The role of the cerebellum in drug-cue associative memory: functional interactions with the medial prefrontal cortex

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ABSTRACT

Drug-induced Pavlovian memories are thought to be crucial for drug addiction because they guide behaviour towards environments with drug availability. Drug-related memory depends on persistent changes in dopamine-glutamate interactions in the medial prefrontal cortex (mPFC), basolateral amygdala, nucleus accumbens core and hippocampus. Recent evidence from our laboratory indicated that the cerebellum is also a relevant node for drug-cue associations. In the present study, we tested the role that specific regions of the cerebellum and mPFC play in the acquisition of cocaine-induced preference conditioning. Quinolinic acid was used to manage a permanent deactivation of lobule VIII in the vermis prior to conditioning. Additionally, lidocaine was infused into the prelimbic and infralimbic (IL) cortices for reversible deactivation before every training session. The present findings show, for the first time, that the cerebellum and mPFC might act together in order to acquire drug-cue Pavlovian associations. Either a dorsal lesion in lobule VIII or an IL deactivation encouraged cocaine-induced preference conditioning. Moreover, simultaneous IL-cerebellar deactivation prevented the effect of either of the separate deactivations. Therefore, similar to in the IL cortex, neural activity in the cerebellum may be crucial for ensuring inhibitory control of the expression of cocaine-related memories.

INTRODUCTION

The strength and persistence of drug-seeking responses in drug addiction are thought to be sustained by long-lasting drug-cue associative memories that compel goal-directed behaviours towards contexts of drug availability (Everitt & Robbins, 2005). The incentive and conditioned reinforcing properties of drug-related cues depend on persistent changes in dopamine-glutamate interactions in the medial prefrontal cortex (mPFC), basolateral amygdala (BLA), nucleus accumbens core (NAcore) and hippocampus (Belin & Everitt, 2008; Volkow *et al.*, 2013).

Remarkably, the cerebellum is closely connected to the functional loops in the striatum–cortico–limbic circuitry, as has been established by tracing techniques, electrostimulation, and optogenetics (Panagopoulos *et al.*, 1991; Ikai *et al.*, 1992; Hoover & Strick, 1999; Ichinohe *et al.*, 2000; Melchitzky & Lewis, 2000; Hoshi *et al.*, 2005; Glaser *et al.*, 2006; Yu *et al.*, 2007; Bostan *et al.*, 2010; Chen *et al.*, 2014; Herrera-Meza *et al.*, 2014). Moreover, different regions in the cerebellum have been demonstrated to be involved in the formation and storage of motor and emotional Pavlovian memory (Steinmetz *et al.*, 1992; Topka *et al.*, 1993; Sacchetti *et al.*, 2002, 2004; Gao *et al.*, 2016; Giovannucci *et al.*, 2017). Additionally, growing evidence has indicated that the cerebellum is a relevant node for drug-cue associations in humans (Moulton *et al.*, 2014) and animals (Carbo-Gas *et al.*, 2014a; Carbo-Gas, *et al.*, 2014b; Carbo-Gas *et al.*, 2017). Neuroimaging studies of cue reactivity in drug addicts have consistently shown activation in the cerebellum when drug-related cues were presented (Grant *et al.*, 1996; Schneider *et al.*, 2001; Bonson *et al.*, 2002; Anderson *et al.*, 2006; Fuentes *et al.*, 2012). Recent research from our laboratory has gone a step further in determining an accurate location for the cerebellar area involved in these drug-cue associations (Carbo-Gas *et al.*, 2014a; Carbo-Gas *et al.*, 2014b; Carbo-Gas *et al.*, 2017). Overall, our findings have indicated

that cocaine-induced preference conditioning selectively increases neural activity and the expression of perineuronal nets in the dorsal region of the granular cell layer in the vermis. Correlations between neural activity and drug-induced conditioned preference were observed in lobules III, VIII and IX. These cerebellar lobules receive dopaminergic projections from the ventral tegmental area (VTA) (Ikai *et al.*, 1992, 1994) and express dopamine transporters (Melchitzky & Lewis, 2000; Carbo-Gas *et al.*, 2014b).

Several studies have observed that the prelimbic (PL) and infralimbic (IL) cortices form different reciprocal loops through the brain (Ongür & Price, 2000; Vertes, 2004; Hoover & Vertes, 2007) and exhibit opposite roles at the functional level (Ongür & Price, 2000; McFarland & Kalivas, 2001; Capriles *et al.*, 2003; Peters *et al.*, 2009; Sierra-Mercado *et al.*, 2011; Ball & Slane, 2012; Pfarr *et al.*, 2015). Specifically, reinstatement of cocaine-seeking behaviour requires the integrity of the PL cortex (McFarland & Kalivas, 2001; McLaughlin & See, 2003), whereas the IL cortex is needed for the suppression of this response, presumably promoting the extinction of this behaviour (LaLumiere *et al.*, 2010; Lalumiere *et al.*, 2012).

Thus, two different reciprocal loops have been proposed for the mPFC. Reinstatement of cue-induced cocaine seeking is driven by close interactions among the PL, NAcore, BLA and VTA. By contrast, the consolidation and expression of extinction of a previously acquired cocaine seeking response is under the control of the IL, NAshell and BLA (McFarland & Kalivas, 2001; McLaughlin & See, 2003; Lalumiere *et al.*, 2012).

