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Turning on and off the cerebellum in drug-induced memory

Report summited by **Ignasi Melchor Eixea** to be eligible for a doctoral degree awarded by the Universitat Jaume I

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Abbreviations list

A	
AAV	Adeno-associated virus
AAVrg	Retrograde adeno-associated virus
Acq	Acquisition
B	
BC	Basket cell
C	
cf	Climbing fiber
cFos	Protein cFos
ChABC	Chondroitinase ABC
CI	Confidence interval
CS	Conditioned stimuli
CS+	Conditioned stimulus not followed by the US
CS-	Conditioned stimulus followed by the US
D	
DA	Dopamine
DCN	Deep cerebellar nuclei
DS	Dorsal striatum
E	
EGFP	Enhanced green fluorescent protein
G	
GABA	Gamma-aminobutyric acid
GCL	Granule cell layer
GFP	Green fluorescent protein
GO	Golgi cell
GO	Granule cell
I	
IL	Infralimbic cortex
Int	Interposed DCN
IO	Inferior olive
L	
Lat	Lateral DCN
LC	Lugaro cell
LVII	Lobule VII
LVIII	Lobule VIII
M	
Mdn	Medial DCN
Med	Medial DCN
mf	Mossy fiber
ML	Molecular layer
MLIs	Molecular layer interneurons
mPFC	Medial prefrontal cortex
N	
NAcC	Nucleus Accumbens
NG	Neurogranin
O	
OFC	OFC
P	
PAG	Periaqueductal gray
PBP	PBP
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline
PC	Purkinje cell
PCL	Purkinje cell layer
pf	Parallel fiber

PN	Pontine nuclei
PNN	Perineuronal net
PL	Prelimbic cortex
PV	Parvalbumin
R	
RN	Red nucleus
ROI	Region of interest
S	
SC	Stellate cell
SUD	Substance use disorders
T	
TH	Tyrosine hydroxylase
Th	
U	
UB	Unipolar brush cell
US	Unconditioned stimuli
V	
vGAT	Vesicular GABA transporter
vGluT1	Vesicular glutamate transporter 1
vGluT2	Vesicular glutamate transporter 2
VTA	Ventral Tegmental area
W	
WFA	Wisteria floribunda agglutinin
wm	White matter
ΔMdn	Difference between medians

PREFACE

This comprehensive thesis encompasses an exploration of the cerebellum's role in drug-induced memories and its relationship with other critical regions of the addiction circuitry. Over the last decade, neuroscience research has illuminated the cerebellum's central involvement in addiction, particularly highlighting its pivotal role in the associative learning processes that link drug use to contextual cues (Miquel et al., 2009, 2016, 2020; Moulton et al., 2014).

Chapter 1 of this thesis builds upon prior research conducted by our team, which convincingly demonstrated that the posterior cerebellum's vermis (lobule VIII) has the capacity for undergoing significant changes in neural activity during the expression of drug-context memories (Carbo-Gas et al., 2014a; Carbo-Gas et al., 2014b; Carbo-Gas et al., 2017, Gil-Miravet et al., 2021; Guarque-Chabrera et al., 2022). While our earlier investigations into the cerebellar bases of drug-induced Pavlovian memory primarily involved mice or rats on unbiased procedures, this study seeks to replicate and expand upon these findings using a biased conditioned place preference procedure with rats. We assessed neural activity patterns using c-Fos expression within specific regions of the cerebellum, focusing intently on the granular layer within lobules VII and VIII of the vermis and paravermis, as well as Crus 1 and Crus 2 of the hemispheres. We compared rats expressing and not expressing preference towards cocaine-related cues, as well as a pseudo-conditioned group called the unpaired group as our experimental subjects. In accordance with our previous findings, results in Chapter 1 reveal consistent differences within the granular layer of lobule VIII between rats that exhibit a preference for cocaine-related contextual cues and those that do not. Remarkably, rats exhibiting a pronounced preference for cocaine-associated contextual cues such as odor and tactile stimuli showed increased neural activity in the granule cell layer of the dorsal posterior cerebellar vermis. Notably, we did not observe an effect of cocaine-induced conditioning on activity either in the paravermis or the hemispheres. Therefore, the findings from Chapter 1 unveil a potential medial-lateral gradient in the involvement of the cerebellar cortex in shaping Pavlovian memories associated with drug use.

The preference for the cocaine rewarded compartment correlated with the increased activity in the granule cell layer only in the posterior vermis but not with changes in the neural activity in neither paravermis nor hemispheres. Collectively, these findings align with results observed in human neuroimaging studies in which the presentation of drug-related cues triggers drug craving and stimulates cerebellar activity (Grant et al., 1996; Wang et al., 1999; Anderson et al., 2006; Schneider et al., 2001; Martin-Sölch et al., 2001; Filbey et al., 2009; Olbrich et al., 2009; Lou et al., 2012; Xuan et al., 2019; Al-Khalil et al., 2020). and complement previous research conducted in our laboratory using different paradigms.

Chapter 2 expands on previous research by our laboratory and the preceding findings. By using lesioning and temporary deactivation techniques, our early studies suggested the involvement of the dorsal posterior vermis in the inhibitory regulation of preferences for stimuli associated with cocaine (Gil-Miravet et al., 2019). However, these interventions lacked cell-specificity. Thus, in the present chapter, we sought to investigate the dorsal cerebellum's role in the acquisition of conditioned preferences for stimuli associated with cocaine availability using DREADDs (Designer Receptors Exclusively Activated by Designer Drugs), a chemogenetic strategy. These experiments encompass both the activation and inhibition of the dorsal vermis in lobule VIII. The descriptive studies that precede this chapter unveiled the existence of two distinct neuronal populations within the cerebellar cortex - granule cells and Golgi interneurons - which may play an instrumental role in the formation of associative drug-context memories. (Carbo-Gas et al., 2014a; Carbo-Gas et al., 2014b; Carbo-Gas et al., 2017). Our observations validate the exclusive expression of the hSyn promoter by Golgi interneurons in the granule cell layer and interneurons within the molecular layer, including basket and stellate cells. The activation of these two inhibitory neuronal populations in the cerebellar cortex increased preference for stimuli associated with cocaine. However, their inhibition reduced conditioned preference for cocaine. These findings align with the results of Gil-Miravet (2019, 2021), which showed that conditioning training under a lesion of lobule VIII dramatically increased the fraction of animals that expressed cocaine-induced conditioned preference, involving the dorsal cerebellum in the inhibitory regulation of cocaine-induced

Pavlovian learning. In the case of the excitatory DREADD, we hypothesize that the facilitation of cocaine-induced conditioning might be attributed to the excitation of interneurons in the dorsal vermis that in turn would reduce activity in Purkinje cells (PC) enhancing Deep Cerebellar Nulcei (DCN) activity. Conversely, the decrease in conditioned preference by the inhibitory DREADD is likely due to Purkinje cell disinhibition that would lead to a reduction in DCN activity. These findings provide valuable insights into how manipulating specific neural pathways in the cerebellum can impact the development of cocaine-induced preference.

To provide further context, Chapter 3 is part of an article already published in *Frontiers in Systems Neuroscience* that offers a theoretical model for understanding the cerebellum's role in cocaine-induced Pavlovian memory. The chapter also presents research using DREADDs, to further investigate and propose a causative working model for the role of the cerebellum in cocaine-induced conditioned memory. In these experiments, we inhibited the Interposed/Lateral DCN that connects Lobule VIII with the addiction pathway. Our findings suggest that inactivation of DCNs prevents the facilitating effect of a posterior vermis lesion on cocaine-induced preference conditioning. While these findings support our previous research (Gil-Miravet et al; 2019, 2021), they also introduce new questions and avenues for exploration.

Finally, in Chapter 4, we focus on the monosynaptic pathway connecting the Interposed DCN to the Ventral Tegmental areal (VTA). This pathway, as previously identified in studies by our research group and others (Carta et al., 2019; Gil-Miravet et al.,2021), also receives axonal input from Purkinje cells in the cerebellar vermis lobule VII (Gil-Miravet et al.,2021). This intricate network is believed to regulate activity and plasticity in the medial prefrontal cortex (mPC) and striatum, playing a pivotal role in cocaine-induced conditioned preference (Gil-Miravet et al., 2021). The primary objective of Chapter 4 is to test the hypothesis that the cerebellum regulates mPC cortex (IL) activity through the VTA using chemogenetic tools. To achieve this, we infused an inhibitory *cre*-dependent DREADD into the interposed nucleus and a *cre*-retrograde virus in the VTA. This allows us to exclusively express these receptors in the DCNs-VTA projections, and we subsequently examine the inhibition of neurons in the

DCNs, VTA, and IL. Inhibition of the Interposed/Lateral DCNs leads to a decrease in neural activity within both the VTA and IL. These findings offer further support for prior research, which has indicated the existence of a monosynaptic excitatory projection from the DCN to the VTA (Gil-Miravet et al.,2021). This result highlights the intricate neural model involving the cerebellum, VTA, and IL.

In conclusion, this thesis advances our understanding of the cerebellum's multifaceted role in addiction, particularly in the context of drug-induced memories. The study elucidates the neural mechanisms, circuits, and pathways involved, shedding light on the cerebellum's influence on conditioned preferences and its potential as a therapeutic target for addiction intervention. These findings contribute to the ongoing efforts to combat addiction and address its enduring behavioral alterations.

RESUMEN

Esta tesis aborda la exploración del papel del cerebelo en las memorias inducidas por drogas y su relación con otras regiones clave de la circuitería de la adicción. A lo largo de la última década, la investigación en neurociencia ha demostrado la participación del cerebelo en la adicción, destacando especialmente su papel fundamental en los procesos de aprendizaje asociativo que vinculan el uso de drogas con señales contextuales (Miquel et al., 2009, 2016, 2020; Moulton et al., 2014).

El Capítulo 1 de esta tesis se basa en investigaciones previas realizadas por nuestro equipo, que demostraron de manera convincente que el vermis del cerebelo, especialmente el lóbulo VIII, experimenta cambios a nivel de actividad neuronal durante la expresión de memorias asociadas al contexto de administración de cocaína (Carbo-Gas et al., 2014a; Carbo-Gas et al., 2014b; Carbo-Gas et al., 2017, Gil-Miravet et al., 2021; Guarque-Chabrera et al., 2022). Mientras que nuestras investigaciones anteriores sobre las bases cerebelosas de la memoria pavloviana inducida por drogas se usaron ratones o ratas en procedimientos que no tienen en cuenta la preferencia innata de los animales por ciertos contextos, este estudio busca replicar y ampliar estos hallazgos utilizando un procedimiento de preferencia condicionada de lugar sesgado contra la preferencia innata en ratas. Evaluamos patrones de actividad neuronal utilizando la expresión de c-Fos en regiones específicas del cerebelo, centrándonos especialmente en la capa granulosa dentro de los lóbulos VII y VIII del vermis y paravermis, así como Crus 1 y Crus 2 de los hemisferios. Empleamos como sujetos experimentales ratas que expresaban y no expresaban preferencia hacia los contextos asociados con la administración de cocaína, así como un grupo control pseudocondicionado llamado grupo desemparejado. De acuerdo con nuestros hallazgos anteriores, los resultados en el Capítulo 1 revelan diferencias consistentes en la capa granular del lobulillo VIII entre las ratas que muestran preferencia por las señales contextuales relacionadas con la cocaína y aquellas que no lo hacen. Observamos que las ratas que mostraban una preferencia pronunciada por señales asociadas a la cocaína, concretamente olores y los estímulos táctiles, mostraron un aumento en la actividad neuronal en la capa de células granulares del vermis cerebeloso dorsal posterior. Cabe destacar que no observamos un efecto del

condicionamiento inducido por cocaína en la actividad ni en el paravermis ni en los hemisferios. Por lo tanto, los hallazgos del Capítulo 1 revelan un posible gradiente medial-lateral en la participación de la corteza cerebelosa en la formación de memorias pavlovianas asociadas con el uso de drogas. La preferencia por el compartimento asociado con cocaína parece estar estrechamente relacionada con el aumento de la actividad en la capa de células granulosas. En conjunto, estos hallazgos se alinean con los resultados observados en estudios de neuroimagen en humanos y complementan investigaciones previas realizadas en nuestro laboratorio utilizando paradigmas diferentes. Este estudio contribuye a una comprensión translacional y ofrece perspectivas adicionales sobre la participación cerebelosa en la preferencia inducida por la cocaína.

El Capítulo 2 amplía investigaciones anteriores de nuestro laboratorio y los hallazgos previos. Mediante el uso de técnicas de lesión y desactivación temporal, nuestros primeros estudios sugirieron la participación del vermis dorsal posterior en la regulación inhibitoria de las preferencias por estímulos asociados con la cocaína (Gil-Miravet et al., 2019). Sin embargo, estas intervenciones carecían de especificidad celular. Por lo tanto, en el presente capítulo, nuestro objetivo fue investigar el papel del cerebelo dorsal en la adquisición de preferencias condicionadas por estímulos asociados con la disponibilidad de cocaína utilizando DREADDs (Receptores Diseñados Exclusivamente Activados por Fármacos de Diseño), una estrategia quimogenética. Estos experimentos abarcan tanto la activación como la inhibición del vermis dorsal en el lobulillo VIII. Los estudios descriptivos que preceden a este capítulo revelaron la existencia de dos poblaciones neuronales distintas dentro de la corteza cerebelosa: las células granulares e interneuronas de Golgi, que pueden desempeñar un papel fundamental en la formación de memorias asociativas al contexto de drogas (Carbo-Gas et al., 2014a; Carbo-Gas et al., 2014b; Carbo-Gas et al., 2017). Nuestras observaciones validan la expresión exclusiva del promotor hSyn por las interneuronas de Golgi en la capa de células granulosas y las interneuronas dentro de la capa molecular, incluyendo células en cesta y células esteladas. La activación de estas dos poblaciones neuronales inhibitorias en la corteza cerebelosa aumentó la preferencia por estímulos asociados con la cocaína. Sin embargo, su inhibición redujo la

preferencia condicionada por la cocaína. Estos hallazgos coinciden con los resultados de Gil-Miravet (2019, 2021), que mostraron que el entrenamiento de condicionamiento bajo una lesión del lóbulo VIII aumentó drásticamente la fracción de animales que expresaron preferencia condicionada inducida por cocaína, involucrando al cerebelo dorsal en la regulación inhibitoria del aprendizaje pavloviano inducido por cocaína. En el caso del DREADD excitatorio, hipotetizamos que la facilitación del condicionamiento inducido por cocaína puede atribuirse a la excitación de interneuronas en el vermis dorsal, lo que a su vez reduciría la actividad en las células de Purkinje aumentando la actividad del núcleo profundo cerebeloso (DCN). Por el contrario, la disminución de la preferencia condicionada por el DREADD inhibitorio se debe probablemente a la desinhibición de las células de Purkinje, lo que llevaría a una reducción en la actividad del DCN. Estos hallazgos ofrecen valiosas perspectivas sobre cómo manipular vías neurales específicas en el cerebelo puede afectar el desarrollo de la preferencia inducida por cocaína.

Para proporcionar un contexto adicional, el Capítulo 3 es parte de un artículo ya publicado en Frontiers in Systems Neuroscience que ofrece un modelo teórico para entender el papel del cerebelo en la memoria pavloviana inducida por cocaína. El capítulo también presenta investigaciones utilizando DREADDs, para investigar y proponer un modelo causal de trabajo para el papel del cerebelo en la memoria condicionada inducida por cocaína. En estos experimentos, inhibimos el núcleo Interpuesto/Lateral de los DCN que conecta el lobulillo VIII con la vía de la adicción. Nuestros hallazgos sugieren que la iNAcctivación de los DCN previene el efecto facilitador de una lesión del vermis posterior en el condicionamiento de preferencia inducido por cocaína. Si bien estos hallazgos respaldan nuestra investigación anterior (Gil-Miravet et al., 2019, 2021), también plantean nuevas preguntas y vías de exploración.

Finalmente, en el Capítulo 4 nos enfocamos en la vía monosináptica que conecta el DCN Interpuesto con el VTA. Esta vía, como se identificó previamente en estudios de nuestro grupo de investigación y otros (Carta et al., 2019; Gil-Miravet et al., 2021), también recibe entrada axonal de las células de Purkinje en el lobulillo VIII del vermis cerebeloso (Gil-Miravet et al., 2021). Se cree que esta red regula la actividad y la

plasticidad en la corteza prefrontal medial y el estriado, desempeñando un papel fundamental en la preferencia condicionada inducida por cocaína (Gil-Miravet et al., 2021). El objetivo principal del Capítulo 4 es probar la hipótesis de que el cerebelo regula la actividad de la corteza IL a través del VTA mediante herramientas quimogenéticas. Para lograr esto, infundimos un DREADD floxeado en el núcleo interpuesto y un virus retrogrado dependiente de cre en el VTA. Esto nos permite expresar exclusivamente estos receptores en las proyecciones DCN-VTA, y posteriormente examinamos la inhibición de neuronas en los DCN, VTA e IL. La inhibición de los nucleos Interpuesto y Lateral conduce a una disminución en la actividad neuronal tanto en el VTA como en la IL. Estos hallazgos respaldan aún más investigaciones anteriores que han indicado la existencia de una proyección excitatoria monosináptica del DCN al VTA (Gil-Miravet et al., 2021, Carta et al., 2019). Este resultado subraya el intrincado modelo neural que involucra al cerebelo, el VTA y la IL.

En conclusión, esta tesis avanza en nuestra comprensión del papel multifacético del cerebelo en la adicción, especialmente en el contexto de las memorias inducidas por drogas. El estudio aclara los mecanismos, circuitos y vías neuronales involucradas, arrojando luz sobre la influencia del cerebelo en las preferencias condicionadas y su potencial como objetivo terapéutico para la intervención en la adicción. Estos hallazgos contribuyen a los esfuerzos continuos para combatir la adicción y abordar sus duraderas alteraciones conductuales.

GENERAL INTRODUCTION

Substance Use Disorder, reward and associative learning

Numerous individuals use drugs, and as a result, millions fall victim to addiction. Thus, drug addiction or Substance Use Disorder (SUD) is one of the most prevalent conditions among mental disorders, particularly in developed nations (NIDA, 2022). Furthermore, due to the adverse health effects stemming from prolonged drug use, SUD stands as one of the most financially burdensome health challenges in these regions (EMCDDA,, 2011).

SUD can be defined as the persistent pursuit and consumption of drugs, even in the face of negative consequences (EMCDDA, 2014). From a clinical standpoint, the American Society for Addiction Medicine characterizes addiction as a chronic brain disorder that affects reward, motivation, memory, and associated neural pathways. It involves the inability to consistently abstain, impaired behavioral control, intense cravings, diminished recognition of the adverse impact on one's actions and relationships, and an altered emotional response (American Society of Addiction Medicine, 2011).

Consuming natural or synthetic substances for their psychoactive effects is a behavior that is commonly observed in humans and other animals (Piazza & Deroche-Gamonet, 2013). The primary incentive for drug use stems from the pleasurable effects of drugs (Wise, 2009). The progression from occasional recreational drug use to addiction involves distinct stages. Initially, sporadic drug use is a learning process that activates the brain's natural reward systems, causing most individuals to find drugs highly rewarding. In the second stage, intensified and sustained drug use occurs in certain vulnerable individuals with a hyperactive dopaminergic system and impaired prefrontal cortex function. Finally, prolonged drug exposure results in a loss-of-control phenotype, characterized by altered synaptic plasticity in reward areas, leading to an inability to control drug intake. This stage involves a pathological attachment to drugs (Piazza & Deroche-Gamonet , 2013).

Through repeated drug experiences, individuals often associate the euphoric and pleasurable effects of a drug with environmental cues present during drug use. This process reflects classical or Pavlovian conditioning, where a neutral conditioned stimulus (CS) acquires incentive properties when it is linked to a biologically significant unconditioned stimulus (US) (Pavlov, 1927). The enduring propensity for relapse is one of the cardinal features of SUD (Koob and Le Moal, 1997; O'Brien, 1997). Relapse occurs in response to different precipitating events, including stress and drug priming (Gerber & Stretch, 1975; de Wit and Stewart, 1981; 1983; Shaham & Stewart, 1994). However, one of the strongest triggers for relapse is exposure to environmental stimuli that have become associated with drugs of abuse (Davis & Smith, 1974; See, 2002; 2005). SUD arises from an abnormal learning process that generates potent instrumental memories, associating specific actions with drug-seeking and drug-taking outcomes, resulting in the development of persistent stimulus-response habits (Milton & Everitt, 2012).

The preferences for specific environmental cues can be effectively conducted in a laboratory setting using the Conditioned Place Preference (CPP) paradigm. CPP is a learned behavior exhibited across vertebrate and invertebrate species, including humans. This phenomenon occurs when an individual develops a preference for a particular location due to its previous association with rewarding experiences. The CPP paradigm serves as a valuable tool for investigating the reinforcing effects of both natural and pharmacological stimuli, including addictive substances. Across species, including humans, there is a natural inclination toward certain places over others. This preference might arise from the sensory attributes of specific locations or from associations with significant events. These events contribute to shaping future behavioral patterns and preferences associated with those places. Under controlled experimental conditions, CPP can be induced in various species, such as flies (Kaun et al.,2011) rodents (Cunningham et al., 2006; Tzschentke et al., 2007) primates (Barros et al., 2013) and even humans (Childs et al., 2009).

The addiction network

Drugs of abuse exhibit a wide range of behavioral effects and possess diverse pharmacological profiles, but they commonly share a characteristic: an increase in mesocorticolimbic dopamine (DA) activity (Wise et al., 1996). However, their interactions with this system can vary at different levels and depends on more neurotransmitters such as glutamate and GABA (Cami & Farre, 2003). This neural circuit, extensively implicated in the reinforcing properties of natural stimuli like food, drinks, and sex, as well as addictive drugs, is composed of dopamine projections originating from the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). These projections target both limbic structures (forming the mesolimbic pathway, including the amygdala, ventral pallidum, hippocampus, and Nucleus Accumbens (NAcc) and cortical areas (constituting the mesocortical pathway, encompassing the prefrontal cortex (PFC) the orbitofrontal cortex (OFC), and the anterior cingulate). Interestingly, these structures with distinct roles work together in what is known as the corticostriatolimbic circuit in addiction (Cami & Farre, 2003) (**Figure 1**). For instance, the NAcc and ventral pallidum seem to be primarily involved in the reinforcing effects of drugs (Volkow et al., 2003), while the amygdala and hippocampus play crucial roles in conditioned learning, a significant aspect of addiction. In the case of the amygdala (See, 2005) and ventral hippocampus (Feltenstein & See, 2008), this learning entails forming associations between specific stimuli and rewards. In contrast, the dorsal hippocampus is responsible for stimulus-stimulus associations, which are particularly important for contextual learning (Fuchs et al., 2005, 2007). On another note, the PFC, OFC, and anterior cingulate regulate emotional responses, cognitive control, and executive functions (Volkow et al., 1993).

Glutamate plays a crucial role in regulating both the development and expression of addictive behaviors, such as behavioral sensitization and drug seeking (Kalivas et al., 2009). Repeated exposure to drugs induces cellular adaptations in the prefrontal-NAcc glutamatergic pathway, contributing to persistent addictive behaviors. The activation of prefrontal and amygdala glutamatergic projections to the NAcc plays a crucial role in the expression of addictive behaviors such as drug-seeking or behavioral sensitization.

However, when examining the development of addiction, there has been a growing interest in identifying the specific glutamatergic afferents to dopamine neurons in the VTA that are essential. Potential candidates in this regard include glutamatergic projections from the prefrontal cortex, bed nucleus of the stria terminalis, and pedunculopontine region. These include impaired cognitive control and heightened responsiveness to drug-related cues (Kalivas and Volkow, 2005). When drug-seeking behavior is reinstated in animals trained with heroin or cocaine, there is a substantial increase in the firing activity of prefrontal neurons, coupled with elevated glutamate release in the NAcc (McFarland et al.,2003).

Furthermore, GABA signaling plays a pivotal role in the complex landscape of drug reward and the development of addiction (Shyu et al., 2022). Recent studies have shed light on the potential of exogenous light activation of inputs from the rostromedial tegmental nucleus (RMTg) and the nucleus accumbens (NAcc) into the VTA during the acquisition of cocaine-conditioned place preference. This activation significantly

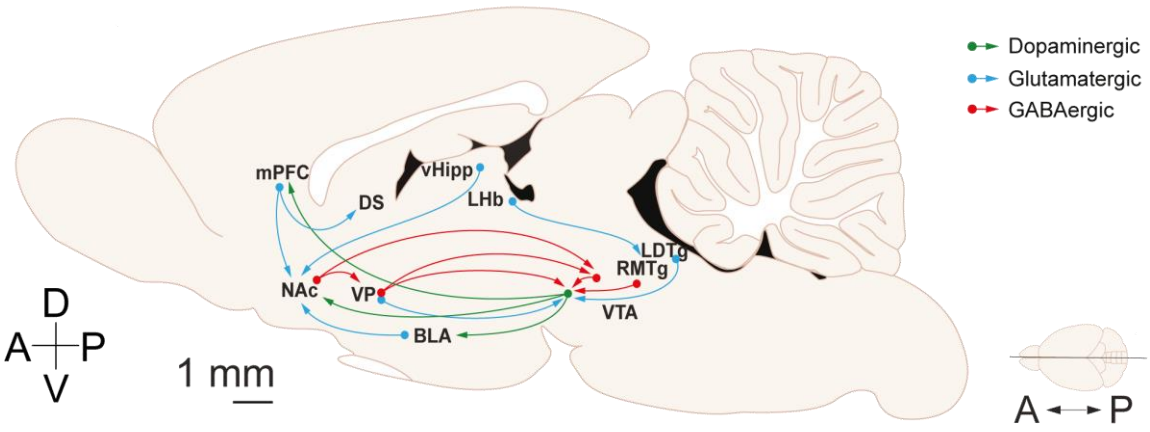

Figure 1. Cortico-striatal-limbic drug addiction circuit without considering the cerebellum. *mPFC: Medial prefrontral cortex; NAcc: Nucleus Accumbes; DS: Dorsal striatum; VP: Ventral pallidum; vHipp: ventral Hippocampus; LHb: Lateral habenula VTA: Ventral tegmental area; RMTg: Rostromedial Tegmental Nucleus; LDTg: Laterodorsal Tegmental Nucleus. Adaptet from Guarque-Chabrera (2023).*

diminishes the rewarding properties of cocaine (Weitz et al., 2021). Activation of GABAergic projections to the VTA or the inhibition of VTA GABA neurons leads to pro-reward behavior, reduced anxiety, heightened motivation, and decreased drug-seeking behavior (Bouarab 2019).

Numerous anatomical and functional investigations have put forward the idea of interconnections between the cerebellum and the striatum-cortico-limbic circuit (Bostan et al., 2013; Bostan and Strick., 2018; Rogers et al., 2011, Gil-Miravet et al.,2019; Carta et al., 2019; Yoshida et al., 2022). In addition to the conventional neuroanatomical model of drug addiction, evidence emerging over a decade ago has begun to highlight the role of the cerebellum in various affected brain functions in individuals with addiction (Miquel et al., 2009, 2016, 2020; Moulton et al., 2014). The research presented here strongly advocates for the inclusion of the cerebellum as a component of the circuitry responsible for the enduring behavioral effects induced by drugs.

Why the Cerebellum?

Anatomy and connections.

The cerebellum, often referred to as the "little brain," is a structure located in the posterior cranial fossa. Despite its smaller size compared to the cerebrum, it contains a significant proportion of the brain's neurons, accounting for about 80% (Herculano-Houzel et al., 2010). The cerebellum is extensively connected to various regions of the brain, including the brainstem, spine, and different cortical and subcortical areas. Traditionally, the cerebellum has been associated with motor control and motor learning, playing a crucial role in coordinating, and refining voluntary movements. Although it does not initiate movements itself, damage to the cerebellum can impair the precise execution and adaptive modification of motor actions. However, there is a growing recognition of the cerebellum's involvement in nonmotor cognitive and emotional functions. Recent advancements in research tools and resources have opened new avenues for investigating the anatomy and physiology of the cerebellum (Schmahmann et al., 1998; Buckner et al., 2013).

In humans, the cerebellum is situated at the posterior part of the brainstem and the fourth ventricle. It is separated from the cerebrum by the tentorium cerebelli, an extension of the dura mater. The cerebellum is composed of two lateral hemispheres and a narrow midline region called the cerebellar vermis, resembling a worm. Its

surface is characterized by numerous parallel transverse folds called folia. The cerebellum consists of an outer layer of convoluted grey matter called the cerebellar cortex. This grey matter surrounds a highly branched white matter structure known as the arbor vitae, which resembles a tree with its branching pattern. Embedded within the central white matter, known as the corpus medullare, are three DCN: the fastigial, Int (including the globose and emboliform nuclei), and dentate nuclei. Anatomically, the cerebellum is divided into three lobes by two transverse fissures. The primary fissure separates the anterior lobe from the posterior lobe (see **Figure 2**), while the posterolateral fissure lies between the posterior lobe and the flocculonodular lobe. In all mammals, the cerebellum is further subdivided into 10 transverse lobules, designated by Roman numerals from I to X. Each lobule encompasses a central portion within the vermis, along with two adjacent lateral segments in the hemispheres (Roostaei et al 2014).

