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INVOLVEMENT OF THE CEREBELLUM IN COCAINE-INDUCED MEMORY

Doctoral Thesis

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La Dra Marta Miquel Salgado-Araujo y la Dra. Carla Sanchis Segura, profesoras titulares de Psicobiología del Departamento de Psicología Básica Clínica y Psicobiología de la Universitat Jaume I, certifican que la tesis Doctoral presentada por Dña Maria Carbó Gas con el título **INVOLVEMENT OF THE CEREBELLUM IN COCAINE-INDUCED MEMORY** ha sido realizada bajo su dirección. Tras haberla examinado hacen constar su autorización para que se realicen los tramites conducentes a su defensa. Y para que conste a los efectos oportunos, firma el presente certificado en Castellón de la Plana a 15 de Mayo del 2017.

Fdo. Dra. Marta Miquel Salgado-Araujo

Fdo. Dra. Carla Sanchis Segura

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TABLE OF CONTENTS

PREFACE

Because of its role in drug-seeking, consumption and addictive behavior, there is a growing interest in identifying the neural circuits and molecular mechanisms underlying the formation, maintenance and retrieval of drug-induced memories. However, very few studies have focused on brain areas beyond the corticostriatal-limbic circuitry. Despite the growing evidence confirming the involvement of the cerebellum in drug-induced alterations, this structure has been traditionally disregarded in the addiction field (Miquel et al, 2009; Miquel et al, 2016).

The general aim of the present research is to address wether the cerebellum is part of the neuronal systems that sustains the plasticity mechanisms underlying drug-induced conditioned memories. We have focused our research in an attempt to clarify if the cerebellum is involved in the acquisition and storage of drug memories. Although previous scattered reports described the involvement of the cerebellum in drug-related memories, this is the first attempt to address a detailed functional analysis about the issue.

The present doctoral thesis contains three different chapters. The first two include two investigations that have been already published (Carbo-gas et al., 2014ab), and the third is one currently under revision. In the first chapter, *Involving the cerebellum in cocaine-induced memory: pattern of cFos expression in mice trained to acquire conditioned preference for cocaine*, we explored the pattern of neuronal activation as revealed by cFos immunoreactivity of mice trained to develop conditioned preference for an olfactory stimulus paired with cocaine. In the second one, entitled *Cerebellar hallmarks of conditioned preference for cocaine*, we used the same behavioral task of the first study in order to further extend the description of cFos expression patterns in cerebellar circuitry, including now the major inputs and one of the output nuclei of the cerebellum.

In the last chapter, *Cerebellar perineuronal nets in cocaine-induced Pavlovian memory: site does matter*, we accomplished a broad analysis of perineuronal nets (PNNs) expression of cerebellar vermis. First, we analysed an outbred mouse strain trained to acquire preference for olfactory stimuli associated with cocaine. Second, α6Cre-Cacna1a mice (Galliano et al, 2013) were used to test if a reduction in the glutamate release of parallel fibres to Purkinje dendrites would alter the acquisition of cocaine-preference conditioning and the expression of PNNs in the cerebellum.

After the presentation of the chapters, there is a section where summarised findings, strengths and pitfalls as well as future directions are provided. References can be also found at the end of the present document.

Finally, we conclude this thesis with an appendix. This document is formed by research not finished yet, but that may be of great relevance to understand the role of the cerebellum in addictive disorders. In that appendix, we explored the effect of a focal lesion in the dorsal cerebellar cortex (Lobule VIII) on the acquisition of food self-administration. This research has to be understood as a part of a broader investigation on the cerebellum's role in drug-related motivation. Our aim was to test whether the role of the cerebellum is specific for drug self-administration or it would be a general modulator of the reward process.

GENERAL INTRODUCTION

1. What is addiction? The role of conditioned memories

Drug addiction is one of the most prevalent neuropsychiatric diseases afflicting society today. Addiction is defined as a chronic brain disease characterized by compulsive drug seeking and use despite harmful consequences, in which craving and relapse episodes persist even after decades of abstinence (Koob and Volkow, 2010). It is considered a brain disease because drugs change the brain; they change its structure and activity (Volkow et al, 2016).It is currently known that drugs of abuse usurp molecular neuronal targets and, when chronically consumed, they produce long-lasting changes in many brain circuits, including those subserving associative learning and memory (Hyman and Malenka, 2001; Hyman, 2005).

A major component of addiction is the strong associations that are developed between environmental stimuli that are predictive of reward (cues) and the reward itself (drugs). By hijacking the neural systems responsible for acquiring and storing reward-related memories, drug-stimuli association become overlearned and highly relevant for addicts (Taylor et al, 2009). Through a Pavlovian conditioning process, context and stimuli closely associated with drug consumption gain progressively greater control over behavior (Jentsch and Taylor, 1999; Everitt and Robbins, 2005). These drug-associated cues have definitory properties which make them especially relevant for addicts. The stimuli related to drug consumption can grab one's attention and elicit approach behavior towards them (Franken, 2003). Additionally, drug-related cues (CS) can act as conditioned reinforcers (Meyer et al, 2014; Pitchers et al, 2017). The acquisition of conditioned reinforcing properties by CS has been demonstrated in different studies, in which some animals will approach CS even though this response does not allow the

animal to obtain the reward (Uslaner et al, 2006; Saunders and Robinson, 2010). On the other hand, drug-associated cues also acquire conditioned motivational properties which are capable of instigating drug-seeking and invigorating ongoing behaviors aimed at obtaining the drug (Saunders and Robinson, 2013). Due to the aforementioned motivational properties, repeated encounters with drug associated cues reactivate salient and long-lasting drug-induced memories, which can produce craving and relapse. (Robinson and Berridge, 1993; O'Brien et al, 1998; Shaham et al, 2003; Taylor et al, 2009; Saunders and Robinson, 2013).

Drug addiction may be understood in terms of the recruitment of neural systems that normally mediate learning and memory processes (Hyman et al, 2006). For this reason, the brain areas involved in the acquisition and expression of drug-induced memories are those responsible for the acquisition and expression of memories associated with natural reinforcers, such as food and sex (Robbins et al, 2008). Most of the focus on neural mechanisms of drug addiction has been on subcortical structures as the amygdala, hippocampus and striatum. The amygdala mediates Pavlovian and stimulus-affect-associative relationships (McDonald and White, 1993), and it is involved in the retrieval of emotional memories (Packard and Teather, 1998). The hippocampus, by contrast, encodes explicit knowledge about the relationship between cues and events in the drug context (Packard and Goodman, 2013). Other brain structures are in charge of linking these emotional memories to behavioral acts. Thus, the dorsal striatum, formed by caudate and putamen, encodes associations between drug-related stimuli and behavioral responses (Goodman and Packard, 2016). This may allow the presentation of a drug-related cue to activate an automatic behavioral response that results in drug seeking. The dorsal striatum has long been assumed to mediate habit formation and thereby, the transition from a goal-directed behavior to an automatized behavioral action (Everitt

and Robbins, 2016). Furthermore, cortical structures exert direct regulatory influences on these subcortical regions (Robbins et al, 2008). Cortical zones involved in drug-induced memories are the orbitofrontal cortex, anterior cingulate cortex, and the dorsolateral prefrontal cortex (White, 1996). The orbitofrontal cortex is involved in processes that range from associative Pavlovian conditioning to decision making (Bechara, 2005). Besides, the anterior cingulate cortex has been linked to the emotional response caused by the exposure to drug-related cues (Robbins et al, 2008). Finally, the dorsolateral prefrontal cortex is controlling the acquisition and storage of drug-induced memories enabling working memory buffer (Tang et al, 2015).

2. Why involve the cerebellum in drug addiction

2.1. The cerebellum in associative learning

As mentioned above, the formation, storage and maintenance of memories is crucial to the development of addiction (Everitt and Robbins, 2016). Indeed, drug-related behaviors acquire emotional value by being associated with contexts and stimuli through Pavlovian conditioning (O'Brien et al, 1998). Such associative memories exert influence on the instrumental behaviors of drug seeking and drug taking (Everitt et al, 2001), and can induce relapse (Shaham et al, 2003). As a result, learning theories of addiction have become more prominent (Everitt et al, 2001; Robinson and Berridge, 2008; Saunders and Robinson, 2013). Moreover, these perspectives are compatible with an increasing focus on neuronal plasticity processes, such as LTP and LTD (Wolf, 2010; Lüscher and Malenka, 2011).

Importantly, the involvement of the cerebellum in these learning processes has been observed by using different kinds of memory paradigms. Animal studies have provided evidence that the cerebellum is involved in eyeblink conditioning, a classical form of

associative motor learning (Bracha, 2004; Thompson, 2005). In addition, there is growing evidence that demonstrate the cerebellum's role in associative learning and consolidation of aversive emotional responses (Leaton, 2003; Sacchetti et al, 2005; Strata, 2015).

It has been demonstrated that in cerebellar-dependent Pavlovian learning, unconditioned stimuli (US) and CS information reach the cerebellum through different pathways. Thus, CS information arrives at the cerebellar cortex via mossy fibers. Nevertheless, US signals arise from the inferior olive and reach the cerebellum through climbing fibers (Thompson and Steinmetz, 2009). To the best of our knowledge, the first report that show an involvement of the cerebellum in eyeblink conditioning is the one by McCormick and colleagues (1982). In this study, an aspiration lesion of half of the cerebellum abolished the conditioned response (CR) in the ipsilateral eye leaving the unconditioned response (UR) unchanged. In posterior studies, it was observed that lesions in the cerebellar cortex impaired the acquisition of conditioned eyeblink responses (Lavond and Steinmetz, 1989), whereas a lesion of the interpositus abolished the acquisition of conditioned eyeblink responses (Garcia et al., 1998).

In addition to this form of motor associative learning, the cerebellum's role in emotional aversive learning has been widely demonstrated. Indeed, the cerebellum is thought to take part in an integrated network that regulates fear learning, formed also by the amygdala, hippocampus and prefrontal cortex (Kim and Jung, 2006). Early experiments with animals have shown that cerebellar lesions weaken fear-related behaviors, while the stimulation of this brain area produce fear-related responses (Snider and Maiti, 1976). In addition, it is well known that cerebellar damage disturbs vegetative and behavioral conditioned fear responses (Supple and Leaton, 1990; Ghelarducci and Sebastiani, 1997). However, the studies of Sacchetti and colleagues were the major

evidence for a causal role of the cerebellum in fear conditioning, specifically its role in fear memory consolidation. In a set of experiments, they demonstrated that reversible inactivation of the cerebellar cortex by injecting tetrodotoxin prevents the consolidation of contextual- and cue-induced fear conditioning (Sacchetti et al, 2002). Moreover, Sacchetti et al, (2004) showed a selective LTP in Purkinje-parallel fibers synapses in lobules V-VI of mice trained in fear conditioning. In addition, they involved GluR2 subunit in plasticity in these synapses because mice that show a selective deficiency in this AMPA subunit exhibit impairments in short- and long-term cue-induced fear conditioning (Sacchetti et al, 2004). These studies, along with studies in humans with cerebellar damage have demonstrated the crucial role of the cerebellum in the consolidation of emotional associative memories (Timmann et al, 2010; Lange et al, 2015; Utz et al, 2015).

Summary of the functional anatomy and synaptic interactions in the cerebellum

The cerebellum is located posterior to the brain hemispheres and the fourth ventricle covering the dorsal part of the brainstem. It hosts around 50 billion of neurons. Surprisingly, the cerebellum includes a much higher number of neurons and a lower amount of glia cells than the rest of the brain (Zagon et al, 1997; Ramnani, 2006; Azevedo et al, 2009; Lent et al, 2012). The dorsal view of the cerebellum shows that morphologically it is a hemispherical ellipse (Figure 1). It is arranged with a central region called vermis and two cerebellar hemispheres on each side of the vermis. The cerebellar outline is a layer of highly convoluted gray matter that surrounds a branched body of white matter called *arbor vitae*, which in turn surrounds 3 pairs of nuclei inside of the cerebellar white matter. In mice, from medial to lateral, the deep cerebellar nuclei are, medial, interposed and lateral nuclei (Roostaei et al, 2014). The cerebellum is attached to the brainstem through 3 pairs of peduncles (inferior, middle and superior). Efferent projections from cerebellum pass through them to reach their targets.

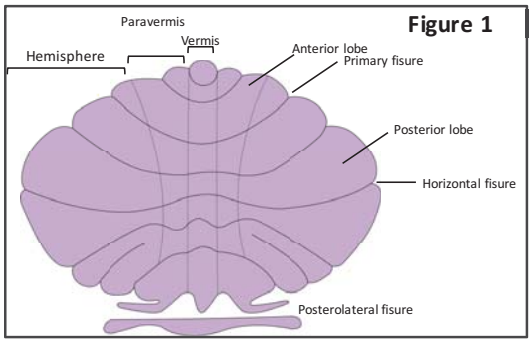

Figure 1

Anatomically, the cerebellum is divided by 2 transverse fissures. The primary fissure separates the anterior from the posterior lobe. The arrangement of fissures in the cerebellar cortical surface allows the identification of 10 different lobules marked by Roman numerals (lobules I-X) (Larsell et al, 1952; Brodal, 1992). Classically, each lobule has been considered to be responsible for a particular sensory motor function (Voogd and Glickstein, 1998). But currently, this dogma is under question (Witter and De Zeeuw, 2015).

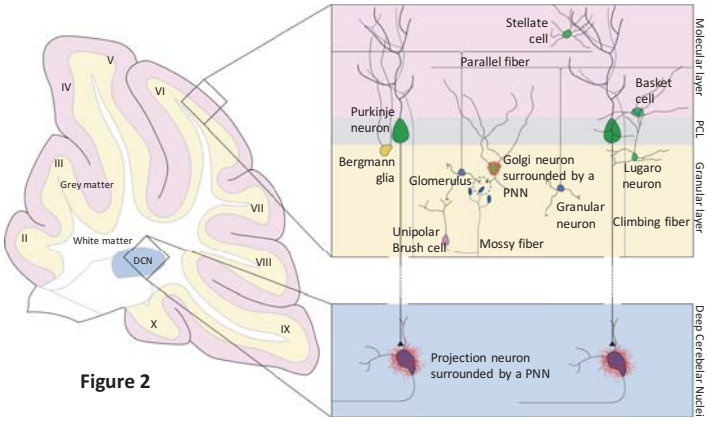

Figure 2

Like the forebrain, the cerebellum has its own cortex that projects to the cerebellar nuclei, which form the output from the cerebellum to other brain areas (Larsell, 1970) (Figure 2). The cerebellar cortical circuitry is highly stereotyped and seems to be identical regardless of the cortical region, functions and connections. It is composed of three different layers organized in a particular way. Dorsally, the molecular layer is composed by Purkinje dendrites, different types of inhibitory interneurons as basket cells and stellate cells and the parallel fibers (PF), the glutamatergic axons of granular neurons. In the middle layer, the Purkinje layer contains Purkinje cells somas and Bergmann glia. Purkinje cells are the exclusive output of the cerebellar cortex. At the inner zone, the granular layer contains a huge number of granular cells modulated by Golgi inhibitory interneurons

(Eccles et al., 1964), unipolar brush cells and Lugaro neurons, as well as different types of glia. This layer is the main input of the cerebellar cortex, because the mossy fibers (MF), from the pontine nuclei, make synapses in this layer with granular cells and Golgi neurons forming structures called glomeruli (Voogd and Glickstein, 1998; Cerminara et al, 2015).

The cerebral cortex and cerebellum are interconnected by a large fibre system (Schwarz and Their, 1999). The cerebellar cortex receives excitatory inputs from two afferent networks (Marr, 1969; Albus, 1971; Gilbert and Thach, 1977). Neuronal information from cerebral cortices, limbic areas and basal ganglia reaches the cerebellum through mossy fibres which have their source in the pontine nuclei. These mossy fibers target the glomerulus at the granular layer making synapses with granular neurons. The other glutamatergic input, climbing fibers (CF) arise from the inferior olive and climb up to the molecular layer to synapt with Purkinje's dendrites. Both mossy and climbing fibers send collaterals to the deep cerebellar nuclei. These two excitatory inputs, besides the activity of parallel fibers, control the Purkinje's GABAergic output onto neurons of the deep cerebellar nuclei (DCN), modifying the release of information out of the cerebellum (Ito, 1984; Roostaei et al, 2011).

2.2. Connexions between the cerebellum and the striato-cortico--limbic circuitry

Another source of evidence hinting a possible role of the cerebellum in addiction is provided by the existence of direct neuroanatomical connections between this structure and the cortico-striatal-limbic circuitry. These anatomic links have been stablished by tracing and stimulation studies in animals, functional imaging investigations in humans and clinical studies in patients as well (Strick et al, 2009; Koziol et al, 2014).

Early stimulation studies in cats and rats showed functional relationship between the cerebellum and ventral tegmental area (VTA) (Snider and Mati, 1976), substantia nigra (Snider and Mati, 1976), and limbic areas as hippocampus and amygdala (Snider et al, 1976; Heath et al, 1978). These techniques revealed the same type of functional relationship between cerebellum and prefrontal areas in both mice and rats (Mittleman et al, 2008; Rogers et al, 2011; Watson et al, 2014). Moreover, tracing studies in rats and monkeys showed anatomical relationships between the cerebellum and VTA (Ikai et al, 1992; Ikai et al, 1994), prefrontal cortices (Middlenton and Strick, 2001; Kelly and Strick, 2003) and striatum (Hoshi et al, 2005; Bostan et al, 2010).

Animal findings regarding cerebellar-cortical and cerebellar-subcortical connections have been confirmed in humans by neuroimaging studies. These studies, which include both functional magnetic resonance and functional connectivity imaging, confirm cerebellar relationships with cortico-striatal-limbic circuitry (Moulton et al, 2014; Moreno-Rius and Miquel, 2017). Functional loops between the cerebellum and different areas of the prefrontal cortex (PFC) have been repeatedly observed. Specifically, the cerebellum is linked to the dorsolateral prefrontal cortex (Habas et al, 2009; Sang et al, 2012), orbitofrontal cortex (Habas et al, 2009; Addis et al, 2016), anterior cingulate (Moulton et al, 2011; Sang et al, 2012), insula (Habas et al, 2009; Sang et al, 2012), and

inferior frontal gyrus (Moulton et al, 2011; Tomasi and Volkow, 2011). Moreover, functional connectivity has been observed between the cerebellum and other subcortical areas as the striatum (Tomasi and Volkow, 2011; Koehler et al, 2013), VTA (Etkin et al, 2009; Kline et al, 2016), amygdala (Sang et al, 2012; Zeng et al, 2012), and hippocampus (Sang et al, 2012; Onuki et al, 2015).

Dopaminergic system plays an important role in the development of addiction. Importantly, it has been observed anatomical and functional connectivity between the dopaminergic system and cerebellum (Snider et al, 1976; Delis et al, 2008). Bidirectional projections between the cerebellar vermis and dopaminergic VTA neurons have been repeatedely shown (Ikai et al, 1992; Ikai et al, 1994; Barili et al, 2000; Schweighofer et al, 2004). The projections from the VTA to the cerebellum include two pathways, a dopaminergic projection to the cerebellar cortex and non-dopaminergic afferents to DCN (Ikai et al, 1994). In addition, the anatomical relationship between the dopaminergic system and the cerebellum has been also confirmed by the presence of dopamine neurotransmitter levels (Glaser et al, 2006), dopamine receptors (Alder and Barbas, 1995; Kiss et al, 2011; Vazquez-Sanroman et al, 2015a), and dopamine transporters in different areas of the cerebellum (Melchitzky and Lewis, 2000). Moreover, increases and decreases in cerebellar activity have been observed after an acute stimulation of susbtancia nigra pars compacta (Herrera-Meza et al, 2014). More important, pathological conditions seem to reorganize the basal ganglia-cerebellum networks. One of the consequences of the nigrostriatal pathway degeneration in Parkinson's disease is an increase in cerebellar activity (Michaelides et al, 2010; da Silva et al, 2011; Flodin et al, 2012; Simioni et al, 2015).

2.3. Effects of addictive drugs on cerebellar functions and plasticity

As a memory and motivational disease, addiction results from aberrant neuroplastic changes within cortico-striatal-limbic circuit. Drug experience can induce both short- and long-term plasticity mechanisms which are thought to be responsible for drug-induced maladaptive behavior (Kalivas et al, 2005; Kasanetz et al, 2010; Wolf, 2010; Gipson et al, 2014; Lüscher, 2016).

As we have just mentioned, addictive drugs produce neuroadaptations in the striato-cortico-limbic circuitry through the direct pharmacological effects on different neurotransmission and neuromodulation systems (Koob and Nestler, 1997; Leyton and Vezina, 2013). Currently, it is widely accepted that plasticity alterations in glutamatergic synapses regulated by dopamine and other neuromodulators as endocannabinoids result from repeated consumption of addictive drugs (Koob and Nestler, 1997; Wolf, 2010; Gipson et al, 2014; Loweth et al, 2014).

Importantly, plasticity mechanisms in cerebellar synapses are also mediated by glutamate and endocannabinoid interactions (Ito, 1984; Salin et al, 1996; Brenowitz and Regehr, 2005; Chevaleyre et al, 2006; Dobson and Bellamy, 2015). Indeed, the cerebellum has numerous molecular targets where addictive drugs can act and modify plasticity mechanisms (see Miquel et al, 2009, for a review). There is growing evidence showing that addictive drugs induce direct effects on cerebellar functioning and plasticity. Drug-related alterations in early genes, neurotransmission and neuromodulation mechanisms, as well as structural changes have been all described after chronic exposure to addictive substances.

Freund and Palmer (1997) observed that alcohol modifies Purkinje neuron firing rates. Also, a chronic exposure to ethanol increases AMPA-dependent calcium signalling in

Purkinje neurons (Netzeband et al, 1999). In other set of experiments, it was found that alcohol modify the interaction between climbing fibers and Purkinje neurons through the activity of glutamate receptors (Carta et al, 2006). Moreover, the function of Golgi interneurons is enhanced by ethanol producing larger GABAergic inhibition of granular cells (Carta et al, 2004). Recently, it has been shown that ethanol exposure reduces the number of interneurons and lobule volume in the mouse cerebellum (Nirgudkar et al, 2016).

Acute and chronic cocaine experience also affect activity and plasticity in the cerebellum. An increased cFos expression (an early transcription factor which is used as a marker of neuronal activity) either in the granular layer or in Purkinje neurons of rodent cerebellum (Klitenick et al, 1995; Vazquez-Sanroman et al, 2015a) has been demonstrated. Additionaly, binding to NMDA receptors has been demonstrated to be enhanced after chronic cocaine (Bhargava and Kumar, 1999). Furthermore, a repeated experience with cocaine produces structural and functional changes in the Purkinje cells (Barroso-Moguel et al, 2002; Jimenez-Rivera et al, 2000; Vazquez-Sanroman et al, 2015ab), and modifies the endocannabinoid system (Rubino et al, 2004; Casu et al, 2005; Palomino et al, 2014). It also alters the balance of cerebellar plasticity-related proteins. Remarkably, the direction of plasticity changes depends on the length of the withdrawal period that precedes a new cocaine experience as we have observed in a series of studies (Vazquez-Sanroman et al, 2015ab). In those studies, after six cocaine injections, we included either a withdrawal period of one week (Vazquez-Sanroman et al, 2015a) or a withdrawal period of one month (Vazquez-Sanroman et al, 2015b); then followed by a new cocaine challenge. After a short period of withdrawal, a new cocaine injection promoted an accumulation of proBDNF and higher levels of its receptor $p75^{NGFR}$ to the detriment of matureBDNF mechanisms. Cocaine-dependent

accumulation of proBDNF was mainly seen in Purkinje neurons that also expressed high levels of the GluR2 AMPA subunit, apparently being internalized in Purkinje dendrites. Interestingly, these changes were associated with pruning in the dendritic spines and, a reduction in size and density of the Purkinje synaptic terminals. Summarizing, a chronic regimen of cocaine followed by a one-week withdrawal period led to a reduction in the Purkinje inhibitory control on the DCN neurons. Purkinje neurons exhibited less cFos expression after the last cocaine experience. As expected, DCN neurons receiving the inhibitory Purkinje input showed higher activity. Moreover, the perineuronal nets surrounding the soma of DCN projection neurons were stronger in sensitized animals, reducing the probability for structural remodelling in the Purkinje-DCN synapses (Vazquez-Sanroman et al, 2015a). When a withdrawal period of one month preceded the last cocaine injection (Vazquez-Sanroman et al, 2015b), proBDNF and mature-BDNF levels were both enhanced. We also found an increase in GluR2 expression, but the GluR2 signal was significantly reduced in the dendritic tree of all lobules. Moreover, dendritic sprouting and increased bouton size in Purkinje neurons accompanied a high BDNF and GluR2 expression. Additionally, we found a reduction in PNNs intensity in the DCN that might facilitate the subsequent remodelling of Purkinje-DCN synapses. It seems that during short-withdrawal periods, Purkinje neurons reduce their capability of inhibiting DCN neurons, whereas during long periods of withdrawal, dendritic and axonal remodelling of Purkinje followed a different trend. Similar plastic modifications have been described in the striatum and linked to the incubation of craving after long periods of withdrawal (Loweth et al, 2014).

2.4. The involvement of the cerebellum in drug-induced cue reactivity: neuroimaging studies

Traditionally, the cerebellum has been neglected as a part of the networks that hold drug-related memories. This is astonishing because several decades of research have demonstrated that the cerebellum mediates consolidation of Pavlovian memories, as we have discussed previously. Moreover, numerous human neuroimaging studies in drug users and addicts have shown cerebellar activations during exposure to drug-associated cues (see Moreno-Rius and Miquel, 2017 for a recent review).

The first study showing the relevance of the cerebellum in response to cocaine cues was a positron emission tomography (PET) study conducted by Grant and colleagues (1996). In this study, there were not significant differences between drug addicts and controls in cerebellar activation after the visualization of drug paraphernalia, but a positive correlation between cerebellar activity and desire for the drug was found. In contrast, in another study from the same lab in which a similar experimental setting was used, no correlation with craving levels was demonstrated (Bonson et al, 2002). Although a correlation between craving and cerebellar activity has not been consistently found, the presentation of drug-related cues to drug addicts induces significant cerebellar activation in alcohol, cocaine, heroin, and cannabis addicts under abstinence when they are presented with drug-related cues (Sell et al, 2000; Kilts et al, 2001; Schneider et al, 2001; Anderson et al, 2006; Olbrich et al, 2006; Filbey et al, 2009; Lou et al, 2012; Tabatabei-Jafari et al, 2014; Tomasi et al, 2015). Interestingly, such activation correlated with D2/D3 receptor availability in the striatum (Tomasi et al, 2015). Adolescent binge drinkers also showed similar activation patterns when they were presented with alcohol-related pictures (Brumback et al, 2015). Of note, cerebellar activity has been demonstrated to be higher in relapsers than in non-relapsers in response

to heroin-related cues (Li et al, 2014). Cue-elicited cerebellar activity in addicts could be restored to control levels after a therapeutic intervention (Schneider et al, 2001).

Importantly, there is an overlap in the cerebellar activation evoked by cocaine and food-related cues, which suggests a general role of this region in the processing of conditioned reinforcing cues in people suffering from compulsive-like disorders (Tomasi et al, 2015).

Functional connectivity studies, in which activation patterns of different brain areas that occur closely in time are correlated, revealed impairment in the striato-cortico-limbic circuitry (Gu et al, 2010; Ramaekers et al, 2016), and a reduced cerebellar functional connectivity between the cerebellum and different cortico-striatal-limbic areas in heroin addicts (Zhang et al, 2015), cocaine abusers (Tomasi et al, 2010) and tobacco smokers (Froeliger et al, 2015). Furthermore, loss of gray matter volume has been described in the cerebellum of cocaine addicts (Sim et al, 2007; Moreno-Lopez et al, 2015), cannabis smokers (Battistella et al, 2014; Nurmedov et al, 2015), heroin addicts (Lin et al, 2012) and alcoholics (Durazzo et al, 2015; Sawyer et al, 2016), paralleling prior observations in the striato-cortico-limbic networks (Fein et al, 2002; Matochik et al, 2005; Kim et al, 2006; Sim et al, 2007).

In summary, neuroimaging studies assessing drug-related cues reactivity also support the notion that the cerebellum is part of the neural circuit mediating drug-seeking. However, as in the preclinical studies discussed above, clinical research has overlooked the role of the cerebellum in addictive behavior, probably because the traditional view of the cerebellum as a brain area exclusively responsible of motor-related functions.

3. Perineuronal nets in brain plasticity

3.1. PNN structure and regulation mechanisms

Perineuronal nets (PNNs) are a specialized extracellular matrix (ECM) found around inhibitory neurons in the central nervous system (Wang and Fawcett, 2012). These structures surround soma and proximal dendrites forming grid-like structures in which holes where synaptic contacts take place (Celio et al, 1998). The formation of PNNs appears late in the development, when synaptic circuits are established (Carulli et al, 2006). PNNs seem to play diverse roles including ionic buffering, neuronal development, neuroprotection, synaptic stabilization and plasticity (Simonetti et al, 2009; Geissler et al, 2013; Sorg et al, 2016; Yamada and Jino, 2017).

PNNs are cartilage-like structures composed by ECM molecules. The main components of PNNs include Hyaluronic Acid (HA), lecticans, link proteins and Tenascin-R (Köppe et al, 1997). Hyaluronic Acid is a linear polymer of N-acetylglucosamine and glurocuronic acid disaccharide units. Notably, this glycosaminoglycan is the only one that is not covalently linked to a protein. Its interaction with other ECM molecules leads to the formation of large aggregates as PNNs (Köppe et al, 1997). HA is synthesized by a group of enzymes called hyaluronan synthase (HAS). This family is composed of three isoforms which synthesize HA chains of different lengths at different speeds and in different brain structures (Carulli et al, 2006; Galtrey et al, 2008). Moreover, lecticans are a kind of chondroitin sulphate proteoglycans (CSPGs) which have the ability to bind to HA and themselves. These molecules are formed by the covalent linkage of chondroitin sulphate (CS) chains to core proteins. There are four types of lecticans; aggrecan, versican, neurocan and brevican which differ in length and function (Kwok et al, 2011). The interplay between HA and lecticans is stabilized by the so-called link

proteins. These family of molecules is composed by three members; Cartilage link protein-1 (Crtl-1); Brain link protein (Bral-1) and Brain link protein-2 (Bral-2). The lack of link proteins prevents the formation of compacted PNNs in different brain areas (Kwok et al, 2010; Carulli et al, 2010). The last component of PNNs is Tenascin-R (Tn-R). This molecule is a glycoprotein which can bind up to three lecticans at the same time resulting in higher PNN stability (Weber et al, 1999).

In order to identify the presence of PNNs, it has been commonly used the staining by *Wisteria Floribunda* agglutinin (WFA) or *Vicia Villosa* agglutinin (VVA) that are lectins which bind to the sugar chains of CSPGs (Brückner et al, 1993). Nevertheless, other staining methods have been used with the same purpose. For example, Carulli and colleagues (2007) have used immunolabelling of different ECMs components for the identification of PNNs.

Notably, although PNNs are highly stable structures resistant to classic enzymatic degradation systems, there are specific enzymes which are able to modify their structure and function (Gogolla et al, 2009; Smith et al, 2014). Matrix metalloproteases (MMPs) are enzymes that degrade the ECMs as well as other molecules expressed on the cell surface. The most studied MMPs in the central nervous system are MMP-2, MMP-3 and MMP-9 (Smith et al, 2015). Besides, an exogenous way to disrupt the PNNs is the use of Chondroitinase-ABC (ChABC). ChABC is a bacterial enzyme able to degrade the CSPGs which has been used in numerous studies in order to ascertain the role of PNNs (Pizzorusso et al, 2002; Corvetti and Rossi, 2005; Balmer, 2016). Thereby, PNNs are dynamic structures susceptible to be modified through the activity of intrinsic enzymes or exogenous drugs, which in turn play an important role in neural plasticity (Wang and Fawcett, 2012).

3.2. PNNs in learning and memory

The involvement of PNNs in neuronal plasticity has been extensively studied over recent years (Wang and Fawcett, 2012). It is known that PNN formation around neurons helps to stabilize synaptic contacts and reduce plasticity changes (Kwok et al, 2011). Accordingly, it is currently thought that PNNs play an important role in associative learning as well as in the formation of memories.

One of the first and most influential studies which evaluated the role of PNNs in the experience-dependent plasticity is the one conducted by Pizzorusso and colleagues (2002). In this study, the depletion of PNNs in the visual cortex mediated by the injection of ChABC restored ocular dominance plasticity, showing that PNNs are a crucial factor in regulating critical periods. More relevant for the present discussion, Gogolla et al, (2009) assessed if PNN related-plasticity is causally involved in the development of associative learning. Specifically, the authors found that an injection of ChABC in the basolateral amygdala (BLA) makes fear memories of adult rats susceptible to extinction in a similar manner to adolescent rats.

Additionally, it has been widely observed that genetic or enzymatic modification of PNNs components are able to modulate different types of memories and associated synaptic plasticity mechanisms (Senkov et al, 2014). For example, genetic deletion of lecticans increases hippocampal LTP and impairs Morris Water Maze (MWM) performance (Niisato et al, 2005). Moreover, it has been found that genetic deletion of link proteins also enhances plasticity mechanisms underlying ocular dominance (Carulli et al, 2010). The same genetic modification produces an improvement of object recognition memory and promotes LTD in the perirhinal cortex (Romberg et al, 2013). In another recent study, Morellini and colleagues (2010) showed that Tenascin-R KO

mice performed better a reversal learning test in the MWM. This genetic manipulation produces an increase of the volume of dentate gyrus and a reduction of LTP in the same brain area.

Regarding enzymatic manipulations of PNNs using ChABC, it has been observed that ChABC infused into the perirhinal cortex enhances object recognition (Romberg et al, 2013), and improves the acquisition of spacial memory in a MWM in the striatum (Lee et al, 2012). The digestion of PNNs in the hippocampus disrupted long-term delayed contextual but not cued fear conditioning, whereas the same modification of PNNs in the mPFC disrupted cued but not contextual fear conditioning (Hylin et al, 2013). It has also been observed that the enzymatic degradation of PNNs in the hippocampus abolishes LTP in hippocampal synapsis (Kochlamazashvili et al, 2010).

Overall, the reviewed studies indicate a causal role of PNNs in memory and associated plasticity mechanisms. Notably, the effect of PNN degradation seems to be region-specific, given that similar manipulation in different brain areas produced opposite results (Sorg et al, 2016). Therefore, these findings point toward different functions of PNNs in memory and plasticity depending on the brain area in which they are expressed (Sorg et al, 2016). In conclusion, PNNs seem to be important modulators of CNS plasticity and thereby, of learning and memory-related processes.

3.3. The involvement of PNNs in drug addiction

Findings point to a prominent role of PNNs in synaptic plasticity, learning and memory, and suggest that alterations in PNN structure can be involved in brain disorders in which aberrant plasticity is a main feature, such as drug addiction (Tsilibary et al, 2014).

Addiction can be described as a pathological form of learning in which overconsolidated drug-associated memories promote drug seeking responses, even after decades of

abstinence (Smith et al, 2015). Importantly, PNN have become one of the more promising targets for drug-related neuroplasticity modifications (Blacktop et al, 2017). The degradation of PNNs by means of ChABC injection in the BLA or central amygdala prior to extinction training of morphine or cocaine-induced CPP and heroin SA decreased reinstatement behavior (Xue et al, 2014). In addition, removal of PNNs using the same enzymatic tool in the prelimbic cortex and dorsal anterior hypothalamus of rats trained to acquired cocaine CPP produced an impairment of acquisition and reconsolidation of these memories (Slaker et al, 2015; Blacktop et al, 2017). Cocaine self-admisnitration also was reduced by PNN degradation. Remarkably, PNN digestion into this hypothalamic region did not affect either sucrose-induced memory or motivation (Blacktop et al, 2017).

As a matter of fact, mice with a permanent downregulation of brevican levels (one of the CSPGs present in PNNs) in the hippocampus showed enhanced preference for the context associated with cocaine, three weeks after training as compared with the preference expressed after one day. These observations might be understood in terms of a possible role of hippocampal PNNs in the “incubation of craving” effect (Lubbers et al, 2016).

Many other studies describe alterations in the levels of MMPs in brain areas classically involved in drug-related behaviors (Lubbers et al, 2014). To the best of our knowledge, the first study reporting drug-induced MMPs change is the one by Wright and colleagues (2003). They found that ethanol exposure impaired MMP-9 activity in the hippocampus and it was associated to a decrease in performance in the MWM. Interestingly, these changes in the MMP-9 activity have also been observed in the hippocampus of cocaine abusers (Mash et al, 2007). Unlike in the hippocampus, MMP-9 activity in the mPFC was increased after exposure to a drug priming which reinstated the preference for a

cocaine-paired context (Brown et al, 2008). Different results have been found when MMPs expression was evaluated after nicotine-induced conditioned place preference (CPP). Natarajan and colleages (2013) observed an upregulation of MMP-9 and MMP-2 in the hippocampus whereas no changes could be detected in mPFC. MMP levels were also assessed in the striatum of rats under withdrawal from cocaine self-administration (SA) and cue-induced reinstatement of drug-seeking behavior. While MMP-2 was upregulated after the withdrawal period, an increase in MMP-9 levels were found during cue-induced reinstatement of different types of drugs (Smith et al, 2014). Studies that have manipulated pharmacologically MMP activity using the infusion of a broad-spectrum MMP inhibitor (FN-439) show that blocking MMP activity before conditioning sessions suppresses cocaine-induced CPP, and abolished cocaine-primed reinstatement when the injection was made just before the reinstatement session (Brown et al, 2007). In a similar manner, cue-induced reinstatement of heroin seeking was also reduced 90 minutes after FN-439 infusion (Van den Oever et al, 2010). In another interesting study, it was observed that either acute or chronic infusion of FN-439, reduced the escalation of ethanol consumption in an alcohol-vapor model of ethanol dependence (Smith et al, 2011).

Other studies have also looked at PNN expression changes after drug exposure. Coleman and colleagues (2014) found that binge-like ethanol exposure in adolescent mice induced an increase in PNN expression in orbitofrontal cortex (OFC) when mice reached adulthood. This effect was also observed in young adult mice under similar experimental conditions (Chen et al, 2015). Furthermore, a cocaine-sensitizing regimen decreased PNN expression in the medial nucleus of the cerebellum (Vazquez-Sanroman et al, 2015a). In a posterior study, it was found that increasing the withdrawal period after the same cocaine-sensitizing regime produces higher expression of PNN around

these neurons (Vazquez-Sanroman et al, 2015b). Moreover, a transient downregulation of PNNs intensity in OFC and VTA was observed after a nicotine SA paradigm which was accompanied by a decrease of the number of PNN+ neurons (Vazquez-Sanroman et al, 2016).

Considering the involvement of PNNs in neural plasticity mechanisms triggered by repeated exposure to drugs of abuse, modifications in these structures might contribute to the development of addiction and persistence of relapse vulnerability (Wright and Harding, 2009). Moreover, counteracting such PNNs modifications may become a new therapeutic approach to treat addictive disorders (Sorg et al, 2016).

GENERAL AIMS

- To investigate the involvement of the cerebellum in cocaine-induced memories using a model of Pavlovian conditioning.
- To validate a cocaine-induced olfactory conditioning protocol for mice.
- To evaluate the pattern of neuronal activation in the cerebellum of mice trained to acquire conditioned preference for a stimulus paired with cocaine using cFos expression.
- To describe the neuronal activity patterns in the cerebellar circuitry, considering afferents and efferents of the cerebellar vermis.
- To assess cerebellar PNN expression in cocaine-induced preference conditioning.
- To test whether the reduction of glutamate released by cerebellar granular cells onto Purkinje neurons can affect the acquisition of cocaine-induced preference and modify cerebellar PNN expression in the cerebellum.

HYPOTHESES

- Repeated experience with cocaine would produce a different pattern of cFos expression in the cerebellum. Differences between mice expressing preference for a cocaine-associated cue and those that do not acquire such conditioned preference will be observed.
- Different regions of the cerebellum would be differentially affected by cocaine exposure and the expression of preference for a cocaine-associated cue.
- Cocaine-induced preference conditioning would increase the activity of granular cells.

- Cocaine-induced preference conditioning would be affect by a reduction of granule cell activity
- Cocaine-induced preference conditioning would increase PNN expression.

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

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Conflict of Interest

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

Authors Contribution

MCG, DVS and LAM conducted behavioural and histological procedures. GACA and JM were involved in the critical review of the experimental design and protocols. MCG, CSS and MM carried out the data interpretation and manuscript writing. All authors critically reviewed content and approved final version for publication.

