the training phase rather to the preference exhibited on the test day. In fact, as can be seen in Table 5, individual correlations between CS+ preference and cFos staining levels in this region were lower than those observed for the apical zone and no longer reached statistical significance in lobules VIII and X.

We also analysed the number of cFos+ Purkinje neurons in the apical and medial regions of the cerebellar vermis for each lobule (for a summary of the results, see Table 6, Fig 6). A one-way MANCOVA in the apical region did not yield any significant effect of the group [Wilk's= 0.18; $F_{24,41}$=1.34, p=0.20] or the number of pairings [Wilk's= 0.50; $F_{8,14}$=1.72, p=0.17]. However, univariate comparisons (Table 7) yielded a significant effect of the treatment group factor on the number of cFos positive Purkinje neurons at lobules V, VI, VIII and IX. A more detailed study of those effects conducted by Tukey HSD tests revealed that the "conditioned" group showed a higher number of cFos staining than the "non-conditioned group" on lobule V ($p<0.05$) and than the "saline", "unpaired" and "non-conditioned" groups in lobule VIII ($p<0.05$ in all cases; see Table 7 and Figure 6). Furthermore, moderate but statistically significant correlation ($r=0.45$, $p<0.05$) between the number of cFos positive Purkinje neurons in this lobule and the preference for the CS+ was also found (see Table 6).

On the other hand, a similar one-way MANCOVA comparing the number of cFos positive Purkinje neurons in the medial region of the cerebellar vermis lobules yielded a significant group effect [Wilk's= 0.10; $F_{24,41}$=2.01, $p<0.05$] but not a covariation with the number of pairings [Wilk's= 0.72; $F_{8,14}$=0.65, p=0.72]. Univariate comparisons revealed that this general effect was due to between group differences on lobule VI [$F_{3,21}$=5.05, $p<0.01$] and, to a lesser extent, lobule VII [$F_{3,21}$=3.38, $p<0.05$] (Tables 6 and 7). Mean comparisons showed that in both lobules, the "conditioned" group exhibited a higher number of cFos positive Purkinje neurons than the other groups but this difference only reached statistical significance at some, but not all, between group comparisons. More specifically, as depicted in Figure 6, the "conditioned" group had more Purkinje cFos positive neurons than the "saline" and "non-conditioned" groups in the medial region of lobule VI ($p<0.05$ in both cases) as

well as than the "saline" group at lobule VII ($p<0.05$). No significant correlations between CS+ preference and Purkinje cFos staining were found.

Finally, we also analysed the number of cFos positively stained neurons in several regions of the prefrontal cortex (Fig 7). A one-way MANCOVA revealed a significant group effect [Wilk's=0.12; $F_{12,50}= 4.55$, $p<0.001$] but not a covariation with the number of pairings [Wilk's=0.97; $F_{4,19}= 0.45$, $p=0.77$]. Univariate comparisons showed that the group effect was observable in all tested regions [cingulate $F_{3,21}=12.68$, $p<0.001$; prelimbic $F_{3,21}=5.77$, $p<0.001$; infralimbic $F_{3,21}=3.73$, $p<0.01$; orbitofrontal $F_{3,21}=7.08$, $p<0.01$] whereas the number of pairings did not reach statistical significance in any of them [cingulate $F_{1,21} =0.23$, $P=0.63$; prelimbic $F_{1,21} =0.06$, $p=0.80$; infralimbic $F_{1,21} =0.002$, $p=0.96$; orbitofrontal $F_{1,21} =0.87$, $p=0.36$] (Table 8). Tukey HSD post-hoc based comparisons demonstrated that between group differences were largely due to the differences between saline- and all cocaine-treated groups. These results are depicted in Figure 7 and seem to indicate that cFos expression in those frontal areas were related to the pharmacological actions of cocaine rather than to the acquisition/ expression of conditioned odour preference. In fact, no significant correlations were found between CS+ preference and cFos expression at the cingulate ($r=0.03$, $p=0.87$), the prelimbic ($r=-0.27$, $p=0.172$), the infralimbic ($r=-0.35$, $p=0.07$) or the orbitofrontal ($r=-0.31$, $p=0.12$) cortices.

Examples of correlations between CS+ preference and cFos expression in the cerebellum and prefrontal cortex are shown in Figure 8.

Discussion

The general purpose of the present research was to address the question as to whether the cerebellum is a part of the neuronal systems that sustain processes

underlying drug seeking and taking behaviours. Specifically, we studied whether cerebellar neuronal activity is related to cocaine-induced conditioned preference memories. Although it has been largely ignored in preclinical research of the drug abuse field, human neuroimaging studies have systematically found enhancements of glucose metabolism in the cerebellum when cocaine and alcohol addicts are exposed to drug-associated cues (Grant et al. 1996; Wang et al. 1999; Schneider et al. 2001; Bonson et al. 2002; Volkow et al. 2003; Anderson et al. 2006). This cerebellar over-activity concurred with reductions in neuronal metabolism of the prefrontal cortex and substantia nigra (Anderson et al. 2006). So, the role of the cerebellum in drug-oriented behaviour deserves more attention and further research, a conclusion further stressed when attending to the fundamental role of this structure for consolidation and storage of long-term emotional and instrumental memories (Sacchetti et al. 2002; Sacchetti et al. 2004; Callu et al. 2007).

For this attempt, we trained mice to acquire a conditioned preference response to an odour associated with cocaine injections. We found that four and eight cocaine-odour pairings produced a robust conditioning in most of the animals, hence allowing us to validate this odour conditioning protocol for cocaine. Remarkably, enhancing the number of odour-cocaine pairings pushed the preference scores distribution up rather than increasing the individual highest preference values (Figure 3). Brabant, Quertemont & Tirelli (2005) observed similar results regarding the magnitude of cocaine-induced place preference. Both findings fit with current notions of conditioning as mediated by an evidence-based decision process, becoming an all-or-nothing phenomenon at the individual level (Gallistel et al. 2004).

