Degradation of perineuronal nets in the cerebellum to prevent relapse of drug seeking

Aitor Sánchez Hernández

Directed by:

Dra. Marta Miquel Salgado-Araujo

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Nombre y apellidos del doctorando:
Aitor Sánchez Hernández
y **FIRMA** original

Nombre y apellidos de la directora de la tesis:
Marta Miquel Salgado-Araujo
y **FIRMA** original

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LICENCIA CREATIVE COMMONS (CC)

“It’s dangerous to go alone! Take this”

Shigeru Miyamoto

The Legend of Zelda, *1986*

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PREFACE

PREFACE

There is a general agreement that drug addiction can be attributed to the ability of certain substances to usurp natural learning and memory mechanisms. Individuals who abuse addictive drugs, such as cocaine, lose control over their actions as they move from recreational to compulsive drug use (Hyman et al., 2006; Penberthy et al., 2010; Vazquez-Sanroman et al., 2015; Garcia et al., 2020). These neuroplastic changes are persistent over time, making the brain inflexible to drugs and facilitating relapse to drug consumption. Beyond cortico-striatal systems, however, very few studies have examined the cerebellum as a component of the addiction circuitry, despite the growing evidence of its involvement (Miquel et al., 2009; Moulton et al., 2014; Miquel et al., 2016; Miquel et al., 2020).

The core of this doctoral thesis is the investigation and manipulation of cerebellar perineuronal nets (PNNs) after cocaine self-administration. Previous studies in our laboratory showed that cocaine-induced Pavlovian memory generates stronger and fully condensed PNNs in the apical region of the granule cell layer. This hallmark signature was only observed when animals expressed a conditioned preference towards an odor associated with the drug (Carbo-Gas et al., 2017). These specializations of the extracellular matrix (ECM) help to maintain (and modify) synaptic architecture and restrict synaptic remodeling stamping in plasticity changes. This is the reason why they have been related to learning and memory and, consequently, to drug addiction (Wright and Harding, 2009; Tsien, 2013; Sorg et al., 2016).

The present doctoral dissertation begins with a theoretical introduction to put into context drug addiction and the role of the cerebellum, followed by a description of PNN functions and their relationship to drug addiction. In the following pages, we present the investigation that managed to explore the expression and role of PNNs in the cerebellar cortex in cocaine addiction. For this purpose, we assessed the effects of different schedules of cocaine self-administration and different withdrawal periods on PNN expression and the level of several of their components. Additionally, we used an enzymatic digestion of PNNs by the bacterial enzyme chondroitinase ABC (ChABC) to investigate the role of cerebellar PNN in drug-seeking and relapse. In the cerebellum, PNNs surround Golgi GABAergic interneurons and Lugaro cells in the cerebellar cortex and both excitatory and inhibitory neurons in the deep cerebellar nuclei (DCN) (Carulli et al., 2007; Crook et al., 2007).

The present doctoral thesis contains four different studies. In the first study, *Cocaine-induced Time-dependent Regulation of PNNs*, which has been already published (Sanchez-Hernandez et al., 2021), we explored PNN expression as revealed by *Wysteria floribunda* agglutinin (WFA) immunoreactivity during three withdrawal periods that followed to two different schedules of cocaine self-administration. In this animal model of escalation (Ahmed and Koob, 1998), rats are allowed to self-administer cocaine for 1 h/day (short-access (ShA)) or 6 h/day (long-access (LgA)) for several days, allowing comparing recreational use in ShA rats with extended addiction-like escalated intake in LgA rats. This paradigm intends to model recreational versus addictive patterns of voluntary cocaine consumption. Our results showed that cocaine self-administration, particularly in an extended access, dynamically regulates conditions for plasticity in the cerebellum during abstinence and increases PNN expression during prolonged abstinence.

In the second study, entitled "*Assessing the impact of different schedules of cocaine self-administration and abstinence on the expression of PNN-related proteins in the cerebellar cortex*", we wanted to explore which PNN components were responsible for the upregulation of PNN expression during protracted abstinence from cocaine self-administration. We included two components of PNNs, brevican (Bcan) and tenascin-R (TN-R), and a synaptic-plasticity regulatory receptor (PTPσ), all of them have been involved in the structure and functions of PNNs. Interestingly, Bcan (50kDa) levels increased in the LgA group after 28 days (28d) of withdrawal. In the case of TN-R and PTPσ levels, we observed a decrease in both the ShA and LgA groups 24 hours (24h) and 28d following the last cocaine self-administration, respectively. These results suggest that extended access to cocaine self-administration enhances plasticity conditions and thereby, encourages drug-induced synaptic remodeling in the cerebellum. However, probability of subsequent synaptic remodeling is progressively restricted during protracted abstinence. These findings support the results obtained in study 1.