Abstract

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

Keywords: Cerebellum, cocaine, conditioning, mice, preference, vermis.

1. Introduction

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

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

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

behaviour such as prefrontal and associative non-motor cortices, the basal ganglia (Bostan, Dum & Strick 2010) and limbic system (Heath et al. 1978). Remarkably, several cerebellar regions have bidirectional connections with the prefrontal and sensorimotor cortices (Dum & Strick 2003; Kelly & Strick 2003), and the striatum (Hoshi et al. 2005; Bostan et al. 2010). Additionally, the medial part of the cerebellum (vermis) connects to DA neurons in the ventral tegmental area (VTA) and substantia nigra (Snider, Maiti & Snider 1976; Middleton & Strick 2000) and the VTA sends dopaminergic projections to the vermis (Snider & Maiti 1976; Ikai et al. 1992; Schweighofer, Doya & Kuroda 2004), forming a reciprocal midbrain-cerebellar circuit. Moreover, activation of the prelimbic subdivision of the medial prefrontal cortex produces electrophysiological responses in the contralateral vermis (Watson, Jones & Apps 2009) and electrical stimulation of the fastigial nucleus, which receives projections from the vermis, evoking neuronal activity in the amygdala and hippocampus (Heath et al. 1978). All of these anatomical findings challenge the traditional view of the cerebellum as a subcortical isolated motor structure and support its involvement in functional networks affected by addictive drugs (Miquel et al. 2009). Indeed, psychostimulant administration increases cFos-like immunoreactivity in the rat granule cell layer of the vermis at a wide range of doses (Klitenick, Tham & Fibiger 1995). Also, sensitization of cFos and jun-B mRNA has been demonstrated in the cerebellar cortex of cocaine-sensitized rats (Couceyro et al. 1994). After cocaine administration, Purkinje soma and dendrites augment the expression of Homer 1b/c and 3a/b (Jimenez-Rivera et al. 2000). These long homer isoforms are a crucial link between mGluR and IP3-dependent intracellular Ca2+ signalling, and they are considered as an important step of synaptic remodelling and spine morphogenesis (Szumlinski, Kalivas & Worley 2006). Furthermore, elevations in the relative cerebral blood volume in the

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

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

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

2. Methods METHODS

2.1. Subjects

Three-week-old Swiss male mice were purchased from Janvier (ST Berthevin Cedex, France) and maintained in our colony room (Jaume I University, Spain) for 30 days prior to experiments (n = 55). Handling was carried out daily for 5 minutes for 21 days before the experiments began. The colony room was kept at a temperature of 22 ± 2°C with lights on from 08:00 to 20:00 hours. Animals were housed in standard conditions with laboratory rodent chow and tap water ad libitum. At the age of 7 weeks, experimental procedures began. Behavioural tests were conducted within the first 5 hours of the light cycle. All animal procedures were performed in accordance with the European Community Council directive (86/609/ECC), Real Decreto 1201/2005 and the local directive DOGV 13/2007.

2.2. Pharmacological agents

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

2.3. Behavioural procedures and experimental design

In a first step, the effect of the number of pairing sessions (2, 4 or 8) between an odour (lavender or papaya) and cocaine (20 mg/kg) was evaluated in three separate groups of mice (n = 12, 16 and 15, respectively). These daily-pairing sessions took place in a specific conditioning environment (a rectangular Plexiglas box of 30 X 15 X 20 cm) and the odours used as CS+ and CS- were counterbalanced between animals and sessions following an ABAB schedule. Thus, one of the odours acted as CS+ and was associated with i.p. cocaine (20 mg/kg). On alternate days, mice were exposed to a different odour

(CS-) associated with saline administration. Cocaine induced odour preference was assessed in a 30-minute drug-free test using a T-maze, in which CS+ and CS- were presented simultaneously but in opposite arms. The preference test took place 24 hours after the last cocaine administration. The animals were habituated to the T-maze apparatus 24 h before the test in order to avoid a novelty effect. All test sessions were videotaped and the time spent (TS) in each arm of the maze was registered manually from the recorded test sessions during the last 20 minutes by a blind observer. Preference score was calculated as TS in CS+/(TS in CS+ + TS in CS-).

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

2.4. Perfusion and dissection protocol

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

for 24 hours. After this time, tissue was cryoprotected in 30% sucrose solution until complete immersion.

2.5. Tissue sections

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

2.6. cFos Immunohistochemistry

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

To obtain a clear view of cFos cellular expression, some additional tissue obtained from the same mice was rinsed and pre-blocked with 5% donkey serum and 0.3% Triton X-

100 for 1 hour. Cerebellar sections were incubated at 4°C for 48 hours with primary antibody rabbit anti-cFos (1:500, Santa Cruz Biotechnology). Thereafter, samples were exposed in the dark to AlexaFluor 647 dye anti-rabbit (1:500; Vector Labs) for 2 hours. To stain Purkinje neurons, sections were reacted with rabbit anticalbindin (1:500, Chemicon, Millipore Corporation, Temecula, CA, USA) for 48 hours, and then with AlexaFluor 488 donkey anti-rabbit (1:500; Invitrogen, Life Technologies SA, Madrid, Spain) for 2 hours. Tissues were rinsed with PBS and mounted with fluorsave reagent (Calbiochem; Millipore).

2.7. Immunostaining analysis

Images were captured in an optical microscope (Nikon E-800; Izasa, Werfen Group, Valencia, Spain) with 40X lens for the cerebellum and 20X lens for the prefrontal cortex. We considered cFos positive (cFos+) peroxidase staining those cells showing a brown labelling in the nucleus (see Fig. 1a).

We counted the first plane of three sagittal sections at the granule cell layer of the vermis cerebellum (L -0.04 to 0.72 mm) (Paxinos & Franklin 2008) in selected regions of interest (ROIs) of 20000 mm2 at the apical (external surface of the internal granular layer) and medial zones (deep portions of lobule) of each cerebellar lobule, for a total area of 40 000 mm2 per lobule and section. Purkinje neurons were counted in an area of 20 000 mm2 in the apical and medial regions and they were considered cFos+ when exhibiting a uniform and constant staining in the soma (see Fig. 1a). For the prefrontal cortex, we counted cFos+ neurons in ROIs of 20 000 mm2 of the cingulate, prelimbic, infralimbic and orbitofrontal medial cortex (from bregma 2.22 mm to bregma 1.94 mm) (Fig. 7). Cell count was performed automatically with ImageJ (now FIJI; NIH sponsored image analysis program) software. Fluorescent microphotographs were taken with an

Olympus FV1000 confocal microscope (Olympus Europa Holding GMBH, Hamburg, Germany) with 60X oil lens (Fig. 1b).

2.8. Statistics

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

3. Results

A one-way ANOVA revealed that the number of pairings during the training phase had a significant effect on the group-averaged preference scores on the test day ($F_{2,36} = 3{,}97$, $P < 0.05$). Tukey's HSD-based comparisons revealed that a training protocol consisting of eight cocaine-odour pairings produced a statistically significant higher group preference than that observed at the two pairings group ($P < 0.05$). These results are displayed in Fig. 2a. On the other hand, Fig. 2b depicts individual preference scores subjected to 2, 4 and 8 conditioning trials. From these data, it is readily observable that almost half of the individuals treated with two pairings during the training phase showed preference scores below the theoretical indifference critical point (50%), whereas this only occurred in one subject (out of 13) of the eight pairings group. Furthermore, a larger number of cocaine odour pairings seem to increase the minimum, but less clearly maximal, preference scores within each group. Thus, it seems that the number of pairings at the training phase displaced the preference scores distribution upwards rather than changing the highest preference values reached by a subset of individuals of each group. Accordingly, a Kruskal–Wallis ANOVA by ranks comparing the proportion of individuals above and below the overall median revealed a significant effect of the number of pairings [$H(2, n = 39) = 7.31$, $P < 0.05$]. Subsequent dyadic chi-square-based comparisons revealed that in the eight pairings group, the proportion of subjects displaying preference scores higher than the overall median value was higher than expected ($c2 = 11.39$, $P < 0.01$). Taken as a whole, these results seem to indicate that the higher the number of pairings, the higher the proportion of subjects surpassing the indifference scores range and, therefore, the higher the group-averaged preference.

In the second step, regardless of their number of pairings at the training phase, these individuals' samples were divided into two groups having CS+ preference scores higher

or lower than the arbitrary cut-off point of 60%. From each one of these two new groups, mice were randomly picked out to conform the thereafter-called ‘conditioned’ (n = 7) and ‘non-conditioned’ (n = 6) groups, respectively. For subsequent analysis, these two groups were compared against the ‘saline’ and the ‘unpaired’ groups (see the Methods section for further details). As expected, an analysis of covariance (ANCOVA) comparing the preference scores of all four treatment groups revealed a significant effect of the treatment factor ($F_{3,33} = 21.53$, $P < 0.001$), whereas the number of pairings, which had been used as covariate, did not affect those scores ($F_{2,33} = 1.39$, $P = 0.24$). Post hoc mean comparisons were performed using theTukey’s HSD test, which showed that the ‘conditioned’ group was different from all the other treatment groups ($P < 0.01$ in all cases) and that the preference scores of the ‘saline’, ‘unpaired’ and ‘nonconditioned’ groups had no difference among them ($P > 0.05$ in all cases). These results are depicted in Fig. 3.

When comparing locomotor activity recorded during the preference test (cm in 20 minutes), no significant differences were seen among any of the four groups ($F_{2,19} = 1,76$, $P = 0.18$). Means and standard error of the mean were as follows: the saline group = 8352.74 ± 966; the unpaired group = 13 935.11 ± 2735; the nonconditioned group = 9266.08 ± 1465; and the conditioned group = 8277.34 ± 2717.67.

Trying to identify evidence for a differential involvement of fronto-cerebellar networks on subjects exhibiting CS+ preference, we examined cFos expression on several cortical and cerebellar regions in each of these four experimental groups. Regarding the cerebellum, we first analysed cFos expression in the granule cell layer of different vermal lobules. As revealed by a one-way multi-variate analysis of covariance (MANCOVA), the treatment group produced an effect that approached, but did not reach, statistical significance (Wilks = 0.14 $F_{24,41} = 1.61$, $P = 0.08$), whereas the

number of pairings did not even have a trend towards producing any relevant effect (Wilks = 0.59, F8,14 = 1.20, P = 0.36).

These results prompted us to analyse cFos expression in further detail, then separating the functionally distinct apical and medial regions of the granule cell layer of different cerebellar lobules (Figs 1, 4 & 5). A one-way MANCOVA revealed a significant effect of the group (Wilks = 0.11, F24,41 = 1.93, P < 0.05) but not of the number of pairings, which was used as covariate (Wilks = 0.56, F8,14 = 1.35, P = 0.29). Subsequent univariate analyses showed a significant effect of the group in all cerebellar vermis lobules (P < 0.01 in all cases; see Table 1 for further details). Interestingly, as revealed by Tukey's HSD post hoc comparisons, in all cases, the 'conditioned' group displayed a significantly higher (P < 0.01) number of cFos+ neurons than the 'saline', the 'unpaired' and the 'non-conditioned' groups, which did not differ among themselves regarding cFos staining. These results are depicted on the different panels of Fig. 4.

Furthermore, as summarized in Table 2, individual levels of cFos staining were significantly and positively correlated with their corresponding CS+ preference scores at lobules, being the correlation indices highest at lobules VIII, IX and X. Taken together, these results seem to indicate that CS+ preference is related to the activity of cells in the apical region of the granule cell layer of the cerebellar vermis and that this relationship might be more prominent in some specific lobules.

On the other hand, a separate one-way MANCOVA comparing cFos expression in the medial region of the granule cell layer also revealed an effect of the group (Wilks = 0.11, F24,41 = 1.94, P < 0.05) but not of the number of pairings (Wilks = 0.63, F8,14 = 1.00, P = 0.47), which was used as a covariate. Follow-up univariate analyses yielded a significant group effect at all cerebellar vermis lobules (P < 0.01 in all cases; see Tables 3 and 4 for further details). However, when post hoc mean comparisons for each

dependent variable were performed, statistically significant differences were focused on the 'unpaired' group, which exhibit significantly lower ($P < 0.01$) cFos staining levels than the other groups in most of these comparisons. These results are presented in detail in the different panels of Fig. 5 and, conversely to what was observed for the apical region, they seem to suggest that cellular activity in the medial region of the granular layer of the cerebellar vermis is related to contingent CS-US administration during the training phase rather than the preference exhibited on the test day. In fact, as can be seen in Table 5, individual correlations between CS+ preference and cFos staining levels in this region were lower than those observed for the apical zone and no longer reached statistical significance in lobules VIII and X.

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

On the other hand, a similar one-way MANCOVA comparing the number of cFos+ Purkinje neurons in the medial region of the cerebellar vermis lobules yielded a

significant group effect (Wilks = 0.10, $F_{24,41} = 2.01$, $P < 0.05$) but not a covariation with the number of pairings (Wilks = 0.72, $F_{8,14} = 0.65$, $P = 0.72$). Univariate comparisons revealed that this general effect was due to between-group differences on lobule VI ($F_{3,21} = 5.05$, $P < 0.01$) and, to a lesser extent, lobule VII ($F_{3,21} = 3.38$, $P < 0.05$) (Tables 6 and 7). Mean comparisons showed that in both lobules, the 'conditioned' group exhibited a higher number of cFos+ Purkinje neurons than the other groups, but this difference only reached statistical significance at some, but not all, between-group comparisons. More specifically, as depicted in Fig. 6, the 'conditioned' group had more Purkinje cFos+ neurons than the 'saline' and 'non-conditioned' groups in the medial region of lobule VI ($P < 0.05$ in both cases) as well as than the 'saline' group at lobule VII ($P < 0.05$). No significant correlations between CS+ preference and Purkinje cFos staining were found.

Finally, we also analysed the number of cFos positively stained neurons in several regions of the prefrontal cortex (Fig. 7). A one-way MANCOVA revealed a significant group effect (Wilks = 0.12, $F_{12,50} = 4.55$, $P < 0.001$) but not a covariation with the number of pairings (Wilks = 0.97; $F_{4,19} = 0.45$, $P = 0.77$). Univariate comparisons showed that the group effect was observable in all tested regions (cingulate $F_{3,21} = 12.68$, $P < 0.001$; prelimbic $F_{3,21} = 5.77$, $P < 0.001$; infralimbic $F_{3,21} = 3.73$, $P < 0.01$; orbitofrontal $F_{3,21} = 7.08$, $P < 0.01$), whereas the number of pairings did not reach statistical significance in any of them (cingulate $F_{1,21} = 0.23$, $P = 0.63$; prelimbic $F_{1,21} = 0.06$, $P = 0.80$; infralimbic $F_{1,21} = 0.002$, $P = 0.96$; orbitofrontal $F_{1,21} = 0.87$, $P = 0.36$) (Table 8). Tukey's HSD post hoc-based comparisons demonstrated that between-group differences were largely due to the differences between saline-treated group and all cocaine-treated groups. These results are depicted in Fig. 7 and seem to indicate that cFos expression in those frontal areas was related to the pharmacological actions of

cocaine rather than to the acquisition/expression of conditioned odour preference. In fact, no significant correlations were found between CS+ preference and cFos expression at the cingulate ($r = 0.03$, $P = 0.87$), the prelimbic ($r = -0.27$, $P = 0.172$), the infralimbic ($r = -0.35$, $P = 0.07$) or the orbitofrontal ($r = -0.31$, $P = 0.12$) cortices.

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

4. DISCUSSION

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

For this attempt, we trained mice to acquire a conditioned preference response to an odour associated with cocaine injections. We found that four and eight cocaine odour pairings produced a robust conditioning in most of the animals, hence allowing us to validate this odour conditioning protocol for cocaine. Remarkably, enhancing the number of odour-cocaine pairings pushed the preference scores distribution up rather than increasing the individual highest preference values (Fig. 3). Brabant, Quertemont & Tirelli (2005) observed similar results regarding the magnitude of cocaine-induced place preference. Both findings fit with current notions of conditioning as mediated by

an evidence-based decision process, becoming an all-or-nothing phenomenon at the individual level (Gallistel, Fairhurst & Balsam 2004).

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

The medial region yielded less consistent results. Nevertheless, it is worth noting that neuronal activity in the medial region seems to be related to contingent CS-US administration as lower activity was seen in medial neurons of the unpaired group as compared with the other groups, which always received cocaine or saline contingently associated with the same odour. We also evaluated activity in Purkinje neurons and observed a higher number of cFos+ Purkinje nuclei in posterior vermal lobules of the conditioned group. Moreover, activity of Purkinje cells in the apical region moderately correlated with the preference for the cocaine-paired stimulus in the same lobules. To date, there is not available information describing the specific role of apical and medial regions in the cerebellar cortex or showing cellular differences between these two areas. Further research is needed to elucidate this functional specificity.

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

lobules of human cocaine addicts; this increase being especially noteworthy in the lobules VIII and IX (but also in lobules II and III).

Unlike the cerebellum, neuronal activity in the prefrontal cortex only allowed to distinguish saline-treated groups from cocaine-treated groups, no matter if cocaine-induced preference was acquired or not. Thus, subjects belonging to each one of the different cocaine treated groups showed a similar number of cFos+ neurons in different cortical regions, being in all cases higher than that observed in saline-treated animals and not showing any statistically significant correlation towards their CS+ preference scores. This pattern of results was especially clear in the cingulate cortex and seems to be in agreement with previous data indicating that activity in this brain area is higher in cocaine than in saline-treated animals subjected to a conditioned place preference (CPP) paradigm (Zombeck et al. 2008), but it is not different between paired and unpaired groups of mice trained in a Pavlovian conditioning protocol (Nordquist et al. 2003). Data indicating that lesions of the cingulate cortex do not affect cocaine, amphetamine or morphine-induced CPP (Tzschentke & Schmidt 1999) seem to provide further support to the notion that the observed differences between groups on cFos staining at the cingulate cortex are probably unrelated to the acquisition/retrieval of CS+ preference. On the other hand, a similar pattern of results was also reproduced in the prelimbic cortex, although in this case, entering in apparent contradiction with the results observed at the lesional study of Tzschentke & Schmidt (1999). Finally, in the infralimbic and orbitofrontal cortex, a non-significant trend towards reduced cFos staining was observed in the conditioned group as compared with the nonconditioned and the unpaired group as well as a trend towards an inverse correlation between the number of cFos+ neurons and the preference for the CS+. Although these trends did not reach statistical significance these observations seem to be in agreement with the inverse

correlation between cocaine-induced CPP preference and cFos in different regions of the prefrontal lobe, including the orbitofrontal cortex, found by Zombeck et al. (2008) as well as with the proposed inhibitory role of the infralimbic cortex in drug-seeking behaviours (Peters, LaLumiere & Kalivas 2008). Nevertheless, it should be taken into account that the last cocaine injection took place 48 hours before the preference test. Hence, cFos expression shown by the cocaine-treated groups could be induced by re-activation of memories about cocaine effects other than those contingently connected to preference. Also, it could be related to withdrawal symptoms after cessation of cocaine regimen.

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

vermis indicates the activation of local neuroplasticity mechanisms required for consolidation and automaticity. Studies on fear memory have supported this conclusion as plasticity changes described within the vermal cerebellar cortex domains strictly correlated with associative processes, but they were absent in unpaired groups (Sacchetti et al. 2005; Zhu et al. 2006; Zhu et al. 2007).

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

Nevertheless, other explanations for the cerebellar cFos expression might arise from the present data and should not be overlooked. On one hand, mice showing cocaine-induced conditioned preference could present a conditioned locomotor response during the preference test that increased cerebellar cFos expression. Studies on functional topography in the cerebellum have suggested that the vermis, which has bidirectional projections to motor cortices and the spinal cord, is mainly involved in balance and head

and eye movements (Cerminara & Apps 2011). In addition, posterior cerebellar vermal lobules control locomotor functions (Barik & de Beaurepaire 2005). However, when we compared with locomotion scores during the test day, we did not find any significant difference between the groups. On the other hand, it seems that repeated long-term cocaine treatment induced Purkinje morphological alterations (Barroso-Moguel et al. 2002), probably due to hypoperfusion and ischaemic lesions that could be accompanied by over-activity of the granule cells. Nonetheless, if it supposes to be the case, we should have found no differences in cFos+ expression between cocaine-treated groups, as there is no reason to assume any relationship between conditioning and Purkinje alterations.

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

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6. Tables

	Treatment group		*Number of pairings*	
Cerebellar lobules	$F_{3,21}$	*P value*	$F_{1,21}$	*P value*
II	5.84	0.004	1.51	0.231 (NS)
III	8.78	<0.001	0.67	0.423 (NS)
V	10.22	<0.001	0.36	0.556 (NS)
VI	15.28	<0.001	0.41	0.521 (NS)
VII	10.73	<0.001	2.59	0.128 (NS)
VIII	15.03	<0.001	0.002	0.962 (NS)
IX	17.14	<0.001	6.27	0.022
X	17.29	<0.001	0.68	0.416 (NS)

Table 1 Main outcomes of univariate analyses of variance assessing the levels of the cFos+ staining in the apical region of the granule cell layer in each cerebellar lobule.

As can be seen, the treatment group factor had a significant effect on the number of cFos positive neurons in all lobules, whereas the number of parings received at the training phase (whichwas used as a covariate in all statistical analyses) only yielded a significant effect at lobe IX. Significant P values are in bold. NS = non-significant effects.

Cerebellar lobules	*Pearson's r*	*P value*
II	0.48	0.012
III	0.59	<0.001
V	0.50	0.009
VI	0.63	0.001
VII	0.60	0.001
VIII	0.68	<0.001
IX	0.66	<0.001
X	0.64	<0.001

Table 2 Correlational analysis between the CS+ preference and the number of cFos positive neurons at the apical region of the granule cell layer in each cerebellar lobule.

As can be seen, CS+ preference was significantly and positively correlated with the levels of cFos expression in all cases, reaching maximal correlation and statistical significance at lobules VI, VIII, IX and X. Significant P values are in bold.

Cerebellar lobules	Treatment group		Number of pairings	
	$F_{3,21}$	*P value*	$F_{1,21}$	*P value*
II	4.53	0.012	1.61	0.216 (NS)
III	8.77	<0.001	2.99	0.098 (NS)
V	8.82	<0.001	1.60	0.218 (NS)
VI	8.75	<0.001	0.40	0.531 (NS)
VII	14.05	<0.001	0.44	0.511 (NS)
VIII	8.95	<0.001	1.47	0.238 (NS)
IX	11.13	<0.001	0.001	0.933 (NS)
X	5.49	0.005	0.79	0.382 (NS)

Table 3 Main outcomes of univariate analyses of variance estimating the levels of the cFos expression in the medial region of the granule cell layer in each cerebellar lobule.

As can be seen, the treatment group factor had a significant effect on the number of cFos positive neurons in all lobules. However, the number of parings at the training phase (which was used as a covariate in all statistical analyses) did not yield any significant effect. Significant P values are in bold. NS = non-significant differences.

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

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

Table 4 Descriptive statistics (mean ± standard error of the mean) corresponding to the levels of the cFos+ labelling at the apical (top) and medial (bottom) regions of the granule cell layer in each lobule in the vermis cerebellum.

Capital letters indicate a significant difference ($P < 0.01$), whereas lowercase letters (a, b, c, d) were used when the same differences were reached at a lower significance level ($P < 0.05$). These differences were assessed by means of a one-way multi-variate analysis of variance (ANOVA), followed by univariate ANOVAs and Tukey's HSD tests when corresponding (see text for details). At the apical region (top), the conditioned group showed significantly higher cFos+ expression than the other groups, thus indicating a clear relationship with the CS+ preference that was corroborated with

the results of the correlational analysis provided in Table 2. On the other hand, at the medial region, differences seem to separate the unpaired group from all the others, suggesting that cFos+ staining in this region could be more related to CS- US contingency than to CS+ preference (see the Discussion section).

Cerebellar lobules	*Pearson's r*	*P value*
II	0.47	0.014
III	0.61	0.001
V	0.53	0.005
VI	0.46	0.016
VII	0.54	0.004
VIII	0.36	0.066 (NS)
IX	0.49	0.010
X	0.35	0.075 (NS)

Table 5 Correlations between the CS+ preference and the number of cFos+ neurons in the medial region of the granule cell layer in each cerebellar lobule.

As can be observed, CS+ preference was significantly and positively correlated with the levels of cFos expression in most of the lobules, although the correlation indices were in general lower to those observed in Table 2 and, in this case, the maximal correlation was found at lobe III. Significant P values are in bold. NS = non-significant differences.

Cerebellar lobules	Treatment group		Number of pairings		Preference correlation	
	$F_{3,21}$	P value	$F_{1,21}$	P value	Pearson's r	P value
II						
Apical	2.21	0.116 (NS)	0.35	0.559 (NS)	−0.14	0.478 (NS)
Medial	1.44	0.257 (NS)	0.0001	0.989 (NS)	−0.20	0.316 (NS)
III						
Apical	3.03	0.051 (NS)	0.007	0.933 (NS)	0.07	0.700 (NS)
Medial	0.87	0.469 (NS)	0.058	0.811 (NS)	−0.10	0.595 (NS)
V						
Apical	4.09	0.019	1.43	0.244 (NS)	−0.04	0.844 (NS)
Medial	1.93	0.155 (NS)	0.09	0.760 (NS)	−0.14	0.481 (NS)
VI						
Apical	3.91	0.022	0.08	0.771 (NS)	0.009	0.967 (NS)
Medial	5.01	0.008	0.99	0.330 (NS)	0.22	0.273 (NS)
VII						
Apical	1.88	0.162 (NS)	0.04	0.162 (NS)	0.25	0.201 (NS)
Medial	0.504	0.683 (NS)	0.02	0.876 (NS)	0.078	0.708 (NS)
VIII						
Apical	4.85	0.010	0.97	0.333 (NS)	0.45	0.021
Medial	1.71	0.683 (NS)	0.342	0.564 (NS)	−0.242	0.232 (NS)
IX						
Apical	3.38	0.037	1.03	0.319 (NS)	−0.08	0.682 (NS)
Medial	1.45	0.256 (NS)	0.34	0.564 (NS)	0.049	0.810 (NS)
X						
Apical	2.26	0.110 (NS)	0.04	0.429 (NS)	−0.05	0.775 (NS)
Medial	2.23	0.113 (NS)	0.41	0.524 (NS)	−0.20	0.316 (NS)

Table 6 Results of univariate analyses of variance assessing the levels of the Purkinje cFos+ labelling in the apical and medial regions in each cerebellar vermis lobule.

The treatment group factor produced a significant effect on the number of cFos+ neurons in the apical regions of lobules V, VI, VIII and IX as well as in the medial region of lobe VI. Conversely, the number of pairings (which was used as a covariate in all these analyses) did not yield any significant effect. The table also shows that cFos+ expression in Purkinje neurons was not clearly correlated with preference for the CS+ and only a

moderate correlation was found when considering the number of cFos+ neurons in the apical region of lobe VIII. Significant P values are in bold. NS = non-significant effects.

	Saline (n = 6)	*Unpaired (n = 7)*	*Non-conditioned (n = 6)*	*Conditioned (n = 7)*
Lobule II	6.00 ± 1.48	13.13 ± 3.93	4.33 ± 2.06	11.41 ± 2.47
Lobule III	7.08 ± 1.81	14.57 ± 4.73	3.67 ± 1.81	14.41 ± 2.05
Lobule V	2.67 ± 1.25	12.14 ± 4.87	1.33 ± 0.95	13.58 ± 2.60[c]
Lobule VI	3.00 ± 1.29	10.3 ± 3.51	1.83 ± 1.13	11.86 ± 2.63
Lobule VII	2.33 ± 1.49	8.00 ± 3.01	1.33 ± 0.98	5.58 ± 2.17
Lobule VIII	3.50 ± 0.84	3.85 ± 1.38	2.83 ± 1.60	9.40 ± 1.65[abc]
Lobule IX	1.17 ± 0.65	5.64 ± 2.04	0.5 ± 0.34	5.32 ± 1.58
Lobule X	0.83 ± 0.54	7.42 ± 2.95	1.33 ± 0.88	6.83 ± 2.79
	Saline (n = 6)	*Unpaired (n = 7)*	*Non-conditioned (n = 6)*	*Conditioned (n = 7)*
Lobule II	8.33 ± 2.67	12.14 ± 3.36	3.67 ± 3.08	8.75 ± 1.87
Lobule III	4.41 ± 2.25	9.14 ± 3.18	4.5 ± 2.39	5.25 ± 1.28
Lobule V	2.83 ± 1.79	10.28 ± 4.06	2.17 ± 1.24	8.58 ± 2.86
Lobule VI	2.83 ± 1.37	7.42 ± 2.42	3.00 ± 1.50	13.33 ± 2.92[ac]
Lobule VII	0.17 ± 0.16	3.07 ± 1.10	1.00 ± 1.00	3.81 ± 0.89[a]
Lobule VIII	2.17 ± 0.79	3.57 ± 2.05	1.33 ± 0.80	2.57 ± 0.75
Lobule IX	1.48 ± 0.79	3.71 ± 1.56	0.83 ± 0.83	4.39 ± 1.85
Lobule X	1.17 ± 0.83	6.57 ± 2.62	1.00 ± 0.81	3.43 ± 1.52

Table 7 Descriptive statistics (mean ± standard error of the mean) corresponding to the number of cFos+ Purkinje neurons at the apical (top) and medial (bottom) regions of each lobule.

Capital letters indicate a significant difference ($P < 0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group, whereas lowercase letters (a, b, c, d) were used when the same differences were reached at a lower significance level ($P < 0.05$). These differences were assessed by means of a one-waymulti-variate analysis of variance (ANOVA), followed by univariate ANOVAs and Tukey's HSD tests when corresponding (see text for details). In this case, very few statistically

significant differences between groups were found and, accordingly, no clear association between cFos+ Purkinje cells and preference for CS+ could be found.

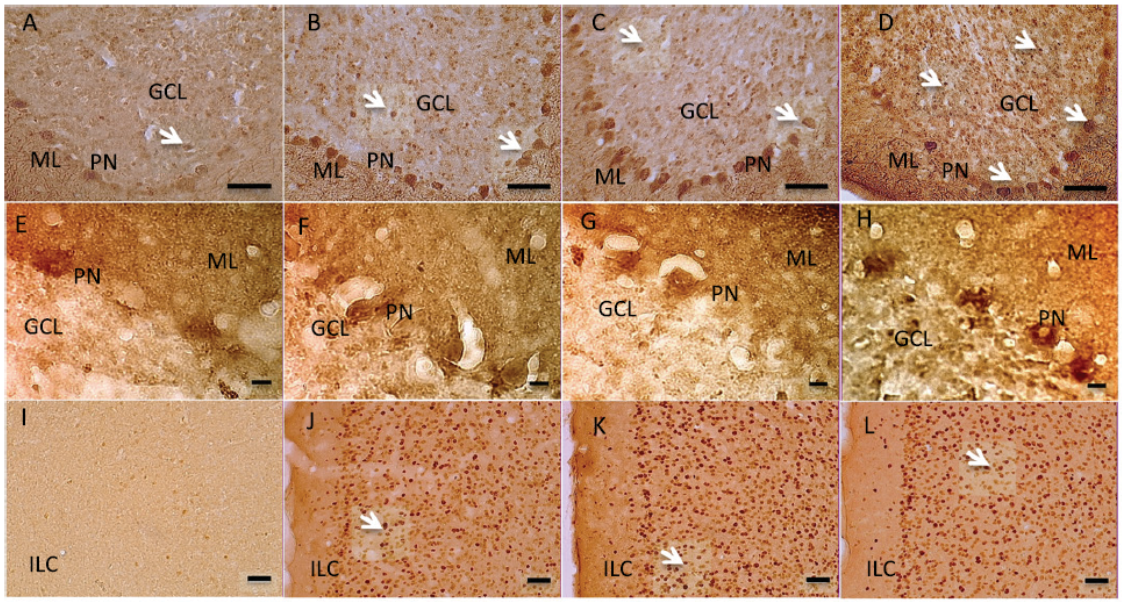
	Saline (n = 6)	*Unpaired (n = 8)*	*Non-conditioned (n = 6)*	*Conditioned (n = 7)*
Cingulate	23.71 ± 5.79[BCD]	101.26 ± 13.13	145.96 ± 17.77	97.62 ± 12.94
Prelimbic	54.58 ± 14.60[B]	149.87 ± 19.63	109.14 ± 16.86	95.83 ± 10.28
Infralimbic	50.22 ± 14.11[b]	146.78 ± 32.30	114.30 ± 18.10	69.09 ± 10.41
Orbitofrontal	42.58 ± 3.74[b]	138.65 ± 25.79	120.17 ± 17.02	71.07 ± 22.00

Table 8 Descriptive statistics (mean ± standard error of the mean) corresponding to the levels of the cFos+ staining at different cortical areas of subjects belonging to each treatment group.

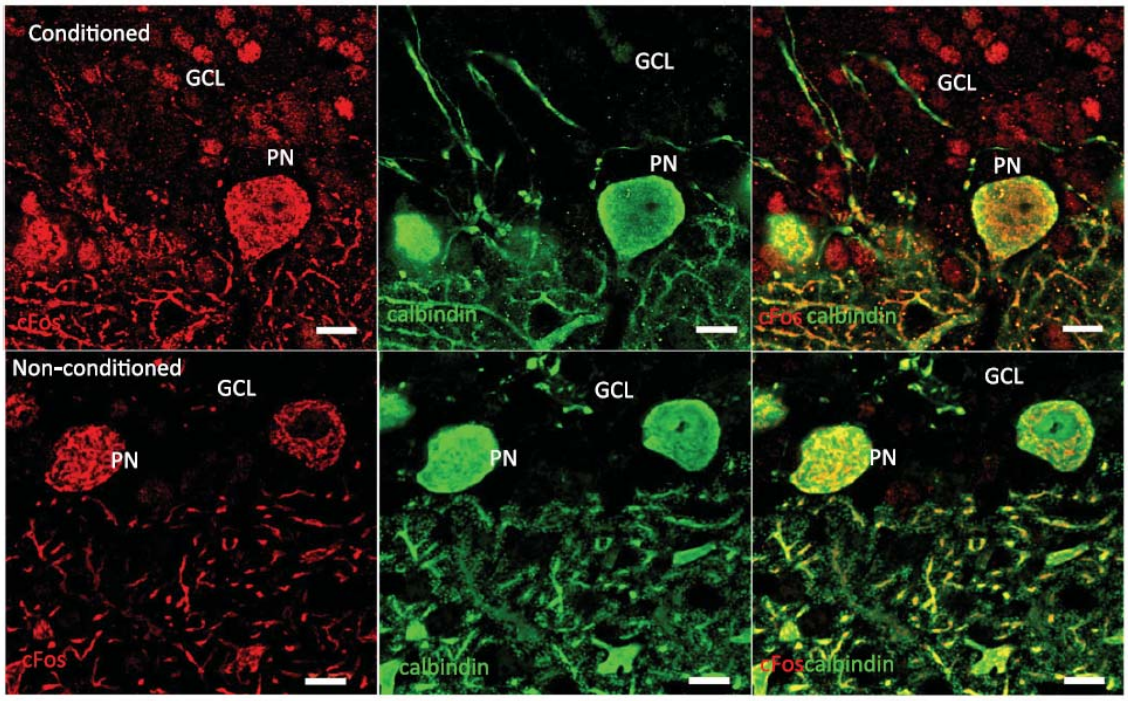
Capital letters indicate a significant difference ($P < 0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group, whereas lowercase letters (a, b, c, d) refers to lower significance level ($P < 0.05$). These differences were assessed by means of a one-way multi-variate analysis of variance (ANOVA), followed by univariate ANOVAs and Tukey's HSD tests (see text for details). As is readily observable from the table, differences in cFos+ expression were mainly associated with differences between the saline-treated group versus the cocaine-treated groups (this pattern is clearly observable at the cingulate cortex and more inconsistently present in the rest of cortical areas). Accordingly, no clear association towards CS+ preference was found (see the Results section for further details).