Interestingly, human studies of drug addicts have indicated that the prefrontal cortex and cerebellum may be recruited in a competitive manner during reward tasks (Martin-Sölch *et al.*, 2001; Desmond *et al.*, 2003; Hester, 2004; Bolla *et al.*, 2005). In these studies, a prefrontal impairment was accompanied by strong activation of the cerebellum. Thus, it seems that the cerebellum acquires higher functional relevance when prefrontal function

is compromised by disease or chronic drug use (Anderson *et al.*, 2006; Miquel *et al.*, 2009).

Very recently, we proposed that the dorsal and ventral regions in the posterior vermis could be functionally related to different prefrontal–striatal–limbic loops in order to initiate or restrain cocaine seeking (Miquel *et al.*, 2016). In the present investigation, we tested for the first time the role that specific regions of the cerebellar cortex play in the acquisition of cocaine-induced conditioned preference. Additionally, we explored the effects of focal deactivation in the IL and PL cortices. Finally, we wondered whether simultaneous IL–cerebellum deactivation would be able to change the effects of deactivating each of the regions separately. Importantly, this work is the first attempt to provide support for a causative role of the cerebellum in the regulation of drug-related behaviours.

METHODS

Subjects

Male Sprague-Dawley rats weighing 175–200 g (N = 151) were obtained from Janvier (ST Berthevin Cedex, France). Rats were individually housed in the animal facility (Jaume I University, Spain) under standard laboratory conditions (12-h light cycle from 8:00 AM to 8:00 PM) with access to food and water *ad libitum*. Handling was performed on a daily basis for two weeks before the experiments began. Rats were subjected to stereotaxic surgery when they reached a weight of 270–350 g. Behavioural protocols took place within the first five hours of the light cycle, two hours after the lights were turned on. All animal procedures were approved by the local Animal Welfare Ethics Committee and Empowered Body and were developed in accordance with the European Community

Council directive (2010/63/EU), Spanish directive BOE 34/11370/2013 and local directive DOGV 26/2010.

Pharmacological agents

Cocaine hydrochloride (Alcaliber S.A., Madrid, Spain) was dissolved in a 0.9% saline solution and administered intraperitoneally (IP). The 0.9% saline solution was used as the control vehicle. Anaesthesia was induced using a cocktail of ketamine (100 mg/kg) (Imalgene 100 mg/ml, Mersal Laboratorios S.A., Barcelona, España) and xylazine (10 mg/kg) (xylazine hydrochloride ≥ 99%, Sigma-Aldrich Co. LLC, Madrid, España). Lidocaine (6%; 60 mg/ml) (lidocaine hydrochloride, Sigma-Aldrich Co. LLC, Madrid, España) and quinolinic acid (90 nmol/µl) (2,3-pyridinedicarboxylic acid, Sigma-Aldrich Co. LLc, Madrid, España) were used for deactivation of the mPFC and the cerebellum, respectively.

Stereotaxic surgery and brain deactivation procedures

All rats weighed between 270 and 350 g before stereotaxic surgery. Surgery was performed using a Kopf stereotaxic apparatus. For the intracranial infusion, a stainless steel guide cannula (length, 10 mm; external diameter, 23 gauge) was targeted at the following coordinates with respect to bregma (Paxinos & Watson, 1998). For the cerebellum, the dorsal area (AP: −14.5; ML: 0; DV: -4.5) and the ventral area (AP: -13; ML: 0; DV: -4.5) of lobule VIII in the vermis were targeted. For the mPFC, the PL (AP: +3.2; ML: +0.6/−0.6; DV: -3) and IL (AP: +3.2; ML: + 0.6/-0.6; DV: -4) cortices were targeted (Fig. 1). After the surgery, all the animals received analgesic treatment with meloxicam (Metacam 5 mg/ml, Boehringer Ingelheim España S.A., Barcelona, España),

repeated every 24 hours for three days. The animals remained undisturbed for three to five days after surgery for recovery **(for the experimental timeline see Fig. 2A).**

Excitotoxic lesions from quinolinic acid were preferred for the lesion of the posterior cerebellum (lobule VIII) because in our past experience, the cannula installation did not remain in place for a long time. In this case, the infusion was performed only once during the initial surgery under anaesthesia. Quinolinic acid (90 nmol/μl) was released through a removable stainless steel injector (length, 11 mm; external diameter, 30 gauge) inserted into the previously implanted guide cannula and connected to an infusion pump (volume, 0.5 μl; infusion ratio, 0.2 μl/min). The infusions were made unilaterally at the middle line of lobule VIII in the vermis (ML: 0), which is in this cerebellar region that we have previously described plasticity changes linked to cocaine-related memory (Carbo-Gas *et al.*, 2014a; Carbo-Gas *et al.*, 2014b; Carbo-Gas *et al.*, 2017). After the infusion was completed, the injector remained in place for 3 min to avoid liquid aspiration. Then, the guide cannula was removed, and the wound was sutured. The same procedure was implemented in the sham group, but in this case, phosphate buffered saline (PBS) was infused.

For mPFC deactivations, the guide cannulas were attached to the skull through stainless steel screws fixed with acrylic dental cement. Stainless steel obturators were kept in the guide cannula to maintain the cannula's integrity. Rats were gently handled while restrained, and 6% lidocaine (60 mg/ml) was infused either into the IL or PL cortex before each training trial (volume, 1 μl; infusion ratio, 0.5 μl/min). Rats were not anaesthetised during the microinjections because this procedure does not involve pain or discomfort for the animals. Behavioural trials began 2 min after the infusion, as deactivation via lidocaine only lasts for 20 min (Martin, 1991). Sham animals underwent the same procedure, but saline was infused instead of lidocaine. Cannula placements for each site

were counterbalanced among the animals in terms of the right and left sides, and infusions were made unilaterally. Bilateral cannula installations were not included in this study as we intended to preserve mPFC functions partially in order to obtain a more realistic picture of what would happen during an early chronic experience with the drug or in vulnerable brains.