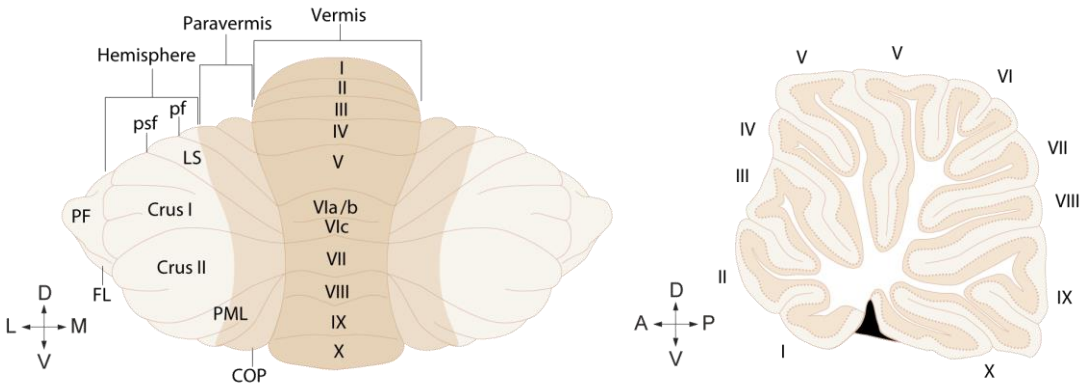

Figure 2. The divisions of the rat's cerebellum. *Antero-posteriorly unfolded cerebellum (left) showinf the vermis, paravermis and hemispheres, and the posterior lobules (COP, copula pyramidis; FL, flocculus; LS, lobulus simplex; PF, paraflocculus; PL, posterior lobe; PML, paramedian lobule; psf, posterior superior fissure pf, primary fissure). The sagittal image (right) shows a medial section and the distribution of all the lobules.*

The entire work of this thesis is done in rat, for this reason we are going to focus on the structure of the rodent's cerebellum. Its cerebellum has a structural arrangement with a thin layer of white matter covered by grey matter, housing three pairs of deep cerebellar nuclei: medial nucleus, interposed nucleus, and lateral nucleus corresponding with fastigial, interposed and dentate nuclei in humans (Voogd and Glickstein, 1998). The grey matter is divided into three layers: the molecular layer,

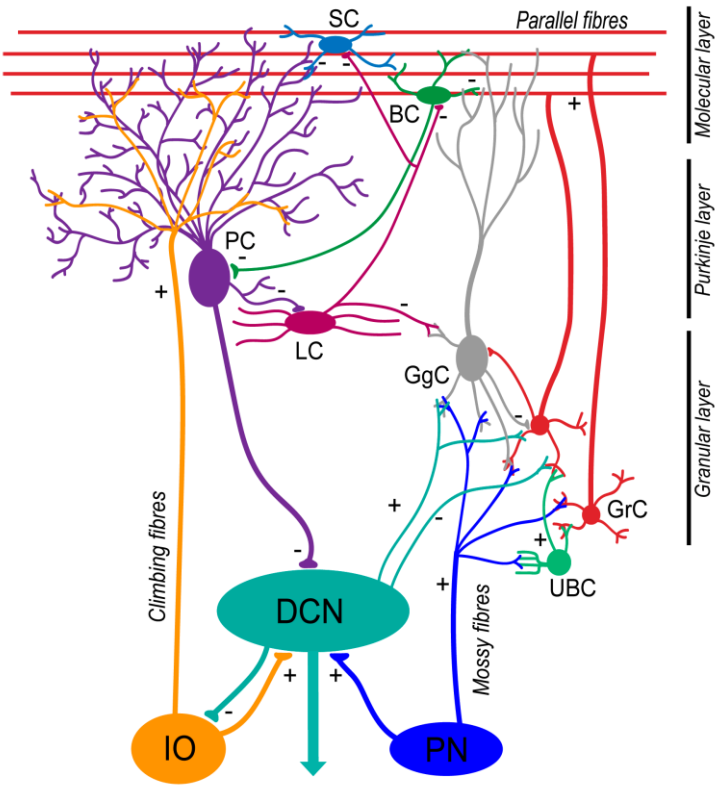

Figure 3. Cerebellar circuitry. *BC: Basket cells; SC; Stellate cells PC; Purkinje cells; LC: Lugaro cells; GC: Golgi cells; GC : Granular cells; UBC: Unipolar brush cells; IO: Inferior Olive; PN: Pontine nuclei; DCN: Deep cerebellar nuclei. Adapted from Gil-Miravet (2019).*

Purkinje layer, and granular layer (Voogd and Glickstein, 1998). The most external layer, called the molecular layer (ML), contains a few neuronal cell bodies of interneurons. The ML interneurons (MLIs) (Kim and Augustine, 2021), canonically subdivided in Stellate (SC) and Basket cells (BC), are in the upper and lower third of the ML respectively and have small cells bodies and their dendrites and axon branched in the sagittal plane (Dorgans et al., 2019; Kim and Augustine, 2021; Palay and Chan-Palay, 1974). In addition, the molecular layer composition is primarily made up of dendrites and axons of other cells like PC, GO, LC and GC (Voogd and Glickstein, 1998). Interestingly, those MLIs of the ML share features between them (SC and BC) (Sultan and Bower, 1998). MLI form GABAergic inhibitory synapses with other MLIs and with PC and receive excitatory input from parallel fibers (Jörntell and Ekerot, 2003) and climbing fibers (Szapiro and Barbour, 2007), and inhibitory input from LC, GC, and PC collaterals (Kim and Augustine, 2021). The molecular layer is also where the dendritic arborizations of PC are located, which are the prominent cells in the cerebellum (Haines & Dietrichs, 2012).

The Purkinje layer consists of the cell bodies of Purkinje GABAergic largest cerebellar neurons. PC have a very extensive dendritic arbor projected in the ML, and a very long axon that descends through the GCL and finishes onto the DCN (Palay and Chan-Palay, 1974).Classically, PC somata were the only constituents of the PCL (Palay and Chan-

Palay, 1974), but there are also identified as part this layer some interneurons as lugaro cells (LC) and other interneurons that make postsynaptic inhibitory GABAergic and glycinergic contacts with GO, PC, BS and SC (Kozareva et al., 2021). PC are arranged in a single layer, and their dendrites extend into the molecular layer (Haines & Dietrichs, 2012). PC is the sole outputs of the cerebellar cortex (Palay and Chan-Palay, 1974; Sillitoe et al., 2012).

The deepest layer of the cerebellar cortex, known as the granular layer, is adjacent to the white matter. It consists of different cell types, including GC, unipolar brush cells (UBC), and most significantly, granule cells (GR), which are the most abundant cells in the cerebellum (Eccles et al., 1964). Among these cells, GR and UBC are glutamatergic neurons, while most other cells in the cerebellar cortex are primarily GABAergic neurons (Apps & Garwicz, 2005). Cerebellar GR are small and are tightly packed in the GCL, with a thin axon that ascends to the ML where bifurcates forming what is known as the parallel fibers (pf) (Sillitoe et al., 2012; Voogd et al., 1996).

The unique output from the cerebellum to the rest of the brain is the DCN formed by a group of neurons in the most internal part of the cerebellar white matter, featuring three pairs—medial/fastigial, interposed, and lateral/dentate nuclei (Goodman et al., 1963). These nuclei, medially distributed, contain diverse excitatory (glutamatergic) and inhibitory (GABAergic and glycinergic) neurons (Kebschul et al., 2020). Recent classifications based on genetic markers revealed nucleus-specific inhibitory and excitatory neuron populations, offering functional diversity confined to distinct DCN subnuclei (Kebschul et al., 2020). DCN neurons, spontaneously active, predominantly receive inhibition from Purkinje cells and excitation from mossy fibers (Shinoda et al., 1992) (see **Figure 3**.), and also receive diverse modulation from various inputs (i.e., DAergic, serotoninergic, cholinergic, noradrenergic), forming the output to the rest of the brain, brainstem, and spinal cord (Carlson et al., 2021; Uusisaari et al., 2007; Novello et al., 2022). Recent insights suggest a more significant role for inhibitory neurons in DCN output (Judd et al., 2021). These neurons project to IO, indicating a nuanced role in the intricate cerebellar circuitry (Bagnall et al., 2009; Prekop et al., 2018; Ruigrok and Voogd, 1990). Additionally, DCN output cells provide feedback to GR

and GC in the cerebellar cortex (Ankri et al., 2015; Eyre and Nusser, 2016; Gao et al., 2016), contributing to a complex nucleocortical network with both excitatory and inhibitory influences (Gao et al., 2016; Houck & Person, 2015; Judd et al., 2021).

Numerous studies in both anatomical and functional realms have established a robust interconnection between the cerebellum and the striatum-cortico-limbic circuit (Bostan et al., 2013; Bostan and Strick, 2018; Rogers et al., 2011; Carta et al., 2019; Gil-Miravet et al., 2021; Yoshida et al., 2022; D'Ambra et al., 2023; Guarque-Chabrera el al; 2023). This intricate network has been supported by several key findings. Bostan et al. (2010) employed a retrograde transynaptic virus to unveil a disynaptic projection from the subthalamic nucleus of the basal ganglia to the cerebellar cortex, illustrating the connectivity between these structures (Bostan et al., 2010). Additionally, Rogers et al. (2011) elucidated two distinct glutamatergic pathways facilitating the link between the cerebellum and the mPFC. The first pathway encompasses a sequence involving the lateral nucleus, reticulotegmental nuclei, pedunculopontine nuclei, and VTA, ultimately reaching the mPFC (Forster & Blaha, 2003). The second pathway, also detailed by Rogers et al. (2011), encompasses the lateral nucleus, along with the mediodorsal and ventrolateral thalamus, eventually connecting to the mPFC. Both pathways contribute equally to the cerebellar modulation of dopamine release in the mPFC. Moreover, electrical stimulation of the DCNs, the main output nucleus of the vermis, evokes local action potentials and regulates the activity in the prelimbic (PL) cortex (Watson *et al*, 2014). Thus, the cerebellum and basal ganglia, traditionally considered independent structures, have direct connections that influence motor coordination, reward processing, and movement vigor (Yoshida et al, 2022; Washburn et al, 2024). In addition, recent electrophysiological evidence has provided valuable insights. Stimulation of cerebellar nuclei in mice of both sexes has been shown to modulate spiking activity in both the NAcc core and medial shell (D'Ambra et al., 2023, Doctoral dissertation).

Numerous anatomical investigations have identified direct dopaminergic projections from the ventral tegmental area to the cerebellar cortex (Ikai et al., 1992, 1994). Moreover, the cerebellum sends direct excitatory projections to the VTA, a brain

region involved in reward processing and encoding (Watabe-Uchida et al., 2012; Carta et al., 2019; et al., Gil-Miravet 2021). Optogenetic activation of the cerebello-VTA projections was rewarding, and these projections were more active when mice explored the social chamber in a three-chamber social task (Carta et al, 2019). A tracing study from our lab, revealed the same direct projection from the DCNs to the VTA, which receives Purkinje axons from lobule VII of the vermis and controls the activity and plasticity of the cortico-striatal circuitry (Gil-Miravet et al., 2021).

Recent research, conducted in collaboration with Khodakhah's lab, has uncovered two previously uncharted IL- lobule VIII descending pathways. These pathways not only enable the direct regulation of cerebellar output (DCN) by the IL but also facilitate communication with the cerebellar cortex via the nucleocortical pathway mossy fibers and IO-ascending climbing fibers. Remarkably, both complementary pathways appear to have similar functional consequences, particularly in amplifying Pavlovian conditioned learning (Guarque-Chabrera et al., 2023, Doctoral dissertation).

When considering these collective findings, it becomes evident that the cerebellum's direct connections with the addiction circuitry are substantial. However, it is worth noting that the intricate ways in which these connections are established and worked are not yet fully comprehended and described. This underscores the need for further research to unravel the complexities of the cerebellum's role in addiction-related processes.

The cerebellum in addiction

Cerebellar responses in individuals with SUD

Cues associated with drug-action-reward connections appear to exert heightened influence in individuals with SUD, even following extended periods of abstinence (Everitt & Robbins, 2005; Hyman et al., 2006). These conditioned memories serve as potent motivational triggers for drug-seeking behavior. Investigations into cue reactivity among SUD patients have explored the ability of drug-associated cues to provoke conditioned emotional responses and craving. Notably, these studies reveal that the presentation of drug-related cues triggers drug craving and stimulates

cerebellar activity, regardless of the sensory modality of the cues (visual, olfactory, auditive, tactile) or the type of drug (cocaine, alcohol, cannabis, nicotine, heroin) (Grant et al., 1996; Wang et al., 1999; Anderson et al., 2006; Schneider et al., 2001; Martin-Sölch et al., 2001; Filbey et al., 2009; Olbrich et al., 2009; Lou et al., 2012; Xuan et al., 2019; Al-Khalil et al., 2020). However, it is important to note that while a positive correlation between self-reported cravings and cerebellar activity has been observed in some studies (Grant et al., 1996; Risinger et al., 2005), conflicting results have also emerged (Bonson et al., 2002). Notably, similar patterns of cerebellar activity have been documented in response to cocaine cues as well as other arousing stimuli, such as scripts evoking anger (Kilts et al., 2003). The cerebellum is commonly believed to function as part of an internal predictive mechanism, involved in associative learning processes related to both reward-seeking and fear responses, where prediction errors arise from disparities between anticipated and actual outcomes (Popa and Ebner, 2019). Additionally, several fMRI studies have noted cerebellar activation during fear extinction, with some highlighting activity in posterolateral regions (Linnman et al., 2012; Faul et al., 2020; Graner et al., 2020; Labrenz et al., 2022), while others emphasize activation in the vermis (Kattoor et al., 2014; Utz et al., 2015). Moreover, research has demonstrated heightened activation in vermal regions in response to both food and cocaine cues compared to neutral cues (Tomasi et al., 2015). These findings suggest that the cerebellum is involved in processing and retaining cues predictive of rewards and other behaviorally significant events, rather than being solely responsible for generating conscious experiences such as drug craving (Moreno-Rius & Miquel, 2017).

It is crucial to acknowledge that cerebellar activity demonstrates a strong correlation with the severity of addiction (Shen et al., 2017; Al-Khalil et al., 2020). Given that cue-reactivity studies are typically conducted during withdrawal periods, the heightened cerebellar activity might arise from a potential mismatch between external cues (predicting drug presence) and the absence of expected internal cues (drug effects) triggered by cue presentation. In this context, cerebellar activity could be linked to a prediction error concerning the internal representation that anticipated sensory and rewarding outcomes of drug effects within a particular context. Notably, brain imaging

studies have indicated that the dorsal caudate, mPFC, and cerebellum exhibit greater activity when individuals with SUD were presented with a mismatch between their mental representation of drug effects and the context of drug consumption (De Pirro et al., 2018). In this study, individuals diagnosed with heroin and cocaine dependence were asked to envision drug use settings (home/outside) while imagining themselves engaging in drug use. The activity of the fronto-striatal-cerebellar network intensified when drug use was imaged in a context not associated with actual drug consumption, such as outside the home for heroin and at home for cocaine (De Pirro et al., 2018). Moreover, recent animal research has indicated that violations of reward expectations activate climbing fibers and specific subpopulations of granule cells in the cerebellum (Heffley and Hull, 2019; Wagner et al., 2017; Larry et al., 2019).

Human cue-reactivity studies have also explored the impact of drug treatment expectancy on cerebellar responses. Findings demonstrate that when drug treatment (methylphenidate) is anticipated, it elicits a more pronounced cerebellar response upon administration compared to an unexpected treatment (Volkow et al., 2003). However, these findings challenge the aforementioned mismatch hypothesis. Notably, research has revealed that the cerebellum remains iNAcctive when participants receive a placebo after expecting a drug (Volkow et al., 2003). Consequently, while expectations can amplify drug effects on the cerebellum, expectancy as a key component of the placebo response, does not involve the recruitment of cerebellar mechanisms.

Conditioned preferences towards drug-related cues play a significant role in shaping neural activity and the expression of perineuronal nets (PNNs) in the cerebellum.

Extensive evidence from preclinical studies has demonstrated the capacity of addictive drugs to induce Pavlovian conditioning (Bardo and Bevins, 2000; Cunningham et al., 2006; Tzschentke, 2007; McKendrick & Graziane, 2020). A widely used animal model for studying Pavlovian conditioning in drug addiction is CPP, wherein distinct contextual cues are paired with the drug, resulting in the development of an associative memory (CS+) (McKendrick & Graziane, 2020). The expression of this conditioned response is reflected in the preference for the drug-associated context.

Notably, the formation of drug-induced conditioned preferences involves various processes such as Pavlovian learning, motivation, and reward (McKendrick and Graziane, 2020).

Research focusing on cocaine-induced Pavlovian olfactory conditioning procedure in rodents consistently shows increased neural activity (cFos) in the granule cells of the cerebellar vermis, accompanied by a higher expression of perineuronal nets (PNNs) around Golgi (GC) interneurons within the same cerebellar region (Carbo-Gas et al., 2014a; Carbo-Gas et al., 2014b; Carbo-Gas et al., 2017). PNNs are extracellular matrix structures, expressed by several neuronal subpopulations, that restrict synaptic remodeling, potentially contributing to the persistence of drug memories (Grimpe & Silver, 2002; Fawcett et al., 2019). These cerebellar changes are specifically linked to animals that exhibit CPP and are not observed when cocaine is randomly paired with cues or in saline-treated animals (Carbo-Gas et al., 2017). Importantly, the increased cerebellar activity and PNN expression are correlated with the preference for drug-related cues in specific cerebellar regions (Carbo-Gas et al., 2014a; Carbo-Gas et al., 2017). Additionally, the impact of cocaine-induced conditioning on cerebellar activity is not replicated when animals are exposed to cocaine-related cues without the opportunity to express the conditioned response (Carbo-Gas et al., 2017).

Given that granule cell activity is known to encode various aspects of reward-related processes, such as reward anticipation and conditioned responses (Wagner et al., 2017), our findings suggest that granule cells might use drug-cue associative engrams to fine-tune action selection (Miquel et al., 2020). Specific subsets of granule cells are tuned to combinations of actions and rewards, allowing distinct subpopulations of Purkinje cells to learn the anticipated rewards for specific actions in particular contexts (Wagner et al., 2019; Kostadinov & Häusser, 2022). However, the extent to which the cerebellar cortex represents the drug-cue association remains unclear. Recent findings from our lab challenge the notion that the initial encoding of drug-cue associative memory involves the vermis. Disrupting glutamatergic transmission from parallel fibers to Purkinje synapses reduced PNN expression around GC interneurons and impaired the consolidation of cocaine-induced CPP without affecting its acquisition (Carbo-Gas

et al., 2017). Furthermore, enzymatic digestion of PNNs around GC interneurons in the posterior vermis affected the short-term memory of cocaine-induced CPP but not its acquisition (Guarque-Chabrera et al., 2022), suggesting that the drug-induced memory engram might initially be encoded elsewhere and subsequently conveyed to the vermis for stabilization.

Previous studies on the role of the cerebellum in Pavlovian conditioning have indicated that specific regions within the cerebellar cortex are involved in the acquisition and short-term expression of conditioned motor responses (Galliano et al., 2013). Stimulation of mossy fibers projecting to the granule cell layer replicates the ability of the conditioned stimulus to trigger conditioned blink responses (Steinmetz et al., 1989; Albergaria et al., 2018). Additionally, fear conditioning processes, including acquisition, consolidation, reconsolidation, and extinction, have been linked to the cerebellar vermis (Sacchetti et al., 2002, 2007; Utz et al., 2015). Several studies have examined the molecular processes involved in fear learning and memory, particularly focusing on Long-Term Potentiation (Sacchetti et al., 2004; Zhu et al., 2007). In mice, after fear acquisition, there is observed heightened GABAergic signaling originated from molecular layer interneurons targeting Purkinje cells (Scelfo et al., 2008) and adjacent molecular layer interneurons (MLIs) in cerebellar lobules V/VI (Dubois and Liu, 2021). Notably, the increased granule cell activity observed in conditioned animals might enhance the inhibitory effect of molecular interneurons on Purkinje cells, subsequently facilitating cerebellar outflow by reducing Purkinje inhibitory control over neurons in the DCN (Dizon & Khodakhah, 2011). Moreover, cocaine may exert an unconditioned effect by reducing Purkinje cell activity (Jiménez-Rivera et al., 2000).

Contribution of the cerebellum-infralimbic interactions to drug-induced conditioned preferences

In recent studies, we have explored the connection between prefrontal cortex dysfunction and cerebellar responses in the context of cocaine-induced conditioning (Gil-Miravet et al., 2019; Guarque-Chabrera et al., 2022). It is worth noting that dysfunction in the medial prefrontal cortex (mPFC) plays a central role in the pathophysiology of SUD (Mc Farl & Kalivas, 2001; Goldstein & Volkow, 2011), as well

as in various neuropsychiatric conditions often comorbid with SUD, such as schizophrenia, autism, obsessive-compulsive disorder, and attention-deficit/hyperactivity disorder (Hester, 2004; Robbins et al., 2012; Winstanley et al., 2010, Couto-Ovejero et al., 2023). Interestingly, individuals suffering from these neuropsychiatric disorders are at a heightened risk of developing SUD (Ersche et al., 2020; Miquel et al., 2019). Many of these patients also exhibit alterations in cerebellar functions (Moulton et al., 2014; Miquel et al., 2019; Brady et al., 2019; Miterko et al., 2019; Kelly et al., 2020).

Our recent studies have focused on understanding cerebellar responses when cocaine-induced conditioning occurs alongside mPFC impairment (Gil-Miravet et al., 2019; 2021, Guarque-Chabrera et al., 2022a). Specifically, deactivation of the infralimbic cortex (IL) - a subregion of the mPFC - promotes the acquisition of a preference for cues associated with cocaine (Guarque-Chabrera et al., 2022a). Importantly, IL deactivation during drug learning has an impact on neural activity and the expression of perineuronal nets (PNNs) in the posterior cerebellar vermis. This IL deactivation selectively increases activity in synapses expressing vesicular glutamate transporter 2 (vGluT2), without affecting vGluT1-related activity. Furthermore, it upregulates PNNs around neurogranin+ PNN-expressing GC (Guarque-Chabrera et al., 2022a). These distinct cerebellar changes appear to result from the interplay between IL impairment and cocaine-induced learning, as neither of these effects is observed when the drug is randomly associated with contextual cues. A decade ago, we hypothesized that prefrontal dysfunction might enhance cerebellar responsiveness when exposed to addictive substances repeatedly (Miquel et al., 2009). Our recent findings lend support to this hypothesis, suggesting that drug use in the presence of prefrontal dysfunction can lead to a functional reorganization of the prefrontal-cerebellar network, amplifying the impact of drugs on behavior.

Over the past decade, research has unveiled the existence of bidirectional anatomical and functional connections between the mPFC and cerebellum (Watson et al., 2014; Pisano et al., 2021; Gil-Miravet et al., 2021). These cerebellar-mPFC interactions are mediated by various brain regions, including the thalamus, basal ganglia, and VTA

(Moers-Hornikx et al., 2009; Watabe-Uchida et al., 2012; Carta et al., 2019). Notably, a dopaminergic projection from the VTA to the cerebellum releases detectable dopamine (DA) levels in specific cerebellar regions, including the posterior vermis (lobules VII–X), the right and left hemispheres, and the fastigial, interposed, and dentate nuclei (Glaser et al., 2006). In return, the cerebellar cortex influences DA activity through several independent pathways. One such pathway involves monosynaptic glutamatergic input from DCN, which targets both dopaminergic and non-dopaminergic neurons in the VTA (Snider et al., 1976; Watabe-Uchida et al., 2012; Beier et al., 2015; Carta et al., 2019; Gil-Miravet et al., 2021) and projects to the mPFC (Gil-Miravet et al., 2021). Additionally, the cerebellum connects with the VTA via the reticulotegmental and pedunculopontine nuclei (Forster & Blaha, 2003) and various thalamic nuclei, including the mediodorsal, ventrolateral, and ventromedial thalamus (Rogers et al., 2011; Kelly et al., 2020).

Functionally, it has been demonstrated that the cerebellum modulates cortical functions within the cerebral cortex (Chen et al., 2014; Gao et al., 2018; Kelly et al., 2020; McAfee et al., 2022) and can regulate activity throughout the striatum-cortico-limbic circuitry (Carta et al., 2019; Gil-Miravet et al., 2021; Yoshida et al., 2022; Washburn et al., 2024). Simultaneous activation of the cerebral cortex and cerebellum enhances activity and plasticity, including long-term potentiation and long-term depression, in the cortico-striatal pathway (Chen et al., 2014). Our recent studies have revealed that deactivation of the posterior vermis (lobule VIII) during the acquisition of cocaine-induced CPP significantly facilitates drug learning. This effect is associated with increased neuronal activity in the Int and Lat deep cerebellar nuclei (DCN), as well as in the mPFC and NAcc, and all striatal subdivisions except the ventrolateral striatum (Gil-Miravet et al., 2019; Gil-Miravet et al., 2021). Another consequence of lesioning the posterior vermis during drug-induced learning is the enhanced expression of PNNs around GABA/Parvalbumin+ interneurons in both the PL and IL cortices. Prior research has shown that the degradation of PNNs around PL parvalbumin+ interneurons can prevent cocaine-induced conditioning (Slaker et al., 2015). Thus, disrupting the cerebellar cortex in the posterior vermis potentiates neural activity and mechanisms

for synaptic stabilization within the addiction circuitry, likely facilitating drug-induced learning.

The use of DREADDs in neuroscience

DREADDs, or Designer Receptors Exclusively Activated by Designer Drugs, encompass a sophisticated method involving the manipulation of receptor proteins through targeted changes in the DNA of endogenous G-protein coupled receptors. This technique involves the modification of proteins to interact with previously unknown small molecule chemical triggers (Forkmann & Dangelmayr, 1980; Strobel, 1998; Sternson & Roth, 2014). Over the last couple of decades, numerous chemogenetic (also referred to as 'chemical genetic') (Bishop et al., 1998; Strader et al., 1991; Chen et al., 2005; Sternson & Roth, 2014) platforms have been developed, proving to be particularly valuable for neuroscientists and researchers in the neurobiological field.

The fields of integrative neurosciences have experienced a transformative shift with the advent of chemogenetic and optogenetic techniques, providing novel tools to selectively manipulate the activity of specific neuronal populations or neurotransmitter systems with enhanced precision (Sternson & Roth, 2014; Roth, 2016; Wiegert et al., 2017). While optogenetics enables rapid and phasic modulation of neuronal activity with high temporal precision, chemogenetics offers the advantage of prolonged modulation, making it particularly suitable for investigating tonic phenomena (Whissell et al., 2016), such as the role of dopamine in motivational processes.

Among the arsenal of chemogenetic tools, have emerged as a widely used approach, often referred to as a biological "lock-and-key" system for selectively manipulating cell activity through G-protein signaling pathways. This innovative technique, originally pioneered by the Roth's research group (Armbruster et al., 2007), involves a G-protein-coupled receptor (GPCR) modelled from a muscarinic receptor that can be efficiently expressed in neuronal cell membranes but lack a natural ligand for activation, which acts as the "lock." This receptor is genetically modified to exclusively respond to clozapine-N-oxide (CNO), they exhibit responsiveness to this inert compound

commonly referred to as the "key." CNO which can be administered systemically or intracranially to interact with DREADD receptors, is a metabolite derived from the atypical antipsychotic clozapine, and under typical circumstances, it might lack significant pharmacological activity (Armbruster et al., 2007).

One particularly well-known variant among DREADDs is hM4Di, a modified version of the M4 muscarinic acetylcholine receptor. Upon binding with CNO, this receptor triggers a process resulting in membrane hyperpolarization. This is achieved by reducing cAMP signaling and enhancing the activation of inward rectifying potassium channels (Armbruster et al., 2007; Rogan & Roth, 2011). Consequently, this leads to a temporary inhibition of neuronal activity akin to the effects observed after natural activation of the M4 receptor. On the other hand, hM3Dq, another commonly employed variant, is an engineered version of the M3 muscarinic receptor. Activation of this receptor by CNO initiates the phospholipase C cascade, causing changes in intracellular calcium levels and resulting in burst-like firing of neurons (Armbruster et al., 2007; Rogan & Roth, 2011). In addition to hM3Dq and hM4Di, there are further options designed to elicit neuronal excitation. rM3Ds, for instance, induces neuronal depolarization through G-protein mediated processes, such as increases in cAMP (Dong et al., 2010; Ferguson, et al., 2013). These advancements in DREADD technology have revolutionized neuroscience by providing researchers with a potent tool for controlling neuronal activity in a controlled and reversible manner.

DREADDs offer a significant advantage in the field of behavioral neuroscience due to their capacity to manipulate brain systems through the administration of an activating ligand via systemic injection, effectively enabling a form of "remote control" over neural activity (Rogan & Roth, 2011). In many instances, this approach minimizes the need for invasive procedures and allows for precise control over the extent of anatomical coverage. In typical scenarios, an initial surgical procedure is necessary for the intracranial delivery of viral constructs that contain the DREADD transgene, along with a promoter and a fluorescent reporter. Consequently, this approach allows for the real-time modulation of targeted neural systems in accordance with the experimental demands, with only a minimal delay associated with the injection.

Additionally, this technique eliminates the need for tethering or the use of cranial implants. As a result, animals can seamlessly engage in various task environments, even those that involve ceilings or guillotine doors, without the necessity of altering the task apparatus to accommodate cranial hardware and connector elements.

Administering CNO through systemic injections presents distinct advantages and disadvantages compared to intracranial microinjections. The efficiency of the injection method results in a reduction in the required personnel time. Furthermore, the adaptability of DREADD-based manipulations makes it possible to combine them seamlessly with other neuroscience methodologies, including intracranial cannulation and electrophysiological recordings. Nevertheless, it is crucial to recognize that local infusion provides a unique advantage in terms of precise temporal control over DREADD activation. Utilizing a cannula for local infusion allows for finer granularity and more precise regulation of when DREADDs are activated. This capability not only facilitates the exploration of neural circuit dynamics but also diminishes the volume of CNO that needs to be administered. Given these considerations, it becomes essential to thoroughly assess our experimental procedure and determine the most suitable administration approach based on the specific requirements of our research goals.

In addiction research, DREADDs offer a valuable tool for investigating various behavioral phenomena such as psychomotor sensitization, drug self-administration, and CPP. For example, in self-administration paradigms, it has been shown that activation of designer receptors that stimulate Gq signaling pathways in dopamine neurons of the VTA enhances cocaine seeking behavior. Conversely, engaging inhibitory Gi/o signaling pathways in dopamine neurons has been shown to weaken the reinforcing and priming effects of cocaine (Mahler et al., 2018). Furthermore, it has been demonstrated how the mPFC and nucleus NAcc influence the perception of an interoceptive cue associated with a combination of nicotine and alcohol (N + A) using DREADDs. The results of the study indicate that the projections from the mPFC-PL to the NAcc play a significant role in regulating the sensitivity to the interoceptive effects of the N + A compound cue (Randall et al., 2019). Another example is the study by Buchta et al. (2017) which investigates how dopamine from the VTA regulates intrinsic

inhibition in the prefrontal cortex PFC combining DREADDs electrophysiology and optogenetic techniques. They have seen that dopamine reduces intrinsic inhibition in PFC neurons, and this effect is potentiated by cocaine.