Because we observed individual differences in the susceptibility for developing conditioned preference for cocaine, in a second step, regardless of their number of

pairings during the training phase, we randomly selected mice either expressing a clear CS+ preference (>60%, conditioned group) or not showing such an acquired preference (<55%, non-conditioned group). We also included two additional control groups: the saline group and the unpaired group. They allowed us to dissect the pharmacological effects of cocaine administration and to provide the most proper control for the acquisition of a Pavlovian association between the CS and the unconditioned stimulus (UCS). We then explored the relationship between the acquired preference for the CS+ and neuronal activation (as measured by cFos expression) in cerebellar and prefrontocortical areas. The most remarkable result is the higher cerebellar neuronal activity in animals expressing cocaine-induced conditioned preference as compared to that observed in subjects from all the other groups. This effect was more clearly observed in the apical region of the granule cell layer in all lobules, but it was especially prominent in the posterior lobules VIII, IX and X. The cFos expression in these neurons in the apical region correlated with cocaine-induced odour preference (Fig 8, 9). **Interestingly, these cerebellar lobules received dopamine projections from VTA (Ikai et al 1992; Melchitzky and Lewis 2000). Moreover, supporting a functional relevance of DA transmission, dopamine-signalling proteins have also been found in the same cerebellar areas (Delis et al. 2008; Kim et al. 2009). In accordance, in a representative sample of conditioned animals we observed about a 280% increase in DAT expression in lobule X as compared to saline mice. However, the non-conditioned group showed smaller increase (56%).**

The medial region yielded less consistent results. Nevertheless, it is worth noting that neuronal activity in the medial region seems to be related to contingent CS-US administration as lower activity was seen in medial neurons of the unpaired group as compared to the other groups, which always received cocaine or saline contingently

associated with the same odour. We also evaluated activity in Purkinje neurons and observed a higher number of cFos+ Purkinje nuclei in posterior vermal lobules of the conditioned group. Moreover, activity of Purkinje cells in the apical region moderately correlated with the preference for the cocaine-paired stimulus in the same lobules. To date there is not available information describing the specific role of apical and medial regions in the cerebellar cortex or showing cellular differences between these two areas. Further research is needed to elucidate this functional specificity.

Previous work has identified the pattern of cFos expression in the rat cerebellum after a repeated treatment with cocaine (Klitenick, Tham & Hans 1995) or amphetamine (Yin et al 2010). Both psychostimulant drugs produced an increase in Fos+ immunoreactivity at the granule cell layer of the vermis, although cFos+ immunostaining in Purkinje cells was sparse. The special relevance of our results is upheld for the finding that this neuronal activity was related to emotional and sensory memories (olfactory) acquired during repeated experience with cocaine, rather than cocaine treatment itself. In this regard, olfactory stimulation with ethanol in alcoholic patients under detoxification, but not in normal healthy controls, activates the cerebellum, right amygdala, hippocampus and insula (Schneider et al. 2001). This cerebellar activation was not observed in response to neutral cues, which is important because it precludes the possibility that the cerebellar activations are due to sensorial or motor processing not related to drug experience. Similarly, Anderson et al. (2006) found that cocaine-associated cues induced an enhancement of neuronal activity in the vermal lobules of human cocaine addicts, this increase being especially noteworthy in the lobules VIII and IX (but also in lobules II and III).

Unlike the cerebellum, neuronal activity in the prefrontal cortex only allowed to distinguish saline treated groups from cocaine-treated groups, no matter if cocaine-

induced preference was acquired or not. Thus, subjects belonging to each one of the different cocaine-treated groups showed a similar number of cFos positive neurons in different cortical regions, being in all cases higher than that observed in saline-treated animals and not showing any statistically significant correlation towards their CS+ preference scores. This pattern of results was especially clear in the cingulate cortex and seems to be in agreement with previous data indicating that activity in this brain area is higher in cocaine than in saline-treated animals subjected to a conditioned place preference paradigm (CPP) (Zombek et al. 2008), but it is not different between paired and unpaired groups of mice trained in a Pavlovian conditioning protocol (Nordquist et al. 2003). Data indicating that lesions of the cingulate cortex do not affect cocaine, amphetamine or morphine-induced CPP (Tzschentke & Schmidt 1999) seem to provide further support to the notion that the observed differences between groups on cFos staining at the cingulate cortex are probably unrelated to the acquisition/ retrieval of CS+ preference. On the other hand, a similar pattern of results was also reproduced in the prelimbic cortex, although in this case, entering in apparent contradiction with the results observed at the lesional study of Tzschentke and Schmidt (1999). Finally, in the infralimbic and orbitofrontal cortex a non-significant trend towards reduced cFos staining was observed in the conditioned group as compared to the non-conditioned and the unpaired group as well as a trend towards an inverse correlation between the number of cFos positive neurons and the preference for the CS+. Although these trends did not reach statistical significance these observations seem to be in agreement with the inverse correlation between cocaine-induced CPP preference and cFos in different regions of the prefrontal lobe, including the orbitofrontal cortex, found by Zombek et al. (2008) as well as with the proposed inhibitory role of the infralimbic cortex in drug-seeking behaviours (Peters, LaLumiere & Kalivas 2008). Nevertheless, it should be

taken into account that the last cocaine injection took place 48 hours before the preference test. Hence, cFos expression showed by the cocaine-treated groups could be induced by reactivation of memories about cocaine effects other than those contingently connected to preference. Also, it could be related to withdrawal symptoms after cessation of cocaine regimen.

Supporting the present findings, previous evidence suggests that the vermis cerebellum might be a key structure for rewarding and aversive memory. Indeed, in a previous study, we observed higher cFos expression in the granule cell layer of female rats allowed to pace copulate (rewarding condition) as compared to females that copulated in nonpaced conditions (non rewarding) or females in pacing chambers with no male to copulate with (Paredes-Ramos et al. 2011). Moreover, consolidation and expression of emotional memories, which are reactivated in an automatic or implicit mode, seem to be controlled by a circuit that includes the vermis cerebellum (Sacchetti et al. 2002; Sacchetti et al. 2004; Anderson et al. 2006; Bonson et al. 2002). Accordingly, vermal connectivity situates the cerebellum within the circuitry responsible for acquiring, maintaining and expressing drug-induced conditioned memories (Snider et al. 1976; Heath et al. 1978; 2010; Ikai et al. 1992; Ikai et al. 1994; Schweighofer et al. 2004; Rossi et al. 2008; Bostan et al. 2010; Zhu et al. 2011; Bernard et al. 2012). The involvement of the cerebellum in emotional behaviour has raised the question of whether this structure is also a site for storage of plasticity related to learning and memory of emotional processes (Sacchetti, Scelfo & Strata 2005; Strata, Scelfo & Sacchetti 2011). It is very likely that the pattern of cFos expression observed in the vermis indicates the activation of local neuroplasticity mechanisms required for consolidation and automaticity. Studies on fear memory have supported this conclusion as plasticity changes described within the vermal cerebellar cortex domains strictly

correlated with associative processes, but they were absent in unpaired groups (Sacchetti et al. 2005, Zhu et al. 2006; Zhu et al. 2007).