Given that our results indicated that extended access to cocaine self-administration increases PNNs expression in the cerebellar cortex during protracted abstinence, in the third study, "*Effects of enzymatic digestion of PNNs in the cerebellar cortex on incubation of drug-seeking*", we wondered whether PNN removal in the cerebellar cortex would affect the incubation of drug-seeking that is developed during prolonged abstinence periods. To this end, we accomplished an enzymatic digestion using the bacterial enzyme ChABC in lobule VII. ChABC has been demonstrated to effectively digest PNNs

in the cerebellar cortex and prevent short-term memory of cocaine-induced conditioned preference (Guarque-Chabrera et al., 2022). We chose this lobule based on a pilot study in which we degraded PNNs in lobules VI, VII and VIII and found the clearest effect on drug-seeking when lobule VII was degraded. Additionally, we explored whether PNN digestion might affect neural activity (C-Fos expression) in the cerebellar cortex. The results showed that removal of PNNs in lobule VII during the incubation period (28d of withdrawal) caused a faster decline in drug-seeking and reduce C-Fos expression in granule cells from the dorsal region of lobule VII in those animals that underwent extended access to cocaine self-administration.

In the last study, "*Effects of enzymatic digestion of PNNs in the cerebellar cortex on extinction of cocaine self-administration*", we wanted to know whether the elimination of cerebellar PNNs facilitated extinction, given that PNN degradation generated a faster decline of drug seeking. For this purpose, after going through cocaine self-administration protocol, self-administration was extinguished under PNN degradation and extinction memory was tested in two reinstatement tests (cue-induced and drug-induced reinstatement). Cerebellar PNNs did not seem to be required for extinction.

Finally, the present doctoral thesis concludes with a general discussion, which further elaborates on the relevance of the results of the four studies and presents a working model for the function of PNNs in lobule VII to encourage future research. We also discuss the strengths and limitations of our findings. References can be found on the last pages. The article that summarizes the results of study 1, published in Psychopharmacology (Sanchez-Hernandez et al., 2021) is shown in appendix I.

RESUMEN

RESUMEN

Existe un consenso general según el cual la drogadicción puede atribuirse a la capacidad de ciertas sustancias para usurpar los mecanismos naturales de aprendizaje y memoria. Los consumidores de drogas adictivas, como la cocaína, pierden el control sobre sus actos a medida que pasan del consumo recreativo al compulsivo (Hyman et al., 2006; Penberthy et al., 2010; Vázquez-Sanromán et al., 2015; García et al., 2020). Estos cambios neuroplásticos son persistentes en el tiempo, haciendo que el cerebro sea inflexible a las drogas y facilitando la recaída en el consumo. Sin embargo, al margen de los sistemas corticoestriatales, muy pocos estudios han examinado el cerebelo como un componente del circuito de la adicción, a pesar de la creciente evidencia de su implicación (Miquel et al., 2009; Moulton et al., 2014; Miquel et al., 2016; Miquel et al., 2020).

El núcleo de esta tesis doctoral es la investigación y manipulación de las redes perineuronales (PNNs) cerebelosas tras la autoadministración de cocaína. Estudios previos en nuestro laboratorio demostraron que la memoria Pavloviana inducida por cocaína genera PNNs más fuertes y completamente condensadas en la región apical de la capa de células granulares. Esta marca distintiva solo se observó cuando los animales expresaron una preferencia condicionada hacia un olor asociado a la droga (Carbo-Gas et al., 2017). Estas especializaciones de la matriz extracelular ("ECM", por sus siglas en inglés) ayudan a mantener (y modificar) la arquitectura sináptica y restringen la impronta de remodelación sináptica en los cambios de plasticidad. Por este motivo, se han relacionado con el aprendizaje y la memoria y, en consecuencia, con la adicción a las drogas (Wright y Harding, 2009; Tsien, 2013; Sorg et al., 2016). La presente tesis doctoral comienza con una introducción teórica para contextualizar la drogadicción y el papel del cerebelo, seguida de una descripción de las funciones de las PNNs y su relación con la drogadicción. En las páginas siguientes, presentamos la investigación realizada para explorar la expresión y el papel de las PNNs en la corteza cerebelosa en la adicción a la cocaína. Para ello, evaluamos los efectos de diferentes patrones de autoadministración de cocaína y diferentes periodos de abstinencia sobre la expresión de las PNNs, así como el nivel de varios de sus componentes. Además, utilizamos una digestión enzimática de las PNNs por la enzima bacteriana condroitinasa ABC (ChABC) para investigar el papel de las PNNs cerebelosas en la búsqueda de drogas y la recaída. En el cerebelo, las PNNs rodean a las interneuronas GABAérgicas