Collectively, the utilization of DREADDs enables researchers to delve into the implicate circuitry and intracellular signaling mechanisms that underlie addictive behavior. This technique provides a mean to address circuit-mapping inquiries that were previously beyond reach due to technological constraints.

OBJECTIVES AND HYPOTHESIS

The objectives of this thesis are grounded in several key premises:

1. **Cerebellar Role in Drug-induced Conditioned Memories:** The cerebellar cortex plays an inhibitory role in the acquisition of cocaine-induced Pavlovian conditioned memory.
2. **Cerebellar activity and cocaine-induced conditioned preference:** Increased neural activity in the posterior cerebellum is observed in rodents that have acquired a preference for contextual cues associated with cocaine.
3. **Cerebellar lesion and cocaine-induced conditioning:** A lesion in the dorsal posterior vermis increases the fraction of animals that express cocaine-induced odor conditioning.
4. **Cerebellar connections with the addiction circuit:** The cerebellum is intricately connected with prefronto-striatal circuits that have previously been implicated in addictive behavior.

The broad objective of this research is to comprehensively investigate the role of the cerebellum during the acquisition of cocaine-induced CPP, shedding light on its interactions with other critical components of the striatum-cortico-limbic system, which is involved in SUD.

To achieve this, we outline the following **specific aims** for each chapter of the thesis:

Chapter 1: To explore the neural activity patterns within various cerebellar regions (dorsal vermis, dorsal para-vermis, Crus1, Crus 2) in rats that exhibit conditioned cocaine preference as compared with rats that do not.

Chapter 2: To investigate the impact of activating and deactivating the posterior cerebellum, specifically lobule VIII of the vermis, using DREADDs on the acquisition of cocaine-induced place preference.

Chapter 3: Propose an explanatory model for the effects of a lesion in LOBULE VIII of the cerebellar vermis on the acquisition of CPP using chemogenetic inhibition of the DCNs.

Chapter 4: To further explore the cerebellar modulation of the VTA and IL activity through the use of DREADDs and *Cre*-dependent strategies.

In line with these specific aims, we propose the following general hypothesis:

General Hypothesis: The modulation of neuronal activity in different parts of the cerebellum affects the acquisition of cocaine-induced CPP.

Building upon this overarching hypothesis, we outline **our predictions** for each chapter:

Chapter 1: We predict that neural activity in the dorsal vermis of the cerebellum will be heightened in animals that develop cocaine-induced CPP.

Chapter 2: We expect that DREADD constructs will exhibit expression across diverse neuronal subpopulations within lobule VIII, encompassing granule cells (GC), Purkinje neurons, and Golgi cells. Our hypothesis posits distinct outcomes based on the specific neuronal targets of DREADD expression. First, if the DREADD selectively localizes within Purkinje neurons, we expect that deactivating these neurons in the dorsal vermis during the acquisition phase will elevate the number of animals exhibiting CPP. Conversely, activating Purkinje neurons during the same phase should diminish CPP. Next, if the DREADD predominantly expresses in granule neurons, we predict contrasting effects. Deactivation of granule neurons should reduce the number of animals exhibiting CPP, while activation should elevate CPP incidence. In the case of DREADD expression within Golgi interneurons, we anticipate that inhibition will decrease CPP, while excitation will increase it. Lastly, if the DREADD is expressed across all neuronal subpopulations, the inhibition or excitation of Purkinje cells should exert a significant influence on behavior. This effect is crucial, as Purkinje cells represent the final neural gateway before the DCNs, which serve as the primary output to the

broader brain circuitry. Thus, manipulating Purkinje cell activity is likely to play a central role in shaping behavior within the context of cocaine-induced CPP.

Chapter 3: The lesion of lobule VIII in the cerebellar vermis by quinolinic acid (QA) is expected to elevate the number of animals developing CPP. Meanwhile, the inactivation of DCNs using the DREADD AAV5-CaMKIIa-hM4D(Gi) is predicted to counteract the facilitation of CPP acquisition induced by the lobule VIII lesion.

Chapter 4: The infusion of pAAV-hSyn-DIO-hM4D(Gi)-mCherry, a *cre*-dependent inhibitory DREADD, in the DCNs and pENN-AAVrg-hSyn-Cre-WPRE-hGH into the VTA, is expected to selectively express DREADDs in the glutamatergic projection from the DCNs to VTA. Inactivating this pathway should decrease activity in the VTA and IL.

By pursuing these specific objectives and testing these hypotheses, our research aims to advance our understanding of the intricate role played by the cerebellum in the development of cocaine-induced CPP and its interactions with crucial components of the addiction circuitry.

Chapter 1: Neural activity changes in the cerebellar cortex of rats trained in cocaine-induced preference conditioning.

Overview. In the last years the field of neuroscience has shed light on the critical role of the cerebellum in drug addiction, particularly emphasizing its involvement in the associative learning processes linking drug use to contextual cues. Our prior research has already demonstrated that the cerebellum undergoes notable changes in plasticity and neural activity during the acquisition of drug-context memories. In this study, we aimed to replicate and expand upon these findings in a biased conditioned preference procedure in which rats were conditioned with cocaine to the non-preferred configuration. We combined a texturized floor and olfactory cues as CS.

Questions and aims. How affect the acquisition of cocaine-induced CPP to the activity of different regions in the cerebellar cortex as the vermis, paravermis or hemispheres? Our investigation addressed neural activity patterns using c-Fos expression within specific regions of the cerebellum. Specifically, we focused on the granular and Purkinje layers in lobules VII and VIII of the vermis, paravermis, and Crus 1 and Crus 2 of the hemispheres.

Hypotheses. We predict that neural activity in the dorsal vermis increases in animals that exhibit cocaine-induced CPP.

Highlights. Rats exhibiting a preference for cocaine-associated cues (odor/tactile) showed increased neural activity in the granule cell layer of the posterior cerebellar vermis (lobule VIII). No effect of cocaine-induced conditioning in cerebellar hemispheres, a notable trend was observable in the paravermis lobule VIII. These results bolster the concept that the posterior vermis activity is modified during the formation of Pavlovian memories linked to drug use. The increased neural activity in the granule cell layer could reveal a context-specific response selection that anticipates drug availability.

Keywords: Addiction, Preference Conditioning, Cocaine, Cerebellum.

METHODOLOGY

Subjects. The present study includes 38 male Sprague Dawley rats weighting 270-350g (Janvier, ST Berthevin Cedex, France) at the time of behavioral experiments. Rats were housed individually in the animal facilities (Jaume I University, Spain) under standard conditions (12-h light cycle from 8:00 AM to 8:00 PM) with access to food and water *ad libitum*. Before beginning with behavioral procedures, rats were handled for 3 days and habituated to experimental protocols. Experiments were conducted during the first 5 hours of the light cycle. All animal procedures were approved by the local Animal Welfare Ethics Committee and Empowered Body (2018/VSC/PEA/0139) and adhered to the European Community Council directive (2010/63/EU), Spanish directive BOE 53/2013, and local directive DOGV 13/26/2007.

Pharmacological agents. Cocaine hydrochloride (Alcaliber S.A., Madrid, Spain) was dissolved in a saline solution and administered intraperitoneally (IP, 10 mg/kg). Saline solution was used as a control vehicle.

***Conditioned Place Preference*.** This procedure was conducted in an opaque, oblong corridor (90 x 20 x 60 cm) that included two lateral black chambers (20 x 20 x 60 cm) located on opposite sides. Two days before the training, animals were habituated to the apparatus in a 15-minute session without texturized floor (stainless-steel floor with perforated small holes or big holes) (Leroy Merlin SL., Alcobendas, ES) or olfactory cues (lemon or almond) (Gran Velada SL., Zaragoza, ES). For *the preconditioning preference test* (**Figure 4A, 4B**), rats were placed in the central chamber and were free to move across the corridor in a 20 min drug-free test in which the two olfactory and tactile cues configurations (lemon + big holes or almond + small holes) were presented simultaneously but in the opposite arms of the corridor. All the test sessions were videotaped and scored by a blind observer. The first five minutes were not considered to allow the animal to explore the location of the cues. In *the conditioning phase* (**Figure 4A, 4B**), the least preferred stimulus configuration for each animal acted as CS+ and was paired with IP cocaine (10 mg/kg). On alternate days, the other configuration was associated with IP saline injections (CS-). For each pairing session, rats were confined in one of the lateral chambers of the apparatus for 25 min. Therefore, four cocaine- and four saline-paired sessions were conducted on alternate days (**Figure 4B**).

The preference test was identical to the preconditioning test and was carried out 24 hours after the last conditioning session and 48 hours following the last cocaine administration (Figure 4A, 1B). The location of the cues in the corridor was counterbalanced between animals. In addition to the paired group, we included a control group in which the least preferred contextual configuration was associated randomly with either cocaine (10 mg/Kg) or saline (**Figure 4B**). This group allowed controlling effects of cocaine in the absence of learning on cerebellar activity.

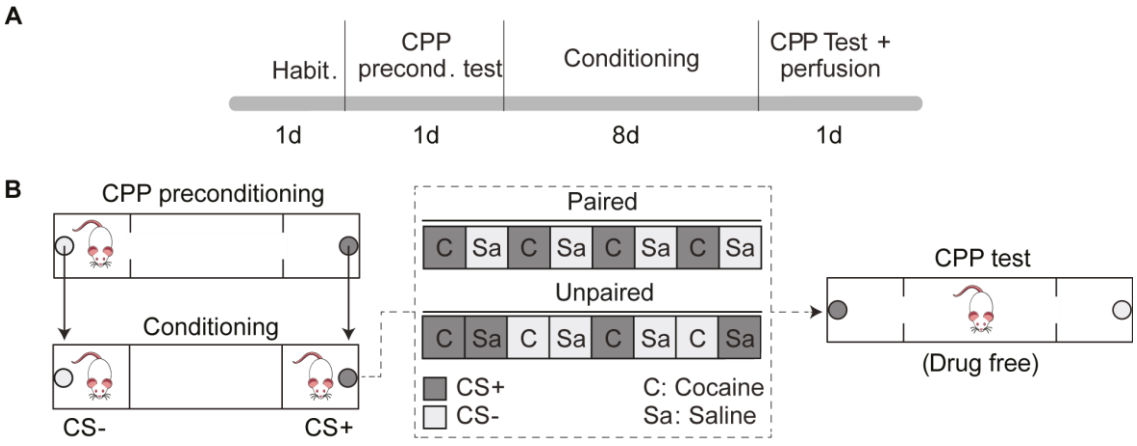

***Figure 4. A) Experimental timeline**. Different stages of the experimental procedure and the behavioral protocol. **B) Schematics of behavioral paradigms**. Schematic representation of the CPP apparatus, the biased procedure, the conditioning schedule for the Paired and Unpaired groups and the preference test.*

***Brain sampling*.** All animals were perfused transcardially 60 min following the last preference test using saline (0.9%), heparin (0.006%) (Heparin sodium salt from porcine intestinal mucosa; CAT# H3393; Sigma-Aldrich, Madrid, Spain) and then 4% paraformaldehyde (Paraformaldehyde powder, 95%; CAT#158127; Sigma-Aldrich, Madrid, Spain), under anesthesia with sodium pentobarbital (30 mg/kg) (Dolethal 100 mL, Vetoquinol E.V.S.A., Madrid, Spain). Brains were extracted and stored with the same fixative for 24h at 4ºC. After that, the tissue was immersed in sucrose solution (30%) with sodium azide (2%) (Sodium azide; Cat# 1.06688.0100; Merck, Darmstadt, Germany) until the brain sank at 4ºC. Sagittal sections were obtained at 40 µm with a cryostat microtome (Microm HM560, Thermo Fisher Scientific, Barcelona, Spain) and stored at −22ºC in cryoprotectant solution.

Immunofluorescence and imaging. Immunolabeling was performed on free-floating sections. We used c-Fos immunofluorescent staining as a neural activity marker. Sections were incubated for 48h at 4ºC with rabbit anti-c-Fos (1:500; Synaptic Systems, Goettingen, Germany; Cat# 226003) in PBST. Next day, brain tissue was exposed to donkey anti-rabbit Alexa Fluor 647 (1:500; Thermo Fisher Scientific, Rockford, IL, USA; Cat# A-31573) as secondary antibody in PBST for two hours.

Image acquisition and analysis. Laser intensity, gain, and offset remained stable across animals. Tile-scan Z-stack images and image magnifications of the vermis (lobule VII and lobule VII) (**Figure 5A**), paravermis (lobule VII and lobule VIII) (**Figure 5B**) and, crus I and crus II (**Figure 5C**) were acquired using confocal microscopy (Leica DMi8, Leica Microsystems CMS GmbH, Wetzlar, Germany). We used Leica Application Suite X (LAS X, Leica Microsystems CMS GmbH, Wetzlar, Germany) to perform maximal projections of the Z-stacks and Fiji free software (Schindelin et al., 2012) for image analysis. cFos+ cells (intense and somatic red labeling) were estimated in two images per rat in all the animals within a ROI of 580 x 580 μm.

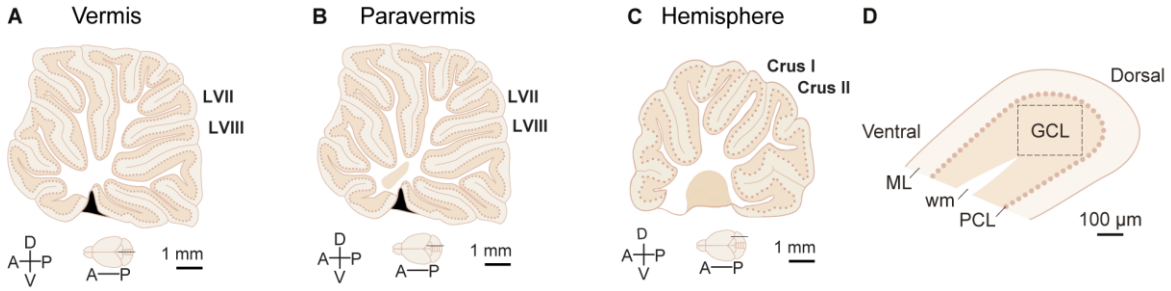

Figure 5. Diagrams of sagittal sections of the cerebellum. A) *Lobules VII and VIII from the vermis (medial region)* ***B)*** *Lobules VII and VIII from the paravermis (medial-lateral).* ***C)*** *Crus I and crus II from the hemisphere (lateral).* ***D)*** *Schematics of sagittal section of the different layers of the cerebellar cortex.ML, molecular layer. WM, white matter. PCL, Purkinje cell layer.*

Experimental design and statistics. We used a total sample size of 37 animals, 7-8 animals per group. From the total sample of paired rats (N=29), we were interested in comparing cerebellar activity of those exhibiting the highest and the lowest preference for cocaine-related cues. To this end, we employed a data-driven approach based on their preference score, dividing our sample into two groups: Rats showing the highest preference score (Q1) and those with the lowest ones (Q4). These two groups were compared with the unpaired control group. Statistical analyses were performed using

GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, USA). Given the non-normal distribution of our data, attributed to the segregation of animals into preferent and non-preferent groups, we opted for non-parametric analysis. To assess pre-post conditioning within group differences we used Wilcoxon matched-pairs signed rank test. Moreover, to assess differences between groups, we employed the Kruskal-Wallis test, followed by multiple comparisons using Dunn's test. Data are presented as individual and group scores (Median and 95% Confidence Intervals; 95% CI). In those cases in which a significant difference was observed, we calculated the effect size of that difference as the Median difference (*MD* = Median Group1 – Median Group2) and its 95% CI, to estimate the magnitude of the difference.

RESULTS

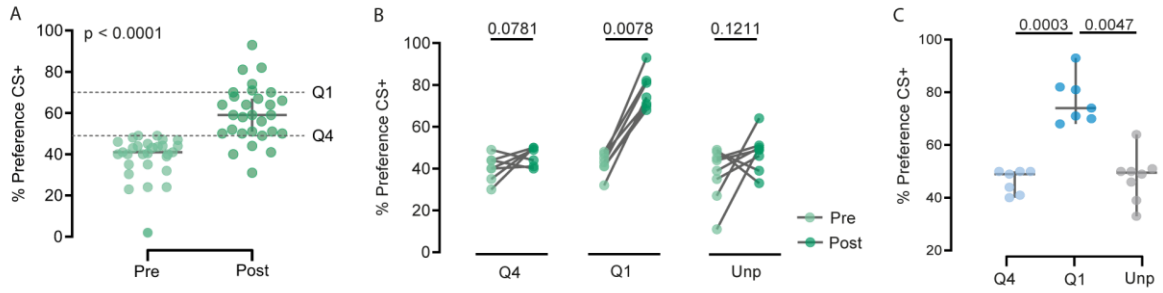

Figure 6. (A) Percentage of preference for the CS+ before and after cocaine-induced conditioning. (B) Percentage of preference for the CS+ in Q1,Q4 and Unp groups pre and post conditioning (C). Percentage of preference for the CS+ in Q1, Q4 and Unp groups post conditioning. *Data are shown as scatterplots (each plot represents the preference score for each animal) and median (horizontal lines) plot with* 95% CI (vertical lines). *P* values are shown on the top for each group comparison.

We used a total of 29 cocaine/CS+ paired animals for this experiment. In the pre-conditioning test, the rats showed a median preference score of 41 with a 95% CI [35, 44] and in the post-conditioning test, the median preference score was 59 with a 95% CI [51, 67]. Moreover, a Wilcoxon matched-pairs signed rank test revelated significant differences between pre-conditioning and post-conditioning, indicating that the animals acquired cocaine-induced conditioning under a biased procedure [$\Delta_{Pre–Post}$ [95% CI] = 21 [14,75, 27.60], p < 0.0001]. Descriptive statistic revelated that within the 1th quartile, rats exhibited a preference score ≥ 69. The 4th quartile encompassed rats showing a preference score for the CS+ in the post-conditioning test ≤ of 50 (**Figure 6A**). After splitting the sample in Q1 and Q4 animals, we compared pre- and post-

conditioning preference scores within each group by means of Wilcoxon matched-pairs signed rank test: Q4 [$\Delta_{Pre-Post}$ [95% CI] = -6 [-20, 5], p = 0.0781], Q1[$\Delta_{Pre-Post}$ [95% CI] =-34 [24, 48], p =0.0078] and Unpaired [$\Delta_{Pre-Post}$ [95% CI] =-8.5 [,-39,16], p = 0.1211]. (**Figure 6B**) Only Q1 group showed a significant pre-post difference in preference score.

Cocaine-induced conditioned preference increases c-Fos expression in lobule VIII of the vermis.

In our analysis, we specifically directed our attention to c-Fos activity within the dorsal region of lobules VIII and VII, spanning vermis sections from ML: 0 to 0.10 mm. The choice of this cerebellar region was based on our prior research in mice which demonstrated that cocaine-induced conditioned preference correlates with an increased neuronal activity in the dorsal region of the posterior vermis (Carbo-Gas et al., 2014a, 2014b, Rodríguez-Borillo et al., 2023.)

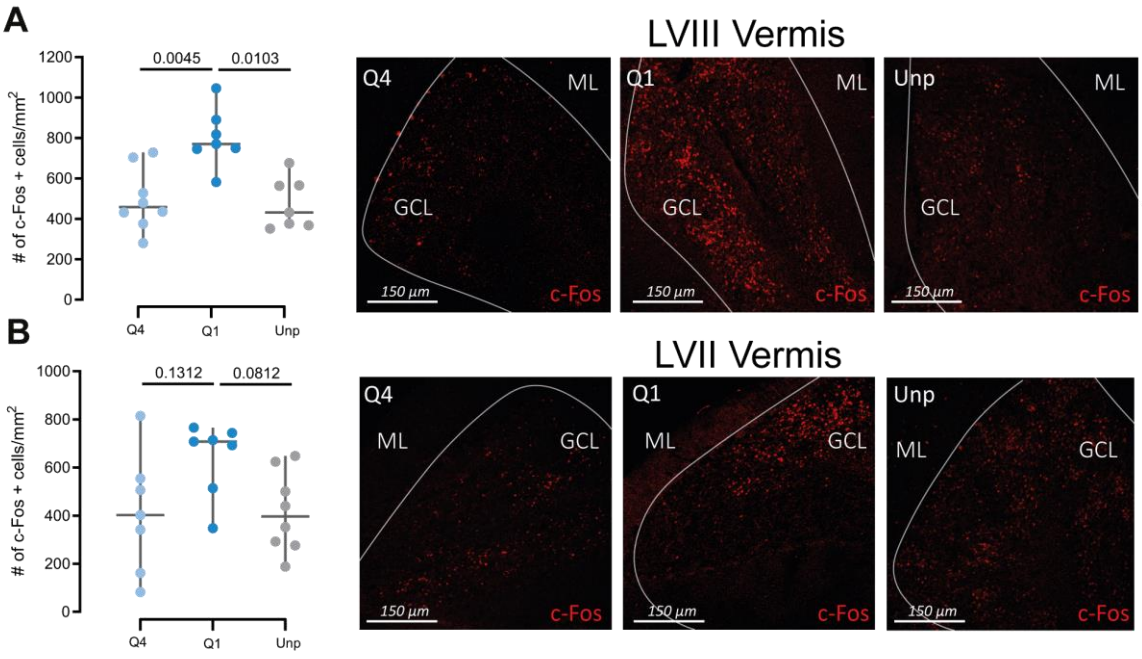

Figure 7. c-Fos expression in the dorsal region of lobules VIII and VII of the cerebellar vermis. *(**A-B**) Number of c-Fos positive cells/mm² in lobule VIII and VII, 60 min after the post-conditioning test in the Q4 (n = 7), Q1 (n = 7), and Unp (n = 8) groups. Data are shown as scatterplots (each dot represents the preference score for each animal) and median (horizontal lines) with 95% CI (vertical lines). Representative images of c-Fos staining in lobule VIII and VII for each experimental group appear on the right. All images were taken at 20x magnification. Scale bar 150 µm. Brightness was standardized across images. P* values are shown on the top for each group comparison.

A Kruskal–Wallis test showed significant effects in the number of c-Fos+ cells in lobule VIII (H (3) = 12.31, P=0.0004) and lobule VII (H (3) = 5.931, P = 0.0460). (**Figure 7A**). In lobule VIII Dunn's multiple comparisons revealed that c-Fos expression increased in Q1 rats regarding the groups not expressing preference [$\Delta_{Q4\text{-}Q1}$ [95% CI] = -338,0 [-480 ,-180,0], P = 0.0046] and $\Delta_{Q1\text{-}Unp}$ [95% CI] = -312 [-470,-102], P = 0,0104] (**Figure 7A**). However, Dunn's multiple comparisons test for lobule VII revealed that the number of c-Fos+ neurons in the Q1 group was not different from that of the Q4 [$\Delta_{Q4\text{-}Q1}$ [95% CI] = -306[-546, -54], P = 0.1312] and Unp [$\Delta_{Q1f\text{-}Unp}$ [95% CI] = -312 [-432, -60], P = 0.0812] groups (**Figure 7B**).

To address whether c-Fos expression in the granular layer might be related to the expression of preference for cocaine-related cues in a biased CPP procedure, we calculated Spearman *r* correlations coefficients (rho). We observed a significant relationship between c-Fos expression and cocaine-induced preference in lobule VIII (r = 0.5223; P=0.0065) and lobule VII (r = 0.5983; P = 0.0016) (**Figure 8A,B**). In summary, the expression of preference for cocaine-related cues was associated with an increase in c-Fos expression in LOBULE VIII and LOBULE VII of the vermis.

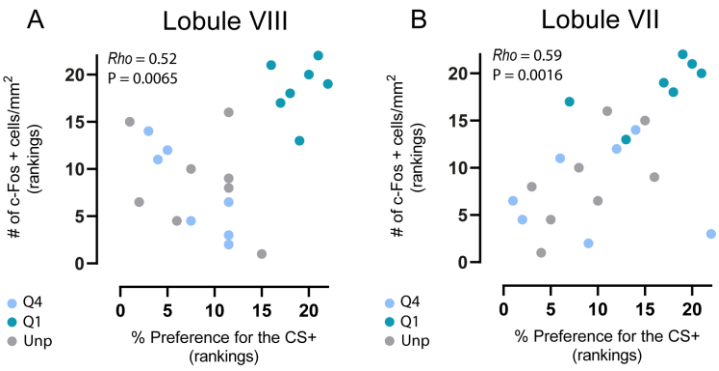

Figure 8. cFos expression in the granular layer of the posterior vermis is related to the expression of preference for cocaine-related cues. *Scatter plots representing the relationship between the % of preference for the CS+ and c-Fos+ neurons/mm² and Spearman r correlations coefficients (Rho) in (**A**) LOBULE VIII and (**B**) LOBULE VII. All groups are pooled together (n = 22); Q4 (n = 7)(clear blue), Q1 (n = 7) (dark blue), and Unp (n = 8)(grey) groups. P* values are shown on the top for each group comparison.

Furthermore, we extended our examination to encompass the same lobules in the paravermis, covering ML: 0.4 to 0.9 mm sections. The paravermis constitutes the central part of the cerebellar hemispheres, situated laterally to the vermis when the medial nucleus of the DCN becomes evident in a sagittal section. We also explored Crus I and Crus II within the cerebellar hemispheres, covering ML: 2.10 to 2.62 mm sections. We selected these cerebellar regions because they are renowned for their involvement in

non-motor functions and a diverse array of cognitive processes, including social behavior (Van Overwalle, et al., 2020). Notably, Crus II aligns with lobule VII in medio-lateral coordinates (Buckner et al. 2011). Moreover, using resting-state functional connectivity between the cerebellum and prefrontal cortex have been found coherence between crus II-lobule VIIB and the dorsolateral prefrontal cortex and crus I-VIIB/VIIIA border and the anterior prefrontal cortex (Krienen and Buckner 2009).

When we went laterally (ML: 0.4 to 0.9) up to the paravermis, Kruskal–Wallis test dit not show significant effects in the number of c-Fos+ cells either in lobule VIII (H (3) = 2.821; p=0.2515) or lobule VII (H (3) = 1.189, p = 0.5709) (**Figure 9A,B**). These results suggest that differences in c-Fos expression are primarily associated with the medial part of the posterior cerebellum.

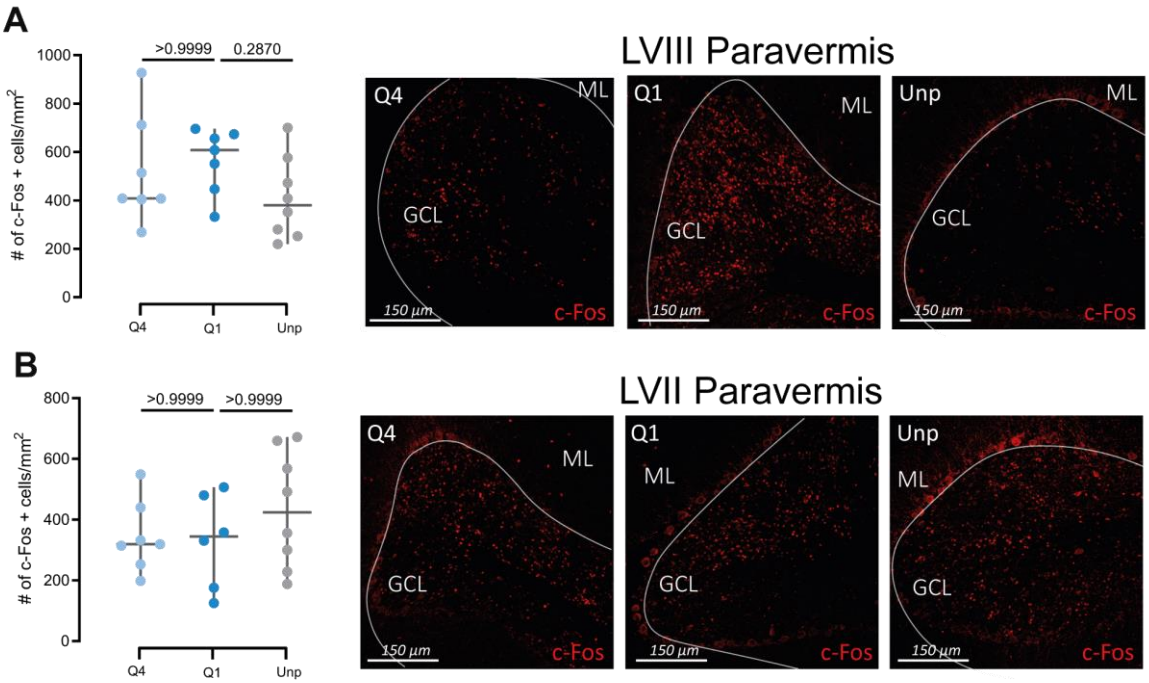

Figure 9. c-Fos expression in the dorsal region of lobules VIII and VII of the cerebellar Paravermis. *(**A-B**) Number of c-Fos positive cells/mm² in lobule VIII and VII, 60 min after the post-conditioning test in the Q4 (n = 7), Q1 (n = 6), and Unp (n = 8) groups. Data are shown as scatterplots (each plot represents the preference score for each animal) and median (horizontal lines) plot with 95% CI (vertical lines). Representative pictures of c-Fos staining in lobule VIII and VII for each experimental group appear on the right. All images were taken at 20x magnification. Scale bar 150 µm. Brightness was standardized across images. P* values are shown on the top for each group comparison.