Why is the vermis cerebellum important for conditioning? Conditioning is a type of learning which, in order to be adaptive, has to allow subjects to predict the occurrence of UCS and to advance the goal-oriented response (Domhan 2005). Thus, what has to be learnt is not only the relationship between stimuli but also a precise temporal relationship between them (Ivry et al. 2002). Interestingly, it seems that one of the main functions of the vermis is related to the ability to provide correct predictions about the temporal relationship between sensory stimuli (Timmann et al. 2010). The vermis cerebellum processes multimodal sensory inputs (Molinari, Filippini & Leggio 2002) and that multimodal sensory processing seems to be closely related to selective attention (Allen et al. 1997), involving context-dependent changes in sensorimotor sets to facilitate motor outputs (Bischoff-Grethe & Ivry & Grafton 2002). These capacities may be very relevant for drug seeking and taking, since a 'hyperattentive state' towards the salient drug-related stimuli is a core characteristic of the drug-induced behaviour, especially once an addictive state has been instituted (Franken et al. 2003).

Nevertheless, other explanations for the cerebellar cFos expression might arise from the present data and should not be overlooked. On one hand, mice showing cocaine-induced conditioned preference could present a conditioned locomotor response during the preference test that increased cerebellar cFos expression. Studies on functional topography in the cerebellum have suggested that the vermis, which has bidirectional projections to motor cortices and the spinal cord, is mainly involved in balance and head and eye movements (Cerminara & Apps 2011). In addition, posterior cerebellar vermal lobules control locomotor functions (Barik & Beaurepaire 2005). However, when we compared locomotion scores during the test day we did not find any

significant difference between groups. On the other hand, it seems that repeated long-term cocaine treatment induced Purkinje morphological alterations (Barroso-Moguel et al. 2002), probably due to hypoperfusion and ischemic lesions that could be accompanied by over-activity of the granule cells. Nonetheless, if it supposes to be the case, we should have found no differences in cFos+ expression between cocaine-treated groups, as there is no reason to assume any relationship between conditioning and Purkinje alterations.

In summary, the relevance of incentive salience gained by a stimulus associated with cocaine is accompanied by an increase in activity of the apical regions of the vermal cerebellar cortex (Fig 9). The present results show similar findings to those of human neuroimaging studies and provide a further description of cerebellar involvement in circuitry that has sustained drug-associated plasticity changes. Future causal research will be essential to elucidate the role of the cerebellum in plasticity alterations leading to compulsive behaviour and addiction-like behaviour.

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Authors' contribution

CGM, VSD, AML conducted behavioural and histological procedures. CAG and MJ were involved in the critical review of the experimental design and protocols. SSC and MM carried out the data interpretation and manuscript writing. All authors have approved the final version of the manuscript.

	Treatment	Group	Number	of Pairings
Cerebellar lobules	**$F_{3,21}$**	**p value**	**$F_{1,21}$**	***p* value**
II	5.84	**.004**	1.51	.231(NS)
III	8.78	**<.001**	0.67	.423 (NS)
V	10.22	**<.001**	0.36	.556 (NS)
VI	15.28	**<.001**	0.41	.521 (NS)
VII	10.73	**<.001**	2.59	.128 (NS)
VIII	15.03	**<.001**	0.002	.962 (NS)
IX	17.14	**<.001**	6.27	**.022**
X	17.29	**<.001**	0.68	0.416 (NS)

Table 1.- Main outcomes of univariate ANOVAs assessing the levels of the cFos+ staining in the apical region of the granule cell layer in each cerebellar lobule. As can be seen, the treatment group factor had a significant effect on the number of cFos positive neurons in all lobules, whereas the number of parings received at the training phase (which was used as a covariate in all statistical analyses) only yielded a significant effect at lobe 9. Significant *p* values are in bold; NS stands for non-significant effects.

Cerebellar lobules	Pearson's *r*	*p* value
II	.48	**.012**
III	.59	**<.001**
V	.50	**.009**
VI	.63	**.001**
VII	.60	**.001**
VIII	.68	**<.001**
IX	.66	**<.001**
X	.64	**<.001**

Table 2.- Correlational analysis between the CS+ preference and the number of cFos positive neurons at the apical region of the granule cell layer in each cerebellar lobule. As can be seen, CS+ preference was significantly and positively correlated to the levels of cFos expression in all cases, reaching maximal correlation and statistical significance at lobules VI, VIII, IX and X. Significant *p* values are in bold.

	Treatment	Group	Number	of Pairings
Cerebellar lobules	**$F_{3,21}$**	**p value**	**$F_{1,21}$**	***p* value**
II	4.53	**.012**	1.61	.216 (NS)
III	8.77	**<.001**	2.99	.098 (NS)
V	8.82	**<.001**	1.60	.218 (NS)
VI	8.75	**<.001**	0.40	.531 (NS)
VII	14.05	**<.001**	0.44	.511 (NS)
VIII	8.95	**<.001**	1.47	.238 (NS)
IX	11.13	**<.001**	0.001	.933 (NS)
X	5.49	**.005**	0.79	.382 (NS)

Table 3.- Main outcomes of univariate ANOVAs estimating the levels of the cFos expression in the medial region of the granule cell layer in each cerebellar lobule. As can be seen, the treatment group factor had a significant effect on the number of cFos positive neurons in all lobules. However, the number of parings at the training phase (which was used as a co-variate in all statistical analyses) did not yield any significant effect. Significant *p* values are in bold; NS stands for non-significant differences.

