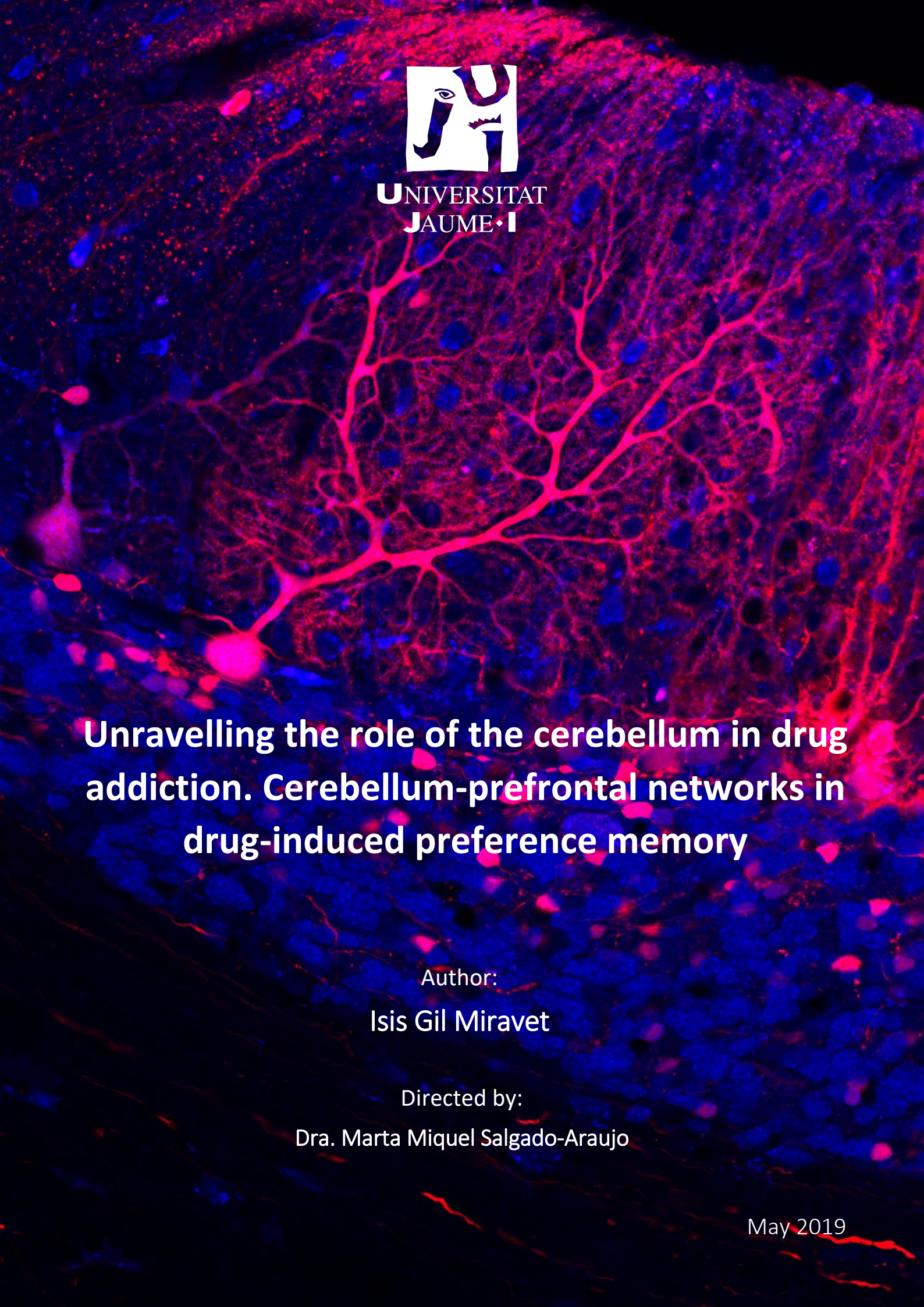

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Unravelling the role of the cerebellum in drug addiction. Cerebellum-prefrontal networks in drug-induced preference memory

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A la meua família

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PREFACE

PREFACE

The cerebellum has been "the long forgotten" in the addiction field for many years. Its study was limited to motor functions and underestimated in the rest of the brain functions. Fortunately, it has changed in the last decades, and the study of the cerebellum has been included in research on language, memory, emotions, decision-making, social behaviour, and drug addiction (Adamaszek *et al*, 2017; Blackwood *et al*, 2004; Broche-Pérez *et al*, 2016; Carbo-Gas *et al*, 2014a, 2014b, 2017; Carta *et al*, 2019; Courchesne and Allen, 1997; Mariën *et al*, 2014; Miquel *et al*, 2016; Moers-Hornikx *et al*, 2009; Moulton *et al*, 2014; Sacchetti *et al*, 2002a, 2004; Strata *et al*, 2011; Turner *et al*, 2007; Vazquez-Sanroman *et al*, 2015b).

Research on the cerebellum's role in addiction is the core of this doctoral thesis. Previous studies in our laboratory involved this structure in drug-induced preference conditioning (Carbo-Gas *et al*, 2014a, 2014b, 2017). This research demonstrated two cerebellar hallmarks of preference for cues linked to the cocaine experience. Both an increased cFos expression and stronger fully condensed perineuronal nets (PNNs) in the apical region of the granule cell layer (the dorsal region of the cerebellar cortex) were observed only when animals expressed a preference towards an odour associated with the drug. These distinctive features were not seen if the animals did not exhibit cocaine-related memory. These findings supported and extended the results of neuroimaging studies on cue-reactivity in addicted cohorts, which found increased greater activation in the cerebellum after the presentation of drug-related cues (Bonson *et al*, 2002; Grant *et al*, 1996; Schneider *et al*, 2001).

Overall, these earlier results suggested that the establishment or expression of cocaine-induced conditioned memories somehow entails cerebellar activation. Moreover, they indicated that cocaine-induced memory encourages, at the cerebellar level, one of the mechanisms for synaptic stabilization, the expression of PNNs. In that way, the cerebellum might be part of the functional networks that represent long-lasting drug-related memories (Sorg *et al*, 2016). Therefore, this doctoral thesis intends to be the first attempt to propose a causative working model for the cerebellum's role in drug-induced memories.

In the following pages, we managed to explore the role of the cerebellum in cocaine-induced conditioned preference and its functional and anatomical relationships with the medial prefrontal cortex. We addressed this goal using temporal or permanent brain deactivations.

The present doctoral dissertation begins with a small theoretical introduction about addiction, followed by a description of the cerebellar anatomy, and a summary of the state of the art in the field.

The first chapter entitled "*The role of the cerebellum in drug-cue associative memory: functional interactions with the medial prefrontal cortex*" is an article already published in the *European Journal of Neuroscience* in which we investigated the role that specific regions of the cerebellum and medial prefrontal cortex (mPFC) play in the acquisition of cocaine-induced preference conditioning. To the best of our knowledge, this is the first study focused on the causative role of the cerebellum in drug addiction. Importantly, the results indicated that the deactivation of these two distal regions generates similar effects on cocaine-related behaviour. Nevertheless, the effects would depend on the specific prefrontal and cerebellar regions deactivated. This first study revealed a close interaction, probably compensatory, between the dorsal cerebellum and the infralimbic cortex in the establishment of cocaine-related memory.

To ascertain the nature of cerebellar-prefrontal relationships and to propose a working functional model for cerebellar-prefrontal interactions, we investigated the consequences of impairing the function in one region for activity and plasticity in the other region. Thus, in the second chapter, "*Changes in neural activity and perineuronal net expression in the cerebellum after deactivation of the medial prefrontal cortex*" we addressed an exhaustive analysis of cFos and PNN expression in the cerebellum and other cerebral regions after mPFC deactivations. Importantly, cerebellar activity and PNN expression increased only after infralimbic deactivation. Based on a more detailed analysis, we discussed whether the observed functional interaction could be seen or not as compensatory.

In the third chapter, "*From back to front: A functional model for the cerebellar modulation in the establishment of conditioned preferences for cocaine-related cues*",

we explored the activity and plasticity in the striatum and mPFC after the cerebellar impairment. Additionally, we accomplished a tracing study using anterograde and retrograde tracers in order to build a working neuroanatomical model to explain the facilitative effect of the cerebellar lesion on cocaine-induced conditioned memory. Our findings hinted at an inhibitory control of the posterior vermis over the striatum and mPFC through the VTA.

Finally, the doctoral thesis concludes with a general discussion, which helps to explain the results of the three chapters, and presents a hypothetical model to encourage future research. We also discuss the strengths and pitfalls of our findings. References can be found at the end of each chapter and on the last pages, after the strengths and pitfalls section.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Addiction and drug reward

Drug addiction is a disorder of the Central Nervous System (CNS) whose main symptoms have been characterized by escalating drug use, loss of control over limiting drug intake, the emergence of chronic compulsive drug-seeking, and a marked reduction of interest in other objectives and rewards (Robinson and Berridge, 2003). Initially, drug reward is the main premise for drug consumption in both humans and animals (Wise, 2009). Thus, addictive drugs compete with other natural stimuli in terms of their ability to initiate goal-directed behaviours, and trigger adaptation and neuroplasticity processes. However, drugs of abuse progressively reduce the ability of individuals to initiate and sustain actions towards natural stimuli (Hyman *et al*, 2006; Kalivas and Volkow, 2005). Although much of the initial studies of drug addiction focused on the critical impact of addictive substances on the CNS, now attention is being directed towards the effects of chronic drug-intake and long-term neuronal brain changes that end in a relapse. A major factor for relapse is the persistence of maladaptive drug-associated memories, which can preserve drug-seeking and taking behaviour. In this respect, there is increasing evidence to support the ability of addictive drugs to promote stable changes in synaptic connections of brain circuits responsible for memories and behaviours established by Pavlovian and instrumental conditioning (Everitt and Robbins, 2005). Indeed, drug addiction results from an aberrant learning that induces the formation of strong instrumental memories, linking actions to drug-seeking and taking outcomes that are finally expressed as persistent stimulus-response habits (Milton and Everitt, 2012). Initially, neutral environmental stimuli become associated with drug highs through Pavlovian conditioning, driving the subsequent interactions between Pavlovian and instrumental memories to influence relapse. Therefore, long-lasting drug-induced modifications in Pavlovian and instrumental learning appear to be the most explanatory mechanism for the establishment of drug addiction.

Everitt *et al.* (2001) showed that the aberrant engagement of Pavlovian and instrumental learning mechanisms leads to enhanced learning about the actions and environmental drug-associated cues or conditioned stimuli (CSs) that predict

opportunities for drug self-administration. They argued that these processes could be produced by the ability of drugs of abuse to increase the release of dopamine in several regions of the striatum-cortico-limbic system. Consequently, repeated drug-taking indeed these CSs to acquire an increasing role in controlling drug-seeking behaviour. As the state of addiction develops, the previously drug-paired CSs induced drug-seeking, independently of goal-directed actions. In consequence, drug consumption becomes controlled by stimulus-response mechanisms that are habitual and automatic, via CS-induced activation of drug-seeking motor programs in the dorsal striatum (Belin *et al*, 2009; Belin and Everitt, 2008). In some individuals, drug-seeking becomes compulsive and persistent and generates an insensitivity to devaluation or punishment (Ahmed, 2012; Belin and Everitt, 2008; Pelloux *et al*, 2007). In this way, the repeated association of a conditioned stimulus with drug effects seems to promote a shift from goal-directed instrumental behaviour to compulsive habits.

The addiction circuitry

Drug-induced memories are mainly stored through molecular interactions between dopaminergic and glutamatergic systems in the prefrontal-limbic-striatal networks (Everitt and Robbins, 2005).

The medial prefrontal cortex (mPFC) is responsible for executive control, which includes the representation of contingencies, representation of outcomes and their value, and

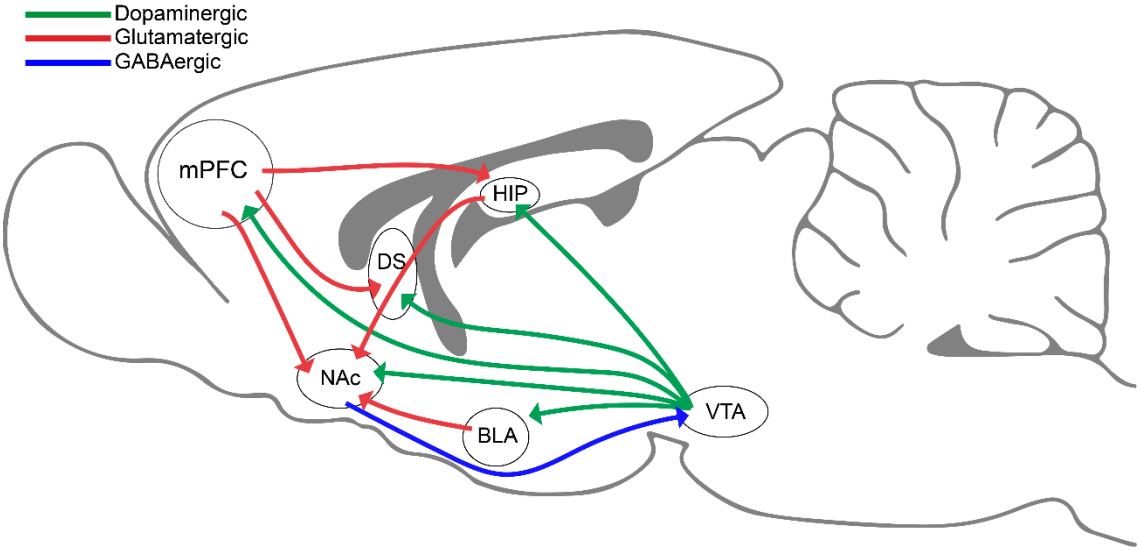

Figure 1. The addiction circuitry. *Medial prefrontal cortex (mPFC), dorsolateral straitum (DL), nucleus accumbens (NAc), hippocampus (HIP), basolateral amygdala (BLA), ventral tegmental area (VTA).*

subjective states associated with drugs (Everitt and Robbins, 2005). The prefrontal cortex, along with the nucleus accumbens (NAc) and the dorsomedial striatum (DMS), orchestrate goal-directed actions, whereas the interactions between some regions in the mPFC and the dorsolateral striatum (DLS) underpin the establishment of habits (Smith and Graybiel, 2013). The reestablishment of drug-seeking after abstinence depends on the mPFC release of glutamate and dopamine in the nucleus accumbens core (NAcC) and the integrity of the ventral pallidum (McFarland *et al*, 2003). The NAc exhibits neuroadaptations following drug experience, particularly changes in glutamatergic signalling (Kalivas and McFarland, 2003) that are hypothesized to increase the salience of drug-associated CSs and thereby their impact on behaviour (Kalivas, 2004). Additionally, long-lasting plasticity changes in the orbitofrontal cortex (OFC), basolateral amygdala (BLA) and NAcC, regions richly interconnected, underlying the capacity of conditioned reinforcers to encourage drug-seeking after long periods of abstinence (Kalivas and McFarland, 2003; Schoenbaum *et al*, 2003; Shaham *et al*, 2003). Dopamine and glutamate interactions in the BLA and NAcC have also been observed in relapse (McFarland *et al*, 2003; See *et al*, 2001). Moreover, the BLA has been shown to be required for sensory-specific conditioned reinforcement and Pavlovian-instrumental transfer (Cardinal *et al*, 2002; Pelloux *et al*, 2013; Stefanik and Kalivas, 2013). The sequential phases of Pavlovian and instrumental learning may be especially relevant for the transition from initial drug use to drug abuse, and finally to compulsive drug-seeking. Pavlovian-instrumental transfer resulting in drug-seeking depends on afferents from specific regions of the mPFC to the DMS (Ostlund and Balleine, 2005; Yin *et al*, 2005). Likewise, the output from the NAcSh can influence the functioning of ascending dopamine projections to the NAcC, and from the NAcC via the substantia nigra to other domains of the DMS (Haber *et al*, 2000). Thus, the potentiation of conditioned reinforcement by stimulant drugs and Pavlovian-instrumental transfer could result from drug's impact on the NAcSh, influencing processing of CSs in the NAcC and DMS.

The amygdala and hippocampus are both key components for mediating the ability of drug-related contexts to trigger drug-seeking and relapse (Bonson *et al*, 2002; Grant *et al*, 1996). Context-induced reinstatement of drug-seeking requires the hippocampus (Carballo-Márquez *et al*, 2009; Sierra-Mercado *et al*, 2011), similar to its role in

contextual fear conditioning (Phillips and LeDoux, 1992). It has been observed that contexts previously associated with drug use can promote relapse in animals with an extensive history of drug self-administration (Bossert *et al*, 2011; Crombag and Shaham, 2002; Fuchs *et al*, 2005). In this way, the hippocampus would represent context as a CS, with the amygdala associating the hippocampal-encoded context with the affective value of the unconditioned stimulus. This is the case for contextual memories associated with aversive outcomes, as well as appetitive conditioning for natural and drug reinforcers (Hitchcott and Phillips, 1997; Matus-Amat *et al*, 2007).

In summary, plasticity mechanisms within the hippocampus, amygdala, and mPFC may all influence drug-seeking through their convergent projections to the NAc, perhaps competing for access to response strategies involving different prefrontal-striatal-limbic networks (Goto and Grace, 2005). It has been hypothesised that the transition from voluntary actions to more habitual modes of responding in drug-seeking involves a transfer from prefrontal to striatal control over responding, as well as from ventral to more dorsal striatal regions (Belin and Everitt, 2008; Everitt and Robbins, 2005), through a progressive recruitment of dopaminergic neurons in the midbrain (Haber *et al*, 2000). Together with an overreliance on striatal mechanisms, the progressive loss of control over drug consumption requires a reduction in the inhibitory control exercised by the prefrontal cortex (Volkow *et al*, 2013). Therefore, drug-seeking becomes less dependent on voluntary control and more prone to be triggered automatically and compulsively (Everitt and Robbins, 2005).

Beyond the traditional neuroanatomical model of drug addiction, an increasing amount of data suggests the involvement of the cerebellum in many of the affected brain functions in addicts (Miquel *et al*, 2009, 2016; Moulton *et al*, 2014). The research we present and discuss in this thesis strongly supports the inclusion of the cerebellum as a part of the circuit responsible for long-lasting drug-induced behavioural effects.

Cerebellum: “the little brain”

Cerebellum anatomy

The cerebellum controls and regulates motor and non-motor functions. It is integrated by the vermis (the central region), the paravermal area (on each side of the vermis), and the hemispheres, which are the two most external regions. Two fissures divide the cerebellum in a rostral-caudal direction into anterior (lobules I to V), posterior (lobules VI to IX), and flocculonodular (lobule X) lobules (Marr, 1969). The cerebellum architecture consists of a thin layer of white matter covered by grey matter and three pair of deep cerebellar nuclei (medial/fastigial, interpositus, and lateral/dentate). The grey matter is composed by three layers: the molecular layer, Purkinje layer, and granular layer (Brodal, 2016; Voogd and Glickstein, 1998). The molecular layer is the outermost layer and contains few neuronal somas (basket and stellate cells), being mainly formed by dendrites and axons of other cells (Voogd and Glickstein, 1998). This layer includes dendritic arborizations of Purkinje cells, whose cell bodies are the predominant component of the Purkinje layer (Haines and Dietrichs, 2011). The granular layer is the deepest layer and limits with white matter. This layer is formed by Golgi, Lugaro, and unipolar brush cells, as well as in greater numbers, granule cells (Eccles *et al*, 1964). The majority of cells in the cerebellar cortex are glutamatergic neurons, including granule cells and unipolar brush cells. The rest of them are GABAergic neurons (Apps and Garwicz, 2005).

The cerebellar cortex receives information mainly from cerebral cortices, limbic areas and basal ganglia through two glutamatergic afferents. Climbing fibres, coming from the

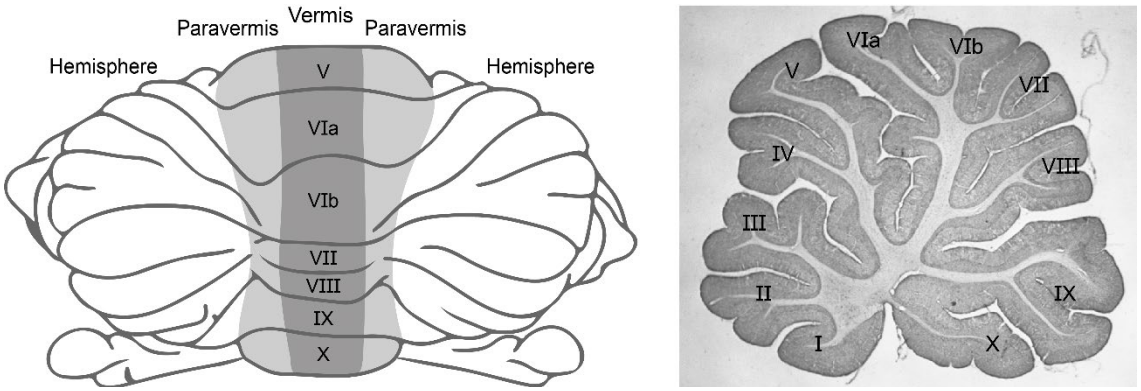

Figure 2. Cerebellum divisions. *The posterior coronal image shows the vermis, paravermis and hemispheres, and the posterior lobules. The sagittal image shows a medial section and the distribution of all the lobules.*

inferior olive, climb up to dendrites of Purkinje neurons. Mossy fibres, originated in the pontine nuclei, synapse within the glomerulus, that included Golgi and granule cells (Albus, 1971; Andersen *et al*, 1992; Gilbert and Thach, 1977; Marr, 1969). The glomerulus is a structure wrapped into a glial lamina that limits the diffusion of the neurotransmitter (Barbour and Häusser, 1997). Golgi cells are the most numerous inhibitory interneuron of the granular layer and control the activity of as many as 100 billion granule cells (Marr, 1969). Golgi cells can reside at different depths in the granular layer, and their basal dendrites usually remain in the granular layer, while apical dendrites ascend into the molecular layer traversing the parallel fibre (Albus, 1971). The axons of granule cells ascend to the molecular layer and branch into parallel excitatory fibres that come into contact with Purkinje somas and dendrites (Brunel *et al*, 2004; Marr, 1969). Basket and stellate cells (inhibitory interneurons) regulate the excitatory inputs on Purkinje cells, and send inhibitory signals to the deep cerebellar nuclei (DCN), which control the final output of the cerebellum (Ito, 1984; White and Sillitoe, 2013). Golgi cells also receive inhibitory innervation from stellate and basket cells, as well as Lugaro cells (Eccles *et al*, 1964). Moreover, DCN receive axon collaterals from the inferior olive and pontine nuclei. The DCN are the main output from the cerebellum and are composed mainly of neurons with large glutamatergic projections, although they harbour a small number of GABAergic neurons that project towards the inferior olivary and pontine nuclei (Gao *et al*, 2016;

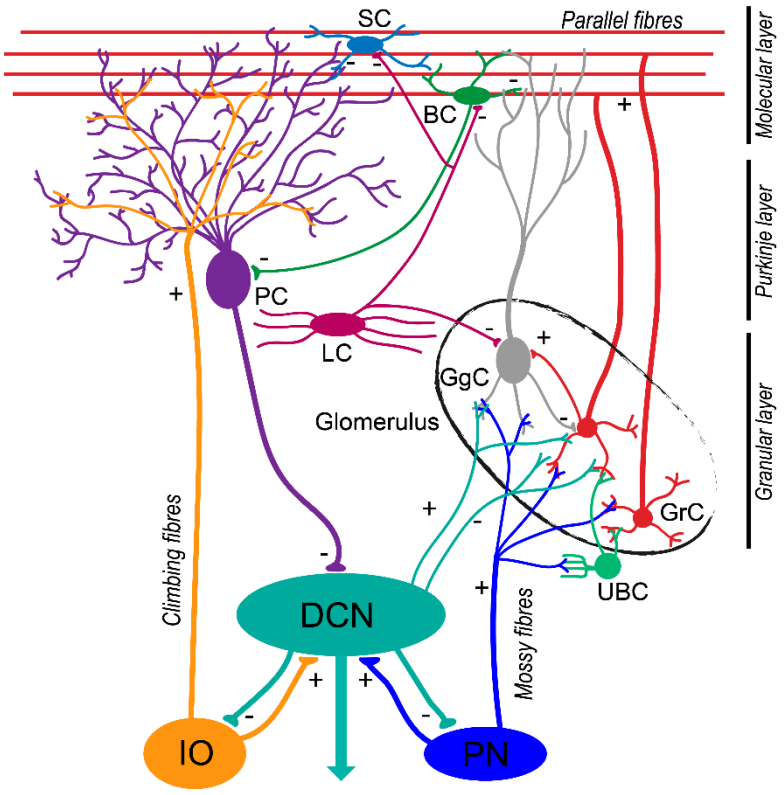

Figure 3. Cerebellar cortex. *Stellate cell (SC), basket cell (BC), Purkinje cell (PC), Lugar cell (LC), unipolar brush cells (UBC), Golgi cell (GgC), granule cell (GrC), deep cerebellar nuclei (DCN), inferior olive (IO), pontine nuclei (PN).*

Voogd and Glickstein, 1998). The activation of these glutamatergic neurons releases the information out of the cerebellum (Shinoda *et al*, 2000).