In the hemispheres (ML: 2.10 to 2.62), Kruskal–Wallis test showed no significant effects in the number of c-Fos+ cells either in Crus 1 left (H (3) = 4.035, P=0.1327) (**Figure 10A**) or right (H (3) = 1.581, P = 0.4690 (**Figure 10B**). In Crus 2, Kruskal–Wallis

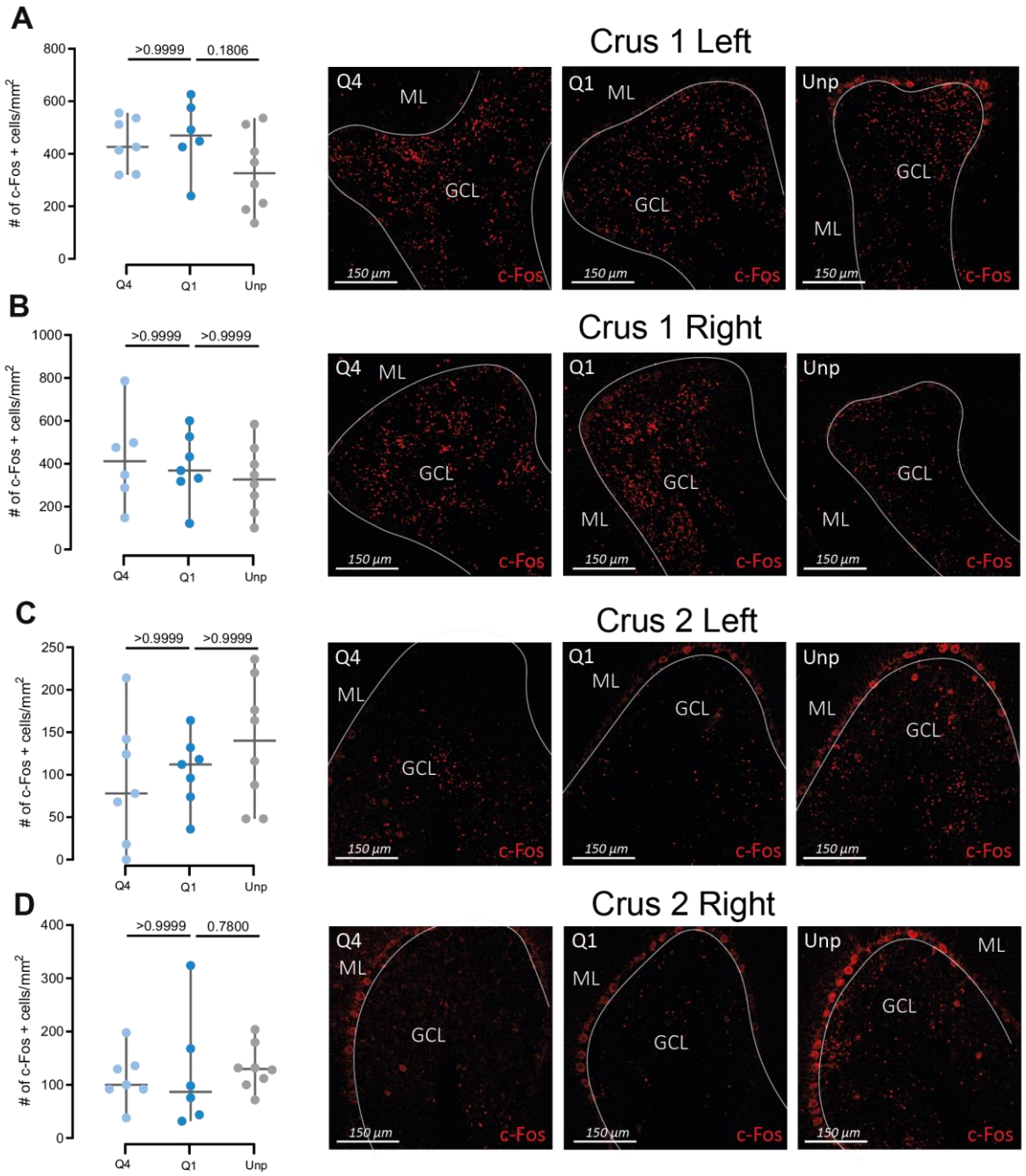

Figure 10. c-Fos expression in the dorsal region of Crus 1 and Crus 2 lobules in the left and right hemispheres. (A to D) *Number of c-Fos positive cells/mm^2 in Crus 1 and Crus 2 60 min after the post-conditioning test in the Q4 (n = 7), Q1 (n = 6), and Unp (n = 8) groups. Data are shown as scatterplots (each plot represents the preference score for each animal) and median (horizontal lines) plot with 95% CI (vertical lines). Representative pictures of c-Fos staining in Crus 1 and Crus 2 for each experimental group appear on the right. All images were taken at 20x magnification. Scale bar 150 µm. Brightness was standardized across images. P* values are shown on the top for each group comparison.

on the left (H (3) =1.382, P=0.5166) and right (H (3) = 1.581, P=0.469) hemispheres result in no significant c-Fos differences (**Figure 10C,D**).

These results suggest that neuronal activity in these more lateral regions of the cerebellar cortex is not related to drug-induced Pavlovian conditioning.

CONCLUSIONS

The present study delved into neural activity patterns assessed through c-Fos expression within specific regions of the cerebellum in rats trained to acquire cocaine-induced conditioned preference. Our findings demonstrate that rats expressing a strong preference for cocaine-associated cues (Q1) exhibited elevated neural activity in the granule cell layer of the dorsal posterior cerebellar vermis (specifically, lobule VIII) in contrast to Q4 and Unpaired groups. Most of the activated cells were identified as granular cells and Golgi interneurons by their size. As expected, our investigation revealed a positive correlation between the strength of preference for cocaine-related contextual cues and neuronal activity observed in lobule VII and lobule VIII. The involvement of lobule VII is less evident than that of lobule VIII, although when considering the medians of each group for c-Fos expression, it is clear that similar trends exist in both lobules. Notably, we observed no effect of cocaine-induced conditioning on neuronal activity either in the paravermis or the hemispheres. These findings suggest that only the posterior vermis appears to be part of the network involved in drug-related conditioned memory.

These findings offer robust support for the concept that neural activity within the posterior vermis undergoes modifications during the formation and retention of Pavlovian memory associated with drug experience no matter the paradigm used. The heightened neural activity in the granule cell layer was not an effect of cocaine treatment per se but of the cocaine-induced learning and could indicate a context-specific response selection that anticipates the availability of the drug. This sheds light on the intricate neural processes underlying drug-associated memory formation and reinforces the role of the cerebellum in appetitive Pavlovian memory.

Chapter 2: Chemogenetic activation and inhibition of the vermis (Lobule VIII) to modulate the acquisition of cocaine-induced conditioned preference.

Overview. In accordance with the results of the previous chapter, early descriptive studies in mice unveiled the involvement of two distinct neuronal populations within the posterior cerebellar cortex, granule cells and Golgi interneurons, in the formation of associative drug-context memories. Other investigations from Miquel's lab, which employed lesions and temporary deactivation techniques, have pointed to the involvement of the posterior vermis in the inhibitory regulation of preferences for stimuli linked to cocaine. However, these interventions lacked cell-specificity.

Questions and aims. Can we prevent or facilitate the acquisition of cocaine-induced CPP inhibiting or activating the vermis LVIII using chemogenetic tools? Our research aimed to evaluate the function of lobule VIII in the establishment of conditioning preferences for stimuli associated with cocaine availability using DREADDs. This research involves both activation and inhibition of lobule VIII in the vermis.

Hypotheses. The effect of chemogenetic activation or inhibition will be contingent upon the specific neuronal subpopulations targeted by the DREADDs. This efficacy will be determined by the neural circuitry involving the DREADD-expressing neurons and their connectivity with Purkinje cells, which serve as the final neural gateway before DCNs. We hypothesize that deactivating these neurons in the vermis during the acquisition phase will increase the number of animals exhibiting. Conversely, activating Purkinje neurons during the same phase is expected to decrease CPP.

Highlights. hSyn promoter seems to be exclusively expressed in interneuron populations, Golgi cells in the granular layer and basket and stellate cells in the molecular layer. Activation of molecular and Golgi interneurons by hm3D (Gq)-CNO increased preference for cocaine-related contextual cues. Inhibition of interneurons of the cerebellar cortex by hm4D(Gi)-CNO prevented the acquisition of cocaine-induced preference conditioning.

Keywords: Addiction, DREADDs, Conditioned Place Preference, Cocaine, Cerebellum

METHODOLOGY

Subjects. The present study includes 35 male Sprague Dawley rats (n=6-8 each group) (Janvier, ST Berthevin Cedex, France) weighting 270-350g at the time of stereotaxic surgery. Rats were housed individually in the animal facilities (Jaume I University, Spain) under standard conditions (12-h light cycle from 8:00 AM to 8:00 PM, with access to food and water *ad libitum*. Before beginning with behavioral procedures, animals were handled for 3 days and habituated to experimental protocols. Animal procedures were approved by the local Animal Welfare Ethics Committee and Empowered Body (2018/VSC/PEA/0139) and adhered to the European Community Council directive (2010/63/EU), Spanish directive BOE 53/2013, and local directive DOGV 13/26/2007.

Pharmacological agents, adenoviruses and DREADDs. Cocaine hydrochloride (Alcaliber S.A., Madrid, Spain) was dissolved in a 0.90% saline solution and administered intraperitoneally (IP, 10 mg/kg). Saline solution was used as a control vehicle. Anesthesia was induced by isoflurane (1000mg/g) (Isoflutek 250ml, Laboratorios Karizoo S.A., Barcelona, Spain) using a Isotec 5 isoflurane anesthesia vaporizer (Datex-Ohmeda Inc., Madison, WI). AAV5-hSyn-hM4D(Gi)-mCherry (Titer≥ 3×10^{12} vg/mL; Cat# 50475)**,** AAV5-hSyn-hM3D(Gi)-mCherry (Titer≥ 3×10^{12} vg/mL; Cat# 50474), and AAV5-hSyn-EGFP (Titer≥ 3×10^{12} vg/mL; Cat# 50465) used in the present study were provided by Addgene (Watertown, MA, USA). DREADD activation was achieved using 1 mg/kg intraperitoneal of Clozapine N-oxide dihydrochloride (CNO, Hello Bio Inc, Princeton, USA; Cat# HB6149, water soluble).

Stereotaxic Surgery. Rats weighing between 270 and 350 g were anesthetized with isoflurane (1000mg/g) (induction at 3.00 % and maintenance through the whole surgery at 2.00 %) using Isotec 5 isoflurane anesthesia vaporizer and placed in a stereotaxic apparatus (Kopf Model 902; David Kopf Instruments, Tujunga, CA, USA). Intracranial infusions of AAV were performed by placing a stainless-steel guide cannula (23-gauge external diameter, 36mm length) in lobule VIII (AP: −14.50; ML: ±0; DV: −4.5) (Paxinos and Watson 1998). Then, a removable stainless-steel injector (30-gauge external diameter, 38 mm length) connected to a 2 µL Hamilton syringe (Microliter

Syringe Model 7102 KH; Cat# 80300) driven by an infusion pump (11 Plus dual Syringe; Harvard Appatarus; Cat# HA702209) was inserted into the previously placed guide cannula to infuse 1 µL of AVV (0.50 µL/min, two infusions of 0.5 µL separated by a period of 3 min) for the viral expression. After the infusion completion, the injector remained in place for 3 min for a better absorption of the substance. Then, the guide cannula and injector were removed, and the wound was sutured. After surgery, animals received analgesic treatment with meloxicam (Metacam 20 mL, 5 mg/mL; Boehringer Ingelheim, Barcelona, Spain, CAT#9993012) every 24 hours for two days. . For behavioral experiments, rats were gently handled, and CNO or saline were IP injected 30 min before each conditioning session (1mg/kg).

***Conditioned Place Preference*.** The procedure was conducted in an oblong corridor apparatus. Rats underwent habituation, followed by a preconditioning preference test,

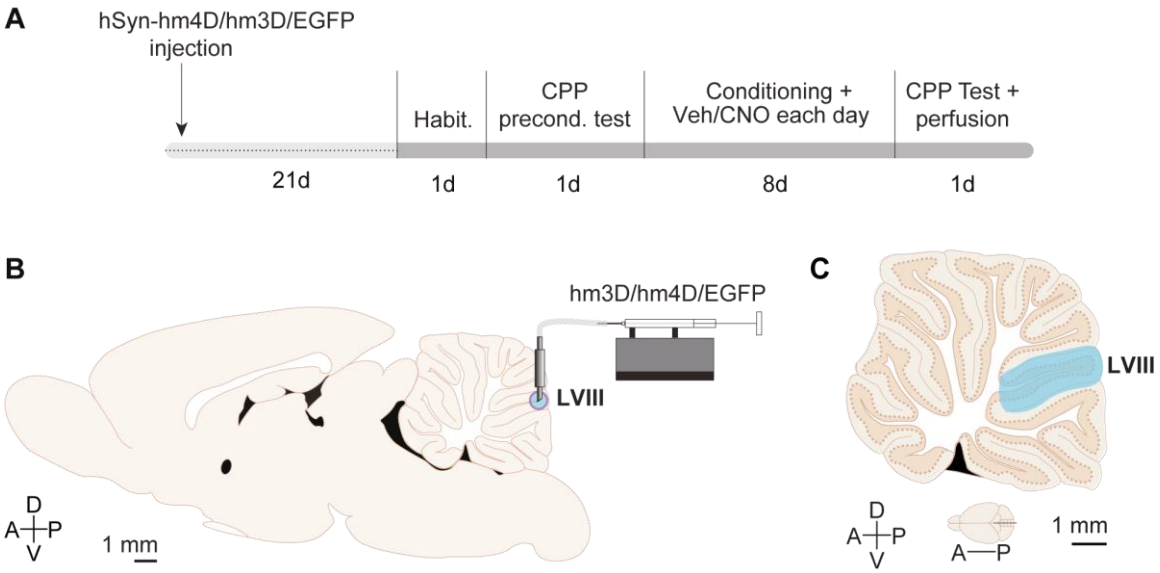

Figure 11. (A) Experimental timeline. *Experimental procedure and behavioral phases.* ***(B)*** *Schematics of a sagittal section of a rat brain depicting the injection site of AAV-hSyn-hM3D/hm4D/EGFP.* ***(C)*** *Schematics of a sagittal section of the DREADD expression region. The expression area was established overlaying each cerebellar real image over the correspondent rat brain digital atlas image (Paxinos and Watson,1998). The blue region shows the largest diffusion area.*

in which the innate preference for the two olfactory and tactile cues configurations (lemon + big holes or almond + small holes) were tested. The conditioning phase involved 4 cocaine and 4 saline pairings. The preference test was identical to the preconditioning test (**Figure 11**). A detailed explanation of the procedure can be found in the methodology of Chapter 1 (**page 40**).

Brain sampling. 60 min after the final preference test, animals were perfused transcardially with saline, heparin, and 4% paraformaldehyde under anesthesia. Brains were stored in the same fixative, followed by immersion in a 30% sucrose solution with sodium azide until they sank. Sections, either sagittal or coronal, were obtained at 40 µm using a cryostat microtome and stored in cryoprotectant at -22°C. For more detailed information of this procedure see Chapter 1 methods (**page 41**)

Immunofluorescence and imaging. Immunolabeling was performed on free-floating sections. To amplify the endogenous mCherry fluorescent signal, sections were exposed to a blocking buffer with donkey serum (Sigma Aldrich, Madrid, Spain; Cat# D9663) for 30 min and then incubated overnight at 4ºC in PBS 0.1 M Triton X-100 (Sigma-Aldrich, Madrid, Spain; Cat# T9284) (PBST) with rabbit anti-RFP (1:500; Rockland Immunochemicals Inc, Philadelphia, Pennsylvania, USA; Cat# 600-401-379). The next day, and after several rinses, tissue was incubated in PBST with anti-rabbit Alexa Fluor 555 (1:500; Thermo Fisher Scientific, Rockford, IL, USA; Cat# A-31572) as secondary antibody.

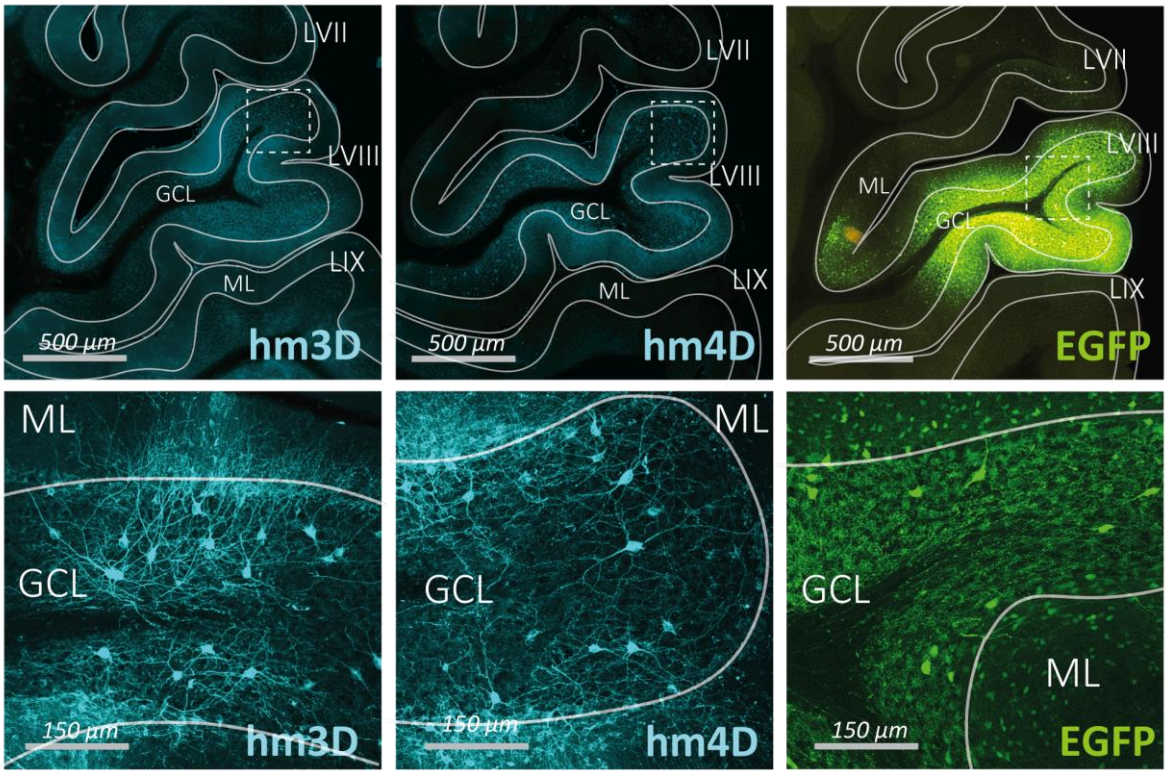

Figure 12. *Representative confocal tile scans images (on the top) and x20 magnifications (on the bottom) of hm3D(Gi) (left, blue) hM4D(Gq) (middle, blue) EGFP (right, green) of the expression in Lobule VIII respectively. Scalebar of 150 µm.*

To phenotype in which neuronal subpopulation AAV5-hSyn-hM4D(Gi)-mCherry, AAV5-hSyn-hM3D(Gi)-mCherry were expressed, sections were exposed to a blocking buffer with donkey serum (Sigma Aldrich, Madrid, Spain; Cat# D9663) for 30 min and then incubated overnight at 4ºC in PBS 0.1 M Triton X-100 (Sigma-Aldrich, Madrid, Spain; Cat# T9284) (PBST) with Rabbit anti-Neurogranin for Golgi interneurons (1:1000, Millipore Merck KGaA, Darmstadt, Germany Cat#AB5620) and Mouse anti-Parvalbumin for Purkinje cells and molecular layer interneurons (1:15.000, Swant, Marly, Switzerland Cat#PV235). The next day, and after several rinses, tissue was incubated in PBST with Donkey anti-Rabbit 647 (1:250 Thermo Fisher Scientific, Geel, Belgium #CatA31573) Donkey anti-Mouse 488 (1:250 Jackson Inmuno Research Laboratories, Inc., West Grove, PA, USA Cat#715-545-150)

Image acquisition and analysis. The expression of the AAV5-hSyn-hM4D(Gi)-mCherry**,** AAV5-hSyn-hM3D(Gi)-mCherry and AAV5-hSyn-EGFP was determined using confocal imaging. Animals with no DREADD expression because of miss injections were not included in the statistical analysis. The acquisition parameters remained stable between animals, for AAV5-hSyn-hM4D(Gi)-mCherry**,** AAV5-hSyn-hM3D(Gi)-mCherry were laser intensity (1.3%), gain (800), and offset (-0,1) and for the AAV5-hSyn-EGFP were laser intensity (4%), gain (768), and offset (-0,1). To phenotype neurons expressing DREADDS, we used parvalbumin (PV) with a laser intensity (0.4%), gain (800), and offset (0) and neurogranin (NG) with a laser intensity (1.6%), gain (800), and offset (0).

Tile-scan, Z-stack images and image magnifications of the posterior cerebellum were acquired using a confocal microscopy (Leica DMi8, Leica Microsystems CMS GmbH, Wetzlar, Germany) to assess DREADD/mCherry/EGFP expression. We used Leica Application Suite X (LAS X, Leica Microsystems CMS GmbH, Wetzlar, Germany) to perform maximal projections of the Z-stacks.

Experimental design and statistics. A total sample size of 32 animals, 6-8 animals per group (CNO-hm3D, SAL-hm3D, CNO-hm4D, SAL-hm4D, EGFP-CNO) was involved in the present study. Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, USA). The acquisition of cocaine-induced conditioning was analyzed by comparing Pre-Post preference scores using paired t-

tests for each group. Then, DREADD effects were analyzed by repeated-measures two-way ANOVAs and multiple comparisons performed using Sidak's tests. Data are presented as individual and group scores (Mean and 95% Confidence Intervals; 95% CI). In the cases in which a significant difference was observed, we calculated the effect size of that difference as the Mean difference (*MD* = Mean Group1 – Mean Group2) and its 95% CI, to estimate the magnitude of those differences.

RESULTS

DREADDs with hSyn promoter seem to be exclusively expressed in interneuron populations, Golgi cells in the granular layer and basket and stellate cells in the molecular layer.

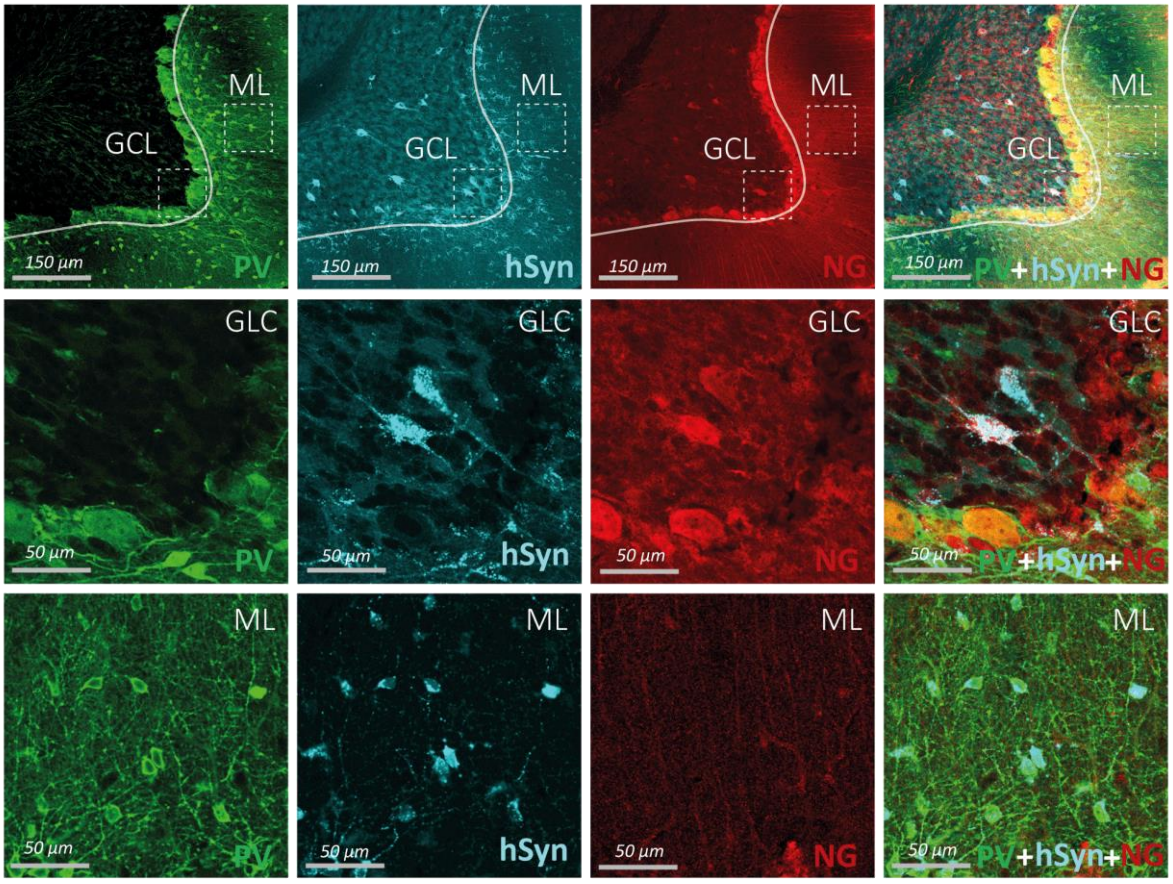

Figure 13. Phenotype of DREADD-expressing cells. *Parvalbumin (PV) labelling (green), AAV5-hSyn-hm4d-mCherry (cyan), Neurogranin (NG) 20x lenses (top). Magnification of granular cell layer (GLC on the middle) and molecular layer (ML on the bottom). 20x lenses, x3 digital zoom.*

To phenotype cells expressing DREADDs, we used two calcium-binding proteins, Parvalbumin and Neurogranin. Parvalbumin is a calcium-binding protein that is a well-known marker for Purkinje cells and molecular layer interneurons (Basket and Stellate) in the cerebellum (Kosaka, et al.,1993; Brown, et al., 2019), whereas neurogranin, predominantly labels Golgi interneurons (Singec et al., 2003). (**Figure 13**). Our results collectively indicate that the DREADD construct under the hSyn promoter and associated with the AAV5 serotype seems to exhibit an exclusive expression in these two subpopulations of interneurons in the cerebellar cortex. Nevertheless, further quantification of DREADD-expressing neurons using cell phenotyping markers might be required to conclusively support this observation. Therefore, despite it was not our initial aim, we decided to explore the effects of manipulations of Golgi and molecular interneurons on cocaine-induced CPP. We expected the acquisition of cocaine-induced CPP to be boosted by stimulating inhibitory interneurons (CNO-hm3D) and prevented when the activity of these neurons was reduced (CNO-hm4D).

Activation of molecular and Golgi interneurons by hm3D (Gq)-CNO increased preference for cocaine-related contextual cues.

In the case of groups with hm3D expression (**Figure 14A**) paired t-tests were used to compare Pre-Post preference scores within each group. The Veh-hm3D group showed a post-conditioning increase in preference for cocaine-associated cues [$\Delta_{Pre–Post}$ [95% CI] = 9.333 [-1.505; 20.17], p = 0.0389] that was boosted in the CNO-hm3D group [$\Delta_{Pre–Post}$ [95% CI] = 25.28 [17.33; 34.27], p = 0.0002]. Regarding the CNO-EGFP group (**Figure 14 A,B**), a paired t-test showed a similar increased in post-conditioning preference for cocaine-associated cues to the vehicle group [$\Delta_{Pre–Post}$ [95% CI] = 9.714 [-0.2138; 19.64], p = 0.0269]. However, although all groups acquired cocaine-induced conditioning, the effect was less consistent in vehicle and EGFP animals considering 95% CI of the difference pre-post in these groups.

To analyze the effect of hm3D(Gq) activated by CNO, a two-way repeated measures ANOVA (test x DREADD) for Veh-hm3D vs CNO-hm3D was performed. We observed a main effect of the test ($F(1, 22) = 29.75$, $p < 0.0001$) and an effect of the treatment (Veh or CNO) ($F(1, 22) = 4.414$, $p = 0.0473$), along with the interaction ($F(1, 22) =$

6.535, p = 0.0180). Post-hoc Sidak's Multiple comparisons demonstrated differences in post-conditioning between Veh and CNO [$\Delta_{Veh-CNO}$ [95% CI] = -15 [-25.93, -4.070], p = 0.0066], suggesting that activation of the main inhibitory neuronal populations in the cerebellar cortex increased the time spent in the cocaine-associated compartment compared to Veh-treated animals. Furthermore, in order to know whether this effect was related with the CNO administration or the viral transfection, we performed a two-way repeated measures ANOVA for CNO-EGFP vs CNO-hm3d (Gq) groups (**Figure 14A, middle**), showing only a main effect of the test (F (1, 24) = 35.56, p < 0.0001). No effect of treatment was found (F (1, 24) = 1.641, p = 0.2125), but an interaction between test and treatment was revealed (F (1, 24) = 7.294, p = 0.0125). Moreover, post hoc Sidak's Multiple comparisons showed differences in the post-conditioning test between hm3d(Gq) and EGFP rats [$\Delta_{EGFP-hm3D}$ [95% CI] = -11.86 [-21.90 to -1.812], p = 0.0191], suggesting that the facilitation effect is specific for the animals in which the stimulatory DREADD was activated. When comparing CNO-EGFP vs Veh-hm3D control groups (**Fig 14A, right**), the two-way repeated measures ANOVA yield a main effect of

the test (F (1, 22) = 7.777, p = 0.0107) but no effect of the treatment (F (1, 22) = 0.7474, p = 0.3966) nor an interaction (F (1, 22) = 0.003111, p = 0.9560). This supports the previous analysis that neither DREADD expression nor the CNO activator per se significantly impacted preference.

Inhibition of interneurons of the cerebellar cortex by hm4D-CNO prevented the acquisition of cocaine-induced preference conditioning.

We performed the same analyses for the groups infused with the inhibitory DREADD hm4D(Gi) (**Figure 14B**). First, paired t-tests were performed to compare pre/post-conditioning preference scores within each group. For the Veh-hm4D group, we observed a significant increase in post-conditioning preference for cocaine-associated cues [$\Delta_{Pre–Post}$ [95% CI] = 12.67 [0.8930; 24.44], p = 0.0198]. In contrast, the CNO-hm4D group showed no significant change in post-conditioning preference [$\Delta_{Pre–Post}$ [95% CI] = 4.374 [-5.103; 13.85], p = 0.1509], indicating that the inhibition of molecular and Golgi interneurons prevented the acquisition of cocaine-induced conditioning.