	SALINE (n=6)	UNPAIRED (n=7)	NON-CONDITIONED (n=6)	CONDITIONED (n=7)
Lobule II	16.41 ± 2.60	13.41 ± 3.93	14.50 ± 2.66	30.83 ± 3.97^{ABC}
Lobule III	14.92 ± 4.20	14.57 ± 4.73	13.50 ± 1.31	37.67 ± 4.34^{ABC}
Lobule V	14.83 ± 2.75	12.14 ± 4.87	13.00 ± 1.67	36.00 ± 3.80^{ABC}
Lobule VI	16.00 ± 2.12	10.30 ± 3.51	16.00 ± 3.44	38.00 ± 3.39^{ABC}
Lobule VII	16.5 ± 4.24	8.00 ± 3.01	17.17 ± 5.36	36.00 ± 2.21^{ABC}
Lobule VIII	17.00 ± 2.69	3.85 ± 1.38	15.83 ± 3.44	37.10 ± 5.53^{ABC}
Lobule IX	17.33 ± 2.62	5.64 ± 2.04	14.33 ± 1.70	33.85 ± 4.29^{ABC}
Lobule X	14.33 ± 2.75	7.42 ± 2.95	13.33 ± 2.30	36.17 ± 3.92^{ABC}

	SALINE (n=6)	UNPAIRED (n=7)	NON-CONDITIONED (n=6)	CONDITIONED (n=7)
Lobule II	25.75 ± 3.39	12.41 ± 3.36^{D}	25.83 ± 5.48	33.17 ± 4.56
Lobule III	28.08± 2.53	9.15 ±3.18^{ACD}	23.83 ± 2.93	29.41 ± 3.80
Lobule V	20.33± 2.07^{d}	10.29 ±4.06^{cD}	26.00 ± 4.18	35.67 ± 3.79
Lobule VI	26.00 ± 1.59	7.43 ±2.42^{ACD}	24.5 ± 3.98	28.83 ± 4.43
Lobule VII	27.33 ± 4.63	3.07 ±1.10^{ACD}	26.17 ± 4.21	34.20 ± 4.27
Lobule VIII	26.00 ± 5.57	3.57 ±2.05^{ACD}	24.17 ± 2.34	24.33 ± 3.85
Lobule IX	23.83± 4.17	3.71 ±1.57^{ACD}	25.00 ± 3.22	26.22 ± 3.82
Lobule X	25.17± 2.38	6.57 ±2.62^{ACD}	20.58 ± 5.05	23.00 ± 4.02

Table 4.- Descriptive statistics (mean ± SEM) corresponding to the levels of the cFos+ labeling at the apical (top) and medial (bottom) regions of the granule cell layer in each lobule in the vermis cerebellum. Capital letters indicate a significant difference ($p<0.01$), whereas lowercase letters (a, b, c, d) were used when the same differences were reached at a lower significance level ($p<0.05$). These differences were assessed by means of a one-way MANOVA, followed by univariate ANOVAs and Tukey HSD tests when corresponding (see text for details). At the apical region (top), the conditioned group showed significantly higher cFos+ expression than the other groups, thus indicating a clear relationship with the CS+ preference that was corroborated with the results of the correlational analysis provided at Table 2. On the other hand at the medial region, differences seem to separate the unpaired group from all the others, suggesting that cFos+ staining in this region could be more related to CS-US contingency than to CS+ preference (see discussion section).

Cerebellar lobules	Pearson's *r*	*p* value
II	.47	**.014**
III	.61	**.001**
V	.53	**.005**
VI	.46	**.016**
VII	.54	**.004**
VIII	.36	.066 (NS)
IX	.49	**.010**
X	.35	.075(NS)

Table 5.- Correlations between the CS+ preference and the number of cFos+ neurons in the medial region of the granule cell layer in each cerebellar lobule. As can be observed, CS+ preference was significantly and positively correlated to the levels of cFos expression in most of the lobules, although the correlation indexes were in general lower to those observed in table 2 and, in this case, the maximal correlation was found at lobe 3. Significant *p* values are in bold; NS stands for non-significant differences.

	Treatment	Group	Number	of Pairings	Preference Correlation	
Cerebellar lobules	**$F_{3,21}$**	**p value**	**$F_{1,21}$**	**p value**	**Pearson's *r***	***p* value**
II						
Apical	2.21	.116 (NS)	0.35	.559 (NS)	-.14	.478(NS)
Medial	1.44	.257 (NS)	0.0001	.989 (NS)	-.20	.316 (NS)
III						
Apical	3.03	.051 (NS)	0.007	.933 (NS)	.07	.700(NS)
Medial	0.87	.469 (NS)	0.058	.811 (NS)	-.10	.595 (NS)
V						
Apical	4.09	**.019**	1.43	.244 (NS)	-.04	.844(NS)
Medial	1.93	.155 (NS)	0.09	.760 (NS)	-.14	.481(NS)
VI						
Apical	3.91	**.022**	0.08	.771 (NS)	.009	.967(NS)
Medial	5.01	**.008**	0.99	.330 (NS)	.22	.273 (NS)
VII						
Apical	1.88	.162 (NS)	0.04	.162 (NS)	.25	.201(NS)
Medial	0.504	.683 (NS)	0.02	.876 (NS)	.078	.708 (NS)
VIII						
Apical	4.85	**.010**	0.97	.333 (NS)	.45	**.021**
Medial	1.71	.683 (NS)	0.342	.564 (NS)	-.242	.232 (NS)
IX						
Apical	3.38	**.037**	1.03	.319 (NS)	-.08	.682(NS)
Medial	1.45	.256 (NS)	0.34	.564 (NS)	.049	.810(NS)
X						
Apical	2.26	.110(NS)	0.04	.429(NS)	-.05	.775(NS)
Medial	2.23	.113(NS)	0.41	.524(NS)	-.20	.316(NS)

Table 6.- Results of univariate ANOVAs assessing the levels of the Purkinje cFos+ labeling in the apical and medial regions in each cerebellar vermis lobule. The treatment group factor produced a significant effect on the number of cFos+ neurons in the apical regions of lobules V, VI, VIII and IX as well as in the medial region of lobe VI. Conversely, the number of pairings (which was used as a co-variate in all these analyses) did not yield any significant effect. The table also shows that cFos+ expression in Purkinje neurons was not clearly correlated with preference for the CS+ and only a moderate correlation was found when considering the number of cFos+ neurons in the apical region of lobe VIII. Significant *p* values are in bold; NS stands for non-significant effects.