de Golgi y a las células de Lugaro en la corteza cerebelosa, así como a las neuronas excitadoras e inhibidoras en los núcleos profundos del cerebelo ("DCN", por sus siglas en inglés) (Carulli et al., 2007; Crook et al., 2007).

La presente tesis doctoral consta de cuatro estudios diferentes. En el primer estudio, "*Cocaine-induced Time-dependent Regulation of PNNs"*, que ya ha sido publicado (Sanchez-Hernandez et al., 2021), exploramos la expresión de las PNNs mediante la inmunorreactividad de la aglutinina de *Wysteria floribunda* (WFA) durante tres periodos de abstinencia que siguieron a dos patrones diferentes de autoadministración de cocaína. En este modelo animal de escalamiento (Ahmed y Koob, 1998), se permite a las ratas autoadministrarse cocaína durante 1 h/día (acceso restringido [ShA]) o 6 h/día (acceso extendido [LgA]) durante varios días, lo que permite comparar el consumo recreativo en ratas ShA con el consumo extendido de tipo adictivo en ratas LgA. Este paradigma pretende simular un consumo voluntario de cocaína recreativo frente a un consumo adictivo. Nuestros resultados mostraron que la autoadministración de cocaína, particularmente en un acceso extendido, regula dinámicamente las condiciones para la plasticidad en el cerebelo durante la abstinencia y aumenta la expresión de PNNs durante la abstinencia prolongada.

En el segundo estudio, titulado "*Assessing the impact of different schedules of cocaine self-administration and abstinence on the expression of PNN-related proteins in the cerebellar cortex*", queríamos explorar qué componentes de las PNNs eran responsables de la regulación al alza de la expresión de las PNNs durante la abstinencia prolongada de la autoadministración de cocaína. Incluimos dos componentes de las PNNs, brevican (Bcan) y tenascina-R (TN-R), y un receptor regulador de la plasticidad sináptica (PTPσ), todos ellos implicados en la estructura y funciones de las PNNs. Resulta interesante observar que los niveles de Bcan (50kDa) aumentaron en el grupo LgA tras 28 días (28d) de abstinencia. En el caso de los niveles de TN-R y PTPσ, observamos una disminución tanto en el grupo ShA como en el LgA 24 horas (24h) y 28d después de la última autoadministración de cocaína, respectivamente. Estos resultados sugieren que el acceso prolongado a la autoadministración de cocaína mejora las condiciones de plasticidad y, por tanto, favorece la remodelación sináptica inducida por la droga en el cerebelo. Sin embargo, la probabilidad de remodelación sináptica posterior se reduce progresivamente durante la abstinencia prolongada. Estos hallazgos apoyan los resultados obtenidos en el estudio 1.

Dado que nuestros resultados indicaban que el acceso prolongado a la autoadministración de cocaína aumenta la expresión de PNNs en la corteza cerebelosa durante la abstinencia prolongada, en el tercer estudio, "*Effects of enzymatic digestion of PNNs in the cerebellar cortex on incubation of drug-seeking*", nos preguntamos si la eliminación de PNNs en la corteza cerebelosa afectaría a la incubación de la búsqueda de drogas que se desarrolla durante los periodos de abstinencia prolongada. Para ello, realizamos una digestión enzimática utilizando la enzima bacteriana ChABC en el lóbulo VII. Se ha demostrado que ChABC digiere eficazmente las PNNs en la corteza cerebelosa y previene la memoria a corto plazo de la preferencia condicionada inducida por la cocaína (Guarque-Chabrera et al., 2022). Elegimos este lóbulo basándonos en un estudio piloto en el que degradamos PNNs en los lóbulos VI, VII y VIII y encontramos el efecto más claro sobre la búsqueda de drogas cuando se degradó el lóbulo VII. Además, exploramos si la digestión de las PNNs podría afectar a la actividad neuronal (expresión de C-Fos) en la corteza cerebelosa. Los resultados mostraron que la eliminación de PNNs en el lóbulo VII durante el periodo de incubación (28d de abstinencia) causó un descenso más rápido en la búsqueda de drogas y redujo la expresión de C-Fos en las células granulares de la región dorsal del lóbulo VII en aquellos animales que se sometieron a un acceso extendido a la autoadministración de cocaína.