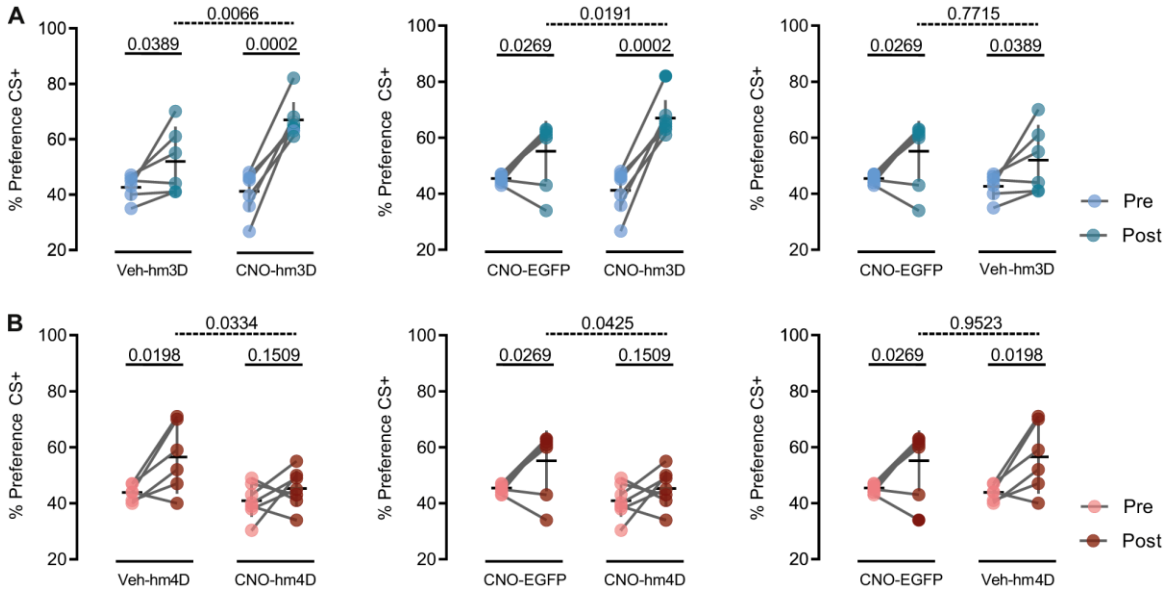

Figure 14. Percentage of preference for the CS+ before (pre) and after (post) cocaine-induced conditioning. (A) *Post-conditioning comparisons (dashed lines) of percentage of time spent in the CS+ (s) between: Veh-hm3d(Gi) (n = 7) vs CNO-hm3d(Gi) (n = 7) (left), CNO-EGFP(Gi) (n = 8) vs CNO-hm3d(Gi) (n = 7) (middle), CNO-EGFP(Gi) (n = 8) vs Veh-hm3d(Gi) (n = 7) (right) and Pre/Post-conditioning within-subject comparisons (continuous line).* ***(B)*** *Post-conditioning comparisons (dashed lines) of percentage of time spent in the CS+ (s) between: Veh-hm4d(Gi) (n = 7) vs CNO-hm4d(Gi) (n = 7) (left), CNO-EGFP(Gi) (n = 8) vs CNO-hm4d(Gi) (n = 7) (middle), CNO-EGFP(Gi) (n = 8) vs Veh-hm4d(Gi) (n = 7) (right) and Pre/Post-conditioning within-subject comparisons (continuous line). Data are shown as scatterplots (each plot represents the preference score for each animal) and mean (horizontal lines) plot with 95% CI (vertical*

Moreover, a two-way repeated measures ANOVA (test x DREADD) for Veh-hm4D vs CNO-hm4D revealed a main effect of the test ($F (1, 22) = 5.314$, $p = 0.0310$), an effect of the treatment ($F (1, 22) = 5.314$, $p = 0.0310$), but no an interaction effect ($F (1, 22) = 1.828$, $p = 0.1900$). Post-hoc Sidak's Multiple comparisons indicated differences in post-conditioning between Veh and CNO [$\Delta_{Veh–CNO}$ [95% CI] = 11.21 [0.8091 to 21.62], $p = 0.0334$], suggesting that hm4D activation by CNO reduced the time spent in the cocaine-associated compartment compared to Veh-treated animals. A two-way repeated measures ANOVA for CNO-EGFP vs CNO-hm4d(Gq) (**Figure 14B, middle**), demonstrated a main effect of the test ($F (1, 24) = 6.181$, $p = 0.0203$) and an effect of the treatment (hm4D or EGFP) ($F (1, 24) = 6.434$, $p = 0.0181$), but no interaction between test and treatment ($F (1, 24) = 0.8880$, $p = 0.3554$). Post hoc Sidak's Multiple comparisons showed that whereas EGFP-treated rats increased their post-conditioning preference hm4D(Gi) [$\Delta_{EGFP–hm4D}$ [95% CI] = 9.57 [0.299; 19.42], $p = 0.0425$] CNO-hm4d(Gq) animals did not. This suggests that the decrease in preference for cocaine-associated cues is primarily caused by the activation of hm4D(Gi), but not by CNO alone, the surgery procedure or the AAV vector infection.

A two-way repeated measures ANOVA (test x treatment) for CNO-EGFP and Veh-hm4d control groups (**Fig 14B, right**) showed a main effect of the test ($F (1, 22) = 10.72$, $p = 0.0035$) but no effect of the treatment ($F (1, 22) = 0.001213$, $p = 0.9725$) or the interaction ($F (1, 22) = 0.1865$, $p = 0.67$). Like with the excitatory DREADD, these results support the previous analysis that neither hm4D expression nor CNO alone significantly impacted drug conditioned preference.

CONCLUSIONS

The present study initially aimed at assessing the role of the granule cells in the formation of conditioned preferences for stimuli linked to cocaine availability. We employed DREADDs to stimulate or inhibit these neurons in lobule VIII of the vermis. However, our critical observations highlighted the exclusive expression of the hSyn promoter exclusively in two specific cerebellar interneuron populations, Golgi cells in the granule cell layer and, basket and stellate cells in the molecular layer.

Interestingly, the activation of these two populations of interneurons facilitates the acquisition of preference for cocaine-related cues in the post-conditioning test compared to the control groups, including EGFP and Vehicle controls, although all groups acquired preference conditioning. As we previously observed (Gil-Miravet et al., 2019; Gil-Miravet et al., 2021), it is the fraction of animals expressing preference that was increased but also the magnitude of the preference for the cocaine-related cue. Our hypothesis is that the facilitating effect on conditioned preference may be attributed to the excitation of interneurons within lobule VIII that in turn lead to a reduction in Purkinje cells activity, enhancing the activity of the DCNs. Conversely, inhibition of interneurons of the cerebellar cortex by increasing Purkinje activity might prevent the acquisition of cocaine-induced preference conditioning. Moreover, given that cocaine-induced preference conditioning stimulates granule cell activity (Carbo-Gas et a., 2014a; Carbo-Gas et al., 2017; Rodríguez-Borillo et al., 2023) in all groups (controls and DREADD), the effect could be attributed, in particular, to interneuron excitation or inhibition. It is essential to note that this hypothesis could not be conclusively proven in the current experiment since the animals did not receive CNO treatment on the test day. To test these hypotheses, it would be necessary to obtain cerebellar samples the last day of conditioning in which CNO was administered. Nevertheless, this working model will be approached in the forthcoming chapters.

These findings shed light on the intricate role of the posterior vermis in the formation of drug conditioned preferences, with DREADDs offering a precise tool to modulate specific neural pathways. It is remarkable that only by manipulating the inhibitory activity in lobule VIII of the posterior vermis, we were able to facilitate or prevent drug-induced Pavlovian learning. The present findings point to a relevant role of the vermis on the Pavlovian learning processes underlying drug addiction.

Chapter 3: Putting forward a model for the role of the cerebellum in cocaine-induced Pavlovian memory.

Adapted from Melchor-Eixea et al., 2023; https://doi.org/10.3389/fnsys.2023.1154014

Overview. Long-lasting molecular and structural changes in brain regions functionally and anatomically linked to the cerebellum, such as the prefrontal cortex, amygdala, hippocampus, basal ganglia, and ventral tegmental area, are characteristic of SUD. Direct and indirect reciprocal connectivity between the cerebellum and these brain regions can explain cerebellar roles in Pavlovian and reinforcement learning, fear memory, and executive functions. It is increasingly clear that the cerebellum modulates brain functions altered in SUD and other neuropsychiatric disorders that exhibit comorbidity with SUD. Other investigations from our lab, which employed lesions techniques, have pointed to the involvement of the posterior vermis in the inhibitory regulation of preferences for stimuli linked to cocaine. In this study, we showed that an excitotoxic lesion with QA in the apical region of lobule VIII dramatically facilitated the acquisition of cocaine-induced conditioned preference

Questions and aims. Can we revert the facilitating effect of an excitotoxic lesion of the dorsal Lobe VIII on cocaine-CPP by inhibiting the main cerebellar output (DCN)? Our aim is to propose an explanatory model for the effects of a lesion in lobule VIII of the cerebellar vermis on the acquisition of CPP using chemogenetic inhibition of the DCNs.

Hypotheses. The lesion of lobule VIII in the cerebellar vermis by QA is expected to elevate the number of animals acquiring CPP. Meanwhile, the inactivation of DCNs using the DREADD AAV5-CaMKIIa-hM4D(Gi) is predicted to counteract the facilitation on CPP acquisition induced by the lobule VIII lesion.

Highlights. Our data showed that inactivation of the region that includes the Int and Lat DCN prevents the facilitating effect of a posterior vermis lesion on cocaine-induced preference conditioning. These findings support our previous research and suggest that posterior vermis damage may increase drug impact on the addiction circuitry by regulating activity in the DCN.

Keywords: Addiction, DREADDs, Conditioned Place Preference,

Methods

Subjects. The present study includes 36 (*n* = 32 in experiment 1; *n* = 4 in experiment 2) male Sprague-Dawley rats weighing 125–225 g (Janvier, ST Berthevin Cedex, France). Rats were housed individually in the animal facilities (Jaume I University, Spain) under standard conditions (12-h light cycle from 8:00 AM to 8:00 PM, with access to food and water *ad libitum)*. Before beginning with behavioral procedures, rats were handled for 3 weeks and habituated to experimental protocols. Animal procedures were approved by the local Animal Welfare Ethics Committee and Empowered Body (2018/VSC/PEA/0139) and adhered to the European Community Council directive (2010/63/EU), Spanish directive BOE 34/11370/2013, and local directive DOGV 26/2010.

Pharmacological agents, adenoviruses and DREADDs. Cocaine hydrochloride (Alcaliber S.A., Madrid, Spain) was dissolved in a 0.90% saline solution and administered intraperitoneally (IP). Saline solution was used as a control vehicle. Anesthesia was induced with isoflurane (1000 mg/g) (Isoflutek 250ml, Laboratorios Karizoo S.A., Barcelona, Spain) using a Isotec 5 isoflurane anesthesia vaporizer (Datex-Ohmeda Inc., Madison, WI). AAV5-CaMKIIa-hM4D(Gi)-mCherry (Titer≥ 3×10^{12} vg/mL; Cat# 50477) used in the present study were provided by Addgene (Watertown, MA, USA). DREADD activation was achieved using 1 mM intracranial 0.50 µL/side and 1 mg/kg intraperitoneal of Clozapine N-oxide dihydrochloride (CNO-100 mg, Hello Bio Inc, Princeton, USA; Cat# HB6149). QA used for permanent damage of lobule VIII (90 nmol/_x03BC;L) (2,3-pyridinedicarboxylicacid, Sigma-Aldrich, Madrid, Spain) dissolved in phosphate-buffered saline (PBS; 0.1M pH 7.4)

Intracranial injections. Rats weighing between 270 and 350 g were anesthetized with isoflurane (1000 mg/g) (induction at 3.00 % and maintenance through the whole surgery at 2.00 %) using an Isotec 5 isoflurane anesthesia vaporizer and placed in a stereotaxic apparatus (Kopf Model 902; David Kopf Instruments, Tujunga, CA, USA).

In the first stereotaxic surgery, intracranial bilateral infusions of AAV were performed by placing a stainless-steel guide cannula (23-gauge external diameter, 36mm length) in the Int (AP: −11.30; ML: ±2.50; DV: −5.80) (Paxinos and Watson 1998) (**Figure 15 A, B**). Then, a removable stainless-steel injector (30-gauge external diameter, 38 mm

length) connected to a 1 µL Hamilton syringe (Microliter Syringe Model 7102 KH; Cat# 80300) driven by an infusion pump (11 Plus dual Syringe; Harvard Appatarus; Cat# HA702209) was inserted into the previously placed guide cannula to infuse bilaterally 0.50 µL of AAV (0.50 µL/min, two infusions of 0.25 µL separated by a period of 3 min) for the viral expression. After the infusion completion, the injector remained in place for 3 min for a better absorption of the substance. Then, the guide cannula and injector were removed, and the wound was sutured (**Figure 15B**). DREADD expression was observed in the Int and Lat DCN.

After 2 weeks from the AVV infusion, a second stereotactic surgery was performed to infuse in the posterior cerebellum (lobule VIII) (AP: −14.50; ML: 0.00; DV: −4.50) 0.50 µL (0.50 µL/min) of QA (90 nmol/µL) dissolved in phosphate-buffered saline (PBS; 0.10M pH 7.40) in order to permanently inactivate the apical region of vermis lobule VIII (Gil-Miravet et al., 2019; Gil-Miravet et al., 2021) (**Figure 15A, B, C**). After the infusion was completed, the injector remained in place for 5 min to avoid liquid aspiration. Then, the guide cannula and injector were removed. In the same surgery, for future infusion of the DREADD activator (CNO) or vehicle (saline), two guide cannulas (30-gauge external diameter, 15.20 mm length) were aimed bilaterally at the Int and attached to the skull with two stainless steel screws and fixed with dental cement (**Figure 15A, B**). Then, a dummy cannula (23-gauge external diameter, 15.20 mm length) was placed and kept inside the guide cannula to maintain the cannula's integrity until experiments were performed. After surgery, animals received analgesic treatment with meloxicam (Metacam 20 mL, 5 mg/mL; Boehringer Ingelheim, Barcelona, Spain, CAT#9993012) every 24 hours for two days. Rats were left undisturbed for four days for recovery. For behavioral experiments with intracranial infusions, rats were gently handled while restrained, and CNO or saline were infused bilaterally into the Int 5 min before each training session (0.50 µL, at 0.50 µL/min). Rats were not anesthetized during the microinjections because this procedure does not involve pain or discomfort for the animals. Behavioral trials began 5 min after the infusion.

***Conditioned Place Preference*.** The procedure was conducted in an oblong corridor apparatus. Rats underwent habituation, followed by a preconditioning preference test,

in which the innate preference for the two olfactory and tactile cues configurations (lemon + big holes or almond + small holes) were tested. The conditioning phase involved 4 cocaine and 4 saline pairings. The preference test was identical to the preconditioning test (**Figure 15A**). A detailed explanation of the procedure can be found in the methodology of Chapter 1 (**pag 40**).

Open field test. We performed this test during habituation (before training) and the last saline-paired day (after training but without CNO; without Int iNAcctivation). Rats were placed in the center of a 60 × 40 × 30 cm acrylic box for 15 min (**Figure 15G**). The open field was cleaned with 20% ethanol before each test to avoid any possible odor contamination that might affect the experimental result. All tests were videotaped, and spontaneous locomotor activity was evaluated blindly, assessing time spent in the periphery/center, number of quadrant crossings, and vertical and lateral rearing (**Figure 15H**). These parameters were recorded in three blocks of 5 min. Only the intermedium 5 min block is represented in figures to avoid confounding effects such as initial exploration and boredom at the end of the period.

***Brain sampling*.** 60 min after the final preference test, animals were perfused transcardially with saline, heparin, and 4% paraformaldehyde under anesthesia. Brains were stored in the same fixative, followed by immersion in a 30% sucrose solution with sodium azide until they sank. Sections, either sagittal or coronal, were obtained at 40 µm using a cryostat microtome and stored in cryoprotectant at -22°C. For more detailed information of this procedure see Chapter 1 methods (**Page 41**). The expression of the AAV5-CaMKIIa-hM4D(Gi) was checked using confocal imaging. Animals with no DREADD expression or cannula misplacement were not included in the statistical analysis (**Figure 15D**).

Immunofluorescence and imaging. Immunolabeling was performed on free-floating sections. To amplify the endogenous mCherry fluorescent signal, sections were exposed to a blocking buffer with donkey serum (Sigma Aldrich, Madrid, Spain; Cat# D9663) for 30 min and then incubated overnight at 4ºC in PBS 0.1 M Triton X-100 (Sigma-Aldrich, Madrid, Spain; Cat# T9284) (PBST) with rabbit anti-RFP (1:500; Rockland Immunochemicals Inc, Philadelphia, Pennsylvania, USA; Cat# 600-401-379). The next day, and after several rinses, tissue was incubated in PBST with anti-rabbit

Alexa Fluor 555 (1:500; Thermo Fisher Scientific, Rockford, IL, USA; Cat# A-31572) as secondary antibody. We used cFos immunofluorescent staining as a neural activity marker. Similarly, sections were incubated for 48h at 4ºC with rabbit anti-cFos (1:500; Synaptic Systems, Goettingen, Germany; Cat# 226003) in PBST, and the next day, in PBST with donkey anti-rabbit Alexa Fluor 647 (1:500; Thermo Fisher Scientific, Rockford, IL, USA; Cat# A-31573) as secondary antibody. To ensure that the QA lesion was affecting Purkinje innervation in the DCN**,** we used Calbindin as a marker for Purkinje terminals and vGAT to estimate GABA-related activity**.** Sections were incubated for 24h at 4ºC with guinea pig anti-vGAT (1:500; Synaptic Systems, Goettingen, Germany; Cat# 131004) and mouse anti-CB (1:1000 Swant, Bellinzona, Switzerland; Cat# CB38) in PBST, and the next day, in PBST with donkey anti-guinea pig Alexa Fluor 647 (1:250; Thermo Fisher Scientific, Rockford, IL, USA; Cat# A-21450) and donkey anti-mouse Cy3 (1:200 Jackson InmunoResearch, West Grove, PA; Cat# 715-165-150) as secondary antibodies. Brain tissue was mounted and covered with Mowiol (Calbiochem, Merck Chemicals and Life Science, Madrid, Spain; Cat# 475904100GM).

Image acquisition and analysis. Laser intensity, gain, and offset remained stable across animals. Tile-scan Z-stack images and image magnifications of the posterior cerebellum or DCN were acquired using confocal microscopy (Leica DMi8, Leica Microsystems CMS GmbH, Wetzlar, Germany) to assess QA/Sham lesions, cannula placements, and DREADD/mCherry expression. Imaging for cFos was perfumed at 20x, and vGAT and Calbindin co-expression around DREADD-expressing DCN neurons was performed at 63x. We used Leica Application Suite X (LAS X, Leica Microsystems CMS GmbH, Wetzlar, Germany) to perform maximal projections of the Z-stacks and Fiji free software (Schindelin et al., 2012) for image analysis. cFos+ cells (intense red labeling) were estimated in 2 images per rat (n = 2) co-expressing the AAV5-CaMKIIa-hM4D(Gi) within a ROI of 580 x 580 μm.

Experimental design and statistics. We used a total sample size of 32 animals, 8 animals per group (Veh-Sham, Veh-QA, CNO-Sham, CNO-QA). Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, USA), and R Statistical language (version 4.1.2; R Core Team, 2021). Data were analyzed by repeated-measures two-way ANOVAs and multiple comparisons were

performed using Sidak's tests. When two groups were compared, we used an unpaired *t*-test. Data are presented as individual and group scores (Mean and 95% Confidence Intervals; 95% CI). In the cases in which a significant difference was observed, we calculated the effect size of that difference as the Mean difference (*MD* = Mean Group1 – Mean Group2) and its 95% CI, to estimate the magnitude of those differences.

To assess for consistent differences between the weight of subjects before and after conditioning, we used the sign tests (Two-sided sign test for matched pairs) to assess the proportion of animals whose time spent (TS) in the CS+ increases (probability of success) with respect to themselves in the previous test. Then we assessed whether that probability of change as a group was different or not from chance (0.5) using the *binom.test* of the R *mosaic* package (Pruim et al., 2017), that returns the probability of success (as a group) with its 95% Confidence Interval (CI) and exact p-value (having as an alternative hypothesis: true probability of success is not equal to 0.5), which is what was reported.

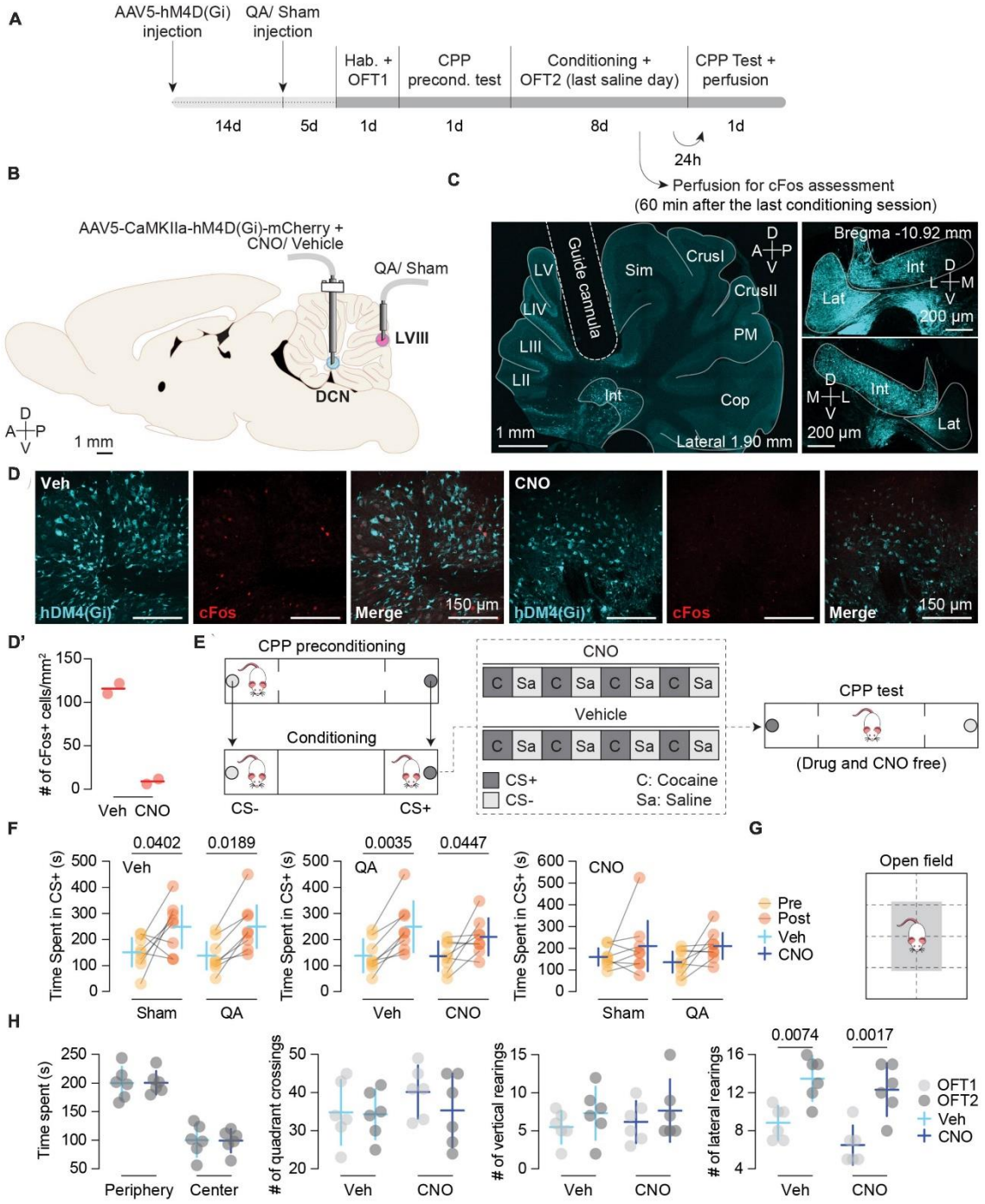

Figure 15. (A) *Experimental timeline. Different stages of the experimental procedure from stereotaxic surgeries to behavioral protocol. (**B**) Schematic depiction of intracranial infusions and cannula placements. (**C**) DREADD expression and cannula implant. Left panel: tilescan image depicting the guide cannula aiming at a region that encompasses the Int and Lateral Lat nuclei where the AAV5-CaMKII-hM4D(Gi) was previously injected showing positive cells expressing the inhibitory DREADD (cyan). Right panels: left (top) and right (bottom) DCN images (magnifications extracted from the same section) showing the bilateral expression of AAV5-CaMKII-hM4D(Gi). **(D)** Representative images of cFos expression in the Int nucleus from hM4D(Gi)+Vehicle (Veh) (left) and hM4D(Gi)+CNO (right) rats. Cerebellar tissue was obtained the last conditioning day in which the Veh and CNO animals received a saline injection (n = 2). (D´) cFos quantification (n = 2). Data are shown as single scores and Mean. **(E)** Schematic representation of the CPP apparatus, biased procedure, and conditioning schedule for the Vehicle and CNO groups.*

Figure 15.(Continuation) ***(F)*** *Pre- and Post-conditioning comparisons of Time spent in the CS+ (s) between: Veh-treated animals with Sham (n = 8) vs QA (n = 8) lesions (left panel), QA-lesioned animals with Veh (n = 8) vs CNO (n = 8) treatments (middle panel), CNO-treated animals with Sham (n = 8) vs QA (n = 8) lesion (right panel). Top left label on each plot reports the common treatment of both groups.* ***(G)*** *Schematic representation of the Open field box.* ***(H)*** *CNO treatment did not have any effect on motor activity. Left to right: time spent in the periphery (white area in G) and center (grey area in G) of the Open field box, number of quadrant crossings, number of vertical rearings, and number of lateral rearings). Vehicle (Veh; n = 6); CNO (n = 6); OFT1: Open field test before conditioning; OFT2: Open field test after 8 days of cocaine-induced conditioning. Data are shown as Mean ± 95% CI.*

RESULTS

Inhibition of the Int and Lat DCN prevented the facilitating effect of a posterior vermis lesion on cocaine-induced CPP.

To test the effectiveness of CNO in inhibiting DCN neural activity, we sampled two AAV5-CaMKIIa-hM4D(Gi)-expressing rats per group and assessed cFos expression in Int and Lat. The average number of cFos+ cells/mm^2 was 8.92 [5.94, 11.89] for CNO-treated (*n* = 2) and 115.90 [40.39, 191.50] for Vehicle (Veh)-treated (*n* = 2) rats (**Figure 15A, D**). Showing that CNO effectively reduces the activity of DREADD-expressing cells.

In two earlier studies, we showed that an excitotoxic lesion with QA in the apical region of lobule VIII dramatically facilitated the acquisition of cocaine-induced conditioned preference (Gil-Miravet et. al, 2019, Gil-Miravet et. al, 2021). Then, the facilitation of drug conditioning did not affect the magnitude but the fraction of subjects that expressed the conditioned response. The present results showed a similar effect.

In the present study, we confirmed by immunofluorescence analysis that QA lesion (**Figure 16B, C**) seems to be effective in reducing Purkinje inputs to DCN neurons (**Figure 16D**). Nevertheless, confirming the reduction in GABAergic Purkinje input would require further quantitative analysis. To verify the facilitation of the acquisition of CPP caused by the vermis lesion, we compared the TS in the CS+ chamber between the Sham- and QA-lesioned animals with Veh treatment. Both groups increased the TS chamber of the initially non-preferred configuration (Pre/Post test: $F(1,14) = 15.78$, $p = 0.0014$) (**Figure 15F, left panel**). Moreover, we observed that from all Veh-treated animals, 100% of the QA-lesioned rats increased their TS in the CS+ in the post test (Sign test: PS_{QA} = 100 [63, 100] %, p = 0. 0078) whereas only 63 % of the Sham-lesioned

group displayed higher scores in the second preference test (Sign test: PS_{Sham} = 63 [24, 91] %, p = 0.7266) (**Figure 15F, left panel**). Then, we wondered whether CNO treatment in the Int and Lat DCN might influence the effects of a QA lesion in lobule VIII. To answer this, we compared the Veh and CNO treatments in QA-lesioned animals. This comparison revealed that DCN inhibition did not affect the acquisition of cocaine-induced CPP; all groups acquired a conditioned preference for the initially non-preferred configuration (Pre/Post test: $F(1,14) = 20.57$, $p = 0.0005$) (**Figure 15F, middle panel**). However, the DCN inhibition using the AAV5-CaMKIIa-hM4D(Gi) seemed to have interfered with the facilitating effect of the QA vermis lesion on drug memory, reducing the proportion of animals that increased their TS in the CS+ chamber in the post-conditioning test in the CNO animals (Sign test: PS_{CNO} = 63 [24, 91] %, p = 0.7266) when compared with the Veh group (Sign test: PS_{Veh} = 100 [63, 100] %, p = 0.0078) (**Figure 15F, middle panel**).

To confirm that DCN inhibition only prevented the facilitation of the acquisition of CPP caused by the vermis lesion, we compared the TS in the CS+ chamber on the post-conditioning test between the Sham and QA groups infused CNO. The QA-CNO group not only spend similar time in the CS+ chamber as the Sham-CNO group (Pre/Post test: $F(1,14) = 4.48$, $p = 0.0532$), but also displayed similar probability of change between tests (PS_{Sham} = 63 [24, 91] %, p = 0.7266; PS_{QA} = 63 [24, 91] %, p = 0.7266) (**Figure 15F; right panel**). Moreover, Sham-Veh and -CNO groups showed similar TS in the CS+ chamber in the post-conditioning test (***p* = 0.6493; Figure 15F, left and right panels**) and proportion of animals increasing their scores in the second test (PS_{Sham} = 63 [24, 91] %, p = 0.7266). Therefore, Int and Lat inhibition seemed to have only influenced the effect of the vermis lesion (QA) on cocaine-induced CPP (**Figure 15F**).