	SALINE (n=6)	UNPAIRED (n=7)	NON-CONDITIONED (n=6)	CONDITIONED (n=7)
Lobule II	6.00 ± 1.48	13.13 ± 3.93	4.33 ± 2.06	11.41 ± 2.47
Lobule III	7.08 ± 1.81	14.57 ± 4.73	3.67 ± 1.81	14.41 ± 2.05
Lobule V	2.67 ± 1.25	12.14 ± 4.87	1.33 ± 0.95	13.58 ± 2.60^{c}
Lobule VI	3.00 ± 1.29	10.3 ± 3.51	1.83 ± 1.13	11.86 ± 2.63
Lobule VII	2.33 ± 1.49	8.00 ± 3.01	1.33 ± 0.98	5.58 ± 2.17
Lobule VIII	3.50 ± 0.84	3.85 ± 1.38	2.83 ± 1.60	9.40 ± 1.65^{abc}
Lobule IX	1.17 ± 0.65	5.64 ± 2.04	0.5 ± 0.34	5.32 ± 1.58
Lobule X	0.83 ± 0.54	7.42 ± 2.95	1.33 ± 0.88	6.83 ± 2.79

	SALINE (n=6)	UNPAIRED (n=7)	NON-CONDITIONED (n=6)	CONDITIONED (n=7)
Lobule II	8.33 ± 2.67	12.14 ± 3.36	3.67 ± 3.08	8.75 ± 1.87
Lobule III	4.41 ± 2.25	9.14 ± 3.18	4.5 ± 2.39	5.25 ± 1.28
Lobule V	2.83 ± 1.79	10.28 ± 4.06	2.17 ± 1.24	8.58 ± 2.86
Lobule VI	2.83 ± 1.37	7.42 ± 2.42	3.00 ± 1.50	13.33 ± 2.92^{ac}
Lobule VII	0.17 ± 0.16	3.07 ± 1.10	1.00 ± 1.00	3.81 ± 0.89^{a}
Lobule VIII	2.17 ± 0.79	3.57 ± 2.05	1.33 ± 0.80	2.57 ± 0.75
Lobule IX	1.48 ± 0.79	3.71 ± 1.56	0.83 ± 0.83	4.39 ± 1.85
Lobule X	1.17 ± 0.83	6.57 ± 2.62	1.00 ± 0.81	3.43 ± 1.52

Table 7.- Descriptive statistics (mean ± SEM) corresponding to the number of cFos+ Purkinje neurons at the apical (top) and medial (bottom) regions of each lobule. Capital letters indicate a significant difference ($p<0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group, whereas lowercase letters (a, b, c, d) were used when the same differences were reached at a lower significance level ($p<0.05$). These differences were assessed by means of a one-way MANOVA, followed by univariate ANOVAs and Tukey HSD tests when corresponding (see text for details). In this case, very few statistically significant differences between groups were found and, accordingly, no clear association between cFos+ Purkinje cells and preference for CS+ could be found.

	SALINE (n=6)	UNPAIRED (n=8)	NON-CONDITIONED (n=6)	CONDITIONED (n=7)
CINGULATE	23.71 ±5.79^{BCD}	101.26 ±13.13	145.96 ± 17.77	97.62 ± 12.94
PRELIMBIC	54.58 ± 14.60^{B}	149.87 ± 19.63	109.14 ± 16.86	95.83 ± 10.28
INFRALIMBIC	50.22 ± 14.11^{b}	146.78 ±32.30	114.30 ± 18.10	69.09 ± 10.41
ORBITOFRONTAL	42.58 ± 3.74^{b}	138.65 ± 25.79	120.17 ± 17.02	71.07 ± 22.00

Table 8.- Descriptive statistics (mean ± SEM) corresponding to the levels of the cFos+ staining at different cortical areas of subjects belonging to each treatment group. Capital letters indicate a significant difference ($p<0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group, whereas lowercase letters (a, b, c, d) refers to lower significance level ($p<0.05$). These differences were assessed by means of a one-way MANOVA, followed by univariate ANOVAs and Tukey HSD tests (see text for details). As is readily observable from the table, differences in cFos+ expression were mainly associated with differences between the saline- *vs.* the cocaine-treated groups (this pattern is clearly observable at the cingulate cortex and more inconsistently present in the rest of cortical areas). Accordingly, no clear association towards CS+ preference was found (see results section for further details).

Figure 1A: Examples of microphotographs of cFos+ peroxidative staining in the cerebellum (panels A-H) (40x; scale bar = 50 μm) and the infralimbic cortex (I-L) (20x; scale bar = 50 μm). Saline (A-E-I), unpaired (B-F-J), non-conditioned (C-G-K), or conditioned (D-H-L). As the figure depicts cFos immunoreactivity was greater in the granule cell layer in the conditioned group (A-D). High-magnification image (100x) depicting cFos+ immunostaining in Purkinje nucleus (scale bar = 10 μm) (E-H). ML: molecular layer; GCL: granule cell layer; PL; Purkinje layer; PN: Purkinje neuron. **Figure 1B**: Confocal images showing an example of cerebellar cFos immunofluorescence from conditioned and non-conditioned animals (magnification 150x). According to what is shown in peroxidative immunostainig, double staining (yellow) for cFos (red) and calbindin (green) was observed in Purkinje soma and dendrites but axons devoid of cFos immunoreactivity. Also, cFos (red) was presented in granule cells (GC), which did not express calbindin. As expected, cFos immunoreactivity seems to be greater in the conditioned than in the non-conditioned animal. GC: granule cell; PN: Purkinje neuron. **Figure1C**: **Representative immunolabelling for dopamine transporter (DAT) in lobule X (**magnification 20x; scale bar= 50 μm).

Figure 2: Effect of the number of pairings on the acquired preference for an odour associated with cocaine administration. Panel A depicts the mean ± SEM of the percentual preference for cocaine-associated odour on the test day as a function of the number of cocaine pairings at the training phase (*$p<0.05$). Panel B represents the distribution of the individual scores of the percentual CS+ preference on the test day. As can be readily observed, a higher number of pairings was associated with an upward

displacement of the subjects' distribution and with a reduction of variability in their preference scores rather than with a change of the maximal values.