Finally, simultaneous deactivations of the cerebellum and IL cortex were achieved using the two abovementioned procedures in the same rat. Therefore, rats were trained under a unilateral IL deactivation together with a neurotoxic lesion in the dorsal region of lobule VIII. The rationale behind this study was to test whether these two regions might act to compensate each other after impairment of any of them. In this case, one could expect that the effect of separate deactivations would be prevented.

Cocaine-induced preference conditioning procedure

Conditioning was developed in an opaque, oblong corridor ($90 \times 20 \times 60$ cm) that included two lateral black chambers ($20 \times 20 \times 60$ cm) located on opposite sides. We evaluated the initial preference for two olfactory stimuli (lavender and rose) of four animals. Because the innate preferences for the odours were not different [Student's t-test for dependent samples: $t(3) = 0.8692$, $p = 0.4487$], these two equally preferred odours were used in the conditioning experiment. Two drops of lavender or rose fragrance were put on gauze and presented inside a steel ball with holes that hung on the walls of the chambers. One of the odours acted as the conditioned stimulus (CS+) and was associated with cocaine (15 mg/kg, IP). On alternate days, rats were exposed to the other odour (CS) and received saline injections. During the training session, the animals remained confined in one of the lateral chambers, and access to the other side was blocked by a panel. Each pairing session lasted for 15 min. A total of eight cocaine-cue paired sessions were

conducted, and the odours used as the CS+ and CS-, as well as the left and right locations in the corridor, were counterbalanced among the animals (Fig. 2B).

Preference for the cocaine-related cue was evaluated 48 h after the last cocaine administration in a 30 min drug-free test in which the CS+ and CS- were presented simultaneously on both sides of the corridor. Importantly, the location of the odours (CS+ and CS-) was opposite to that in the training. Therefore, for the first 10 min of the test session, the animals were allowed to explore the new location of the cues, and thus, this period was not included in the analysis. Then, the time spent (TS) in each chamber was recorded for the last 20 min. All the test sessions were videotaped and scored by a blind observer. The preference score was calculated as [TS in (CS+) / TS in (CS+) + TS in (CS-)] × 100. Additionally, we included a pseudo-conditioning group (Unp group) that was treated with the same number of cocaine injections but was randomly associated with both olfactory stimuli (Fig. 2C). These unpaired groups allowed us to test for memory-related effects of our brain deactivations.

Locomotor activity

Activity was scored by a blind observer in the videos obtained from the preference test session. The 20-min testing period was split into 4 segments of 5 min. The number of crossovers was registered by dividing the corridor into four equal quadrants on the screen. A locomotion score was assigned each time an animal crossed over from one quadrant to another on all four legs. Locomotion was assessed only during the preference test. During conditioning, motor activity was not considered, as rats were confined to one of the lateral chambers for the entire session. Thus, despite the fact that free movement was possible inside these boxes, the movement was limited to a very short distance.

Perfusion protocol and brain sampling

Animals were deeply anaesthetised with sodium pentobarbital (30 mg/kg) (Dolethal 100 ml, Vetoquinol E.V.S.A., Madrid, España) 90 min after the preference test and were perfused transcardially, first with saline solution (0.9%) and then with paraformaldehyde (4%). After perfusion, the brain and cerebellum were quickly dissected and placed in a container with the same fixative for 24 h at 4ºC. After this time, the tissue was immersed in a 30% sucrose solution in PBS until the brain sank. The brain tissue was rapidly frozen by quick immersion in liquid nitrogen, and 40-µm sections were performed with a cryostat microtome (Microm HM560, Thermo Fisher Scientific, Barcelona, Spain). Four series of tissue sections were collected and stored at $-80°C$ in cryoprotectant solution with ethylene glycol. Sagittal sections of the cerebellum and brainstem were selected according to the lateral coordinates from -0.72 mm to 0.72 mm, comprising the whole vermis. For the prefrontal cortex, coronal sections were collected according to bregma coordinates from 4.70 mm to 1.70 mm (Paxinos & Watson, 1998). Several sections were stained with cresyl violet for assessment of the cannula locations. Lesion sites were identified and represented using light microscopy and camera lucida drawings. Rats with cannula misplacement were used as negative controls and were not included in the statistical analysis (Fig 1).

Experimental design and statistics

All behavioural data were based on the preference scores obtained on the test day. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). As a first step, we analysed the effect of cerebellar and prefrontal deactivations on cocaine-induced preference conditioning. In this analysis, because the normality requirements were met, the results were presented as the mean ±

SEM and were analysed by one-way ANOVA or Student's t-tests for independent samples. Then, post hoc comparisons were performed using Tukey's HSD tests. As a second step, we used an arbitrary cut-off point of 60% to cluster sham rats in two subgroups: the preference ($\geq$ 60%, Sham P) and no preference ($<$ 60%, Sham NP) groups. The rationale behind the use of a cut-off point to conform these two different subgroups was based on our previous findings that indicated a completely different kind of cocaine-induced plasticity when comparing mice expressing preference with those that did not (Carbo-Gas et al., 2014a; Carbo-Gas et al., 2014b; Carbo-Gas et al., 2017). Comparisons of the variances in these groups were carried out using Kruskal-Wallis nonparametric analyses tests with post hoc Dunn's multiple comparison test. The results were depicted by scatterplots and median scores. For the data regarding the proportion of rats expressing preference scores higher than 60%, a Chi-square test was used to determine differences between the expected versus observed frequencies. In all analyses, the statistical level of significance was set at $p < 0.05$.