7. Figures

(a)



(b)



(c)

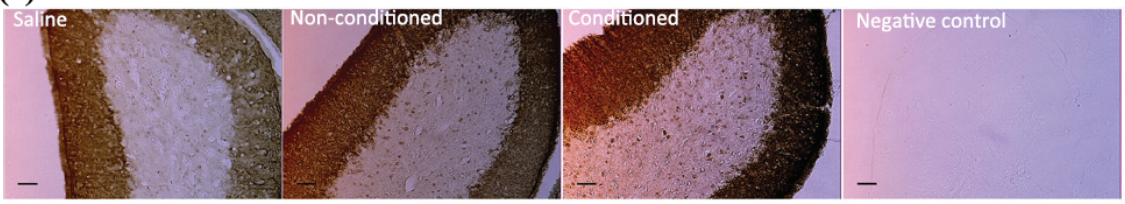


Figure 1 (a) Examples of microphotographs of cFos+ peroxidative staining in the cerebellum (panels A–H) (40X; scale bar = 50 μm) and the infralimbic cortex (I–L) (20X; scale bar = 50 μm). Saline (A, E, I), unpaired (B, F, J), non-conditioned (C, G, K) or conditioned (D, H, L). As the figure depicts,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

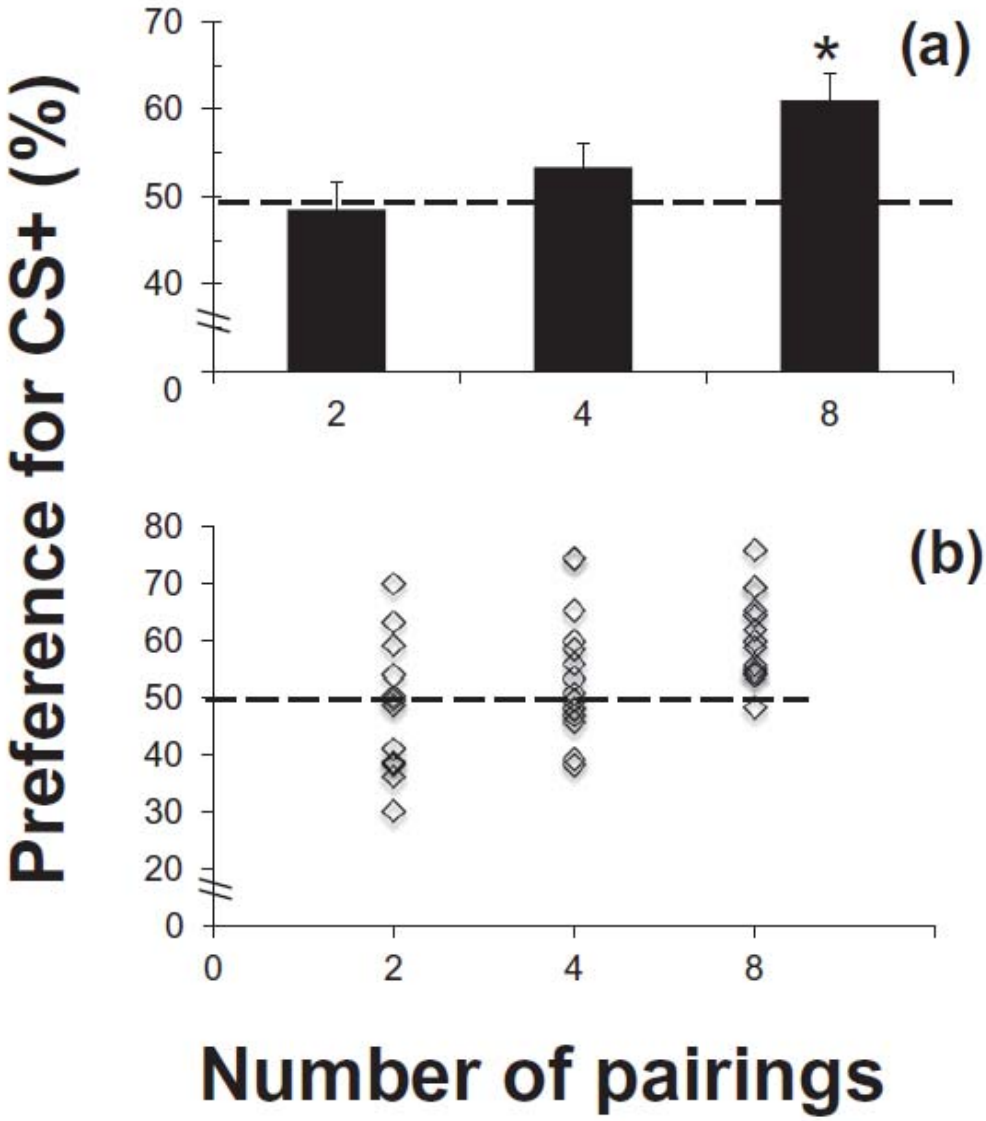


Figure 2 Effect of the number of pairings on the acquired preference for an odour associated with cocaine administration. Panel (a) depicts the mean ± standard error of the mean of the percentual preference for cocaine-associated odour on the test day as a function of the number of cocaine pairings at the training phase (*P < 0.05). Panel (b) represents the distribution of the individual scores of the percentual CS+ preference on the test day. As can be readily observed, a higher number of pairings was associated with an upward displacement of the subjects' distribution and with a reduction of variability in their preference scores rather than with a change of the maximal values

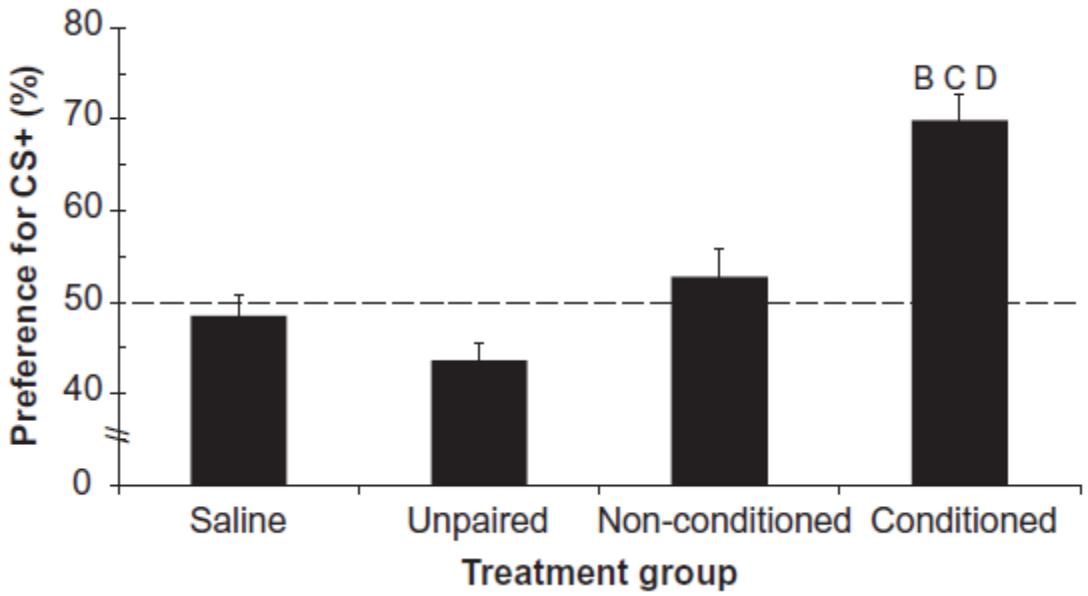


Figure 3 Preference for the CS+ in the experimental groups used for the study of the cFos staining in prefronto–cortical and cerebellar regions. The 'conditioned' and 'non-conditioned' groups were randomly picked up from those having a preference higher/lower than the arbitrary 60% cut-off point, respectively.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

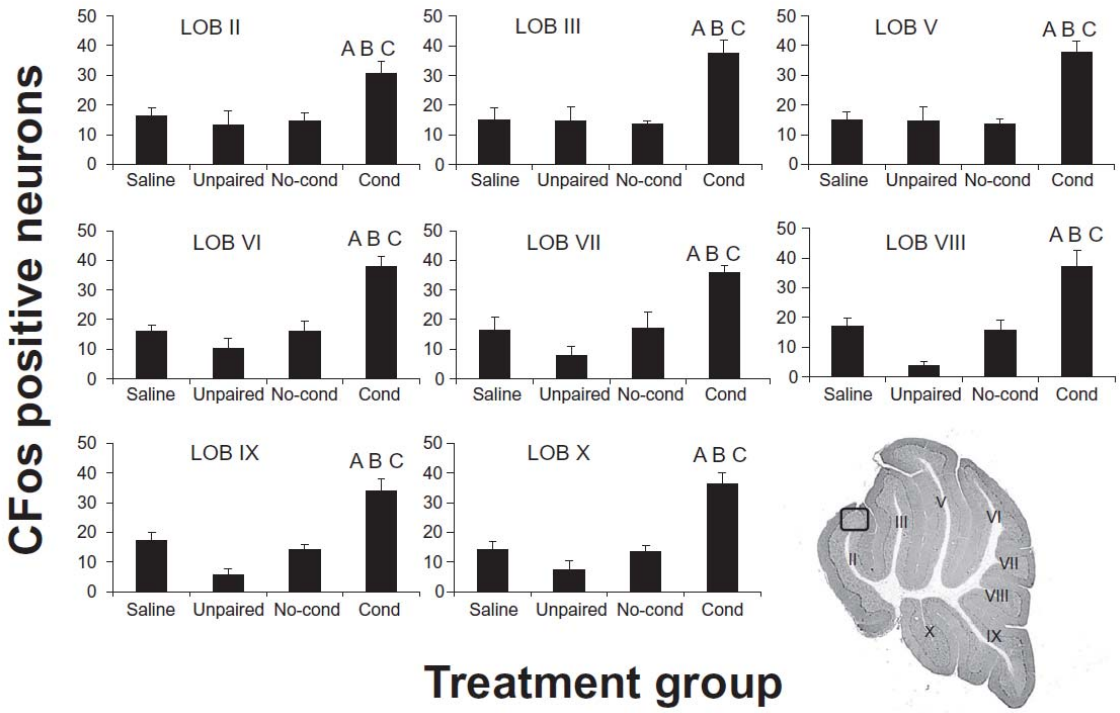


Figure 4 Group effects on cFos+ staining in the apical region of the granule cell layer (black square) for each cerebellar vermis lobule. Each panel corresponds to a different lobule for which the mean ± standard error of the mean of cFos positive neurons is depicted. Capital letters indicate a significant difference ($P < 0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group. Additional details on these data can be found at the top panel of Table 4

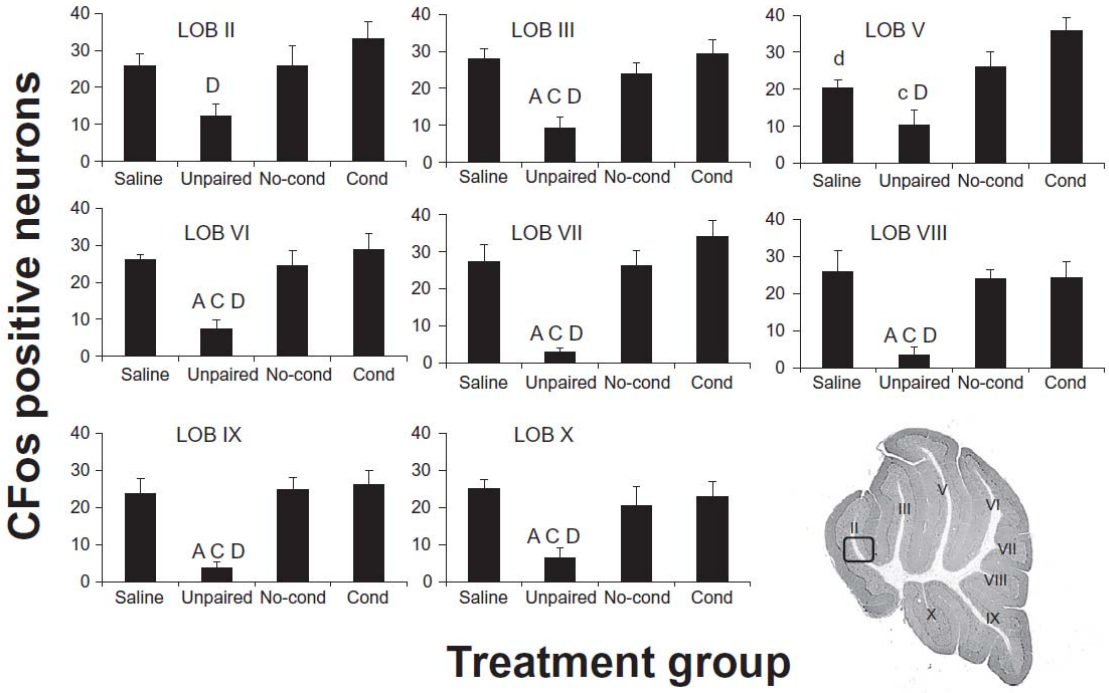


Figure 5 Group effects on cFos staining at the medial region of at the granule cell layer (black square) for each cerebellar vermis lobule. Each panel corresponds to a cerebellar lobule for which the mean ± standard error of the mean of cFos positive neurons is depicted. Capital letters indicate a significant difference ($P < 0.01$) towards the saline (A), unpaired (B), non-conditioned (C) or conditioned group, whereas lowercase letters (a, b, c, d) were used when the same differences were reached at a lower significance level ($P < 0.05$). Additional details on these data can be found at the bottom panel of Table 4

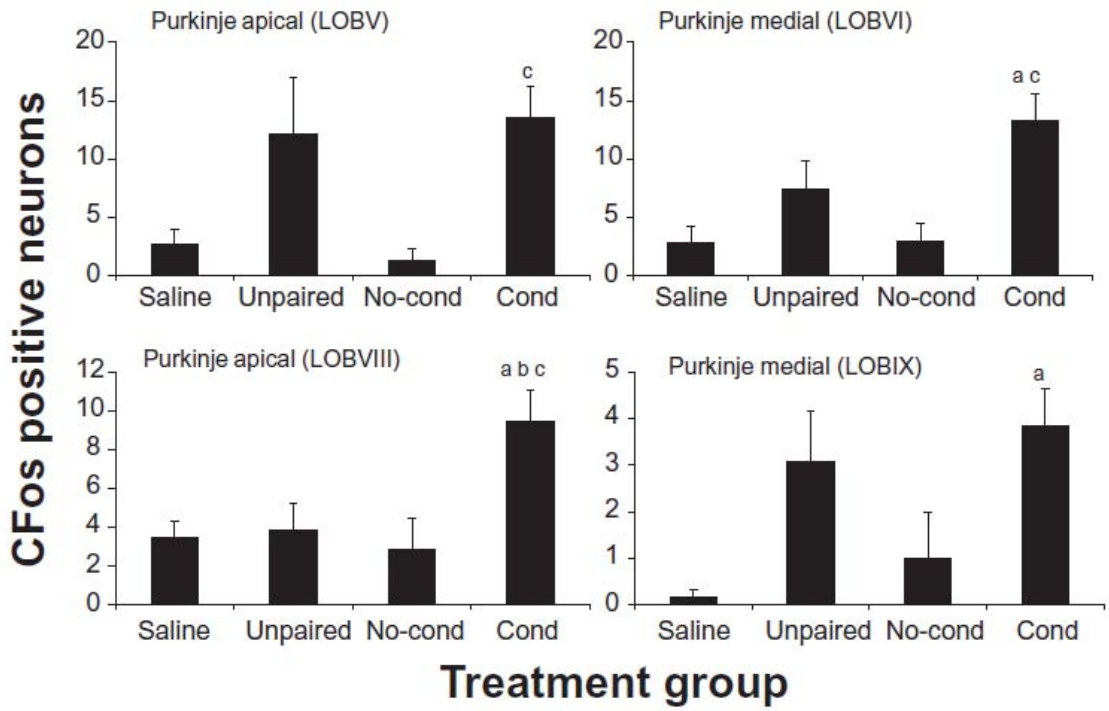


Figure 6 Group effects on cFos expression in the apical and medial regions of the Purkinje cell layer of the lobule VI (panel A) and lobe VII (panel B) of the vermis cerebellum. The results for these two lobules are shown because they were the only ones at which statistically significant differences between groups were found (see Table 3 for further details). Lowercase letters indicate a significant difference ($P < 0.05$) towards the saline (a), unpaired (b), non-conditioned (c) or conditioned group (see Tables 6 and 7)

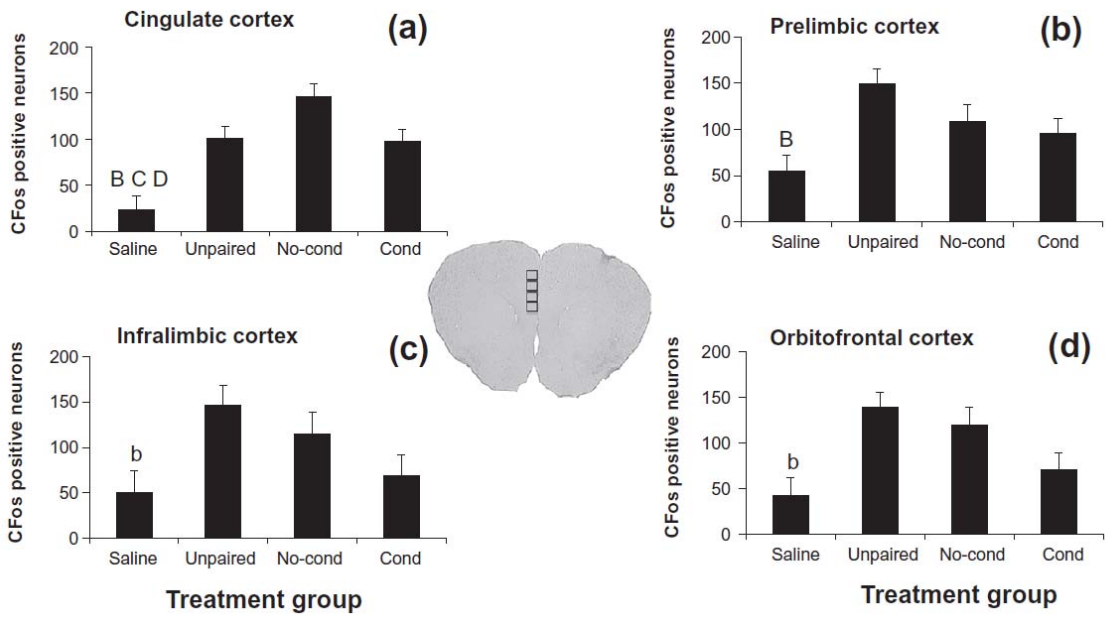


Figure 7 Group effects on cFos+ staining in the different cortical regions of the prefrontal cortex (black squares). Panels display the mean ± standard error of the mean of cFos positive neurons at the cingulate (a), prelimbic (b), infralimbic (c) and orbitofrontal (d) cortices of each treatment group.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

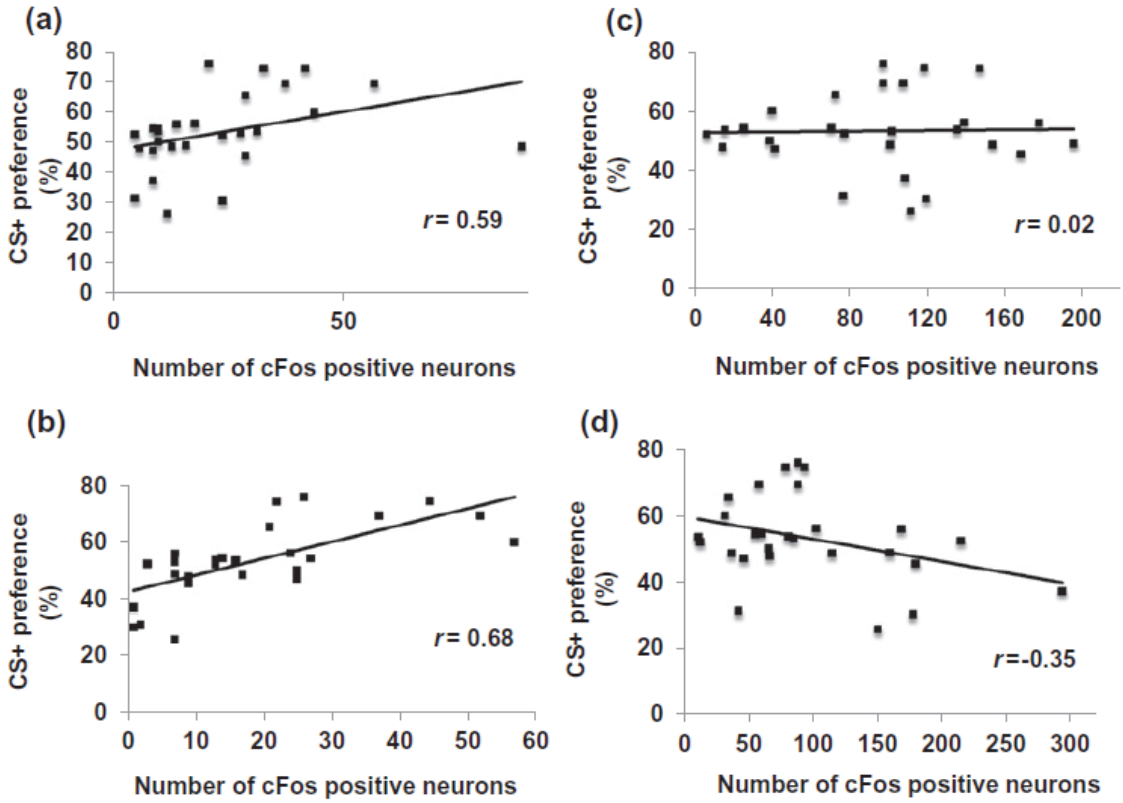


Figure 8 Representative correlations between c-Fos expression in the apical region of the granule cell layer and preference for CS+ obtained: (a) lobule III, (b) lobule VIII, (c) cingulate cortex and (d) infralimbic cortex

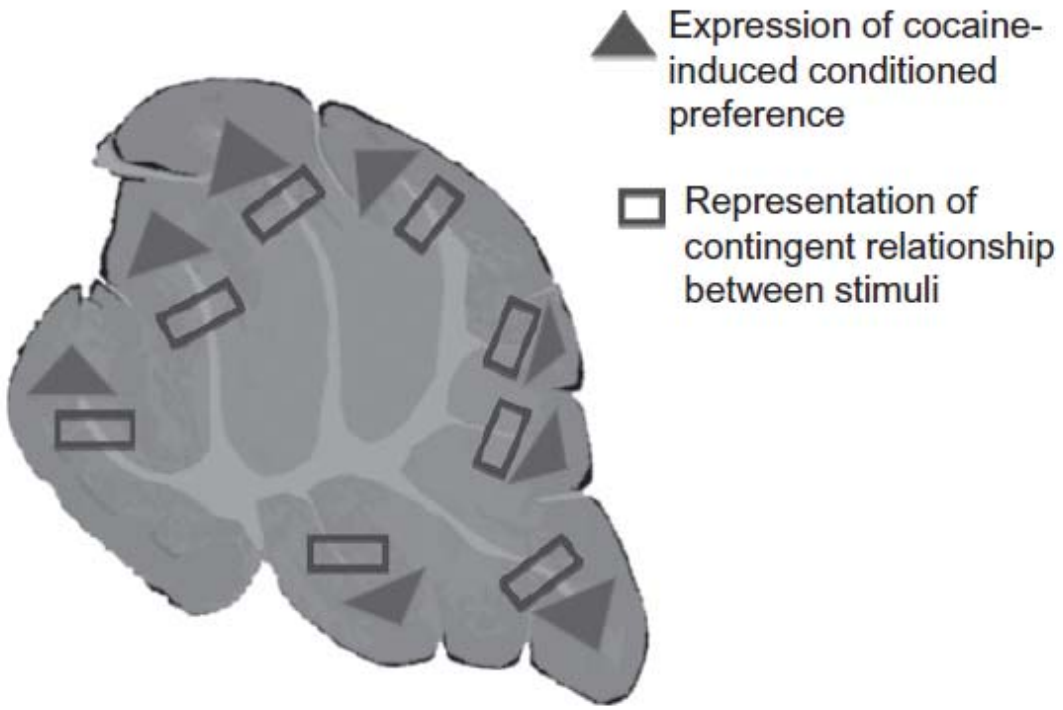


Figure 9 Schematic representation of the hypothetical functional role of different regions in the cerebellar cortex.

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Cerebellar hallmarks of conditioned preference for cocaine

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Conflict of interests (COI) statement

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

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Abstract

Pavlovian conditioning tunes the motivational drive of drug-associated stimuli, fostering the probability of those environmental stimuli to promote and trigger drug seeking and taking. Interestingly, different areas in the cerebellum are involved in the formation and long-lasting storage of Pavlovian emotional memory. Very recently, we have shown that conditioned preference for an odour associated with cocaine was directly correlated with cFOS expression in cells at the dorsal region of the granule cell layer of the cerebellar vermis. The main goal of the current investigation was to further extend the description of cFOS-IR patterns in cerebellar circuitry after training mice in a cocaine-odour Pavlovian conditioning procedure, including now the major inputs (the inferior olive and pontine nuclei) and one of the output nuclei (the medial deep nucleus) of the cerebellum. The results showed that the cerebellar hallmark of preference towards an odour cue associated to cocaine is an increase in cFOS expression in the dorsal part of the granule cell layer. cFOS-IR levels expressed in the granule cell layer of mice that did not show cocaine conditioned preference did not differ from the basal levels. Remarkably, mice subjected to a random cocaine-odour pairing procedure (the unpaired group) exhibited higher cFOS-IR in the inferior olive, the pontine nuclei and in the deep medial nucleus. Therefore, our findings suggest that inputs and the output of cerebellar circuitry are enhanced when contingency between the CS+ and cocaine is lacking.

Keywords: Cocaine, Cerebellum, Mice, Pavlovian conditioning, cFOS

Highlights

• Activity in the dorsal granular layer was related to preference for cocaine.

• Non-conditioned mice showed different cerebellar activity patterns.

• The unpaired group exhibited high activity in the olive, pontine and medial nuclei.

• Dorsal granular activity was the cerebellar hallmark of cocaine-induced preference.

1.Introduction

Long-lasting storage of drug related memories has been revealed as one of the key processes that contribute to orienting the organism response towards drug-related stimuli [1–4]. Particularly, Pavlovian conditioning tunes the motivational drive of drug-associated stimuli, fostering the probability of those environmental stimuli to promote and trigger drug seeking and taking [1]. Previous studies have strongly suggested that drug-cue associative memories are stored and reactivated by dopamine–glutamate interactions in the basal ganglia, basolateral amygdala, hippocampus and prefrontal cortex [5,6]. Interestingly, different areas in the cerebellum are involved in the formation and long-lasting storage of Pavlovian emotional memory [7,8].

Remarkably, increasing evidence has demonstrated close anatomical and functional relationships between the cerebellum and the prefrontal–striatal–limbic networks [9–18]. Both cerebellar–striatal and cerebellar–prefrontal connectivity is bidirectional, forming reciprocal prefrontal midbrain–cerebellar loops. Importantly, dopamine–glutamate interactions have also been described in the cerebellum [9,10,13,15,19,20]. Recently, we have shown that conditioned preference towards an odour associated with cocaine was directly correlated with cFOS expression in cells at the dorsal region of the granule cell layer of the cerebellar vermis [21]. These findings are coincident with those of some clinical reports. In human cocaine addicts, cerebellar activations during exposure to drug-associated cues have been found [22–26]. These findings challenge the conventional perspective of the cerebellum as a subcortical isolated motor structure and they would suggest its involvement in functional networks affected by addictive drugs [27].

In the present study, we aimed at further extending our previous description of neuronal activity patterns in cerebellar circuitry (through cFOS-IR) after training mice in a

cocaine-associated odour cue conditioning. Thus, in this case we included the two major inputs to the cerebellum (the olivary complex and pontine nuclei) and the output of the vermis (the deep medial nucleus) in order to suggest an initial picture of the cerebellar hallmarks of conditioned preference for cocaine.

2. Methods

2.1. Subjects

Three-week-old Swiss male mice were purchased from Janvier (ST Berthevin Cedex, France) and maintained in the colony room (Universitat Jaume I, Spain) for 30 days prior to experiments. Handling was carried out daily for 5 min before experiments began. The colony room was kept at 22 ± 2 °C with lights on from 08:00 to 20:00 h. Animals were housed in standard conditions with laboratory rodent chow and tap water ad libitum. At the age of 7 weeks, experimental procedures began. Behavioural tests were conducted within the first 5 h of the light cycle. All animal procedures were performed in accordance with the European Community Council directive (86/609/ECC), Real Decreto 1201/2005 and the local directive DOGV 13/2007. Of the total number of 51 mice involved in the behavioural protocols 21 were used for the purposes of determining cFOS activity in the cerebellum.

2.2. Pharmacological agents

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

2.3. Behavioural procedures and experimental design

Two equally preferred odours (lavender and strawberry) [21] were used as conditioned stimuli in the present study. A gauze was scented with four drops of lavender or strawberry fragrance and presented inside a steel ball with holes, which overhung on one of the maze arms walls. One of the odours acted as CS+ and was associated to cocaine (20 mg/kg, IP). On alternate days, mice were exposed to the other odour (CS−) and received saline injections. These pairing sessions lasted for 15 min and took place

in a specific conditioning environment (a rectangular plastic box of 30 × 15 × 20 cm). A total of 8 cocaine paired sessions were conducted using an ABAB design and the odours used as CS+ and CS− were counterbalanced between animals. Additionally, we included two control groups: The so-called "unpaired group", which was composed of animals receiving the same number of cocaine injections but randomly associated with the odours and the "saline group" included mice that were subjected to the same conditioning sessions but received saline in all of them. Preference was evaluated 48 h after the last cocaine administration in a 30-minute drug- free test using a T- maze in which CS+ and CS− odours were present simultaneously but in opposite arms. The animals were habituated to the t-maze apparatus 24 h before the test in order to avoid a novelty effect. Time spent in each arm was automatically registered. All test sessions were videotaped and the time spent (TS) in each arm of the maze was registered manually from the recorded test sessions during the last 20 min by a blind observer. Preference score was calculated as [TS in CS+/ (TS in CS++TS in CS−)] × 100.

2.4. Perfusion protocol and tissue section

Animals were deeply anesthetized with sodium pentobarbital (30 mg/kg) 70 min following the preference test and perfused transcardially, first with 0.9% saline solution and then with 4% paraformaldehyde. After perfusion, the brainstem and the cerebellum were quickly dissected and placed in a container with 4% paraformaldehyde for 24 h. After this time, tissue was cryoprotected in 30% sucrose solution until complete immersion.

Brain tissue was rapidly frozen by immersion in liquid nitrogen and sections were performed at 40 μm with a cryostat microtome (Microm HM560, Thermo Fisher Scientific, Barcelona, Spain). Six series of tissue sections were collected and stored at−80 °C in cryoprotectant solution. Sagittal sections of the cerebellum and the

brainstem were selected according to the lateral coordinates from −0.04 mm to 0.72 mm, comprising the vermis cerebellum, the medial cerebellar nucleus, the inferior olive and the pontine nuclei [28].

2.5. cFOS immunohistochemistry

Immunohistochemistry was performed on free-floating sections. For peroxidative immunostaining, tissue peroxidases were eliminated with 0.3% of H2O2 and methanol 20%, during a period of 30 min. Tissue was incubated for 48 h with a polyclonal primary antibody, rabbit anti-cFOS (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in smooth agitation at 4 °C. In a second step, sections were exposed to an affinity purified secondary biotinylated antibody, donkey anti-rabbit (1:400; BA-2000; Vector Laboratories, Inc., Burlingame, CA, USA) 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). Sections were exposed to DAB solution free of nickel component until the tissue developed an intense brown staining, and then the tissue was rinsed and mounted.

2.6. Immunostaining analysis

Images were captured in an optic microscope (Nikon E-800, Izasa Werfen Group, Valencia, Spain) with 20× or 40× lenses. We considered cFOS+ those cells exhibiting a uniform and constant brown labelling in the nucleus (see Figs. 1, 2).

We counted the first plane of three sagittal sections at the granule cell layer of the vermis cerebellum (L −0.04 to 0.72 mm) at the dorsal and medial zone of each cerebellar lobule [28], in selected regions of interest (ROIs) of 20,000 μm2 for a total area of 40,000 μm2 per lobule. Purkinje neurons were estimated in an area of 80,000 μm2 in the dorsal and ventral regions, for a total area of 160,000 μm2 per lobule. The ROI for the medial nucleus was 80,000 μm2.

For the olivary complex, ROIs were 20,000 μm2 of the dorsal, ventral and medial parts for a total area of 60,000 μm2. cFOS+ neurons in the pontine nuclei were considered in an area of 40,000 μm2. Cell count was performed automatically with FIJI (1.47 h; NIH) software by properly identifying every cFOS+ cell.

2.7. Statistics

Data presented as mean±SEM were analysed by one-way ANOVAs or Student t-tests using the treatment group as the comparison factor. Follow-up comparisons if necessary were conducted by Fisher's LSD tests. Statistical level of significance was set at $p < 0.05$.

Although it is not a common practice in this kind of studies, by calculating the Cohen's d statistic (and corresponding confidence intervals), we estimated the effect size for each and every dyadic comparison of means that yielded statistical significance. Reporting effect sizes enables the interpretation of the effect magnitud, then complementing the conclusions drawn from inferential statistics based on the rejection of the null hypothesis at a particular p value [29]. In this way, we were able to provide information about the magnitude of the effects of interest as well as about the precision on these estimates. Finally, we analysed the pattern of intercorrelations among the cFOS levels at different cerebellar anatomical sites of interest. These correlations were calculated on the percentual increases/decreases on cFOS levels over saline and estimated by means of the non-parametric Spearman's Rho index, which does not incorporate an a priori assumption of a linear (but just a monotonic) relationship between the variables of interest.

3. Results

3.1. Preference for cocaine-paired odor cue

A one-way ANOVA comparing the preference for the CS+ yielded a significant effect for the experimental group ($F_{2,18}= 7,44$, $p < 0.01$). As revealed by subsequent posthoc comparisons the contingently trained group (the paired group) (n=10) exhibited significantly higher preference ($p < 0.01$ in both cases) for the maze arm containing the CS + than the unpaired (n = 6) and saline (n = 5) groups, which did not differ among them (Fig. 3A).

However, as it can be observed in the scatterplot (Fig. 3A), not all subjects receiving cocaine injections paired with the CS+ exhibited a preference score higher than the indifference point (50%). Therefore, we used an arbitrary cut-off preference score of 60% to split this group into two subgroups "conditioned" (n = 5) and "non-conditioned" (n = 5). Then in a first step, we accomplished comparisons between the saline, conditioned and unpaired groups. As expected, a new one-way ANOVA yielded a significant effect of the experimental group ($F_{2,13} = 8.24$, $p < 0.01$). Post-hoc based tests revealed that the conditioned group exhibited a higher preference for the arm containing the CS+ than the unpaired and saline groups ($p < 0.01$ in both cases). These results are displayed in Fig. 3A, B.

As there was a subgroup of animals that in spite of being trained under contingent odour-cocaine associations never developed preference for CS+, in a second step we addressed the comparison between these two groups (the conditioned and non-conditioned group) by means of Student t-test for independent samples. As expected, the Student t-test showed a significant higher preference score in the conditioned group as compared to the non-conditioned group ($T8 = 4.80$, $p < 0.02$) (Fig. 3C).

We also addressed a one-way ANOVA to evaluate to what extent locomotion displayed during the preference test could be one of the relevant variables in order to explain between-group differences. None of the four groups differed significantly from each other ($F_{3,17}= 0.18$; $p = 0.47$). Mean and standard error of cm in 30 min were as follows: the saline group = 12,472 ± 2691; the conditioned group = 8276 ±2105; the non-conditioned group = 8861±795; the unpaired group = 9851 ± 1718.

3.2. cFOS-IR in the granule cell layer

First, we examined cFOS expression in several cerebellar regions of the three experimental groups. We were able to replicate our previous findings [21] indicating selective changes on the level of cFOS-IR in the dorsal and the ventral regions of the granule cell layer of the vermis cerebellum (see Fig. 4A, B, D, E, G, H) (Tables 1 and 2 for further details). More specifically, a series of one-way ANOVAs confirmed a group effect on the number of cFOS+cells in the dorsal region of all lobules ($p < 0.01$ in all cases, see Table 1 for further details). As expected, post-hoc comparisons revealed that this effect was driven by a significant increase in cFOS staining levels in the conditioned group as compared to the saline and unpaired groups, which had a similar number of cFOS+ cells ($p < 0.01$ in all cases). In lobes VII and VIII, differences between the conditioned and the unpaired group were additionally boosted by a statistically significant reduction on cFOS expression in the unpaired group, falling below that of the saline group ($p < 0.05$ in both cases). On the other hand, a second series of one-way ANOVAs demonstrated a group effect on the cFOS-IR in the ventral region of all vermal lobules. In this case, between-group differences were achieved by a generalized reduction of cFOS levels in the unpaired group, which was statistically significant ($p < 0.01$) in all lobules except in lobule V ($p > 0.05$ in this case). Also in this lobule, the

conditioned group exhibited a significant increase in cFOS+ cells towards the saline group ($p < 0.05$).

Next, we addressed a further exploration into the cerebellar signatures of drug-induced preference memory by assessing cFOS expression in conditioned animals as compared to non-conditioned ones (Fig. 5). When conditioned preference for cocaine was not expressed, the dorsal region of the granule cell layer showed significant lower cFOS-IR levels in lobules II ($T8= 3.00$, $p < 0.02$); III ($T8 = 4.06$, $p < 0.005$); V ($T8 = 3.96$, $p < 0.006$); VI ($T8 = 3.26$, $p < 0.02$); VIII ($T8 = 3.38$, $p < 0.02$); IX ($T8 = 2.86$, $p < 0.03$); X ($T8 = 3.49$; $p < 0.02$). Rather, in the ventral region of the granule cell layer cFOS expression was similar in either of two groups trained under contingency.

Therefore, it appears that at the level of the granular layer the signature of conditioned preference for cocaine is a higher activity in those neurons in the dorsal region. As we also showed in a previous study [21], ventral regions of the granular layer seem to represent contingency between stimuli rather than emotional memory associated to the drug, because it is the unpaired group which are those exhibiting less activity.

3.3. cFOS-IR in Purkinje cells

cFOS staining in Purkinje cells, the main target of the granule cell output through the parallel fibers, was initially evaluated by comparing the three experimental groups. A series of ANOVAs revealed a group effect that was restricted to the posterior lobules VIII, IX and X, each one of them displaying a characteristic pattern of results. Thus, in lobule VIII, the group effect [$F_{2,13}= 7.02$, $p < 0.01$] was mainly the result of a reduction in the number of cFOS+ neurons in the unpaired group (mean ± SEM: 3.16 ± 1.49), which was lower than that observed in the conditioned (11.80 ± 2.45, $p < 0.01$) and saline (9.06 ± 0.80, $p < 0.05$) groups. On the other hand, in lobule IX the group effect [$F_{2,13} = 4.21$, $p < 0.05$] was due to a significant increase in the number of cFOS+

neurons in the conditioned group (7.60 ± 1.39) as compared to the unpaired (3.83 ± 1.10) and saline (3.75 ± 1.11) groups (Fig. 4C, F, I). Finally, in lobule X, the group effect [$F_{2,13} = 13.51$, $p < 0.01$] was probably a consequence of cocaine treatment, as it reflected a significant ($p < 0.01$) increase in cFOS staining levels for the unpaired (13.38 ± 2.32) and conditioned (11.20 ± 1.38) groups versus those observed in the saline-treated mice (1.25 ± 0.73).

In a second stage, we estimated cFOS-IR in Purkinje neurons in the conditioned and non-conditioned groups (Fig. 5C, F, I). We did not observe any difference in Purkinje cells apart from lobule VII ($T8 = -2.69$, $p < 0.05$), where non-conditioned animals showed higher number of cFOS+ Purkinje cells.

3.4. cFOS-IR in the olivary complex, pontine nuclei and the medial nucleus of the cerebellum

Having confirmed the existence of a different pattern of cFOS expression in the conditioned and unpaired groups, we extended our cFOS analysis to the brainstem nuclei that involve the two major input sources to the parallel fiber-Purkinje ensemble (Fig. 6). Thus, we included the pontine nuclei (Fig. 6A, D) (which is the origin of the mossy fibers providing excitatory input to the granule cells) and the olivary complex (Fig. 6B, E) (source of climbing fibers excitatory inputs reaching Purkinje cells). Also, we assessed that cFOS-IR in the medial nucleus (Fig. 6C, F) considered the main destination of Purkinje cells' axons in the vermis and reciprocally connected to the pontine and olivary nuclei. As summarized in Table 3, separate ANOVAs revealed the existence of statistically significant differences between groups in all these structures. More specifically, we observed that the unpaired group exhibited a significant increase in the number of cFOS+ cells as compared to the conditioned and saline groups ($p < 0.01$ in all cases).

cFOS expression in the mossy and climbing fiber inputs to the cerebellum as well as in the medial nucleus was similar in the conditioned and the non-conditioned groups. No significant differences arose from Student t-test when comparing these two groups.

3.5. Effect sizes

Trying to sort out everything between group differences by their relative relevance, we decided to calculate their respective effect sizes by means of the Cohen's d statistic. The results of these estimations are summarized in Tables 4, 5, 6 and 7. Of note, in almost all cases analysed, d values were higher than/2/for those effects showing significant differences. In this regard, according to Cohen's own proposal, d values higher than 0.8 (or lower than−0.8) are considered as “large effects”, although this and other similar bench marks must be viewed with caution [29,30]. To obtain a valid indication of the precision of these estimates, we also calculated the standard errors and 95% confidence intervals (CI) corresponding to each one of these effects. Remarkably, in several cases, the lower limit of the 95% CI corresponding to positive differences (increases) or the upper limit of the 95% CI corresponding to negative differences (decreases) still yielded d values > 0.8 or <−0.8, respectively. That is, a substantial proportion of the between-groups differences identified in the present study should be considered as “large effects” even though we used a more conservative estimation of the cacna.

3.6. Correlational analysis

Interestingly, most of the largest d estimates were found on the comparisons involving the lobules III, VIII and IX, in particular at the level of the dorsal and ventral regions of the granule cell layer. As this and other observations seem to point out these lobules as especially relevant for reacting to cocaine-paired cues, we decided to investigate the reciprocal correlations between the levels of cFOS-IR observed in the different cerebellar regions. Thus, coupling the known anatomical connections and the obtained

correlational values, we built up three separate working models that summarize the interrelationships between all the components of this cerebellar circuit as well as towards the preference for the cocaine-paired odour. These models are displayed in Figs. 7 and 8 commented on in further detail in the discussion section.

4. Discussion

Our earlier findings involved the vermis cerebellum in the preference memory towards an odour-cue paired with repeated cocaine administrations [21]. Here, we extend the analysis to inputs and outputs of the vermis circuitry in order to draw a wider picture of the involvement of the cerebellum in preference towards a cue that predicts availability of cocaine.

Meaningfully, preference towards a cocaine-paired odour and contingency during training induced a different pattern of cFOS-IR (Fig. 9). As we previously observed [21], the cerebellar signature of conditioned preference was an increased expression of cFOS in the dorsal region of the granule cell layer of the cerebellar vermis. This enhanced cFOS expression was not seen when contingent training was provided to animals that did not become conditioned (the non-conditioned group). Neither was it seen when odour-cocaine pairings were not contingent as both stimuli were randomly presented (the unpaired group). Moreover, the lack of a contingent relationship between CS and US was specifically associated to a reduced expression of cFOS in the dorsal and ventral regions of the granule cell layer in several lobules, including the lobules VIII and IX. Therefore, a reduction in the neuronal activity of the granule cell layer may be tentatively regarded as a part of the hallmarks associated with lack of contingency in the relationship between CS and US.

Less clear is the association between the expression of preference towards a cocaine-associated cue and the cFOS-IR in Purkinje cells. Nevertheless, such association was seen in lobule VIII. It is also in this lobule where we observed a significant correlation between cFOS expression at the granule cell layer and that observed in Purkinje neurons. Furthermore, the number of activated Purkinje cells in lobule VIII was inversely

correlated with that at the medial deep nucleus, supporting an inhibitory Purkinje modulation over the deep cerebellar neurons (Fig. 7B).

Extending our assessment to an unprecedented analysis, we observed that cFOS levels at the olivary complex and pontine nuclei (which provide the principal inputs to the cerebellum) were raised in the unpaired group. Interestingly, the same result was found in the medial nucleus, the main cerebellar output from the vermis. As a matter of fact, cFOS expression levels at these three cerebellar areas were highly inter-correlated. The results suggest that neurons in the input and output nuclei of the cerebellum increase their activity when contingency between cues and cocaine is lacking and, therefore, the appropriate behavioural alternative for the on-going contextual situation is uncertain. This suggestion seems to be coherent with the role of the cerebellum in prediction about internal events related to external cues [31–33]. The cerebellum accomplishes prediction in order to trigger preparatory actions that involve neuronal readiness of the brain networks that are going to be needed for the upcoming events. It is a probabilistic task that requires previous learning [32]. Thus, it could be expected that as the relationship between external cues and internal events become more and more predictable, and behavioural reactions are progressively more properly tuned to environmental demands, the inputs might be progressively suppressed. It is known that deep nuclear neurons are able to induce a powerful GABAergic inhibition over the olivary complex [34–36], so climbing fibers could be inhibited once learning has been optimised and behaviour tuned to environmental demands [31].