En el último estudio, "*Effects of enzymatic digestion of PNNs in the cerebellar cortex on extinction of cocaine self-administration*", queríamos saber si la eliminación de las PNNs cerebelosas facilitaba la extinción, dado que la degradación de las PNNs generaba una disminución más rápida de la búsqueda de drogas. Para ello, tras pasar por el protocolo de autoadministración de cocaína, se extinguió la autoadministración bajo degradación de PNNs y se evaluó la memoria de extinción en dos pruebas de reinstauración (reinstauración inducida por señales y reinstauración inducida por drogas). Las PNNs cerebelosas no parecían ser necesarias para la extinción.

Por último, la presente tesis doctoral concluye con una discusión general, que profundiza en la relevancia de los resultados de los cuatro estudios y presenta un modelo de trabajo de la función de las PNNs en el lóbulo VII para fomentar futuras investigaciones. También discutimos los puntos fuertes y las limitaciones de nuestros hallazgos. Las referencias se encuentran en las últimas páginas. El artículo que resume los resultados del estudio 1, publicado en *Psychopharmacology* (Sanchez-Hernandez et al., 2021) se muestra en el apéndice I.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Current state of art on cocaine addiction in Europe and Spain

The most recent report of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) highlights the increasing role that psychostimulants, such as cocaine, now play in the European drug problem. In the last few years, cocaine has been the most widely used illegal drug in Spain (45.4% of total) and the second, only after cannabis, in Europe (Observatorio Español de las Drogas y Adicciones, 2022). More than 2.2% (2,2 million) of young European adults (15-34 years old) have consumed cocaine in the last year and around 15% of all first-time treatment demand for drug problems in 2020 was related to cocaine (European Drug Report, 2022). Indeed, within the first year of use, around 5% of cocaine users develop dependence (Wagner and Anthony, 2002).

It is important to note that among users entering treatment, approximately 50% had been previously entered in treatment, revealing the high rate of relapse into cocaine consumption (European Drug Report, 2022). In 2020 the total number of drug users who enrolled into treatment for drug consumption in Europe decreased 14% compared to 2019. This drop in drug use is most likely due to difficulties in case reporting resulting from the COVID-19 pandemic, as the latest indicators for 2021 show alarming increases from the previous year and a 'return to normal' in substance consumption. All together indicators suggest that cocaine availability and consumption remain very high compared to historical levels (European Drug Report, 2022; Observatorio Español de las Drogas y Adicciones, 2022)

Addiction and long-lasting memories

Drug addiction is one of the most prevalent disorders of the Central Nervous System (CNS). Its main symptoms include increased drug use, loss of control over limiting drug intake, chronic compulsive drug seeking and relapse episodes that persist even after long periods of abstinence (Koob and Volkow, 2016). Early theories of addiction, such as the traditional dependence or hedonic view (Kornetsky et al., 1979), postulate that drug pleasure and subsequent unpleasant withdrawal symptoms are the main causes of addiction. In other words, addictive drugs are taken because they are pleasurable, but with repeated use neuroadaptations lead to tolerance and dependence. Thus, drugs are not taken to achieve pleasant drug “highs” but to avoid withdrawal “lows” (Solomon and Corbit, 1974; Koob et al., 1997). Decades of research into drug addiction point to a