RESULTS

The injections sites are shown in Fig 1. As can be seen, focal infusions with very small diffusion areas were achieved in the present study. Neither of the sham deactivations produced significant effects on cocaine-induced preference conditioning, as demonstrated by Student's t-tests for independent samples [Sham Dorsal versus Sham Ventral: ($t_{(19)}$ = 0.6104, p = 0.5489); Sham IL versus Sham PL: ($t_{(17)}$ = 0.7523, p = 0.4622)]. Therefore, sham animals were collapsed for each brain region to shape two different control groups, namely, the Sham cerebellum and Sham mPFC groups. Then, these two groups were split into preference (P) and no preference (NP) groups, as explained above. Additionally, we tested for significant differences between the effects of deactivation on

the left and right sides of the mPFC. Neither the sham [(t (17) = 1.05, p = 0.3085)] nor the lidocaine groups [IL (t (6) = 1.299, p = 0.2418); PL (t (5) = 0.06548, p = 0.9503)] exhibited any kind of lateralisation effect.

An excitotoxic lesion in the dorsal region of lobule VIII facilitates cocaine-induced preference conditioning

A one-way ANOVA of the preference scores yielded a significant group effect (F (2,32) = 4.672, p = 0.0166). As shown by subsequent post hoc comparisons using Tukey's HSD tests, the quinolinic acid dorsal group (QA Dors) (n = 6) exhibited a significantly higher preference for the CS+ than the control (Sham) (n = 21) (p = 0.0143) and unpaired dorsal (Unp Dors) (n = 8) (p = 0.0492) groups (Fig. 3A).

As seen in figure 3A, only a subgroup of the sham rats showed a clear preference for the cocaine-related odour cue. Therefore, the sham animals were split into two subgroups, namely, the Sham NP (n = 15) and Sham P (n = 6) groups, by using the arbitrary preference cut-off point of 60%. A Kruskal-Wallis test demonstrated a significant effect of the group factor (H (4) = 23.06, p < 0.0001). Post hoc comparisons revealed that all lesioned animals (QA Dors) showed the same preference level as that of the Sham P group (p > 0.99) (Fig. 3B), and both groups exhibited an increased preference for the CS+ compared to that of the Sham NP group (p < 0.001). Then, a Chi-square test was conducted to compare the proportion of animals that met our criteria for preference in each group. Remarkably, the excitotoxic lesions in the dorsal lobule VIII promoted the acquisition/expression of cocaine-induced preference conditioning in 100% of the trained animals (χ^2 (2) = 10.89, p = 0.0043). However, the percentage of preference animals in the sham group was 28.57% (Fig. 3C).

Rats with a ventral region of lobule VIII do not show cocaine-induced preference conditioning

A one-way ANOVA comparing the preference for the CS+ did not demonstrate a significant effect of the group factor $(F_{(2,36)} = 1.301, p = 0.2848)$ (Fig. 3D). Nevertheless, the nonparametric analysis, which split the sham group into the NP (n = 15) and P (n = 6) groups, yielded a significant effect of the group factor $(H_{(4)} = 16.31, p < 0.001)$ (Fig. 3E). Dunn's multiple comparisons test revealed that ventrally lesioned animals (QA Vent) showed a similar preference score to those of the Sham NP (p > 0.99) and unpaired ventral (Unp ventral) groups (p > 0.99). In addition, the Sham P group exhibited a higher preference than the Sham NP (p < 0.001) and QA Ventral (p < 0.004) groups (Fig. 3E). Despite the fact that no lesioned animal reached the preference score of 60%, a Chi-square test revealed no significant differences in the proportions of rats that acquired cocaine-induced conditioned preference after ventral lesions $(\chi^2_{(2)} = 3.954, p = 0.138)$ (Fig. 3F).

A temporary deactivation of the IL cortex promotes cocaine-induced preference conditioning

A temporal deactivation of the IL cortex facilitated the acquisition of cocaine-induced preference conditioning, as indicated by a one-way ANOVA $(F_{(2,31)} = 8.879, p = 0.0009)$. As shown by a subsequent post hoc comparison using Tukey's HSD tests, the lidocaine IL group (Lido IL) (n = 8) exhibited a significantly higher preference for the CS+ than the sham (n = 19) (p = 0.0021) and unpaired lidocaine groups (Unp IL) (n = 7) (p = 0.0021) (Fig. 4A). The Kruskal-Wallis test showed a significant effect of the group factor $(H_{(4)} = 21.3, p < 0.0001)$ (Fig. 4B). Dunn's post hoc comparisons revealed that a repeated IL deactivation before each training session increased preference to the same

level as that shown by the Sham P group ($p > 0.99$). Additionally, both groups were different from the Sham NP group ($p < 0.001$ and $p < 0.03$, respectively), but only the animals in the lidocaine IL group exhibited a significantly higher preference than the unpaired group ($p < 0.02$) (Fig. 4B). Moreover, 100% of deactivated animals expressed a preference score higher than 60% ($\chi^2 (2) = 10.6$, $p = 0.005$) (Fig. 4C).