Cerebellum-cerebro reciprocal loops

Several anatomical and functional studies have proposed that the cerebellum and the striatum-cortico-limbic circuit are interconnected (Bostan *et al*, 2013; Bostan and Strick, 2018; Rogers *et al*, 2011). Bostan *et al.* (2010) demonstrated, with a virus transporter, that the subthalamic nucleus of the basal ganglia projects disynaptically to the cerebellar cortex (Bostan *et al*, 2010). Additionally, Rogers *et al.* (2011) proposed two glutamatergic pathways to connect the cerebellum and mPFC. The first network involves the lateral nucleus, reticulotegmental nuclei, pedunculopontine nuclei, ventral tegmental area (VTA), and finally mPFC (Forster and Blaha, 2003). The second arrives at the mPFC through the lateral nucleus and mediodorsal and ventrolateral thalamus (Rogers *et al*, 2011). Both neuronal circuities seem to contribute equally to cerebellar modulation of mPFC dopamine release. Studies in humans have observed that lesions or alterations of the cerebellum can generate similar deficits to those caused by alterations of the prefrontal cortex (Schmahmann and Sherman, 1998; Strata *et al*, 2011; Timmann *et al*, 2010). A greater release of dopamine in the cerebellar vermis has also been observed in humans after a hyperstimulation of the prefrontal cortex (Yoon *et al*, 2006). In addition, several anatomical studies in animals have found a dopaminergic direct VTA-cerebellar projection (Ikai *et al*, 1992, 1994) and non-dopaminergic direct projections from the DCN to the VTA (Carta *et al*, 2019; Ikai *et al*, 1992; Watabe-Uchida *et al*, 2012). Some of these studies observed a connection between the prefrontal cortex and cerebellum through dopaminergic projections to the cerebellar cortex and non-dopaminergic to the DCN (Ikai *et al*, 1992). Ikai *et al.* (1994) showed that VTA dopaminergic fibres reach both the granular layer and the Purkinje layer. On the other hand, the non-dopaminergic fibres from the VTA reached the DCN, mainly the lateral and interpositus nuclei. These non-dopaminergic projections were also observed in the opposite direction, from the DCN to the VTA, with a contralateral predominance (Carta *et al*, 2019; Ikai *et al*, 1994). Additionally, the medial and interpositus nuclei send projections to the thalamus (Stanton, 1980) and the lateral nucleus project to the caudate and putamen (Hoshi *et al*, 2005).

The cerebellum in addiction

The role of the cerebellum has traditionally been related to posture, motor control, and coordination. However, as it has been demonstrated in the last decades, the cerebellum also participates in brain functions such as emotional memory and behaviour (Adamaszek *et al*, 2017; Sacchetti *et al*, 2002a, 2004; Strata *et al*, 2011; Turner *et al*, 2007), linguistic processing (Mariën *et al*, 2014), planning, prediction and temporal perception (Courchesne and Allen, 1997), reward (Wagner *et al*, 2017), and decision-making (Blackwood *et al*, 2004; Broche-Pérez *et al*, 2016; Moers-Hornikx *et al*, 2009).

As above mentioned, several anatomical studies have shown that the cerebellum connects anatomically and functionally with the addiction circuitry (Bostan *et al*, 2018; Buckner *et al*, 2011; Carta *et al*, 2019; Chen *et al*, 2014; Hoshi *et al*, 2005; Ichinohe *et al*, 2000; Ikai *et al*, 1992; Middleton and Strick, 2000, 2001; Panagopoulos *et al*, 1991; Sang *et al*, 2012; Stanton, 1980; Watabe-Uchida *et al*, 2012; Xiao *et al*, 2018). Moreover, it is known that drug use promotes neuroplasticity processes and reorganization in the prefronto-cerebellar circuits (Miquel *et al*, 2009, 2016; Moulton *et al*, 2014).

Volumetric studies of magnetic resonance imaging (MRI) have observed smaller cerebellums in drug addicts (Barros-Loscertales *et al*, 2011; Gallinat *et al*, 2006; Lin *et al*, 2012; Shear *et al*, 1996; Sim *et al*, 2007). A study conducted in methamphetamine-addicted individuals showed an increase in grey matter volume in multiple cortical regions (angular and temporal gyrus, precuneus, insula, and occipital pole), but a reduction in the cerebellum as compared with the normal sample, even after one month of abstinence (Morales *et al*, 2012).

Additionally, functional magnetic resonance imaging (fMRI) studies have repeatedly demonstrated activations in the cerebellum of addicted individuals during cue-reactivity tasks for all drugs of abuse. In a group of cocaine abusers exposed to drug-related cues, metabolic increases were described in the dorsolateral prefrontal cortex, amygdala, and cerebellum (Grant *et al*, 1996). Another study in cocaine addicts showed left hemispheric activation of lateral amygdala, lateral orbitofrontal cortex, and rhinal cortex, as well as right hemispheric activation of the dorsolateral prefrontal cortex and cerebellum after presentation of drug-related cues (Bonson *et al*, 2002). Cocaine-

associated cues elicit selectively activation in lobules II, III, VIII, and IX of the vermis (Anderson *et al*, 2006). Increases in baseline fMRI activity have also been observed bilaterally in the ventral striatum and cerebellum in heroin addicts (Li *et al*, 2015). In alcoholic patients, craving-elicited odour cues activate the right amygdala, hippocampus, insula, and cerebellum (Schneider *et al*, 2001). Remarkably, opioid-dependent patients present decreased functional connectivity at rest between reward structures (NAc and amygdala) and the cerebellum, including Crus I (Upadhyay *et al*, 2010).

It has been assumed that a main role of the basal ganglia in reward prediction and reward-based learning, while the cerebellum would be involved in adaptive modification of behaviour and error-based learning (Doya, 2000). However, fMRI research showed that reward prediction error in a Pavlovian reward association task correlated not only with striatum but also with cerebellar activity (O'Doherty *et al*, 2003). More importantly, granule cell activity appeared to encode the expectation of reward (Wagner *et al*, 2017). In this way, the cerebellum may play an influential modulatory role in reward/saliency, as it also shares reciprocal connections with dopaminergic systems in the basal ganglia (Bostan and Strick, 2010). Strongly supporting the cerebellum's role in reward, Carta *et al.* (2019) have very recently shown that an optogenetic stimulation of the cerebellar axons in the VTA was as rewarding as direct optogenetic stimulation of dopaminergic neurons within the VTA.

Recent research from our lab found a specific and distinctive cerebellar hallmark of preference for cues linked to chronic cocaine experience. We have shown that cocaine-induced conditioned preference significantly increases the activity in neurons located in the most dorsal part of the cerebellar cortex in the vermis (the apical part) (Carbo-Gas *et al*, 2014a, 2014b). This pattern was not observed when animals did not develop conditioned preference, despite being treated with the same cocaine dose (Carbo-Gas *et al*, 2014a, 2014b). More specifically, the cerebellar activity correlated with preference only in lobule VIII. Thus, lobe VIII could be especially relevant for addiction since it is one of the components of the sensorimotor (Bostan *et al*, 2013; Schmahmann, 1991) and the limbic networks (Adamaszek *et al*, 2017), working as an interface among sensory processing, emotional states, and motor responses. The posterior vermis has

been proposed as "the limbic cerebellum" (Bostan *et al*, 2013; Strata *et al*, 2011; Timmann *et al*, 2010; Turner *et al*, 2007). Therefore, lobe VIII would exhibit an advantageous position when making predictions using drug-conditioned memories to evoke preparatory operations in motor networks that may automatically activate drug-seeking (Carbo-Gas *et al*, 2014b). Supporting this hypothesis, the posterior vermis has been involved in the automation of behaviour repertoires towards drug-related cues (Yalachkov *et al*, 2010).

In conclusion, all these findings indicate that different cerebellar regions participate in executive control, drug-induced memory, response selection, and salience (Goldstein and Volkow, 2002, 2011; Habas *et al*, 2009; Volkow *et al*, 2010) further highlighting the cerebellum's potential role in addiction.

Drug-induced plasticity changes in the cerebellum

It has been observed that the acute and chronic experience with cocaine affects both the activity and plasticity in the cerebellum. Chronic cocaine use is related to the brain interactions between glutamate and dopamine, which have also been found in the cerebellum (Schweighofer *et al*, 2004). More concretely, it has been observed that levels of the NR2C subunits of the glutamate receptor were reduced in the rat cerebellum during the late withdrawal from cocaine (Yamaguchi *et al*, 2002). In addition, extracellular activity records in the cerebellar cortex showed that cocaine administration can decrease the spontaneous activation of Purkinje cells and glutamate-evoked discharges (Jiménez-Rivera *et al*, 2000). Other studies observed an increase of cFos levels in the granular layer of the cerebellar vermis (Carbo-Gas *et al*, 2014a, 2014b), mediated via D1 receptors in rats treated chronically with d-amphetamine or cocaine (Klitenick *et al*, 1995).

The changes that cocaine causes in molecular and structural plasticity of the cerebellum appear already at the short term, but they seem to require an incubation time (Vazquez-Sanroman *et al*, 2015a, 2015b). In mice, the direction of cerebellar plasticity changes depends on the duration of the withdrawal period that precedes a new cocaine experience (Vazquez-Sanroman *et al*, 2015a, 2015b). After a one-week withdrawal, a new cocaine administration promoted an increase in proBDNF levels and its expression

in Purkinje neurons, while no changes were observed in the expression of mature BDNF (Vazquez-Sanroman *et al*, 2015a). Moreover, cocaine-treated mice showed an increase in D3 receptor levels and internal expression of Glu2 subunit of the AMPA receptor. Interestingly, these changes were associated with pruning of dendritic spines and a reduction in the size and density of the Purkinje synaptic terminals. The cocaine-induced effects impaired the inhibitory Purkinje function over the DCN associated with a decrease in the probability of remodelling in the Purkinje-DCN synapses, due to an upregulation of perineuronal nets (PNNs) that surround the medial nuclear neurons (Vazquez-Sanroman *et al*, 2015a). Conversely, one-month withdrawal period induced an increase in both proBDNF and mature BDNF levels in the cerebellum (Vazquez-Sanroman *et al*, 2015b). Externalization of GluR2 expression was selectively increased in the soma and dendrites of Purkinje cells in the posterior cerebellum. Additionally, we found more dendritic branching and larger axon terminals in Purkinje neurons to be associated with the increased balanced expression of BDNF. This kind of plasticity accompanied a reduction in PNN expression in the DCN that might facilitate the subsequent remodelling of Purkinje-DCN synapses.

PNNs are a specialised extracellular matrix, composed of chondroitin sulphate proteoglycans surrounding the soma and proximal dendrites of several neuronal populations, which expression restricts neuronal plasticity to stabilize circuits (Brückner *et al*, 1993; Carulli *et al*, 2006; Foscarin *et al*, 2011). Consequently, PNN expression has been proposed as a stabilization mechanism for plasticity changes in learning and memory (Gogolla *et al*, 2009; Romberg *et al*, 2013; Tsien, 2013).

In addition, PNNs have been involved in the long-lasting expression of drug plasticity changes. Similar effects of PNN disruption through the use of chrondroitinase ABC have been observed in different brain regions, and a large number of studies support a PNN's role in drug-induced memories (Blacktop *et al*, 2017; Van den Oever *et al*, 2010; Slaker *et al*, 2015, 2016; Xue *et al*, 2014). PNN degradation in the prelimbic, but not in the infralimbic cortex, reduced acquisition and reconsolidation of cocaine-induced conditioned place preference (CPP) (Slaker *et al*, 2015), whereas the digestion of PNNs in the amygdala prevented priming-induced drug reinstatement as long as degradation was made before extinction (Xue *et al*, 2014). Furthermore, it has been shown that

deletion of PNNs in the anterior dorsal lateral hypothalamic area abolished the acquisition of cocaine-induced CPP and significantly diminished self-administration of cocaine (Blacktop *et al*, 2017). In a recent study in our laboratory, animals exposed to cocaine-induced preference conditioning showed that the formation of cocaine-related preference memories increased the expression of PNNs surrounding Golgi inhibitory interneurons and the activity of these Golgi cells surrounded by strong and fully condense PNNs in the dorsal region of lobule VIII. However, the expression of PNNs in the medial nucleus was reduced in all cocaine-treated groups and thereby was not memory related (Carbo-Gas *et al*, 2017). All these results suggest that the regulation of PNNs around Golgi neurons in the cerebellar cortex might be a relevant mechanism for the stabilization of drug-related memories.

Overall, findings using drugs of abuse support the previously demonstrated role of the cerebellum in the formation of Pavlovian memories (Boele *et al*, 2010; Sacchetti *et al*, 2002b, 2005; Thompson and Steinmetz, 2009), suggesting that the posterior vermis is an important region for the persistence of drug-related memories. More importantly, as we previously proposed (Miquel *et al*, 2009), the relevance of the cerebellum in addiction may increase as far as the activity of the prefrontal cortex is reduced. In the present doctoral thesis, we proposed for the first time a hypothetical model to explain prefrontal-cerebellar interactions for the establishment of cocaine-related conditioned memory.

AIMS AND HYPOTHESIS

AIMS AND HYPOTHESIS

The premises underpinning the present thesis are the following:

- The cerebellum is a fundamental region for the consolidation of Pavlovian and instrumental conditioned memories.
- The cerebellum has connections with the prefronto-striatal circuits previously related to addictive behaviour.
- Addictive drugs, and specifically cocaine, promote stable neuroplastic changes in the cerebellum.
- Activity in the posterior cerebellum increases in mice that developed preference for cocaine-related cues.

The general aim of the thesis is to investigate the role of prefronto-cerebellar networks in cocaine-induced conditioned memory. More specifically, the present thesis is aimed at: (1) studying the function of the prefronto-cerebellar circuits in the acquisition of cocaine-induced conditioned preferences; (2) investigating the effects of cerebellar-prefrontal manipulations on neural activity and plasticity; and (3) proposing a working hypothetical model to explain cerebellum-prefrontal relationships in drug addiction.

Specific aims:

- To explore the effects produced by deactivations of the medial prefrontal cortex, comparing prelimbic and infralimbic cortices on the acquisition of cocaine-induced preference conditioning.
- To explore the effects produced by deactivations of the posterior cerebellum, specifically lobe VIII of the vermis on the acquisition of cocaine-induced preference conditioning.
- To evaluate the effects of simultaneous prefrontal-cerebellar deactivations on cocaine-induced preference conditioning.
- To investigate whether mPFC deactivations could promote plasticity changes in the cerebellum by analysing neuronal activity (cFos) and one of the mechanisms for synaptic stabilization, the expression of PNNs, associated with the acquisition of cocaine-induced conditioned memory.

- To investigate whether impairment in the posterior cerebellar cortex may affect activity and PNN expression in the mPFC and the striatum.

The present thesis proposes, as a general hypothesis, that the posterior cerebellum plays a fundamental role in the storage of cocaine-related memories. In this way, our predictions were:

- Infralimbic deactivation will facilitate the acquisition of cocaine-induced conditioned memory, while prelimbic impairment will prevent its acquisition.
- Animals with deactivations of lobe VIII in the vermis will not acquire cocaine-related memory.
- No effect of the deactivations will be observed in animals trained under a random cocaine-cue association.
- Activity and plasticity in the cerebellum will be promoted after mPFC deactivations.
- Deactivation of lobule VIII will change the activity and plasticity in the mPFC and the striatum.

EXPERIMENTAL STUDIES

Chapter 1

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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 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 and 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 and Everitt 2008; Volkow *et al.* 2013).

Remarkably, the cerebellum is closely connected to the functional loops in the striatum–cortico–limbic circuitry, which has been established by tracing techniques, electrostimulation, and optogenetics (Panagopoulos *et al.* 1991; Ikai *et al.* 1992; Hoover and Strick 1999; Ichinohe *et al.* 2000; Melchitzky and 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,b, 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,b, 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 and 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 and Price 2000; Vertes 2004; Hoover and Vertes 2007) and

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exhibit opposite roles at the functional level (Ongür and Price 2000; McFarland and Kalivas 2001; Capriles *et al.* 2003; Peters *et al.* 2009; Sierra-Mercado *et al.* 2011; Ball and Slane 2012; Pfarr *et al.* 2015). Specifically, reinstatement of cocaine-seeking behaviour requires the integrity of the PL cortex (McFarland and Kalivas 2001; McLaughlin and 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 are under the control of the IL, NAshell and BLA (McFarland and Kalivas 2001; McLaughlin and 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 2 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 5 hours of the light cycle, 2 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) and quinolinic acid (90 nmol/μl) (2,3-pyridinedicarboxylic acid: Sigma-Aldrich Co. LLc) 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 and 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 3 days. The animals remained undisturbed for 3 to 5 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,b, 2017). After the infusion was completed, the injector remained in place for 3 minutes 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 minutes after the infusion, as deactivation via lidocaine only lasts for 20 minutes (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

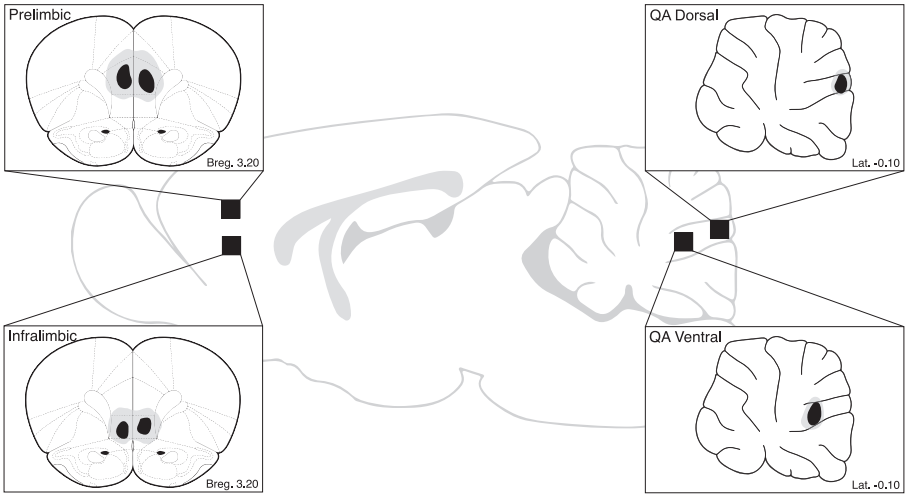

FIG. 1. Diagrams of the cannula locations. Schematic diagrams depicting the largest (grey) and smallest (black) diffusion areas in the PL and IL cortices, as well as in the dorsal and ventral regions of the cerebellar vermis. The extent of the diffusion areas was assessed using light microscopy and lucida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

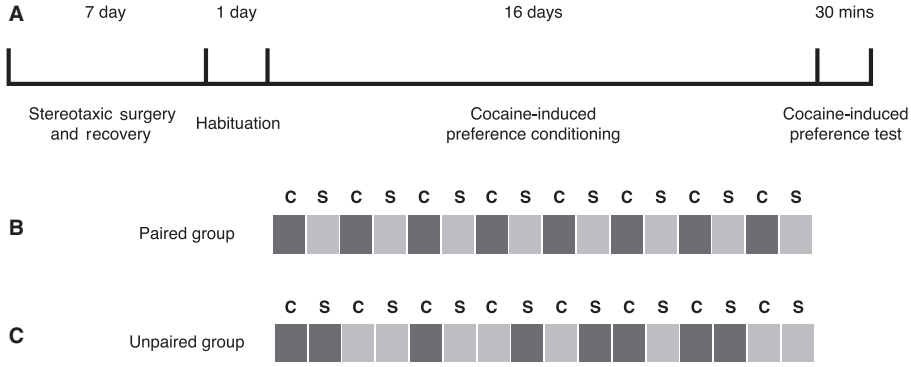

FIG. 2. (A) Experimental timeline. Different stages of the experimental procedure from the stereotaxic surgery to the animal perfusion. (B) Cocaine-induced preference conditioning protocol for the paired group. For 16 training days, rats received eight cocaine 'C' and eight saline 'S' administrations 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.

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 × 20 × 60 cm) that included two lateral black chambers (20 × 20 × 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 hours after the last cocaine administration in a 30 minutes 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 minutes 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-minute testing period was split into four 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 minutes 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 hours at 4 °C. 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 to 0.72 mm, comprising the whole vermis. For the prefrontal cortex, coronal sections were collected according to bregma coordinates from 4.70 to 1.70 mm (Paxinos and 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 (≥ 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,b, 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 scatter plots 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 vs. observed frequencies. In all analyses, the statistical level of significance was set at $P < 0.05$.

Results

The injection 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 vs. Sham Ventral: (t (19) = 0.6104, P = 0.5489); Sham IL vs. 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 (χ^2 (2) = 3.954, P = 0.138) (Fig. 3F).

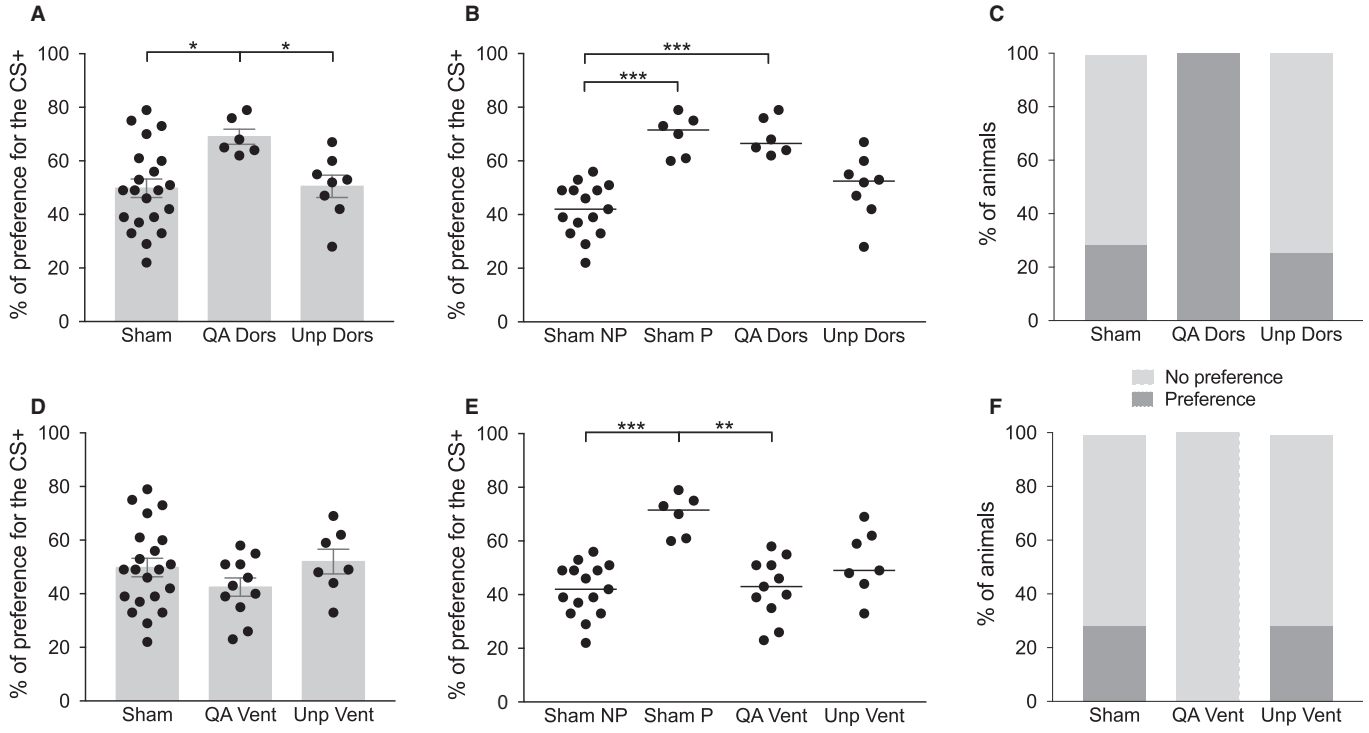

FIG. 3. Effect of an excitotoxic lesion in the cerebellum on cocaine-induced preference conditioning. (A) Preference scores for the CS+ on the test day in the control (Sham) (n = 21), quinolinic acid dorsal (QA Dors) (n = 6) and unpaired dorsal (Unp Dors) (n = 8) groups. Data are shown as the mean ± 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).

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% (χ^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 scatter plots (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 (χ^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).