Figure 3: Preference for the CS+ in the experimental groups used for the study of the cFos staining in prefronto-cortical and cerebellar regions. The "conditioned" and "non-conditioned" groups were randomly picked up from those having a preference higher/ lower than the arbitrary 60% cut off point, respectively. The "saline" and the "unpaired" groups were specifically designed to provide matched controls for drug and contingency effects (see text for further details). Capital letters indicate a significant difference ($p<0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group.

Figure 4: Group effects on cFos+ staining in the apical region of the granule cell layer (black square) for each cerebellar vermis lobule. Each panel corresponds to a different lobule for which the mean $\pm$ SEM of cFos positive neurons is depicted. Capital letters indicate a significant difference ($p<0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group, whereas lowercase letters (a, b, c, d) were used when the same differences were reached at a lower significance level ($p<0.05$). Additional details on these data can be found at the top panel of table 4.

Figure 5: Group effects on cFos staining at the medial region of at the granule cell layer (black square) for each cerebellar vermis lobule. Each panel corresponds to a cerebellar lobule for which the mean $\pm$ SEM of cFos positive neurons is depicted. Capital letters indicate a significant difference ($p<0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group, whereas lowercase letters (a, b, c, d) were used

when the same differences were reached at a lower significance level ($p<0.05$). Additional details on these data can be found at the bottom panel of table 4.

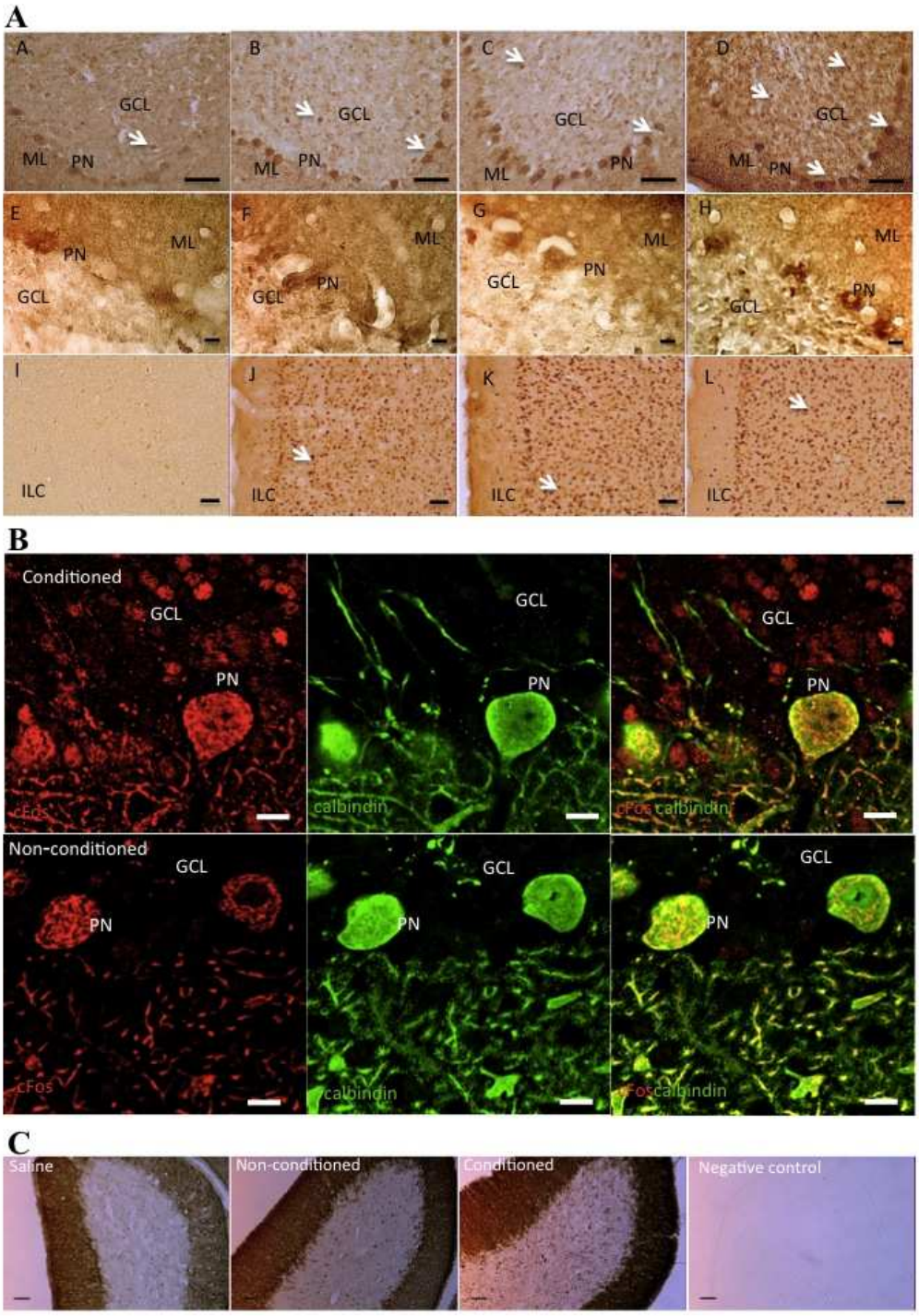
Figure 6: Group effects on cFos expression in the apical and medial regions of the Purkinje cell layer of the lobule VI (panel A) and lobe VII (panel B) of the vermis cerebellum. The results for these two lobules are shown because they were the only ones at which statistically significant differences between groups were found (see table 3 for further details). Capital letters indicate a significant difference ($p<0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group, whereas lowercase letters (a, b, c, d) were used when the same differences were reached at a lower significance level ($p<0.05$) (see tables 6, 7).

Figure 7: Group effects on cFos+ staining in the different cortical regions of the prefrontal cortex. Panels display the mean $\pm$ SEM of cFos positive neurons at the cingulate (A), prelimbic (B), infralimbic (C) and orbitofrontal (D) cortices of each treatment group. Capital letters indicate a significant difference ($p<0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group, whereas lowercase letters (a, b, c, d) were used when the same differences were reached at a lower significance level ($p<0.05$). Additional details on these data can be found table 8.

Figure 8: Representative correlations between c-Fos expression in the apical region of the granule cell layer and preference for CS+ obtained. (A) Lobule III, (B) Lobule VIII, (C) cingulate cortex, (D) infralimbic cortex.