Rats with a temporary deactivation of the PL cortex do not show cocaine-induced preference conditioning

The transient deactivation of the PL cortex did not produce a significant effect on cocaine-induced conditioned preference ($F (2,31) = 1.152$, $p = 0.3293$) (Fig. 4C). Nevertheless, as seen in the scatterplots (Fig. 4D), PL-deactivated animals (n=7) showed a preference score similar to that of the Sham NP group (n=12). A Kruskal-Wallis test demonstrated a significant effect of the group factor ($H (4) = 13.14$, $p = 0.0043$). Both the Sham NP ($p < 0.01$) and lidocaine PL ($p < 0.05$) groups were different from the Sham P group (n=7), as revealed by post hoc tests (Fig. 4D). However, a Chi-square test of the proportions of animals that met the criterium for preference revealed no significant differences ($\chi^2 (2) = 3.579$, $p = 0.167$) (Fig. 4F).

Simultaneous deactivation of the IL cortex and dorsal lobule VIII prevents the facilitative effect on cocaine-induced preference conditioning

Remarkably, the effect of IL deactivation was very similar to that observed after dorsal lesions of the cerebellar cortex. Therefore, we managed to deactivate both regions simultaneously in order to ascertain if these regions might outweigh the lack of activity in the other region after impairment. As expected if they were functionally related, the facilitative effect of the separate deactivations was prevented by combining both a

unilateral deactivation of the IL cortex and a dorsal lesion of lobule VIII. Student's t-test

for independent samples supported no differences in the preference scores between the

animals with deactivation and sham animals (t (9) = 0.8126, p = 0.4374) (Fig 5A). Thus,

the proportion of rats expressing preference was rescued to control levels [(χ^2 (1) =

0.5051, p = 0.4773)] (Fig. 5B).

Motor activity during the preference test is unaffected by either prefrontal or

cerebellar deactivations

Locomotion was assessed during the preference test by dividing the 20-min testing period

into 5 min segments. Neither of our manipulations affected locomotion during the

preference test, as was demonstrated by two-way repeated measures ANOVAs of each

region. In all cases, locomotion decayed during the session for all groups independent of

the group factor [Dorsal cerebellum: Group $F_{(2,10)}$ = 0.888, p = 0.4415; Time $F_{(3,15)}$

= 35.72, p < 0.0001; Interaction $F_{(6,30)}$ = 2.338; p = 0.0569]; [Ventral cerebellum: Group

$F_{(2,12)}$ = 2.16 p = 0.1581; Time $F_{(3,18)}$ = 25.85, p < 0.0001; Interaction $F_{(6,36)}$ =

2.212, p = 0.0642]; [IL: Group $F_{(2,12)}$ = 1.293, p = 0.31; Time $F_{(3,18)}$ = 33.04, p <

0.0001; Interaction $F_{(6,36)}$ = 0.728, p = 0.0629]; [PL: Group $F_{(2,12)}$ = 0.489, p =

0.6247; Time $F_{(3,18)}$ = 18.43, p < 0.0001; Interaction $F_{(6,36)}$ = 2.282, p = 0.0573] (Fig.

6).

DISCUSSION

It is widely accepted that mPFC impairment is a crucial part of the physiopathology of

drug addiction (McFarland & Kalivas, 2001; Van den Oever *et al.*, 2010; Goldstein &

Volkow, 2011). However, not until recently has the cerebellum been considered a

relevant structure in understanding the persistent drug-induced behavioural alterations in addiction (Miquel *et al.*, 2009, 2016; Moulton *et al.*, 2014).

The present results show, for the first time, that the dorsal region of the posterior cerebellum plays a role similar to that of the IL cortex in the establishment of drug-cue Pavlovian memory. The loss of activity in either of these regions dramatically increased the number of animals that expressed cocaine-induced conditioned preference. The effects of a lesion in the ventral region of lobule VIII or a deactivation of the PL cortex are less clear. In both cases, the inactivation seems to reduce the proportion of rats that show preference for the cocaine-related cue, although statistics do not provide full support for the significance of the effects. Thus, further research is needed in order to propose any functional interactions between these two regions. Importantly, all the effects were memory-related and specific for the formation of drug-cue associations since none of our manipulations were shown to be effective in the pseudo-conditioned rats (unpaired groups).

We noticed that in our procedure only a small group of control rats (29%) developed a clear preference for the cocaine-related cue. Several methodological issues, such as the high cocaine dose used (15 mg/kg), the use of a discrete odour cue instead of a place preference procedure or the elevated number of drug-cue pairings, might explain the reduced number of conditioned animals found in the sham groups. Nevertheless, both IL and dorsal cerebellar impairment caused a consistent and robust effect, increasing by up to 100% the number of animals expressing conditioned preference (Fig. 3C-4C).

As the IL deactivation was unilateral, the intact contralateral IL or even the PL cortex might increase its activity, promoting the acquisition of cocaine-related memory. As a matter of fact, the PL cortex may be inhibited by the IL cortex (McFarland & Kalivas, 2001; Lalumiere *et al.*, 2012), and thus, the facilitative effect on cocaine-induced

conditioning, caused by a partial IL deactivation, might then be explained by a reduced inhibition of PL activity. IL deactivation could also enhance activity in the cerebellum. Indeed, it has been shown that the prefrontal dysfunction observed in drug addicts is accompanied by an increase in cerebellar activity (Martin-Sölch *et al.*, 2001; Desmond *et al.*, 2003; Hester, 2004; Bolla *et al.*, 2005). Similarly, lesions of the dorsal cerebellum could boost neural activity in the mPFC, striatum and limbic regions and thereby facilitate cocaine-induced preference conditioning. Supporting a close functional loop, our results also revealed that simultaneous cerebellum-IL impairment prevents the facilitative effect of separate deactivations. This finding suggested that both the IL cortex and the dorsal cerebellum might increase their relevance during conditioning when the other region has been compromised. Subsequently, if both areas are impaired at the same time, this compensatory function will not be possible, and the propensity to acquire cocaine-induced conditioned preference will resemble that of the control group.