In previous studies addressing the cerebellar role in conditioning, it has been established that conditioned (CS) and unconditioned stimuli (US) reach the cerebellum by two separate pathways (for a review, [8, 37,38]). CS information arrives at the granule cell layer from cerebral cortices and other brain areas via mossy fibers originated in the

pontine nuclei. In turn, granule cells send information to Purkinje dendrites via parallel fibers. Also, climbing fibers projecting from the inferior olive convey US information to Purkinje dendrites. In addition, climbing and mossy fibers send direct excitatory inputs to the deep nuclear neurons [39,40]. In the present protocol, we used two odours as CS. The vermis cerebellum has been found consistently activated during odour perception tasks [41–44]. It is known that an extended prefrontal–limbic network sustains olfactory processing and memory [45] so odour information may reach the cerebellum throughout the pontine nuclei via mossy fibers. Unconditioned effects of cocaine are a more complex configuration of interoceptive and central stimuli. Thus, during conditioning US information could arrive at the cerebellum from both the pontine nuclei and the inferior olivary complex. Importantly, the cerebellum connects anatomically and functionally to the circuitry responsible for acquiring, maintaining and expressing drug induced conditioned memories [14,18–20,46,48]. Specifically, the posterior vermis has been identified as the "limbic cerebellum" acting as an interface area between sensorimotor circuitry and emotional neural systems [8,18,20,47].

Moreover, cocaine may act locally in the cerebellum and trigger in situ aberrant plasticity [27]. Indeed, dopamine transporter (DAT) and receptors have been repeatedly described in the cerebellar cortex and deep nuclei [9,10,13,15,20]. As a further matter, we previously observed enhanced DAT levels in the granule cell layer of the animals exhibiting preference for cocaine-paired cues [21].

Overall, the differences seen when comparing the three cocaine treated groups lead us to suggest that the observed cerebellar pattern of neuronal activity resulted from plasticity reorganization in the cerebellar circuitry associated with memory induced by cocaine. Herein, although highly speculative at the moment, our findings point to the possibility that the dorsal region of the granule cell layer is the possible locus for the

storage of conditioned emotional memory induced by cocaine. Notwithstanding, future causal research will be essential to elucidate the role of cerebellar areas in alterations leading to addiction-like behaviour as the present approach using c-FOS expression is not more than a correlational marker of neuronal activity.

5. References

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6. Tables

	Saline	Conditioned	Unpaired	$F_{2,13}$	p
Lobule II	15.20 ± 2.81	32.56 ± 3.87[A,C]	12.17 ± 4.51	7.71	<0.006
Lobule III	11.60 ± 3.17	40.33 ± 5.80[A,C]	13.00 ± 5.28	10.22	<0.002
Lobule V	14.80 ± 3.33	36.60 ± 4.78[A,C]	12.33 ± 5.76	7.20	<0.007
Lobule VI	16.60 ± 2.50	39.20 ± 4.74[A,C]	9.69 ± 4.09	15.19	<0.001
Lobule VII	18.20 ± 4.77	36.20 ± 3.00[A,C]	6.50 ± 3.10[a]	17.01	<0.001
Lobule VIII	17.20 ± 3.29	43.34 ± 5.48[A,C]	4.33 ± 1.54[a]	30.87	<0.001
Lobule IX	16.80 ± 3.15	34.79 ± 5.91[A,C]	5.09 ± 2.22	14.84	<0.001
Lobule X	12.00 ± 1.79	37.23 ± 5.41[A,C]	7.67 ± 3.49	17.08	<0.001

Table 1

Descriptive statistics (mean ± SEM) and main outcomes of univariate ANOVAs assessing the number of the c-Fos+ neurons at the dorsal region of the granule cell layer in each cerebellar lobule. As can be seen, the treatment group factor had a significant effect on the number of c-Fos positive neurons in all. Capital letters indicate a significant difference ($p < 0.01$) towards the saline (A), conditioned (B) or unpaired group (C). Lowercase letters indicate a significant difference ($p < 0.01$) towards the saline (a), conditioned (b) or unpaired group (c).

	Saline	Conditioned	Unpaired	$F_{2,13}$	p
Lobule II	26.4 ± 4.08	30.03 ± 4.56	10.16 ± 3.22^{A,B}	15.60	<0.001
Lobule III	28.60 ± 3.04	30.18 ± 5.07	9.00 ± 5.07^{A,B}	7.67	<0.006
Lobule V	19.6 ± 2.37	33.33 ± 4.73^{a}	9.00 ± 4.73^{B}	9.03	<0.003
Lobule VI	25.8 ± 1.93	33.56 ± 4.47	5.92 ± 2.24^{A,B}	23.53	<0.001
Lobule VII	30.20 ± 4.45	35.48 ± 6.03	2.59 ± 1.17^{A,B}	19.49	<0.001
Lobule VIII	27.80 ± 6.46	26.67 ± 5.15	1.66 ± 0.91^{A,B}	11.48	<0.001
Lobule IX	21.40 ± 4.14	30.50 ± 3.22	3.33 ± 1.80^{A,B}	21.32	<0.001
Lobule X	23.00 ± 1.22	24.40 ± 5.40	6.83 ± 3.09^{A,B}	7.71	<0.006

Table 2

Descriptive statistics (mean ± SEM) and main outcomes of univariate ANOVAs assessing the number of the c-Fos+ neurons at the ventral region of the granule cell layer in each cerebellar lobule. As can be seen, the treatment group factor had a significant effect on the number of c-Fos positive neurons in all. Capital letters indicate a significant difference ($p < 0.01$) towards the saline (A), conditioned (B) or unpaired group (C). Lowercase letters indicate a significant difference ($p < 0.01$) towards the saline (a), conditioned (b) or unpaired group (c).

	Saline	Conditioned	Unpaired	$F_{2,13}$	p
Pontine n	44.37 ± 5.05	68.00 ± 11.81	199.83 ± 56.66[A,B]	5.14	<0.02
I. olive	28.60 ± 3.04	23.40 ± 5.28	45.69 ± 18.65[A,B]	10.63	<0.001
Medial n	25.50 ± 5.48	27.80 ± 6.52	74.83 ± 10.01[A,B]	12.70	<0.001

Table 3

Descriptive statistics (mean ± SEM) and main outcomes of univariate ANOVAs assessing the number of the c-Fos+ neurons in the brainstem nuclei and the medial deep nucleus. As can be seen, the treatment group factor had a significant effect on the number of c-Fos positive neurons in all of them. Capital letters indicate a significant difference ($p < 0.01$) towards the saline (A), conditioned (B) or unpaired group (C).

	Cohen's d	SE	96%CI lower bound	96%CI upper bound
Dorsal				
Lob II. conditioned vs. unpaired	2.25	0.77	0.73	3.76
Lob II. conditioned vs. saline	1.91	0.76	0.42	3.41
Lob III. conditioned vs. unpaired	2.38	0.79	**0.83**	3.93
Lob III. conditioned vs. saline	2.50	0.84	**0.85**	4.16
Lob V. conditioned vs. unpaired	2.14	0.76	0.65	3.62
Lob V. conditioned vs. saline	1.92	0.76	0.42	3.42
Lob VI. conditioned vs. unpaired	3.24	0.92	**1.44**	5.04
Lob VI. conditioned vs. saline	2.48	0.84	**0.83**	4.13
Lob VII. conditioned vs. unpaired	3.52	0.96	**1.63**	5.42
Lob VII. conditioned vs. saline	2.14	0.76	0.65	3.62
Lob VII. unpaired vs. saline	−1.39	0.67	−2.71	−0.07
Lob VIII. conditioned vs. unpaired	4.71	1.17	**2.41**	7.01
Lob VIII. conditioned vs. saline	3.16	0.95	**1.30**	5.02
Lob VIII. unpaired vs. saline	−1.55	0.69	−2.91	−0.20
Lob IX. conditioned vs. unpaired	3.29	0.93	**1.47**	5.11
Lob IX. conditioned vs. saline	1.99	0.77	0.48	3.51
Lob X. conditioned vs. unpaired	3.35	0.94	**1.51**	5.18
Lob X. conditioned vs. saline	2.85	0.90	**1.09**	4.62
Ventral				
Lob II. unpaired vs. conditioned	−2.23	0.77	−3.74	−0.72
Lob II. unpaired vs. saline	−1.80	0.72	−3.20	−0.39
Lob III. unpaired vs. conditioned	−2.28	0.78	−3.80	−0.75
Lob III. unpaired vs. saline	−2.11	0.75	−3.58	−0.63
Lob V. unpaired vs. conditioned	−2.55	0.81	−4.14	**−0.95**
Lob V. conditioned vs. saline	1.44	0.71	0.05	2.83
Lob VI. unpaired vs. conditioned	−3.98	1.04	−6.03	**−1.94**
Lob VI. unpaired vs. saline	−2.86	0.86	−4.55	**−1.18**
Lob VII. unpaired vs. conditioned	−3.47	0.96	−5.35	**−1.60**
Lob VII. unpaired vs. saline	−2.45	0.80	−4.02	**−0.88**
Lob VIII. unpaired vs. conditioned	−2.42	0.80	−3.98	**−0.86**
Lob VIII. unpaired vs. saline	−2.53	0.81	−4.12	**−0.94**
Lob IX. unpaired vs. conditioned	−3.85	1.02	−5.85	**−1.85**
Lob IX. unpaired vs. saline	−2.56	0.82	−4.16	**−0.96**
Lob X. unpaired vs. conditioned	−2.11	0.75	−3.59	−0.63
Lob X. unpaired vs. saline	−1.94	0.73	−3.38	−0.50

Table 4

Effect sizes of between-group differences found at the dorsal and ventral areas of the granular cell layer. Cohen's d statistics, with its corresponding standard error (SE) and 96% confidence intervals (CI) are provided. The lower limit of the 95% CI corresponding to positive differences (increases) or the upper limit of the 95% CI corresponding to negative differences (decreases) yielded d values >0.8 or <−0.8 are highlighted (see text for further details).

	Cohen's d	SE	96%CI lower bound	96%CI upper bound
Purkinje				
Lob VIII. conditioned vs. unpaired	**2.20**	0.77	0.70	3.70
Lob VIII. unpaired vs. saline	**−1.50**	0.68	−2.84	−0.16
Lob IX. conditioned vs. unpaired	**1.36**	0.67	0.04	2.67
Lob IX. conditioned vs. saline	**1.38**	0.70	0.00	2.76
Lob X. conditioned vs. saline	**2.47**	0.84	0.82	4.11
Lob X. unpaired vs. saline	**3.13**	0.90	1.37	4.90
Brainstem inputs and medial nucleus				
N. pontine unpaired vs. conditioned	**1.51**	0.69	0.16	2.85
Pontine n. unpaired vs. saline	**1.78**	0.71	0.38	3.18
Olive n. unpaired vs. conditioned	**2.13**	0.76	0.65	3.62
Olive n. unpaired vs. saline	**1.95**	0.73	0.51	3.39
Medial n. unpaired vs. conditioned	**2.74**	0.84	1.09	4.38
Medial n. unpaired vs. saline	**2.87**	0.86	1.18	4.56

Table 5

Effect sizes of between-group differences found at Purkinje, cerebellar inputs and the medial nucleus. Cohen's d statistics, with its corresponding standard error (SE) and 96% confidence intervals (CI) are provided. According to Cohen's own proposal, d values higher than 0.8 (or lower than−0.8) are considered as “large effects”. Cases in which the lower limit of the 95% CI corresponding to positive differences (increases) or the upper limit of the 95% CI corresponding to negative differences (decreases) yielded d values >0.8 or <−0.8 are highlighted.

	Cohen's d	SE	96%CI lower bound	96%CI upper bound
Dorsal				
Lob II. conditioned vs. non-conditioned	**2.08**	**0.78**	**0.54**	**3.62**
Lob III. conditioned vs. non-conditioned	**2.88**	**0.90**	**1.11**	**4.65**
Lob V. conditioned vs. non-conditioned	**2.74**	**0.88**	**1.02**	**4.47**
Lob VI. conditioned vs. non-conditioned	**1.53**	**0.71**	**0.12**	**2.94**
Lob VII. conditioned vs. non-conditioned	1.32	0.69	−0.04	2.69
Lob VIII. conditioned vs. non-conditioned	**2.36**	**0.82**	**0.75**	**3.98**
Lob IX. conditioned vs. non-conditioned	**2.03**	**0.77**	**0.50**	**3.55**
Lob X. conditioned vs. non-conditioned	**2.48**	**0.84**	**0.83**	**4.13**
Medial				
Lob II. conditioned vs. non-conditioned	0.10	0.63	−1.34	1.13
Lob III. conditioned vs. non-conditioned	0.37	0.63	−0.87	1.62
Lob V. conditioned vs. non-conditioned	0.42	0.63	−0.83	1.67
Lob VI. conditioned vs. non-conditioned	0.54	0.64	−0.71	1.80
Lob VII. conditioned vs. non-conditioned	0.42	0.63	−0.82	1.67
Lob VIII. conditioned vs. non-conditioned	0.15	0.63	−1.09	1.39
Lob IX. conditioned vs. non-conditioned	0.23	0.63	−1.00	1.47
Lob X. conditioned vs. non-conditioned	0.01	0.63	−1.24	1.23

Table 6

Effect sizes of between-group differences found at the dorsal and ventral areas of the granular cell layer when comparing the conditioned to the non-conditioned group. Cohen's d statistics, with its corresponding standard error (SE) and 96% confidence intervals (CI) are provided. According to Cohen's own proposal, d values higher than 0.8 (or lower than−0.8) are considered as “large effects”. Cases in which the lower limit of the 95% CI corresponding to positive differences (increases) or the upper limit of the 95% CI corresponding to negative differences (decreases) yielded d values >0.8 or <−0.8 are highlighted.

	Cohen's d	SE	96%CI lower bound	96%CI upper bound
Purkinje				
Lob II. conditioned vs. non-conditioned	−0.29	0.63	−1.54	0.95
Lob III. conditioned vs non-conditioned	−1.15	0.68	−2.49	0.18
Lob V. conditioned vs. non-conditioned	−0.84	0.66	−2.14	0.44
Lob VI. conditioned vs. non-conditioned	−0.34	0.63	−1.58	0.90
Lob VII. conditioned vs. non-conditioned	**−1.70**	**0.73**	**−3.15**	**−0.25**
Lob VIII. conditioned vs. non-conditioned	−1.18	0.68	−2.53	0.15
Lob IX. conditioned vs. non-conditioned	−1.30	0.69	−2.66	0.06
Lob X. conditioned vs. non-conditioned	−0.41	0.63	−1.66	0.84
Brainstem inputs and medial nucleus				
N. pontine conditioned vs. non-conditioned	−0.08	0.63	−1.32	1.15
Olive n. conditioned vs. non-conditioned	−0.68	0.65	−1.95	0.59
Medial n. conditioned vs. non-conditioned	0.75	0.65	−0.52	2.03

Table 7

Effect sizes of between-group differences found at Purkinje, cerebellar inputs and the medial nucleus when comparing the conditioned to the non-conditioned group. Cohen's d statistics, with its corresponding standard error (SE) and 96% confidence intervals (CI) are provided. According to Cohen's own proposal, d values higher than 0.8 (or lower than−0.8) are considered as “large effects”. Cases in which the lower limit of the 95% CI corresponding to positive differences (increases) or the upper limit of the 95% CI corresponding to negative differences (decreases) yielded d values >0.8 or <−0.8 are highlighted in bold.

7. Figures

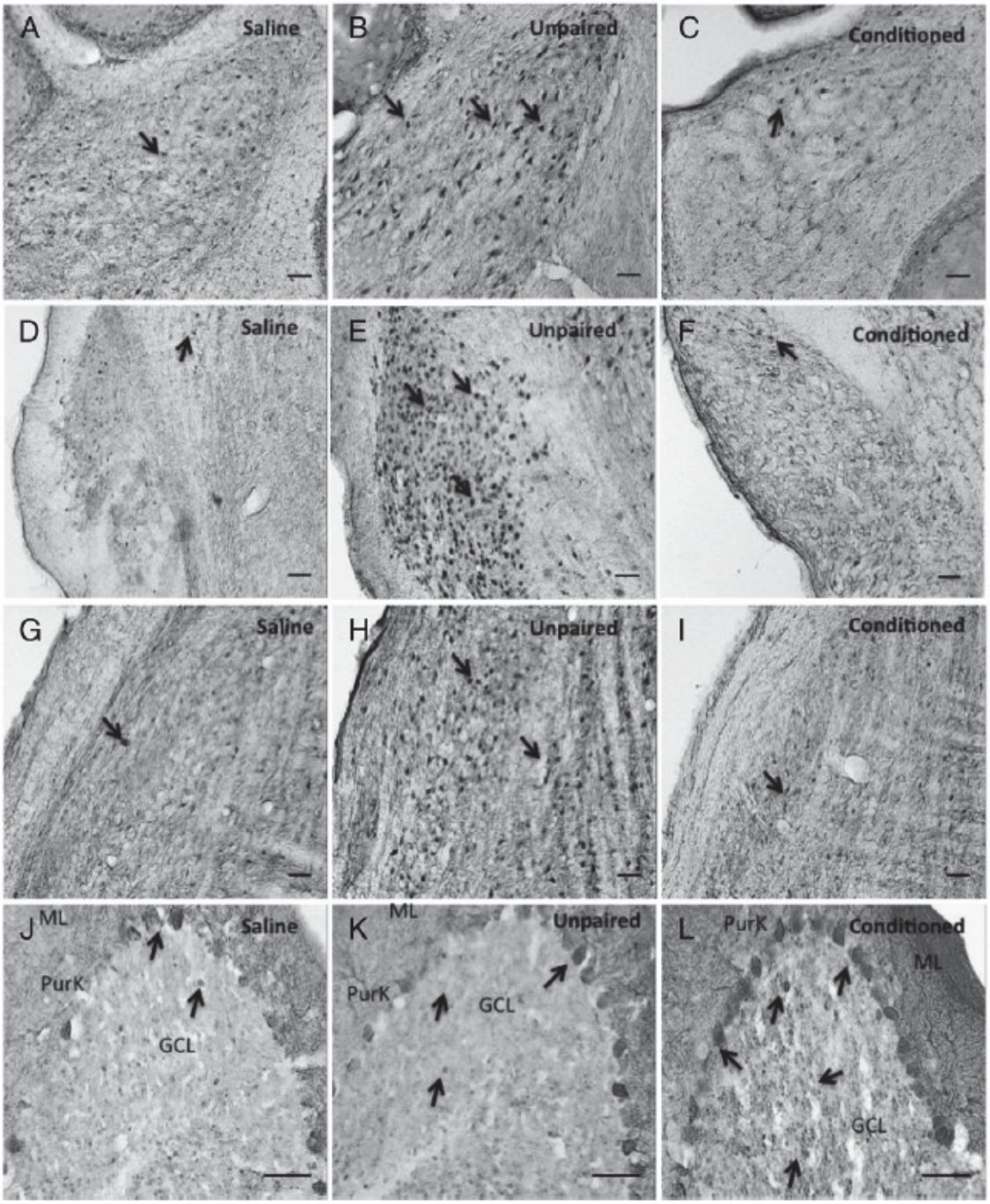

Fig. 1. Representative microphotographs of cFOS-IR in mice from the saline, conditioned and unpaired groups. The medial nucleus of the cerebellum (A, B, C); the pontine nucleus (D, E, F); the inferior olive (G, H, I) and the cerebellar cortex (J, K, L). Arrows indicate examples of cFOS+ cells. ML: molecular layer; PurK: Purkinje neurons; GCL: granule cell layer. Scale bar: 50 µm.

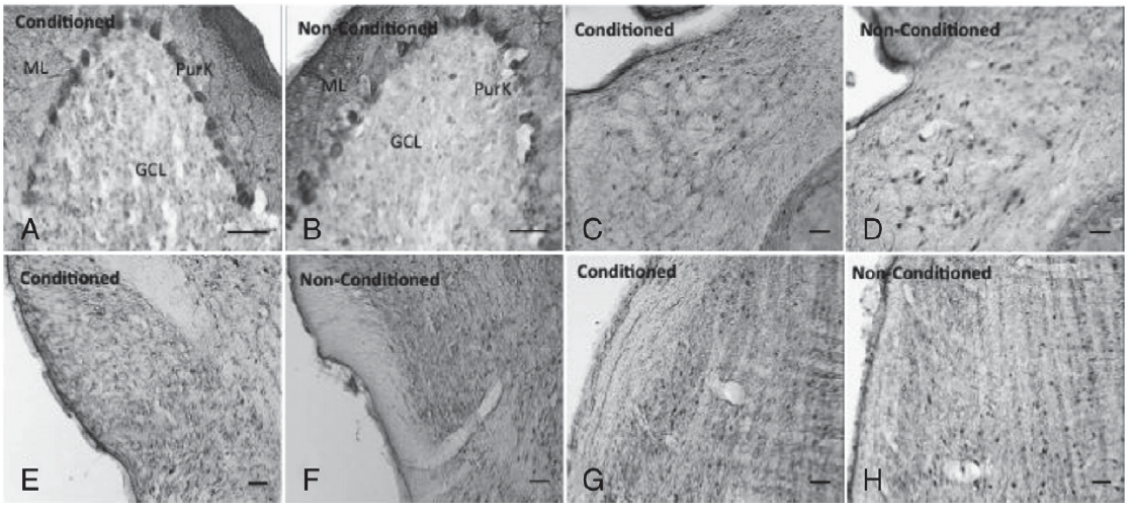

Fig. 2. Representative microphotographs of cFOS-IR from the conditioned and non-conditioned mice. The granule cell layer (A, B); the medial nucleus of the cerebellum (C, D); the pontine nucleus (E, F) and the inferior olive (G, H). ML: molecular layer; PurK: Purkinje neurons; GCL: granule cell layer; WM: white matter. Scale bar: 50 μm.

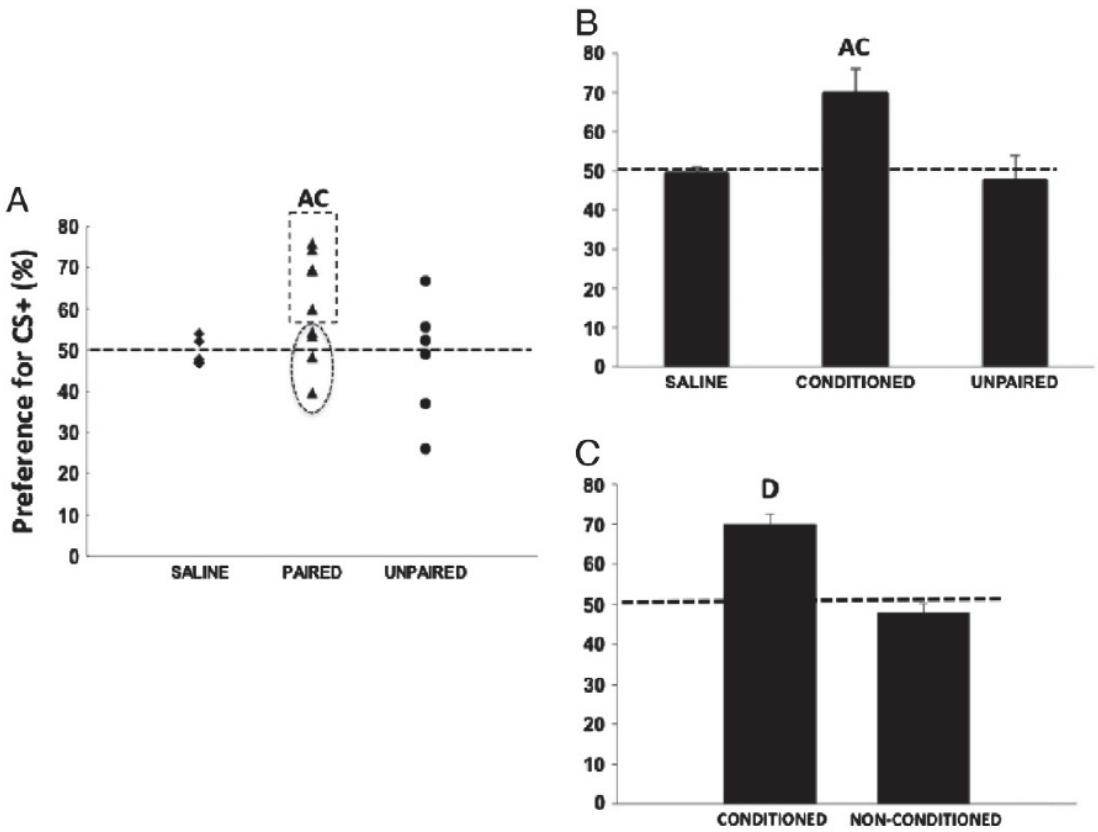

Fig. 3. (A) Scatterplot of cocaine-induced odour preference score in the three experimental groups. (B) Percentage of preference for cocaine-associated odour-cue on the test day in the saline, conditioned and unpaired groups. (C) Percentage of preference for cocaine-associated odour-cue in the conditioned and non-conditioned groups. Data are shown as mean± (SEM) of preference on the test day in each treatment group. Capital letters indicate a significant difference (p b 0.01) towards the saline (A), unpaired group (C) or non-conditioned (D), respectively.

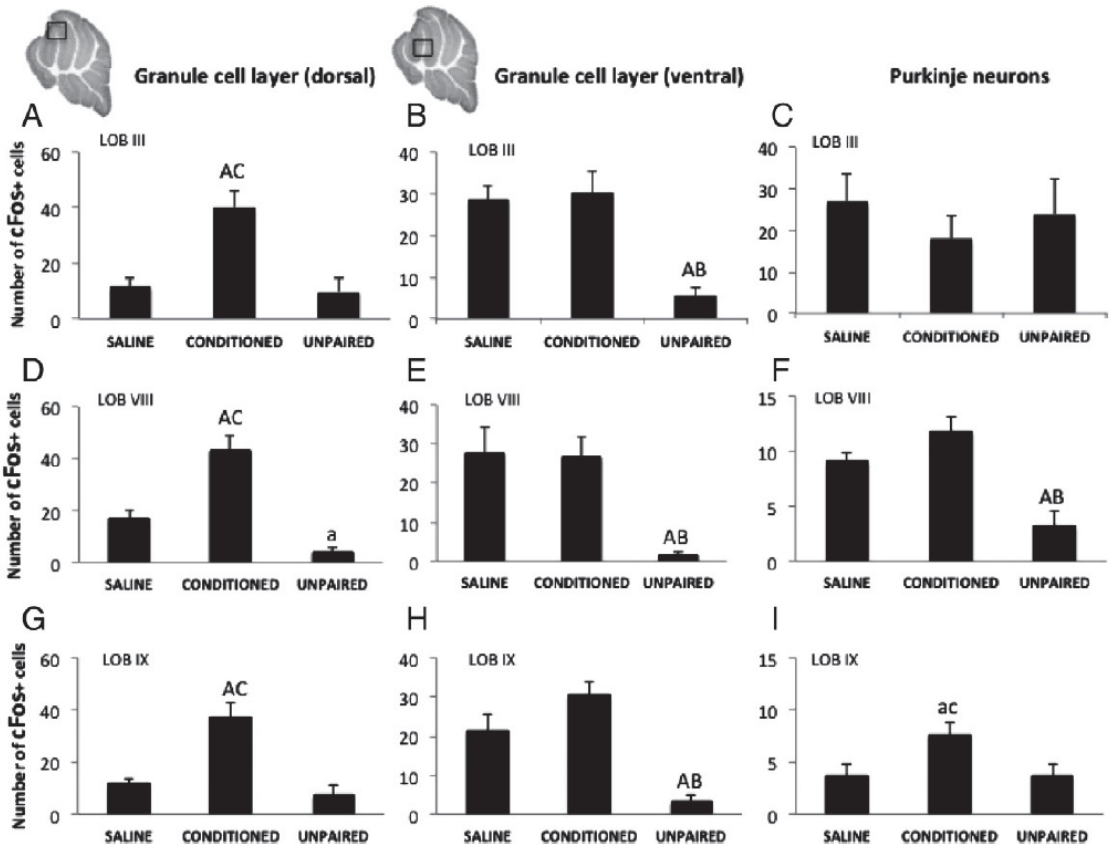

Fig. 4. cFOS-IR in different areas of the cerebellar cortex lobules III (A, B, C), VIII (D, E, F) and IX (G, H, I) of the saline, conditioned and unpaired groups. Panels depict mean± (SEM) of the number of cFOS+ neurons in the dorsal (A, D, G) and ventral (B, E, H) regions of the granule cell layer. Panels (C, F, I) represent mean ± (SEM) of cFOS+ Purkinje cells. Capital letters indicate a significant difference ($p < 0.01$) towards the saline (A), conditioned (B), or unpaired group (C), respectively. Minor letters indicate a significant difference ($p < 0.05$) towards the saline (a), conditioned (c) or unpaired group (c), respectively.

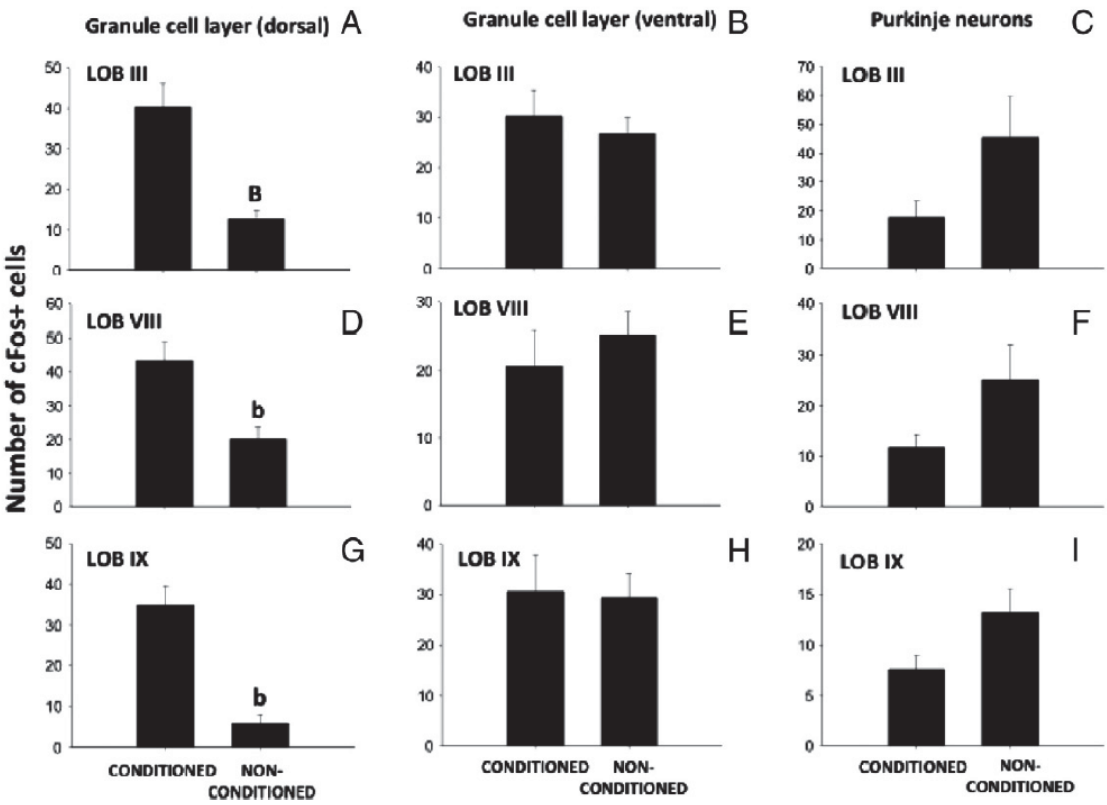

Fig. 5. cFOS-IR in lobules III (A, B, C), VIII (D, E, F) and IX (G, H, I) of the conditioned and non-conditioned groups. Panels showed mean ± (SEM) of the number of cFOS+ neurons in the dorsal (A, D, G) and medial (B, E, H) regions of the granule cell layer. Panels (C, F, I) represent mean ± (SEM) of cFOS+ Purkinje cells. Capital letters indicate a significant difference ($p < 0.01$) towards the conditioned group (B). Minor letters indicate a significant difference ($p < 0.05$) towards the conditioned group (b).

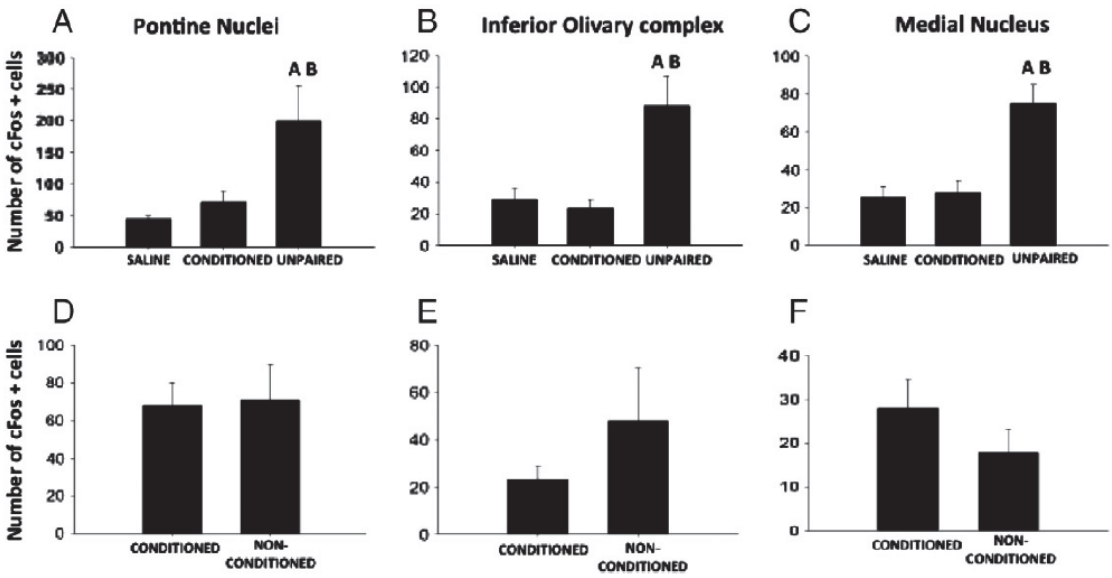

Fig. 6. cFOS-IR in the pontine nucleus (A, D); the olivary complex (B, E) and the medial nucleus (C, F). Panels A, B and C depict the results in the saline, conditioned and unpaired groups. Panels D, E and F showed cFOS expression in the conditioned and non- conditioned groups. Data are expressed as mean± (SEM) of the number of cFOS+ neurons in the ROIs evaluated. Capital letters indicate a significant difference ($p < 0.01$) towards the saline (A), or conditioned (B), respectively.

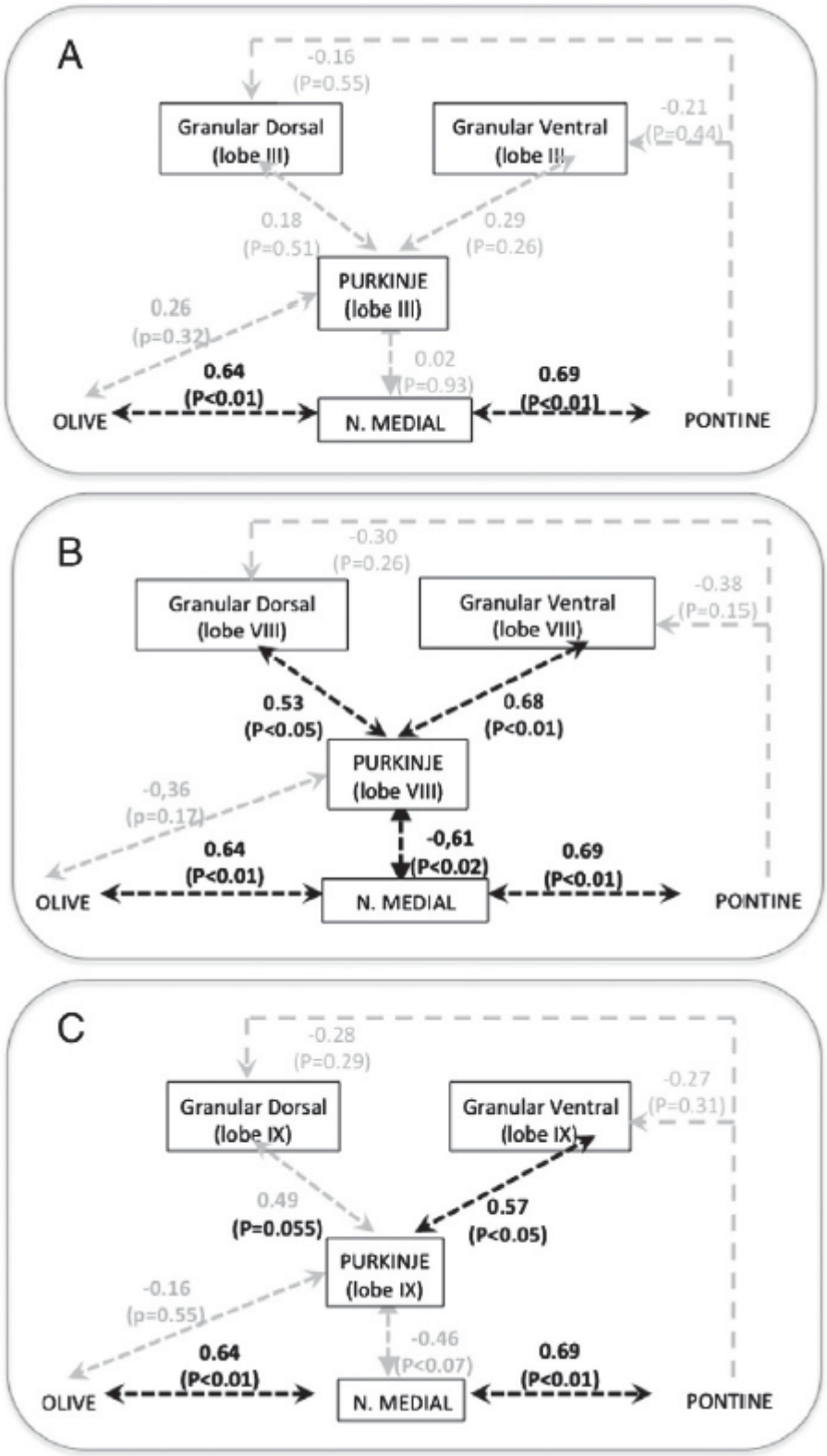

Fig. 7. Correlation matrices among the number of the c-Fos+ neurons found in different regions of the cerebellar cortex in lobule III (A); lobule VIII (B); lobule IX (C) and other anatomical regions of interest (ROIs). Dotted lines connect pairs of ROIs in which

correlation was analysed through the Spearman's Rho index (black lines $p \leq 0.05$, grey lines $p > 0.05$).