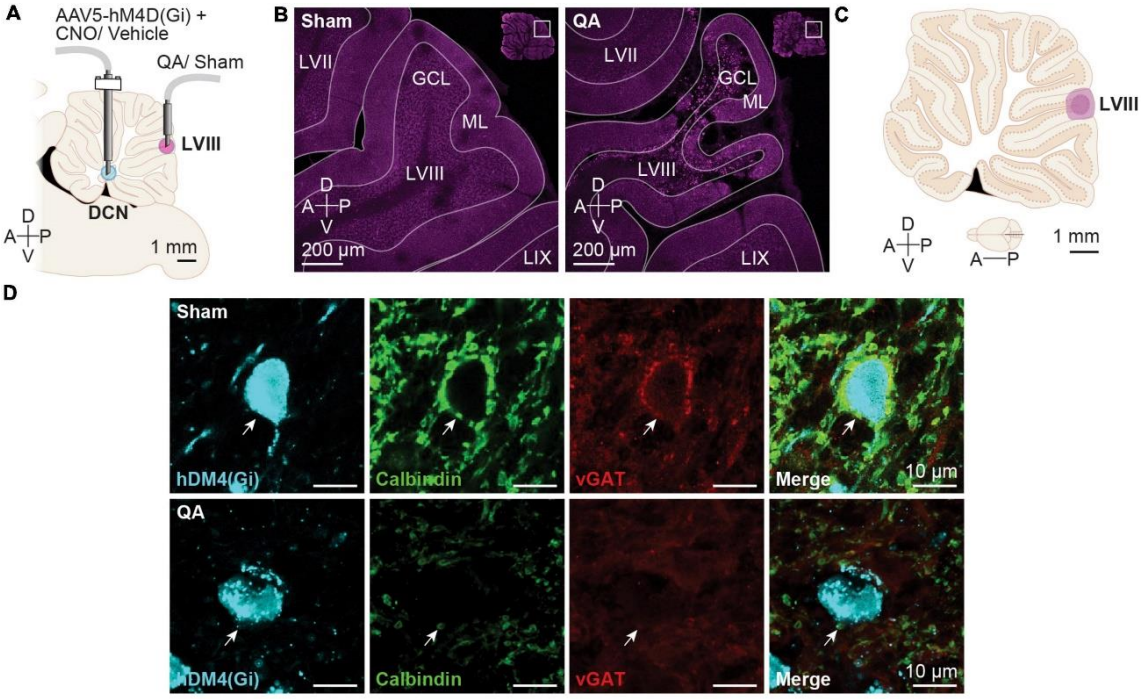

***Figure 16. DCN effect of sham and quinolinic acid infusions**. **(A)** Schematic depiction of intracranial infusions and cannula placements. **(B)** Representative confocal images of Sham and QA injections. **(C)** Schematic diagram depicting the largest (light purple) and smallest (dark purple) diffusion areas in the apical region of lobule VIII in the vermis. The diffusion areas were established overlaying each cerebellar image over the correspondent rat brain digital atlas image (Paxinos and Watson,1998). **(D)** Representative images of hM4D(Gi)+ cells in the Int nucleus from of Sham- and QA-infused animals. QA reduced the number of Purkinje cell axon terminals (Calbindin+; green) and de vesicular GABA transporter (vGAT+; red) onto DCN neurons expressing the hM4D(Gi)-mCherry DREADD (cyan). White arrows display examples of a Purkinje cell axon terminal.*

CNO infused in the Int and Lat did not affect motor activity after eight infusions.

To rule out a general motor impairment caused by inhibition of the DCN or intracerebellar infusions of CNO, we tested motor activity in the open field before (OFT1) and after conditioning (OFT2) (n = 6) (**Figure 15G, H**). We did not observe any effect of the treatment in TS in the periphery or center (F(1, 10) = 0.00, p = 1.000), number of quadrant crossings (F(1, 10) = 0.70, p = 0.4230), vertical (F(1, 10) = 0.11, p = 0.7474) or lateral rearing (F(1, 10) = 3.74, p = 0.0818) (**Figure 15H; left to right panels respectively**). However, we did find an effect of the test factor on lateral rearing (F(1, 10) = 35.89, p = 0.0001) that increased after conditioning (OFT2) regardless of CNO treatment (**Figure 15H; right panel**).

CONCLUSIONS

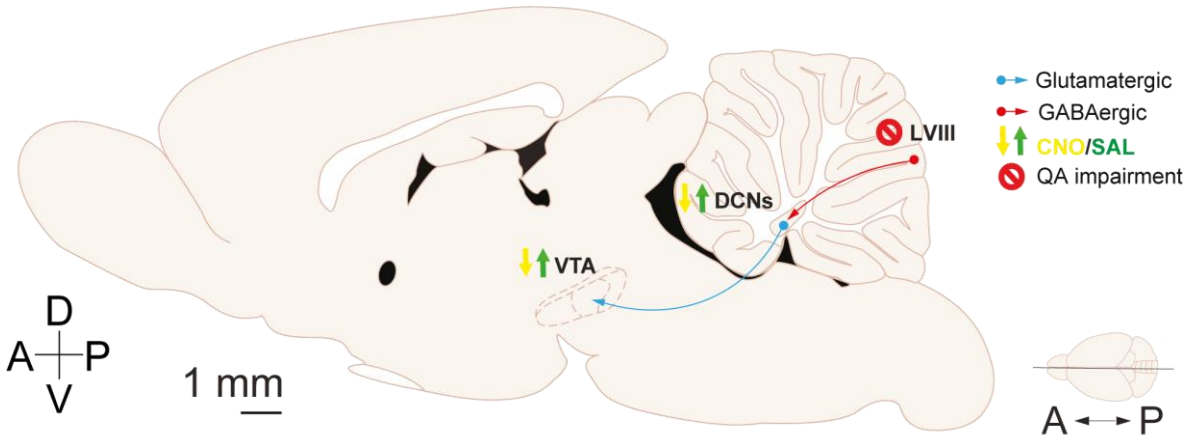

Figure 17. Working neuroanatomical model for the function of lobule VIII on drug-related memory. *The lesion of lobule VIII by increasing activity in Int and Lat nucleus can heighten glutamate release within the VTA. The lesion in lobule VIII would block the inhibitory tonic control exerted by the cerebellum over the VTA while the inhibition of the Int and Lat block the facilitative effect of the lesion.*

With the present exploratory study, we aimed to further support our working model using a chemogenetic inhibition of the more lateral DCNs. In line with this model (**Figure 17**), our preliminary findings suggest that DCN inhibition reduces the facilitating effect of a vermis lesion on learning cocaine-induced conditioned preference. Inhibition of the Int and Lat DCN left intact the ability to acquire conditioned preference for drug-related stimuli and did not cause any motor impairment or anxiogenic effects. Nevertheless, we did find an increase in lateral rearing after conditioning. This result points to motor sensitization following four cocaine administrations despite having conducted the motor test in a different chamber from that used for conditioning. This effect was not affected either by QA or CNO administrations. Our QA lesion permanently damages the most external region of the granule cell layer, Purkinje neurons, and molecular layer interneurons in the same region, reducing the inhibitory control of a subpopulation of Purkinje neurons onto DCN neurons **(Fig. 16B).** Consequently, lacking Purkinje inhibition, the activity in the cerebellar output neurons and the other regions in the striatum-cortico-limbic circuitry may increase and encourage drug-induced learning. The lesion of lobule VIII (QA-Veh) increased the proportion of animals that expressed conditioned preference up to 100%, and the inhibition of the DCN decreased the fraction of rats that expressed cocaine-induced conditioning to 63%. Interestingly, our rats still learned to prefer the

cocaine-associated configuration despite Int and Lat inhibition, which suggests that the formation of the drug-cue memory trace does not require these cerebellar nuclei. Even so, the DCN may fuel drug memory acquisition when there is posterior vermis dysfunction, hypothetically, through the modulation of activity in the VTA.

Altogether, these results confirm the modulatory role of the cerebellum on cocaine-induced conditioned memory and support the causal link between vermis dysfunction and the facilitation of drug effects. Therefore, the present findings may open new avenues to consider the cerebellum as a therapeutic target for stimulation in SUD and other comorbid mental disorders such as ADHD.

Chapter 4: Turning off the Interposed-VTA monosynaptic pathway.

Overview. In previous studies conducted by our research group, we identified a monosynaptic pathway connecting the DCN to the VTA, which also receives axonal input from Purkinje cells in the cerebellar vermis lobule VIII. This pathway is thought to regulate activity and plasticity in the mPFC and striatum, playing a role in cocaine-induced conditioned preference. The lesion of lobule VIII led to an increase in neural activity and PNN expression in IL and PL cortices, as well as in all striatal regions except the ventromedial striatum. Furthermore, deactivation of the IL, but not the PL, resulted in heightened activity and PNN expression in the posterior cerebellar cortex.

Questions and aims. Can we modulate the activity of the IL by changing the DCN-VTA pathway activity? In the current investigation, our objective was to test the working hypothesis that the cerebellum regulates IL activity through the VTA using chemogenetic tools. To achieve this, we used a double injection strategy expressing an inhibitory floxed-DREADD into the Int and a retrograde Cre AAV into the VTA to exclusively express the DREADD in the Int-VTA projections and activate it by an IP CNO injection. C-Fos activity was analyzed in the DCNs, VTA and IL.

Hypotheses. The infusion of pAAV-hSyn-DIO-hM4D(Gi)-mCherry, a cre-dependent inhibitory DREADD, in the DCNs and pENN-AAVrg-hSyn-Cre-WPRE-hGH into the VTA, is expected to selectively express DREADDs in the glutamatergic projection from the DCNs to VTA. Inactivating this pathway should decrease activity in the VTA and IL.

Highlights. Inhibition of the DCNs (IP and Lat) led to decreased neural activity within the VTA and IL. These findings provide further support for earlier research, which demonstrated the existence of a monosynaptic excitatory projection from the DCN to the VTA.

Keywords: Cerebellum, Ventral Tegmental Area, Infralimbic, DREADDs.

Methods

Subjects. The present study included 16 male Sprague-Dawley rats weighing 125–225 g (Janvier, ST Berthevin Cedex, France) at the time of stereotaxic surgery. Rats were housed individually in the animal facilities (Jaume I University, Spain) under standard conditions (12-h light cycle from 8:00 AM to 8:00 PM, with access to food and water *ad libitum*.) Animal procedures were approved by the local Animal Welfare Ethics Committee and Empowered Body (2018/VSC/PEA/0139) and adhered to the European Community Council directive (2010/63/EU), Spanish directive BOE 53/2013, and local directive DOGV 13/26/2007.

Pharmacological agents, adenoviruses and DREADDs. Anesthesia was induced with isoflurane (1000mg/g) (Isoflutek 250ml, Laboratorios Karizoo S.A., Barcelona, Spain) using a Isotec 5 isoflurane anesthesia vaporizer (Datex-Ohmeda Inc., Madison, WI). pENN-AAVrg-hSyn-Cre-WPRE-hGH (titer ≥ 7×10^{12} vg/mL; Cat#105553) and pAAV5-hSyn-DIO-hM4D(Gi)-mCherry (titer ≥ 7×10^{12} vg/mL; Cat#44362) used in the present study were provided by Addgene (Watertown, MA, USA). DREADD activation was achieved using 1mg/kg intraperitoneal of Clozapine N-oxide dihydrochloride (CNO-100 mg, Hello Bio Inc, Princeton, USA; Cat# HB6149). Saline solution was used as a control vehicle.

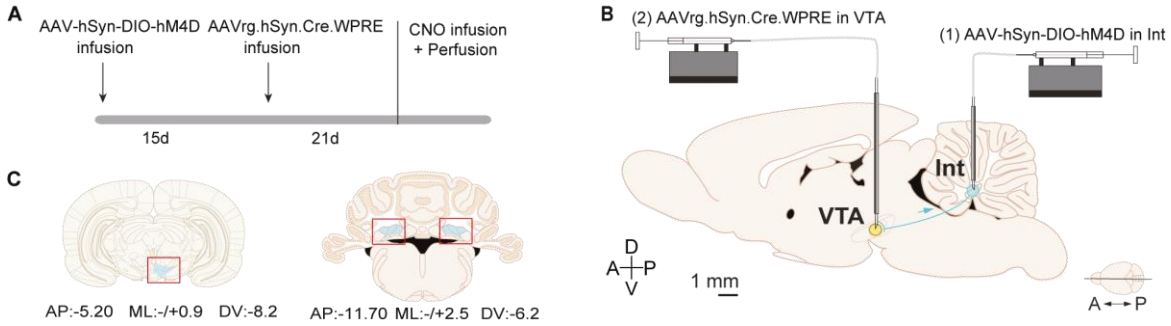

Figure 18. (A) Experimental timeline. *Experimental timeline of the surgeries and CNO injection.* ***(B)*** *Schematics of a sagittal section of a rat brain depicting the injection site of pENN-AAVrg-hSyn-Cre-WPRE-hGH and pAAV5-hSyn-DIO-hM4D(Gi)-mCherry.* ***(C)*** *Schematics of a coronal section of the DREADD expression region (blue) in Int and the injection sites in the VTA.*

Intracranial injections. Rats weighing between 270 and 350 g were anesthetized with isoflurane (1000mg/g) (induction at 3.00 % and maintenance through the whole surgery at 2.00 %) using an Isotec 5 isoflurane anesthesia vaporizer and placed in a stereotaxic apparatus (Kopf Model 902; David Kopf Instruments, Tujunga, CA, USA).

In the first stereotaxic surgery, intracranial infusions of AAV were performed by placing a stainless steel guide cannula (23-gauge external diameter, 36mm length) in the Int (AP: −11.70; ML: ±2.50; DV: −6,2) (Paxinos and Watson 1998). Then, a removable stainless-steel injector (30-gauge external diameter, 38 mm length) connected to a 2 µL Hamilton syringe (Microliter Syringe Model 7102 KH; Cat# 80300) driven by an infusion pump (11 Plus dual Syringe; Harvard Apparatus; Cat# HA702209) was inserted into the previously placed guide cannula to infuse bilaterally 0.50 µL of pAAV-hSyn-DIO-hM4D(Gi)-mCherry (0.50 µL/min, two infusions of 0.25 µL separated by a period of 3 min) for the viral expression. After the infusion completion, the injector remained in place for 3 min for a better absorption of the substance. Then, the guide cannula and injector were removed, and the wound was sutured. After 2 weeks from the pAAV-hSyn-DIO-hM4D(Gi)-mCherry infusion, a second stereotaxic surgery was performed to infuse unilateral (8 animals on the left hemisphere and 8 on the right) in the VTA (into the pigmented parabraquial area PBP) (AP: −5,2; ML: ±0,9; DV: −8,2) of pENN-AAVrg-hSyn-Cre-WPRE-hGH 0.5µL (same rate an timing than the IntP infusion). After the infusion was completed, the injector remained in place for 5 min to avoid liquid aspiration. Then, the guide cannula and injector were removed. After surgery, animals received analgesic treatment with meloxicam (Metacam 20 mL, 5 mg/mL; Boehringer Ingelheim, Barcelona, Spain, CAT#9993012) every 24 hours for two days.

Brain sampling. 120 min after the CNO injection animals were perfused transcardially with saline, heparin, and 4% paraformaldehyde under anesthesia. Brains were stored in the same fixative, followed by immersion in a 30% sucrose solution with sodium azide until they sank. Sections, either sagittal or coronal, were obtained at 40 µm using a cryostat microtome and stored in cryoprotectant at -22°C. For more detailed information of this procedure see Chapter 1 methods (**pag. 41**). The expression of the pAAV-hSyn-DIO-hM4D(Gi)-mCherry was checked using confocal imaging. Animals with

no DREADD expression or cannula misplacement were not included in the statistical analysis.

Immunofluorescence and imaging Immunolabeling was performed on free-floating sections. To amplify the endogenous mCherry fluorescent signal, we used rabbit anti-RFP (1:500; Rockland Immunochemicals Inc, Philadelphia, Pennsylvania, USA; Cat# 600-401-379) and anti-rabbit Alexa Fluor 555 (1:500; Thermo Fisher Scientific, Rockford, IL, USA; Cat# A-31572) as secondary antibody. For more detailed information of this procedure see Chapter 2 methods (**pag 53**). We used c-Fos immunofluorescent staining as a neural activity marker, we used rabbit anti-c-Fos (1:500; Synaptic Systems, Goettingen, Germany; Cat# 226003) donkey anti-rabbit Alexa Fluor 647 (1:500; Thermo Fisher Scientific, Rockford, IL, USA; Cat# A-31573) as secondary antibody. For more detailed information of this procedure see Chapter 1 methods (**pag 42**). We used Calbindin as a marker for Purkinje terminals. Sections were incubated for 24h at 4ºC with mouse anti-CB (1:1000 Swant, Bellinzona, Switzerland; Cat# CB38) in PBST, and the next day, in PBST with and donkey anti-mouse 647 (1:200 Jackson InmunoResearch, West Grove, PA; Cat# 715-165-150) as secondary antibody. In the last fluorescence immunostaining, tissue was incubated with rabbit anti-Tyrosine Hydroxylase (1:500; Millipore Merck KGaA, Darmstadt, Germany) and guinea pig anti-vGluT2 (1:500; Synaptic Systems, Goettingen, Germany), and then exposed to goat anti-rabbit 488 (1:200; Vector Laboratories, Bulingame, Ca, USA) and goat anti-guinea pig Alexa 647 (1:500; Thermo Fisher Scientific, Geel, Belgium).

Image acquisition and analysis. Laser intensity, gain, and offset remained stable across animals for the DREADD acquisition were as follows: laser intensity (1.3%); gain (800), and offset (-0,1). Tile-scan Z-stack images and image magnifications of the posterior cerebellum or DCN were acquired using confocal microscopy (Leica DMi8, Leica Microsystems CMS GmbH, Wetzlar, Germany) to assess DREADD/mCherry expression. Imaging for c-Fos was performed at 20x (laser intensity (1.62%), gain (800), offset (-0)). We used Leica Application Suite X (LAS X, Leica Microsystems CMS GmbH, Wetzlar, Germany) to perform maximal projections of the Z-stacks and Fiji free software (Schindelin et al., 2012) for image analysis. The number c-Fos+ cells (intense red labeling) were estimated in 2 images per rat (*n* =7 for Veh, n=6 for CNO) co-

expressing pAAV-hSyn-DIO-hM4D(Gi)-mCherry and pENN-AAVrg-hSyn-Cre-WPRE-hGH within a ROI of 500 x 500 μm.

Experimental design and statistics. We used a total sample size of 16 animals, 8 animals per group (SAL, CNO), three animals showed no DREADD expression and were excluded for the analysis resulting in a final n=7 in the Vehicle group and n=6 in the CNO group. Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, USA). Student t-tests for independent samples were carried out to analyse the number of positive c-Fos cells per mm^2. Data were presented as mean ±95% CI. The statistical level of significance was set at $P < 0.05$.

RESULTS

The projection from the DCNs to the VTA is mainly contralateral.

Following bilateral infusions of pAAV-hSyn-DIO-hM4D(Gi)-mCherry into the Int nuclei and pENN-AAVrg-hSyn-Cre-WPRE-hGH into the VTA during a subsequent surgery, our investigation revealed predominant DREADD expression within the contralateral Int and Lat nuclei. Furthermore, we did not observe substantial DREADD expression in the medial (Med) nucleus. Interestingly, a lower number of cells expressed the DREADD on the ipsilateral side within the same region (**Fig. 19A**).

Additionally, with an enhanced fluorescence assessment utilizing Anti-RFP labeling, we discerned axonic terminals in the region where we infused the retrograde Cre adenovirus, specifically within the parabrachial pigmented nucleus of the VTA (PBP), which represents the most caudal part of the VTA (**Fig. 19B**).

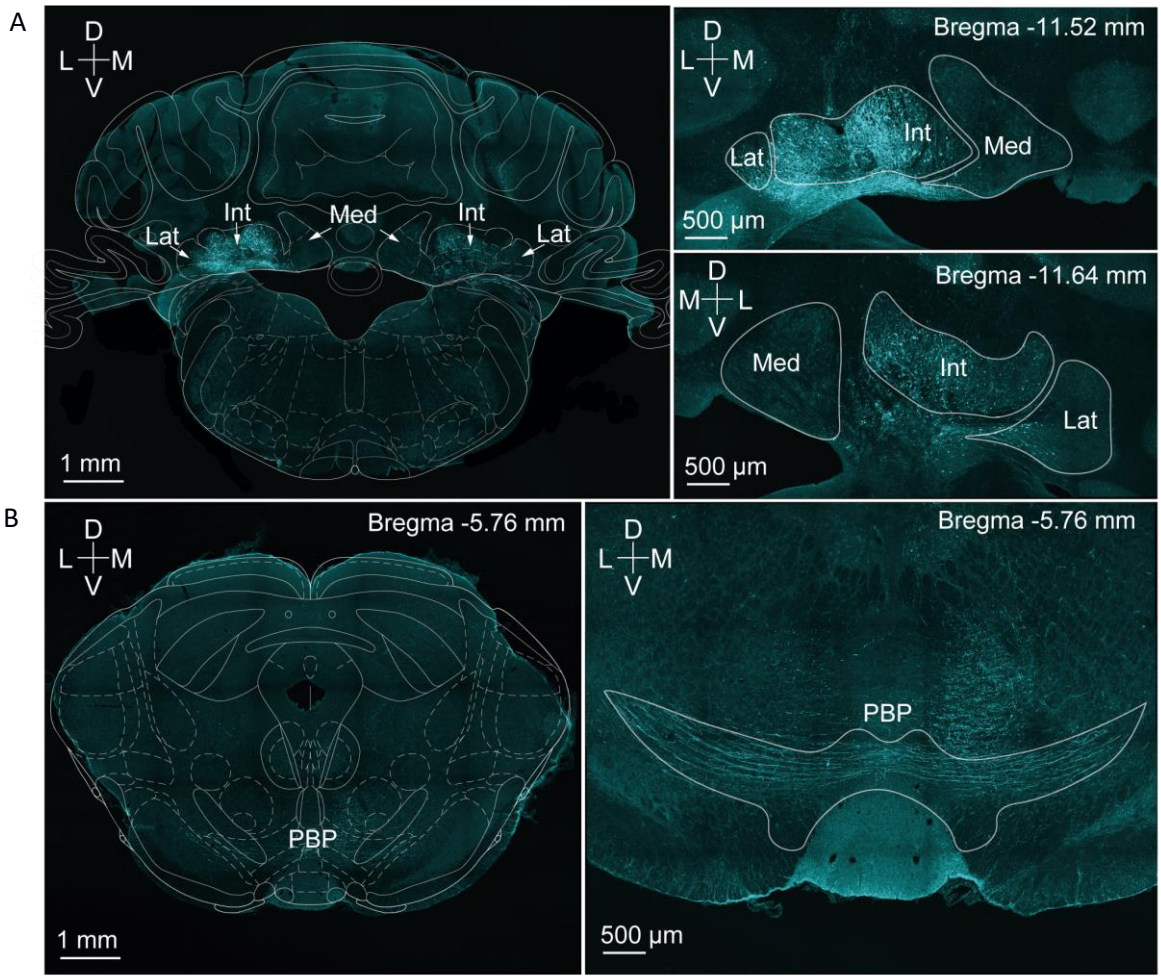

Figure 19. Anti-RFP immunofluorescence showing DREADD expression. *(**A**) Tilescan of DREADD expression. Left panel: tilescan image representative figure of the region that encompasses the Int and Lateral Lat nuclei where pAAV-hSyn-DIO-hM4D(Gi)-mCherry was injected showing positive cells expressing the inhibitory DREADD (cyan) images were taken at 10x magnification. Right panels: left (top) and right (bottom) DCN images (magnifications extracted from the same section) showing the bilateral expression of DREADD images were taken at 20x magnification. (**B**) Tilescan of the axonic terminals in PBP. Left panel: tilescan image representative figure of the coronal section that encompasses the PBP where the pENN-AAVrg-hSyn-Cre-WPRE-hGH was previously injected showing the axonic terminals of the inhibitory DREADD (cyan). images were taken at 10x magnification. Right panel: tilescan magnification extracted from the same section showing in which is shown the axonic terminals in both hemispheres, images were taken at 20x magnification.*

Int inhibition led to a reduction in neural activity within both the VTA and IL

Upon examining c-Fos expression within the Int and Lat nuclei, independent samples Student's t-tests unveiled a significant decrease in c-Fos expression in the Int region when comparing animals treated with CNO to those injected with Veh. The change [$\Delta_{veh–CNO}$ [95% CI] = -53.33 [-73.47; -41.20], $p<0.0001$] **(Figure 20A-C)** suggests that with

hSyn-DIO-hM4D(Gi)-CNO effectively suppresses neuronal activity in the DCN output neurons projecting to the VTA.

Our observations also revealed a significant reduction in c-Fos expression within the VTA in rats injected with CNO as compared to Veh, as demonstrated by independent samples Student's t-tests [$\Delta_{veh-CNO}$ [95% CI] = -58.38 [-77.78; -38.98], p<0.0001] (**Figure 20D-F**). As expected, this result implies the existence of a monosynaptic excitatory projection originating from the Int and Lat nuclei to the VTA. The decrease in activity within the Int and Lat nuclei consequently leads to reduced activity in the VTA.

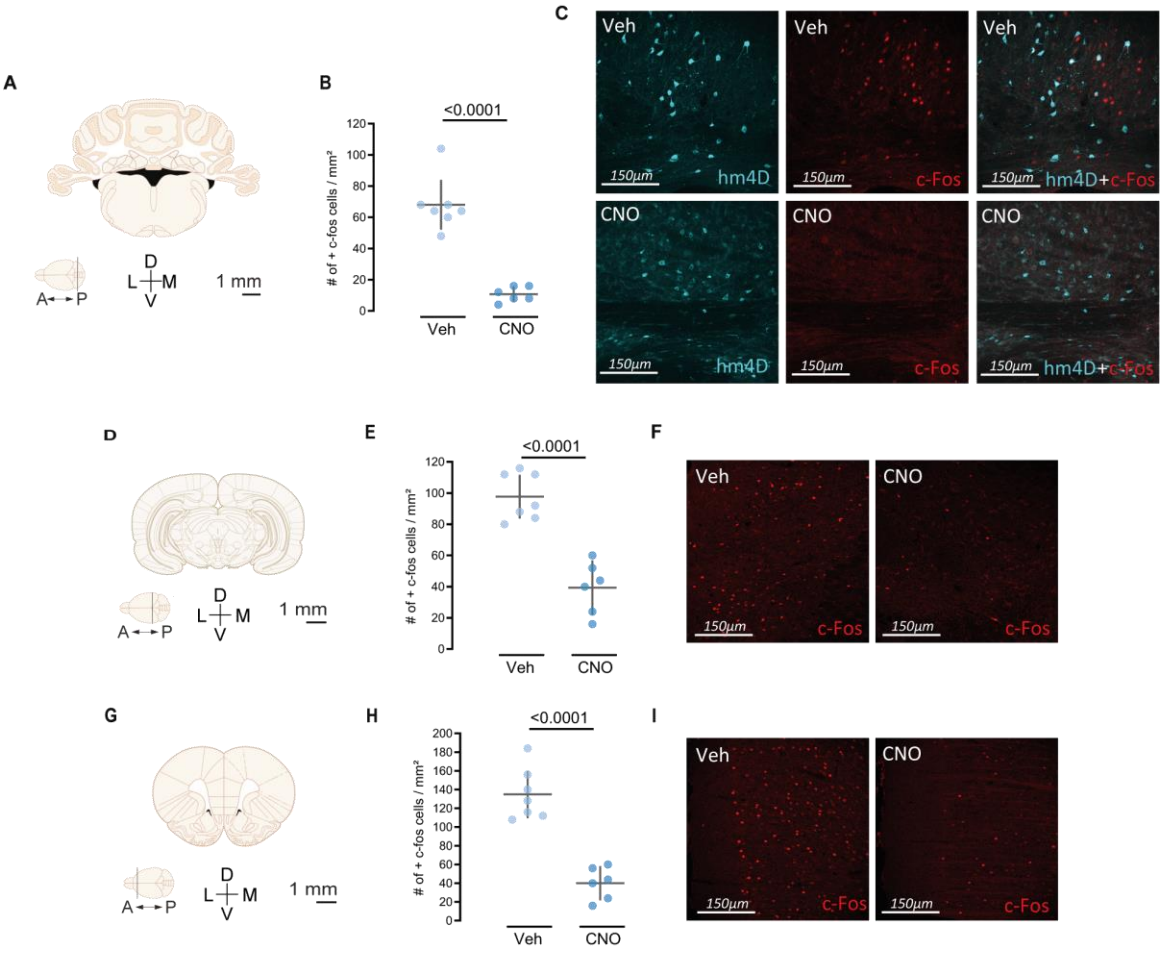

Figure 20. c-Fos and hm4D expression in the Int. *(**A,D,G**)Schematic representations of coronal sections of brain slices selected for the c-Fos analysis. (**Int**, Bregma: -11.16 to -11.70, **VTA** Bregma: -5.20 to -6, **IL** Bregma: 3.72 to 3.0) (**B, E, H**) Number of c-Fos positive cells/mm² in the Int (top), VTA (middle) and IL (bottom) 120 min after the CNO/Veh injection in the Veh(n = 7), CNO (n = 6). Data are shown as scatterplots (each plot represents number of c-Fos+ cells for each animal) and mean (horizontal lines) and 95% CI (vertical lines). (**C, F, I**) Representative pictures of c-Fos staining for each experimental group in the Int, VTA and IL respectively, DREADD expression and overlay with c-Fos expression added in the case of Int (**C**). All images were taken at 20x magnification. Scale bar 150 µm. Brightness was standardized across images.*

Furthermore, independent samples Student's t-tests highlighted a significant decrease in cFos expression within the IL when comparing animals treated with CNO to the control group. The change [$\Delta_{veh-CNO}$ [95% CI] = -94.86 [-123.5; -66.16], p<0.0001] (**Figure 20G-I**) suggests that the inhibition of the VTA not only impacts its own activity but also reduces the activity of the IL, likely as a result of the inhibition of the dopaminergic projection from the VTA to the IL.