The present findings argued in favour of our recent hypothesis proposing that the dorsal regions of the posterior vermis are part of the IL-NAshell-BLA network (Miquel *et al.*, 2016). Our previous work established that the plasticity hallmark signatures of cocaine-induced preference conditioning are expressed in the dorsal region of the cerebellar cortex (Carbo-Gas *et al.*, 2014a; Carbo-Gas *et al.*, 2014b). Strikingly, we showed here that the acquisition of drug-cue associations is facilitated when the same region of the cerebellar cortex is damaged. Taken together, our findings suggested that the dorsal region of the posterior vermis might inhibit drug seeking using previously learned Pavlovian associations that involve other additional regions in the striatum–cortico–limbic circuitry. Interestingly, behavioural inhibition has been one of the functions ascribed to the cerebellum (Moers-Hornikx *et al.*, 2009; Picazio & Koch, 2015). Cerebellar lesions promote behavioural disinhibition (Schmahmann & Sherman, 1998; Tanaka *et al.*, 2003),

whereas increasing activity in the cerebellum improves inhibitory control (Brunamonti *et al.*, 2014).

Numerous studies have found reciprocal loops between the prefrontal cortex and the cerebellum that may provide anatomical evidence to explain the present results (Middleton & Strick, 1994, 2001; Schmahmann & Pandya, 1997; Sang *et al.*, 2012; see Bostan & Strick, 2018 for a recent compelling review). Moers-Hornikx *et al.* (2009) observed an increase in cFos expression in the deep cerebellar nuclei and the prefrontal cortex after deep brain stimulation of the mediodorsal and ventrolateral thalamic nuclei in rats. Furthermore, cortical regulation of striatal activity can be modulated by the cerebellum (Chen *et al.*, 2014). A direct dopaminergic VTA-cerebellar projection has also been demonstrated (Ikai *et al.*, 1992, 1994). Detectable DA levels were found in the posterior lobules of the vermis (VII–X), the right and left hemispheres and the fastigial, interpositus and dentate nuclei (Glaser *et al.*, 2006). In addition, it has been shown that the cerebellar cortex may regulate dopamine release in the mPFC by several independent pathways. First, the cerebellum connects to the VTA through the reticulotegmental and pedunculopontine nuclei (Forster & Blaha, 2003). Second, the cerebellum projects to the VTA through the mediodorsal and ventrolateral thalamus (Rogers *et al.*, 2011). Finally, and more relevant for the present discussion, is the finding of a direct projection from the deep cerebellar nuclei to the VTA (Watabe-Uchida *et al.*, 2012). This projection would be crucial for explaining the present results as it provides a direct pathway for the cerebellum to control the cortico-striatal circuitry through an increase in dopaminergic activity.

Nevertheless, a number of caveats and limitations of the present study should be considered. Our sample only included rats with focal lesions in lobule VIII. It is noteworthy that other anterior or posterior cannula locations (lobule VII or IX) did not

seem to reproduce the facilitative effect on cocaine-induced preference conditioning. Recent evidence indicated that the cerebellum is subdivided into different specialised regions to regulate specific behaviours (Glickstein *et al.*, 2011; Witter & De Zeeuw, 2015). However, it has also become clear that a cerebellar lobule is not the main functional unit. First, a lobule can contain several functional areas; second, cerebellar functions can encompass several lobules (Witter & De Zeeuw, 2015). This raises the question of which functional characteristics and connectivity patterns make the dorsal region of lobule VIII in the vermis somehow relevant to associative memory and behavioural inhibition. The dorsal cerebellar cortex receives sensorimotor corticopontine and exteroceptive components of the mossy fibre afferent system, providing neural information from cortical sensorimotor networks to the cerebellum (Ekerot & Larson, 1972; Voogd & Ruigrok, 2004). In addition, a recent study of motor associative learning established a prominent nucleocortical excitatory projection of mossy fibres to the most superficial region of the granule cell layer that optimised the conditioned response (Gao *et al.*, 2016). Granule cell activity in this area is present during unconditioned and conditioned stimuli, as well as during the conditioned response (Giovannucci *et al.*, 2017). This activity also encodes the expectation of reward (Wagner *et al.*, 2017). Classically, lobule VIII in the vermis was considered part of the skeletomotor divisions of the cerebellum, projecting to motor cortices through the fastigial nucleus and also to the descending motor pathways (Glickstein *et al.*, 2011). It is important to highlight that the present cerebellar lesion did not cause a generalised and unspecific motor disinhibition since locomotor activity during the preference test was not affected. Lobules VII–X of the vermis have also been proposed to serve as an interface among sensory processing, emotional states and motor responses, due to the anatomical and functional connectivity with the amygdala and other areas of the emotional brain (Adamaszek *et al.*,

2017). Therefore, it is plausible for the cerebellum to modulate the reward response in other areas of the striatum–cortico–limbic circuitry.

In conclusion, our findings open new avenues to understanding the role of the cerebellum in drug addiction. Further research using specific experimental approaches is needed to determine the control of different neuronal populations in the dorsal and ventral regions of the vermis.

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AUTHOR DISCLOSURE

All authors declare no conflicts of interest.