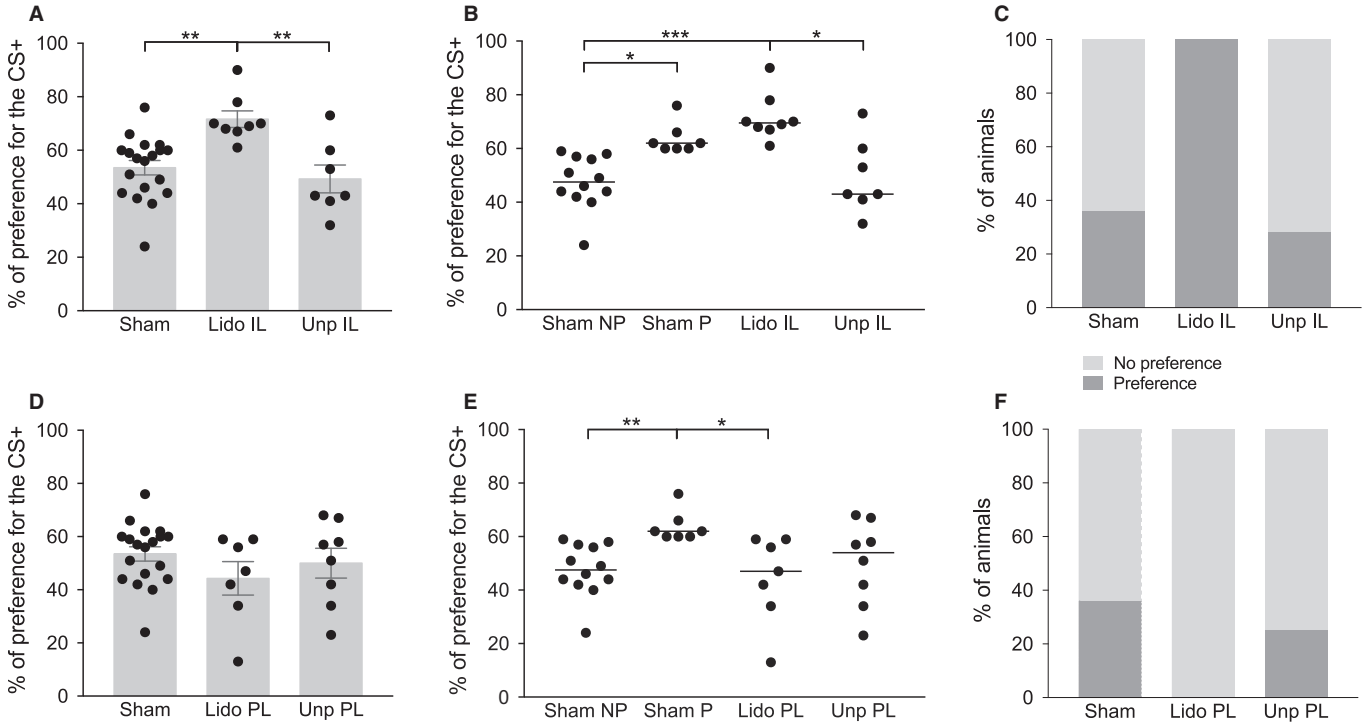

FIG. 4. Effect of a temporary deactivation in the mPFC before each training session on cocaine-induced conditioned preference. (A) Preference scores for the CS+ on the test day in the Sham (n = 19), lidocaine infralimbic (Lido IL) (n = 8) and unpaired infralimbic (Unp IL) (n = 7) groups. Data are shown 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).

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-minute testing period into 5 minute 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 and Kalivas 2001; Van den Oever *et al.* 2010; Goldstein and 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, as all the effects were memory-related and specific for the formation of drug-cue associations, 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 (Figs 34C–C).

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 and Kalivas 2001; Lalumiere *et al.* 2012), and thus, the facilitative effect on cocaine-induced

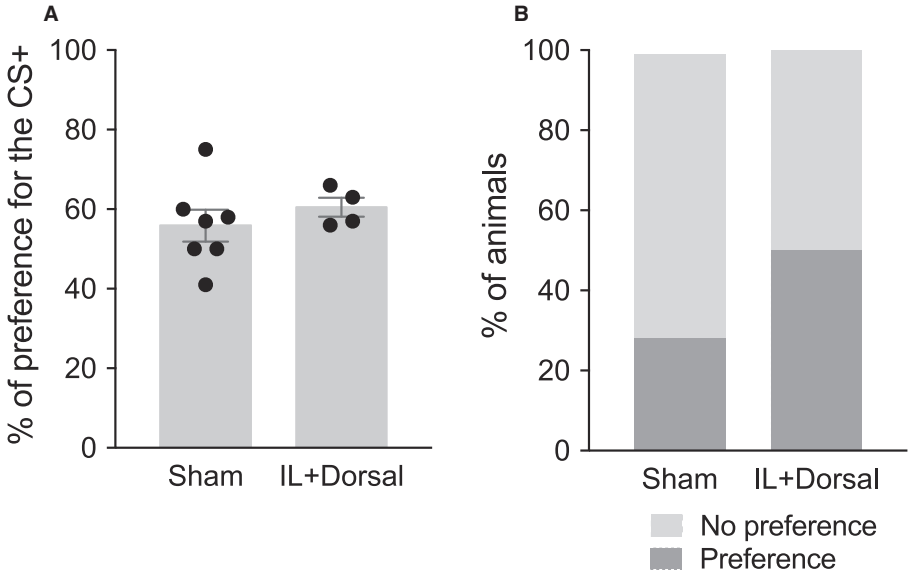

FIG. 5. Effect of a simultaneous deactivation of the IL cortex and dorsal cerebellum on cocaine-induced preference conditioning. (A) Preference scores for the CS+ on the test day in the Sham (n = 7) and IL + Dorsal (n = 4) groups. Data are shown as the mean ± SEM. The facilitative effect of separate 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.

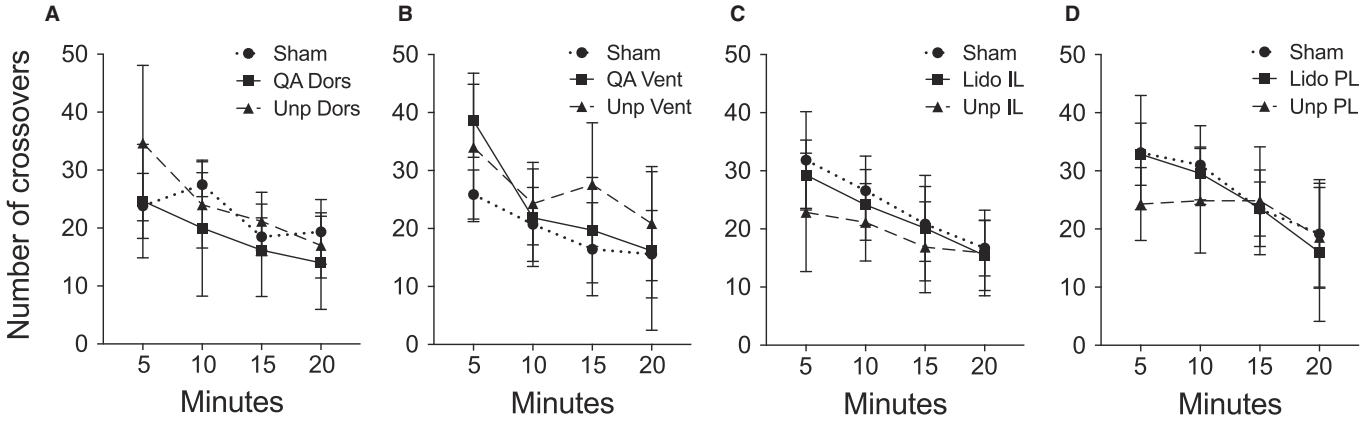

FIG. 6. Effects of deactivation in the cerebellum and mPFC on locomotor activity during the preference test. (A) A dorsal excitotoxic lesion in the cerebellum [(Sham: n = 21); (QA Dors: n = 6); (Unp Dors: n = 8)]. (B) Ventral excitotoxic lesion in the cerebellum [(Sham: n = 21); (QA Vent: n = 11); (Unp 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.

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,b). 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 and Koch 2015). Cerebellar lesions promote behavioural disinhibition (Schmahmann and 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 and Strick 1994, 2001; Schmahmann and Pandya 1997; Sang *et al.* 2012; see Bostan and 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 and 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 and 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 and 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 and Larson 1972; Voogd and 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 because 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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Conflict of interest

All authors declare no conflicts of interest.

Data accessibility

Data are available from the corresponding author upon request.

Author Contributions

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.

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EXPERIMENTAL STUDIES

Chapter 2

Chapter 2: Changes in neural activity and perineuronal net expression in the cerebellum after deactivation of the medial prefrontal cortex

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ABSTRACT

Prelimbic (PL) and infralimbic (IL) cortices exhibit opposite roles in drug-related behaviour. The PL cortex is involved in initiating cocaine-seeking, while the IL cortex is responsible, among other functions, for the inhibitory control over drug-seeking. Importantly, several findings support the existence of reciprocal loops between the medial prefrontal cortex (mPFC) and the cerebellum. Neuroimaging studies in human addicts with prefrontal damage have shown an overactivation of the cerebellum during reward and cognitive tasks. Thus, the cerebellum may become more relevant when prefrontal function is compromised by disease or chronic drug use. In the present research, we investigated whether impairment of the mPFC during the acquisition of cocaine-induced preference conditioning may increase drug impact on neural activity and PNN expression in the cerebellum, as well as different striatal regions. Before every training session, lidocaine was infused into PL and IL for a reversible deactivation. The results showed that IL deactivation increased the probability of developing cocaine-induced preference conditioning, while PL deactivation prevented it. Moreover, the formation of cocaine-related preference memories was associated with an increase in cFos expression and PNNs intensity in the dorsal region of the posterior cerebellum. Additionally, cocaine-induced preference memory increased cFos expression in the nucleus accumbens shell. Therefore, our findings suggest that impairment of the mPFC function increases susceptibility to the acquisition of drug-induced Pavlovian memory, but do not exhibit a direct effect of mPFC deactivations on cerebellar activity and plasticity. All this data suggest that the cerebellum might be a critical region for the storage or reactivation of conditioned associations that predict drug availability.

Keywords: cocaine, mPFC, cerebellum, lidocaine, PNNs

INTRODUCTION

The rat medial prefrontal cortex (mPFC) includes four different functional regions: the medial precentral cortex (PrCm), anterior cingulate cortex (AC), prelimbic (PL) cortex, as well as infralimbic (IL) cortex (Edward, 1992; Heidbreder and Groenewegen, 2003; Hoover and Vertes, 2007; Ongür and Price, 2000; Vertes, 2004). The different subdivisions have been linked to a wide range of brain functions dramatically affected in drug addiction, such as working memory, decision-making, executive control, instrumental learning, and emotion (Cardinal *et al*, 2002; Delatour and Gisquet-Verrier, 2000; Dias and Aggleton, 2000; Heidbreder and Groenewegen, 2003; Milad and Quirk, 2002; Mogensen and Holm, 1994; Morgan *et al*, 1993; Ragozzino *et al*, 1998, 1999). Particularly, several studies have observed that the PL and IL cortices exhibit opposite roles in drug-related behaviours (Ball and Slane, 2012; Capriles *et al*, 2003; McFarland and Kalivas, 2001; Ongür and Price, 2000; Peters *et al*, 2009; Pfarr *et al*, 2015; Sierra-Mercado *et al*, 2011). The PL cortex is involved in initiating cocaine-seeking (Martín-García *et al*, 2014; McFarland and Kalivas, 2001; Zavala *et al*, 2003), while the IL cortex is responsible, among other functions, for the inhibitory control over drug-seeking (Lalumiere *et al*, 2012; LaLumiere *et al*, 2010; McFarland and Kalivas, 2001; Peters *et al*, 2008). Thus, infusions of dopaminergic antagonists or pharmacological inactivators into the PL cortex prior to the reinstatement of cocaine self-administration decreased lever pressing for the drug (McFarland *et al*, 2004; McFarland and Kalivas, 2001; McLaughlin and See, 2003). Oppositely, the IL deactivation before extinction promoted cocaine-seeking (Lalumiere *et al*, 2012; Peters *et al*, 2008). Consistent with an inhibitory role of the IL region, its pharmacological stimulation before relapse decreased drug-seeking (Peters *et al*, 2008). Notwithstanding these findings, inhibitory control seems also to require PL function, since optogenetic stimulation of the PL cortex decreased compulsive cocaine-seeking in rats (Chen *et al*, 2013).

The PL and IL cortices form separated reciprocal loops through the striatum-limbic circuitry (Hoover and Vertes, 2007; Ongür and Price, 2000; Vertes, 2004). Indeed, the nucleus accumbens core (NAcC) receives inputs primarily from the PL cortex, whereas the nucleus accumbens shell (NAcSh) receives afferences from the IL cortex (Sesack *et al*, 1989; Voorn *et al*, 2004). The activation of the glutamatergic projection from the PL

cortex to the NAcC seemed to be critical for the reinstatement of cocaine-seeking after extinction (McFarland *et al*, 2003, 2004). Conversely, the inactivation of the glutamatergic projection from the IL cortex to the NAcSh restored cocaine-seeking after extinction (Lalumiere *et al*, 2012; Peters *et al*, 2008). Furthermore, the manipulation of the plasticity mechanism for synaptic stabilization within the PL and IL cortices impact differently on cocaine-related behaviour. Thus, the degradation of perineuronal nets (PNNs) around GABAergic inhibitory interneurons in the PL, but not in the IL cortex, attenuated acquisition and reconsolidation of cocaine-induced conditioned place preference (Slaker *et al*, 2015).

Importantly, several findings support the existence of reciprocal loops between the mPFC and the cerebellum (Bostan and Strick, 2018; Middleton and Strick, 1994, 2001; Sang *et al*, 2012; Schmahmann and Pandya, 1997). Electrical stimulation of the fastigial nucleus, the main output nucleus of the vermis, evokes local action potentials and regulates the activity in the PL cortex (Watson *et al*, 2014). Moreover, cortical regulation of striatal activity can be modulated by the cerebellum (Chen *et al*, 2014). 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. Neuroimaging studies in human addicts with prefrontal damage have shown an overactivation of the cerebellum during reward tasks, especially with increased cognitive demands (Bolla *et al*, 2005; Desmond *et al*, 2003; Hester and Garavan, 2004; Martin-Sölch *et al*, 2001). These results indicated that the cerebellum may become more relevant when prefrontal function is compromised by disease or chronic drug use, suggesting that both regions can be recruited to functionally compensate each other (Anderson *et al*, 2006; Miquel *et al*, 2009).

In a previous study (the first chapter), we investigated the functional relationships between the PL and IL subdivisions of the mPFC and the cerebellum in a model of cocaine-induced conditioned preference (Gil-Miravet *et al*, 2018). Our findings showed that a deactivation of either the apical region of lobule VIII in the vermis or the IL cortex encouraged the acquisition of cocaine-induced preference conditioning. Simultaneous deactivation of both regions prevented this facilitative effect on cocaine-related memory. However, opposite results were found after a deactivation of the PL cortex or

deeper regions of lobule VIII. In both cases, the acquisition of preference for the cocaine-related cue was prevented.

In the present research, we investigated whether impairment of the mPFC during the acquisition of cocaine-induced preference conditioning may increase drug impact on neural activity and PNN expression in the cerebellum as well as different striatal regions.

METHODOLOGY

Subjects

The present study included 27 male Sprague-Dawley rats (Janvier, ST Berthevin Cedex, France) randomly selected from the total number of rats included in the first study with mPFC deactivation (N = 49) (Gil-Miravet *et al*, 2018) (Chapter 1). Rats, weighing between 175 and 200 g, were individually housed under standard laboratory conditions with temperature and humidity controlled (12 h light cycle from 8:00 a.m. to 8:00 p.m.). Access to food and water was *ad libitum* (Jaume I University, Spain). Animals were handled on a daily base one week before the experiment began and were habituated to all of the experimental procedures. Animal procedures were approved by the local Animal Welfare Ethics Committee and Empowered Body (2014/VSC/PEA/00208) and adhered to 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). Saline solution was used as a control vehicle. Anaesthesia was induced using a cocktail of ketamine (100 mg/kg) (Imalgene 100 mg/ml, Mersal Laboratorios S.A., Barcelona, Spain), and xylazine (10 mg/kg) (Xylazine hydrochloride ≥99%, Sigma-Aldrich, Madrid, Spain). Lidocaine 6% (60 mg/ml) (Lidocaine hydrochloride, Sigma-Aldrich, Madrid, Spain) was used for a transient deactivation of the PL or IL.

Stereotactic surgery and temporal deactivation

Animals weighing between 270 and 350 g were anaesthetised with a cocktail of ketamine/xylazine (100/10 mg/kg) (IP). Surgery was performed using a Kopf stereotaxic apparatus. For the intracranial infusion, a stainless steel guide cannula (10 mm length; 23-gauge external diameter) was placed in the PL (AP: +3.2; ML: + 0.6/−0.6; DV: −3) or IL (AP: +3.2; ML: + 0.6/−0.6; DV: −4) cortices (Paxinos and Watson, 1998) (Fig. 1). The guide cannula was applied to the skull with screws and acrylic dental cement. After surgery, all animals received analgesic treatment with meloxicam (Metacam 5 mg/ml, Boehringer Ingelheim, Barcelona, Spain). Administration was repeated every 24 hours

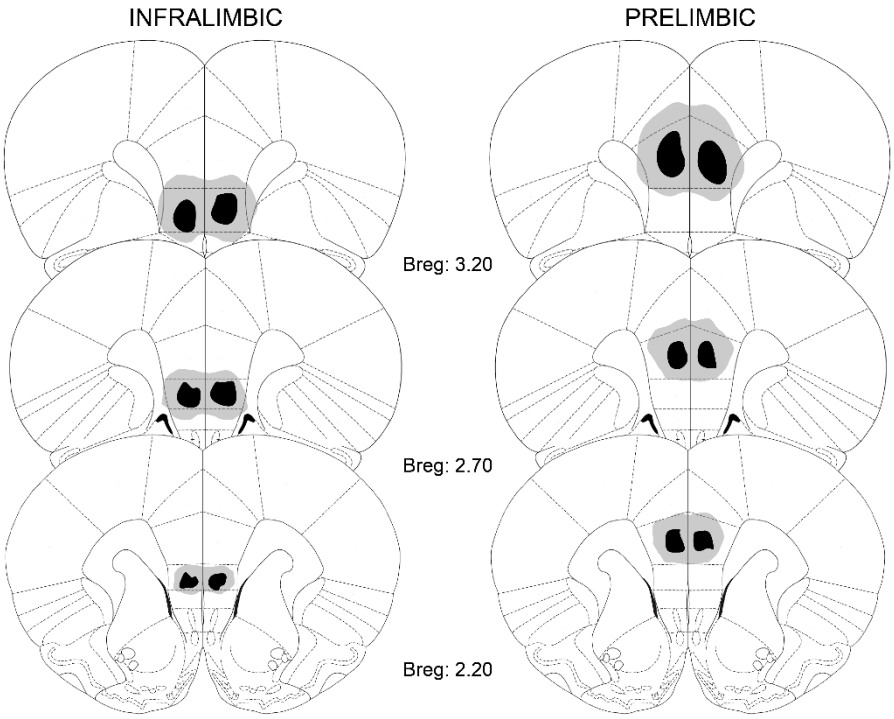

Figure 1. Diagrams of the cannula locations. *Schematic diagrams depicting the largest (grey) and smallest (black) diffusion areas in the PL and IL cortices. The extent of the diffusion areas was assessed using light microscopy and camera lucida drawings.*

for three days. The rats were left undisturbed for five days after surgery for recovery.

Before each training session, lidocaine 6% (60 mg/ml) was infused in the PL or IL using an infusion pump (1 µl volume; infusion ratio of 0.5 µl/min). Microinjections were conducted in awake animals because this procedure is painless. Behavioural testing started two minutes after the lidocaine infusion. Sham surgery followed the same protocol, but saline was infused instead of lidocaine. This procedure was repeated before every conditioning trial (16 days). Cannula installations were unilateral in all the

experiments, counterbalancing the right and left hemispheres. The rationale was to partially preserve the functions of the mPFC in order to model an early chronic experience with the drug in susceptible brains.

Cocaine-induced preference conditioning procedure

Conditioning was developed in an opaque corridor (90 × 20 × 60 cm) containing two black chambers (20 × 20 × 60 cm) located on the opposite arms (Gil-Miravet *et al*, 2018). We evaluated the initial preference for two odours (lavender and rose) in four animals. A Student t-test for independent samples [t (6) = 0.2843; p = 0.7856] revealed no preference towards any of these olfactory stimuli. Then, these two equally preferred odours were used in the conditioning experiment as previously described (Gil-Miravet *et al*, 2018). One of the odours was associated with cocaine (15 mg/kg, IP) (CS+) and the other one with saline (CS-). The odours were counterbalanced between animals. For each pairing session, the rats were confined in one of the chambers for 15 min. Eight cocaine- and eight saline-paired sessions were conducted on alternate days. Odour locations were also counterbalanced in the corridor.

Preference for the CS+ was evaluated 48 h after the last cocaine administration in a 30 min drug-free test in which CS+ and CS− odours were present simultaneously but in opposite arms in the corridor. All the test sessions were videotaped and scored by a blind observer. The first ten minutes were not considered in order to allow the animal to explore the location of the odours. Preference score was calculated as [Time Spent in CS+ / (Time Spent in CS+ + Time Spent in CS−)] × 100. An additional group of rats underwent the same procedure as the deactivated animals, but cocaine injections were randomly associated with the odours (Gil-Miravet *et al*, 2018) (the Unp group). This pseudo-conditioned group allowed us to test for memory-related effects of prefrontal deactivations.

Perfusion protocol and brain sampling

Animals were perfused 90 min following the preference test. Animals were deeply anaesthetised with sodium pentobarbital (30 mg/kg) (Dolethal 100 ml, Vetoquinol E.V.S.A., Madrid, Spain) and perfused transcardially with saline (0.9%) and paraformaldehyde (4%). After perfusion, the brain and cerebellum were quickly

dissected and placed in a container with the same fixative for 24 h at 4 ºC. Then, the tissue was immersed in sucrose solution (30%). The tissue was rapidly frozen by quick immersion in liquid nitrogen. Sagittal (cerebellum) and coronal (brain) sections were performed at 40 µm 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. Lesion sites were localized and represented using light microscopy and camera lucida drawings. Animals with cannula misplacement were not included in the statistical analysis.

Immunohistochemistry and immunofluorescence

cFos immunohistochemistry was performed on free-floating sections. For peroxidative immunostaining, tissue peroxidases were eliminated with 0.3% of H_2O_2 and methanol 20%, during a period of 30 min. Tissue was incubated for 48 h with polyclonal primary antibody rabbit anti-cFos (1:1000; Synaptic Systems, Goettingen, Germany) in PBS 0.1M tween X-100 (PBSt) at 4 °C. In a second step, sections were exposed to affinity purified secondary biotinylated antibody goat anti-rabbit (1:400; Jackson InmunoResearch Laboratories, Inc., West Grove, PA, USA) in PBSt for 120 min at room temperature. For magnification, we used preassembled biotin–avidin peroxidase complex according to the Vector Labs recommendations (ABC Elite; Vector Laboratories, Bulingame, Ca, USA). Sections were exposed to DAB solution with nickel. Then, the tissue was rinsed and mounted in Eukitt (Sigma-Aldrich, Madrid, Spain).

PNNs were labelled by incubating cerebellar tissue with the lectin Wisteria Floribunda Agglutinin (WFA) (1:200; Sigma-Aldrich, Madrid, Spain) at 4 °C overnight in PBS 0.1M triton X-100. Samples were then exposed to Cy3-conjugated Streptavidin (1:200; Jackson InmunoResearch Laboratories, Inc., West Grove, PA, USA) for 120 min at room temperature. The sections were mounted in Mowiol.

Image analysis

Images from cFos immunostaining were acquired using an optic microscope (Nikon E-800, Izasa Werfen Group, Valencia, Spain) with 20x lenses and a resolution of 1,360 x 1,024 dpi. We acquired images of the following regions: the apical region of lobules VIII and IX of the cerebellar vermis, dorsomedial striatum (DMS), dorsolateral striatum (DLS),

ventrolateral striatum (VLS), NAcC, and NAcSh. Three images were taken by structure and hemisphere in coronal sections. We used bregmas between 1.60 mm to 0.70 mm for the striatum and NAc, as well as three sagittal sections between lateral 0.40 mm to -0.40 mm for the cerebellum. Unmanipulated images were used to estimate the density of cFos+ neurons. The estimation was made in selected regions of interest (ROIs) of 80,000 μm² for striatal structures and 20,000 μm² for the cerebellum. We considered cFos positive neurons only those cells exhibiting a uniform and constant black labelling in the nucleus. Results are given as number of cFos positive neurons per mm².