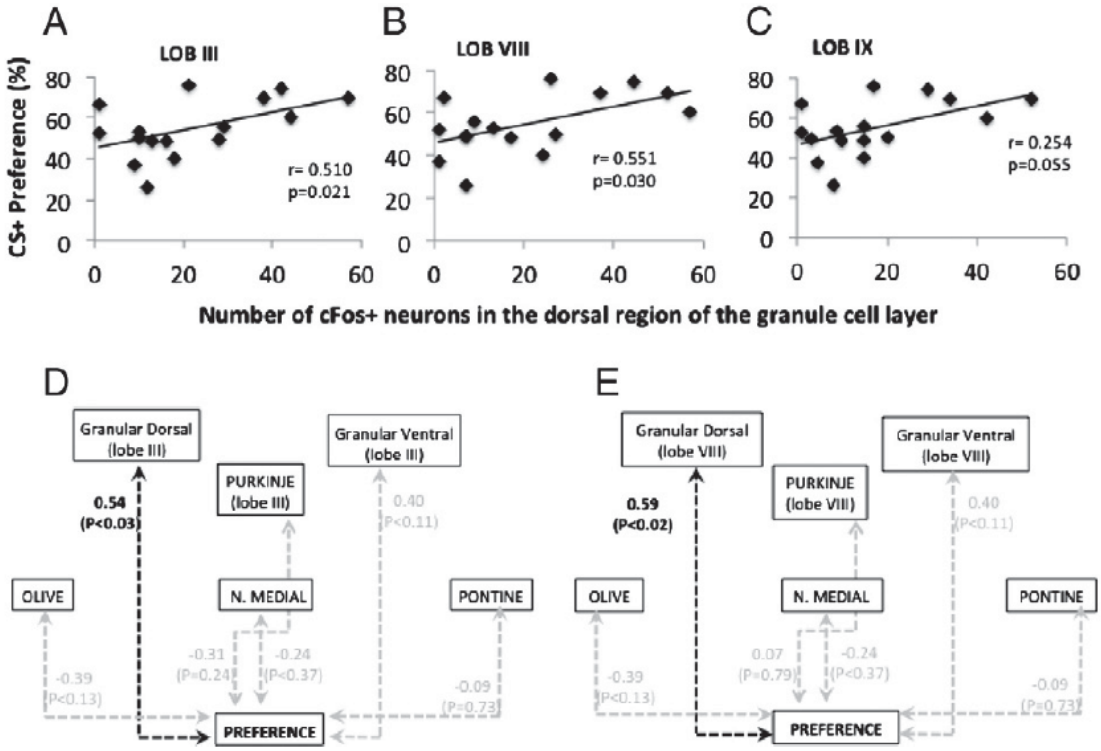

Fig. 8. Panels A, B and C show correlations between the number of the c-Fos+ neurons observed in the granule cell layer and the preference exhibited for the CS+ on the test day. Panels D and E depict correlation matrices among the number of the c-Fos+ neurons found in the anatomical regions of interest (ROIs) and the preference exhibited for CS+on the test day. Dotted lines were used to illustrate each one of the performed correlations using the Spearman's Rho index (black lines $p \leq 0.05$, grey lines $p > 0.05$).

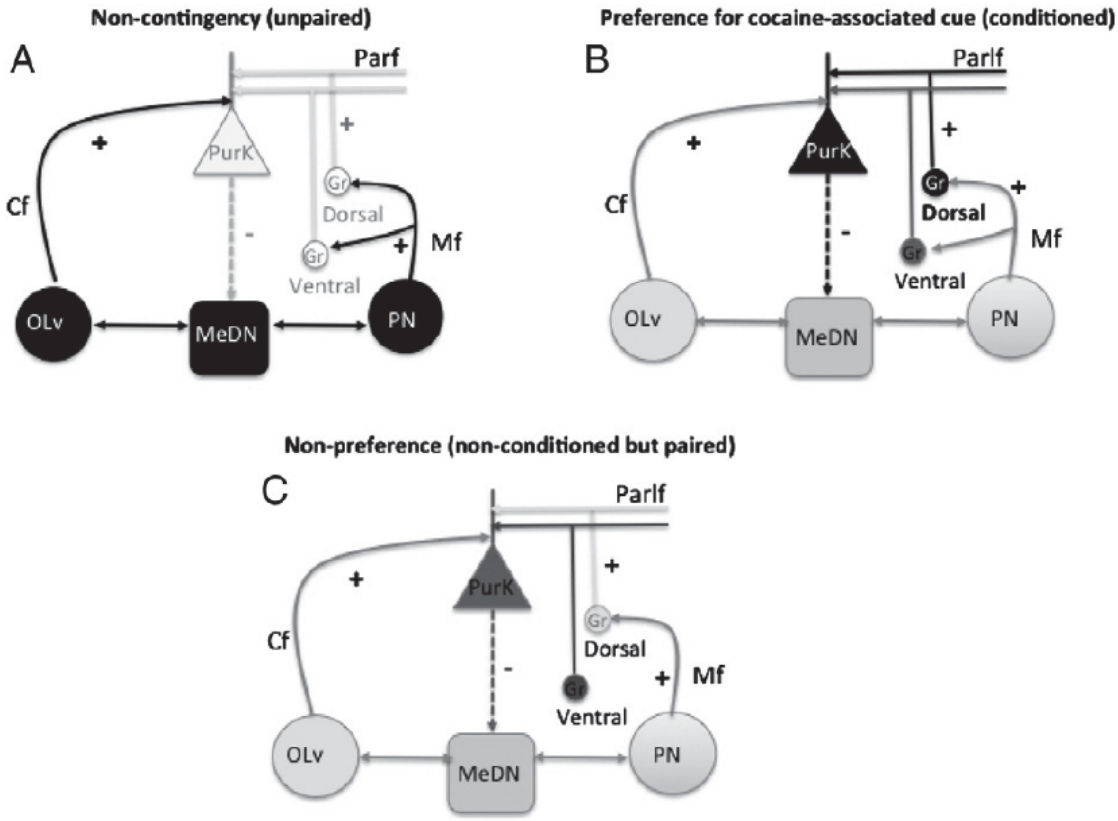

Fig. 9. Working models depicting the present findings. (A) The lack of a contingent relationship between CS and US was associated with increased excitatory inputs to the cerebellum (climbing and mossy fibers) (black diagrams). In turn, this could lead to higher activity in the medial cerebellar nucleus. Also, the lack of contingency was featured by a reduced expression of cFOS in the dorsal and ventral regions of the granule cell layer (white diagrams). After contingent training (B), the hallmark of preference towards a cue paired with cocaine was a higher activity in neurons located at the dorsal region of the granule cell layer (black diagrams). In lobule VIII, in addition, cocaine-induced learning appeared to also be related to higher activity in Purkinje cells (black diagrams), which could induce an inhibitory control onto the medial nucleus activity. Despite contingent cue-cocaine associative training, there is a subgroup of animals that did not express preference for the cue paired with cocaine (C). In this case, we did not observe any of the signatures of cocaine-induced preference. Non-conditioned animals

neither exhibited higher activity in the granule cell layer nor in Purkinje neurons. However, activity in the climbing and mossy excitatory inputs and in the deep medial nucleus remained similar to that of the group that develop preference for cocaine-associated cue.

Cerebellar perineuronal nets in cocaine-induced pavlovian memory: site does matter

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Abstract

One of the key mechanisms for the stabilization of synaptic changes is the formation of specific lattice extracellular matrix structures (ECM) surrounding several neuronal populations near the end of critical periods for experience-dependent plasticity during postnatal development. They are called perineuronal nets (PNNs). Few studies have focused on PNN in animal models of drug addiction. Findings in this field suggest that PNNs play diverse functional roles in various regions of the brain. Interestingly, recent data have indicated that the local circuits in the apex of the cerebellar cortex may be relevant to the formation of drug memories. Thus, the present research targeted PNN expression in the cerebellum using an animal model of cocaine-induced preference conditioning. Our results indicated that cocaine-related preference memory increased PNN expression surrounding Golgi inhibitory interneurons in the apex of the cerebellar cortex. Also, granule cells in this area were activated (higher cFos levels) selectively in animals that acquired cocaine reward memory. However, the PNNs surrounding projection neurons in the medial deep cerebellar nucleus (DCN) were reduced in all cocaine-treated groups, independently of whether animals expressed a preference for cocaine-related cues. A discriminant function analysis indicated that stronger PNNs in Golgi neurons and higher cFos levels in granule cells of the apex might be considered as the cerebellar hallmarks of cocaine-induced preference conditioning. A α6Cre-Cacna1a mutant mouse line was included in the present investigation to test the consequences of reducing granule cell capacity for glutamatergic transmission in PNN cerebellar expression. The acquisition of cocaine-induced preference conditioning was not affected by the reduction of output from granule cells, but retraining did not maintain cocaine-related pavlovian memory in KO mice. This consolidation impairment was selectively accompanied by a reduction in PNNs expression around Golgi cells in the

apex of the granule cell layer. Overall, PNN surrounding Golgi interneurons may play a role in consolidating drug-related memories.

Key words: cocaine, cerebellum, perineuronal nets, Golgi cell, preference conditioning.

Highlights

1. Cocaine preference memory increases PNNs surrounding Golgi cells in the cerebellar apex
2. PNNs in DCN projection neurons are unrelated to cocaine preference memory
3. The persistence of drug pavlovian memory is impaired in α6Cre-cacna1a KO mice
4. α6-Cacna mice show fainter PNNs around Golgi cells in the dorsal cerebellar cortex

1. Introduction

It is now accepted that addiction results from the capacity of drugs to produce neuroplasticity changes that render the brain "inflexible" and "enslaved" to drug-related contexts and stimuli (Solinas et al., 2010). Therefore, one of the main issues in the addiction field is to be able to turn the clock back and restore the brain plasticity potential. It is expected that by changing the conditions for drugs to activate long-term brain plasticity addicts will be capable of responding appropriately to future environmental challenges.

One of the key mechanisms for the stabilization of synaptic changes is the formation of specific extracellular matrix structures surrounding several neuronal populations at the end of the brain development, so called perineuronal nets (PNNs) (Kwok et al., 2011). A PNN is a cartilage-like structure consisting of molecules of extracellular matrix (versican, aggrecan, neurocan, brevican, hyaluronan, tenascin-R, link proteins, and semaphorin 3A) that wraps the perikaryon and proximal dendrites of particular types of neurons and is believed to create restrictive conditions for the emergence of new synaptic contacts and synaptic plasticity modifications in the adult brain (Brückner et al., 1993; Grimpe and Silver, 2002; Carulli et al., 2005; Carulli et al., 2006; Carulli et al., 2010). The latticed PNN structure leaves several gaps in which synapses are contained. PNNs help to maintain the synaptic architecture critical for synaptic plasticity (Frischknecht et al. 2009; Carstens et al., 2016), and regulate learning and memory (Gogolla et al., 2009; Romberg et al. 2013). Moreover, PNN structure and configuration has been proposed as a candidate mechanism for very long-term memory storage because of the stability of its components (Tsien, 2013).

The suggestion that PNNs might underlie the stability of drug plasticity changes is receiving increasing support in the last years (Wright and Harding, 2009; Van den Oever

et al., 2010; Xue et al., 2014; Slaker et al., 2015; Slaker et al., 2016; Vazquez-Sanroman et al. 2016 Sorg et al., 2016; Blacktop et al., 2017). Still, very few studies have focused on PNNs in animal models of drug addiction. Two pharmacological strategies have been used to target PNNs. The inhibition of metalloproteinases that could restore PNN expression demonstrated to decrease sensitivity to drug-related cues, preventing reinstatement (Brown et al., 2007; Van den Oever et al., 2010). In these two studies, however, the MMP inhibitor was administered i.c.v., affecting the whole brain. Other studies published more recently have pointed to the anatomical and functional selectivity in the effects of PNN disruption. Accordingly, PNN degradation in the prelimbic but not in the infralimbic cortex attenuated acquisition and reconsolidation of cocaine-induced conditioned place preference (Slaker et al., 2015). Moreover, the digestion of PNNs in the amygdala was capable of preventing subsequent priming-induced drug reinstatement if degradation was made before extinction (Xue et al., 2014). In this case, extinction effects increased. Very recently, Blacktop and colleagues (2017) have observed that removal of PNNs in the anterior dorsal lateral hypothalamic area using chrondroitinase ABC (ChABC) administration abolished the acquisition of cocaine-induced CPP and significantly attenuated cocaine self-administration (SA). Overall, these findings suggest that PNNs play diverse roles across brain regions. Plausibly, the consequences of PNN manipulation for drug-induced behavioural effects would rely on the functional specialization of the circuits in which those neurons expressing the PNN work.

Recently, we have found that PNNs surrounding DCN neurons appear to be under dynamic control during withdrawal periods (Vazquez–Sanroman et al., 2015ab). Prior findings from our lab also indicated that the local circuits in the apex of the cerebellar cortex might be an important and largely overlooked part of the networks involved in

forming, maintaining and/or retrieving drug memories (Carbo-Gas et al., 2014ab). Indeed, the acquisition of preference towards an olfactory cue associated with a repeated cocaine experience increased activity in the granule cells at the apex of the cerebellar cortex. This effect was not found when mice did not exhibit preference for the cocaine-related cue. Supporting data from animal models, human research also involves the cerebellum in drug-conditioned memories. Indeed, the activation of the cerebellum when drug-associated cues are presented has been a common finding in the majority of cue-reactivity neuroimaging studies (Grant et al., 1996; Kilts et al., 2001; Bonson et al., 2002; Anderson et al., 2006; Smolka et al., 2006; Filbey et al., 2009; Tomasi et al., 2015; Noori et al., 2016).

Given all these previous findings, the present research was aimed at assessing cerebellar PNN expression in an animal model of cocaine-induced preference conditioning (Carbo-Gas et al., 2014ab). This investigation included two different studies. First, we addressed a broad analysis of PNN expression encompassing the anterior and posterior cerebellar vermis in outbred mice trained to acquire preference for olfactory stimuli associated with cocaine. Second, α6Cre-Cacna1a KO strain mice (Galliano et al., 2013a) were used to test whether silencing the bulk of cerebellar granule cells would alter cocaine-preference conditioning and the expression of PNNs in the cerebellum.

2. Methods

2.1. Subjects

For the first experiment, five-week-old Swiss male mice were purchased from Janvier (ST Berthevin Cedex, France) and housed in our animal facilities (Jaume I University, Spain). Experimental procedures started at the age of 7 weeks. Handling was carried out daily for 5 min before the experiment began. For the second experiment, female and male mice of the following genotypes were used: Cacna1a$^{LoxP/LoxP}$/Cre+ (α6Cre-Cacna1a KO mice lacking P/Q-type VGCC in most of GC and Cacna1a$^{wt/wt}$/Cre- (i.e., wild-type) (N=14) (Galliano et al., 2013a). Briefly**,** α6Cre-Cacna1a KO mice were generated by cross-breeding of the floxed mouse line targeted for the gene encoding the pore-forming 1 subunit of P/Q-type VGCCs (Cacna1a$^{LoxP/LoxP}$ mice) with mice expressing Cre recombinase in the transgene of the α6 subunit of the $GABA_A$-receptors, which in the cerebellum is uniquely expressed in granule cells (Aller et al., 2003). The resulting deletion of functional CaV2.1-channels, which mediate ~90% of glutamate release from granule cell axon terminals (D'Angelo et al., 1997), results in a silencing of ~75% of all granule cells (Galliano et al., 2013a). Mice were transferred from the Department of Neuroscience, Erasmus MC Rotterdam (3015GE Rotterdam, The Netherlands) and maintained in our colony room for 7 days prior to experiments.

The colony room was maintained at 22 ± 2ºC temperature with lights on from 08:00 to 20:00 hours. Animals were housed under standard conditions with laboratory rodent chow (Panlab S.L, Barcelona, Spain) and tap water *ad libitum*. Behavioural tests were conducted within the first five hours of the light cycle. All animal procedures were approved by the local ethical committee and performed in accordance with the European Community Council directive (86/609/ECC), Spanish directive BOE 34/11370/2013,

and local directive DOGV 26/2010.

2.2. Pharmacological agents

All drugs were administered intraperitoneally (IP). Cocaine hydrochloride (Alcaliber S.A., Spain) was dissolved in 0.9% w/v saline (1 mg/ml), and injected immediately before each conditioning trial. Saline solution 0.9% w/v was used as the vehicle control.

2.3. Behavioural procedures

2.3.1. First study: Cocaine-induced preference conditioning in Swiss mice

Conditioning phase

Conditioning was carried out in a corridor that included two boxes (a rectangular Plexiglas box of 30 x 15 x 20 cm) at the end of each arm. Two equally preferred olfactory stimuli (papaya and strawberry) were used and counterbalanced between animals as CS+/CS-. One of the odours acted as CS+ and was associated with cocaine (20 mg/kg, IP) (US). The other one (CS-) was associated with saline injections. Four drops of papaya or strawberry scents were presented inside a steel ball with holes, which overhung on the walls at the ends of the corridor. The pairing sessions lasted for 15 minutes. A total of 8 cocaine-paired sessions were run (n=18). Additionally, we included two control groups: The unpaired group (UNP) (n=9), which was composed of animals receiving the same number of cocaine injections but randomly associated with the olfactory stimuli, and the "saline group" (SA) (n=9) including animals that were subjected to the same conditioning sessions but received only saline in all of them.

Preference Test

Preference was evaluated 24 hours after the last conditioning session in a 30-minute drug- free test using the same corridor as for the conditioning phase, but in which CS+

and CS- were present simultaneously in opposite arms and counterbalanced. All test sessions were videotaped and the time spent (TS) in each box was registered manually from the recorded test sessions during the last 20 minutes by a blind observer. Preference score was calculated as [Time spent in CS+/(Time spent in CS^{+}+Time spent in CS-)] x 100. The whole procedure has been described previously (Carbo-Gas et al. 2014ab). Locomotion during the test was estimated in the videotapes by drawing three sections through the corridor on the screen. A locomotion score was assigned every time the mouse crossed from one section to another on all four legs. Mice were perfused 70 minutes after the preference test.

Re-exposure to CS+

Twenty-four hours following the preference test, a different group of mice was confined to the CS+ box for 15 min with the CS+ olfactory cue present (n=10). Animals were perfused 70 minutes later. This new test was addressed to assess the effect of cue exposure on cerebellar plasticity in absence of any behavioural selection.

2.3.2. Second study: Cocaine-induced preference conditioning in α6Cre-Cacna1a KO mice

α6Cre-Cacna1a KO mice (n=7) and wild-type controls (n=7) were trained in cocaine-induced preference conditioning by using a bias procedure. It was a bias method because animals were conditioned (cocaine pairings) to the non-preferred olfactory cue. This method was selected to avoid counterbalance that would imply the use of a higher number of animals. The whole procedure included four steps as described below. Although we did not register the estrous cycle in female mice, no difference was observed between male and female in innate preference.

Innate preference test (preconditioning)

Innate preference test for both scents (lavender and jasmine) was conducted in a corridor that included two goal boxes at the end of each site. Each box contained one stainless steel ball with one of the two scents, and could be freely accessed through a doorway. Placement of the olfactory stimuli on the right or left arm was counterbalanced. The test lasted for 30 minutes and was videotaped. The time spent (TS) in each goal box of the corridor was registered manually on videotape during the last 20 minutes of the session by a blind observer. During the first ten minutes, animals were allowed to explore for odour cues. Preconditioning preference score was calculated as TS in CS+/ [(TS in CS+) + (TS in CS-)] x 100.

Conditioning phase

Because these mice exhibited an innate preference for jasmine, lavender was selected as the CS+, and thereby paired to cocaine (10 mg/kg, IP) for all the animals. On alternate days, mice were exposed to jasmine (CS-) and received saline injections. The pairing sessions lasted for 15 minutes and took place in one of the two goal boxes of the corridor on alternant days. Thus, CS+ and CS- occurred in different compartments. A total of 8 cocaine-paired sessions were conducted.

Preference Test

Preference tests were identical to the innate preference test. They were conducted 24, 96 hours, and 7 days after the last conditioning session.

Memory Reactivation

Then, we re-trained mice for two additional cocaine-paired sessions (4 days), 48 hours after the last preference test. Preference for CS+ was evaluated 24 hours later.

2.4. Perfusion and dissection protocol

Animals were deeply anesthetised with sodium pentobarbital (30mg/kg) 70 min after the last tests and perfused transcardially, first with 0.9% saline solution and then with 4% paraformaldehyde. After perfusion, the brain was immediately dissected and placed in a container with 4% paraformaldehyde for 24 h. After this time, tissue was cryoprotected in 30% sucrose solution until complete immersion. Brain tissue was rapidly frozen by immersion in liquid nitrogen and sections were performed at 40 µm with a cryostat (Microm HM560, Thermo Fisher Scientific, Barcelona, Spain). Four series of tissue sections were collected and stored at -80°C in cryoprotectant solution. Sagittal sections of the cerebellum and brainstem were selected according to lateral coordinates ranging from -0.04 mm to 0.72 mm. (Paxinos and Franklin, 2008).

2.5. Immunolabelling

PNN immunolabelling was performed on free-floating sections. After several rinses with PBS 0.1 M triton X-100 (1%), cerebellar sections were exposed to a blocking buffer with donkey serum, and then they were incubated overnight at 4ºC in 1.5% donkey serum dissolved in PBS 0.1 M Triton X-100 with biotinylated Wisteria floribunda agglutinin (WFA) (1:200; Sigma Aldrich, Madrid, Spain), and the following antibodies: anti-calretinin (1:500; Swant, CH1723 Marly, Switzerland), anti-metabotropic glutamate receptor 2 subunit (mGlu2) (1:1000; Abcam, Cambridge, UK), and anti-neurofilament H Non-phosporilated (SMI32) (1:500 Stenberger, Covance, USA). WFA binds to the glycosaminoglycan (GAG) chains of the proteoglycans (CSPGs) and was used to label the PNN structure (Hartig et al., 1992). Calretinin is expressed by brush cells, Lugaro neurons, and granule cells in the cerebellar cortex (Rogers, 1989). We also used mGlu2 antibody to label Golgi neurons (Neki et al., 1996), and SMI32 antibody to

identify projection neurons in DCN (Vazquez-Sanroman et al., 2015ab). Tissue samples were incubated for 2 hours at room temperature with the following secondary antibodies conjugated with fluorochromes: donkey anti-mouse Alexa Fluor 647 (1:500 Fisher Scientific, Life Technologies Limited, Paisley, UK), and Cy3-streptavidin (1:200 Jackson Immunoresearch Europe Ltd, Suffolk, UK). Once fluorescence reaction occurred, the sections were mounted using Mowiol (Calbiochem, Merck Chemicals and Life Science, Madrid, Spain).

For peroxidative immunostaining, tissue peroxidases were eliminated with 0,3% of H_2O_2 and 20% of methanol for 30 min. Tissue was serially incubated with rabbit anti-cFos (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 48 h, and then with WFA overnight. Tissue was then exposed to donkey anti-rabbit secondary antibody (1:400; Vector Labs, Inc., Burlingame, CA, USA) for 120 min. Amplification was accomplished with preassembled biotin-avidin peroxidase complex (ABC Elite. Vector Labs, Inc., Burlingame, CA, USA). Slices were revealed in a 3,3'-Diaminobenzidine (DAB) solution for 5 min (Vector Labs, Inc., Burlingame, CA, USA).

2.6. Image analysis

PNN were labelled and analysed in two cerebellar regions, the granule cell layer of the vermis and the DCN. In the granule cell layer, we took images of 15 PNN per mouse and lobule, in lobules 3, 8, and 9. These lobules were selected in order to obtain a representative sampling of the anterior and posterior vermis. A 40x objective with a 2.5 zoom was used for a final magnification of 100x. Due to the low PNN density in the dorsal region of the granule cell layer, 15 PNNs per lobule involved a broad sample of PNNs surrounding Golgi cells in this region. Also, 50 SMI32+ DCN projection neurons bearing a PNN were evaluated. In this case, a 40x objective with a 2.0 zoom was used

for a final magnification of 80x. Brightness intensity of every PNN was estimated by randomly selecting 15 pixels in the net and calculating their average intensity. Data were expressed as arbitrary units of intensity (AU of PNN intensity) with a maximum intensity of 255. Pictures of fluorescent-labelled sections were taken with a Leica SP8 confocal microscope at a resolution of 1024x1024 and 100 Hz speed. Laser intensity, gain and offset were maintained constant in each analysis. All quantitative evaluations were made using the FIJI software *(*Schindelin et al., 2012*).*

cFos expression was analysed in three sagittal sections per mouse and lobule, in the same animals and aforementioned regions of the cerebellum. For quantification, we selected a ROI of 60.000 μm^2 in which every cFos+ neuron was tagged. Cells were quantified using the cell-counter plug-in from FIJI software *(*Schindelin et al., 2012*).*

3. Statistical analysis

All behavioural and biochemical experiments were performed blind. For statistical analyses, we used the Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS (IBM) software packages. Data were expressed as mean and standard error of the mean (SEM). They were analysed by means of univariate one-way or two-way repeated measures ANOVAs, as well as unpaired Welch-corrected two-tail t-tests. Tukey HSD tests were used as parametric *post-hoc* tests, when required. The level of significance was set at $p < 0.05$.

We used a discriminant function analysis to unravel the architecture of the reciprocal differences between groups according to their cFos and WFA immunolabelling at several cerebellar areas. Discriminant function analysis is a statistical procedure that might be used to determine which variables discriminate between two or more (K) naturally occurring groups. When only two groups are involved, discriminant function

analysis is analogous to multiple regression and the interpretation of the standardized coefficients of the obtained discriminant function is analogous to that of the weights (beta) of multiple regression equations. However, when more than two groups are included, this procedure provides a number (K-1) of discriminant functions. For example, when there are three groups, a function is estimated to discriminate between Group 1 and Groups 2 and 3 combined, and another function for discriminating between Group 2 and Group 3. Again, the interpretation of the standardized coefficients of these functions is straightforward and similar to these of multiple regression equations and, therefore, this procedure allows the identification of the unique contribution of each variable to the discrimination of multiple groups. More specifically, a forward stepwise approach was taken to step-by-step build a statistical model that would hierarchically reveal how much each one of these predictors contributed to the discrimination between groups

4. Results

4.1. First study: Cerebellar signatures of cocaine-induced preference conditioning in Swiss mice

Preference scores

According to our previous findings (Carbo-Gas et al., 2014ab), eight CS+/cocaine pairings induced a significant preference conditioning in 60% of the mice that were contingently trained to associate an olfactory cue with 8 cocaine administrations. However, the rest of the animals in this group did not exhibited any preference for the CS+ despite having been trained under the same experimental conditions. As it is clear in Figure 1A, there are mice that showed an opposite preference pattern in the paired group (PA), and thereby, when they were collapsed no preference could be demonstrated [$F(2,21)=1.03$, $p>0.05$]. Because we were interested in identifying the cerebellar signatures of cocaine-induced memory, the paired group was split into two groups, the preference (PREF) and no preference (NOPRE) groups, by using an arbitrary cut-off point of 60% of time spent in the CS+. These two groups were then compared to the saline (SA) and unpaired (UNP) controls. In this case, a one-way ANOVA yield significant differences [$F(3,20)=28.55$, $p<0.01$]. As expected, post-hoc comparison showed that the PREF group ($p<0.01$) was the only group that showed preference for the CS+ (Figure 1B). The SA, UNP, and NOPRE groups exhibited indifference and did not differ from each other. The number of entries to CS+ box was slightly higher in the PREF group [SAL= 19.17±3.49; UNP=29.50±3.48; NOPRE=19.66±3.08; PREF=33.17±3.5]. A one-way ANOVA yield marginally significant differences between groups [$F(3,20)=3.06$; $p=0.052$], although Tukey tests were not able to demonstrate differences between group means. The number of entries to the CS- compartment did not show any difference between groups.

Motor activity during the preference test

We estimated motor activity during the whole preference test session by splitting the 30-minutes period into 6 periods of 5 min. The number of crossovers was registered within every of these 5-min periods. A two-way ANOVA demonstrated that motor activity decayed for all groups throughout the test session, as shown by significant differences observed for the time factor (min) [$F(5,100)=75.51$; $p<0.001$]; but neither group factor [$F(3,20)=1.28$; $p>0.05$] nor interaction [$F(15,100)=1.17$; $p>0.05$] were significant (Figure 1C).

Perineuronal nets in the cerebellum

The evaluation of PNN was addressed in two regions of the cerebellum: the granule cell layer and the deep medial nucleus. In the granule cell layer, we observed PNN exclusively expressed surrounding Golgi interneurons. As is shown in Figure 2, a loose matrix surrounds other cell types. Our observations replicated previous findings (Corvetti and Rossi, 2005; Carulli et al., 2006). Interestingly, the pericellular nets surrounding the soma and basal dendrites of Golgi neurons were significantly stronger and more prominent in PREF mice ($p<0.01$ for all comparisons) (Figure 3) in all lobules assessed [Lob 3 $F(3,19)=27.24$, $p<0.001$; Lob 8 $F(3, 19)=28.29$, $p<0.001$; Lob 9 $F(3, 19)=7.28$, $p<0.01$]. The SAL, UNP, and NOPRE groups showed fainter PNN and, thereby a lower average intensity of WFA. The majority of the strong fully condense Golgi nets in the PREF group were found in the apex of the cerebellar cortex (Figure 2), over the plexus, the most superficial region of the granular layer (Ramón and Cajal, 1911).

Remarkably, eight cocaine administrations reduced WFA staining surrounding projection neurons (SMI32+ cells) of the deep medial nucleus [$F(3,19)=5.07$, $p<0.01$],

in all the cocaine-treated groups independently whether or not they expressed preference for the cocaine-associated cue ($p<0.05$ as compared to the SAL group) (Figure 4). Thus, unlike the PNN surrounding Golgi neurons, nets in the deep medial nucleus appeared to be uncorrelated to cocaine pavlovian memory.

cFos expression

As previously observed (Carbo-Gas et al., 2014ab) in the present study, cocaine-induced preference conditioning was associated with a higher cFos expression in granule cells at the apex of the cerebellar cortex (Figure 5). The increase in cFos in lobules 3 [$F(3,20)=6.90$, $p<0.01$], and 8 [$F(3,20)=13.70$, $p<0.0001$] was selective for PREF mice ($p<0.01$ for all comparison), and it was not found in any of the other three groups. In lobule 9, the pattern was almost the same as the above mentioned [$F(3,20)=39.35$, $p<0.0001$], except for the NOPRE group that exhibited larger cFos levels than the SA and UNP groups ($p<0.01$), but lower than PREF mice ($p<0.01$).

In addition, we evaluated the deep medial nucleus, and the two cerebellar afferent regions from the brainstem: the inferior olive and pontine nuclei (Figure 5). These regions have been demonstrated to show elevated levels of cFos expression specifically in the UNP group (Carbo-Gas et al., 2014b). In the present study, we observed very similar results. Indeed, the three regions showed larger cFos staining when the association between cues and drug was random and no contingencie could be learned (UNP) [Medial N ($F(3,20)=3.82$, $p<0.05$); I Olive ($F(3,20)=4.42$, $p<0.05$); and Pontine N ($F(3,20)=5.26$, $p<0.01$)]; (Tukey HSD tests: The Pontine N, $p<0.01$; Medial N and I. Olive, $p<0.05$) (Figure 6).

Discriminant function analysis

To build up our discriminant model between PREF, NOPRE and UNP groups, 18 independent predictors were used. These predictors corresponded to: cFos levels in cerebellar lobules 3, 8 and 9; in deep medial; inferior olive and pontine nuclei. Also, WFA expression in cerebellar lobules 3, 8 and 9, and the deep medial nucleus were included in our model. After 4 iterations, the resulting model explained the 100% of the observed variance (see table 1) and two discriminant functions were obtained: *function 1* mostly distinguished the PREF from the NOPRE and UNP groups (explained variance 81.4%; eigenvalue: 28.12); and *function 2* distinguished between these two later groups (explained variance 18.6%; eigenvalue: 6.41). The two functions achieved statistical significance [Wilks-Lambda (8)= 0.005, $p<0.001$], and [Wilks-Lambda (3)= 0.135, $p<0.001$], respectively. The canonical correlation was very high in both cases (0.983 and 0.930, respectively), hence indicating that both of them had more than adequate discriminate capabilities.

Discriminant function 1 mainly involved the distinction between the PREF vs. the NOPRE and the UNP groups, with the former being associated with high scores in this function (Figure 6). Accordingly, the PREF group was featured by high levels of WFA intensity and cFos staining at lobule 9, accompanied by low levels of cFos staining at the pontine nucleus and, to a minor extent, by elevated levels of WFA staining in lobule 3 (see table 1). On the other hand, discriminant function 2 was especially useful to discriminate between the NOPRE and UNP groups (Figure 6). More specifically, subjects belonging to the UNP group showed high (positive) values on this function and the subjects belonging to the NOPRE group showed low (negative) values on this function (members of the conditioned group had scores close to zero). In this second discriminant function, high scores were associated with: low WFA staining in the

lobules 3 and 9; low levels of cFos staining in the granular layer of lobule 9; and high cFos expression in the pontine nuclei (see Table 1)

Cerebellar PNN and cFos expression after re-exposure to the CS+

Twenty-four hours following the preference test [t (7)=6.33, p<0.001], a different subgroup of PREF and NOPRE mice (n=5) were confined to the training box and presented only with the CS+ in absence of cocaine (Figure 7). The nets surrounding Golgi neurons were still significantly more prominent and stronger only in lobules 8 [t (7)=2.39, p<0.05], and 9 [(t (7)=2.38, p<0.05] of PREF confined animals (Figure 7). No significant differences were found in the nets surrounding DCN neurons [t (6)=1.28, p=0.25] (Figure 7).

Interestingly, the CS+ presentation abolished the differences between PREF and NOPRE groups in cFos expression in all lobules evaluated [t (7)=0.89, p=0.40]. The number of cFos+ neurons lowered in both groups (Figure 7) regarding the levels observed in the above-mentioned group of mice after the preference test (NOPRE=44%; PREF=67% of reduction) (see also Figure 5). As can be observed, the decrease was more noticeable in the PREF group after being confined under the sole presence of the CS+. Of note, there was no significant difference between PREF and NOPRE mice in motor activity during the preference test previous to the CS+ re-exposure [NOPRE= 89.60±13.24; PREF=92.60±14.02] [Unpaired t-test: [t (8)=0.19, p=0.85].

4.2. Second study: Cocaine-induced preference conditioning and PNN in the cerebellum of α6Cre-Cacna1a KO mice

The targeted mutation for the gene encoding the pore-forming α1 subunit of P/Q-type VGCC is expressed in most (from 75% to 86%) but not all granule cells, allowing only a partial glutamatergic control over Purkinje activation from parallel fibres-Purkinje

synapses (see Galliano et al., 2013a for a full description). Worthy of mention, granule cells are those which expressed higher levels of activity (cFos+) exclusively in mice that developed conditioned preference for cocaine-related cues (the PREF group). Therefore, α6Cre-Cacna1a mutant mouse line was included in the present investigation as a causal approach to test whether by reducing the granule cells capacity for glutamatergic transmission cocaine-induced preference memory and PNN in Golgi might be affected.

The acquisition of cocaine-induced preference conditioning was not affected by the reduction of the output from granule cells (Figure 8). Both WT (control littermates) and KO (α6Cre-Cacna1a) mice spent more time during the first preference test (24h) in the CS+ compartment in spite this was the arm with the initially non-preferred cue. Preference tests were repeated after 96 hours and then, after 1 week, to estimate memory decay. Preference for the CS+ was maintained in WT mice even after a period as long as 12 days. However, the KO group showed a marginal decay after the same period. A two-way ANOVA with repeated measures yield significant differences only for the test factor [$F(3,36)=5.46$, $p<0.01$]. The group factor was marginally significant [$F(1, 12) = 3.31$, $p=0.09$]. The interaction group x tests was non-significant [$F(3, 36) = 1.06$; $p=0.37$].

Following the last preference test, we retrained animals to evaluate whether reactivating the CS+/US association could improve memory in KO mice (Figure 8). While WT mice maintained intact their preference for the cocaine-related cue, retraining did not strengthen cocaine-related pavlovian memory in KO mice, and thereby preference declined to pre-conditioning levels [The group factor: $F(1, 24)=9.87$, $p<0.01$; Tests: $F(1, 24)=8.70$, $p<0.01$; group x tests interaction was non-significant: $F(1, 24) = 1.55$; $p=0.22$] (Figure 8).

Remarkably, KO mice showed a significant reduction in PNN expression surrounding Golgi neurons in the apex of the granule cell layer [Unpaired t-test: Lob 3: $t(10)=8.32$, $p<0.001$; Lob 8: $t(9)=18.03$, $p<0.001$; Lob 9: $t(7)=9.29$, $p<0.01$] (Figure 8). There was no significant difference in the number of Golgi+ neurons for WFA staining [Unpaired t-test: Lob 3: $t(12)=1.83$, $p=0.09$; Lob 8: $t(12)=0.31$, $p=0.76$; Lob 9: $t(12)=0.54$, $p=0.59$]. Unlike the PNNs around Golgi cells, the nets surrounding the large projection neurons in the DCN were not different from those of the WT group [Unpaired t-test: $t(8)=0.16$, $p>0.05$] (Figure 8).

5. Discussion

The aberrant strength and persistence of drug-induced plasticity and associated memories are thought to have a primary role in drug seeking and relapse because they compel goal-directed behaviours towards contexts with drug availability (Everitt and Robbins, 2005; Kalivas et al., 2005; Hyman et al., 2006). In the present study, we found that cocaine-related preference memory produced an up-regulation of the perineuronal nets surrounding Golgi inhibitory interneurons in the apex of the cerebellar cortex. PNNs surrounding Golgi neurons were strong and fully condensed in mice that expressed preference for the olfactory stimulus associated with cocaine. Moreover, neurons in this region of the granule cell layer were activated when cocaine reward memory was expressed. We also found that the PNN regulation by cocaine in the cerebellum presents regional specificity. Unlike Golgi cells, the PNNs surrounding projection neurons in the deep medial nucleus decreased in all cocaine-treated groups, independently of preference conditioning. A succinct summary of these results has been discussed in a recent review by Sorg and co-workers (2016) as unpublished data. We used a discriminant function analysis to clarify the architecture of the reciprocal differences between groups according to their WFA expression and cFos levels in different cerebellar regions. Two discriminant functions were obtained, and the resulting model explained the 100% of the variance. The model revealed that stronger PNNs in Golgi neurons and higher cFos levels in granule cells of the apex might be considered as the cerebellar hallmarks signatures of cocaine-induced preference conditioning. Indeed, neither of these features was found when mice did not express preference for the cocaine-related cue (SAL, NOPRE and UNP groups).

The increased cFos expression in the granule cells of this cerebellar region could result from elevated levels of motor activity during the preference test session. As a matter of

fact, even though there were not significant differences between groups, PREF mice exhibited moderately higher levels of activity during the test. Nevertheless, these higher levels were mainly due to a larger number of visits to the CS+ arm. Therefore, our results suggest that this neural activity might reflect the behavioural selection driven by the cocaine-related cue. In support of this conclusion, when behavioural selection was not possible, as happened during confinement in the only presence of CS+, cFos levels in PREF mice were not different from that exhibited by the NOPRE group. Unlike cFos, the PNN expression around Golgi interneurons were found unaltered after confinement, thereby indicating a stable mechanism.

Golgi neurones play a crucial role in modulating the activity and plasticity of local circuits in the cerebellar cortex (see D'Angelo et al., 2013 for a review). This regulation comprises: synchronization of the activity of granule cell clusters; control of spike timing and bursts in granule cells (Eccles et al., 1964); as well as the modulation of the direction of plasticity in mossy fibre-granule cell synapses (D'Angelo et al., 1999; Armano et al., 2000). Golgi cell activity is controlled by glutamatergic inputs from granule cells (GC) and mossy fibres (MFs) (Palay and Chan-Palay, 1974), as well as by GABAergic and glycinergic inhibitory signals from other populations of cerebellar interneurons (Sotelo and Llinas, 1972; Dumoulin et al., 2001). Golgi-mediated inhibition appears to be experience-dependent. When a sensory input arrives at the cerebellum through MF, it activates Golgi and granule cells, thus triggering the feedforward inhibitory loop (D'Angelo and De Zeeuw, 2009). Moreover, Golgi cells are key elements to controlling synaptic plasticity at the level of the granule cell layer. When Golgi inhibition is blocked LTP is induced in vitro (Mapelli and D'Angelo, 2007), and in vivo (Roggeri et al., 2008). On the contrary, when inhibition is higher than excitation, LTD is developed in granule cells (D'Angelo and De Zeeuw, 2009).

Consequently, Golgi cells appear to be crucial to information processing in the circuitry of the cerebellar cortex.

In the present research, interfering partially with the glutamatergic signal in parallel fibers-Purkinje synapses impaired consolidation but not acquisition of cocaine-induced preference conditioning. Remarkably, Golgi PNN in the cerebellum of α6Cre-Cacna1a mice were fainter and less prominent than those of WT littermates. We would speculate that fainter PNNs around Golgi neurons could be the result of the reduced glutamatergic input from granule cells. It is clear that neural activity affects PNN formation (Pizzorruso et al., 2002). It is possible that the targeted mutation for the gene encoding the pore-forming α1 subunit of P/Q-type VGCC (α6Cre-Cacna1a) affected not only the direct input to Purkinje cells, but also the indirect route to inhibitory interneurons (Galliano et al., 2013a). Then, the excitatory control of granule cells over Golgi neurons might be compromised in KO mice reducing PNN formation, and hence the persistence of drug memory. Prior findings using motor, emotional and cognitive tasks found that α6Cre-Cacna1a KO mice show a normal cerebellar cytoarchitecture, no impairment in motor performance, intact acquisition or consolidation of explicit spatial memory, and a normal olfactory perception (Galliano et al., 2013ab). However, they have demonstrated impairment of motor learning and consolidation (Galliano et al., 2013a). Importantly, memory deficits in these KO mice were associated with impaired synaptic plasticity processes (LTP/ LTD) in parallel fibres-Purkinje synapses (Galliano et al., 2013a). Strikingly, α6Cre-Cacna1a KO mice did not exhibit deficits in either cue or contextual fear conditioning (Galliano et al., 2013b), despite the cerebellar cortex has shown to be crucial to the consolidation of fear conditioning (Sacchetti et al., 2002, 2005).