dysfunction of frontal cortical systems that fail to regulate optimal inhibitory control and decision-making, leading to impaired judgment and impulsivity (Jentsch and Taylor, 1999). Nevertheless, these studies fail to correctly explain how the risk of relapse can persist for years, even from a drug-free state (Hyman et al., 2006). The Incentive Sensitization theory focuses on how drug cues trigger excessive incentive motivation for drugs, leading to drug-seeking, compulsive drug-taking and relapse (Robinson and Berridge, 1993). It is consistent with the view that associative learning mechanisms bind specific drug-related cues to drug wanting and seeking (Hyman et al., 2006). The current understanding is that drugs of abuse compete with natural stimuli interfering with their molecular neuronal targets. They cause long-lasting changes in brain circuits involved in associative learning and memory for the acquisition and expression of reinforcement learning (Hyman, 2005; Kalivas et al., 2005; Robbins et al., 2008). Drug-associated cues may acquire conditioned motivational properties, leading to drug seeking and enhancing behaviors in pursuit to obtain the drug. (Saunders and Robinson, 2013). Because of such motivational properties, repeated encounters with drug-associated cues reactivate salient and persistent drug-induced memories that can lead to drug addiction and relapse (Saunders et al., 2014).

In this regard, there is growing evidence of the ability of addictive drugs to affect the plasticity of synaptic connections in brain circuits responsible for memories and behaviors that arise through Pavlovian and instrumental conditioning (Everitt and Robbins, 2005). Drugs are well known for their acute rewarding properties due to the activation of prefrontal-limbic-striatal networks, which are modulated by the mesolimbic dopamine system. In fact, the formation of drug-induced memories requires the interplay of molecular interactions between glutamatergic and dopaminergic systems in these circuits (Everitt and Robbins, 2005). Thus, addiction brain circuitry traditionally includes regions of the medial prefrontal cortex (mPFC), striatum, amygdala, and the ventral tegmental area (VTA). Briefly, the mPFC is responsible for executive control, contingencies and value representation (Weissenborn et al., 1997; Dalley et al., 2004; O'Doherty et al., 2001) (**Fig. 1**). The dorsomedial striatum (DMS) plays a critical role in the transition from recreational to regular, compulsive drug use (Everitt et al, 2008). The nucleus accumbens (NAc) exhibits neuroadaptations following drug experience, particularly changes in glutamatergic signaling (Kalivas and McFarland, 2003) that increase the salience of drug-associated conditioned stimulus and thereby their impact on behavior (Kalivas, 2004). This region is situated strategically to receive important limbic information from the prefrontal cortex and amygdala which is required for sensory-

specific conditioned reinforcement and Pavlovian-instrumental transfer (Cardinal et al., 2002; Pelloux et al., 2013; Stefanik and Kalivas, 2013). Dopaminergic modulation from the VTA crucially mediates reinforcement learning of drugs (Mameli et al., 2009; Wolf and Tseng, 2012; Lammel et al., 2014) and natural rewards (Mitchell and Stewart, 1990; Schultz et al., 1993; Meye and Adan, 2014).

In animal models, the intravenous self-administration paradigm is generally considered the best model to assess psychoactive substance abuse liability. Compared to other animal models aimed at mimicking human drug-taking behavior, this paradigm has numerous advantages, for example: (i) the drug is administered voluntarily by the animal; (ii) it models pharmacokinetics of intravenous drug administration in humans; (iii) it allows experimental control of variables such as dose, frequency and duration of drug access; (iv) extinction and reinstatement procedures can be incorporated to model drug-seeking and relapse (Watterson and Olive, 2017; Belin-Rauscent et al., 2018; Schwendt and Knackstedt, 2021). Another hallmark of the transition to cocaine addiction is an escalation in cocaine consumption. In the most widely used animal model, rats gradually escalate their intake of 0.75 mg/kg/i.v. cocaine infusion over time when permitted to self-administer cocaine in extended access sessions. In contrast to extended access, rats that self-administer the same dose of cocaine under restricted access conditions (i.e., 1h sessions) tend to display lower, stable patterns of drug intake over many sessions (Ahmed and Koob, 1998).

The cerebellum in addiction

The cerebellum has been classically involved in sensorimotor planning, execution, control, automation, and learning. However, in the last three decades, a growing number of studies have expanded its role to include cognitive and emotional processing (Schmahmann and Sherman,1998; Strick et al., 2009; Stoodley et al., 2012). Anatomical studies, human clinical data, and functional imaging have clearly demonstrated the important role of the cerebellum in cognition and emotion (Habas, 2021). There is a phylogenetic coupling between telencephalization and cerebellar development with progressive complexity of motor skills requiring increasing executive control (attention, anticipation, and regulation) and behavioral integration (emotion-related behaviors) until the cerebellum can monitor non-motor tasks (Habas, 2021). The neocerebellar cortex, especially the prominent lobule VII and the dentate nuclei (corresponding to the lateral nuclei in rodents) form a supramodal zone connected to the main associative brain regions (O'Reilly et al., 2010) and, to a lesser extent, to affective and associative