PNNs images were captured in a confocal microscope (Leica DMi8, Leica Microsystems CMS GmbH, Wetzlar, Germany) with 20× lenses and resolution 2,048 x 2,048 dpi. Laser intensity (1%), gain (750), and offset (-1) were maintained constant in each acquisition. Three images in sagittal sections of the dorsal region of lobules VIII and IX were taken from the vermis. We assessed the intensity (brightness range 0-255) of all PNNs in unmodified images by randomly selecting 15 pixels (approximately equidistant from each other) in the net surrounding the neuronal soma and calculating their average. We also counted the number of PNNs and calculated their average manually with ImageJ's software cell counter plugin. We used FIJI free software (Schindelin *et al*, 2012) for all analyses.

Experimental design and statistics

Behavioural data relied on preference scores obtained on the test day. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). First, we analysed the effect of mPFC deactivations on preference scores and immunohistochemistry data by means of one-way ANOVAs and post hoc Tukey's HSD tests. Results were presented as mean ±SEM. Second, we applied an arbitrary cut-off point of 60% to cluster sham rats in two subgroups: the preference group (≥60%, Sh P) and the no preference group (<60%, Sh NP) (Gil-Miravet *et al*, 2018). Our previous findings have shown the utility of such clustering to predict cocaine-induced cerebellar plasticity (Carbo-Gas *et al*, 2014a, 2014b, 2017) and the effects of mPFC lesions (Gil-Miravet *et al*, 2018). Therefore, we compared cFos and PNN expression among the Sham P, Sham NP, mPFC deactivation (PL or IL) and Unp groups. Comparisons between variances and frequencies in these groups were carried out using Kruskal-Wallis for non-

parametric distributions. Post hoc analyses were performed by Dunn's multiple comparison tests. Spearman's correlation analyses were calculated to investigate the correlation between cFos expression and preference scores. The statistical level of significance was set at $p < 0.05$. Scatterplots were depicted in all figures.

RESULTS

Histological infusion sites and their respective diffusion areas can be seen in figure 1. The comparison of preference scores between the sham IL and sham PL groups did not yield significant differences (Student's t-tests ($t(12) = 0.06335$, $P = 0.9505$). For this reason, we pooled both groups to form a single sham group for the analysis. Then, the sham group was split using the cut-off point of 60% into the preference (Sh P) and no preference (Sh NP) groups.

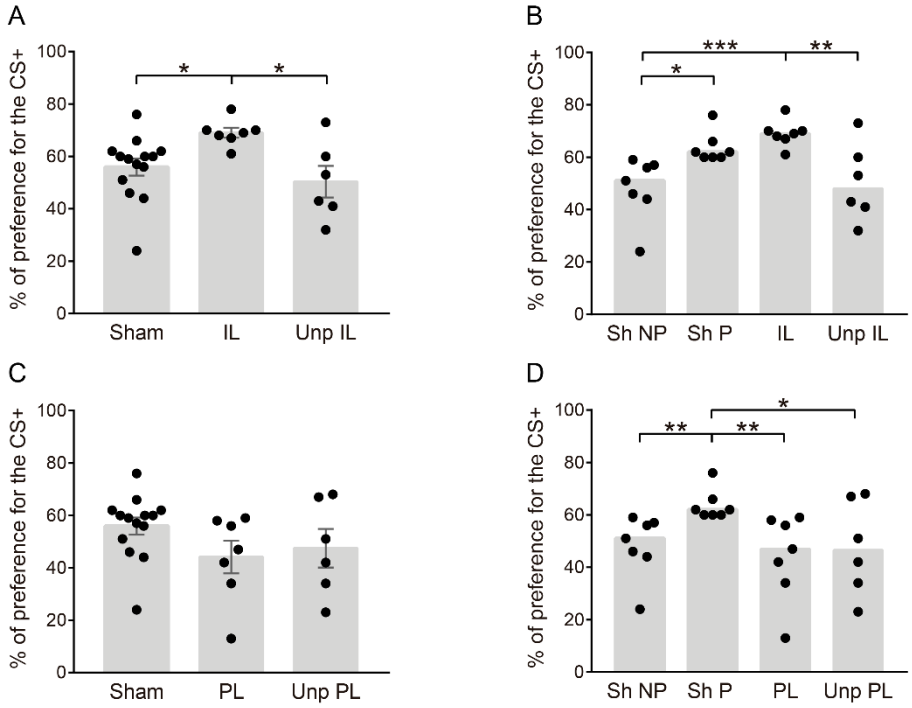

Figure 2. Effect of IL or PL temporal deactivation before each training session on cocaine-induced conditioned preference *The IL deactivation increased the proportion of rats that expressed cocaine-induced conditioned preference, while the PL deactivation prevented it. (A) IL deactivations. Data are shown as the mean ± SEM and scatterplots of the preference scores for the CS+ on the test day in the sham (n=14), infralimbic deactivation (IL) (n = 7) and unpaired IL deactivation (Unp IL) (n = 6) groups. (C) PL deactivations. Mean ± SEM and scatterplots of preference scores for the CS+ on the test day in the sham (n=14), prelimbic deactivation (PL) (n = 7) and unpaired PL deactivation (Unp PL) (n = 6) groups. (B-D) Results after clustering the sham group in the sham no preference (Sh NP) (n = 7) and sham preference (Sh P) (n = 7) groups. Data are shown as the median and scatterplots. (*P < 0.05; **P < 0.01; ***P < 0.001).*

A one-way ANOVA showed significant effects for the group factor ($F(2,24) = 4.774$, $p = 0.0180$). As shown by a subsequent post hoc comparison using Tukey's HSD tests, the IL deactivation facilitated the acquisition of cocaine-induced preference conditioning. The IL group (n = 7) exhibited a significant higher preference for the CS+ than the sham (n = 14) ($p = 0.049$) and unpaired groups (Unp IL) (n = 6) ($p = 0.019$) (Fig. 2A). On the contrary, the PL deactivation did not produce a significant effect on cocaine-induced conditioned preference ($F(2,24) = 1.718$, $p = 0.2007$) (Fig. 2C). No lateralisation effect was found in any of the groups when comparing left and right deactivation sides: Sham ($t(12) = 0.5654$, $P = 0.5822$); IL ($t(5) = 1.076$, $P = 0.3310$); PL ($t(5) = 0.0655$, $P = 0.9503$).

After clustering rats using the cut-off point for preference (60%), a Kruskal–Wallis analysis confirmed that IL deactivation increased the number of animals that exhibited a preference score higher than 60% ($H(4) = 15.66$, $P = 0.0013$) (Fig. 2B). Dunn's post hoc comparisons showed that the IL group (n = 7) exhibited significantly higher preference for the CS+ than the Sh NP (n = 7) ($P = 0.0005$) and Unp IL (n = 6) ($P = 0.0042$) groups. However, post hoc comparisons revealed no differences between the IL and Sh P groups ($P > 0.2$) (Fig. 2B).

Oppositely, PL deactivation seemed to block the acquisition of cocaine-induced preference conditioning. A Kruskal–Wallis test showed a significant effect of the group factor ($H(4) = 10.71$, $P = 0.0134$), being the Sh P group that exhibited a higher preference as compared to the Sh NP ($P = 0.0081$), PL ($P = 0.0033$) and Unp PL ($P = 0.0278$) groups (Figure 2D).

Deactivations of mPFC generate differential effects on cerebellar activity

We focused our analysis on cFos activity in the apical (dorsal) region of lobules VIII and IX, as previous research from the group indicated that this is the cerebellar region that showed significant differences linked to the expression of cocaine-induced conditioned preference (Carbo-Gas *et al*, 2014a, 2014b). Therefore, we were interested in investigating whether the effects of mPFC deactivations on cerebellar activity could be modulated by the expression of preference for the cocaine-related cue. A Kruskal–Wallis test showed significant effects in the number of cFos+ cells in lobule VIII ($H(4) = 22.01$,

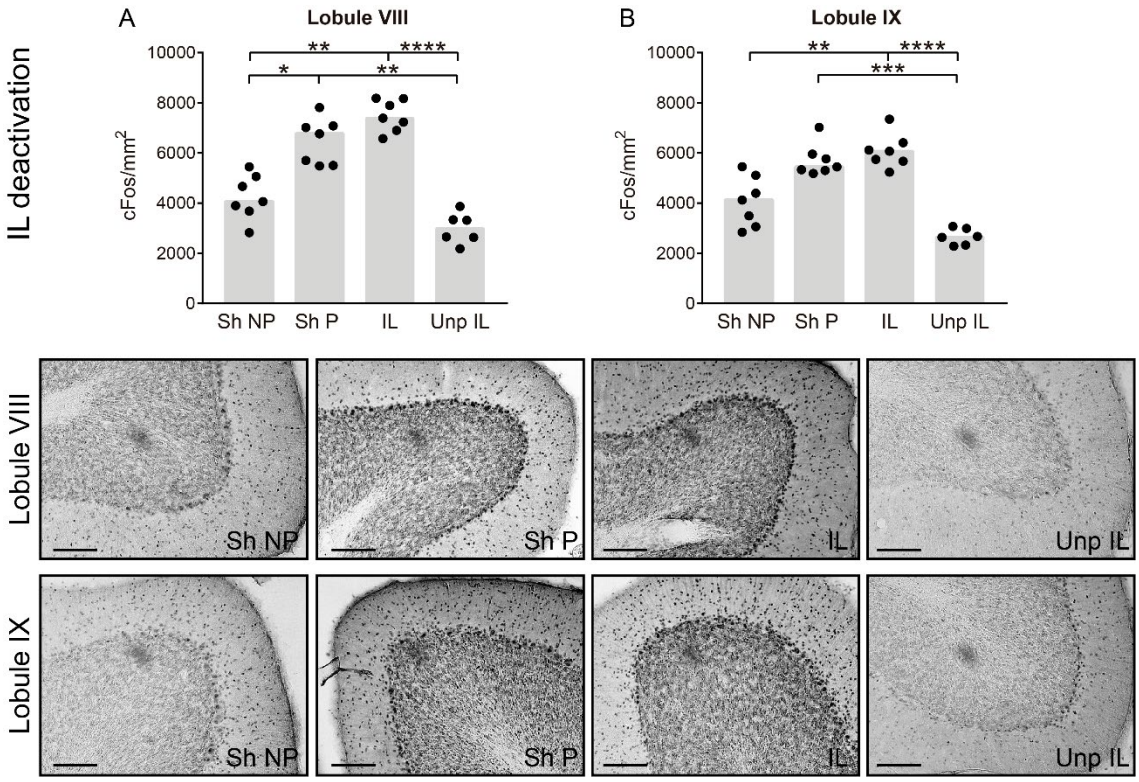

Figure 3. Effect of PL deactivation on cFos expression in the dorsal region of lobules VIII and IX of the cerebellar vermis. *(A-B) Number of cFos positive cells/mm² in lobule VIII and IX 90 min after the preference test in the Sh NP (n = 7), Sh P (n = 7), prelimbic deactivation (PL) (n = 7) and unpaired PL deactivation (Unp PL) (n = 6) groups. Representative pictures of cFos staining in lobule VIII and IX for each group are depicted on the bottom panel. All images were taken at 20x magnification. Scale 50 µm. (*P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001). Brightness was standardised across images.*

P < 0.0001) and lobule IX (H (4) = 19.99, P = 0.0002). In lobule VIII, cFos expression increased in the IL and Sh P groups regarding the groups not expressing preference (Sh NP: P = 0.0017 and P = 0.0368, respectively) and (Unp IL: P < 0.0001 and P = 0.0014, respectively) (Fig. 3A). Similar increase in the number of cFos+ neurons was found in lobule IX as an effect of the IL deactivations (Sh NP (P = 0.0071) and Unp IL (P < 0.0001) groups). However, in this case, the Sh P group was only different from the Unp IL (P = 0.0010) group (Fig. 3B).

The expression of cFos in the cerebellum after PL deactivations showed a different pattern. Kruskal–Wallis test also demonstrated a significant effect for lobule VIII (H (4) = 18.52, P = 0.0003) and IX (H (4) = 18.35, P = 0.0004). However, Dunn's multiple comparisons test for lobule VIII revealed that the number of cFos+ neurons in the PL group was not different from that of the Sh NP (P > 0.3) and Unp PL (P > 0.3) groups. Only the Sh P group exhibited higher cFos expression than the other groups (Sh NP (P = 0.022), PL (P = 0.0016), Unp PL (P < 0.0001)) (Fig. 4A). Similar results were observed for

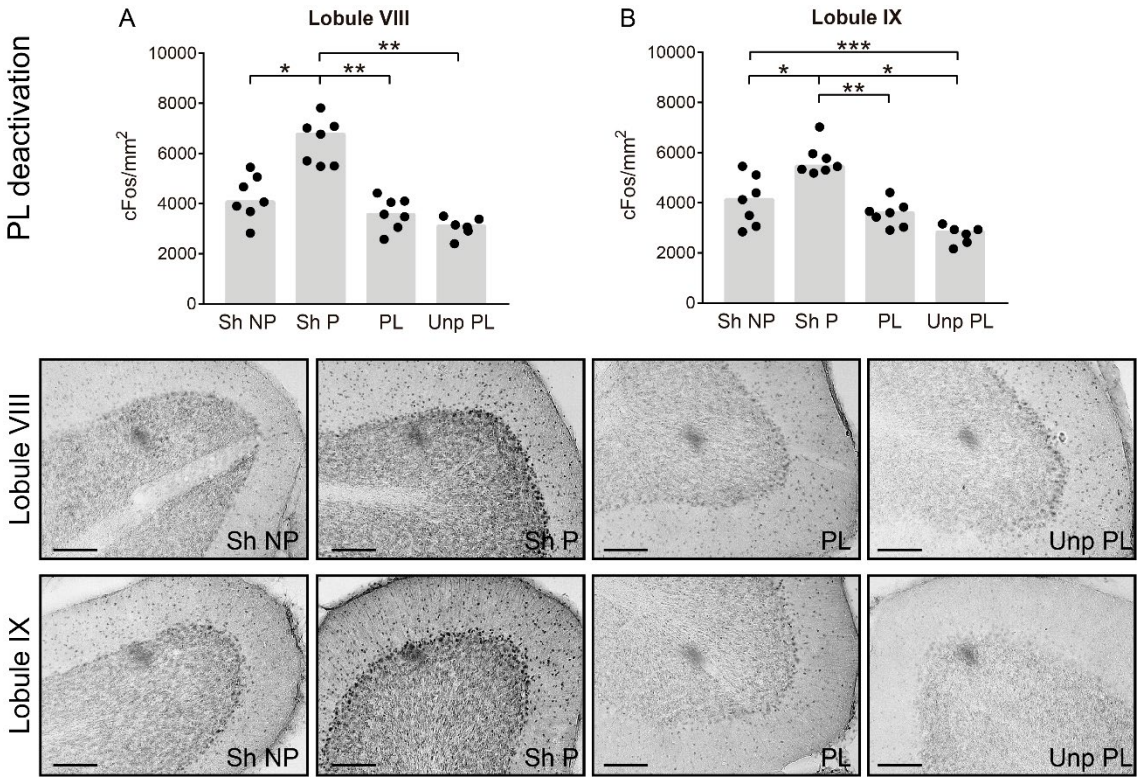

Figure 4. Effect of IL deactivation on cFos expression in the dorsal region of lobules VIII and IX of the cerebellar vermis. *(A-B) Number of cFos positive cells/mm² in lobule VIII and IX, 90 min after the preference test in the Sh NP (n = 7), Sh P (n = 7), IL deactivation (n = 7) and Unp IL deactivation (n = 6) groups. Representative pictures of cFos staining in lobule VIII and IX for each experimental group appear below. All images were taken at 20x magnification. Scale bar 50 µm. (*P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001). Brightness was standardised across images.*

lobule IX. The expression of cocaine-induced preference (Sh P) increased cFos levels as compared to those groups that did not express preference (Sh NP (P = 0.0339), PL (P = 0.0071), Unp PL (P < 0.0001)) (Fig. 4B).

Significant correlations were observed between cFos expression and preference in lobule VIII (r = 0.8216; P < 0.0001) and IX (r = 0.7956; P < 0.0001) (Fig. 5A-B).

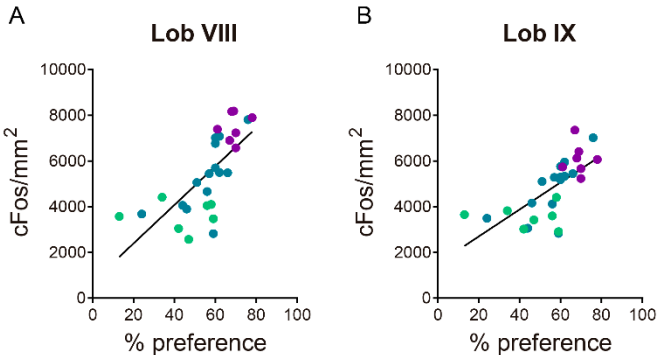

Figure 5. Correlations between cFos expression and preference scores in lobules VIII and IX *(A) Correlation between cFos positive cells/mm² and percentage of preference in lobule VIII. (B) Correlation between cFos positive cells/mm² and preference percentage in lobule. IL group (purple dots), PL group (green dots), sham group (blue dots).*

In summary, the expression of preference for cocaine-related cues was associated with an increase in cFos expression in the

posterior cerebellum. IL and PL deactivations oppositely influenced cerebellar activity, and their effects seem to be modulated by the expression of cocaine-induced conditioned preference. While all rats with IL deactivation showed conditioned preference and an increase in cerebellar activity, those that underwent PL deactivation did not.

Different impact of mPFC deactivations on striatal activity

The Kruskal–Wallis tests for the results of the IL experiment showed significant effects on cFos expression in the DMS (H (3) = 7.75, P = 0.0208) and NAcSh (H (3) = 12.79, P = 0.0017)), but not in the DLS (H (3) = 2.08, P = 0.3539), VLS (H (3) = 2.57, P = 0.2773), and NAcC (H (3) = 1.21, P = 0.5672) (Fig. 6A). Significantly greater numbers of cFos+ neurons were observed in the DMS and NAcSh for the IL (P = 0.0264, P = 0.0071) and Sh P (P = 0.0103; P = 0.0007) groups as compared to the Sh NP group, respectively.

Significant effects were observed in the DMS (H (3) = 7.03, P = 0.0.297), NAcC (H (3) = 9.11, P = 0.0.105) and NAcSh (H (3) = 11.26, P = 0.0011), but not in the DLS (H (3) = 1.79, P = 0.4077) and VLS (H (3) = 3.29, P = 0.1932) in the PL experiment (Fig. 6B). For the DMS,

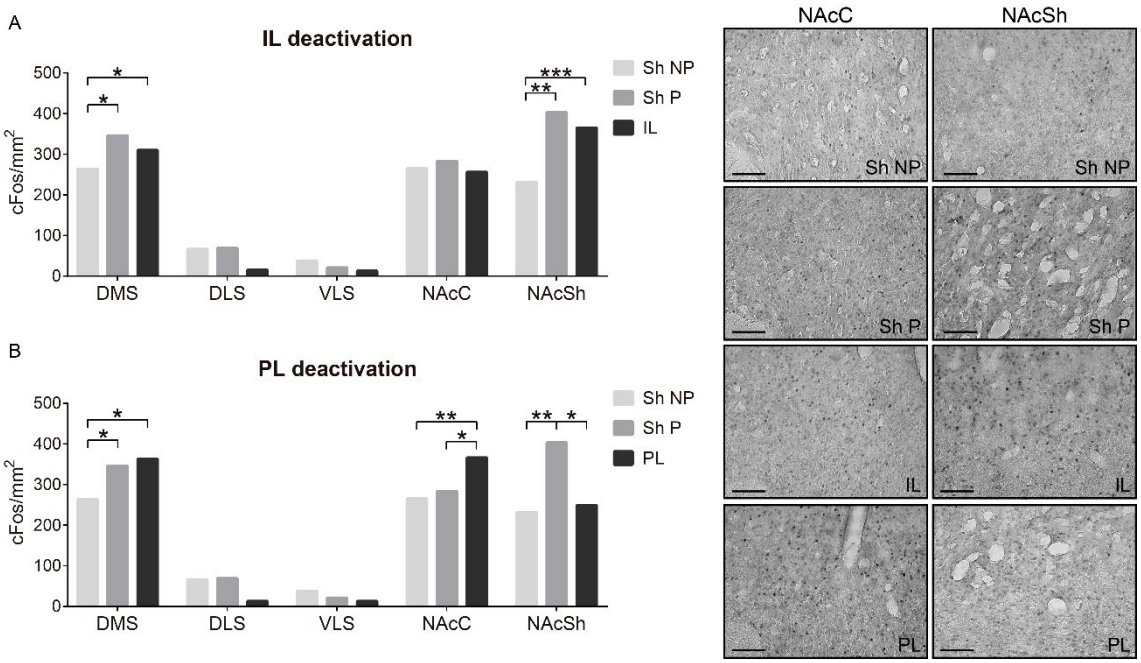

Figure 6. Mean number of cFos positive cells/mm2 in DMS, DLS, VLS, NAcC and NAcSh for IL and PL deactivations. *(A) IL deactivation. DMS, DLS, VLS, NAcC and NAcSh in Sh NP (n = 7), Sh P (n = 7) and IL (n = 7) groups. (B) PL deactivation. DMS, DLS, VLS, NAcC and NAcSh in Sh NP (n = 7), Sh P (n = 7) and PL (n = 7) groups. On the right, representative pictures of cFos staining in the NAcC and NAcSh for the four experimental groups. All images were taken at 20x magnification. Scale bar 50 µm. (*P < 0.05; **P < 0.01; ***P < 0.001). Brightness was standardised across images. Data are shown as the median score*

the Sh NP group showed lower cFos activity than the Sh P (P = 0.0251) and PL (P = 0.0189) groups. In the NAcC, PL deactivation increased the number of cFos+ neurons as compared to the Sh NP (P = 0.0032) and Sh P (P = 0.0429) groups. Finally, in the NAcSh, the level of cFos expression in PL deactivated animals was very similar to that of the Sh NP group. Only the Sh P group showed a significant increase in cFos activity with respect to the other groups (Sh NP: P = 0.0014; PL: P = 0.0125).

Taken together, the present results indicated that mPFC deactivations showed differential effects in the NAc. After IL deactivation, activity in the NAcSh increased at the same level as that of those animals expressing preference. On the contrary, PL deactivation increased neural activity within the NAcC, but did not change activity in the NAcSh. Moreover, after the PL deactivation, neural activity in the NAcSh was very similar to that exhibited by those rats not expressing preference for cocaine-related cues. Deactivation of both mPFC regions impacted DMS activity similarly and did not change neural activity in other striatal regions.

Cocaine-induced conditioned preference upregulates PNN expression around Golgi interneurons in the cerebellar cortex

The expression of PNNs surrounding Golgi interneurons showed significant effects for the group factor in both experiments. (IL) lobule VIII (H (4) = 19.87, P = 0.0002) and IX (H (4) = 19.58, P = 0.0002); (PL) lobule VIII (H (4) = 12.25, P = 0.0066), and IX (H (4) = 14.69, P = 0.0021).

Cerebellar PNNs in those groups expressing preference (IL and Sh P) were stronger than PNNs in the Sh NP (P = 0.0011; P = 0.0184, respectively) and Unp IL (P = 0.0002 and P = 0.0039, respectively) groups (Fig. 7A). In Lobule IX, the results were very similar, and more intense PNNs were found in the IL and Sh P groups as compared to the Sh NP (P = 0.0005; P = 0.0311, respectively) and Unp IL (P = 0.0002; P = 0.0125, respectively) groups (Fig. 7B).

Oppositely, PL deactivation induced fainter expression of PNNs in lobules VIII and IX resembling the expression of those groups that did not express cocaine-induced conditioned preference. Thus, in this case, only the Sh P group showed PNNs more intense than the rest of groups in lobule VIII (P < 0.02) and IX (P < 0.05) (Fig. 8A-B).