In the mature brain, PNN components stabilise neuronal circuits at the cost of plasticity (Pizzorusso et al., 2002; Gundelfinger et al., 2010; Valenzuela et al., 2014). Nevertheless, the mechanisms through which PNN affect synaptic plasticity are still under discussion (Kwok et al., 2011). The majority of molecules that composed a PNN are resistant to the intracellular degradation systems (Tsien, 2013). However, there are intrinsic molecular mechanisms to regulate and convert non-permissive cues into permissive ones (Valenzuela et al., 2014). It is known that by degrading PNNs or altering PNN composition it is possible to turn the clock back in cortical plasticity (Pizzorusso et al., 2002; Carulli et a. 2010; Beurdeley et al. 2012). Sprouting and formation of new synaptic contacts in intact or injured CNS are promoted after ECM enzymatic degradation by chondrotinase (Corvetti and Rossi, 2005; Barritt et al. 2006; Soleman et al. 2012; Starkey et al. 2012) or when PNNs are genetically reduced (Carulli et al. 2010). Also, AMPA receptor motility falls near the PNN area, and it can be restored after PNN degradation (Frischknecht et al., 2012). Finally, ECM may act as a scaffold for elements that inhibit synaptic remodelling (Valenzuela et al., 2014).

In the present research, we show that cocaine-related preference memory enhanced selectively PNN expression around Golgi interneurons. Recently, we have proposed that a fully condensed PNN surrounding Golgi cells might “stamp in” the synaptic arrangement related to cue-drug associations, then preventing ulterior synaptic changes in the local circuits of the granule cell layer (Sorg et al., 2016). On the contrary, fainter PNNs around these neurons would facilitate the probability of reconfiguration of drug-cue neural representations and thereby, affect memory stability and reconsolidation. As a matter of fact, the degradation of PNNs in the prelimbic cortex using the bacterial enzyme Chrondotinase-ABC before memory reactivation blunted reinstatement of

cocaine-induced place preference, indicating impairment in drug memory reconsolidation (Slaker et al., 2016).

Interestingly, PNNs surrounding projection neurons in DCN were reduced in all cocaine-treated mice, and then they were not linked to cocaine-induced preference conditioning. Moreover, the expression of DCN PNNs remained unaffected in α6Cre-Cacna1a mice. Hence, consolidation deficits were also dissociated from PNN expression in DCN neurons. Our previous findings indicate that nets wrapping DCN neurons change during withdrawal periods following a repeated cocaine experience (Vazquez–Sanroman et al., 2105ab). Short withdrawal increased these PNN (Vazquez-Sanroman et al., 2015a), whereas long withdrawal decreased them (Vazquez-Sanroman et al., 2015b). This opposite regulation occurred in both cases 24 hours after relapse (modelled by a new cocaine challenge), and was linked to restrictive (Vazquez-Sanroman et al., 2015a) or permissive (Vazquez-Sanroman et al., 2015b) plasticity events in Purkinje neurons. Therefore, PNNs in DCN seem to work as a mechanism to dynamically regulate the cerebellar output depending on the state of local circuits in the cerebellar cortex (Sorg et al., 2016).

Taken together, the present findings suggest that PNNs would be a mechanism for memory stabilisation. Nevertheless, two important issues remain unexplored. First, at which point in time PNNs were upregulated around Golgi cells is unknown. It is clear that 24 hours following the last conditioning trial the PNN structure already showed a highly condense configuration in PREF mice. However, a reduced PNN expression did not prevent from exhibiting cocaine-induced preference conditioning at that time point, as was seen in α6Cre-Cacna1a KO mice. Second, it is also unknown through which pathways CS+ (olfactory stimulus) and US (cocaine) signals reach the cerebellar cortex (Miquel et al., 2016). Cocaine effects include different interoceptive signals but also

direct drug central actions on striatum cortico-limbic and cerebellar molecular targets (Carbo-Gas et al., 2014ab). The apex of the cerebellar cortex receives sensorimotor corticopontine and extereoceptive components of the MF afferent system (Ekerot and Larson, 1972; Voogd and Ruigrok, 2004; Voogd, 2014). This pathway provides neural information from cortical sensorimotor networks to the cerebellum, forming cortico-thalamic-cerebellar loops (Suzuki et al., 2012; Bostan et al., 2013). Also, vermis-prelimbic cortex functional connectivity has been demonstrated in rodents through the fastigial nucleus (Watson et al., 2014). Moreover, the cerebellum seems to be modulated by dopamine signals. An acute stimulation of dopaminergic neurons increases neural activity in the vermis (Herrera-Meza et al., 2014). Finally, a direct ventral tegmental area (VTA)-cerebellar projection pathway has been described (Ikai et al, 1992; Ikai et al., 1994), and DA changes can be detected in posterior lobules of the vermis VII–X, right and left hemispheres and the fastigial, dentate and interpositus deep nuclei (Glaser et al., 2006).

6. Conclusions

Supporting previous data regarding the cerebellar role in Pavlovian memory (Sacchetti et al., 2002, 2005; Thompson and Steinmetz, 2009; Boele et al., 2010), our results support that the cerebellum is an important region for the persistence of drug-related memories. Moreover, the present findings indicate that the regulation of PNNs around Golgi neurons in the cerebellar cortex might be a relevant mechanism for the stability of drug-related memories. Nevertheless, a definitive explanation for PNN functions in Golgi neurons requires new experiments addressing the enzymatic degradation of CSPG in the cerebellar PNN by Ch-ABC during various stages of drug-induced learning and memory.

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8. Figures

STEP	PREDICTOR	GROUP			
			Pref	No Pre	Unp
1	WFA L9	PREF F value P value	---	154.757 0.000	137.870 0.000
		NOPRE F value P value	154.757 0.000	---	0.757 0.398
		UNP F value P value	137.870 0.000	0.757 0.398	---
	F1: 0.395 F2: -1.328				
2	cFos L9	PREF F value P value	---	72.233 0.000	81.317 0.00
		NOPRE F value P value	72.233 0.000	---	18.203 0.000
		UNP F value P value	81.317 0.00	18.203 0.000	---
	F1: -0.856 F2: 0.534				
3	cFos pontine	PREF F value P value	---	69.475 0.000	110.693 0.000
		NOPRE F value P value	69.475 0.000	---	19.069 0.000
		UNP F value P value	110.693 0.000	19.069 0.000	---
	F1: 0.192 F2: 0.898				
4	WFA L3	PREF F value P value	---	57.520 0.000	6.693 0.000
		NOPRE F value P value	57.520 0.000	---	21.183 0.000
		UNP F value P value	76.693 0.000	21.183 0.000	---
	F1: 0.940 F2: 0.419				
			Centroid	Centroid	Centroid
	Discriminant	Function 1	6.743	-2.348	-4.395
	Discriminant	Function 2	0.564	-3.071	2.507

Table 1. Step-wise construction of the discriminant functions between the PREF, NOPRE and UNP groups First column (left) displays the ordinal entry of the four

predictors (second column) required to construct the two discriminant functions effectively separating our three experimental groups. Within the second column, the predictors' names and their corresponding standardized coefficients in each discriminant function are included. The third column (group) displays the F and p values for all dyadic between-group comparisons at each step of the discriminant analysis. As it can be seen on the first row of this table, a statistically significant discrimination of the PREF vs. the NOPRE and UNP groups (but not between the last two) was achieved already at the first iteration. A statistically significant discrimination among all three groups was obtained at the second iteration of the model (second row). However, the addition of two more predictors (third and fourth rows) was required to satisfactorily explain the 100% of the observed variance. On the other hand, the last two rows of this table display the centroid of the PREF, NOPRE and UNP groups in each discriminant function. From these centroid values, it is readily appreciable that discriminant function 1 was especially useful to separate the PREF from the NOPRE and UNP groups, while the discriminant function 2 mainly discriminated between these last two groups (see main text and figure 6 for further details).

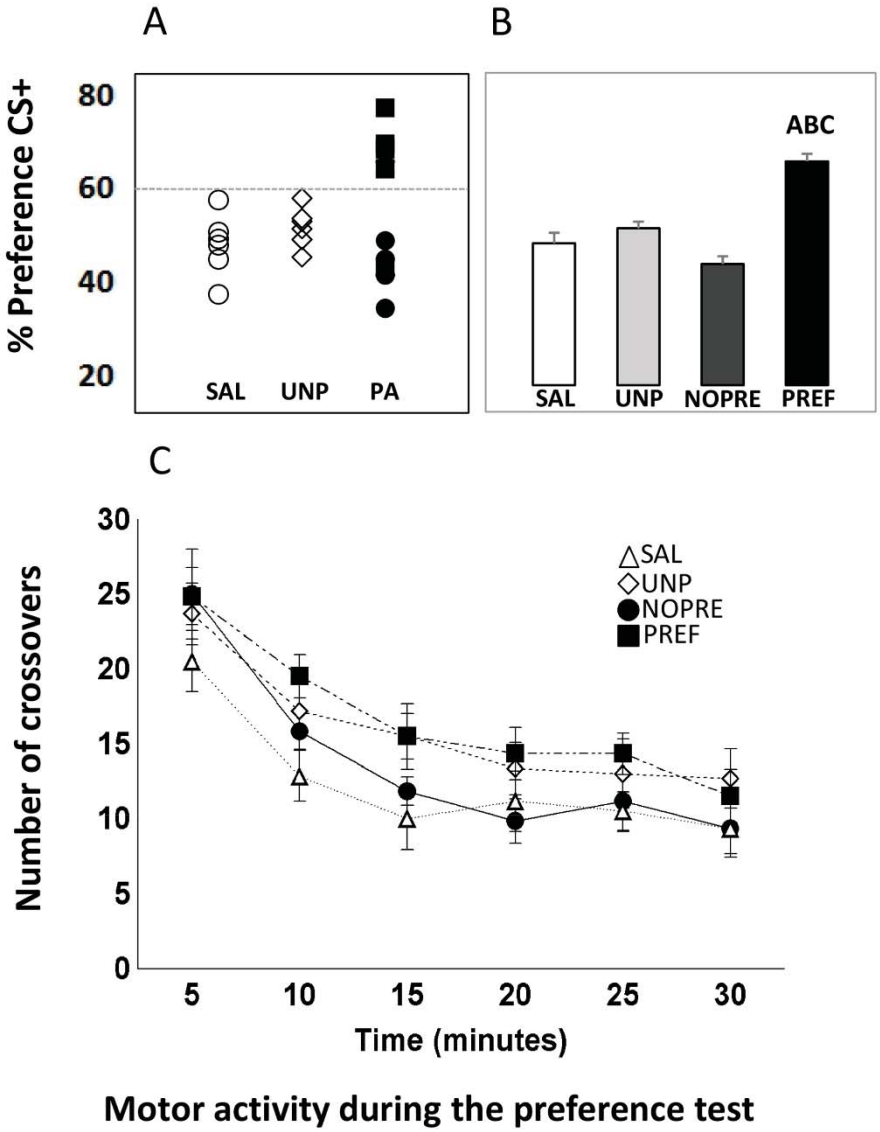


Fig. 1 (A) Scatterplot of cocaine-induced olfactory preference in the three experimental groups: saline (SAL), unpaired (UNP) and paired (PA). The panel depicts individual scores of time spent in CS+ compartment on the test day (%). Mice expressing a preference for CS+ higher than 60% (cutting-off point represented as dotted grey lines) were assigned latter to the preference group (PREF) **(B)** Percentage of preference for the cocaine associated odour-cue (CS+) on the test day in the saline (SAL), unpaired (UNP), no preference (NOPRE) and preference (PREF) groups. Data are shown as mean ± SEM. Capital letters indicate a significant difference of $p<0.01$ **(C)** Locomotion

during the preference test. Mean ± SEM of number of crossover every 5 minutes. No significant differences were found between groups in motor activity during the test session.

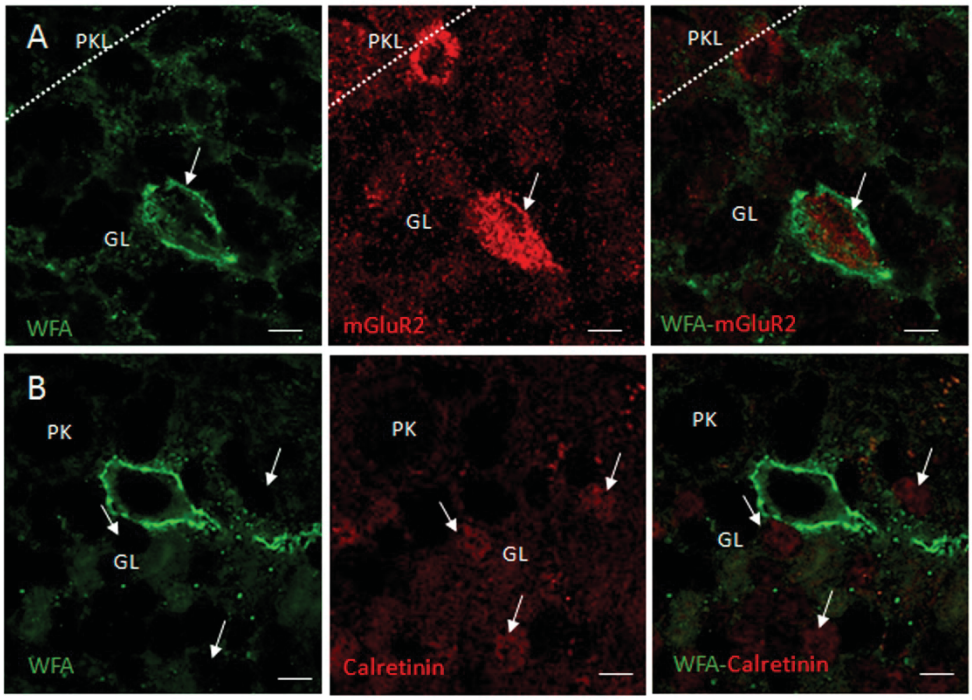


Fig. 2 Phenotype of neurons surrounded by a PNN in the cerebellar corex. **(A)** A representative microphotograph of the apical region of lobule IX from a sagittal section stained with Wisteria floribunda agglutinin (WFA) (green) and mGluR2 (red), an immunomarker for Golgi cells. As can be observed, PNNs are expressed surrounding Golgi neurons near the plexus, the more superficial region of the granule cell layer. Purkinje layer (PKL), Granular layer (GL). The confocal images were acquired with a 40x lens and a digital zoom of 2.5 for a final amplification of 100x. Arrows point to examples of Golgi interneurons surrounded by a PNN. **(B)** A sagittal section of the apex in lobule IX stained with WFA (green) and calretinin (red), a calcium-binding protein expressed by Lugaro, brush and granule cells. Neurons surrounded by a PNN did not express calretinin. White arrows point to examples of calretinin positive cells. Scale bars of 20 µm.

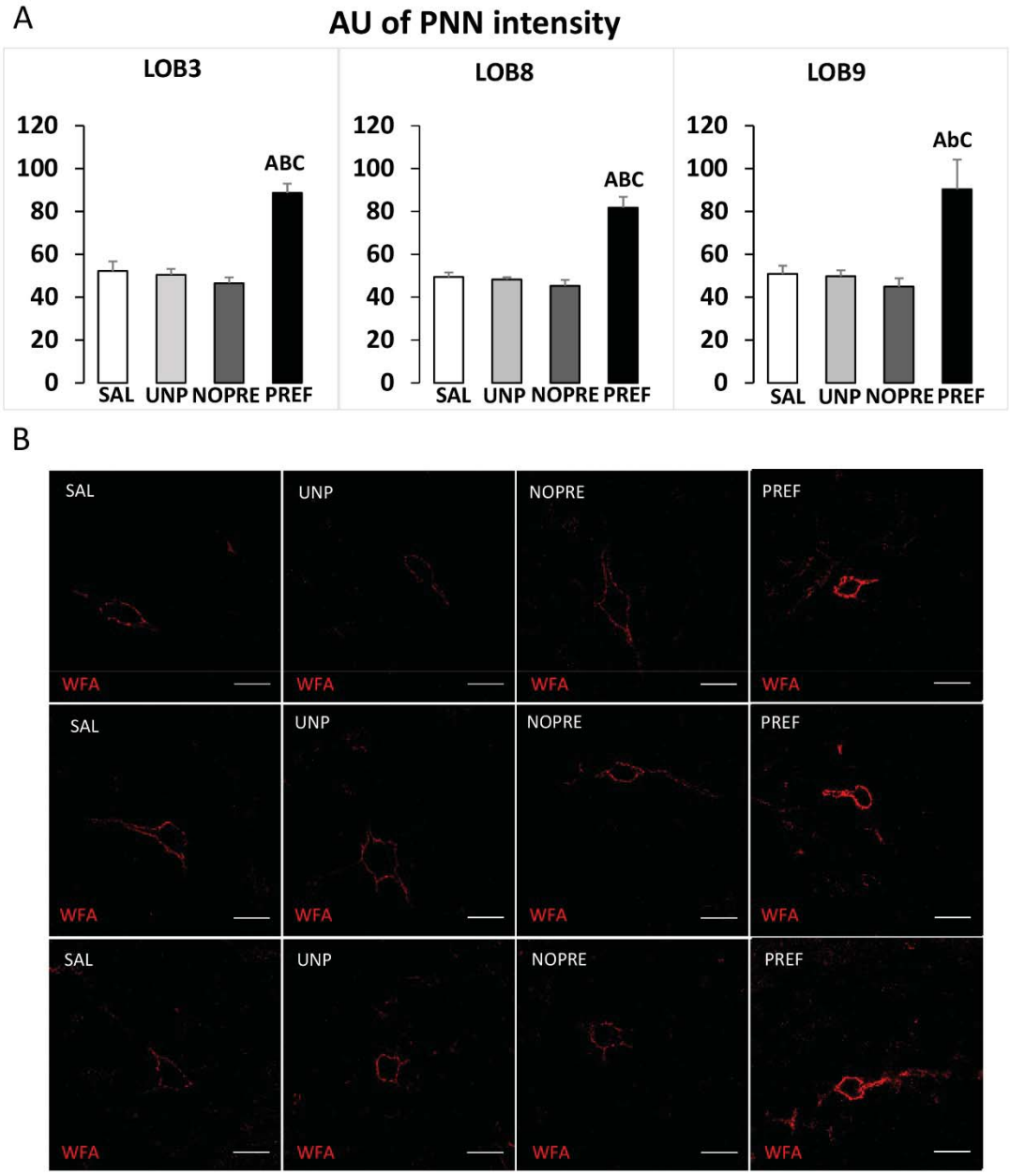


Fig. 3 PNN expression surrounding Golgi neurons in the cerebellar cortex. **(A)** Average intensity of WFA in the apex of the granule cell layer of lobules 3, 8, and 9. Saline (SAL), unpaired (UNP), no preference (NOPRE) and preference (PREF) groups. Data are shown as mean ± SEM. Lowercase letters indicate $p<0.05$, whereas capital letters indicate $p<0.01$. **(B)** Representative microphotographs of PNNs in the apical region of cerebellar cortex stained with Wisteria floribunda agglutinin (WFA). The confocal

images were acquired at 40x with a 2.5x zoom for a final amplification of 100x. Scale bar of 20 µm. Only animals that acquired preference for cocaine-associated cue demonstrated to have strong fully condense PNNs.

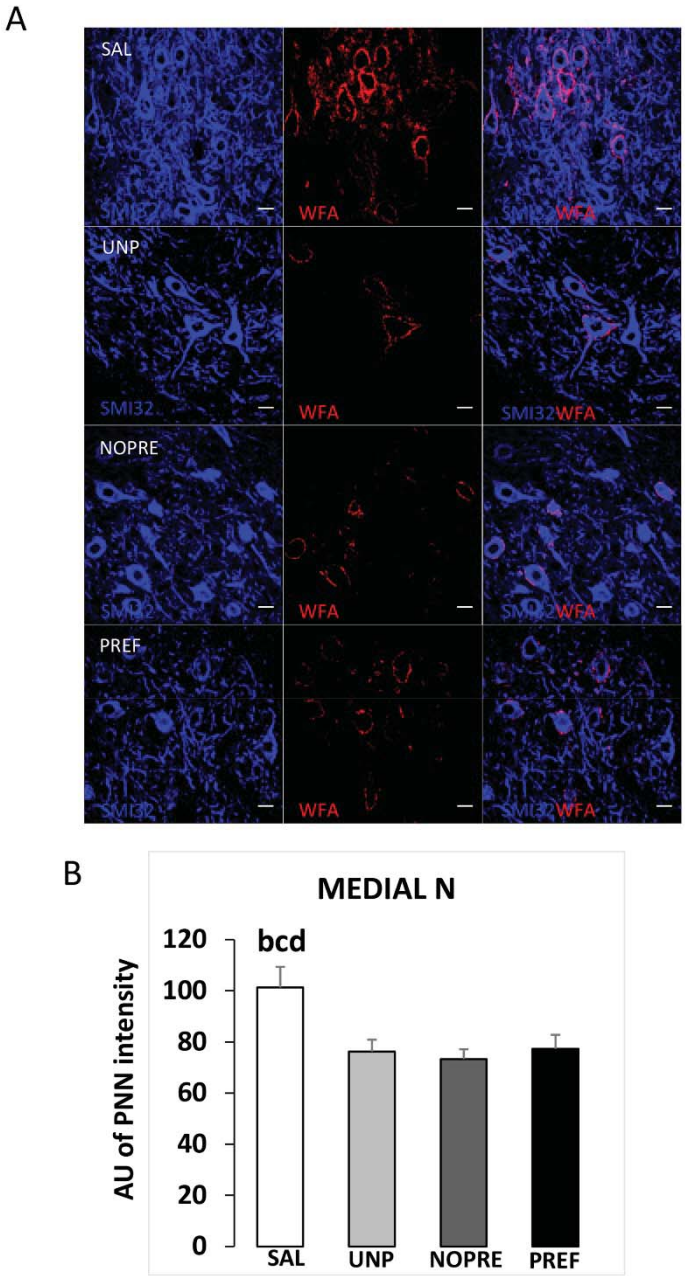


Fig. 4 PNN in the medial nucleus (DCN). **(A)** Representative microphotographs of PNNs stained with Wisteria floribunda agglutinin (WFA) (red) in DCN projection neurons identified by SMI32 stained (blue). The confocal images were acquired at 40x with a 2x zoom for a final amplification of 80x. Scale bar of 20 µm. **(B)** Average

intensity of WFA in DCN in saline (SAL), unpaired (UNP), no preference (NOPRE) and preference (PREF) groups. Data are shown as mean (columns) ± SEM (bars). Lowercase letters indicate a significant difference of $p<0.0.5$. Cocaine down-regulated PNN expression in DCN.

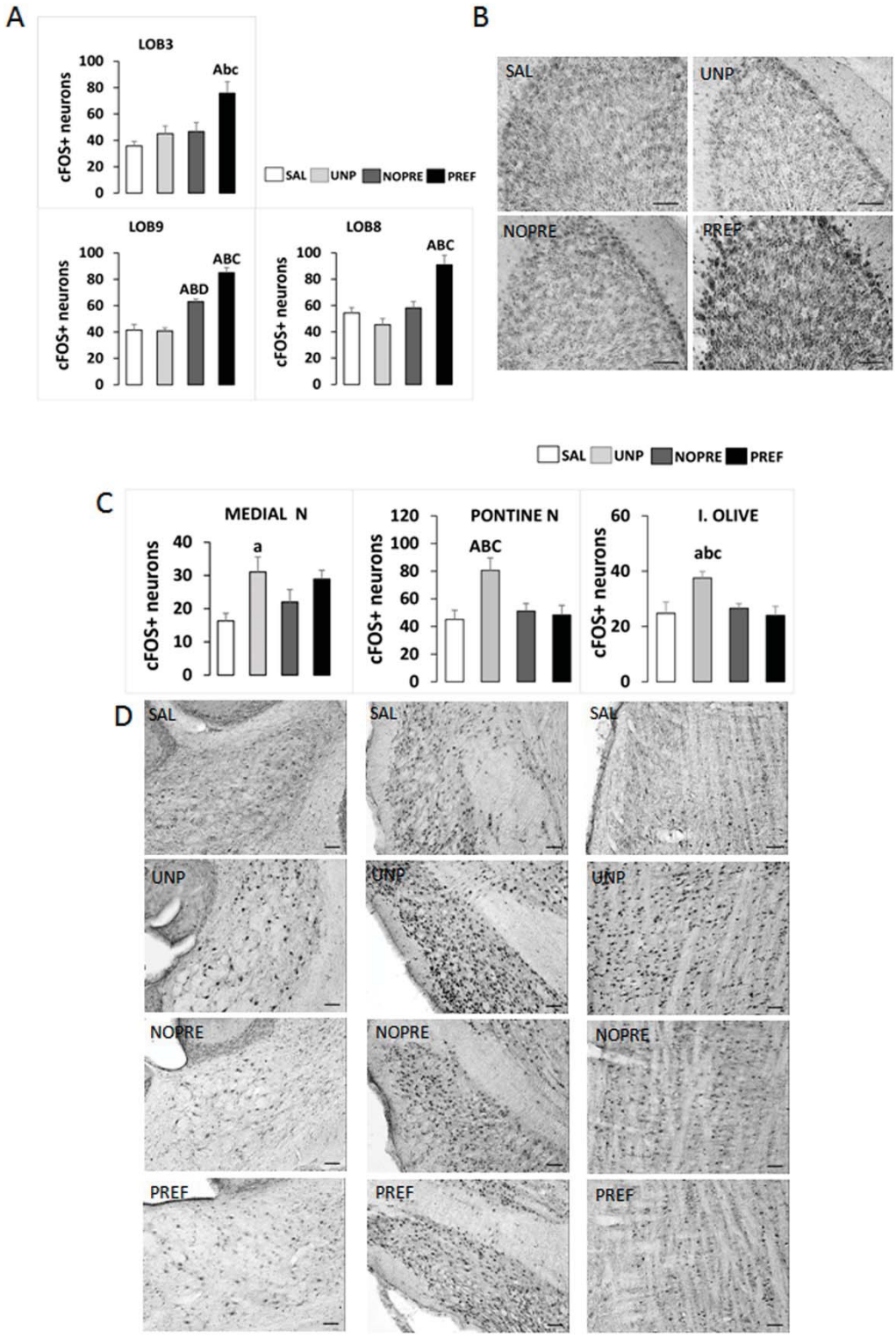


Fig. 5 (A) Average number of cFos+ neurons in a selected ROI of 60.000 μm^2 at the apex of the granular cell layer in lobules 3, 8, and 9. Saline (SAL), unpaired (UNP), no

preference (NOPRE) and preference (PREF) groups; ($p<0.05$, lowercase letters) ($p<0.01$, capital letters). Cocaine-induced preference conditioning increased cFos expression in the apical region of the cerebellar cortex. **(B)** Representative pictures of cFos expression in the apex of lobule IX. Images were taken with a 40x lens. Scale bar of 50 μm. **(C)** Average number of cFos+ neurons in a selected ROI of 60.000 μm^2 in the medial nucleus, pontine nuclei, and inferior olive. Data are shown as mean± SEM; (lowercase letters: $p<0.05$), (uppercase letters: $p<0.01$). cFos expression increased in the cerebellar afferent and efferent regions after a non-contingent cocaine administration (UNP). (D) Representative images of cFos expression in the medial nucleus (left), pontine nuclei (middle), and inferior olive (right). Images were taken with a 20x lens. Scale bar of 50 μm.

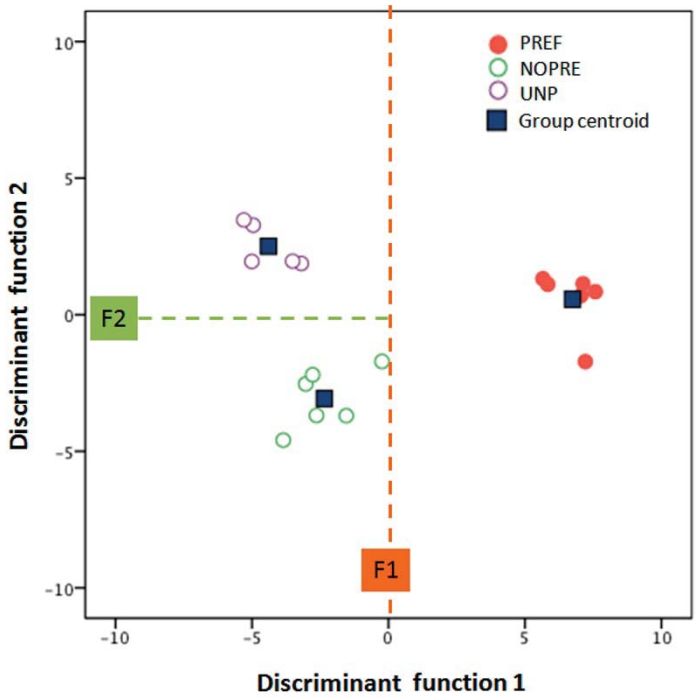
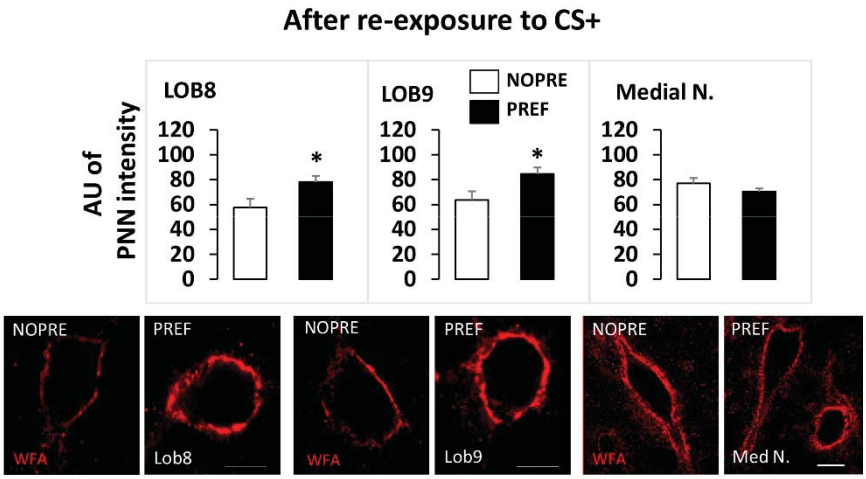
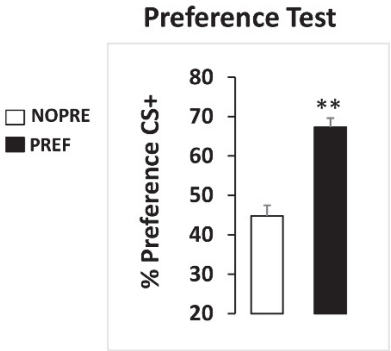


Fig. 6 Dispersion diagram of cases in the plane defined by the two obtained discriminant functions. Individual values are represented as circles (red, green and purple for PREF, NOPRE and UNP group members, respectively) while group centroids are depicted as blue squares. For illustrative purposes, dashed lines demarcating the 0 value of each discriminant function were manually added. As it can be readily appreciated in this diagram, discriminant function 1 (X axis) distinguishes between the PREF group (with all its members obtaining positive values on this function) from the NOPRE and UNP groups (all of their members obtaining negative values on function 1). Complementarily, discriminant function 2 (Y axis) separates the NOPRE and UNP groups, which members obtain negative and positive values on this function, respectively. See text for further details.

A





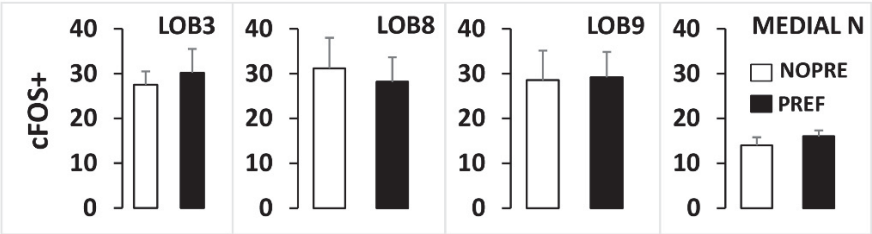


Fig. 7 (A) Percentage of preference for the cocaine associated olfactory cue on the test day before re-exposure to CS+. No preference (NOPRE) and preference (PREF) groups. Data are shown as mean ± SEM; (**p<0.01). **(B)** PNN expression in Golgi neurons after re-exposure to CS+. Top panel: Average intensity of WFA at the apex of the granule cell layer in lobules 8 and 9, as well as in the medial nucleus. Data are shown as mean ± SEM; (*p<0.05; **p<0.01). Bottom panel: Representative microphotographs of PNNs in the apex of cerebellar cortex and DCN stained with Wisteria floribunda agglutinin

(WFA) (red). The confocal images were acquired at 40x with a 2.5x or 2x zoom for a final amplification of 100x/80x, respectively. Scale bar of 20 μm. PNN surrounding Golgi cells were still stronger and more prominent in the PREF group after CS+ re-exposure. **(C)** Average number of cFos+ neurons after re-exposure to CS+ in a selected ROI of 60.000 μm^2 at the apex of the granular cell layer, and DCN (Medial N). Data are shown as mean ± SEM. Differences in cFos expression between PREF and NOPRE groups were occluded after CS+ re-exposure.

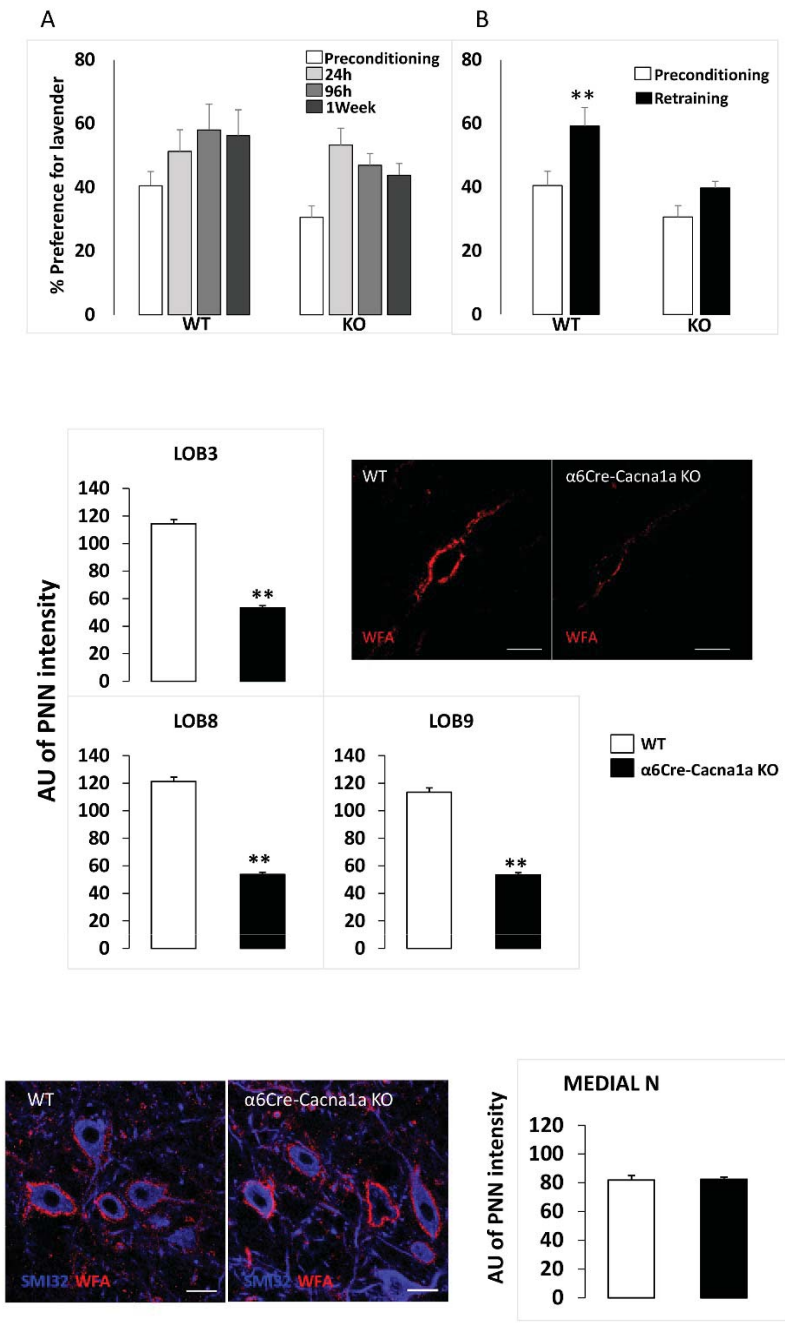


Fig. 8 (A) Percentage of preference for lavender (CS+) in the wild-type littermates (WT) and α6Cre-Cacna1a knockout mice (KO) throughout tests: preconditioning, 24h, 96h, and 1 week after conditioning. **(B)** Percentage of preference for lavender (CS+) in WT and KO mice before conditioning and after retraining (memory reactivation) (**p<0.01). Acquisition of cocaine preference conditioning was not affected by the reduction in the glutamatergic transmission from granule cells, but retraining did not maintain cocaine-induced memory. (C) PNNs surrounding Golgi neurons in WT and

KO mice. Average intensity of WFA at the apex of the cerebellar cortex in lobules 3, 8, and 9. Data are shown as mean± SEM; (**p<0.01). Representative microphotographs of PNN surrounding Golgi neurons at the apex of the granule cell layer in WT and KO mice. The confocal images were acquired at 40x with a 2.5x zoom for a final amplification of 100x. The intensity of the staining in the nets surrounding Golgi inhibitory interneurons was fainter in KO mice. **(D)** Representative microphotographs of PNNs in DCN in WT and KO mice. The confocal images were acquired at 40x with a 2x zoom for a final amplification of 80x. No differences were observed between WT and KO mice. Scale bar of 20 µm.

GENERAL DISCUSSION

The general objective of the present research was to investigate whether the cerebellum is part of the neuronal systems that sustain drug-induced conditioned memories. Due to the fact that such memories play a crucial role in craving and relapse (Shaham et al, 2003; Saunders and Robinson, 2013), we have focused our research on an attempt to clarify whether the cerebellum is involved in the acquisition and storage of such memories. The traditional omission of the cerebellum as a part of the networks that sustain drug-related conditioned memories had been previously challenged by numerous human neuroimaging studies in drug users and addicts which demonstrated cerebellar activations during exposure to drug-associated cues (Miquel 2009; Moulton et al, 2014; Moreno-Rius and Miquel, 2017). Other important source of evidence for our initial proposal was the previously demonstrated cerebellar role in conditioned memories. Several decades of research have shown that the cerebellum mediates consolidation of aversive Pavlovian memories as we have discussed previously (Strick et al, 2009; Strata, 2015). Despite these antecedents, the present doctoral thesis is the first explicit formulation of the role of the cerebellum in the establishment of drug-induced memories.

In the present investigation, the results obtained have been structured in three articles in which we presented and discussed three main findings. First, we have found that cocaine-induced preference conditioning produces an increase in activity (using cFos expression) in the dorsal region (apex) of the granular cell layer in the cerebellar vermis (Carbo-Gas et al, 2014ab). Second, neurons in the inputs and output nuclei of the cerebellum seem to enhance their activity during the learning phase (Carbo-Gas et al, 2014b). Finally, we also found an up-regulation of PNNs surrounding Golgi neurons in

the same dorsal region of the cerebellar cortex that could be regulated by granule cell activity (Carbo-Gas et al, under revision).

Notably, the increased activity in the apical region of the granular cell layer was found especifically in the animals that develop conditioned preference for a cue associated with cocaine. cFos is an immediate early-gene which also acts as transcription factor that encodes the cFos protein. Several forms of Fos-like proteins including cFos are induced in neurons by acute stimuli. Because of its temporal expression pattern, it is traditionally used as a neuronal activity marker (Curran and Morgan, 1995; Nestler, 2004). It has been observed that in the cerebellum, the expression peaks between 60 and 90 minutes after the occurrence of the stimulus (Tian and Bishop, 2002). Due to this temporal pattern, we assessed the levels of cFos 70 minutes after the preference test.