subcortical nuclei such as the amygdala and striatum (Habas, 2021). Also, cognitive and emotional processing regions in the cerebellum have been described primarily in the lobule VII (Stoodley et al., 2012; Stoodley and Schmahmann, 2018). Studies on functional topography in the cerebellum have suggested that the vermis, which has bidirectional projections to motor cortices and the spinal cord, is mainly involved in balance and head and eye movements (Cerminara and Apps, 2011). On the other hand, the limbic cerebellum appears to have an anatomical signature in the fastigial nucleus and the cerebellar vermis, particularly the posterior vermis (Stoodley and Schmahmann, 2010). In animal models, the principal region involved in emotional functions has been identified in lobules III to VIII of the vermis (Strata, 2015). There is mounting evidence indicating that the cerebellum, in particular the cerebellar vermis and its connections to several cerebral regions, including the limbic system, are involved in emotional functions, including emotional perception, emotional recognition, emotional processing, and fear conditioning (Imaizumi et al., 1997; Schmahmann et al., 2009; Stoodley and Schmahmann, 2009; Stoodley and Schmahmann, 2010; Sang et al., 2012).

Given the amount of evidence that has been accumulating over the last few years, it is difficult to ignore the strong connection of the cerebellum to areas of the addiction circuitry (**Fig. 1**). The subthalamic nucleus of the basal ganglia is the origin of a large number of projections to the cerebellar cortex forming an integrated network (Bostan and Strick, 2018). Furthermore, activation of the DCN-VTA monosynaptic pathway could control the activity of the cortico-striatal circuit through dopaminergic and nondopaminergic mechanisms (Carta et al., 2019; Gil-Miravet et al., 2021). Carta et al., (2019) showed that optogenetic activation in the VTA of DCN terminals causes a strong rewarding effect, promoting social behavior. Moreover, recent findings from our group demonstrated a role of the dorsal region of the posterior vermis similar to the infralimbic prefrontal cortex (IL) in the establishment of rewarding drug memories that projects to the VTA and the mPFC (Gil-Miravet et al., 2021).

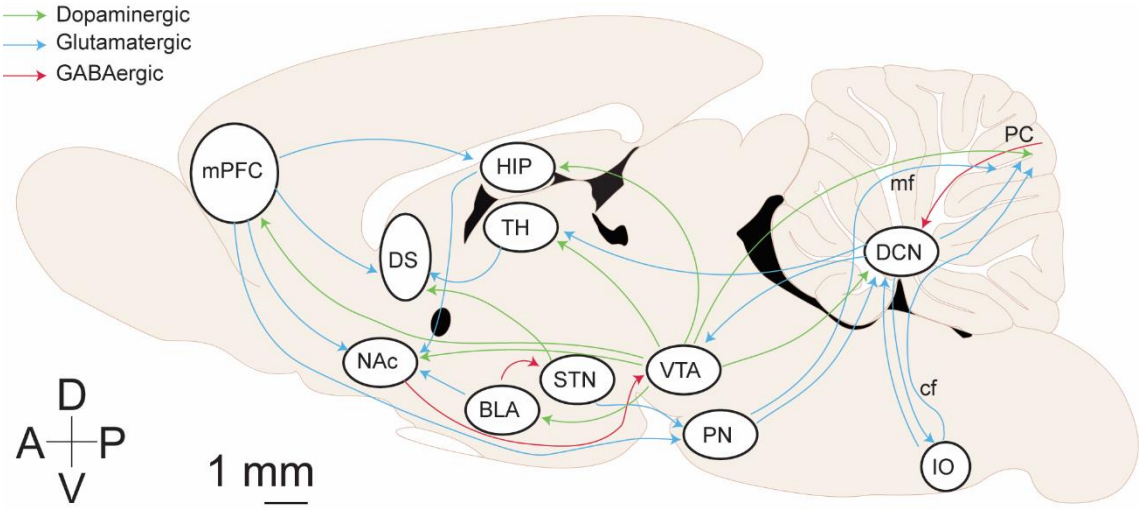

Figure 1. *Modified from Miquel et al., 2020.The addiction circuitry. mPFC, medial prefrontal cortex; DS, dorsal striatum; NAc, nucleus accumbens; HIP, hippocampus; BLA, basolateral amygdala; TH, thalamus; STN, subthalamic nucleus; VTA, ventral tegmental area; PN, pontine nucleus; IO, inferior olive; DCN, deep cerebellar nucleus; PC, purkinje cells; mf, mossy fibers; nmf, nuclear mossy fibers; cf, climbing fibers. Direct and indirect reciprocal connectivity between the cerebellum and other brain regions as the VTA, amygdala, basal ganglia, and mPFC.*