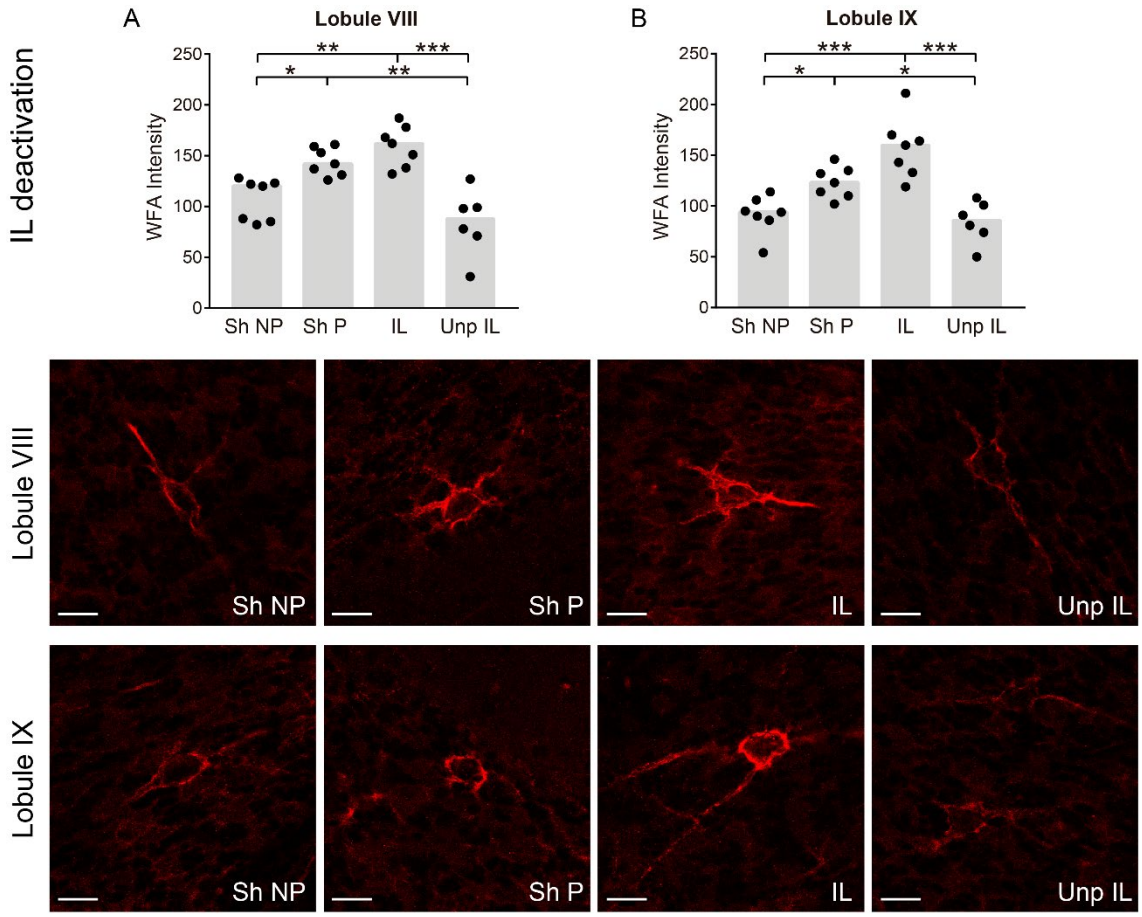

Figure 7. PNN expression surrounding Golgi interneurons in the posterior vermis after IL deactivation*. (A-B) Average intensity of WFA at the dorsal region of the granule cell layer in lobules VIII and IX in the Sh NP (n = 7), Sh P (n = 7), IL (n = 7) and Unp IL (n = 6) groups. On bottom panels, representative microphotographs of PNNs around Golgi interneurons stained with Wisteria floribunda agglutinin (WFA) (red). The confocal images were acquired at 20x with a 2x zoom for a final amplification of 80x, respectively. Scale bar 20 µm. PNN surrounding Golgi cells were still stronger and more prominent in the Sh P and IL groups. (*P < 0.05; **P < 0.01; ***P < 0.001).*

Finally, the correlation between preference and PNNs intensity were significant in lobule VIII (r = 0.6521; P = 0.0002) and in the lobule IX (r = 0.5817; P = 0.0012) (Fig. 9A-B).

In conclusion, the expression of preference for the cocaine-related cue was associated with stronger and fully condense PNNs. As was previously described for neural activity, the key factor in explaining the upregulation of PNNs around Golgi interneurons was the acquisition of cocaine-induced conditioned memory that seemed to be mediating the effects of mPFC deactivations on cerebellar plasticity.

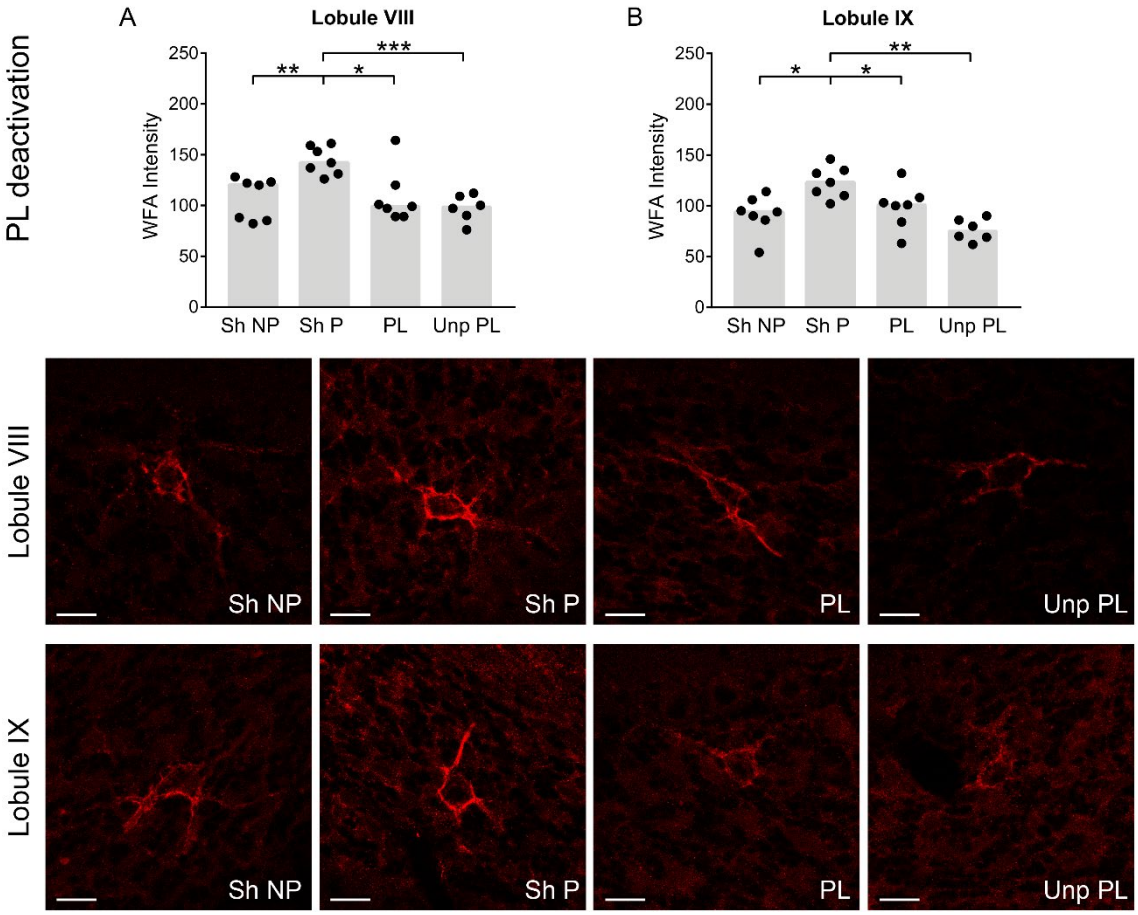

Figure 8. PNN expression around Golgi interneurons the posterior vermis after PL deactivations. *(A-B) Average intensity of WFA at the dorsal region of the granule cell layer in lobules VIII in the Sh NP (n = 7), Sh P (n = 7), PL (n = 7) Unp PL (n = 6) groups. Representative microphotographs of PNNs in the dorsal region of cerebellar cortex stained with Wisteria floribunda agglutinin (WFA) (red). The confocal images were acquired at 20x with a 2x zoom for a final amplification of 80x, respectively. Scale bar 20 µm. PNN surrounding Golgi cells were stronger and more prominent only in the Sh P group. (*P < 0.05; **P < 0.01; ***P < 0.001).*

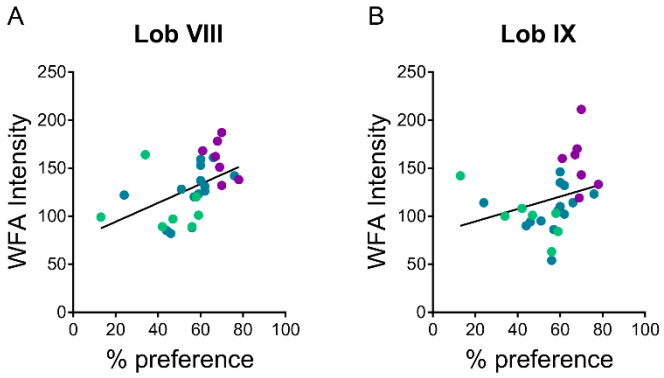

Figure 9. Correlations between PNNs intensity and preference scores in lobules VIII and IX *(A) Correlation between WFA intensity and percentage of preference in lobule VIII. (B) Correlation WFA intensity and preference percentage in lobule IX. IL group (purple dots), PL group (green dots), sham group (blue dots).*

DISCUSSION

A large number of studies have shown that prefrontal cortex plays a crucial role in drug addiction in animals and humans (Chen *et al*, 2013; Goldstein and Volkow, 2011; Lasseter *et al*, 2010; McFarland and Kalivas, 2001; Volkow *et al*, 2015). Functional impairment of the prefrontal cortex underlies loss of inhibitory control over drug-seeking (Chen *et al*, 2013; Jentsch and Taylor, 1999). Remarkably, the cerebellum, always being considered a motor structure, has revealed itself as an important region for the addiction field during the last decade (Miquel *et al*, 2009, 2016; Moulton *et al*, 2014). In previous studies, we showed that cocaine-induced preference conditioning enhanced cFos expression and upregulates PNN expression in the apical region of the posterior cerebellum in mice (Carbo-Gas *et al*, 2014a, 2014b, 2017). In the present study, we wanted to explore whether mPFC hypofunction would influence cocaine-dependent activity and plasticity in the cerebellum. Thus, we trained rats to acquire a cocaine-cue conditioned association under temporal deactivation of either the IL or PL cortex. Our findings indicated that impairment of these two regions in the mPFC may have a different impact on cocaine-related memory. Then, such an impact modulates neuronal activity and plasticity in the cerebellum.

In the first chapter (Gil-Miravet *et al*, 2018), we demonstrated that an IL deactivation produces a robust increase in the percentage of animals that develop a preference for the cocaine-related cue. The analysis of the subsample of rats we selected for the immunohistochemistry study yielded the same results. Therefore, we can conclude that IL deactivation during conditioning increased the likelihood of acquiring drug-induced memories. As lidocaine administrations were performed before each training session, but not on the test day, the IL deactivation could only affect the acquisition phase. In this way, on the test day, IL deactivated animals were under similar conditions to those in the sham group. Our results, together with the already existing literature (Lalumiere *et al*, 2012; LaLumiere *et al*, 2010; Peters *et al*, 2008; Rocha and Kalivas, 2010), suggest that the IL cortex can be part of an inhibitory route for the acquisition of drug-seeking.

IL deactivation enhanced neural activity in the apical region of the granule cell layer in the posterior vermis. This increase was not seen in animals with PL deactivation. Neither was it seen in those cerebella from control animals that did not express preference for

the cocaine-related cue. However, a more careful analysis of the present data did not support a direct effect of the IL deactivation. Neural activity in this region of the cerebellar cortex also increased in control rats expressing preference. Hence, our results suggest that IL deactivation by facilitating the acquisition of cocaine-induced conditioned preference increased neural activity in the cerebellum. The present results are consistent with our previous observations in mice and support also our hypothesis about the function of the posterior cerebellar cortex in drug reward (Carbo-Gas *et al*, 2014a, 2014b, 2017; Moreno-Rius and Miquel, 2017). We have proposed that the posterior cerebellar cortex generates unconscious predictions of drug availability after cue presentation while monitoring the internal state under drug abstinence (Moreno-Rius and Miquel, 2017). Cue exposure would then trigger a cerebellum-generated prediction of drug availability, thereby activating the preparation of the brain networks (striatal-limbic loops) responsible for drug-seeking and taking. Moreover, we observed that when animals are confined in the only presence of the cocaine-related cue without the possibility of selecting the other alternative (CS-), the increase in cerebellar activity is prevented (Carbo-Gas *et al*, 2017). Therefore, our past and present results indicate that neural activity in the granule cell layer of the cerebellar cortex may reflect the behavioural selection driven by the cocaine-related cue. They also indicate that if drug experience occurs under IL impairment, the probability for the acquisition of cocaine-induced conditioned memory, as well as the development of associated cerebellar changes raises. Interestingly, neuroimaging studies showed that greater cerebellar activity accompanied prefrontal dysfunction in drug addicts (Bolla *et al*, 2005; Desmond *et al*, 2003; Hester and Garavan, 2004; Moulton *et al*, 2014).

In the present research, the acquisition of cocaine-induced preference was also associated with an upregulation of PNNs around Golgi inhibitory interneurons in the apical region of the cerebellar cortex. Stronger and fully condense PNNs were found after IL deactivation, but also in control animals that acquired the conditioned memory. These findings suggest that cocaine-induced conditioned memory, and not IL hypofunction, is the key factor in increasing PNNs expression around Golgi interneurons. Indeed, those groups that did not express conditioned preference, such as the PL deactivation group, pseudo-conditioned groups, and the no preference group all exhibit

faint PNNs around Golgi interneurons. Similar results were described previously in mice (Carbo-Gas *et al*, 2017). The expression of PNNs surrounding Golgi inhibitory interneurons in the apical region of the cerebellar cortex were stronger in animals that exhibit preference for the cue associated with cocaine. A reduced PNN intensity might correspond to an immature PNN with increased capacity for plasticity, whereas higher intensity would correspond to a mature PNN with decreased capacity for plasticity (Wang and Fawcett, 2012). In this way, the animals that acquired preference and, therefore, generated cocaine associated memories, developed fully condense PNNs and consequently their capacity for plasticity was reduced. Sorg *et al*. (2016) have proposed that stronger PNNs could "stamp in" synaptic connections that represent drug-cue associations, preventing future synaptic remodelling. With stronger PNNs, the new synapses formed during drug-induced conditioning would be more stable and difficult to modify. Accordingly, a recent study observed that the degradation of PNNs in the PL cortex in cocaine-induced CPP memory impaired acquisition and reconsolidation of cocaine-induced place preference memories (Slaker *et al*, 2015). Moreover, PNNs degradation in the amygdala following drug exposure, but before extinction training, augments extinction and inhibits subsequent reinstatement of drug-seeking behaviour (Xue *et al*, 2014). Other recent investigations have observed that the degradation of PNNs in the lateral hypothalamic area abolishes the acquisition of cocaine-induced conditioned place preference, reduces cocaine self-administration, and blocked the expression of cue-induced reinstatement of cocaine- but not sucrose-seeking behaviour (Blacktop *et al*, 2017; Blacktop and Sorg, 2018).

In the cerebellum, PNNs are also developed around glutamatergic and GABAergic projection in the deep cerebellar nuclei (DCN) (Carulli *et al*, 2006). These PNNs did not change after the acquisition of cocaine-induced preference memory, at least as far as the medial nucleus concerns (Carbo-Gas *et al*, 2017). They have demonstrated, however, to be regulated by plasticity events affecting Purkinje neurons during periods of cocaine abstinence. Thus, stronger and more intense PNNs are found after a short period of abstinence associated with a kind of molecular and structural plasticity which reduce Purkinje cell capacity to inhibit DCN neurons (Vazquez-Sanroman *et al*, 2015). On the contrary, PNNs are downregulated in the medial nucleus when the synaptic capacity

of Purkinje neurons increased after longer periods of abstinence (Vazquez-Sanroman *et al*, 2015).

Deactivations of the IL and PL cortices generated different effects on activity in the NAc. Literature suggests that the PL-to-NAcC pathway promotes drug-seeking behaviour and the IL-to-NAcSh pathway is responsible for the extinction of drug-seeking behaviour (Lalumiere *et al*, 2012; McFarland and Kalivas, 2001; Peters *et al*, 2008). As observed in the cerebellum, neural activity in the NAcSh was related to cocaine-induced conditioned preference. NAcSh activity increased only in animals expressing preference. Several studies suggest that NAcSh is involved in the suppression of cocaine-seeking behaviour (Di Ciano *et al*, 2008; Peters *et al*, 2008). Lalumiere *et al.* (2012) found that AMPA receptor blockade in the NAcSh restored cocaine-seeking previously inhibited by a positive modulator of AMPA receptor activation of IL afferents to the NAcSh (Lalumiere *et al*, 2012). Oppositely, AMPA receptor blockade in the NAcC prevented cue-induced reinstatement of cocaine-seeking (Bäckström and Hyytiä, 2007; Di Ciano and Everitt, 2001; Cornish and Kalivas, 2000; McFarland and Kalivas, 2001). The NAcSh and NAcC receive glutamatergic input from the IL and PL, respectively, and dopaminergic inputs from the VTA (Fallon and Moore, 1978; Sesack *et al*, 1989). A recent study observed that optical inhibition of VTA-to-NAcC afferents prevented cocaine-seeking reinstatement (Stefanik and Kalivas, 2013). These dopaminergic projections seem to be critical for learning and maintenance of goal-directed responding, including drug-seeking behaviour (Ettenberg, 1989; Koob and Swerdlow, 1988). Moreover, dopaminergic and non-dopaminergic projections from the VTA to cerebellum have been described (Ikai *et al*, 1992, 1994), supporting the presence of dopamine transporters and receptors in the cerebellar cortex and deep nuclei (Ikai *et al*, 1992; Melchitzky and Lewis, 2000; Panagopoulos *et al*, 1991). Previous studies in our laboratory observed increases in levels of dopamine transporter in the posterior cerebellum of animals that develop cocaine-induced conditioned preference (Carbo-Gas *et al*, 2014a).

In conclusion, our laboratory studies do not support a direct effect of mPFC deactivations on cerebellar activity and plasticity. On the contrary, they indicate that impairment of the mPFC function increases or decrease susceptibility to the acquisition of drug-induced Pavlovian memory thereby modulating cerebellar activity. In addition,

they suggest that the cerebellum might be a critical region for the storage or reactivation of conditioned associations that predict drug availability.

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AUTHOR DISCLOSURE

All authors declare no conflicts of interest.

AUTHORSHIP

All authors made a notable 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 and Ignasi Melchor-Eixea were involved in image and data analysis. Finally, Marta Miquel designed the study, supervised the surgeries and behavioural experiments, was involved in data analysis, and drafted the manuscript. All authors approved the present version of the manuscript.

DATA ACCESSIBILITY

Raw data are available from the corresponding author upon request.

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EXPERIMENTAL STUDIES

Chapter 3

Chapter 3: From back to front: A functional model for the cerebellar modulation in the establishment of conditioned preferences for cocaine-related cues

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ABSTRACT

It is now clear that the cerebellum may modulate brain functions altered in drug addiction. We previously demonstrated that cocaine-induced conditioned preference increased activity at the apical region of lobule VIII in the vermis. Activity in lobule VIII was significantly correlated with the level of preference towards cocaine-conditioned cues. Unexpectedly, a neurotoxic lesion of lobule VIII raised by up to one hundred the percentage of animals that acquired cocaine-induced conditioned preference. The present research aimed at providing an explanatory model for the facilitative effect of the cerebellar lesion on cocaine-induced conditioned memory. We evaluated cFos expression in different regions of the medial prefrontal cortex and striatum after a lesion in lobule VIII before conditioning. Additionally, to explore whether the cerebellar lesion might affect synaptic stabilization mechanisms in the medial prefrontal cortex, PNN expression was assessed. Damage in this region of the vermis induced a general disinhibition of the mPFC and striatal subdivisions that receive dopaminergic projections, mainly from the ventral tegmental area (VTA). Moreover, cerebellar impairment induced an upregulation of PNN expression in the mPFC. Finally, we addressed a tracing study using anterograde and retrograde tracers in order to build a working neuroanatomical model to explain the present results. We found a direct projection from the lateral nucleus to the VTA that also receives Purkinje axons from lobule VIII in the vermis. Hypothetically, this pathway might control activity and plasticity of the cortico-striatal circuitry through an increase in dopaminergic activity.

Keywords: cocaine, cerebellum, quinolinic acid, PNNs, VTA

INTRODUCTION

For decades, the cerebellum's role has been restricted only to motor functions. Fortunately, in recent years, numerous investigations have described the involvement of the cerebellum in non-motor functions including language, spatial and emotional processing, reward, working memory, and executive functions (Ball *et al*, 1974; Carta *et al*, 2019; Corbett *et al*, 1982; Sacchetti *et al*, 2002; Schmahmann and Pandya, 1997; Turner *et al*, 2007; Wagner *et al*, 2017; Watson *et al*, 2014; Zhu *et al*, 2011). Anatomical and functional studies in rodents and non-human primates have shown extensive pathways that connect the cerebellum to the prefrontal cortex, striatum, amygdala, thalamus, hippocampus and basal ganglia (Bostan *et al*, 2018; Buckner *et al*, 2011; Chen *et al*, 2014; Hoshi *et al*, 2005; Ichinohe *et al*, 2000; Ikai *et al*, 1992; Middleton and Strick, 2000, 2001; Panagopoulos *et al*, 1991; Sang *et al*, 2012; Stanton, 1980; Xiao *et al*, 2018). More recently, two findings pointed to a direct control of the cerebellum over the ventral tegmental area (VTA) (Carta *et al*, 2019; Watabe-Uchida *et al*, 2012). All these results suggest that the cerebellum is part of cortical-striatal-limbic loops and may modulate brain functions altered in drug addiction (Miquel *et al*, 2009, 2016; Yalachkov *et al*, 2010).

Indeed, the cerebellum plays an important role in the consolidation of emotional memory, as well as in the establishment of automatic behavioural protocols (Callu *et al*, 2007; Sacchetti *et al*, 2002). Moreover, neuroimaging studies of drug-induced cue reactivity in drug addicts described cerebellar activation after the presentation of drug-related cues (Anderson *et al*, 2006; Bonson *et al*, 2002; Fuentes *et al*, 2012; Grant *et al*, 1996; Moulton *et al*, 2014; Schneider *et al*, 2001). In a mice model of cocaine-induced conditioned preference, we showed that only those animals that developed preference for cocaine-related cues exhibited increased activity at the apical region of the cerebellar vermis (Carbo-Gas *et al*, 2014a, 2014b, 2017). Although this effect was found throughout the cerebellar cortex, only activity in lobule VIII was significantly correlated with the level of preference towards cocaine-related cues (Carbo-Gas *et al*, 2014b). Furthermore, cocaine-induced conditioned preference also increased the expression of perineuronal nets (PNNs) surrounding Golgi inhibitory interneurons located in the same region of the vermis (Carbo-Gas *et al*, 2017), suggesting that drug-induced Pavlovian memory

encouraged one of the main mechanisms for synaptic stabilization (Sorg *et al*, 2016). On that basis, one could expect a neurotoxic lesion localised in lobule VIII to prevent the acquisition of cocaine-induced conditioned preference. On the contrary, the cerebellar lesion dramatically raised by up to 100 the percentage of rats that acquired cocaine-induced conditioned preference (Gil-Miravet *et al*, 2018). The same effect was observed after a reversible deactivation of the infralimbic (IL) cortex (Gil-Miravet *et al*, 2018). Moreover, simultaneous IL-cerebellar deactivation prevented the effect of either of the separate manipulations (Gil-Miravet *et al*, 2018). These results were in agreement with findings reporting that the IL cortex is required for the suppression of cocaine-seeking response and expression of extinction memory (Lalumiere *et al*, 2012; LaLumiere *et al*, 2010). Overall, our findings suggested that both the cerebellum and IL cortex might act together in regulating the establishment of drug-cue Pavlovian associations.

In the present work, we aimed at: (1) further investigating cerebellum-infralimbic functional relationships for the acquisition of cocaine-induced conditioned preference; and (2) proposing a functional model to explain the effects of the cerebellar lesion in cocaine-conditioned memory. We assessed cFos expression in different regions of the medial prefrontal cortex (mPFC) and striatum after a neurotoxic lesion in the apical region of lobule VIII before conditioning. Also, to explore whether the cerebellar lesion might affect synaptic stabilization mechanisms in the mPFC, PNN expression was evaluated. Finally, we addressed a tracing study using anterograde and retrograde tracers in order to build a working neuroanatomical model to explain the present results.

METHODOLOGY

Subjects

Twenty-two male Sprague-Dawley rats (Janvier, ST Berthevin Cedex, France) weighing between 175 and 200 g were randomly selected from the rats included in the first study (Gil-Miravet *et al*, 2018) (Chapter 1). Animals were individually housed under standard laboratory conditions, with controlled temperature and humidity (12 h light cycle from 8:00 a.m. to 8:00 p.m.) and access to food and water *ad libitum* (Jaume I University, Spain). Rats were handled and habituated to all of the experimental procedures. All

animal procedures were approved by the local Animal Welfare Ethics Committee and Empowered Body (2014/VSC/PEA/00208) and developed in accordance with the European Community Council directive (2010/63/EU), Spanish directive BOE 34/11370/2013 and local directive DOGV 26/2010.