AUTHORSHIP

All authors made a substantial contribution to the manuscript, and they were involved in critically revising the present version. Isis Gil-Miravet performed the stereotaxic surgeries and behavioural experiments; Isis Gil-Miravet, Julian Guarque-Chabrera, María Carbo-Gas and Francisco Olucha-Bordonau were involved in data analysis and the editing of the manuscript. Finally, Marta Miquel designed the study, supervised the surgeries and behavioural experiments, and drafted the manuscript. All authors approved the present version of the manuscript.

DATA ACCESSIBILITY

Data are available from the corresponding author upon request.

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LEGENDS

FIG. 1 Diagrams of the cannula locations. Schematic diagrams depicting **the largest (grey) and smallest (black) diffusion areas** in the PL and IL cortices, as well as in the dorsal and ventral regions of the cerebellar vermis. The extent of the diffusion areas was assessed using light microscopy and lucida camera drawings.

FIG. 2 (A) Experimental timeline. Different stages of the experimental procedure from the stereotaxic surgery to the animal perfusion. (B) Cocaine-induced preference conditioning protocol for the paired group. For sixteen training days, rats received eight cocaine "C" and eight saline "S" administrations on alternate days that were associated with olfactory stimuli that acted as the CS+ (dark grey) or CS- (light grey). (C) Cocaine-induced preference conditioning protocol for the unpaired group. The number of cocaine "C" and saline "S" injections was the same as previously mentioned, but they were randomly associated with the odours.

FIG. 3 Effect of an excitotoxic lesion in the cerebellum on cocaine-induced preference conditioning. (A) Preference scores for the CS+ on the test day in the control (Sham) (n = 21), quinolinic acid dorsal (QA Dors) (n = 6) and unpaired dorsal (Unp Dors) (n = 8) groups. Data are shown as the mean ± SEM. (B) Scatterplots of preference scores for the CS+ on the test day in the Sham NP (n = 15), Sham P (n = 6), QA Dors and Unp Dors groups. Data are shown as the median and individual preference scores. (C) Percentages of rats expressing a preference score above and below 60% after dorsal lesions of lobule VIII. Dorsal cerebellar lesions increased the number of rats with a preference score ≥ 60 by up to 100%. (D) Preference scores for the CS+ on the test day in the control (Sham) (n = 21), quinolinic acid ventral (QA Vent) (n = 11) and unpaired ventral (Unp Vent) (n

= 7) groups. Data are shown as the mean ± SEM. (E) Scatterplots of the preference scores for the CS+ on the test day in the Sham NP (n = 15), Sham P (n = 6), QA Vent and Unp Vent groups. Data are shown as the median and individual preference scores. (F) Percentages of rats expressing a preference score above and below 60% after ventral lesions of lobule VIII. The lesions prevented rats from expressing a preference towards the cocaine-related cue. (*p < 0.05; **p < 0.01; ***p < 0.001).

FIG. 4 Effect of a temporary deactivation in the mPFC before each training session on cocaine-induced preference. (A) Preference scores for the CS+ on the test day in the Sham (n = 19), lidocaine infralimbic (Lido IL) (n = 8) and unpaired infralimbic (Unp IL) (n = 7) groups. Data are shown as the mean ± SEM (**p < 0.01). (B) Scatterplots of the preference scores for the CS+ on the test day in the Sham NP (n = 12), Sham P (n = 7), Lido IL and Unp IL groups. Data are shown as the median and individual preference scores. (C) Percentage of rats expressing a preference score above and below 60% after the deactivation of the IL cortex. The IL deactivation increased the number of rats showing a preference score ≥ 60 by up to 100%. (D) Preference scores showed by the control (Sham) (n = 19), lidocaine prelimbic (Lido PL) (n = 7) and unpaired prelimbic (Unp PL) (n = 8) groups on the test day. Data are shown as the mean ± SEM. (E) Preference scores for the CS+ on the test day in the Sham NP (n = 12), Sham P (n = 7), Lido PL and Unp PL groups. Data are shown as the median and individual preference scores. (F) The percentage of rats expressing a preference score above and below 60% after the deactivation of PL. The IL lesion dramatically reduced the proportion of rats that expressed cocaine-induced conditioned preference. (*p < 0.05; **p < 0.01; ***p < 0.001).

FIG. 5 Effect of a simultaneous deactivation of the IL cortex and dorsal cerebellum on cocaine-induced preference conditioning. (A) Preference scores for the CS+ on the test day in the Sham (n = 7) and IL + Dorsal (n = 4) groups. Data are shown as the mean ± SEM. The facilitative effect of separate deactivations was blocked after combining both a unilateral deactivation of the IL cortex and a dorsal lesion of lobule VIII. (B) The percentage of rats expressing a preference score above and below 60% after the simultaneous IL + Dorsal cerebellar deactivation. The number of rats that acquired preference for the cocaine-related olfactory stimulus was similar to that in the control group.