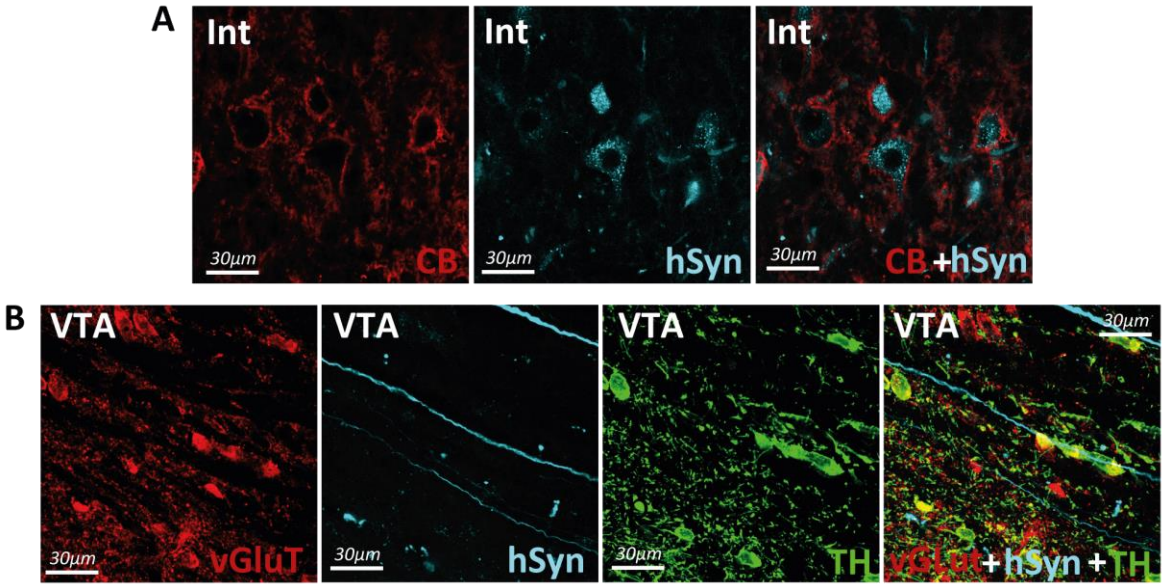

Figure 21. Coronal sections from the Int and VTA. (A) *Purkinje axons were identified by calbindin in red (first panel) and hSyn expression in blue (cyan) and the overlay (right).* ***(B)*** *Vesicular glutamate transporter (vGlut2) in red (first panel) Axons expressing the DREADD in cyan (second panel), dopamine neurons identified as expressing tyrosine hydroxylase (TH) in green (third panel) and the channel overlay (last panel). All images were taken at 20x4 magnification. Scale bar 30 µm.*

DREADD expressing neurons in the Int are surrounded by calbindin positive terminals from Purkinje cells that project to the VTA **(figure 21A).** Moreover, this projections from the Int and Lat nucleus seem to be glutamatergic (vGlut2+) and contacted with TH+ but also with TH- cells in the VTA **(figure 21B).** Nevertheless, further quantification is required to estimate the number of both subpopulations that are regulated by the cerebellar output.

CONCLUSIONS

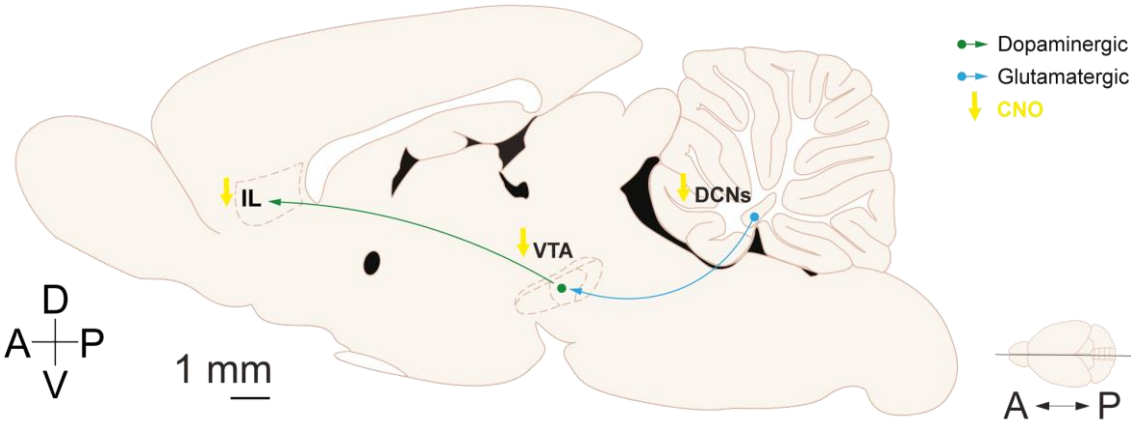

Figure 22. Working neuroanatomical model for the projection from the DCNs to the VTA and IL. *Inhibition of the Int and Lat may decrease glutamate release within the VTA. This inhibition blocks the dopaminergic projection from VTA to IL and decrease its activity.*

In our current investigation, we aimed to test the hypothesis that the cerebellum modulates the activity of the IL through the VTA using chemogenetic tools. To achieve this, we employed a double injection strategy, involving the expression of an inhibitory floxed-DREADD in the Int nucleus and a retrograde Cre AAV in the VTA, specifically targeting the DCNs-VTA projections. Subsequently, we examined the impact of this inhibition on neural activity within the DCNs, VTA, and IL.

Our findings showed a predominant expression of DREADDs within the contralateral Int and Lat nuclei, with limited expression in the Med nucleus. Interestingly, we also observed a small number of cells expressing the DREADD in the ipsilateral hemisphere within these same regions. Moreover, an enhanced fluorescence assessment using Anti-RFP labeling revealed axonic terminals in the region of the second infusion, particularly within the parabrachial pigmented nucleus of the PBP.

Notably, inhibiting the DCNs led to a reduction in neural activity within both the VTA and IL. These results support the existence of a monosynaptic excitatory projection originating from the Int and Lat nuclei to the VTA previously demonstrated (Carta et al., 2019; Gil-Miravet et al., 2021). The decrease in activity within the Int and Lat nuclei subsequently resulted in reduced activity within the VTA. Additionally, our findings suggested that by inhibiting the VTA not only impacted its own activity but also

reduced the activity of the IL, likely due to the inhibition of the dopaminergic projection from the VTA to the IL.

Additionally, we noted that the neurons expressing the DREADD are glutamatergic projecting cells that receive axons from calbindin-positive Purkinje cells. Furthermore, within the VTA, the dopaminergic TH+ cells receive DREADD+/vGluT2+ terminals from Int and Lat DCNs.

A proposed double immunostaining of tyrosine hydroxylase with c-Fos in the IL cortex would demonstrate that neurons receiving TH axons are inhibited in the CNO group compared to Veh. Additionally, utilizing the same cre-dependent strategy to manipulate the VTA-IL dopaminergic projection could ensure that IL activity variation is exclusively due to VTA inhibition, although the existence of this connection is evident.

For future exploration, training animals under the CPP paradigm following this specific cre-dependent strategy could offer enhanced specificity to manipulate this pathway compared to the approach used in Chapter 3. These results contribute further validation to previous research, affirming the existence of a monosynaptic excitatory projection from the DCNs to the VTA involved in cocaine-induced conditioning, emphasizing the intricate neural circuitry regulating this process.

GENERAL DISCUSSION

The primary aim of this research is to comprehensively explore the role of the cerebellum in the acquisition of CPP and its dynamic interactions with the striatum-cortico-limbic system, the circuitry involved in drug-induced brain and behavioral alterations.

In the first chapter, we focused on identifying neural activity patterns in specific cerebellar regions of rats undergoing training for cocaine-induced CPP by assessing c-Fos expression. This initial phase elucidated the enhanced activity in the dorsal part of lobule VIII of the cerebellar vermis. The results not only supported our hypothesis but also corroborate previous findings from our laboratory with different CPP paradigms and type of conditioned stimuli.

Building upon the insights from Chapter 1, the second chapter extended our investigation, employing DREADDs to delve into the role of lobule VIII in conditioned preferences for cocaine-associated stimuli. This approach allowed us to confirm specific DREADD expression in inhibitory interneurons of the cerebellar cortex. The observed inhibition of lobule VIII inhibitory interneurons prevented CPP acquisition, while increasing the inhibitory tone in lobule VIII facilitated it, validating our specific hypothesis and showing the inhibitory role of lobule VIII in drug-induced CPP acquisition.

With a clearer understanding of lobule VIII's inhibitory role, we delved into explore its connectivity with the main output to rest of the addiction circuitry. In the third chapter, we also employed chemogenetic tools to inhibit the activity of the Int and Lat DCNs. This experimental strategy aimed to neutralize the facilitating effect of lobule VIII impairment on cocaine-induced CPP. The results support our working model of an inhibitory regulation of the posterior vermis over the striatum-cortico-limbic circuitry through the modulation of the Int and Lat DCNs.

The final phase of our investigation targeted how the cerebellum modulates the activity of two key regions in SUD, namely the VTA and IL. The fourth chapter advanced

our exploration, experimentally assessing the hypothesis that the cerebellum regulates the IL through the VTA using chemogenetic tools. A dual-injection strategy based on a *Cre*-recombinase chemogenetic approach facilitated exclusive DREADD expression in the glutamatergic projections from the DCNs to the VTA, offering a better understanding of how the cerebellum modulates activity in these key regions. The observed decrease in activity in both the VTA and IL upon inhibiting this pathway show the connectivity and regulatory role of the cerebellum.

Taken all experiments together, the sequential chapters systematically addressed the overarching hypothesis, each contributing a crucial piece to the puzzle. Together, the findings provide a better understanding of how the modulation of neural activity in different cerebellar regions significantly influences the acquisition of cocaine-induced CPP. This work sheds light on the role of the cerebellum in Pavlovian drug-induced memories and its intricate connections within the addiction circuitry. However, it raises further questions that will also be discussed.

The specific results will be discussed in detail below based on the existing theoretical framework and their relevance for future advances in addiction research.

Cocaine-induced conditioned preference increases c-Fos expression in lobule VIII of vermis.

Our findings reveal that rats expressing a strong preference for cues associated with cocaine exhibited heightened neural activity in the granule cell layer of the dorsal posterior cerebellar vermis, specifically within lobule VIII. This finding points to a specific direct relationship between neuronal activity in the posterior vermis and the strength of preference for drug-related cues. First, this aligns with studies conducted by our team mentioned before using different procedures of Pavlovian conditioning procedure in mice and rats that consistently demonstrated similar increased neural activity in granule cells and Golgi interneurons of the posterior cerebellar vermis (Carbo-Gas et al., 2014a; Carbo-Gas et al., 2014b; Carbo-Gas et al., 2017; Guarque-Chabrera et al., 2022, Rodriguez-Borillo et al., 2023).

While classical views of cerebellar learning have suggested that this structure predominantly operates according to an error-based supervised learning rule to refine movements, emerging evidence suggests that the cerebellum may also harness a wider range of learning rules to contribute to a variety of behaviors, including cognitive processes (Hull, 2020). Granule cells in the cerebellum have been found to encode various aspects of reward-related processes, such as reward anticipation and conditioned responses. Research has shown that subsets of granule cells are activated by reward delivery and encode reward predictively, while others selectively encode reward anticipation or respond preferentially to reward or reward omission (Wagner et al., 2017, Wagner et al., 2019; Kostadinov & Häusser, 2022). These findings challenge the traditional view that granule cells only encode sensory and motor context, indicating that they also convey information about the expectation of reward. The discovery of predictive, non-sensorimotor encoding in granule cells enriches the contextual information available to postsynaptic Purkinje cells, with important implications for cognitive processing in the cerebellum (Wagner et al., 2017). Taking these findings in the context of our results, one could speculate that the posterior cerebellar cortex is activated during the expression of cocaine-induced CPP to predict and anticipate drug-effects to guide the selection of reward-related compartment. We focused our attention in the dorsal part on the lobules because it is the cerebellar region in which cocaine-dependent changes have been previously described (Carbo-Gas et al., 2014a; Carbo-Gas et al., 2014b; Carbo-Gas et al., 2017; Guarque-Chabrera & Gil-Miravet et al., 2022). As a matter of fact, activity in the ventral regions of the vermis were demonstrated to be related to contingency between US and the CS (Carbo-Gas et al., 2014b). This specificity likely reflects an anatomical organization of the inputs to the cerebellar cortex. Existing literature has documented the concentric arrangement of mossy fibers in the cerebellum (Voogd, 2014). Notably, corticopontine and exteroceptive components of mossy fibers target the dorsal part of the lobules, while proprioceptive components terminate in the ventral part of cerebellar lobules (Voogd & Ruigrok, 2004). This is also the cerebellar cortical region where the mossy fiber corticonuclear pathways, which facilitated Pavlovian conditioning, project (Gao et al., 2016). Notably, granule cell activity in this specific region is observed not only during unconditioned and conditioned stimuli but also throughout the conditioned

response (Giovannucci et al., 2017; Wagner et al., 2017). Moreover, previous studies have observed dopaminergic innervation from the VTA to these the cerebellar lobules (Ikai et al., 1992; Melchitzky & Lewis, 2000) that express dopamine transporter and receptors (Delis et al., 2008; Kim et al., 2009; Shimizu et al., 2014; Vazquez-Sanroman et al., 2015a).

Our findings are consistent with cue reactivity observed in individuals with SUD. As have been told in the introduction, studies among individuals with SUD consistently demonstrate that the presentation of drug-related cues triggers drug craving and stimulates cerebellar activity, irrespective of the sensory modality or the type of drug (Grant et al., 1996; Wang et al., 1999; Anderson et al., 2006; Schneider et al., 2001; Martin-Sölch et al., 2001; Filbey et al., 2009; Olbrich et al., 2009; Lou et al., 2012; Xuan et al., 2019; Al-Khalil et al., 2021). We observed a significant relationship between c-Fos expression and cocaine-induced preference in lobule VIII. This evidence, however, does not support the assertion of a direct causal relationship between cerebellar activity and drug craving. While some studies have reported a positive association between self-reported craving and cerebellar activity (Grant et al., 1996; Risinger et al., 2005), others have not found such correlations (Bonson et al., 2002). Additionally, similar patterns of cerebellar activity have been observed in response to cocaine cues and other arousing stimuli, such as anger-related scripts (Kilts et al., 2003). Furthermore, cerebellar activity increases in response to fear- (Utz et al., 2015) or food-related stimuli (Tomasi et al., 2015). These findings suggest a broader role of the cerebellum in processing and storing cues predicting rewards and other behaviorally relevant events, rather than being specifically tied to the conscious experience of drug craving (Moreno-Rius and Miquel, 2017). It is crucial to note, however, that cerebellar activity is strongly correlated with addiction severity (Shen et al., 2017; Al-Khalil et al., 2021). Given that cue-reactivity studies were conducted under withdrawal conditions, heightened cerebellar activity might result from a perceived mismatch between external cues (drug-predicting cues and context) and the absence of expected internal signals (drug effects) triggered by cue presentation. For fear conditioning, these internal models are believed to forecast the arrival of an unpleasant US. During extinction learning, if the aversive US is unexpectedly withheld, the cerebellum utilizes

prediction errors to revise and actualize the internal model (Doubliez et al., 2023). In this scenario, cerebellar activity could be associated with a prediction error concerning the internal representation anticipating the sensory and rewarding consequences of drug effects in a given context. Consistent with this, blood oxygen level-dependent imaging in the dorsal caudate, mPFC, and cerebellum showed increased activity when individuals with substance use disorders (SUD) experienced a mismatch between mentally represented drug effects and drug-taking contexts (De Pirro et al., 2018). In this study, individuals diagnosed with heroin and cocaine dependence were instructed to mentally visualize drug use settings (home/outside) while imagining themselves engaging in heroin or cocaine use. The activity of the fronto-striatal-cerebellar network was more pronounced when drug taking was mentally represented in a non-associated context for drug consumption (outside-home for heroin and the home context for cocaine) (De Pirro et al., 2018). Furthermore, animal research has demonstrated that violations of reward expectations activate climbing fibers (Heffley & Hull, 2019) and specific subpopulations of granule cells (Wagner et al., 2017).

Cocaine-induced CPP does not increase c-Fos expression in the paravermis and cerebellar hemispheres.

It is worth noting that we did not observe any effect of cocaine-induced conditioning on neuronal activity in the paravermis or the hemispheres. Crus 1 and Crus 2 of the cerebellar hemispheres are renowned for their involvement in non-motor functions and a diverse array of cognitive processes, including social behavior (Van Overwalle et al., 2020). Nevertheless, it is important to highlight that there is no clear evidence or studies indicating that Crus I and Crus II are directly related to Pavlovian conditioning. Therefore, our findings suggest that only the posterior vermis appears to be a part of the network involved in drug-related conditioned memory. Numerous investigations have illustrated the cerebellar vermis as the convergence site for US and CS stimuli in fear-conditioned responses, such as fear-induced freezing behavior and bradycardia (Supple et al., 1987, 1988; Supple and Leaton, 1990; Sebastiani et al., 1992, Dubois and Liu, 2021). Rodent studies have demonstrated that both electrical stimulation (Steinmetz et al., 1989) and optogenetic activation (Albergaria et al., 2018) of mossy fiber inputs originating from the pontine nuclei and projecting to the granular cell layer

can replicate the conditioned stimulus, leading to conditioned eyeblink responses. The acquisition and short-term expression of the conditioned motor response rely on specific regions within the cerebellar cortex (Galliano et al., 2013). However, long-term consolidation appears to take place in the interposed nucleus (Carulli et al., 2020). Additionally, the cerebellar vermis contributes to the acquisition and extinction of conditioned fear. Research has shown that the cerebellum is not only associated with the acquisition and retention of conditioned motor and emotional responses, but also with the extinction of conditioned fear. Human fMRI studies have highlighted the robust activation of lobule VI in the vermis and hemispheres during the early phases of fear acquisition and extinction learning, respectively (Utz et al., 2015). In people with SUD, the presentation of drug-related cues has been also shown to elicit activity mainly in the vermis (Grant et al., 1996; Wang et al., 1999; Anderson et al., 2006; Schneider et al., 2001; Martin-Sölch et al., 2001; Filbey et al., 2009; Olbrich et al., 2009; Lou et al., 2012; Xuan et al., 2019; Al-Khalil et al., 2020).

DREADDs with hSyn promoter is expressed in interneuronal populations, Golgi cells in the granular layer and basket and stellate cells in the molecular layer.

Regarding chapter 2, our critical observations revealed a noteworthy aspect - the exclusive expression of the hSyn promoter within two distinct cerebellar interneuron populations: Golgi cells in the granular layer and basket and stellate cells in the molecular layer. The hSyn promoter is known for its neuronal-specific expression and is typically used to express DREADDs in a wide range of neurons, effectively making it a pan-neuronal promoter (Campbell et al., 2018). The choice of viral serotype also plays a crucial role in driving protein expression. A review has highlighted that different serotypes of adeno-associated virus exhibit varying efficiency and cell tropism when delivered to different central nervous system regions (Srivastava, 2016). Additionally, AAV serotypes 1, 5, 8, and 9 have been reported to transduce astrocytes, glial cells, and neurons (Burger et al., 2004; O'Carroll et al., 2021; Gleichman et al., 2023), while AAV2 predominantly transduces neurons (Kaplitt et al., 1994; McCown et al., 1996; During et al., 1998; Mandel et al., 1998).

For the hSyn-hM4D DREADD, its cell-type selectivity for MLIs was reported for AAV8 vectors containing the human synapsin promoter (Kuhn et al., 2012; Badura et al., 2018, Zamudio et al.,2023). However, an AAV1 vector with the CaMKIIα promoter was reported to result in expression in MLIs, but also in Purkinje neurons in adult mice (Kim et al., 2015). As far as we concern, our study is the first to demonstrate that the AVV5 serotype, when associated with the hSyn promoter, exhibits a cell-specific affinity for infecting Golgi interneurons in addition to molecular layer interneurons in the cerebellar cortex of rats. Nevertheless, a pilot study carried out in our lab using the serotype AAV8 yielded a similar expression to that of the AVV5 serotype.

Some investigations illustrate that the hSyn promoter's effectiveness varies across different brain regions. For instance, in the substantia nigra's dopaminergic neurons, the hSyn promoter associated with adenoviral vectors fails to sustain long-term expression, as these neurons seem to selectively reduce transgene expression by deleting the adenoviral genome (Kügler, 2003). Concerning Syn expression during the postnatal development of the rat cerebellum, the onset of the synapsin I gene's expression coincides with the neurogenic differentiation pattern of Purkinje and granule neurons. By postnatal day 21, the neurogenic and morphogenic development concludes, and the cerebellar layers assume their adult appearance. At this stage, in situ hybridization indicates a significant increase in synapsin I gene expression in the internal granule cell layer (Melloni & DeGennaro, 1994). While this finding corresponds with our observations in adult rats, where we observed expression in granular layer interneurons, it fails to elucidate the observed expression in molecular layer interneurons.

In our study, we utilized AAV5, which has been demonstrated to selectively label GABAergic neurons in a transgenic mouse strain where these neurons are marked by the red fluorescent reporter protein tdTomato (Aschauer et al., 2013). Moreover, AAV5 efficiently also transduces cortical parvalbumin-expressing interneurons (Tkatch et al., 2022). Corresponding with our study, however, basket and stellate cells are parvalbumin positive cells, but also Purkinje cells that do not express the DREADDs. Moreover, Golgi interneurons do not express parvalbumin, but they were infected by

our AVV5 vector. Overall, the selection of a viral serotype, coupled with the choice of a promoter, is crucial for directing the expression of DREADDS. This study marks the pioneering demonstration that AAV5, when associated with hSyn, exhibits a distinct preference for inhibitory interneurons within the cerebellar cortex.

Excitation of interneurons in lobule VIII increased preference for cues associated with cocaine while their inhibition prevented its acquisition.

Several studies have employed DREADDs in the context of CPP experiments. For instance, Wayman & Woodward (2018) utilized hM3Dq DREADDs to explore the involvement IL neurons projecting to the NAcc in toluene-induced CPP, suggesting the necessity of involvement of specific medial prefrontal cortex (mPFC)-NAcc pathways for CPP expression. Another study employed hM4Di DREADDs in the ventral VTA to investigate the effects of cocaine on locomotion and reward in mice. While hM4Di-mediated inhibition did not impact cocaine-induced CPP, it did affect its extinction (Runegaard et al., 2018).

In the cerebellar cortex, Zamudio et al. (2023) used DREADDs to chemogenetically inhibit molecular layer interneurons in the posterior cerebellum, observing a reduction in voluntary ethanol consumption in male mice. This aligns with our findings showing that inhibiting cerebellar cortex interneurons prevented the acquisition of cocaine-induced preference conditioning, and their activation increased preference for cocaine-associated cues. Consistent with the present findings are also our previous results (Gil-Miravet et al.,2019, Gil-Miravet et al., 2021) indicating that lesions in lobule VIII facilitate the acquisition of cocaine-induced conditioning. In this case, the excitation of inhibitory interneurons, induced by hSyn-hM3D(Gq)-mCherry, could cause similar effects to blocking Purkinje cell inhibitory output by a QA lesion. In both cases, the outcome involves the reduction of Purkinje- dependent inhibition onto the DCNs. Collectively, these studies emphasize the inhibitory role of the posterior vermis in the acquisition and expression of addiction-like behaviors.

Inhibition of the Int and Lat DCN prevented the facilitating effect of a posterior vermis lesion on cocaine-induced CPP.

Our results also indicate that inhibiting the Int and Lat DCNs blocks the facilitating effect of a vermis lesion on the acquisition of cocaine-induced conditioned preferences. Notably, the inhibition of these DCNs did not hinder the ability to acquire conditioned preferences for drug-related stimuli and did not induce motor impairment or anxiogenic effects. Previous research demonstrated that a lesion in the dorsal region of lobule VIII (lobule VIII) of the vermis enhances the learning of cocaine-induced conditioned preferences and increases neuronal activity in the Lat nucleus, mPFC, NAcc, and all striatal subdivisions, except the ventrolateral striatum (Gil-Miravet et al., 2019; 2021). Both sets of findings suggest an inhibitory modulation of the posterior vermis on the activity of the addiction circuitry through the Int and Lat DCN.

Additionally, studies in rats with cerebellar vermal lesions targeting lobules IV and V or VIII revealed deficits in innate fear-evoked freezing to a predator (cat) with normal contextual fear memory retrieval (Supple, et al 1987), showing animals with vermal lesions displayed apparent disinhibited behavior, failing to express innate fear towards the cat. This aligns with our findings supporting the inhibitory function of the vermis.

Compromised inhibitory control under cerebellar vermis dysfunction may potentiate the effects of drugs on behavior (Miquel et al., 2019). These results confirm the modulatory role of the cerebellum in cocaine-induced conditioned memory and support the causal link between vermis dysfunction and the facilitation of drug effects. The concept of "cerebellar brain inhibitory function" has been proposed, with patients exhibiting posterior cerebellum impairment, particularly in the vermis, showing difficulties in controlling behavior, emotions, and cognition—referred to as "cerebellar cognitive-affective syndrome" (Schmahmann & Sherman, 1998). Dysregulation of the cerebellar vermis has also been implicated as a potential etiological factor for attention deficit hyperactivity disorder and other impulsive spectrum disorders, contributing to comorbidity with substance use disorders (Mulder et al., 2008; Durston et al., 2011; Miquel et al., 2019). Consequently, these findings suggest potential

therapeutic avenues, considering the cerebellum as a target for stimulation in substance use disorders and comorbid mental health conditions such as ADHD.

The DCN-VTA projection is mainly contralateral.

The findings from the current study revealed a predominant expression of DREADDs within the contralateral Int and Lat nuclei, with limited expression in the medial nucleus. Interestingly, a small number of cells expressing the DREADD were observed in the ipsilateral hemisphere within these same regions. Notably, enhanced fluorescence assessment using Anti-RFP labeling unveiled axonic terminals in the region of the second infusion, particularly within the PBP of both hemispheres. These results align with other traditional tracer studies in which infusing Fluoro-Gold (FG) into the VTA, somata labeled with FG were identified within the contralateral IntA, IntP, Lat nuclei, while no labeling was found within the Med nucleus. Furthermore, confirmation of cerebellar afference to the VTA was obtained by infusing biotinylated dextran amine (BDA) into the Lat. Unilateral BDA infusions into the Lat resulted in the labeling of the ipsilateral cerebellar hemisphere, lobules VIII and IX of the vermis and the contralateral PBP nucleus of the VTA. This comprehensive labeling pattern supports the cerebellar projection to the VTA, as indicated by both FG and BDA tracing methods (Gil-Miravet et al., 2021).

Nearly five decades ago, certain anatomical studies hinted at cerebellar projections to the VTA (Snider et al.,1976; Phillipson et al., 1979; Perciavalle et al., 1989), but subsequent investigations into this pathway were lacking until the recent advent of advanced tracing methods. The first study to show this, discovered that the medial nucleus in both cats and rats possesses ipsilateral projections to the VTA via the superior cerebellar peduncle. However, in these animals, the interposed and dentate nuclei were identified as having connections to the contralateral VTA (Snider et al., 1976). Other study in rats that used horseradish peroxidase (HRP) injections into the VTA found retrograde labels in both the contralateral dentate and interposed nuclei. The same procedure was repeated in an anterograde fashion, with results showing that tracer injections int he dentate and interposed nuclei both traveled to the contralateral VTA (Perciavalle et al., 1989). For instance, newer studies using modern

viral tracers in mice revealed bilateral projections between the interposed nucleus and the VTA (Judd et al., 2021; Watabe-Uchida et al., 2012). Moreover, optogenetic approaches in mice (Carta et al., 2019) has confirmed this bilateral cerebello-VTA connections. These studies support the idea that the DCNs-VTA projection in rodents and cats goes mainly to the contralateral side.

A recent human study in humans delved into these connections with a higher level of granularity reveals that these connections are most robust in the ipsilateral direction, although contralateral connections also exist (Hoffman et al., 2023). Moreover, when examining the monosynaptic tracts from the DCN to the VTA, the Int exhibited the highest density, followed by the fastigial and dentate nuclei. These findings suggest potential differences between rat and human DCN-VTA connections in terms of the number of ipsilateral/contralateral fibers and the specific nuclei of the DCNs involved.

Inhibiting the DCN-VTA projection led to a reduction in neural activity within both the VTA and IL.

Inhibition of the Int and Lat DCNs resulted in a notable reduction in neural activity within both the VTA and IL, providing evidence for the presence of a monosynaptic excitatory projection originating from the Int and lateral Lat nuclei to the VTA. The decline in activity within the Int and Lat nuclei subsequently led to diminished activity within the VTA. Interestingly, inhibiting the DCN-VTA projections not only impacted in the VTA activity but also diminished the activity of the IL, potentially due to the suppression of the dopaminergic projection from the VTA to the IL. Notably, neurons expressing the hSyn DREADD in the DCNs were found to be enveloped by calbindin originating from Purkinje cells that in turn, target the VTA. Furthermore, the Int and Lat projecting neurons appeared to be glutamatergic, as indicated by co-labeling vGlut2 and contact tyrosine TH+ but also several TH- cells in the VTA.

Building on previous findings from our laboratory, BDA in lobule VIII resulted in numerous BDA-labeled terminals from Purkinje cells within the DCN, particularly in the the IntA, IntP, and Lat nuclei. Additionally, when FG was infused into the IL cortex and BDA into the Lat nucleus, close apposition was observed between anterogradely labeled BDA fibers and retrogradely labeled FG neurons in the PBP of the VTA.

Projections from the Lat nucleus appeared to be glutamatergic and contacted both TH+ and TH− cells within the VTA (Gil-Miravet et al., 2021). Additionally, physiological characterization of the DCN projections to the VTA exclusively revealed excitatory connections (Carta et al., 2019). Alterations in the DCN-VTA pathway in mice can modulate VTA activity, influencing dopamine release (Low et al., 2021). Moreover, viral tracers demonstrated that the DCN sends direct projections to VTA DA, GABAergic, and glutamatergic neurons (Baek et al., 2022; Beier, 2022; Beier et al., 2019, 2015; Watabe-Uchida et al., 2012; Gil-Miravet et al, 2021). In summary, these findings further validate and emphasize the existence of a monosynaptic excitatory projection from the DCNs to the VTA.

Anatomical and functional research has shown evidence of VTA-to-mPFC DAergic projections (Breton et al., 2019; Lammel et al., 2008; Morrens et al., 2020; Popescu et al., 2016; Yan et al., 2019). Noteworthy, studies have also explored the connections between the medial prefrontal cortex and the cerebellum, revealing novel prefrontal-cerebellar connections. Stimulation of deep layers in the PL resulted in distinctive field potentials in the cerebellar cortex. These responses exhibited a specific topography, being most prominent in lobule VII of the contralateral vermis. (Watson et al., 2009). Additionally, simultaneous recordings of ongoing local field potential activity in the medial nucleus and PrL during rest or active exploration in open field arena demonstrated significant network coherence, particularly in the theta frequency range (Watson et al., 2014). Additionally, Mittleman et al. (2008) demonstrated long-lasting DA efflux in the prefrontal cortex of mice following cerebellar Lat nucleus stimulation.