Brain infusions and stereotactic surgery

The animals were anaesthetized using a cocktail of ketamine (100 mg/Kg) (Imalgene 100 mg/ml, Mersal Laboratorios S.A., Barcelona, Spain) and xylazine (10 mg/kg) (Xylazine hydrochloride ≥99%, Sigma-Aldrich, Madrid, Spain) (IP), and placed in a Kopf stereotaxic apparatus for the surgery. We use a stainless-steel guide cannula (10 mm length; 23-gauge external diameter) for the intracranial infusion of quinolinic acid (QA) (90 nmol/µl) (2,3-Pyridinedicarboxylic acid, Sigma-Aldrich, Madrid, Spain) dissolved in phosphate buffered saline (PBS). The coordinates for the dorsal region of lobe VIII in the vermis were AP: −14.5; ML: 0; DV: −4.5 (Paxinos and Watson, 1998) (Fig. 1). QA infusion (0.5 µl volume; infusion ratio of 0.2 µl/min) was released through a removable stainless-steel injector (length, 11 mm; external diameter, 30-gauge) inserted into the previously implanted guide cannula. After infusion, the cannula remained in place for 3 min to allow for diffusion. The same procedure was implemented in the sham group infusing PBS. After the surgery, all the animals received analgesic treatment with meloxicam (Metacam 5 mg/ml, Boehringer Ingelheim, Barcelona, Spain) for 24 hours for three days. Cannula locations were verified by Nissl immunostaining and camera lucid. More detailed information can be found in (Gil-Miravet *et al*, 2018).

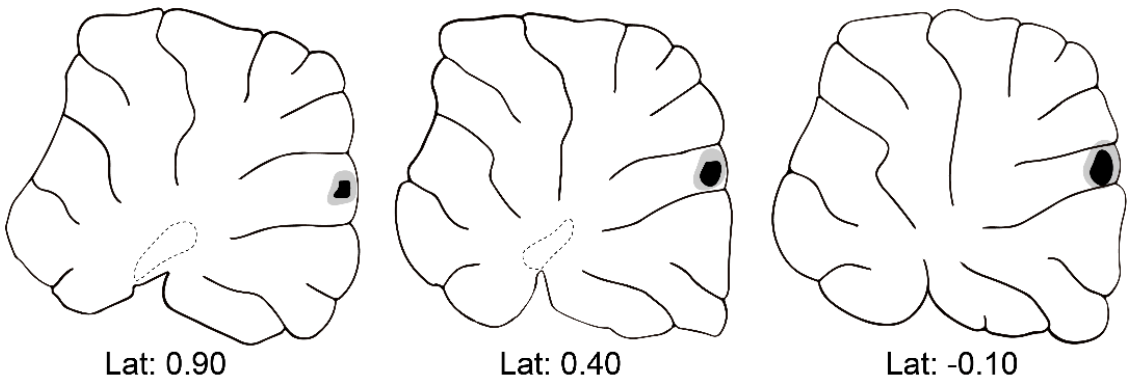

***Figure 1. Diagrams of the cannula locations**. Schematic diagrams depicting the largest (grey) and smallest (black) diffusion areas in the apical region of lobule VIII in the vermis. The extent of the diffusion areas was assessed using light microscopy and camera lucida drawings.*

For the tracing study, retrograde and anterograde tracers were infused in different regions of the brain. As a retrograde tracer, we used FluoroGold with DAPI (FG) (Hydroxystilbamidine, Biotium, Barcelona, Spain). The anterograde tracing was accomplished using Dextran Biotin (BDA) (10,000 MW, Lysine Fixable, Thermo Fisher Scientific, Barcelona, Spain). The following coordinates were used: IL (AP: +3.2; ML: + 0.6/−0.6; DV: −5); VTA (AP: -5.2; ML: + 0.9/−0.9; DV: −8.3); lateral nucleus (Lat) (AP: -11.4; ML: + 3.6/−3.6; DV: −6.2); interpositus nucleus, anterior part (IntA) (AP: -11.3; ML: + 2.5/−2.5; DV: −5.8); interpositus nucleus, posterior part (IntP) (AP: -11.7; ML: + 2.5/−2.5; DV: −6.2); medial nucleus (Med) (AP: -11.4; ML: + 1/−1; DV: −6.2); and the apical area of lobe VIII in the vermis (AP: −14.5; ML: 0; DV: −4.5) (Paxinos and Watson, 1998). FG or BDA infusion volumes were 0.5 µl in the IL and 0.3 µl in the rest of regions with an infusion ratio of 0.2 µl /min. After infusions, the rats remained undisturbed for ten days before perfusion.

Cocaine-induced preference conditioning procedure

The cocaine-induced conditioning procedure has been published previously (Gil-Miravet *et al*, 2018). Briefly, conditioning was conducted using two equally preferred olfactory stimuli located in the walls of a black chamber (20 × 20 × 60 cm) at the opposite arms of a corridor. One of the odours acted as the conditioned stimulus (CS+) and was associated with an IP injection of cocaine hydrochloride (15 mg/kg, IP) (Alcaliber S.A., Madrid, Spain). On alternate days, rats were exposed to the other scent (CS−) placed at the opposite black chamber in the corridor and received 0.9% saline injections. During pairing sessions (15 min) animals remained confined in the chamber. A total of eight cocaine-paired sessions were conducted. The olfactory cues and locations in the corridor were counterbalanced between animals. Preference for the cocaine-related cue was evaluated 48 h after the last cocaine administration in a 30 min drug-free test in which CS+ and CS− odours were present simultaneously but in opposite arms of the corridor. The first ten minutes were not considered in order to allow the animal to explore the location of the odours, which was the opposite to the conditioning phase. The preference score was calculated as [TS in CS+ / (TS in CS+ + TS in CS−)] × 100. Additionally, we included a pseudo-conditioning group (the Unp group) that was treated with the same number of cocaine injections, but randomly associated with the olfactory stimuli.

The Unp group allowed us to test for memory-related effects of our cerebellar deactivations. Animals were perfused transcardially 90 min following the preference test.

Immunohistochemistry and immunofluorescence

Animals were deeply anaesthetised with sodium pentobarbital (30 mg/kg) (Dolethal 100 ml, Vetoquinol E.V.S.A., Madrid, Spain) and perfused transcardially with saline (0.9%) and paraformaldehyde (4%) 90 min following the preference test. After perfusion, the brain and cerebellum were quickly dissected and placed in a container with the same fixative for 24 h at 4 ºC. Then, the tissue was immersed in sucrose solution (30%). The brain tissue was frozen with liquid nitrogen, and sections were performed at 40 µm with a cryostat microtome (Microm HM560, Thermo Fisher Scientific, Barcelona, Spain). Eight series of tissue sections were collected and stored at −80 °C in cryoprotectant solution. Lesion sites were localized and represented using light microscopy and camera lucida drawings. Animals with cannula misplacement were not included in the statistical analysis.

Immunolabelling was performed on free-floating sections. For cFos peroxidative immunostaining, tissue peroxidases were eliminated and the brain tissue was incubated for 48 h with a polyclonal primary antibody, rabbit anti-cFos (1:1000; Synaptic Systems, Goettingen, Germany) and then, for 120 min with an affinity purified secondary biotinylated antibody, goat anti-rabbit (1:400; Jackson InmunoResearch Laboratories, Inc., West Grove, PA, USA). For magnification, we used preassembled biotin–avidin peroxidase complex according to the Vector Labs recommendations (ABC Elite; Vector Laboratories, Bulingame, Ca, USA). Sections were exposed to DAB solution with nickel. Then the tissue was rinsed and mounted in Eukitt (Sigma-Aldrich, Madrid, Spain).

For the double fluorescence immunolabelling of PNNs and cFos, brain tissue was incubated with lectin from Wisteria Floribunda Agglutinin (WFA) (1:200; Sigma-Aldrich, Madrid, Spain) and the polyclonal primary antibody, rabbit anti-cFos (1:1000; Synaptic Systems, Goettingen, Germany) at 4 °C for 48 hours in PBS 0.1M triton X-100. In a second step, brain samples were exposed to FICT-Streptavidin (1:50; Jackson InmunoResearch

Laboratories, Inc., West Grove, PA, USA) and goat anti-rabbit Cy5 (1:200, Synaptic systems, Goettingen, Germany).

The anterograde tracer BDA was revealed using Cy3-conjugated Streptavidin (1:300; Jackson InmunoResearch Laboratories, Inc., West Grove, PA, USA). Different transporters were marked at the same time as the tracers. In a first step, brain tissue was incubated with rabbit anti-Calbindin 28k (1:1500; Swant, Marly, Switzerland) and guinea pig Anti-VGAT cytoplasmic (1:100; Synaptic Systems, Goettingen, Germany), and then exposed to donkey anti-rabbit Alexa 488 (1:500; Thermo Fisher Scientific, Geel, Belgium) and goat anti-guinea pig Alexa 647 (1:500; Thermo Fisher Scientific, Geel, Belgium). Different sections were incubated with rabbit anti-Tyrosine Hydroxylase (1:500; Millipore Merck KGaA, Darmstadt, Germany) and mouse anti-Synapsin1 (1:500; Synaptic Systems, Goettingen, Germany), and then exposed to goat anti-rabbit FITC (1:200; Vector Laboratories, Bulingame, Ca, USA) and donkey anti-mouse Alexa 647 (1:500; Jackson InmunoResearch Laboratories, Inc., West Grove, PA, USA). In the last fluorescence immunostaining, tissue was incubated with rabbit anti-Tyrosine Hydroxylase (1:500; Millipore Merck KGaA, Darmstadt, Germany) and guinea pig anti-vGluT2 (1:500; Synaptic Systems, Goettingen, Germany), and then exposed to goat anti-rabbit FITC (1:200; Vector Laboratories, Bulingame, Ca, USA) and goat anti-guinea pig Alexa 647 (1:500; Thermo Fisher Scientific, Geel, Belgium). All the sections were mounted with Mowiol.

Image acquisition and analysis

Images of immunoperoxidase cFos expression were acquired using an optic microscope (Nikon E-800, Izasa Werfen Group, Valencia, Spain) with 20x lenses and a resolution of 1,360 x 1,024 dpi. Three photos were taken by structure (IL, PL, dorsomedial striatum (DMS), dorsolateral striatum (DLS), ventrolateral striatum (VLS), nucleus accumbens core (NAcC) and shell (NAcSh)), and hemisphere in coronal sections. We included bregma coordinates between 3.20 mm to 2.20 mm for PL and IL, and for the striatum and NAc between 1.60 mm to 0.70 mm.

Fluorescence images of cFos and PNNs were captured in a confocal microscope (Leica DMi8, Leica Microsystems CMS GmbH, Wetzlar, Germany) with 20× lenses and

resolution 1,024 × 1,024 dpi. Laser intensity (1%), gain (600) and offset (-4) were maintained constant in each acquisition. Each image was formed by a stack of ten images. Image stacks were pre-processed applying a maximal projection process with Leica Application Suite LAS X (Leica Microsystems CMS GmbH, Wetzlar, Germany). Three image stacks in coronal sections for brain regions and in sagittal sections for deep cerebellar nuclei were acquired by structure and hemisphere.

FIJI free software (Schindelin *et al*, 2012) was used for all quantifications. The cFos expression was evaluated using the cell-counter plugin of FIJI software. Additionally, PNN expression was estimated using a densitometry assessment of WFA intensity (brightness range 0-255) in all the PNNs that were found in three sections of each ROI (Carbo-Gas *et al*, 2017; Vazquez-Sanroman *et al*, 2015a, 2015b). Unmodified images were used for all the analyses.

Fluorescent images of the infusion locations, tracer diffusion and transporters were acquired as eight confocal stacked images using the tile-scan tool in order to obtain complete coronal sections in which ROIs were presented.

Experimental design and statistics

All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). One-way ANOVAs and Student t-tests for independent samples were carried out to analyse preference scores and immunohistochemistry data. Data were presented as mean ±SEM. Post hoc comparisons were performed using Tukey's HSD tests. The statistical level of significance was set at $P < 0.05$.

RESULTS

In chapter 1 (Gil-Miravet *et al*, 2018), we showed that an excitotoxic lesion in the apical region (dorsal) of lobule VIII before conditioning facilitated cocaine-induced conditioned preference. Indeed, the whole sample of lesioned animals developed a preference for the cocaine-related cue, as compared to only one third of the sham group. In the present study, we confirmed the results with the smaller sample of rats (N=18) in which immunohistochemistry studies were conducted (the sham group (n = 6), the lesioned

group (QA) (n = 6), and the pseudo-conditioned group (Unp) (n = 6)). As expected, the cerebellar lesion promoted the acquisition of cocaine-induced conditioned preference (F (2,15) = 5.337, P = 0.0178). Post hoc analysis revealed that the QA group showed significant higher preference that the sham (P = 0.0463) and Unp (P = 0.0235) groups. No difference was found between the sham and Unp groups (P = 0.9350). Remarkably, the neurotoxic lesion was ineffective in affecting the behaviour of the Unp group, indicating that any effect of the lesion was learning-related (Fig. 2).

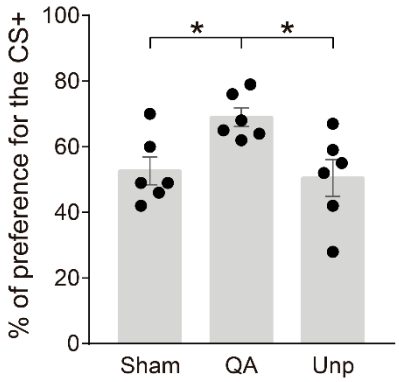

Figure 2. Effect of AQ lesion before each training session on cocaine-induced conditioned preference. *The QA lesion increased the proportion of rats that expressed cocaine-induced conditioned preference. Scatterplots of the preference scores for the CS+ on the test day in the sham (n = 6), QA lesion (QA) (n = 6) and unpaired (Unp) (n = 6) groups. Data are shown as the mean ± SEM and individual preference scores (*P < 0.05).*

A lesion of lobule VIII in the vermis increases neural activity and PNN expression in the medial prefrontal cortex

Then, we explored the impact of the cerebellar lesion on neural activity (cFos) and PNN expression (WFA) in the medial prefrontal cortex of these rats. As shown in figure 3, the cerebellar lesion increased cFos expression in both the PL (F (2,15) = 13.4, P = 0.0005) and the IL (F (2,15) = 12.23, P = 0.0007) subdivisions of the mPFC. In the IL cortex, Tukey tests showed higher number of cFos+ neurons in the lesion group as compared to the sham (P = 0.004) and unpaired (P = 0.0009) groups. The number of cFos+ neurons also increased significantly in the PL region of the lesioned rats as compared to the sham (P = 0.0018) and unpaired (P = 0.0008) groups.

Moreover, the lesion in lobule VIII enhanced WFA intensity in PNNs around GABAergic interneurons either in the PL (F (2,15) = 7.262, P = 0.0062) or IL (F (2,15) = 18.03, P = 0.0001) cortex. Post hoc tests indicated that prefrontal PNNs were stronger after the

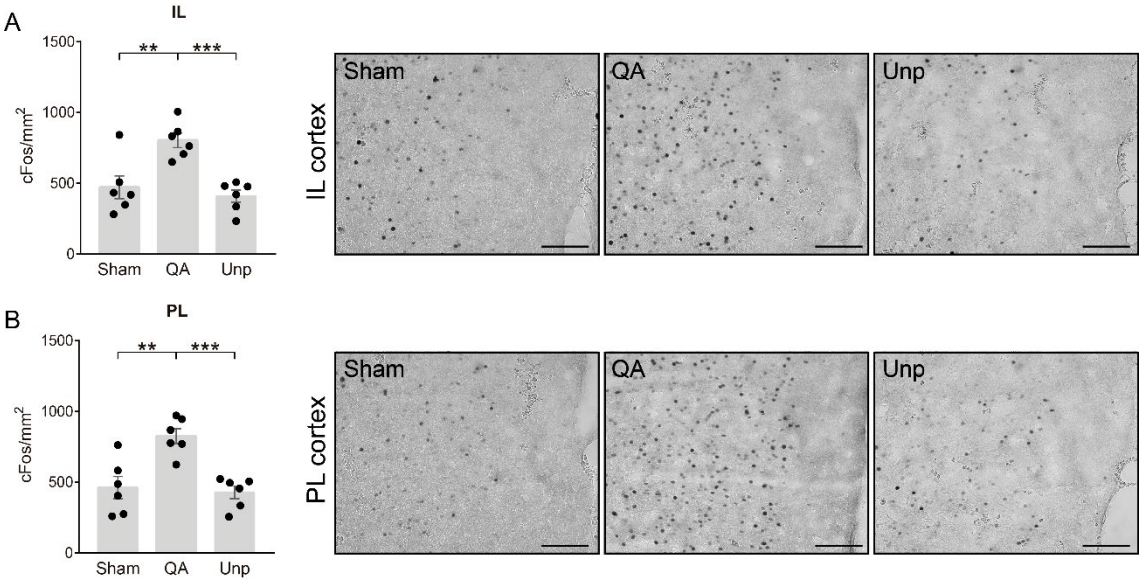

Figure 3. Effect of an excitotoxic lesion in lobule VIII of the vermis on cFos activity in the mPFC*. The cerebellar lesion was made before cocaine-induced conditioning training. cFos expression in PL and IL cortices for Sham (n = 6), QA (n = 6) and Unp (n = 6) groups. (A) the PL and (B) IL cortices for Sham (n = 6), QA (n = 6) and Unp (n = 6) groups. The cerebellar lesion increased cFos expression in both subdivisions of the mPFC. The lesion was ineffective in producing both effects when cocaine was randomly associated with the odour cues (Unp). Data are shown as the mean ± SEM and individual scores (**P < 0.01; ***P < 0.001). All images on the right panels were taken at 20x magnification. Scale bar 100 µm.*

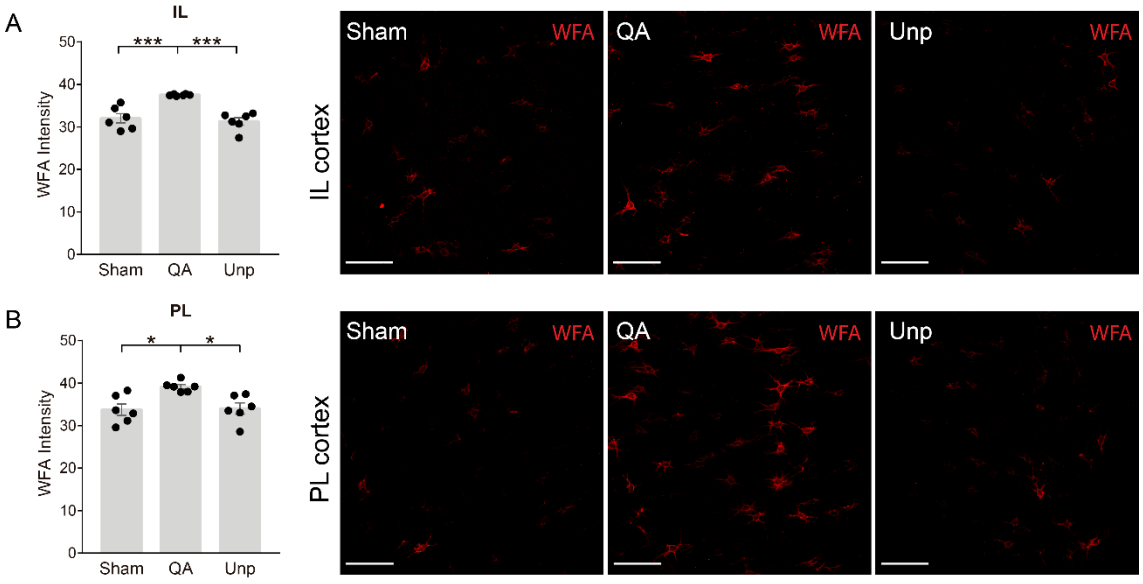

Figure 4. Effect of an excitotoxic lesion in lobule VIII of the vermis on PNNs expression in the mPFC. *WFA intensity in PNNs in the PL and IL cortices for the Sham, QA and Unp groups (n = 6). The cerebellar lesion increased WFA intensity in both subdivisions of the mPFC. The lesion was ineffective in producing both effects when cocaine was randomly associated with the odor cues. Data are shown as the mean ± SEM and individual scores (*P < 0.05; ***P < 0.001).* Right***,*** *representative microphotographs of PNNs in the IL and PL cortex stained with Wisteria Floribunda Agglutinin (WFA) (red). The confocal images were acquired at 20x. Scale bar of 100 µm.*

cerebellar lesion as compared to the sham (PL (P = 0.0108); IL (P = 0.0006)) and Unp (PL (P = 0.0151); IL (P = 0.0002)) groups (Fig. 4).

Neural activity in the NAc and striatum increased after the lesion of lobule VIII

Additionally, we wonder whether cerebellar impairment during the acquisition of cocaine-induced conditioned memory might affect striatal activity. Student's t-test for independent samples showed that the cerebellar lesion raised cFos expression in the NAc and the majority of subdivisions of the striatum as compared to the sham group (NAcC (t (10) = 3.479, P = 0.0059); NAcSh (t (10) = 5.062, P = 0.0005); DMS (t (10) = 2.676, P = 0.0233); and DLS (t (10) = 4.761, P = 0.0008)). The only striatal region unaffected by the cerebellar lesion was the VLS (T (10) = 1.526, P = 0.158) (Fig. 5).

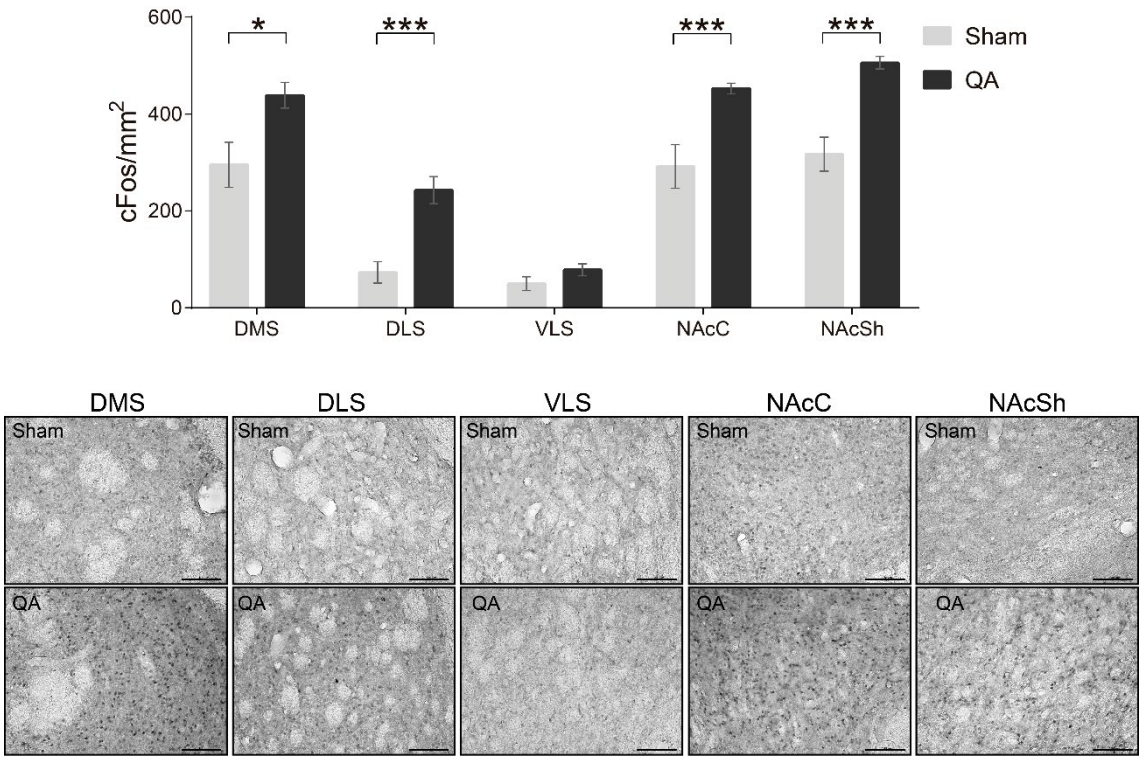

Figure 5. Effect of an excitotoxic lesion in lobule VIII on cFos expression in several striatal regions and the nucleus accumbens*. DMS, DLS, VLS, NAcC and NAcSh in the sham and QA groups (n=6). Data are shown as the mean ± SEM. The cerebellar lesion increased cFos expression in DMS, DLS, NAcC and NAcSh but not in the VLS (*p < 0.05; ***p < 0.001). All images were taken at 20x magnification. Scale bar is equal to 100 µm.*

A lesion in lobule VIII increases neural activity and PNNs expression in the lateral nucleus

Student's t-tests revealed that the lesion of the vermis did not significantly change the cFos expression in the interpositus nucleus, either anterior (IntA) (t (10) = 1.48, P = 0.1697) or posterior (IntP) (T (10) = 2.037, P = 0.069) (Fig. 6). Neither PNNs in the IntA (P = 0.3384) nor in the IntP (P = 0.5832) were affected by the lesion (see also Fig. 7).

However, it is remarkable that the lesion in the posterior vermis increased cFos (t (10) = 2.597, P = 0.0266) in the lateral nucleus (Lat) (Fig. 6). Moreover, WFA intensity was stronger in PNNs of this nucleus after the lesion of the posterior vermis (t (10) = 2.614, P = 0.0259) (Fig. 7).