Although increased activity was described in every cerebellar lobule, the effect was especially prominent in posterior lobules VIII, IX and X. Previously, it was observed that those cerebellar lobules receive DA innervation from VTA (Ikai et al, 1992; Melchitzky and Lewis, 2000), and express dopamine transporter and receptors (Delis et al, 2008; Kim et al, 2009; Shimizu et al, 2014; Vazquez-Sanroman et al, 2015a). Interestingly, we detected DAT expression in posterior lobules in cocaine-treated animals. This increase was larger in lobule X of animals that exhibited preference for the drug-related cue (Carbo-Gas te al, 2014a). Our results are in accordance with those of Anderson and colleagues (2006), who observed higher binding of a DAT-like ligand in the cerebellar vermis of cocaine addicts.

Our study is not the first that show drug-related cFos expression in the cerebellum. Previously, a few reports described an enhancement in this expression in the granular cell layer and in Purkinje neurons after repeated injections of cocaine or amphetamine (Clark et al, 1992; Klitenick et al, 1995; Yin et al, 2010; Vazquez-Sanroman et al,

2015a). Nevertheless, one of the contributions of the present research is to demonstrate that the increased activity might represent a cerebellar hallmark of cocaine-induced preference conditioning. That is, it occurs exclusively in those animals that express preference for the cocaine-related cue. As expected, the higher the granule cell activity the higher the preference score. Another important finding is the regionalization of the neuronal activation, which was limited to the dorsal part of the granule cell layer. This selectivity probably represents an anatomical regionalization of the inputs to the cerebellar cortex. Indeed, it has been reported that mossy fibers in the cerebellum are distributed in a concentric arrangement (Voogd, 2014). Whereas corticopontine and extereoceptive components of the mossy fibers reach the apical part of the lobules, proprioceptive components of the same system terminate in the ventral part of the cerebellar lobules (Voogd and Ruigrok, 2004).

In our research, the inclusion of two additional control groups, the saline and unpaired groups allowed us to dissect the pharmacological effects of cocaine and to provide the most proper control for the acquisition of Pavlovian association between CS and US. Studies in emotional and motor associative learning support the hypothesis that memories responsible for these conditioned responses can be formed and stored in the cerebellar cortex (Freeman and Steinmetz, 2011; Strata et al, 2011; De Zeeuw and Ten Brinke, 2015). Besides, other studies that address the cerebellar involvement in conditioning have demonstrated that CS and US reach the cerebellar cortex by two different pathways (Ruigrok and Voogd, 2000). CS information arrives at the cerebellar cortex via mossy fibers originated in the pontine nuclei whereas US information arises from the inferior olive and arrives to the cerebellum through climbing fibers (Thompson and Steinmetz, 2009). Both mossy and climbing fibers send collaterals to the deep cerebellar nuclei. These two excitatory inputs, control the Purkinje's GABAergic output

to the deep cerebellar nuclei (DCN), modifying the release of information out of the cerebellum (Ito, 1984). Based on this evidence, cFos expression in the inferior olive and the pontine nuclei, as well as in the medial nucleus was also assessed (Roostaei et al, 2014). In this case, we observed that cFos levels in those brain areas were raised in the unpaired group after the preference test. That result could indicate that neurons in the inputs and output nuclei of the cerebellum enhance their activity when there is not contingency between environmental stimuli and cocaine and, consequently, the appropriate behavioural pattern for the contextual situation is uncertain. This suggestion seems to be coherent with the involvement of the cerebellum in prediction about internal events related to external cues (D'Angelo and Casali, 2013; Peterburs and Desmond, 2016). Hence, it might be expected that when the relationship between external stimuli and internal events becomes more predictable, and behavioural reactions are more adjusted to environmental demands the inputs might be progressively suppressed.

It should also be taken into account that the observed pattern in the cerebellar expression of cFos was not merely due to a neuropharmacological effect of cocaine. First, the last cocaine injection took place 48 hours before the preference test. Thus, it was a drug free test. Second, the other two cocaine-treated groups did not show the same pattern of activity in any of the studies developed in the present research. Hence, the first explanation we proposed was that the activity pattern exhibited by cocaine-preferring animals could be induced by the re-activation of drug-related memories. Unexpectedly, this idea was not supported by the results observed in the group of animals confined 24 hours after the preference test (Carbo-Gas et al, under revision). In this case, the pattern of cFos expression did not differ between mice showing preference conditioning and those that did not. Therefore, the selective increase in activity in the granule cells could be due not only to the reactivation of the CS+/US association but to the selection of the

action required to approach the cue previously associated with cocaine that was preferred over the other option. This interpretation would link the dorsal cerebellum to action selection during a reward process, due to the fact that animals under confinement had no choice. Nevertheless, we cannot rule out other alternative explanations, such as a putative memory decay induced first, by the preference test and then, by the confinement, since they are essentially extinction trials. Definitely, future research is needed to clarify which of these two alternatives might be the right one.

Given that one of the main results of the present research was the selective enhancement of activity found in granular cells, we introduced a causal approach to test the role of granular cell function in cocaine-induced preference conditioning: the use of α6Cre-Cacna1a mice. These mice show impaired granular glutamatergic function produced by the lack of P/Q-type voltage-gated calcium channels (Galliano et al, 2013a). These mice showed normal cerebellar cytoarchitecture, normal acquisition but impaired consolidation of a motor learning task which was associated with deficits in synaptic plasticity in parallel fibers-Purkinje synapses (Galliano et al, 2013a). Furthermore, no impairment was observed in olfactory perception, MWM performance, acquisition of fear memories and anxiety-related behavior in these KO mice (Galliano et al, 2013b). Our results show that KO mice acquired cocaine-induced preference conditioning, but they were not able to maintain such memories despite being retrained for a few additional sessions. This result points to impairment in consolidation mechanisms, as it has been previously observed employing different conditioning paradigms (Galliano et al, 2013ab).

The other important finding derived of the present doctoral thesis is that cocaine-induced preference conditioning upregulates selectively PNN expression around Golgi cells located in the dorsal region of the granular cell layer. PNNs have gained relevance in

the last years as a fundamental plasticity mechanism when a new conceptual construct called “the tetrapartite synapse” emerged (Dityatev and Rusakov, 2011; Smith et al, 2015). Going beyond the traditional point of view on the synaptic function, this emerging concept included two new elements in the synaptic architecture along with the presynaptic and postsynaptic compartments: astrocites and the extracellular matrix (ECM). PNNs are specialized structures consisting of ECM components that surround the soma and proximal neurites of several interneuronal populations. They have been demonstrated to be crucial to the maintenance of previously existing synaptic contacts and the prevention of the emergence of new ones, possibly contributing to the stability of memories (Botta et al, 2014). The increase in the expression of PNN was only observed in the animals that expressed preference for the drug-related cue. Such upregulation was of special interest for the present research because of the well-known relationship between Golgi interneurons and granular cells. Indeed, Golgi neurons play a fundamental role in modulating the activity of granular cells (D’Angelo et al, 2013). It has been found that Golgi neurons control granular cells firing patterns, synchronize the activity of granular cell clusters (Eccles et al, 1964), and modulate the plasticity of granular cell synapses (D’Angelo et al, 1999; Armano et al, 2000). In turn, Golgi cell activity is regulated by glutamatergic activity of granular cells and mossy fibers (Palay and Chan-Palay, 1973) together with the inhibitory control exerted by other interneurons (Sotelo and Llinas, 1972). Furthermore, synaptic plasticity in the granular cell layer is highly controlled by Golgi cell activity. As an example, blockade of Golgi function induces LTP in vitro (Mapelli and D’Angelo, 2007) and in vivo (Roggeri et al, 2008). Conversely, when its function is enhanced, LTD is observed (D’Angelo and De Zeeuw, 2009). To the best of our knowledge, there is no information regarding the effects of repeated drug exposure on Golgi function and plasticity. The scattered available data

only described an increased Golgi cell excitability and decreased granular activity in response to acute ethanol administration in vitro (Carta et al, 2004), and in vivo (Huang et al, 2012).

Importantly, we also demonstrated that the α6Cre-Cacna1a mice displayed reduced PNN expression. Such downregulation could be associated with the consolidation impairment seen in these mice. The reduced PNN expression may be related to the reduced granular cell modulation of Golgi neurons caused by the disconnection of the P/Q-type voltage gated calcium channels in granular cells. Altogether, this result directly links PNN expression surrounding Golgi cells with both cocaine-induced memory and granular cell-mediated glutamatergic function. However, animals that preferred the drug-related cue, and were confined with the CS+ 24 hours after the preference test, still showed an increased PNN expression as compared to the animals that did not prefer the cocaine-associated cue. As above mentioned, however, the increased dorsal granular activity seen in animals showing drug-related preference conditioning was abolished after confinement. This decorrelation also allowed us to discard Golgi PNN upregulation as an epiphenomenon resulted merely from the hyperactivity of granule cells. Therefore, one can speculate that cerebellar-dependent mechanisms of cocaine-associated memory directly involve PNNs around Golgi cells. Accordingly, Golgi PNNs could be responsible for the stabilization of synaptic contacts and prevention of a posterior rearrangement in the granular cell layer, thereby providing a mechanism by which drug-cue associations may be maintained in the cerebellum (Sorg et al, 2016).

Cocaine-induced changes in cerebellar PNNs show regional specificity (Sorg et al, 2016). Indeed, PNNs that surround projection neurons in the medial cerebellar nucleus were not affected by the acquisition of cocaine-induced preference memory. All cocaine

treated groups showed a reduced PNN expression as compared to saline-treated animals. This effect may be caused by a neuropharmacological effect of cocaine rather than being related to cocaine-related memory. It has been shown that cocaine is sufficient to modify synaptic architecture in the DCN (Vazquez-Sanroman et al, 2015ab). Consistently, both α6Cre-Cacna1a mice and wild-type littermates showed similar levels of PNN expression in the DCN given that both groups were treated with cocaine, further dismissing an involvement of PNNs around DCN in a memory process.

Based on the selective increase in Golgi PNN expression in animals that show preference for the CS+ and in the fact that disrupting PNNs in other brain regions has been able to attenuate drug-induced memories and enhance extinction of such memories, we propose that Golgi PNN manipulation should be considered as a future target of manipulation in animal models of drug addiction.

In summary, the results of the present doctoral thesis confirm that the cerebellum is part of the neural circuit underlying addictive behavior. More specifically, we have shown that, far from being "just a sensoriomotor structure", the cerebellum is crucially involved in the formation of drug-associated memories. Thus, the relevance gained by a stimulus associated with cocaine is accompanied by stronger PNNs in Golgi neurons and higher cFos levels in granular cells of the apical part of the posterior cerebellum. Those effects might be considered as the cerebellar distinctive signatures of cocaine-induced preference conditioning.

STRENGHTS, PITFALLS AND FUTURE DIRECTIONS

STRENGHTS

1. One of the major contributions of this research is to enable the inclusion of the cerebellum as a part of the networks involved in drug-related memories.

2. Our findings identify two cerebellar hallmarks of cocaine-related memories for the first time.

3. We used a very specific genetic manipulation in order to determine causally the role of the granular neurons in cocaine-induced preference conditioning.

4. This research points to the dorsal part (apex) of the granular cell layer as the locus for drug-induced plasticity changes related to preference conditioning.

5. This is the first investigation linking PNNs expressed around Golgi interneurons to drug-related memories, and showing regional specificity for cocaine-induced changes.

6. Another important aspect of this thesis is the validation of a protocol to produce cocaine-induced preference odor conditioning.

7. Finally, other key strength of this doctoral thesis is the use of innovative statistical methods as the discrimination function analysis and the estimation of effect sizes to better ascertain the functional relevance of drug-induced cerebellar changes.

PITFALLS, WEAKNESS AND FUTURE DIRECTIONS

1. The present studies exclusively focused on the involvement of the cerebellar vermis in cocaine-induced memories. However, it would be interesting to explore whether plasticity and neuronal activity is different in the cerebellar hemispheres due to the fact that human neuroimaging studies have also shown changes in the cerebellar hemispheres after the exposure to drug cues.

2. Further investigation is required to clarify the involvement of Purkinje neurons in cocaine-induced memory. In the previous research of the laboratory, it has been demonstrated that Purkinje plasticity is clearly affected by cocaine (Vazquez-Sanroman et al., 2015ab). However, in the present research, we obtained inconsistent results about the involvement of these neurons.

3. It would be very enlightening to explore whether the described plasticity changes in the cerebellar cortex were modulated by extinction training.

4. cFos expression has been extensively used as a marker of neuronal activity. However, electrophysiology would be a better approach to test cocaine-induced activity changes related to preference conditioning.

5. It is also necessary to go further in the characterization of cerebellar PNNs including the evaluation of different CSPGs expressed in Golgi PNNs.

6. It is also needed to explore cerebellar plasticity and neuronal activity in other behavioral paradigms relevant for drug addiction, such as cocaine self-administration.

7. The results presented here are relevant but have been obtained using mainly a correlational approach. Therefore, it is necessary a causal approach to explain the role

that the cerebellum plays in drug-related memories. For example, lesion studies would allow to clarify the cerebellums's role.

8. Both degradation and restoration of PNNs in the cerebellum using the bacterial enzyme ChABC and MPP inhibitors are also required.

9. The use of DREADDs to specifically manipulate neuronal activity around Golgi PNNs would allow us to properly describe the regulation of cerebellar PNNs and its functional consequences.

10. Finally, it would be of importance to evaluate the cerebellar involvement in food-induced preference, in order to understand whether the role of the cerebellum in cocaine-induced preference is selective for addictive drugs or for natural rewards as well.

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APPENDIX I

PREFACE

In the previous part of the present doctoral thesis, we focused our research on an attempt to clarify whether the cerebellum is involved in the acquisition and storage of drug memories. For this purpose, we investigated the involvement of the cerebellum in cocaine-induced memories using a model of Pavlovian conditioning. The next step in the explanation of the cerebellum´s role in addiction was to study its involvement in cocaine self-administration. For this purpose, we conducted a set of experiments in collaboration with Marcello Solinas (University of Poitiers), expert in self-administration of substances and operant behavior, focused on unravelling the effect of a focal lesion at the dorsal region of lobe VIII in the cerebellar cortex. We performed the lesion in that region since our previous studies revealed the specific involvement of this area in cocaine-induced cue-related memory (Carbo-Gas et al, 2014ab; Carbo-Gas et al, under revision). We managed to use an addiction-like animal model of cocaine self-administration "the escalation model". Also, it was required to test whether the effects of such cerebellar lesion were selective for addictive drugs or they were also observed in self-administration of natural reinforcers such as food. Unfortunately, due to several unexpected problems in the animal facilities bulding of the University of Poitiers, the cocaine-self administration experiment is still on going. Nevertheless, we could satisfactorily implement the food self-administration study. In the next appendix thereby, we present the results of the food study. We consider these findings as an important piece of evidence that enlightens the cerebellum role's on reward and motivation.

1. Introduction

Traditionally the cerebellum has been seen merely as a motor structure related to motor coordination and learning (Marr, 1969; Llinas and Welsh 1993). However, in the last 3 decades, neuroimaging and neuropsychological research has provided evidence that the cerebellum has also important non-motor functions (Schmahmann and Sherman, 1998; Strick et al, 2009; Timmann et al, 2010). Indeed, the cerebellum is critical for executive functions (Koziol et al, 2012; Koustenis et al, 2016), learning and memory (Strata et al, 2011; D'Angelo et al, 2016), motivation (Belkhiria et al, 2017), behavioral flexibility (Thoma et al, 2008) and habit formation (Salmon and Butters, 2005).

The behavioral tuning from a recreational use of drugs to an addictive phenotype involves brain networks underpinning Pavlovian and instrumental rewarded learning as well as decision making. Drug-induced habit formation has been proposed as a key feature in the transition to addiction (Everitt and Robbins, 2005). Interestingly, several data provide evidence of cerebellar contribution to repetitive sequential learning and habit formation. Clinical studies have suggested skill-learning impairments (Mulhern et al, 2004) and decision-making deficits (Cardoso et al, 2014) occur after cerebellar injury. However, hemicerebellectomy rather than preventing acquisition of sequential learning seems to delay the transition to response automatization (Mandolesi et al, 2010). Moreover, a bilateral lesion in the interpositus nucleus prevents rats from developing habits with overtraining (Callu et al, 2007). However, the lesion does not affect any learning process of an instrumental task (Callu et al, 2007). Therefore, contrary to correlational findings lesion studies suggest that the integrity of the cerebellum is not critical to learning goal-directed behaviours, but it is a hub of the brain process underlying habit formation.

Moreover, motivated behaviour is characterized by a high degree of behavioural activation and it is involved with the instigation and maintenance of instrumental responding (Nicola, 2010; Floresco, 2015). A high degree of motivation towards drug and drug-related stimuli is essential for the emergence and maintenance of addiction (Robinson and Berridge, 1993). Considerable evidence from both animal and human research has revealed that motivational processes are mediated by a distributed network of brain structures. Recently, it has been observed that the cerebellum is also involved in that function (Belkhiria et al, 2017). Cerebellar damage produce deficits on motivation-related behavior (D'Agata et al, 1993; Bauer et al, 2011), and these deficits seem to be due to the disruption of the functional networks formed by the cerebellum and cerebellar cortex (Middlenton and Strick, 2001).

Besides, an essential process to an effective behavioral regulation is behavioral flexibility. It has been defined as the ability to modify and adjust behavior in response to changes in external conditions, maximizing gains or minimizing losses (Darrah et al, 2008; Stalnaker et al, 2009). In preclinical models, behavioral inflexibility can be defined as the inability to adapt their behavior when external or internal contingences change (Istin et al, 2016). In the context of addiction, behavioral flexibility deficits could be involved in the transition from recreational to compulsive use of drugs (Koob and Volkow, 2010). In fact, impairments in behavioral flexibility are associated with addiction (Lyvers and Yakimoff, 2003; Lucantonio et al, 2012). Notoriously, behavioral flexibility is a complex process mediated by an array of brain areas including also the cerebellum. (Thoma et al, 2008; De Bartolo et al, 2009; Dickson et al, 2010; Dickson et al, 2017).

The involvement of the cerebellum not only appears to be a common factor in all drug addictions but also seems to be shared with other behavioral addictions. Drugs and food

seem to share common brain circuits (Tomasi et al, 2015). Both exhibit potent rewarding properties and both can be abused (Volkow et al, 2013). Importantly, the cerebellum is activated when cocaine addicts are exposed to food- and cocaine-related cues (Tomasi et al, 2015). Interestingly, cocaine users showed an overactivation of the cerebellum during the observation of food- and cocaine-related cues as compared to neutral cues. Nevertheless, further research is needed to elucidate the involvement of the cerebellum in food and drug addiction.

The aim of the present study is to evaluate the effect of a local lesion in lobule VIII on motivation for food and behavioral flexibility tasks. For this purpose, a cerebellar lesion was performed using infusions of quinolinic acid at the dorsal region of lobule VIII in the cerebellar vermis. We performed the lesion at the apical part of lobule VIII because it is one of the cerebellar region in which cocaine-dependent changes have been described in the present thesis. Also, lobule VIII is a component of the sensorimotor network (Schmahmann, 1991; Bostan et al., 2013), and it has been included as part of "the limbic cerebellum" (Timmann et al., 2010; Strata et al.,2011). Finally, it seems to be crucial in automatizing behavioural repertories towards drug-related cues (see Yalachkov et al, 2010 for a review).

2.Methods

2.1.Subjects

Fourteen male Sprague-Dawley rats weighing 250-275g (Charles River, France) were housed in pairs under standard laboratory conditions (12h light cycle from 8:00 to 20:00), at constant temperature (21±1°C), and free access to laboratory chow and water. One week before starting the experiments, rats were fed 20g of food each day. Food was always given shortly after the end of daily experimental sessions. Water was always

freely available in the home cage. Despite food restriction, rats were allowed modest weight gain throughout the experiment. When the animals weigh between 270-350g, they were subjected to stereotaxic surgery. All experiments were conducted in accordance to European Union directives (2010/63/EU) for the care of laboratory animals and all experimental protocols were approved by the local ethics committee (COMETHEA).

2.2.Stereotaxic Surgery

Six days before the start of the behavioral procedures, stereotaxic surgery was performed. To allow the stereotaxic procedure the animals had to weigh between 270 and 350 g. Rats were anesthetized under exposure to isoflurane 4% (Baxter, Maurepas, France) and were placed into the stereotaxic apparatus. The cannula was aimed at the dorsal part of Lobule VIII of the cerebellar vermis at the following coordinates; AP: -14.5 DL: 0 DV: -4.5 (Paxinos and Watson, 2006) and infused through a surgical-grade injector connected to tubbing attached to a 10 µl Hamilton syringe. Using an infusion pump, a volume of 0.5µl of quinolinic acid (90nmol/µl) with a Ratio of 0.2µl/min was injected over a period of 150 seconds. Following the infusion, the injector remained in place 3 minutes before the extraction for a correct diffusion of the substance. The cannula was removed and the wound was sutured. This same procedure was also performed with the infusion of PBS for the Sham group. Before to start the surgery all the animals received an intramuscular injection of ketoprofen (10mg/kg). Rats were allowed to recover for 5 days before the start of the experiment. All lesion placements were verified at the end of the experiment. Please, see figure 1.

2.3.Behavioral Procedures

All Behavioral sessions were conducted in standard operant conditioning chambers (28×23×23 cm^3; Med Associates) enclosed in sound attenuating compartments. Each chamber contained a white house light, two levers and two lights above each lever so-called "cue lights". Both levers were separated by a feeder into which a pellet dispenser could deliver 45-mg food pellets (1811155, TestDiet). The operant-conditioning chambers were controlled by microcomputers using Med-PC software package. For details on the experimental timeline, please see Figure 2.

Fixed Ratio Schedule

In order to assess the acquisition of the operant response, rats were trained to lever press on a continuous reinforcement schedule of FR1 during 5 days. The procedure was as follows: at the start of the session, the house light was turned on, the two levers were available and in their presence the rats were required to make 1 response on the correct lever. Responses on the correct lever produced the delivery of a 45-mg food pellet, the retraction of both levers and the illumination of the cue light above of the correct lever for 5 seconds. After these 5 seconds, a 10-second time-out period started during which lever-press responses had no programmed consequences and the house light was turned off. Responses on the incorrect lever had no programmed consequences. After each time-out, the house light was again turned on and the next trial began. Each session ended after either completion of 100 FR trials or 60 min elapsed, whichever occurred first.

Following this initial training period of 5 days under FR1 schedule, the rats were trained in ascending FR schedule before moving to Progressive Ratio. The animals were trained

3 days in a FR3 schedule, followed by 2 days at FR5 schedule and finally 2 additional days at FR10 (Figure 2).

Progressive Ratio Schedule

After the sessions under the Fixed Ratio schedule, rats were switched to the Progressive Ratio schedule. Under the Progressive Ratio schedule, the response requirement increased with each successive food pellet obtained. The response Ratio schedule during PR using the following formula extracted from Richardson and Roberts (1996):=[$5e^{(R*0.2)}$]-5 where *R* is equal to the number of food rewards already earned plus 1. Thus, the number of responses required to earn a food reward followed the order: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, 737, 901, 1102, 1347 and so on. Under the PR schedule the time-out period was of 30 seconds. The final Ratio completed was the breakpoint. Sessions under the Progressive Ratio schedule lasted as maximum of 5 h or until 10 min passed without a response, which almost always occurred within less than 5 h. The animals performed the test 3 consecutive days.

To assess the effects of changes of the motivational state for food reward in the PR task, rats perform two additional sessions of PR. In the first session, rats were tested after 24h of ad libitum feeding (satiation state), whereas in the second session the animals performed the PR task under 24h of fasting.

Reversal learning

To assess behavioral flexibility, rats were trained to lever press on a continuous reinforcement schedule of FR5 during one session. The Fixed Ratio procedure was as we previously explained. Following this FR5 training session, the rats were tested on a

reversal of reward contingencies. Parameters for the reversal phase were identical to the FR5 session with the exception that the reward contingencies were reversed. This is, responding in the previously active lever had no consequences and completing the Ratio in the previously inactive lever resulted in the delivery of a food pellet. This condition lasted for 2 consecutive sessions, and the day after, rats were trained on a FR5 session where the reward contingencies were the same as in the first FR5 training session.

2.4.Statistical Analyses

Data was analysed using Prism version 7 (GraphPad Software, La Jolla, CA). A repeated-measures two-way ANOVA was used to analyse the acquisition of the operant response under the 5 consecutive FR1 sessions, and the performance on the 3 consecutive PR sessions in which no feeding manipulations were performed. The rest of the data were analysed by two-way ANOVA with Sidak's multiple comparisons test as a post-hoc. Significance level was set at $p<0.05$. The Šídák method assumes that each comparison is independent of the others, and thus it is recommended when multiple comparisons have to be performed due to its higher statistical power (Abdi, 2007).

3.Results

Fixed Ratio Schedule

1. FR1 schedule

Results of the acquisition of operant response under FR1 condition are depicted on the Figure 3. A two-way repeated measures ANOVA for correct lever presses showed a main effect of time ($F[4,48]=37.4$, $p<0.0001$). Neither an effect of lesion ($F[1,12]=0.1833$, $p=0.6761$) nor interaction ($F[4,48]=1.026$, $p=0.4033$) were demonstrated. In the same direction, the analyses of incorrect lever presses yielded a

main effect of time ($F[4,48]=9.505$, $p<0.0001$), but no effect of lesion ($F[1,12]=3.692$, $p=0.0787$) or interaction ($F[4,48]=2.291$, $p=0.0732$). Nevertheless, the cerebellar lesion increased the number of incorrect responses during the first day of training ($p=0.0117$). The analyses of total lever presses showed only a main effect of time ($F[4,48]=16.29$, $p<0.0001$), without any effect of lesion ($F[1,12]=0.3456$, $p=0.5675$) or interaction ($F[4,48]=0.7286$, $p=0.5768$). It suggests that the increase in the number of incorrect responses given by lesioned rats was not due to a 'hiperactive' state but an initial transitory deficit in adjustmenting behaviour to environmental conditions. When we analysed the number of the reinforcers obtained by rats, we did observe no effect of lesion ($F[1,12]=0.1833$, $p=0.6761$), a main effect of time ($F[4,48]=37.4$, $p<0.0001$) and no interaction ($F[4,48]=1.026$, $p=0.4033$). Therefore, the lesioned rats did not present any deficit in the acquisition of the operant response.

2. FR schedules collapsed

Similar conclusions can be reached when all the FR schedules were analysed together using a two-way ANOVA (Figure 4) for correct and incorrect lever pressing. We observed a main effect of Ratio ($F[3,48]=42308$, $p<0.0001$) but neither a lesion ($F[1,48]=0.1253$, $p=0.7249$) nor an interaction effects were significant ($F3,48]=0.1253$, $p=0.9447$). So, the cerebellar lesion did not affect the required increase in the number of lever presses in order to complete the ratio. Similarly, the analyses for incorrect lever presses yielded a main effect of Ratio ($F[3,48]=24.18$, $p<0.0001$) possibly due to the effect of learning, but not an effect of lesion ($F[1,48]=1.954$, $p=0.1686$) or interaction ($F[3,48]=2.865$, $p=0.0463$). On the other hand, the analyses of the discrimination between both levers showed a main effect of Ratio ($F[3,48]=51.39$, $p<0.0001$), but no lesion ($F[1,48]=0.7466$, $p=0.3918$) nor interaction effects ($F[3,48]=0.8723$, $p=0.4620$), suggesting that cerebellar lesion did not impar discrimination between the correct and

the incorrect lever. The number of reinforcers obtained was also unaffected by the lesion of the cerebellum [Ratio ($F[3,48]=31.67$, $p<0.0001$), lesion ($F[1,48]=0.1253$, $p=0.7249$), interaction ($F[3,48]=0.1253$, $p=0.9447$)].

Progressive Ratio Schedule

1.Under a standard restriction condition

Results of the performance on PR schedule in which no feeding manipulations were performed are showed on the Figure 5. A two-way repeated measures ANOVA for correct lever presses showed a significant effect of time ($F[2,24]=10.5$, $p=0.0005$), lesion ($F[1,12]=54.56$, $p<0.0001$) and interaction ($F[2,24]=7.602$, $p=0.0028$). Sidak post-hoc tests showed that lesioned rats performed more correct lever presses on day 2 ($p<0.0001$ and day 3 ($p<0.0001$), suggesting that lesioned rats are more motivated for achieving the reinforcer than the sham rats. However, the analyses for the incorrect lever pressing also yielded an effect of lesion ($F[1,12]=5.319$, $p=0.0397$), though neither time ($F[2,24]=2.493$, $p=0.1038$) nor interaction were significant ($F[2,24]=2.579$, $p=0.0967$). This finding pointed to a certain behavioural desinhibiton. Additionally, the analyses of the number of reinforcers obtained showed a main effect of time ($F[2,24]=7.013$, $p=0.0040$), an effect of lesion ($F[1,12]=39.44$, $p<0.0001$) and interaction between both factors ($F[2,24]=5.343$, $p=0.0120$). When we performed Sidak post-hoc tests, we found that lesioned rats receive more reinforcers than sham rats on day 2 ($p<0.0001$) and day 3 ($p=0.0002$). Finally, the analyses of the Breakpoint showed a significant effect of time ($F[2,24]=9.897$, $p=0.0007$), lesion ($F[1,12]=52.56$, $p<0.0001$) and interaction ($F[2,24]=7.199$, $p=0.0036$). Sidak post-hoc tests confirmed that lesioned rats achieved higher breakpoints than sham rats on day 2 ($p<0.0001$) and day 3 ($p<0.0001$). In summary, under standar motivational conditions a lesion in the dorsal region of lobule

VIII promotes a higher motivational state in which animals are able to work harder to obtain food rewards.

2. Three different conditions of feeding

The performance on the PR schedule under three different conditions of feeding were analysed using a two-way ANOVA (Figure 6). When correct lever pressing was analysed, a significant effect of feeding state ($F[2,36]=16.48$, $p<0.0001$) and lesion ($F[1,36]=14.43$, $p=0.0005$) were found, but interaction was not significant ($F[2,36]=1.425$, $p=0.2538$). Sidak post-hoc tests indicated that lesion rats pressed more the correct lever under restricted condition than the sham rats ($p=0.004$) . Moreover, an intragroup comparision showed that both groups seem to dispay a different motivational profile. While in sham rats differences were observed between restricted and fasting conditions ($p=0.0387$), in lesioned rats differences were seen between restricted and satiation conditions ($p=0.0043$).

The analisys for incorrect lever pressing showed a main effect of feeding state ($F[2,36]=3.708$, $p=0.0343$), an effect of lesion ($F[1,36]=6.409$, $p=0.0159$), but not interaction effect ($F[2,36]=1.71$, $p=0.1952$). When we performed the Sidak post-hoc tests, significant differences were found when comparing sham and lesion groups in the restricted condition ($p=0.0318$), showing that lesioned rats performed more incorrect responses than sham rats under such condition. Also, in this group, there were rats made more errors on the restricted condition as compared to the satiation ($p=0.0073$).

Similar results were found regarding the number of reinforcers obtained. There were effects of feeding state ($F[2,36]=21.8$, $p<0.0001$), lesion ($F[1,36]=21.76$, $p<0.0001$), but not interaction ($F[2,36]=1.344$, $p=0.2736$). Again, the effects of the lesion could be seen only under restricted conditions ($p=0.0009$). Similarly, sham and lesioned groups

exhibited different profiles when comparing the number of reinforces obtained under the different motivational states. In sham rats differences were demonstrated between restricted and fasting conditions ($p=0.0186$). However, lesioned rats obtained more reinforcers under the restricted condition than under satiation ($p=0.0023$).

Finally, the effect of the lesion on the breakpoint across different motivational states was also considered. We observed a main effect of feeding state ($F[2,36]=15.65$, $p<0.0001$), and an effect of lesion ($F[1,36]=14.16$, $p=0.0006$), but not interaction between both factors ($F[2,36]=1.723$, $p=0.1929$). In this case, Sidak post-hoc tests confirmed differences between sham and lesion groups on the restricted condition as can be expected ($p=0.0025$). The breakpoint analysis also showed that are sham animals those that increased motivation under fasting conditions ($p=0.0396$). In lesioned rats, however, no differences were observed between restricted and a high motivation state like fasting.

Reversal learning

The performance parameters on the reversal learning task are depicted in Figure 7, and were analysed using a two-way ANOVA in which each session was computed as an independent test. The analysis of the number of correct responses demonstrated that there was a marginal effect of lesion ($F[1,48]=3.801$, $p=0.0571$), an effect of session ($F[3,48]=3.801$, $p=0.0159$), and a significant interaction ($F[3,48]=3.801$, $p=0.015$). In this case, Sidak post-hoc tests confirmed that lesioned rats performed higher number of correct responses than sham rats on the first day in which reward contingences were reversed ($p=0.0012$), suggesting that lesioned rats learned faster the change of contingences. Supporting these results, the analysis of the number of incorrect lever presses demonstrated a significant effect of session ($F[3,48]=57.31$, $p<0.0001$), lesion ($F[1,48]=5.38$, $p=0.0247$), and interaction between factors ($F[3,48]=3.641$, $p=0.0191$).

The lesioned group produced less incorrect lever pressing the first day in which reward contingences were reversed than the sham group ($p=0.0014$). Therefore, lesioned rats seem to learn faster the task then, producing less errors. In addition, when total lever pressing was analysed (correct plus incorrect responses), a main effect of session ($F[3,48]=59.82$, $p<0.0001$), lesion ($F[1,48]=4.559$, $p=0.0379$) and interaction were also found ($F[3,48]=2.961$, $p=0.0414$). In this case, lesioned rats performed less total responses than sham rats as they made less errors. This result suggests that the lesioned group presented a better behavioural adjustment when contingencies are modified. Finally, regarding the number of reinforcers obtained, the ANOVA yielded a marginal significance for the lesion ($F[1,48]=3.54$, $p=0.065$), and a main effect of session $F[3,48]=3.544$, $p=0.0213$), and interaction ($F[3,48]=3.54$ $p=0.021$)]. In this regard, lesioned rats obtained more total reinforcers than the sham group.

4.Discussion

The present study aimed to consider motivation and cognitive flexibility as possible functions of the cerebellum. Our findings indicate that a lesion at the apical region of lobule VIII increases motivation on a progressive ratio schedule. Furthermore, the lesioned group apparently exhibited increased flexibility on a reversal learning task.

In the progressive ratio task, the breaking point was defined as the value of the ratio (the number of lever presses required to earn a rewarding sugar pellet) toward which the animal was working but failed to achieve, determined by a 10-minute period of inactivity (absence of lever pressing). Lesioned rats exhibited high breaking points, and thereby they were considered to display a higher level of motivation for the reinforcer, a very salient feature of the addicted subjects, as we mentioned before (Robinson and Berridge, 1993). Also, we observed that the lesion did not modify the number of correct

responses under a FR task, but did change the incorrect lever pressing during the first day (Figure 4), somewhat suggesting an initial behavioral disinhibition.

Motivation is a complex process that is critical for survival, which involves multiple behavioral functions mediated by a number of interacting neural circuits, including the cerebellum (Peterson et al, 2012). It has been found that cerebellar-dentate lesioned rats demonstrated reduced motivation on a progressive ratio task and reduced exploration in an open field exploration task (Swain et al, 2010). In other study, a lesion in the dentate nucleus produced a reduction in the motivation shown by a decrease in the effort-based decision making (Peterson et al, 2012). These findings are incongruent with our observations. However, lesions in these studies were performed in a different region, the dentate nucleus, the output nucleus of the cerebellar hemispheres. Importantly, when the cerebellar lesion was made in the cerebellar vermis, rats increased responses in an apetitive operant paradigm (Callu et al, 2007).

Furthermore, in the present study, we have found that a lesion of the apical region of lobule VIII enhances behavioral flexibility. After a FR5 training session, the rats were tested on a reversal of reward contingencies (i.e. change of the position of the active lever). Lesioned rats demonstrate to exhibit higher flexibility in all the parameters analysed on the first day in which reward contingences were reversed. These findings, although preliminary, showed that lobule VIII in the vermis could be involved in behavioural inflexibility and persistence.

Behavioral flexibility has been considered traditionally as a frontal and striatal function. However, converging evidence suggests an involvement of a larger brain circuit which includes the cerebellum (Gottwald et al, 2004). The degree to which the cerebellum contributes to restrict or enhance distinct forms of cognitive and behavioural flexibility as well as rule learning is unknown (Dickson et al, 2017). Mice with a massive reduction

of Purkinje cells in the whole cerebellum presented performance deficits in a reversal learning task (Dickinson et al, 2010). Previous studies in which cerebellar lesions affected the hemispheres have also suggested impairments in behavioral flexibility. For example, De Bartolo and coworkers (2009) found a reduction in behavioral flexibility in rats after the suppression of the cerebellar hemispheres. Additionally, it has been observed deficits in behavioral flexibility after vascular lesions of the lateral cerebellum in humans (Thoma et al, 2008). Therefore, taking together prior and present findings, it seems to exist a functional dissociation in the cerebellum regarding the regulation of reward-based reversal learning and goal-directed behaviour.

Overall, the findings of the present thesis suggest that the dorsal region of the posterior vermis is part of a functional network that restrain goal-directed behaviour when contingencies between stimuli have been established and learnt. Maybe, it could explain why that is the precise region where hallmarks signatures of drug-related memory have been found. One can speculate that a lesion in this area would release behaviour from previously learnt associations, then flexibility to acquire new contingencies would be regained. This hypothesis opens new avenues to explore the inhibitory role of the dorsal cerebellar cortex. Further animal studies are required to clarify whether the apical part of cerebellar lobule VIII is involved in substance abuse and binge eating.

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6.Figures

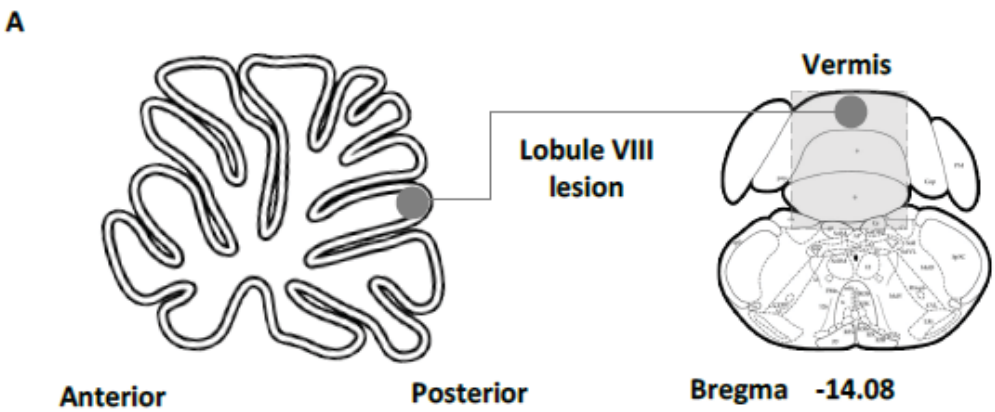

Figure 1. Representation of the cerebellar lesion in lobule VIII in the vermis. Image by courtesy of Julian Guarque.

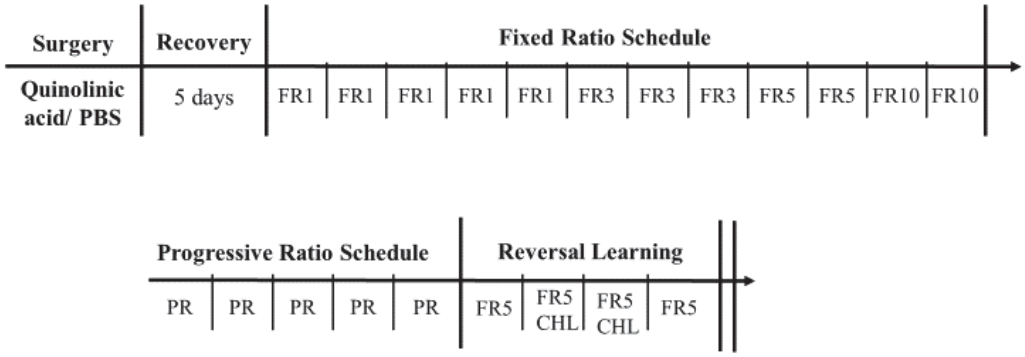

Figure 2. Experimental timeline. Time course of the different steps of the experimental procedure.