Figure 9: Schematic representation of the hypothetical functional role of different regions in the cerebellar cortex.

Fig 1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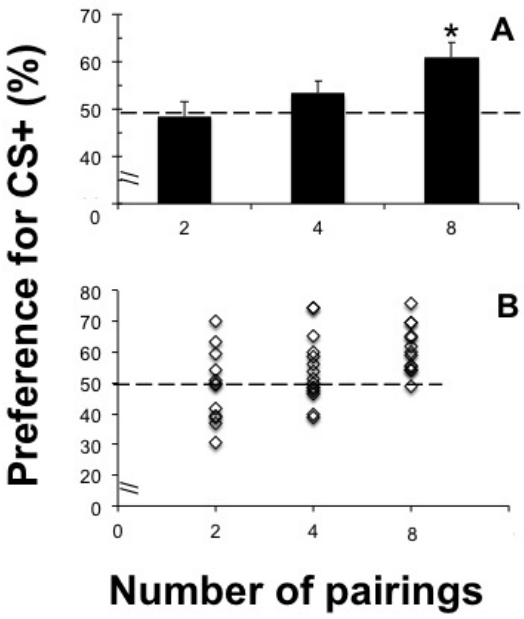
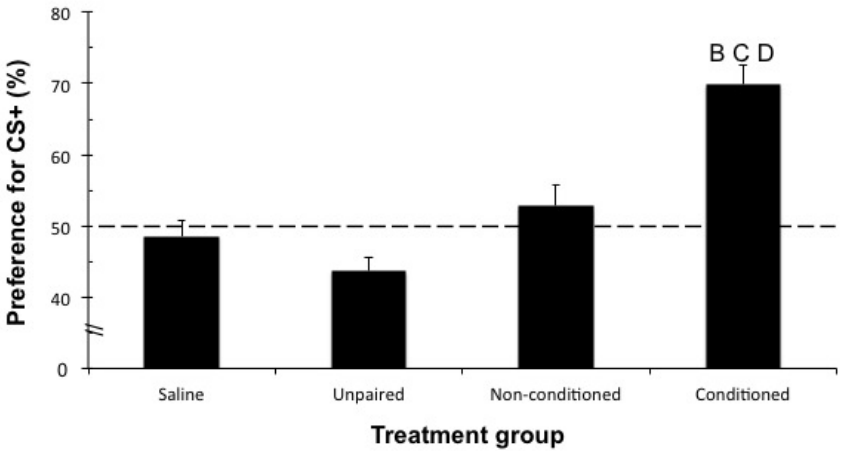


Fig 2

Fig 3



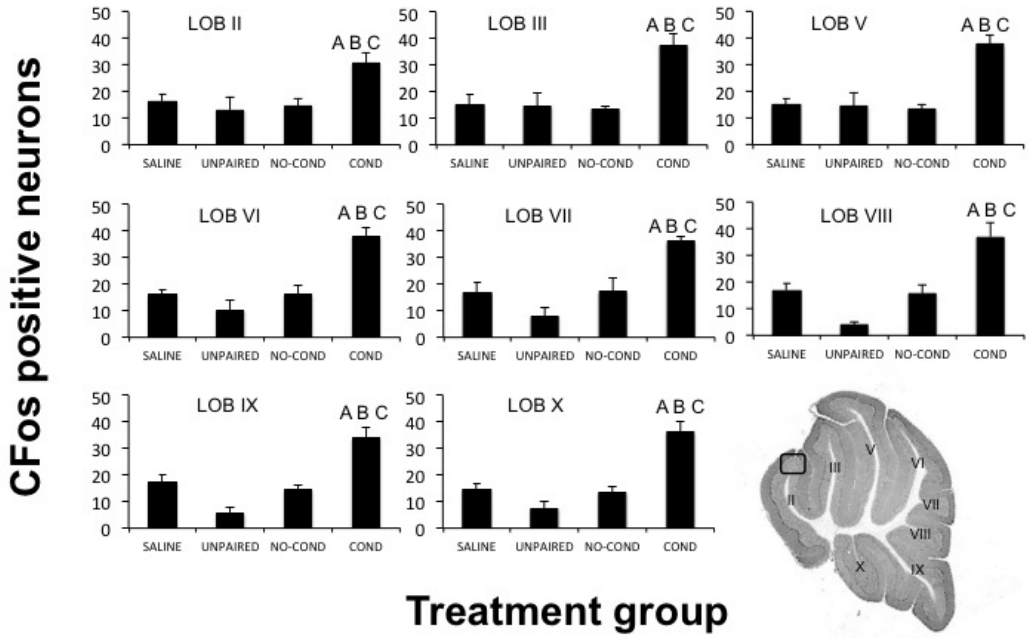
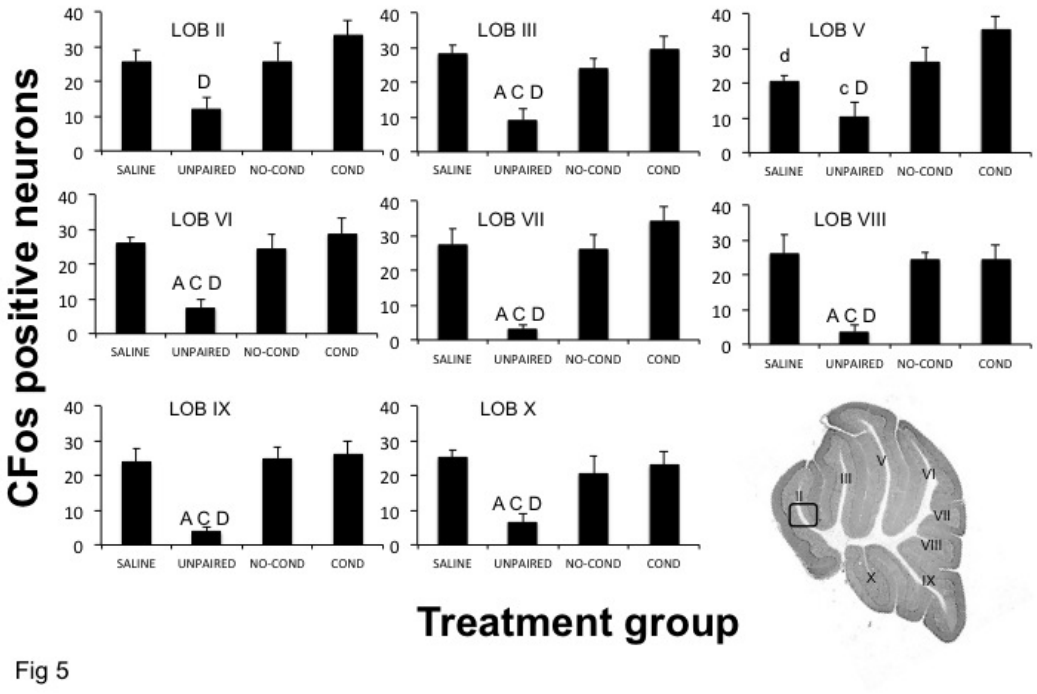