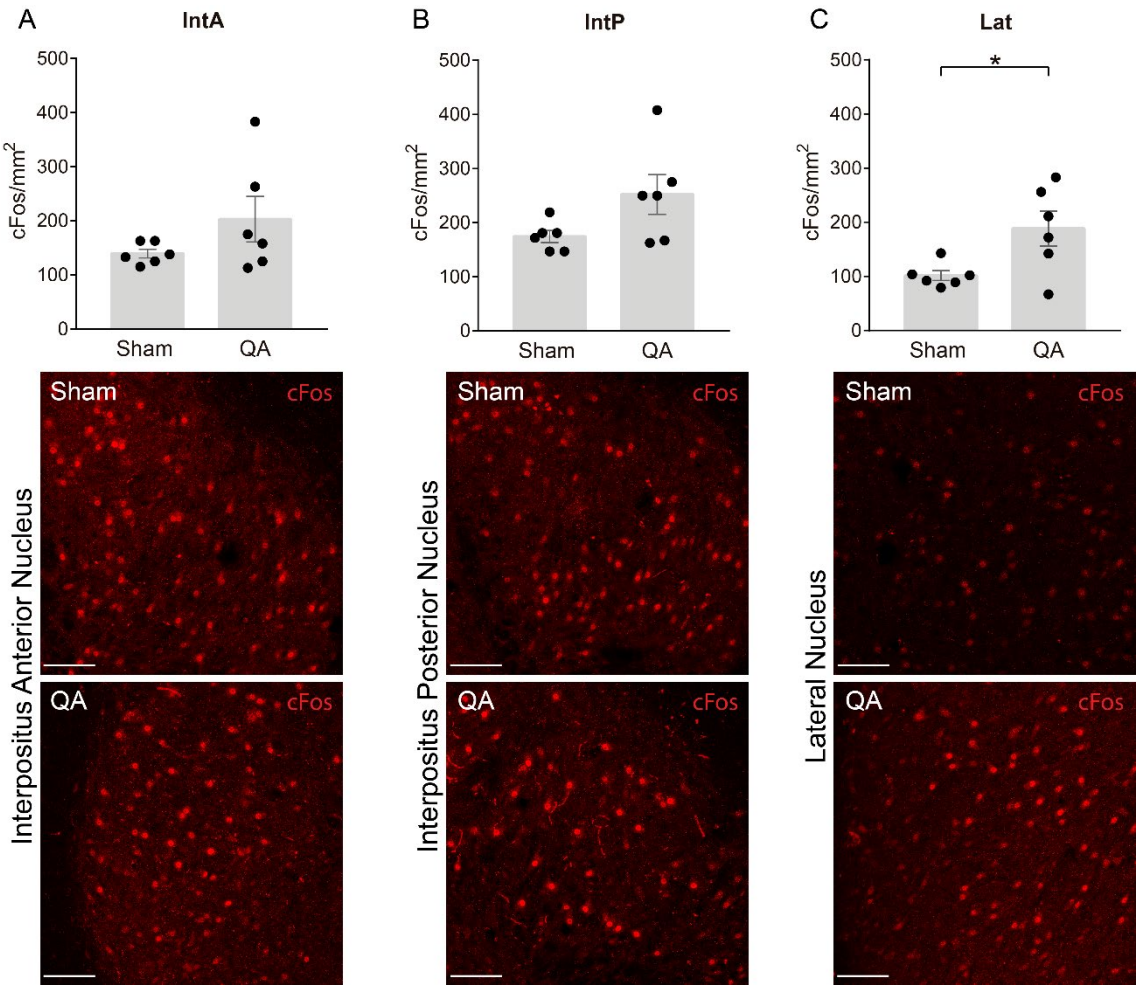

Figure 6. Effect of a lesion of lobule VIII in the vermis on cFos activity in the DCN*. cFos expression rose in the Lat nucleus as an effect of the lesion of the posterior vermis (n=6). Bottom panels depict representative pictures of cFos staining (red) for each group. All images were taken at 20x magnification. Scale bar 100 µm. Data are shown as the mean ± SEM. Scatterplots were overlapped for each group (*P < 0.05).*

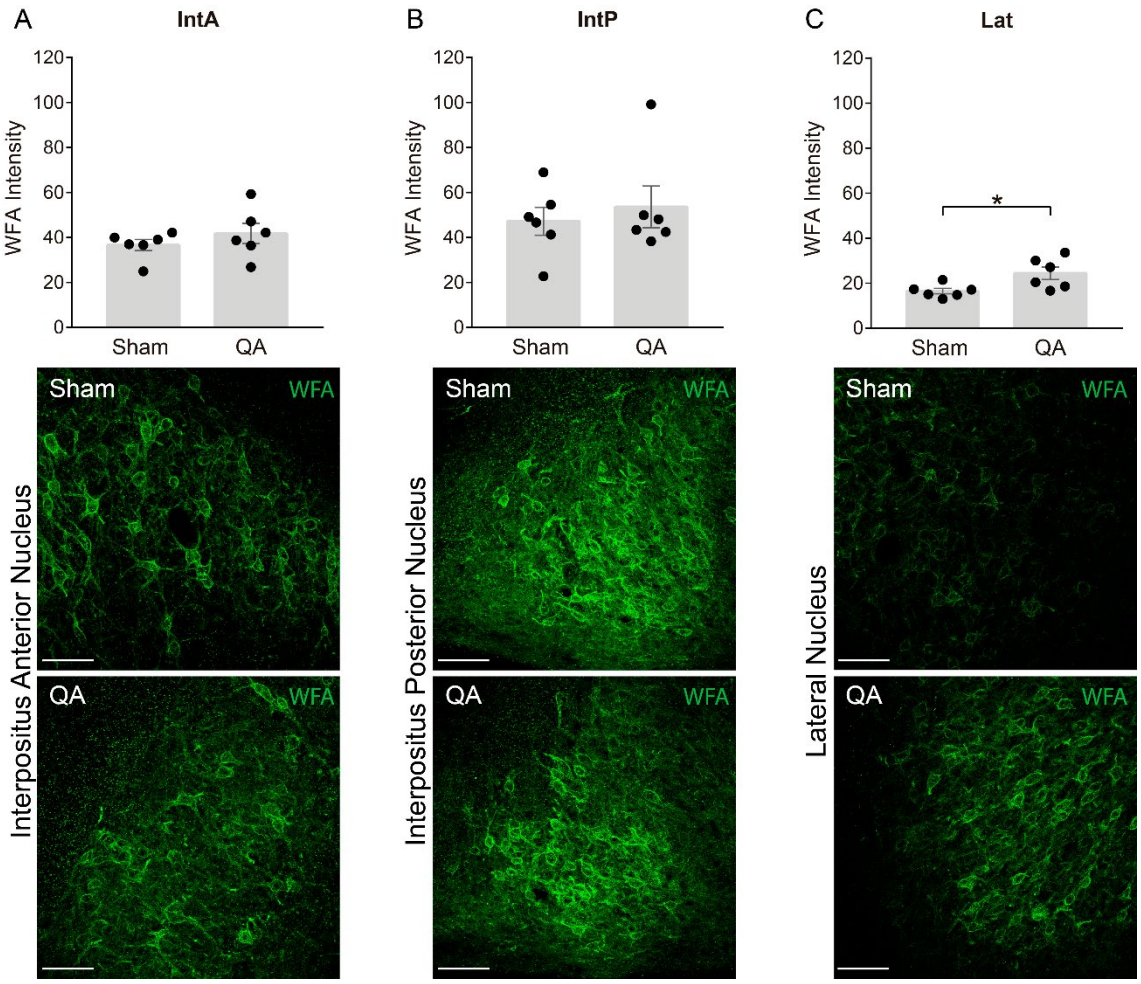

Figure 7. Effect of a lesion of lobule VIII in the vermis on WFA intensity in the lateral nucleus. *PNN expression increased in the Lat nucleus as an effect of the lesion in the posterior vermis (n=6). Bottom: representative microphotographs of PNNs stained with Wisteria Floribunda Agglutinin (WFA) (green). The confocal images were acquired at 20x. Scale bar 100 µm. Data are shown as the mean ± SEM. Scatterplots were overlapped for each group (*P < 0.05).*

A working neuroanatomical model for the cerebellar regulation of cocaine-induced conditioned preference

To propose a working neuroanatomical model to explain the effects of the lesion in the posterior vermis, we carried out a tracing study using anterograde (BDA/red) and retrograde (FG/blue) tracers. BDA was infused into the apical region of lobule VIII in the vermis (the same location as the excitotoxic lesion) and the lateral nucleus. In turn, we infused FG into the VTA and IL cortex. We searched for colocalization between BDA and FG within the VTA and lateral nucleus (n=3) (Figs 8-9).

Despite the fact that the infusion point was restricted to lobule VIII in the vermis (Fig. 8), BDA-labelled projections were found throughout the whole vermis, but also the hemispheres reaching Crus I. However, the molecular layer was devoid of BDA labelling.

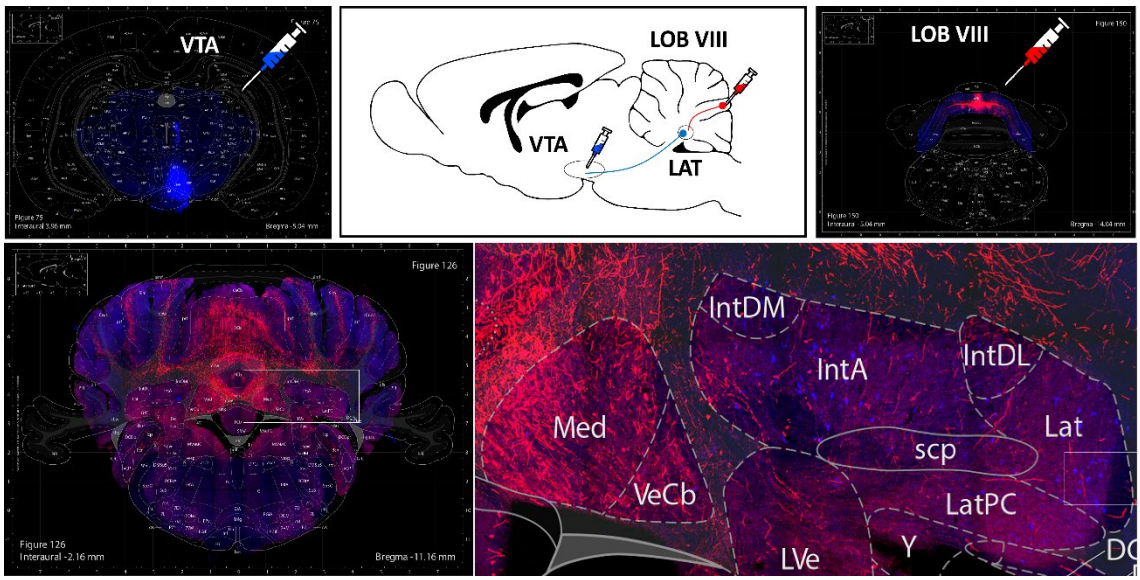

Figure 8. Infusions of FG (blue) in the VTA and BDA (red) in the dorsal region of lobule VIII in the vermis. *Upper left, the infusion point for FG in the VTA. Upper right, the infusion point for BDA in lobule VIII of the vermis. BDA diffusion was found not only in the vermis but also throughout the hemispheres. Colocalization was only found within the IntA and Lat nuclei.*

BDA is an anterograde tracer that identifies only a neural projection from their source to their point of termination. Hence, one plausible explanation for these results is that Purkinje-to-Purkinje collaterals would have spread the tracer laterally from the middle line along the cerebellar cortex. Purkinje collateral branches originate within the parasagittal plane and their maximum length can reach 2 mm towards the apex and the base of the lobule in adult rodents (Witter *et al*, 2016). Importantly, these findings suggest that lobule VIII is interconnected with the whole cerebellum. As expected, we

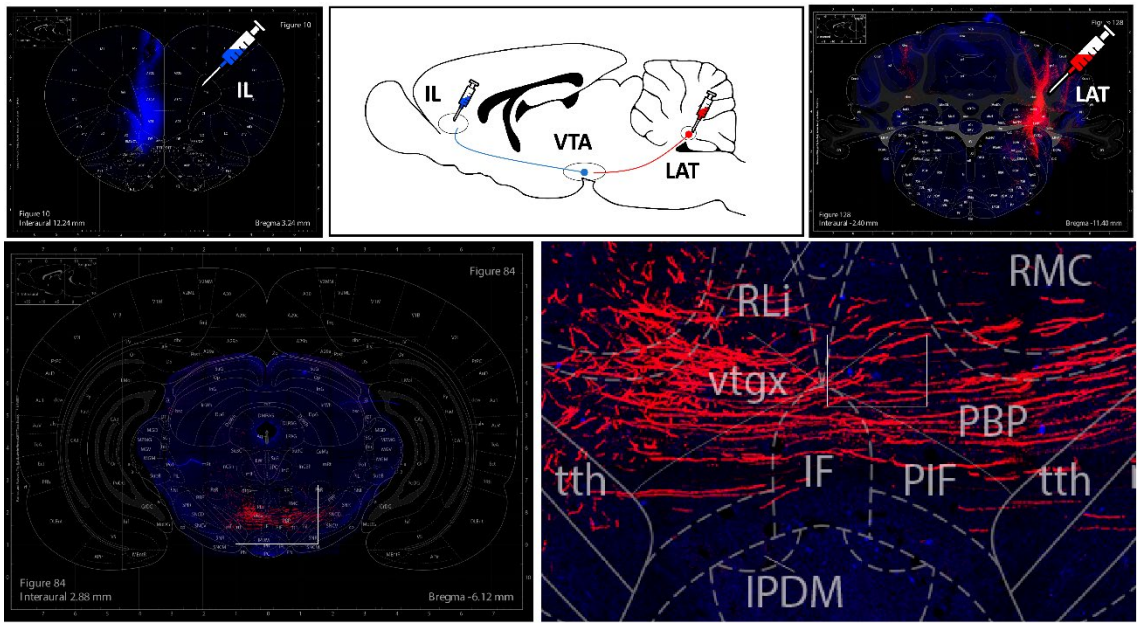

Figure 9. Infusions of FG (blue) in the IL cortex and BDA (red) in the lateral nucleus. *Colocalization was observed within the contralateral parabrachial pigmented nucleus of the VTA (PBP).*

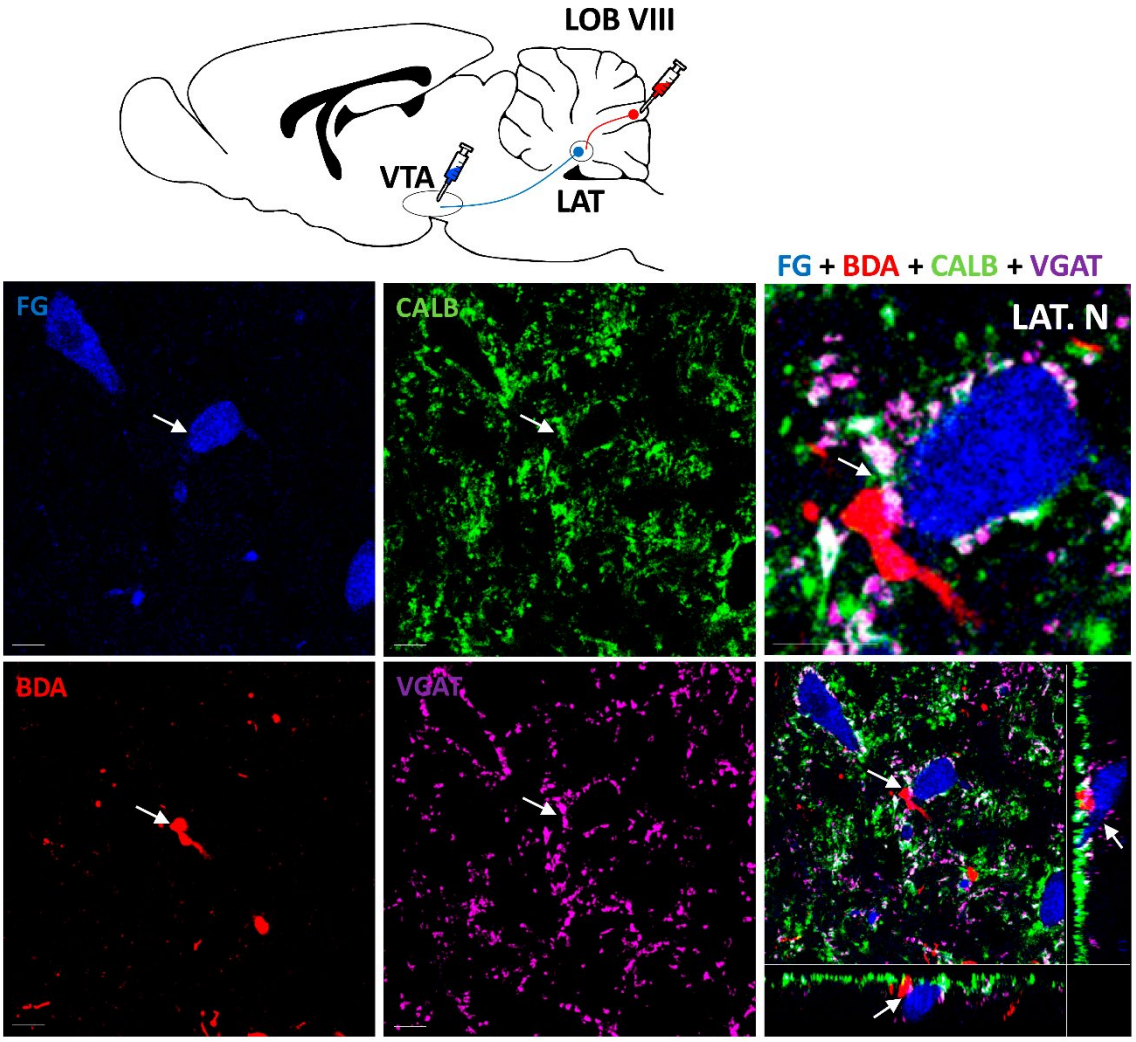

Figure 10. A coronal section of the lateral nucleus. *Infusion of BDA (red) into the apical region of the vermis and FG (blue) in the VTA. The arrows indicate an example of a synaptic contact between a Purkinje terminal from lobule VIII of the vermis (BDA+) and one output neuron in the Lat (FG+). Purkinje puncta was identified by calbindin (CALB/green) and the GABA vesicular transporter (vGAT/purple). All confocal images were taken at 40x with 2.5x zoom. Scale bar 10 µm. Right top panel: Digital amplification of 300x.*

observed a great number of BDA-labelled terminals within the medial nucleus, but also in the interpositus (Int) and lateral (Lat) nuclei (Fig. 8).

When FG was infused into the VTA, FG-labelled somas were found within the contralateral IntA, IntP, and Lat nuclei, but not in the Med nucleus (Fig. 8). This cerebellum-VTA projection was confirmed by infusing BDA into the Lat nucleus (Fig. 9). Unilateral BDA infusions in the Lat nucleus reached ipsilateral cerebellar hemisphere, lobule VIII and IX of the vermis, and contralateral parabrachial pigmented nucleus of the VTA (PBP), the most caudal part of the VTA (Fig. 9). We also observed FG+ somas in mPFC and NAcSh, among other structures, replicating the results observed in different studies (Geisler and Zahm, 2005; Kasanetz *et al*, 2008). A magnification image within the Lat

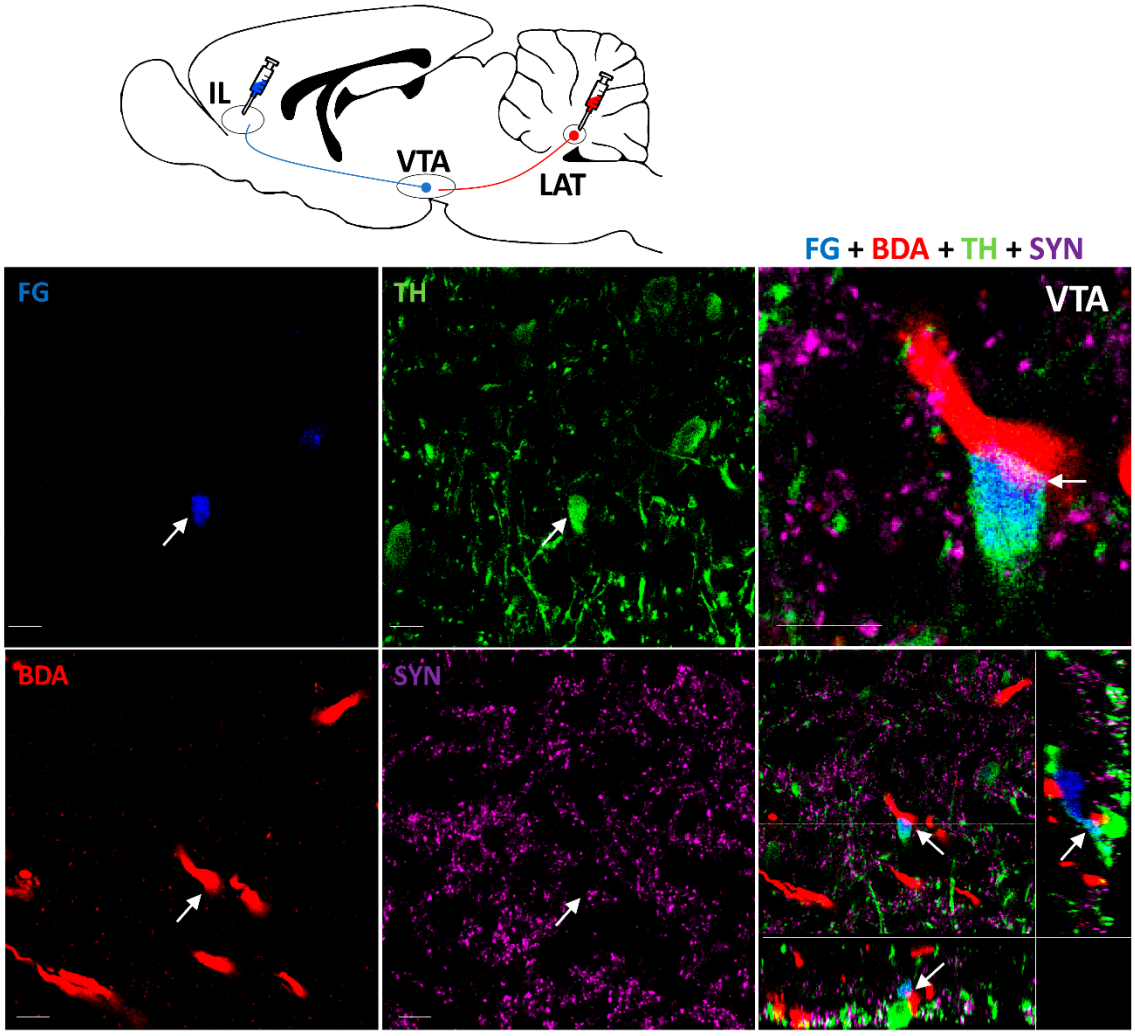

Figure 11. Coronal section of the VTA. *Infusions of BDA (red) in the lateral nucleus and FG (blue) in the IL cortex. The arrows indicate an example of a synaptic contact between the terminal of a projection neuron from Lat nucleus (BDA+) and one TH+ neuron in the VTA (FG+). Dopamine neuron was identified as expressing tyrosine hydroxylase (TH/green). Synapsin is a presynaptic protein (SYN/purple). All the pictures are taken a 40x and 2.5x zoom in a confocal. Scale bar of 10 µm. Digital co-labelling amplification of 300x.*

nucleus showed an example of a synaptic contact between a Purkinje BDA+ terminal from lobule VIII and FG+ cerebellar-VTA projection (Fig 10).

Additionally, FG was infused in the IL cortex and BDA in the Lat (n=2). We observed FG and BDA co-labelling within the PBP (Fig. 9), supporting the mentioned observations and pointing to the caudal VTA as an interface through which the cerebellum would regulate prefrontal activity and striatal function. Projections from the lateral nucleus seemed to be glutamatergic and contacted both TH+ and TH- cells (Fig. 11, 12, 13).