Overall the evidence presented in this chapter highlights the significance of the DCN-VTA-mPFC interface as a regulatory pathway through which the cerebellum modulates prefrontal activity through the VTA.

A functional neuroanatomical model

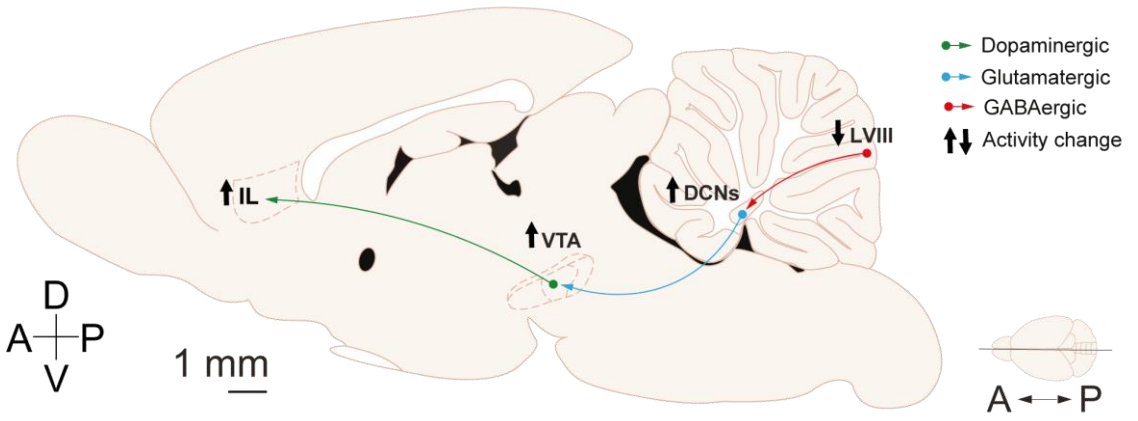

Figure 22. Working neuroanatomical model of lobule VIII-IL projections

This thesis used chemogenetic tools and lesion techniques to investigate the functional dynamics of the above neuroanatomical model and its influence on drug-cue associations. This model posits that cocaine-induced CPP results in heightened activity in the dorsal part of the granular layer of lobule VIII, enhancing the activity of molecular layer interneurons. This, in turn, diminishes the inhibitory control exerted by a specific subpopulation of Purkinje neurons onto DCN neurons. As a consequence of the reduced Purkinje inhibition, activity in cerebellar output neurons of the Int and Lat DCNs increases, modulating dopaminergic activity in VTA neurons and enhancing neuronal activity in the IL and other regions in the striatum-cortico-limbic circuitry. This increased activity in the circuit might in turn encourages drug-induced learning.

Clinical, neuroimaging, and anatomical tracing studies in several species support the existence of "closed-loop" connections between the cerebellum and neocortical brain regions, including the prefrontal cortex (Middleton and Strick, 1994, 2000; Kelly and Strick, 2003; Schmahmann, 2004; Allen et al., 2005; Krienen and Buckner, 2009; Strick et al., 2009; O'Reilly et al., 2010; Stoodley and Schmahmann, 2010; Buckner et al., 2011; Stoodley, 2012). Additionally, still unpublished work from our lab in collaboration with Khodakhah's lab revealed a novel descending pathway where IL sends monosynaptic projections to the Int and the Inferior Olive, possibly through collateral projections and then Int and Inferior Olive reach lobule VIII (Guarque-Chabrera, 2023 Doctoral Dissertation). This would close the circuit of the proposed model.

As is known, mPFC is responsible for executive control, which includes the representation of contingencies, representation of outcomes and their value, and subjective states associated with drugs (Everitt and Robbins, 2005). This aligns closely with the cerebellar role in drug-induced memories described in this work. Progressive loss of control over drug consumption involves a reduction in inhibitory control exercised by the prefrontal cortex (Volkow et al, 2013). This supports the idea that mPFC functions closely parallel the role of the cerebellum in SUD. Furthermore, evidence suggests the rewarding nature of cerebello-VTA projections, essential for social preference in mice and implicated in reward-based learning (Carta et al., 2019; Yoshida et al., 2022). Alterations in the DCN-VTA pathway in mice can modulate VTA activity, influencing dopamine release and potentially regulating reward-driven behavior (Low et al., 2021). The VTA plays a pivotal role in generating reward prediction error signals (Hollerman and Schultz, 1998; Schultz, 1998; Schultz, 2016), but also in aversion and stress, being a crucial hotspot in the fear network (Lammel et al., 2012; Bouarab et al., 2019). The VTA, critical in SUD, undergoes alterations in response to drug consumption, indicating a pivotal role for VTA afferents in mediating drug effects (Oliva & Wanat, 2016). The influence of VTA DA projection to the infralimbic cortex on addiction has been extensively studied, highlighting the importance of VTA synapses in addiction (Volkow et al., 2019; Polter & Kauer, 2014, Cooper et al., 2017; Kalivas et al., 2008). Moreover, it has been shown that there is a preferential expression of D2 receptors (D2Rs) in Purkinje cells, influencing the synaptic efficacy onto these cells (Cutando et al.,2022). Furthermore, they indicate that variations in D2R levels within PCs of adult male mice can impact sociability and the preference for social novelty, establishing a direct connection between the expression levels of cerebellar D2Rs and social behaviors.

The identified cerebello-VTA-IL pathway bears potential relevance for understanding the pathophysiology of socio-affective disorders marked by disrupted reward-seeking behavior, including social anxiety, schizophrenia, autism, eating and substance use and mood disorders (Miquel et al., 2019). Patients with posterior cerebellum impairment, especially in the vermis, exhibit difficulties in behavior control (Schmahmann and Sherman, 1998). As have been told, dysregulation of the cerebellar vermis has been

linked to impulsive spectrum disorders, contributing to comorbidity with substance use disorders (Mulder et al., 2008; Durston et al., 2011; Miquel et al., 2019). Moreover, neuroimaging studies frequently report vermis abnormalities in conditions like schizophrenia, with interventions targeting the vermis showing efficacy in alleviating cognitive and emotional symptoms (Okugawa et al., 2007; Lawyer et al., 2009; Henze et al., 2011; Heath, 1977; Demirtas-Tatlidede et al., 2010).

This thesis aligns with the concept of the "cerebellar brain inhibitory function" (Schmahmann & Sherman, 1998). The hypothetical model (lobule VIII-DCNs-VTA-IL) demonstrates significant predictive potential, suggesting that the cerebellum's role extends beyond regulating cocaine-induced preference conditioning. The cerebellum as has been told, modulates the internal predictive system involved in associative learning for reward-seeking and fear responses. Prediction errors occur when anticipated outcomes differ from actual ones (Popa and Ebner, 2019). It may play a vital role in restraining ongoing actions during changes in environmental conditions, adjusting prefrontal activity through the VTA in response to new stimuli. This adjustment could facilitate flexible behavioral control. Developing appropriate internal models to navigate in context-specific changing situations may rely on the cerebellum's connectivity and interactions with cortico-limbic regions. These models might be particularly vulnerable to misalignment during development (Kelly et al., 2021; Stoodley and Tsai, 2021). Disrupted activity along this pathway could lead to inappropriate formation of internal models within the cerebellum (Katsanevaki et al., 2020). Alterations in this pathway may occur in neuropsychiatric conditions commonly comorbid with SUD, such as schizophrenia, autism, obsessive-compulsive disorder, and attention-deficit/hyperactivity disorder (Hester, 2004; Robbins et al., 2012; Winstanley et al., 2010, Couto-Ovejero et al., 2023), and offers potential explanation for observed phenomena where cerebellar alterations are associated with behavioral disinhibition, impulsivity, and compulsivity.

Potential therapeutic applications may involve precise interventions to modulate the activity of specific neuronal populations within the posterior cerebellar cortex. Fine-tuning neural activity associated with drug-induced memories could mitigate the

reinforcing effects of drugs and disrupt conditioned responses contributing to addictive behaviors. This approach aligns with the emerging trend in neuroscience to explore innovative interventions beyond traditional pharmacological treatments.

In conclusion, the insights gained from this research lay the foundation for exploring targeted interventions involving the cerebellum in SUD treatment. While further studies are necessary to validate and refine these concepts, the potential therapeutic applications to stress the importance of considering the cerebellum as a viable target for innovative and effective strategies in the multidimensional landscape of addiction therapy.

STRENGTHS AND PITFALLS

Strengths

This thesis attempts to test a neuroanatomical model that delineates the pivotal role of the cerebellum in the context of drug addiction. Specifically, it provides a comprehensive understanding of how the posterior cerebellum modulates prefrontal-striatal circuitry, shedding light on the circuits governing the establishment of cocaine-associated memories. This contribution significantly advances the field of addiction neuroscience.

To our knowledge, our investigation is the initial demonstration highlighting that the AAV5 serotype, in conjunction with the hSyn promoter, exhibits a specific affinity for Golgi interneurons, in addition to molecular layer interneurons, within the cerebellar cortex of rats.

Furthermore, the research introduces additional evidence supporting the concept of direct cerebellar control over the VTA. By delving into this complex neural circuitry, the thesis extends our comprehension of the cerebellum's impact on VTA activity, offering a nuanced perspective on its role in addiction-related processes.

Additionally, the thesis establishes the translational nature of the increase in activity in lobule VIII associated with cocaine-induced place preference across species. This enhancement in activity, observed in both mice and rats, demonstrates the stability of this phenomenon between paradigms, with other studies utilizing cocaine-induced odor conditioning and our study employing tactile and odor-conditioned place preference.

Pitfalls, Weaknesses, and Future Directions

While the strengths of this thesis are evident, acknowledging certain pitfalls and weaknesses is crucial for refining methodologies and guiding future exploration.

In Chapters 2 and 4, the administration of CNO for the DREADD activator varied between intraperitoneal and intracranial approaches, creating a challenge in

comparing results. The shift in approach in Chapter 3, from intraperitoneal to intracranial, was prompted by concerns about potential behavioral side effects of CNO. The difficulty in maintaining intracranial cannulas and emerging evidence suggesting low doses of CNO have minimal adverse effects led to a return to the initially employed administration method.

The exclusive use of male subjects in the studies represents a limitation that warrants consideration. Future research should strive for gender diversity to ensure a more comprehensive understanding of the neurobiological mechanisms underpinning SUD.

During preference conditioning, activations and inhibitions were limited to the acquisition period. To enhance our comprehension of the cerebellum's role in cocaine-induced memories, future investigations should explore the activation and inhibition of different cerebellar regions, such as lobule VIII or DCNs (specifically, Lat and Int) during expression (post-conditioning test), extinction, and reconsolidation processes.

The specific Cre-dependent approach employed in the final chapter offers a focused method, selectively influencing the cerebello-VTA projection. This contrasts with the preceding chapter, which manipulated activity across all regions connected to the DCNs, including the inferior olive, pontine nuclei, and the cerebellar cortex.

In the last chapter, conducting a double immunostaining of tyrosine hydroxylase with c-Fos in the IL cortex would provide evidence that neurons receiving TH+ axons are inhibited in the CNO group. Employing the same Cre-dependent strategy to modulate the VTA-IL dopaminergic projection ensures that any variations in IL activity would result exclusively from VTA inhibition. Likewise, it would be enlightening for our hypothesis the assessment of DA activity markers within the IL cortex after DCN activation and inhibition.

Furthermore, it could be of interest to manipulate the activity of lobule VIII and assess c-Fos activity in Int and Lat DCN. This would help to verify the proposed explanations in this chapter. However, due to the absence of CNO administration during the CPP expression test, we are unable to demonstrate the inhibition/excitation of the DCNs through the inhibitory control of Purkinje projections. This verification could be

achieved by sacrificing a group of animals on the last conditioning day, similar to the approach employed in the experiment of Chapter 3.

An alternative and potentially more informative approach could involve using optogenetics combined with electrophysiology instead of the DREADDs/c-Fos approach. This shift would provide superior temporal resolution during the activation/inhibition of cells and recording of activity in our experiments.

The specific Cre-dependent strategy used in the last chapter provides a targeted approach, exclusively manipulating the cerebello-VTA projection, in contrast to previous chapter manipulating the activity in all regions projecting from the DCNs, such as the inferior olive, pontine nuclei, or the cerebellar cortex. With this approach could be interesting to test the animals under the CPP paradigm.

Finally, beyond the establishment of cocaine-induced Pavlovian memory, future research endeavors should investigate how our deactivations influence other paradigms of drug self-administration. This broader exploration could yield insights into the generalizability and specificity of the observed effects in different addiction-related contexts.

In conclusion, while this thesis makes significant strides in unraveling the cerebellum's role in SUD, the identified pitfalls and weaknesses pave the way for refined methodologies and future directions. These improvements promise to further enrich our understanding of the intricate neural mechanisms associated with drug addiction.

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ANNEXES

Throughout the development of this thesis, the following contributions were made, including scientific articles, posters presented at international congresses, and informative publications:

Annex 1. Melchor-Eixea, I., Guarque-Chabrera, J., Sanchez-Hernandez, A., Ibáñez-Marín, P., Pastor, R., & Miquel, M. (2023). Putting forward a model for the role of the cerebellum in cocaine-induced pavlovian memory. Frontiers in Systems Neuroscience, 17. https://doi.org/10.3389/fnsys.2023.1154014

Annex 2. Melchor-Eixea I., Guarque-Chabrera J., Marcho-Rochina A., Marta Miquel. (2021). Turning On and off the cerebellum-infralimbic-cortical network [Poster]. 49th Meeting of the European Brain and Behaviour Society (Congress), Lausanne, Switzerland.

Annex 3. Melchor-Eixea I., Guarque-Chabrera J., Marta Miquel. (2022). Modulating the efects of a lesion of the cerebellar vermis on cocaine-induced cpp through chemogenetic inhibition of the interposed nucleus activity [Poster]. Federation of European Neuroscience Societies (Congress), Paris, France.

Annex 4. Melchor-Eixea I., Guarque-Chabrera J., Marta Miquel. (2022 INAcctivation of deep cerebellar nuclei reverts the effect of a neurotoxic lesion in the apical vermis on the acquisition of cocaine-induced CPP [Poster]. IV International Congress of Psychobiology (Congress), Valencia, Spain.

Annex 5. Melchor-Eixea, I. M., & Miquel, M. (2023). Encendiendo y apagando el cerebelo para cambiar la preferencia por los contextos relacionados con las drogas. Àgora De Salut, 9,11–12. https://doi.org/10.6035/agorasalut.2023.9.2.

Annex 1

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Putting forward a model for the role of the cerebellum in cocaine-induced pavlovian memory

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Substance Use Disorder (SUD) involves emotional, cognitive, and motivational dysfunction. Long-lasting molecular and structural changes in brain regions functionally and anatomically linked to the cerebellum, such as the prefrontal cortex, amygdala, hippocampus, basal ganglia, and ventral tegmental area, are characteristic of SUD. Direct and indirect reciprocal connectivity between the cerebellum and these brain regions can explain cerebellar roles in Pavlovian and reinforcement learning, fear memory, and executive functions. It is increasingly clear that the cerebellum modulates brain functions altered in SUD and other neuropsychiatric disorders that exhibit comorbidity with SUD. In the present manuscript, we review and discuss this evidence and present new research exploring the role of the cerebellum in cocaine-induced conditioned memory using chemogenetic tools (designer receptor exclusively activated by designer drug, DREADDs). Our preliminary data showed that inactivation of a region that includes the interposed and lateral deep cerebellar nuclei reduces the facilitating effect of a posterior vermis lesion on cocaine-induced preference conditioning. These findings support our previous research and suggest that posterior vermis damage may increase drug impact on the addiction circuitry by regulating activity in the DCN. However, they raise further questions that will also be discussed.

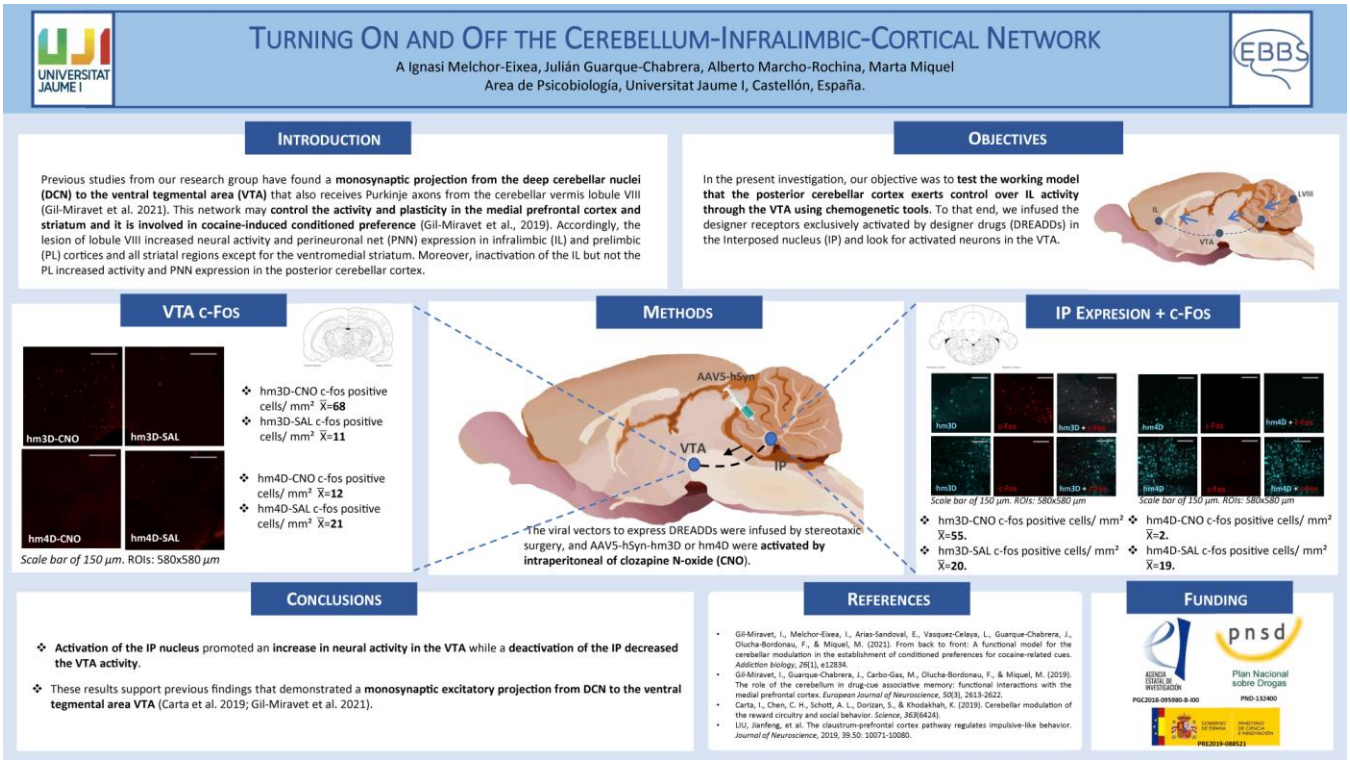
KEYWORDS

cerebellum, drug addiction, drug memory, cocaine, DREADDs

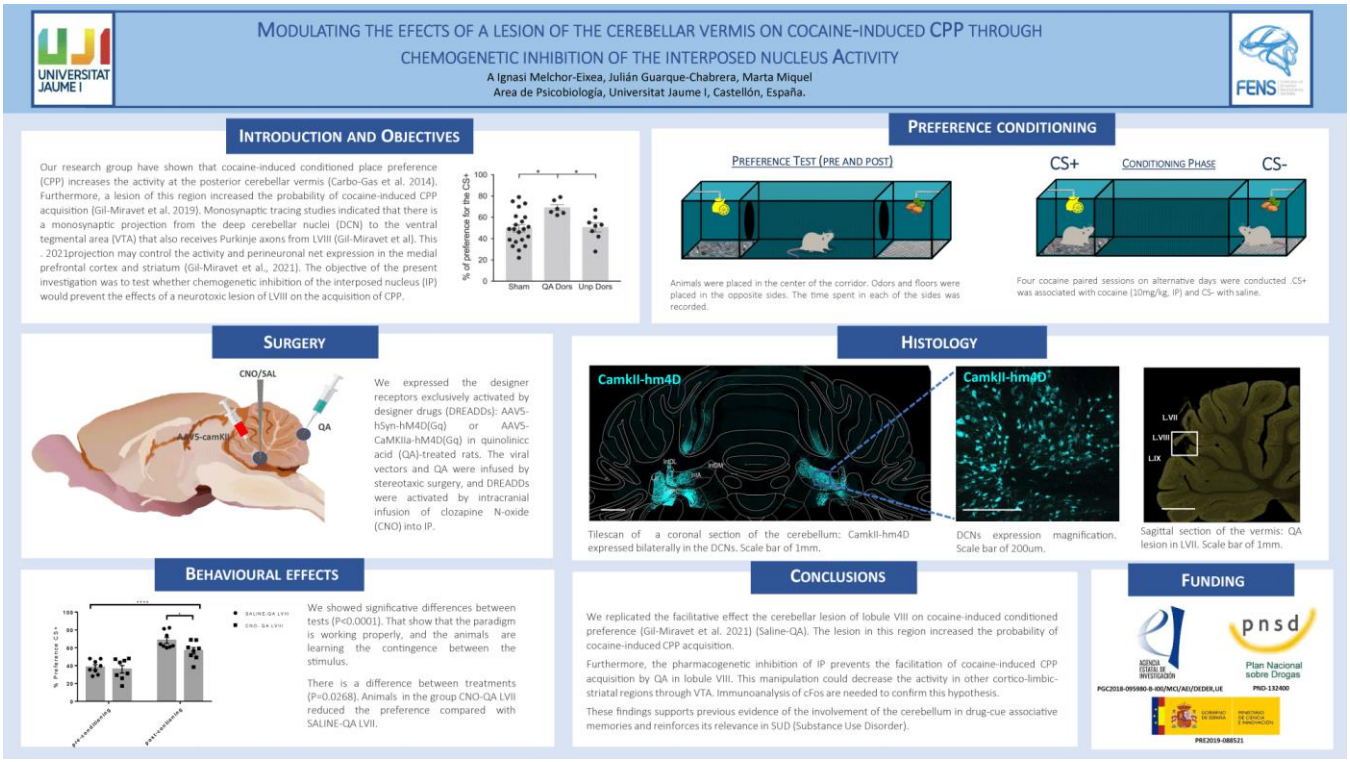
Introduction

Substance Use Disorder (SUD) is a devastating mental disorder associated with brain circuit alterations that lead to emotional, motivational, and cognitive dysfunction. SUD involves long-lasting molecular and structural plasticity in brain regions anatomically and functionally related to the cerebellum, including the prefrontal cortex, amygdala, hippocampus, basal ganglia, including the nucleus accumbens (NAc), and ventral tegmental area (VTA) (Sacchetti et al, 2002; Bostan et al, 2010; Chen et al, 2014; Carta et al, 2019; Holloway et al, 2019; Rondi-Reig et al, 2022; D'Ambra et al., 2023). Although the canonical view linked the cerebellum to motor control, growing evidence points to a broader modulatory function of this region (McAfee et al, 2022). It is now evident that the cerebellum modulates brain functions impaired in SUD and other neuropsychiatric

Annex 2



Annex 3



Annex 4

Inactivation of Deep Cerebellar Nuclei Reverts the Effect of a Neurotoxic Lesion in the Apical Vermis on the Acquisition of Cocaine-Induced CPP

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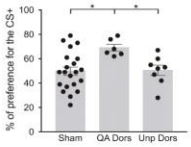
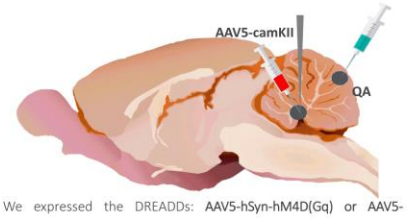
Introduction and Objectives

Our research group have shown that cocaine-induced conditioned place preference (CPP) increases the activity at the posterior cerebellar vermis (Carbo-Gas et al. 2014). Furthermore, a lesion of this region increased the probability of cocaine-induced CPP acquisition (Gil-Miravet et al. 2019). Monosynaptic tracing studies indicated that there is a monosynaptic projection from the deep cerebellar nuclei (DCN) to the ventral tegmental area (VTA) that also receives Purkinje axons from LVIII (Gil-Miravet et al 2021). This projection may control the activity and perineuronal net expression in the medial prefrontal cortex and striatum (Gil-Miravet et al., 2021). The objective of the present investigation was to test whether chemogenetic inhibition of the interposed nucleus (IP) would prevent the effects of a neurotoxic lesion of LVIII on the acquisition of CPP.



Surgery



We expressed the DREADDs: AAV5-hSyn-hM4D(Gq) or AAV5-CaMKIIa-hM4D(Gq) in quinolinicc acid (QA)-treated rats. The viral vectors and QA were infused by stereotaxic surgery, and DREADDs were activated by intracranial infusion of clozapine N-oxide (CNO) into IP.

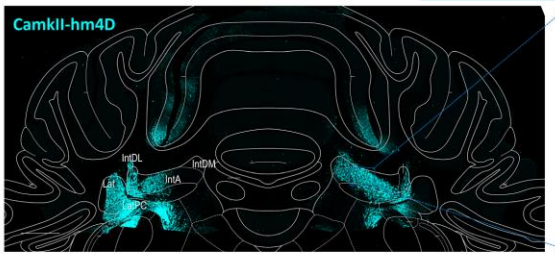
Behavioural Procedure

Preference Test (pre and post)

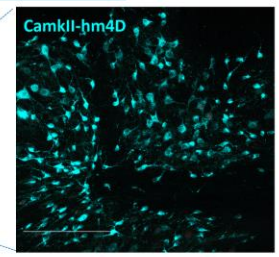
Conditioning Phase

- Animals were placed in the center of the corridor. Odors and floors were placed in the opposite sides. The time spent in each of the sides was recorded.
- Four cocaine paired sessions on alternative days were conducted .CS+ was associated with cocaine (10mg/kg, IP) and CS- with saline.

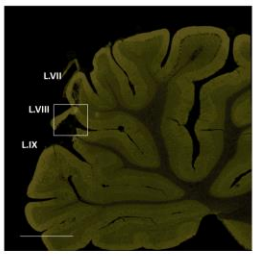
Histology



Tilescan of a coronal section of the cerebellum: CamkII-hm4D expressed bilaterally in the DCNs. Scale bar of 1mm.



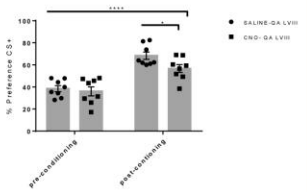
DCNs expression magnification. Scale bar of 200um.



Sagittal section of the vermis: QA lesion in LVII. Scale bar of 1mm.

Behavioural Effects

- We showed significative differences between tests (P<0.0001). That show that the paradigm is working properly, and the animals are learning the contingence between the stimulus.
- There is a difference between treatments (P=0.0268). Animals in the group CNO-QA LVII reduced the preference compared with SALINE-QA LVII.



Conclusions

- We replicated the facilitative effect the cerebellar lesion of lobule VIII on cocaine-induced conditioned preference (Gil-Miravet et al. 2021) (Saline-QA). The lesion in this region increased the probability of cocaine-induced CPP acquisition.
- Furthermore, the pharmacogenetic inhibition of IP prevents the facilitation of cocaine-induced CPP acquisition by QA in lobule VIII. This manipulation could decrease the activity in other cortico-limbic-striatal regions through VTA. Immunoanalysis of cFos are needed to confirm this hypothesis.
- These findings supports previous evidence of the involvement of the cerebellum in drug-cue associative memories and reinforces its relevance in SUD (Substance Use Disorder).

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Annex 5

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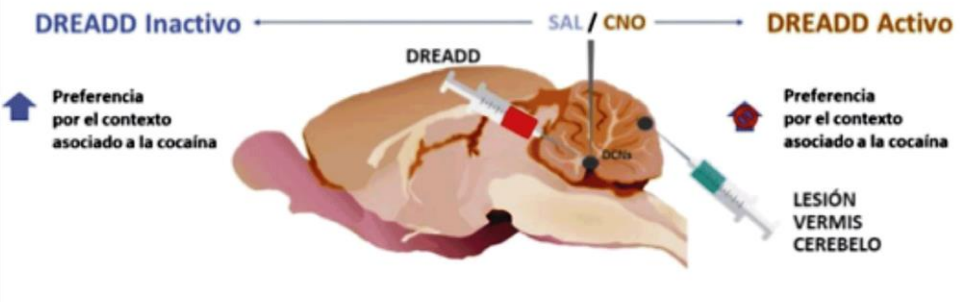
Encendiendo y apagando el cerebelo para cambiar la preferencia por los contextos relacionados con las drogas

IGNASI MELCHOR-EIXEA[1*], MARTA MIQUEL

Desde el año 2018 pertenezco al grupo Adicción y Neuroplasticidad de la Universitat Jaume I, dirigido por la Dra. Marta Miquel. En el año 2020 conseguí un contrato predoctoral de «Personal Investigador en Formación (FPI-MINECO)» a cargo del Ministerio de Ciencia e Innovación.

Nuestra investigación se centra en los mecanismos neurobiológicos de la adicción y, en particular, en los relacionados con los cambios cerebelosos inducidos por las drogas.



Uno de los motivos principales de la recaída en personas adictas es la exposición a contextos que rodean el consumo de drogas. En otras palabras, las personas que consumen drogas son hipersensibles a los estímulos que les recuerdan a estas, como lugares, personas o la propia parafernalia relacionada con el consumo. Los resultados previos de nuestro grupo de investigación han demostrado que áreas concretas del cerebelo regulan la preferencia por los contextos asociados a la cocaína, es decir, regiones del cerebelo se «encienden» cuando se perciben estos contextos. Además, si lesionamos estas regiones aumenta la preferencia por dichos contextos, lo que sugiere que estas zonas del cerebelo pueden tener que ver con el bloqueo de los impulsos dirigidos al consumo. Las neuronas de la corteza del cerebelo regulan la actividad de los núcleos profundos del mismo, que son la salida al resto del cerebro, incluyendo un

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FI/FIN/THE END.

Nacho. 😊