Fig 4



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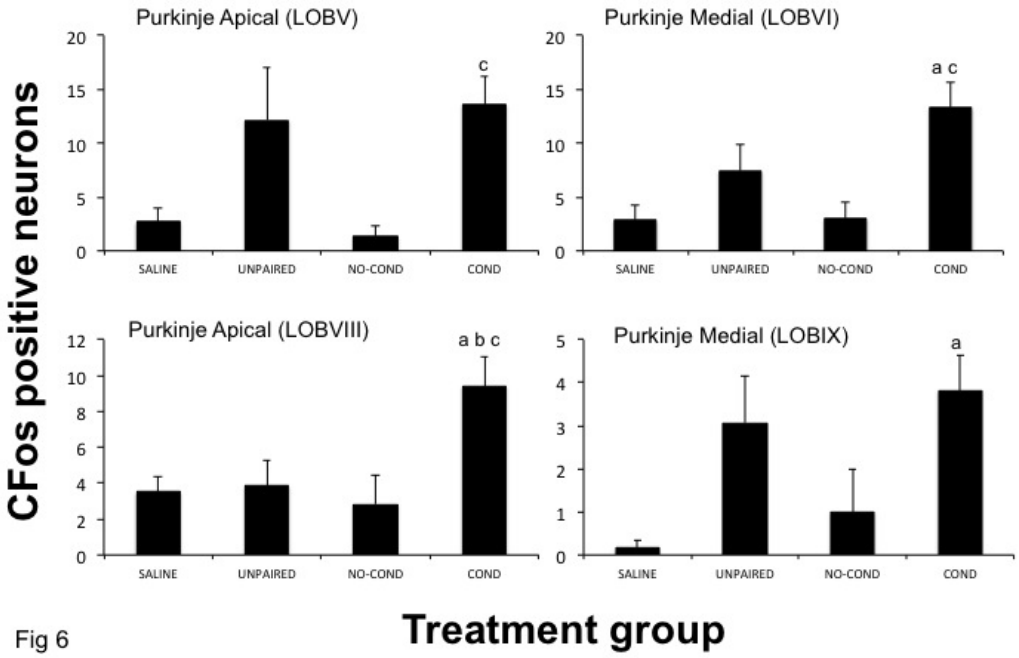

Fig 6

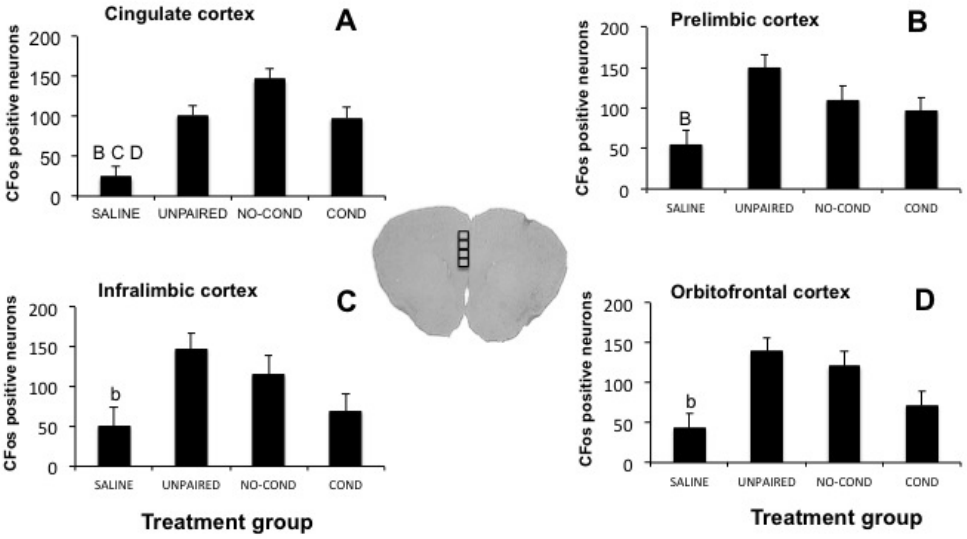

Fig 7

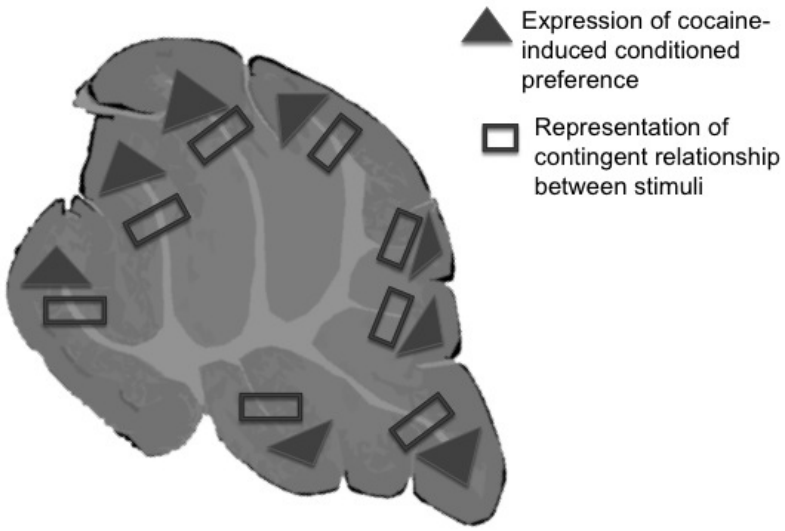

Fig 9