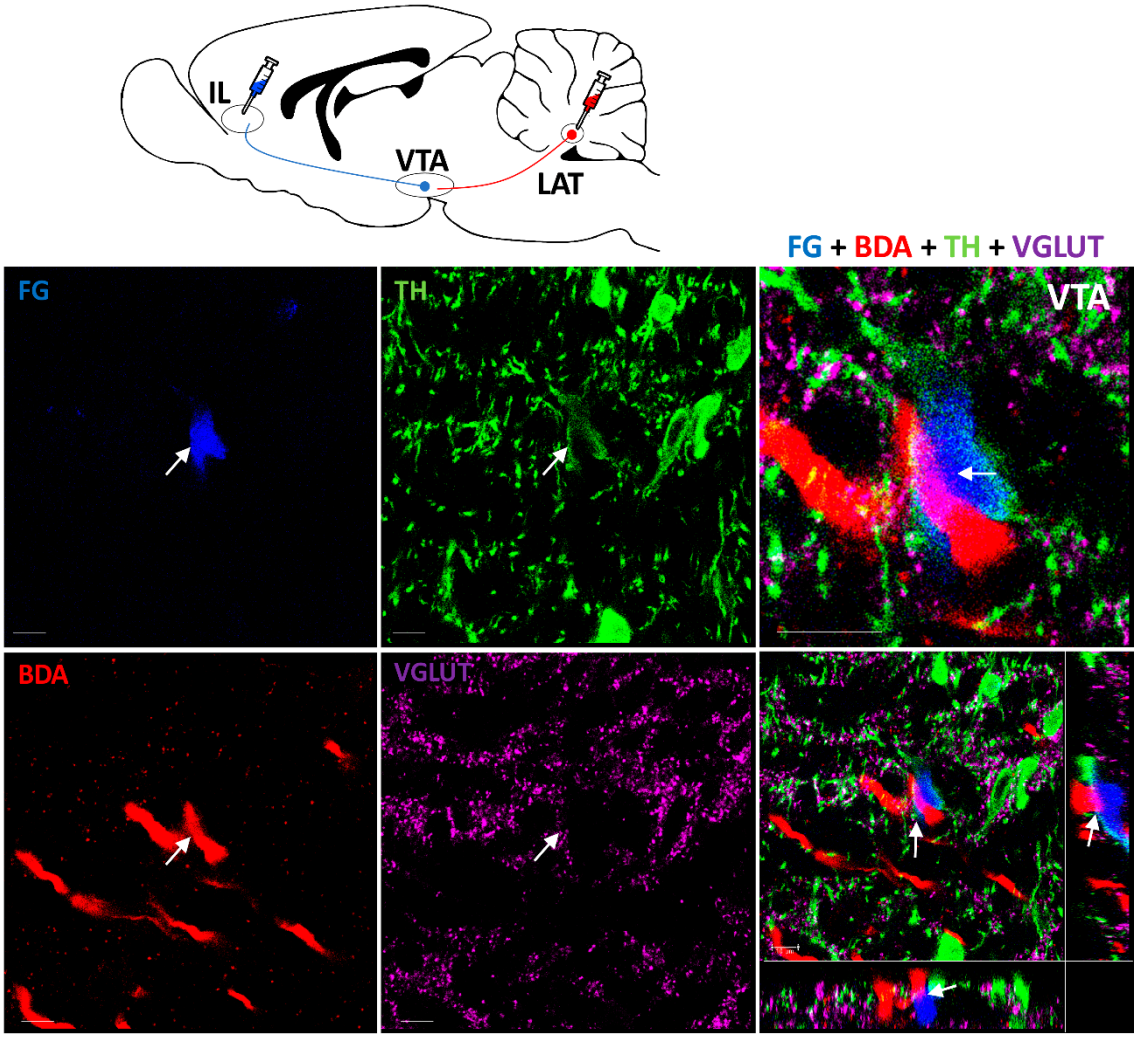

Figure 12. Coronal section of the VTA*. Infusions of BDA (red) in the lateral nucleus and FG (blue) in the IL cortex. The arrows indicate an example of a synaptic contact between the terminal of a projection neuron from Lat nucleus (BDA+) and one TH+ neuron in the VTA (FG+). The dopamine neuron was identified as expressing tyrosine hydroxylase (TH/green). Vesicular glutamate transporter (vGluT2/purple). All confocal images were taken at 40x with 2.5x zoom. Scale bar of 10 µm. Right top panel: Digital amplification of 300x.*

DISCUSSION

The present research is aimed at providing an explanatory model for the facilitative effect of a lesion in the apical region of lobule VIII on cocaine-induced conditioned memory. First, our findings showed that damage in this region of the vermis induced a general disinhibition in the mPFC and striatal subdivisions that receive dopaminergic projections mainly from the VTA. Second, they also showed that impairment of the posterior vermis induced an upregulation of PNN expression in the mPFC. Finally, we found a direct projection from the lateral nucleus to the VTA that also receives Purkinje

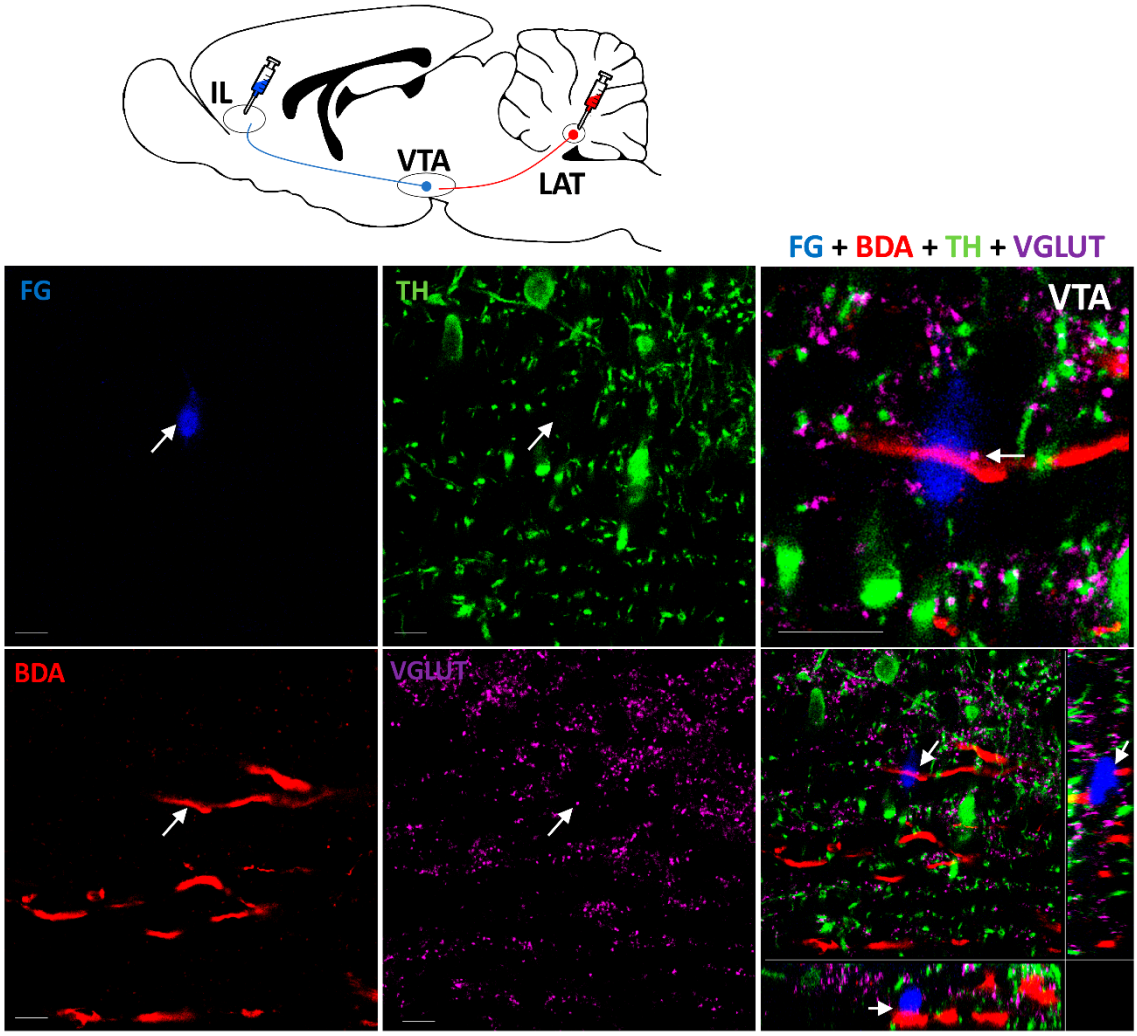

Figure 13. Coronal section of the VTA. *Infusions of BDA (red) in the lateral nucleus and FG (blue) in the IL cortex. The arrows indicate an example of a synaptic contact between the terminal of a projection neuron from Lat nucleus (BDA+) and one TH+ neuron in the VTA (FG+). Dopamine neuron was identified as expressing tyrosine hydroxylase (TH/green). Vesicular glutamate transporter (vGluT2/purple). All the pictures are taken a 40x and 2.5x zoom in a confocal. Scale bar of 10 µm. Digital co-labelling amplification of 300x.*

axons from lobule VIII in the vermis. Hypothetically, this pathway might control activity and plasticity of the cortico-striatal circuitry through an increase in dopaminergic activity. The working hypothetical model that emerged from the present findings predicts that the impairment of the posterior cerebellar cortex, by increasing activity in the lateral nucleus would heighten glutamate release within the VTA and facilitate the release of DA in the mPFC and striatal regions.

The idea of a cerebellar modulation of the VTA is not new. It has been grounded in a few previous findings which described indirect cerebellar-VTA pathways (Forster and Blaha, 2003; Rogers *et al*, 2011), but also a direct control of the cerebellum onto the VTA (Carta

et al, 2019; Watabe-Uchida *et al*, 2012). Indeed, the cerebellum could reach the VTA through the reticulotegmental and pedunculopontine nuclei (Forster and Blaha, 2003) and the mediodorsal and ventrolateral thalamus (Rogers *et al*, 2011). More importantly, there is a direct cerebellar-VTA pathway (Watabe-Uchida *et al*, 2012), whose functional properties have been recently delineated in an elegant study (Carta *et al*, 2019). Supporting the present working model, the optogenetic stimulation of the cerebellar-VTA pathway increased firing in one third of VTA neurons in vivo, elicited excitatory synaptic currents, and induced strong place preference for the location in which mice received the optogenetic stimulation of the cerebellar projection (Carta *et al*, 2019). Moreover, the optogenetic stimulation of the cerebellar axons was as rewarding as the direct optogenetic activation of dopaminergic neurons in the VTA (Carta *et al*, 2019).

The fact that a small lesion in the posterior vermis was able to induce an upregulation of PNN expression in the mPFC was remarkable. PNNs have been proposed as one of the mechanisms for the stabilization of drug-induced memories (Sorg *et al*, 2016). Interestingly, previous findings demonstrated that drug-induced conditioned preference and drug self-administration increase PNN expression in the cerebellum (Carbo-Gas *et al*, 2017) and different prefrontal areas (Blacktop *et al*, 2017; Vazquez-Sanroman *et al*, 2017). Furthermore, PNN digestion within the prelimbic cortex and the anterior lateral hypothalamus prevented the expression of cocaine-induced conditioned place preference (Slaker *et al*, 2015) and cocaine self-administration (Blacktop *et al*, 2017). The present data suggest that the cerebellar lesion by increasing synaptic stabilization mechanisms in the mPFC could facilitate the acquisition of cocaine-cue associations and strengthen drug-induced memories.

In our opinion, the major finding of the present research is to provide an explanatory model for the function of the posterior cerebellar vermis on drug-related memory. In this model (Figure 14), damage of the posterior vermis would release striatum-cortical networks from the inhibitory tonic control exerted by the cerebellum over the VTA, thereby promoting drug effects. The present findings may help to explain why patients with lesions or diseases affecting the posterior cerebellum presented difficulties in controlling their behaviour and emotions (Kim *et al*, 2013; Schmahmann and Sherman, 1998; Silveri *et al*, 1994; Tessier *et al*, 2015). Moreover, our model predicts that the

stimulation of the posterior cerebellar vermis would reduce drug-related effects and improve behavioural inhibition.

In summary, the present results indicate that: (1) the posterior cerebellar cortex may exert an inhibitory control over the striatum and mPFC; (2) the lateral nucleus seems to be the most plausible exit route for the cerebellar cortex to modulate activity and plasticity in the prefrontal-striatal network; and (3) the VTA could be a candidate to mediate cerebellar influences on activity and plasticity in prefrontal-striatal loops that in turn can regulate cocaine-related behaviour.

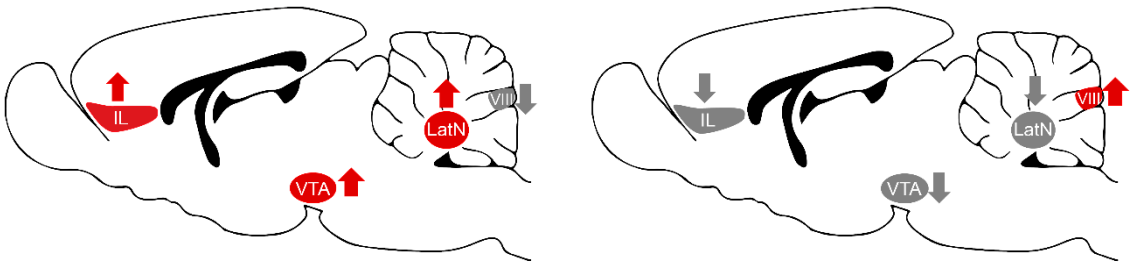

Figure 14. Working neuroanatomical model for the function of lobule VIII on drug-related memory. *Left panel: The lesion of lobule VIII by increasing activity in the lateral nucleus can heighten glutamate release within the VTA and facilitate the release of DA in the mPFC and striatal regions. The lesion would release striatum-cortical networks from the inhibitory tonic control exerted by the cerebellum over the VTA, thereby promoting drug effects. Right panel: The model predicts that the stimulation of the posterior cerebellar vermis would reduce drug-related effects and improve behavioural inhibition.*

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AUTHOR DISCLOSURE

All authors declare no conflicts of interest.

AUTHORSHIP

All authors made a notable 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 and Francisco Olucha-Bordonau performed the tracing study; Isis Gil-Miravet, Edgar Arias de Saavedra-Sandoval, and Lizbeth Vásquez-Celaya were involved in image and data analysis. Finally, Marta Miquel designed the study, supervised the surgeries and behavioural experiments, was involved in data analysis, and drafted the manuscript. All authors approved the present version of the manuscript.

DATA ACCESSIBILITY

Raw data are available from the corresponding author upon request.

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GENERAL DISCUSSION

GENERAL DISCUSSION

The present research focused on providing an explanatory model of the cerebellum's role in cocaine-induced conditioned memory. As we have already said, addiction is a neuroplasticity disorder that involves mechanisms similar to those proposed for learning and memory (Hyman *et al*, 2006; Miles *et al*, 2003; Milton and Everitt, 2012). It is well established that the mPFC is involved in reward learning and addiction (Tzschentke and Schmidt, 2000). Several studies have observed that excitotoxic lesions of mPFC enhance cocaine-seeking and facilitate cocaine self-administration by increasing sensitivity to effects of cocaine reinforcement (Schenk *et al*, 1991; Weissenborn *et al*, 1997), which may be due to a behavioural disinhibition after mPFC impairment. On the contrary, the implication of the cerebellum in addiction is very recent. A large number of studies have demonstrated the involvement of the cerebellum in memory, decision-making, emotional processing, and executive functions (Sacchetti *et al*, 2002b; Schmahmann and Pandya, 1997; Turner *et al*, 2007; Watson *et al*, 2014; Zhu *et al*, 2011), all of them affected in addicted subjects. Moreover, reciprocal loops between the cerebellum and the prefrontal-striatal-limbic circuity have been clearly demonstrated (Bostan *et al*, 2018; Buckner *et al*, 2011; Chen *et al*, 2014; Hoshi *et al*, 2005; Ichinohe *et al*, 2000; Ikai *et al*, 1992; Middleton and Strick, 2000, 2001; Panagopoulos *et al*, 1991; Sang *et al*, 2012; Stanton, 1980; Xiao *et al*, 2018). Taken together, the background information underpin the hypothesis of a significant cerebellar role in learning, memory, and addiction.

The present thesis proposes a functional relationship between the IL and the dorsal area of lobe VIII. Impairment of either of these regions increases the percentage of animals that developed a preference for the cues associated with cocaine. In both cases, the deactivation affected the number of animals expressing cocaine-induced conditioned preference, but not the magnitude of the conditioned response that was very similar to the sham individuals expressing conditioned preference. Behavioural similarities were also found when we deactivated the PL cortex or the ventral region of lobule VIII. However, the functional relationship between these areas could not be explored in depth and thereby we have not been able to establish any hypothesis about their functions and relationships, beyond the behavioural effects described in the first chapter.

To propose a hypothetical working model that can explain the effects of brain and cerebellar deactivations, we then explored changes in neuronal activity and plasticity as an effect of deactivations. The results of mPFC deactivations were consistent with those already published in the previous literature. As previous studies have shown, IL and PL have opposite functions in drug-seeking (Ball and Slane, 2012; Capriles *et al*, 2003; McFarland and Kalivas, 2001; Ongür and Price, 2000; Peters *et al*, 2009; Pfarr *et al*, 2015; Sierra-Mercado *et al*, 2011). In the present thesis, we observed that the inactivation of the IL cortex, in addition to promoting cocaine-induced memory, also increased activity in the posterior cerebellum and NAcSh. Similar results were observed with respect to the intensity of PNNs, which were more intense and stronger in lobule VIII and IX from all animals expressing preference, regardless of the IL deactivation. These findings suggest that the cerebellar activity and plasticity modifications are not directly related to the IL impairment but the development of preference. Thus, the increase in cerebellar activity would be linked to the reactivation of previous acquired drug-cue association in order to select between CS+ and CS-. In fact, when the behavioural selection was prevented as occurs when the animal is confined in the CS+ compartment, cerebellar activation was not seen (Carbo-Gas *et al*, 2017). Moreover, stronger PNNs around Golgi interneurons in the posterior vermis might promote a stabilization of the new connections representing cocaine-related memories. Contrary to what was observed after the IL deactivation, PL impairment during conditioning induced a similar expression of cFos and PNNs to that of the groups that did not show preference. Importantly, mPFC deactivations did not have effects when rats were trained under a random association between the cue and drug. Consequently, these animals did not develop cocaine-induced conditioned preference, and their cFos and PNN expression did not differ from the no preference groups. These data argue in favour of memory-related effects on cerebellar activity and plasticity after mPFC deactivations.

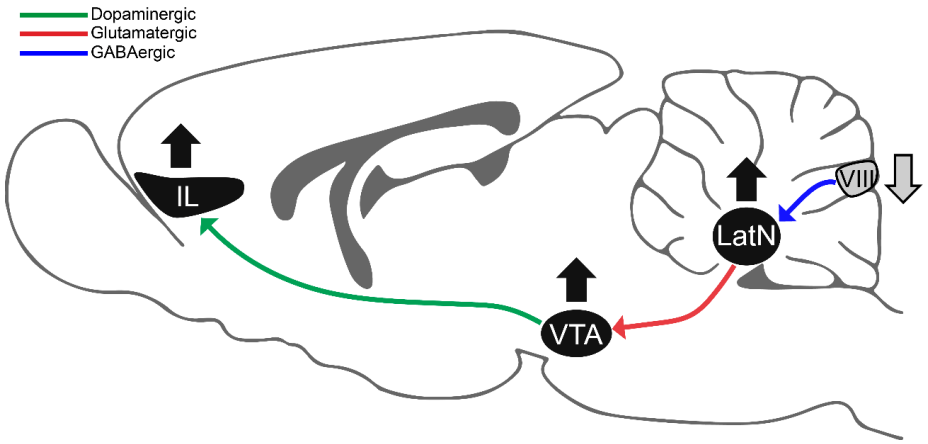
Worthy of mention, the expression of cocaine-induced preference was associated with stronger PNNs around Golgi cells in the dorsal cerebellar cortex, supporting previous studies in mice (Carbo-Gas *et al*, 2017). Golgi cells are inhibitory interneurons (Eccles *et al*, 1964) that crucially control the temporal dynamics and the spatial distribution of information through the cerebellar cortex (D'Angelo, 2009). Golgi cells regulate the

synchronization of granule cell activity, as well as spike timing and burst transmission, determining the sign, intensity, and duration of long-term synaptic plasticity at the mossy fibre-granule cell relay (D'Angelo, 2009; Eccles *et al*, 1964). Palay and Chan-Palay (1974) showed that Golgi cells receive glutamatergic inputs from granule cells and mossy fibres within the cerebellar glomerulus (Palay and Chan-Palay, 1974). Conversely, Golgi cells receive GABAergic and glycinergic inhibitory signals from stellate, basket, and Lugaro cells (Sotelo and Llinás, 1972). Thus, Golgi cells are essential in the control of synaptic plasticity at the level of the granular layer. Here, we described that cerebellar-dependent mechanisms of cocaine associative memory are involved in the upregulation of PNNs around Golgi cells. The majority of Golgi neurons that express stronger PNNs are active (Carbo-Gas *et al*, 2017). It has been shown that blocking inhibition of Golgi cells turns the balance in favour of long-term potentiation in the cerebellar cortex (Mapelli and D'Angelo, 2007). On the contrary, when inhibition is higher than excitation, long-term depression may dominate (D'Angelo and De Zeeuw, 2009). Therefore, one can speculate that the establishment of cocaine memory may encourage the feedforward inhibitory control by Golgi cells.

A neurotoxic lesion of the dorsal cerebellar cortex in lobule VIII generated a global disinhibition in prefrontal and striatal regions, as well as stronger PNNs around GABAergic interneurons of the mPFC. Previous research demonstrated that the degradation of PNNs in the PL cortex blocked conditioned place preference (Slaker *et al*, 2015) and prevented reinstatement to drug-seeking (Slaker *et al*, 2018). Our findings also suggest that the lateral nucleus is the most likely cerebellar output to mediate the present effects. Both activity and PNN expression increased specifically in this DCN after the lesion. No significant lesion effects were seen on the other DCN. Remarkably, as the tracing study demonstrated, the dorsal region of lobule VIII in the vermis sends projections to all of the DCN, including the most lateral portions. However, when infusing the retrograde tracer into the VTA, we only were able to find colocalization in the interpositus and lateral nuclei, but not in the medial nucleus. More importantly, the most caudal region of the contralateral VTA (the parabrachial pigmented nucleus/PBP) receives direct projections from the lateral nucleus that seem to be glutamatergic. Retrograde tracing from the IL cortex indicated that cerebellum-VTA projections make

synaptic contacts with dopaminergic neurons that reach the IL cortex. Altogether, the present findings strongly claim in favour of a key role of the cerebellum in the establishment of drug-cue associations (Miquel *et al*, 2009, 2016) and point to the caudal VTA as the interface through which the cerebellum would regulate the activity and plasticity of the prefrontal-striatal loops. Our results support previous studies that also found direct projections from the DCN to VTA (Carta *et al*, 2019; Watabe-Uchida *et al*, 2012). The role of the VTA and dopaminergic projections to the prefrontal cortex in reward and drug addiction are well established (Chao and Nestler, 2004; Geisler and Zahm, 2005). However, only very recently, it has been shown that optogenetic stimulation of cerebellar projections activate VTA neurons and induce powerful rewarding effects (Carta *et al*, 2019).

The resulting hypothetical neuroanatomical model indicates that the posterior cerebellar cortex may exert a direct inhibitory modulation of the prefrontal-striatal circuitry controlling dopaminergic activity of the VTA neurons. Under impairment of the posterior cerebellar cortex, the lateral nucleus, by increasing glutamatergic activity release within the VTA, encourages activity of dopaminergic neurons within the mPFC and striatal regions.



In our view, this hypothetical model has a high predictive power. Specifically, the cerebellum may be crucial for restraining ongoing actions when environmental conditions change by adjusting prefrontal activity in response to the new external and internal stimuli, thereby promoting flexible behavioural control. Beyond the regulation of cocaine-induced preference conditioning, our model may explain why cerebellar lesions have been shown to generate behavioural disinhibition, impulsivity, and compulsivity (Miquel et al., in press).

STRENGTHS AND PITFALLS

STRENGTHS AND PITFALLS

Strengths

- The main contribution of this thesis is to propose a working neuroanatomical model for the role of the cerebellum in drug addiction with a high predictive power. Moreover, this work represents the first experimental approach to explain the modulatory function of the posterior cerebellum on the prefrontal-striatal circuitry for the establishment of cocaine-associated memories.
- Another significant contribution is the demonstration of a direct cerebellar control over the VTA.
- This thesis also provides support for a functional relationship between the cerebellum and the medial prefrontal cortex in drug-related memory.

Pitfalls, weaknesses, and future directions

- The difficulties to maintain cannulas in the cerebellum forced us to perform permanent injuries in this region. This approach generated a difference between the animals with mPFC deactivations and the animals with lesions in the cerebellum on the test day. In future research, both regions should be studied under the same conditions during the acquisition and expression phases.
- The tracer study shows connection networks between the cerebellum and the prefrontal cortex. It would be interesting to study further the outputs of neurons from the cerebellum, as well as the targeted neurons in the VTA.
- The direct pathway Lateral nucleus-VTA should be blocked and stimulated to confirm the model.
- It would also be interesting to use DREADDs in future research to activate and deactivate all these regions and be able to alter the entire circuit at certain times.
- Finally, beyond the establishment of cocaine-induced Pavlovian memory, it would be interesting to observe how our deactivations affect other paradigms of self-administration, as well as models of extinction and reconsolidation.

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