FIG. 6 Effects of deactivation in the cerebellum and mPFC on locomotor activity during the preference test. (A) A dorsal excitotoxic lesion in the cerebellum [(Sham: n = 21); (QA Dors: n = 6); (Unp Dors: n = 8)]. (B) Ventral excitotoxic lesion in the cerebellum [(Sham: n = 21); (QA Vent: n = 11); (Unp Vent: n = 7)]. (C) Deactivation of the IL cortex [Sham: n = 19; (Lido IL: n = 8); (Unp IL: n = 7)]. (D) Deactivation of the PL cortex [(Sham: n = 19); (Lido PL: n = 7); (Unp PL: n = 8). Data are shown as the mean ± SEM. Locomotion decayed during the session for all groups independent of the group factor and the region deactivated.
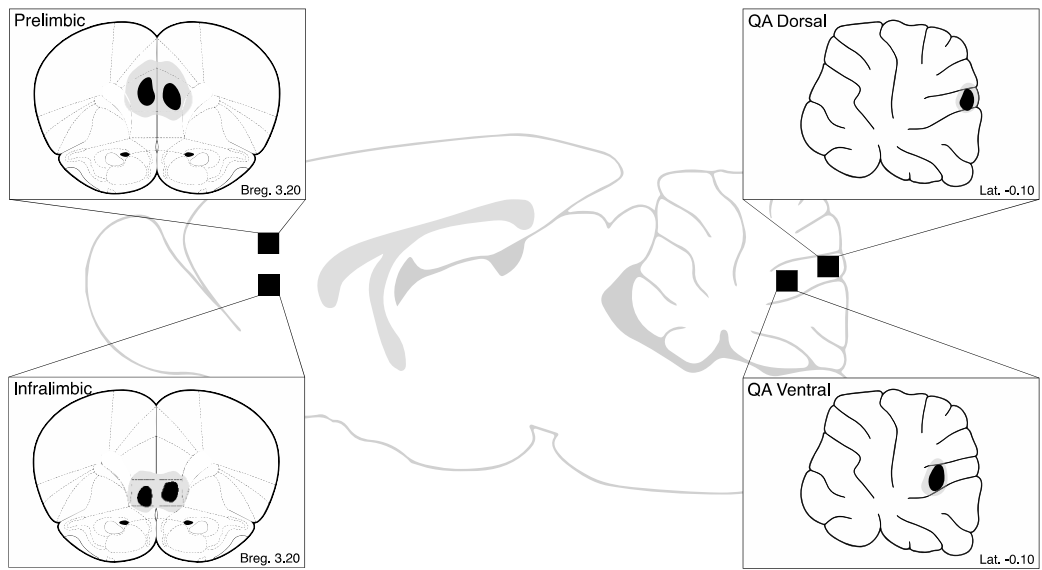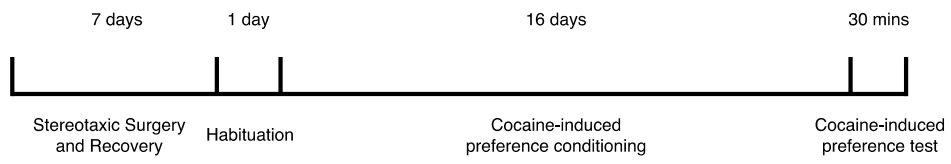
Figure 1

Figure 2

A

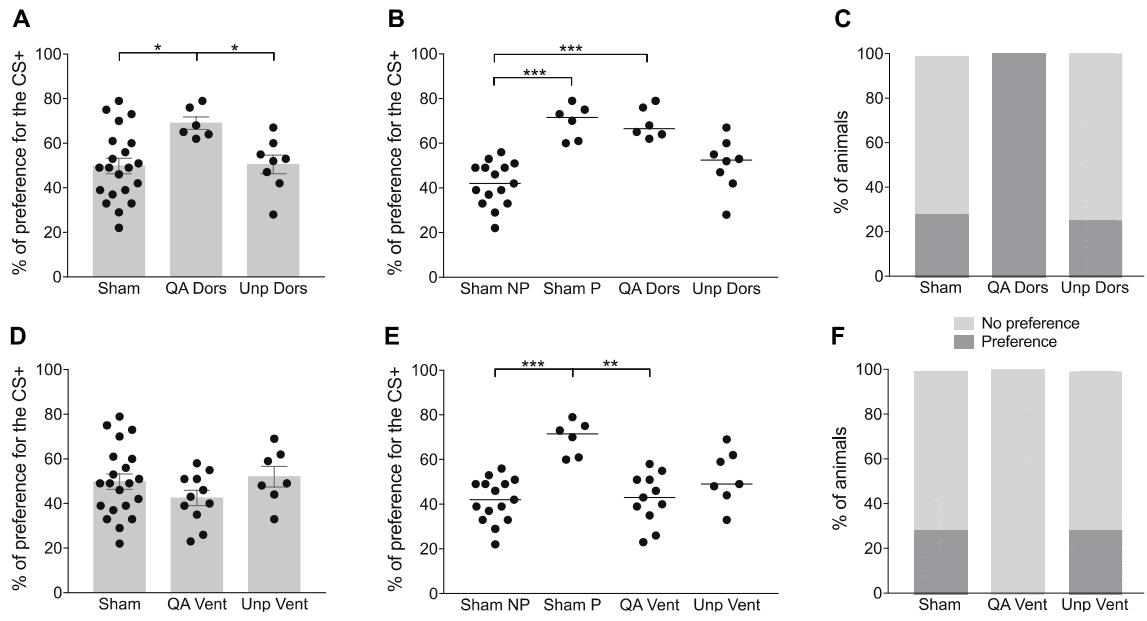7 days   1 day   16 days   30 mins

Stereotaxic Surgery   Habituation   Cocaine-induced   Cocaine-induced
and Recovery   preference conditioning   preference test

B

C S C S C S C S C S C S C S C S

Paired group

C

C S C S C S C S C S C S C S C S

Unpaired group

Figure 3

Figure 4

Figure 5

A

% of preference for the CS+

Sham   IL+Dorsal

B

% of animals

Sham   IL+Dorsal

No preference
Preference

Figure 6