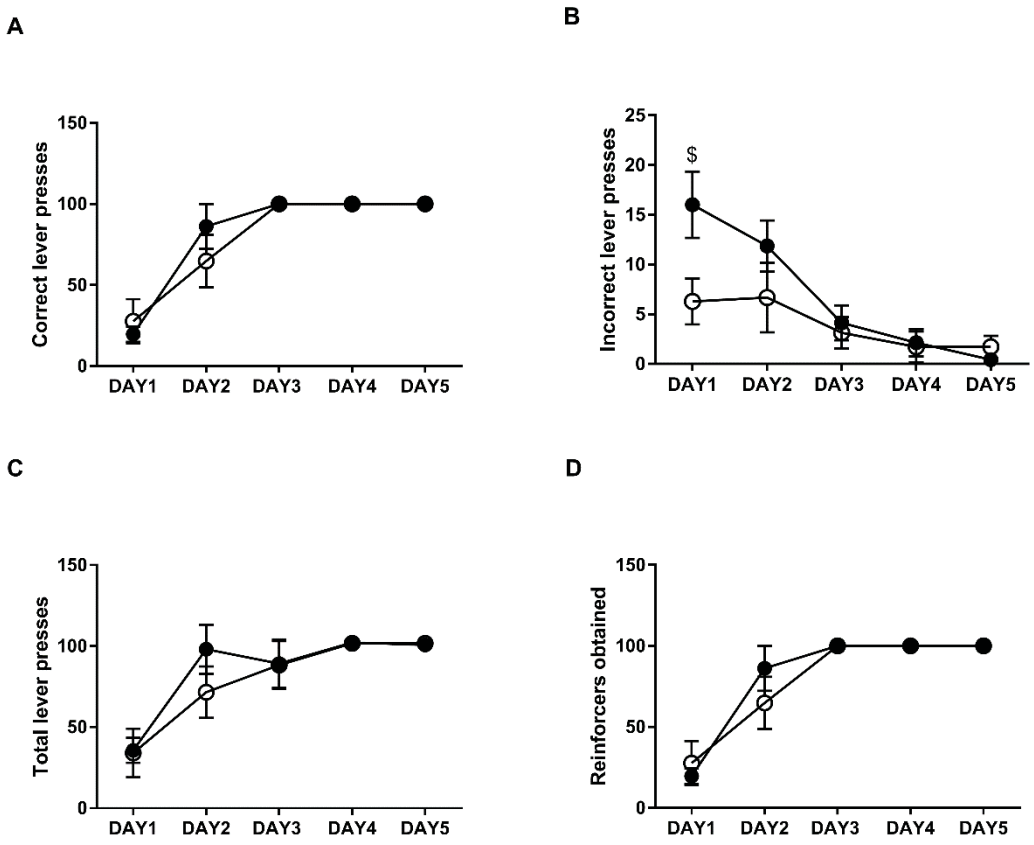

Figure 3. Food self-administration under FR1 schedule. Empty circles represent the sham group, filled circles represent the lesion group. Data are expressed as mean (±SEM) of (A) correct lever presses, (B) incorrect lever presses, (C) total lever presses and (D) reinforcers obtained. $= $p<0.05$ between Sham and Lesion groups.

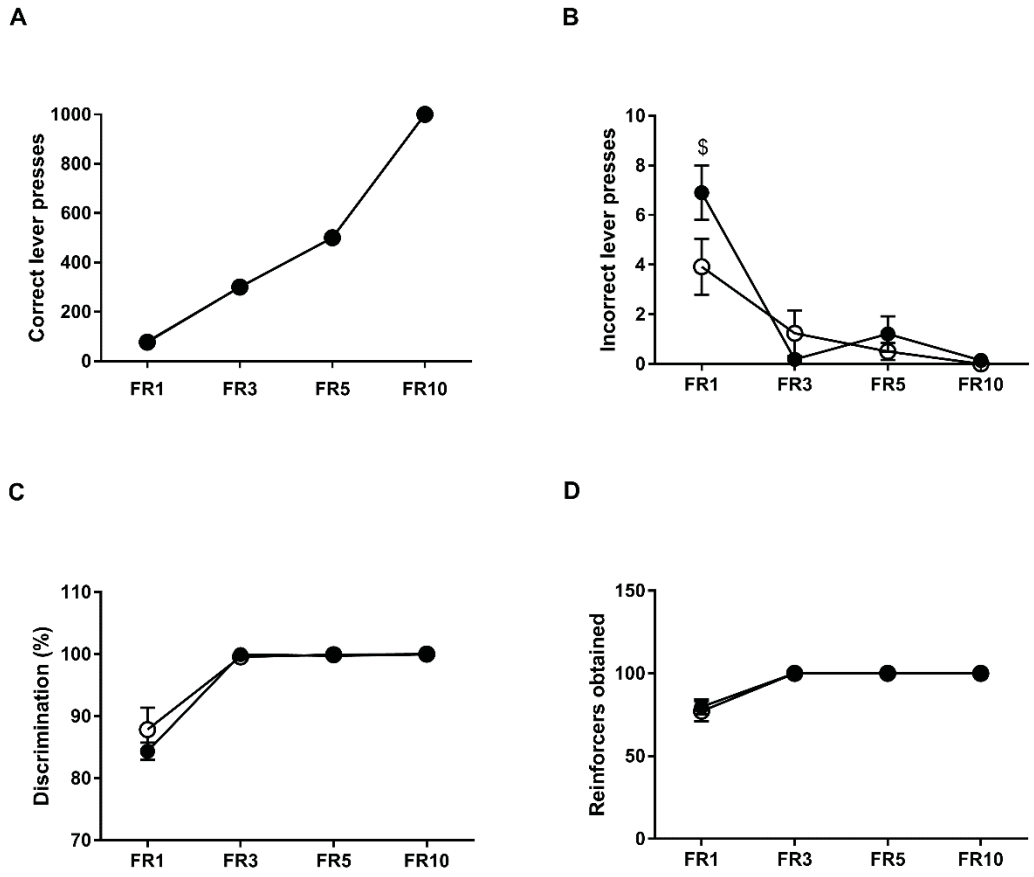

Figure 4. Food self-administration under different FR schedules. Empty circles represent the sham group, filled circles represent the lesion group. Data are expressed as mean (±SEM) of (A) correct lever presses, (B) incorrect lever presses, (C) discrimination between both levers and (D) reinforcers obtained. $= $p<0.05$ between Sham and Lesion groups.

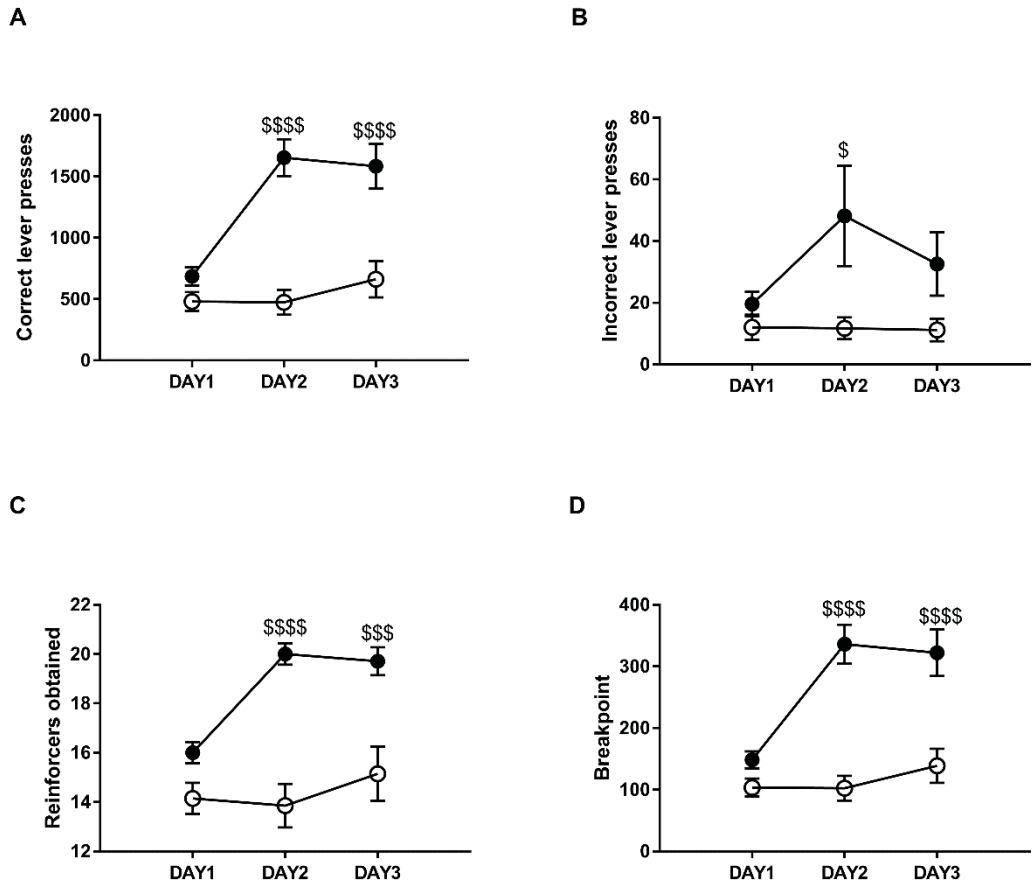

Figure 5. Food self-administration in a PR schedule under standard food restriction condition. Empty circles represent the sham group, filled circles represent the lesion group. Data are expressed as mean (±SEM) of (A) correct lever presses, (B) incorrect lever presses, (C) reinforcers obtained and (D) Breakpoint. $=p<0.05; $$$=p<0.001; $$$$=p<0.0001 between Sham and Lesion groups.

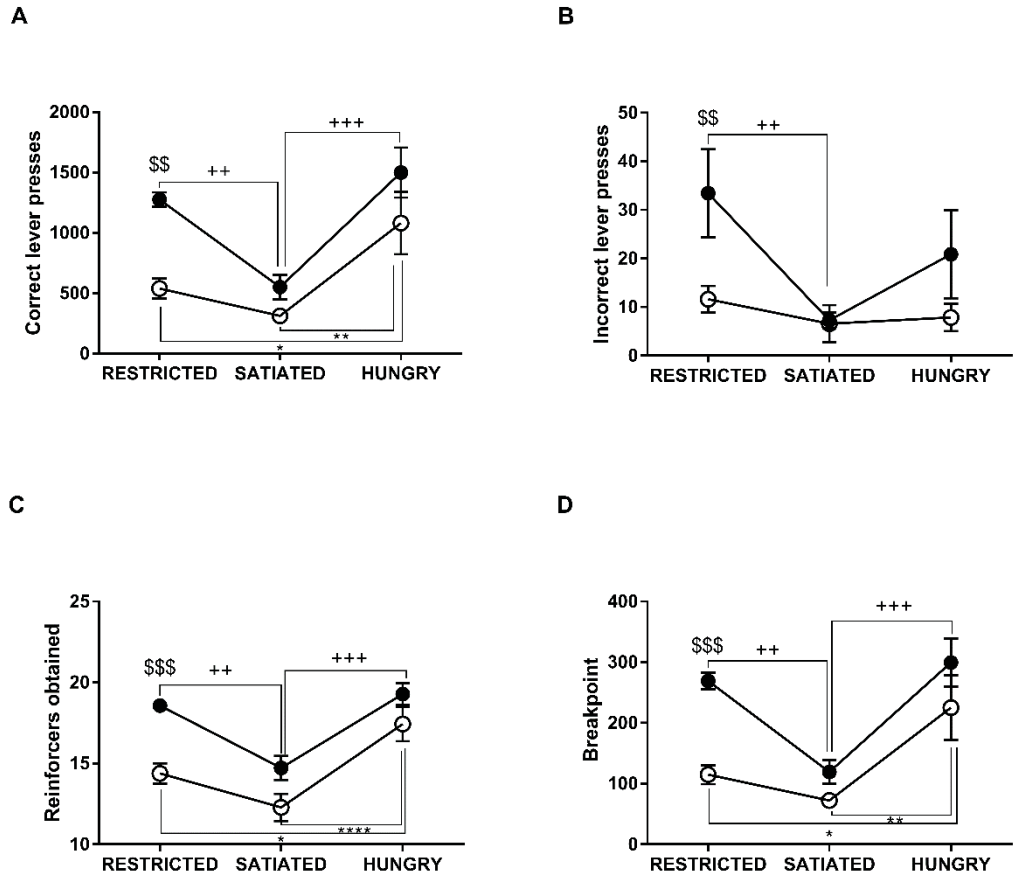

Figure 6. Food self-administration in PR sessions under three different feeding conditions. Empty circles represent the sham group, filled circles represent the lesion group. Data are expressed as mean (±SEM) of (A) correct lever presses, (B) incorrect lever presses, (C) reinforcers obtained and (D) Breakpoint. $$=p<0.01; $$$=p<0.001 between Sham and Lesion groups. *=p<0.05; **=p<0.01; ****=p<0.0001 between different feeding conditions in the sham group. ++=p<0.01; +++=p<0.001 between different feeding conditions in the lesion group.

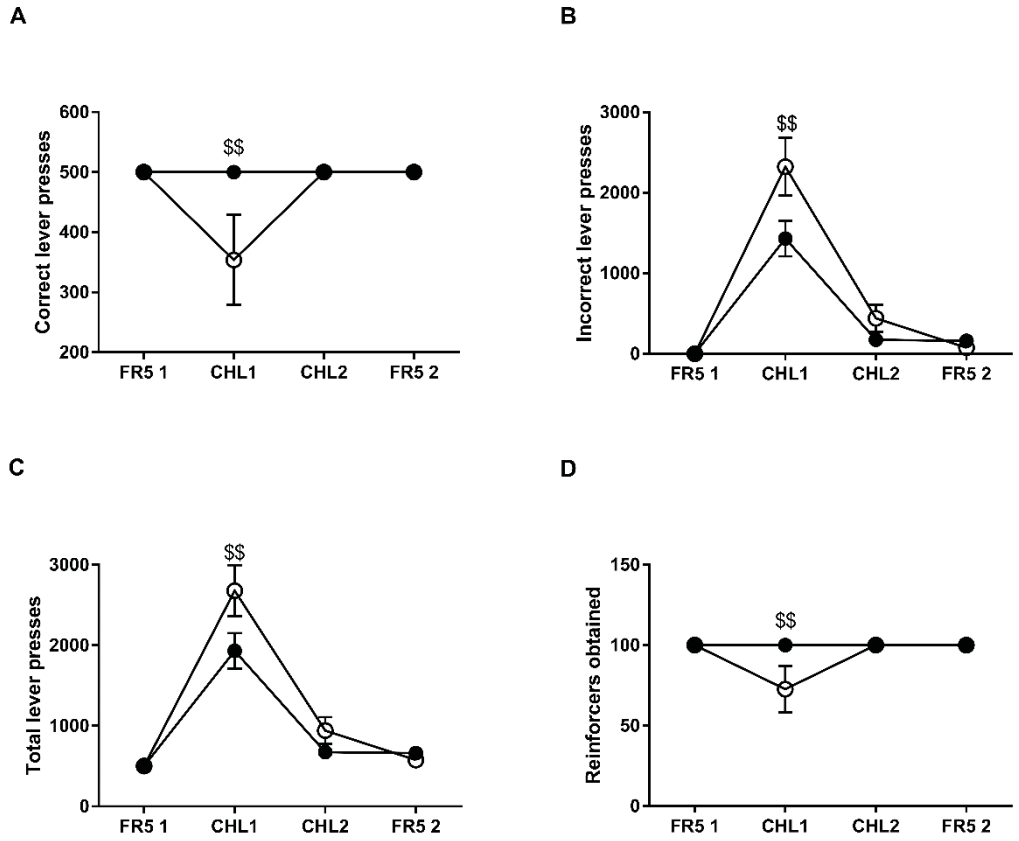

Figure 7. Food self-administration in a behavioral flexibility task. Empty circles represent the sham group, filled circles represent the lesion group. Data are expressed as mean (±SEM) of (A) correct lever presses, (B) incorrect lever presses, (C) total lever presses and (D) reinforcers obtained. $=p<0.05; $$=p<0.01 between Sham and Lesion groups.

APPENDIX II

IMPLICACIÓN DE CEREBELO EN LAS MEMORIAS INDUCIDAS POR LA COCAÍNA

PREFACIO

Debido a su papel en la búsqueda de drogas, el consumo y la conducta adictiva, hay un creciente interés en la identificación de los circuitos neuronales y mecanismos moleculares subyacentes en la formación, el mantenimiento y la recuperación de las memorias inducidas por drogas. Sin embargo, muy pocos estudios se han centrado en áreas cerebrales más allá de los circuitos corticoestriatal-límbicos. A pesar de la creciente evidencia que confirma la participación del cerebelo en las alteraciones inducidas por drogas, esta estructura ha sido tradicionalmente descartada en el campo de la adicción (Miquel et al, 2009; Miquel et al, 2016).

El objetivo general de la presente investigación es abordar si el cerebelo forma parte de los sistemas neuronales que sostienen los mecanismos de plasticidad subyacentes a las memorias condicionadas inducidas por drogas. Hemos centrado nuestra investigación en un intento de aclarar si el cerebelo está involucrado en la adquisición y almacenamiento de memorias asociadas a drogas. Aunque hay estudios anteriores que describen la participación del cerebelo en las memorias inducidas por drogas, este es el primer intento de abordar un análisis funcional detallado sobre el tema.

La presente tesis doctoral contiene tres capítulos diferentes. Los dos primeros han sido publicados (Carbo-gas et al, 2014ab), y el tercero está siendo revisado. En el primer capítulo: *Involving the cerebellum in cocaine-induced memory: pattern of cFos expression in mice trained to acquire conditioned preference for cocaine*, exploramos el patrón de activación neuronal revelado por inmunorreactividad de cFos en ratones

entrenados para desarrollar preferencia condicionada por un estimulo olfatorio asociado a la cocaína. En el artículo, titulado *Cerebellar hallmarks of conditioned preference for cocaine*, se utilizó la misma tarea comportamental del primer estudio con el fin de ampliar la descripción del patrón deexpresión de cFos en la circuitrería cerebelar, incluyendo ahora los principales aferentes y uno de los núcleos de salida del cerebelo. En el último capítulo, *Cerebellar perineuronal nets in cocaine-induced Pavlovian memory: site does matter*, se realizó un amplio análisis de la expresión de PNNs en el vermis cerebeloso. En primer lugar, se realizó el análisis en ratones entrenados para adquirir preferencia por un estímulo olfatorio asociado con la cocaína. En segundo lugar, se utilizaron ratones modificados genéticamente de la cepa α6Cre-Cacna1a (Galliano et al, 2013) para probar si una reducción en la liberación de glutamato de las células granulares a las dendritas de Purkinje alteraría la adquisición de preferencia por estímulos asociados con la cocaína y la expresión de PNNs en el cerebelo.

Después de la presentación de los capítulos, hemos incluido una sección donde se resumen los hallazgos resumidos, las fortalezas y los puntos débiles, así como las direcciones futuras de nuestra investigación. También se encuentran las referencias bibliográficas al final del presente documento.

Finalmente, concluimos esta tesis con un apéndice. Este documento está formado por investigaciones aún no terminadas, pero que pueden ser de gran relevancia para entender el papel del cerebelo en los trastornos adictivos. En el dicho apéndice, se exploró el efecto de una lesión local en la parte apical de la corteza cerebelosa en la auto-administración de comida, así cómo investigamos si el papel del cerebelo es específico para la motivación por la cocaína o sería un modulador general del proceso de recompensa. Así, se mostró el efecto de la misma lesión cerebelosa en la adquisición de auto-administración de comida, así como en la motivación por dicho reforzador.

OBJETIVOS GENERALES

- Investigar la implicación del cerebelo en las memorias inducidas por la cocaína utilizando un modelo de condicionamiento pavloviano.
- Validar un protocolo de condicionamiento olfativo inducido por cocaína para ratones.
- Evaluar el patrón de activación neuronal en el cerebelo de ratones entrenados para adquirir preferencia condicionada por un estímulo asociado con la cocaína, usando la expresión de cFos.
- Describir los patrones de actividad neuronal en el circuito cerebelar, considerando proyecciones aferentes y eferentes del vermis cerebeloso.
- Evaluar la expresión de PNNs cerebelares tras el condicionamiento de preferencia inducido por cocaína.
- Verificar si la reducción del glutamato liberado en la sinapsis entre las células granulares y las neuronas de Purkinje puede afectar a la adquisición de preferencia inducida por la cocaína y modificar la expresión del PNNs en el cerebelo.

HIPÓTESIS

- La experiencia repetida con cocaína producirá un patrón diferente en la expresión de cFos entre los ratones que muestran preferencia por un estímulo asociado a la cocaína y aquellos ratones que no adquieren dicha preferencia condicionada.
- Diferentes componentes de la organización micromodular del cerebelo se verán afectados diferencialmente por la exposición a la cocaína y la expresión de preferencia por un estímulo asociado a la cocaína.

- La preferencia inducida por la cocaína podría ser modulada por la actividad de las neuronas granulares del cerebelo.
- La expresión de PNNs se alteraría en animales que muestran preferencia inducida por cocaína.

DISCUSIÓN GENERAL

El objetivo general de la presente investigación fue investigar si el cerebelo forma parte de los sistemas neuronales que sostienen los mecanismos de plasticidad subyacentes a las memorias condicionadas inducidas por drogas. Debido a que estas memorias desempeñan un papel crucial en el deseo de consumo y la recaída (Shaham et al, 2003; Saunders y Robinson, 2013), hemos enfocado nuestra investigación en un intento de determar si el cerebelo está involucrado en la adquisición y almacenamiento de dichas memorias. La omisión tradicional del cerebelo como parte de las redes que sostienen las memorias condicionadas inducidas por drogas es cuestionada por numerosos estudios de neuroimagen en humanos consumidores de drogas y adictos que han demostrado activaciones cerebelares durante la exposición a estímulos asociadas a drogas de abuso (Miquel et al, 2009; Moulton et al, 2014, Moreno-Rius y Miquel, 2017). Otra fuente importante de evidencia para nuestro estudio ha sido el papel del cerebelo previamente demostrado en las memorias condicionadas. Varias décadas de investigación han demostrado que el cerebelo modula la consolidación de memorias pavlovianas aversivas (Strick et al, 2009, Strata, 2015).

En la presente investigación, los resultados obtenidos se han estructurado en tres artículos diferentes en los que presentamos y discutimos tres conclusiones principales. En primer lugar, hemos observado que la preferencia condicionada inducida por la cocaína produce un aumento de la actividad (utilizando la expresión de cFos) en la región dorsal de la capa granular del vermis cerebeloso (Carbo-Gas et al, 2014ab). En segundo lugar, las neuronas de las aferentes y de los núcleos de salida del cerebelo parecen aumentar su actividad durante la fase de aprendizaje (Carbo-Gas et al, 2014b). Por último, también encontramos una regulación de PNNs alrededor de las neuronas de

Golgi en la misma región dorsal de la corteza cerebelosa que podría ser regulada por la actividad de las células granulares (Carbo-Gas et al, en revisión).

En particular, el aumento de la actividad en la región apical de la capa granular del cerebelo se encontró en los animales que desarrollan preferencia condicionada por el estímulo asociado con la cocaína. CFos es un gen de expresión temprana que también actúa como factor de transcripción, que, a su vez codifica la proteína cFos. Varios tipos de proteínas similares al Fos, incluyendo el cFos son inducidas en las neuronas por estímulos agudos. Debido a su patrón de expresión temporal se utiliza tradicionalmente como marcador de actividad neuronal (Curran y Morgan, 1995; Nestler, 2004). Se ha observado que, en el cerebelo, la expresión máxima se produce entre 60 y 90 minutos después de la aparición del estímulo (Tian y Bishop, 2002). Debido a este patrón de expresión temporal, se evaluaron los niveles de cFos 70 minutos después de la prueba de preferencia.

Aunque se observó un aumento en la actividad de todos los lóbulos cerebelares, el efecto fue especialmente prominente en los lóbulos posteriores VIII, IX y X. Anteriormente, se observó que estos lóbulos cerebelosos reciben inervación dopaminérgica a traves del VTA (Ikai et al, 1992; Melchitzky y Lewis, 2000). Además, expresan el transportador y receptores de dopamina (Delis et al, 2008, Shimizu et al, 2014, Vazquez-Sanroman et al, 2015a). Curiosamente, se detectó la expresión de DAT en lóbulos posteriores en los animales tratados con cocaína. Este aumento fue mayor en el lóbulo X en aquellos animales que mostraron preferencia condicionada por la cocaína (Carbo-Gas et al, 2014a). Nuestros resultados concuerdan con los de Anderson et al (2006), quienes observaron una mayor unión de un ligando similar al DAT en el vermis cerebeloso de adictos a la cocaína.

Sin embargo, nuestro estudio no es el primero que muestra la expresión de cFos inducido por drogas en el cerebelo. Anteriormente, algunos estudios describieron un aumento en la expresión de cFos en la capa granular y en las neuronas de Purkinje después de inyecciones repetidas de cocaína o anfetamina (Clark et al, 1992; Klitenick et al, 1995, Yaz et al, 2010; Vazquez-Sanroman et al, 2015a). Sin embargo, una de las contribuciones de la presente investigación es demostrar que el aumento de la actividad podría representar una característica particular de la expresión de preferencia condicionada por la cocaína. Es decir, que dicho aumento en la expresión de cFos se produce exclusivamente en aquellos animales que expresan preferencia por el estímulo asociado con la cocaína. Como esperábamos, cuanto mayor sea la actividad de las células granulosas, mayor será la expresión de preferencia. Otro hallazgo importante es la regionalización de la activación neuronal, limitada a la parte dorsal de la capa granular. Esta selectividad probablemente representa una regionalización anatómica de las aferencias de la corteza cerebelosa. De hecho, se ha observado que las fibras musgosas del cerebelo se distribuyen en una disposición concéntrica (Voogd, 2014). Mientras que las redes corticopontinas y los componentes extereoceptivos de las fibras musgosas alcanzan la parte apical de los lóbulos, los componentes propioceptivos del mismo sistema terminan en la parte ventral de los lóbulos cerebelosos (Voogd y Ruigrok, 2004).

En nuestra investigación, la inclusión de dos grupos control adicionales, el grupo salino y el grupo no emparejado nos permiten diseccionar los efectos farmacológicos de la cocaína y proporcionar el control más apropiado para la adquisición de la asociación pavloviana entre EC y EI. Los estudios realizados en aprendizaje emocional y aprendizaje motor apoyan la hipótesis de que las memorias responsables de estas respuestas condicionadas pueden ser formadas y almacenadas en la corteza cerebelosa

(Freeman y Steinmetz, 2011; De Zeeuw y Ten Brinke, 2015). Además, otros estudios que abordan la implicación del cerebelo en el condicionamiento han demostrado que EC y EI alcanzan la corteza cerebelosa por dos vías diferentes (Ruigrok y Voogd, 2000). La información del estímulo condicionado llega a la corteza cerebelosa a través de las fibras musgosas originadas en el núcleo pontino. Sin embargo, la información del estímulo incondicionado proviene de la oliva inferior y llega al cerebelo a través de fibras de trepadoras (Thompson y Steinmetz, 2009). Las fibras musgosas y trepadoras envían colaterales a los núcleos profundos cerebelosos. Estos dos aferentes excitatorios, controlan la salida GABAérgica de Purkinje hacia los núcleos cerebelosos profundos, modificando la liberación de información del cerebelo (Ito, 1984). En base a estas evidencias, se evaluó la expresión de cFos en la oliva inferior y en el núcleo pontino, así como en el núcleo medial (Roostaei et al, 2014). En este caso, observamos que los niveles de cFos en estas áreas cerebrales aumentaron en el grupo no emparejado después de la prueba de preferencia. Dicho resultado podría indicar que las neuronas en las aferencias y eferencias del cerebelo aumentan su actividad cuando no hay contingencia entre estímulos ambientales y la administración de cocaína y, por consiguiente, el patrón de comportamiento adaptado a la situación contextual es incierto. Esta idea parece ser coherente con la participación del cerebelo en la predicción de eventos internos relacionados con señales externas (D'Angelo y Casali, 2013; Peterburs y Desmond, 2016). Por lo tanto, se podría esperar que cuando la relación entre los estímulos externos y los eventos internos son más predecibles, y las reacciones conductuales estén más ajustadas a las demandas ambientales, la entrada de información podría ser suprimida progresivamente.

También se debe tener en cuenta que el patrón de expresión de cFos observado no fue debido meramente a un efecto neurofarmacológico de la cocaína. Primero, porque la

última inyección de cocaína tuvo lugar 48 horas antes de la prueba de preferencia. En segundo lugar, porque ninguno de los otros dos grupos tratados con cocaína mostró el mismo patrón de actividad en ninguno de los estudios desarrollados en la presente investigación. Por lo tanto, nuestra primera explicación fue que el patrón de actividad exhibida por los animales que prefieren el estímulo asociado con la cocaína podría ser inducida por la reactivación de las memorias relacionadas con la droga. Inesperadamente, esta idea no fue apoyada por los resultados observados en el grupo de animales confinadoscjunto con el EC 24 horas después de la prueba de preferencia (Carbo-Gas et al, en revisión). En este caso, el patrón de expresión de cFos no fue diferente entre los ratones que mostraron preferencia condicionada por la cocaína y los que no lo hicieron. Por lo tanto, el aumento selectivo de la actividad en las células granulares podría deberse no a la reactivación de la asociación EC/EI, sino a la selección de la acción requerida para abordar la señal previamente asociada con la cocaína. Esta interpretación ligaría el cerebelo dorsal a la selección de acción durante un proceso de recompensa, debido a que la selección de acción no era posible para estos animales bajo confinamiento.

Sin embargo, no podemos descartar otras explicaciones alternativas, como un supuesto decaimiento de la memoria inducida primero, por la prueba de preferencia y luego, por el confinamiento, ya que ambos son esencialmente ensayos de extinción.

Dado que uno de los principales resultados de la presente investigación fue el aumento selectivo de la actividad en las células granulares, se introdujo un enfoque causal para probar el papel de la función celular granular en la cocaína inducida por condicionamiento de preferencia: el uso deratones modificados genéticamente de la cepa α6Cre-Cacna1a. Estos ratones muestran una disminución en la función glutamatérgica producida por la falta de canales de calcio controlados por voltaje P/Q

(Galliano et al, 2013a). Estos ratones mostraron una citoarquitectura cerebelosa normal, pero problemas en la consolidación de una tarea de aprendizaje motor que se asoció con un déficit de en la plasticidad sináptica entre las fibras paralelas y las células de Purkinje (Galliano et al, 2013a). Además, no se observó ningún deterioro en la percepción olfativa, el rendimiento en el MWM, la adquisición de memorias de miedo y el comportamiento relacionado con la ansiedad en estos ratones (Galliano et al, 2013b). Nuestros resultados muestran que los dichos ratones adquirieron preferencia condicionada inducida por la cocaína, pero no fueron capaces de mantener estas memorias a pesar de ser reentrenados en unas pocas sesiones adicionales. Este resultado apunta a un deterioro en los mecanismos de consolidación, como se ha observado anteriormente empleando diferentes paradigmas de condicionamiento (Galliano et al, 2013ab).

El otro hallazgo importante derivado de la presente tesis doctoral es que la preferencia condicionada inducida por la cocaína incrementa la expresión selectiva de PNNs alrededor de las células de Golgi localizadas en la región dorsal de la capa granular. El estudio de los PNNs ha ganado relevancia en los últimos años como mecanismo de plasticidad fundamental cuando surgió una nueva construcción conceptual denominada "la sinapsis tetrapartita" (Dityatev y Rusakov, 2011; Smith et al, 2015). Más allá del punto de vista tradicional sobre la función sináptica, este concepto emergente incluyó dos nuevos elementos en la arquitectura sináptica junto con los clásicos elementos presinápticos y postsinápticos: astrocitos y MEC. Los PNNs son estructuras especializadas compuestas por moléculas de la MEC que rodean el soma y las neuritas proximales de varias poblaciones interneuronales. Se ha demostrado que son cruciales para el mantenimiento de los contactos sinápticos previamente existentes y la prevención de la aparición de nuevos, posiblemente contribuyendo a la estabilidad de

las memorias (Botta et al, 2014). El aumento de la expresión de PNN sólo se observó en los animales que expresaron preferencia el estímulo asociado con la cocaína. Dicho aumento fue de especial interés para la presente investigación debido a la relación entre las interneuronas de Golgi y las células granulares. De hecho, las neuronas de Golgi desempeñan un papel fundamental en la modulación de la actividad de las células granulares (D'Angelo et al, 2013). Se ha observado que las neuronas de Golgi controlan los patrones de disparo de las células granulares, sincronizan la actividad de los grupos de células granulares (Eccles et al, 1964) y modulan la plasticidad de las sinapsis de las células granulares (Armano et al, 1999). A su vez, la actividad de las células de Golgi está regulada por la actividad glutamatérgica de las células granulares y las fibras musgosas (Palay y Chan-Palay, 1973) junto con el control inhibitorio ejercido por otras interneuronas (Sotelo y Llinas, 1972). Además, la plasticidad sináptica en la capa granular está altamente controlada por la actividad de las células de Golgi. Como ejemplo, el bloqueo de la función de Golgi induce LTP in vitro (Mapelli y D'Angelo, 2007) e in vivo (Roggeri et al, 2008). Por el contrario, cuando se aumenta su actividad, se observa LTD (D'Angelo y De Zeeuw, 2009). Hasta donde sabemos, no hay información sobre los efectos de la exposición repetida a fármacos sobre la función de Golgi y la plasticidad. Los pocos datos disponibles sólo describen un aumento de la excitabilidad de las células de Golgi y una disminución de la actividad granular en respuesta a la administración aguda de etanol in vitro (Carta et al, 2004), e in vivo (Huang et al, 2012).

Es importante destacar que también hemos observado que los ratones α6Cre-Cacna1a mostraron una expresión de PNNs reducida. Esta regulación a la baja podría estar asociada con el deterioro en la consolidación visto en estos ratones. La disminución en la expresión de PNNs puede estar relacionada con la reducción de actividad de las

neuronas de Golgi causada por la desconexión de los canales de calcio controlados por voltaje de tipo P/Q en células granulares. En conjunto, este resultado vincula directamente la expresión de PNNs en las células de Golgi tanto con la memoria inducida por la cocaína como con la función glutamatérgica mediada por las células granulares. Sin embargo, los animales que mostraron preferencia por el estímulo asociado con la cocaína y estaban confinados con el EC 24 horas después de la prueba de preferencia, todavía mostraban una mayor expresión de PNNs en comparación con los animales que no preferían el estímulo asociado a la cocaína. No obstante, como se mencionó anteriormente, el aumento de la actividad granular observada en animales que muestran preferencia condicionada se abolió después del confinamiento. Esta falta de correlación también nos permite descartar el aumento en la expresión de los PNNs de Golgi como un epifenómeno que resultó meramente de la hiperactividad de las células granulares. Por lo tanto, se puede especular que los mecanismos de memoria asociadas a la cocaína dependientes del cerebelo, implican directamente a los PNNs que rodean a las células de Golgi. En consecuencia, los PNN de Golgi podrían ser responsables de la estabilización de los contactos sinápticos y la prevención de una modificación posterior en la capa granular, proporcionando así un mecanismo mediante el cual las asociaciones entre estímulos pueden mantenerse en el cerebelo (Sorg et al, 2016).

Los cambios inducidos por la cocaína en los PNNs cerebelosos muestran una especificidad regional (Sorg et al, 2016). De hecho, los PNNs que rodean a las neuronas de proyección en el núcleo medial del cerebelo no se vieron afectados por la adquisición de preferencia condicionada por la cocaína. A su vez, todos los grupos tratados con cocaína mostraron una expresión de PNNs reducida en comparación con los animales tratados con solución salina. Este efecto puede ser causado por el efecto farmacológico de la cocaína en lugar de estar relacionado con la memoria inducida por la cocaína. Se

ha demostrado que la cocaína es suficiente para modificar la arquitectura sináptica en los núclos profundos del cerebelo (Vazquez-Sanroman et al, 2015ab). Consistentemente, los ratones modificados genéticamente α6Cre-Cacna1a y sus compañeros de camada no modificados genéticamente mostraron niveles similares de expresión de PNNs en los núcleos profundos del cerebelo dado que ambos grupos fueron tratados con cocaína, descartando así una participación de los PNNs de los núclos profundos en un proceso de memoria.

Basándonos en el incremento selectivo de la expresión de los PNNs de Golgi en animales que muestran preferencia por el EC y en el hecho de que la interrupción de PNNs en otras regiones cerebrales ha sido capaz de atenuar las memorias inducidas por drogas y mejorar la extinción de dichas memorias, debe ser considerado como un objetivo futuro la manipulación de estas estructuras en modelos animales de adicción a las drogas.

En resumen, la relevancia obtenida por un estímulo asociado con la cocaína es acompañada por PNNs más intensos en las neuronas de Golgi y mayores niveles de cFos en las células granulares de la parte apical del cerebelo posterior. Estos efectos podrían considerarse como las características particulares del cerebelo en un proceso de preferencia por un estímulo asociado con la cocaína.

FORTALEZAS, PUNTOS DÉBILES Y DIRECCIONES FUTURAS

FORTALEZAS

1. Una de las principales contribuciones de esta investigación es permitir la inclusión del cerebelo como parte de las redes involucradas en las memorias relacionadas con las drogas.

2. Nuestros hallazgos identifican dos características particulares de las memorias relacionadas con la cocaína en el cerebelo por primera vez.

3. Utilizamos una manipulación genética muy específica para determinar causalmente el papel de las neuronas granulares en el condicionamiento de preferencia inducido por la cocaína.

4. Esta investigación apunta a que la parte dorsal de la capa granular del cerebelo es un locus para los cambios de plasticidad inducida por drogas relacionados con el condicionamiento de preferencia.

5. Esta es la primera investigación que vincula los PNNs expresados alrededor de las interneuronas de Golgi a memorias relacionadas con las drogas, y que muestra la especificidad regional para los cambios inducidos por la cocaína.

6. Otro aspecto importante de esta tesis es la validación de un protocolo para producir condicionamiento olfativo de preferencia inducido por cocaína.

7. Por último, otro punto clave de esta tesis doctoral es el uso de métodos estadísticos innovadores como el análisis de la función de discriminación y la estimación de los tamaños del efecto para determinar mejor la relevancia funcional de los cambios cerebelosos inducidos por drogas.

PUNTOS DÉBILES Y DIRECCIONES FUTURAS

1. Los presentes estudios se enfocaron exclusivamente en la implicación del vermis cerebeloso en las memorias inducidos por la cocaína. Sin embargo, sería interesante explorar si la plasticidad y la actividad neuronal es diferente en los hemisferios cerebelosos debido al hecho de que los estudios de neuroimagen humanos también han mostrado cambios en los hemisferios cerebelosos después de la exposición a estímulos asociados a las drogas.

2. Es necesaria una investigación adicional para aclarar la participación de las neuronas de Purkinje en la memoria inducida por la cocaína. En una investigación anterior del laboratorio, se ha demostrado que la plasticidad de Purkinje está claramente afectada por la cocaína (Vázquez-Sanroman et al., 2015ab). Sin embargo, en la presente investigación, obtuvimos resultados inconsistentes sobre la participación de estas neuronas.

3. Sería muy instructivo explorar si los cambios de plasticidad descritos en la corteza cerebelosa serian modulados por el entrenamiento en extinción.

4. La expresión de cFos se ha utilizado ampliamente como un marcador de actividad neuronal. Sin embargo, la electrofisiología sería un mejor enfoque para probar los cambios de actividad inducidos por la cocaína relacionados con el condicionamiento de preferencia.

5. También es necesario ir más allá en la caracterización de los PNNs cerebelosos, incluyendo la evaluación de diferentes GPCG expresados en los PNNs de Golgi.

6. También es necesario explorar la plasticidad cerebelosa y la actividad neuronal en otros paradigmas conductuales relevantes para la adicción a las drogas, como la auto-administración de la cocaína.

7. Los resultados aquí presentados son relevantes, pero se han obtenido utilizando principalmente un enfoque correlacional. Por lo tanto, es necesario un enfoque causal para explicar el papel que desempeña el cerebelo en las memorias relacionadas con la droga.

8. Tanto la degradación como el fortalecimiento de los PNNs en el cerebelo utilizando la enzima bacteriana ChABC y los inhibidores de MPP también son necesarios.

9. El uso de DREADDs para manipular específicamente la actividad neuronal alrededor de los PNNs de Golgi nos permitiría describir adecuadamente la regulación de los PNNs cerebelosos y sus consecuencias funcionales.

10. Finalmente, sería importante evaluar la participación cerebelosa en la preferencia inducida por los alimentos, a fin de comprender si el papel del cerebelo en la preferencia inducida por la cocaína es selectivo para las drogas adictivas o también para los reforzadores naturales.