One of the main functions of the cerebellum is to implement internal models for prediction and behavioral adaptation. The prefrontal cortex generates and updates cognitive representations of the world that are integrated in the sensorimotor cortices; in turn, the cerebellum works as an implicit and unconscious template of this representation (Ito, 2008). In case of a conflict between “what I want to do” (prefrontal cortex) and “what is being done” (sensorimotor responses), the cerebellum generates an error signal that is essential to update the internal model and make behavioral adjustments (Fautrelle et al., 2011). In drug addiction, stimuli associated with drug effects can trigger craving and compulsive drug seeking (Pickens et al., 2011; Bossert et al., 2013). Our laboratory proposed that drug-induced synaptic changes in the cerebellum are central for the development of a compulsive behavioral phenotype (Miquel et al., 2009). It appears that drug-associated contexts are more likely to be selected in a drug-free state, which may reactivate the prediction of drug availability. The perception of drug-related cues under abstinence could generate an error signal and the mismatch between the prediction and the sensorial experience may lead to drug seeking (Miquel et al., 2019). Accordingly, neuroimaging studies of cue reactivity in drug addicts under abstinence have consistently shown cerebellar activations when drug-related cues are presented (Grant et al., 1996; Anderson et al., 2006; Filbey et al., 2009; Moulton et al., 2014). Preclinical studies from our laboratory demonstrated that cocaine-induced conditioned preference increases neuronal activity and PNN expression in the cerebellar cortex (Carbo-Gas et al., 2014ab;

Carbo-Gas et al., 2017). Both cerebellar signatures of conditioning correlate with the preference response towards the cocaine-related context when conditioned memory is tested 48 hours after the last cocaine pairing session.

We recently showed that cerebellar cortex impairment encourages behavioral selection directed towards the context that predicts drug availability and increases neural activity in the mPFC and striatum (Gil-Miravet et al., 2019, Gil-Miravet et al., 2021). Similar effects were observed when the IL portion of the mPFC was inactivated before the acquisition of conditioned preference for cocaine (Gil-Miravet et al., 2019; Guarque-Chabrera and Gil-Miravet et al., 2022). Interestingly, a simultaneous impairment of the IL and cerebellum prevents this facilitating effect on cocaine-induced conditioning (Gil-Miravet et al., 2019). Therefore, the cerebellum and IL might work together in order to acquire and store drug-cue Pavlovian associations.

It is remarkable that cerebellar dysfunction appears to be a common feature in all compulsive disorders (Miquel et al., 2019). The evidence is still incomplete and partial, but these patients exhibit structural alterations in the cerebellum and impaired prefrontal-cerebellar connectivity while basal ganglia-cerebellum connectivity has been encouraged (Miquel et al., 2019). Moreover, neuroimaging studies demonstrated that patients with a history of cocaine self-administration, as compared to controls, recruit the cerebellum but not the prefrontal cortex when cognitive task demands increase (Hester and Garavan, 2004; Bolla et al., 2005; Goldstein et al., 2007).

In light of all this evidence, it is crucial to understand how the cerebellar storage of long-term memory contributes to the transit to a compulsive phenotype and subsequent relapse. It is known that several cocaine-dependent modifications in neural plasticity appear to be incubated during withdrawal periods after repeated experiences with the drug not only in areas historically related with drug-addiction such as the striatum (Li et al., 2003), but also in the cerebellum (Vazquez-Sanroman et al., 2015b)

Perineuronal nets, brain plasticity and drug addiction

Structural remodeling in brain circuits appears to be modulated ECM regulatory molecules that restrict neuronal plasticity in order to stabilize circuits (Foscarin et al., 2011). Addictive drugs hijack natural learning and memory processes through neuroplasticity mechanisms. One of these mechanisms are the PNNs. PNNs are lattice cartilage-like structures consisting of ECM molecules that wrap several neuronal populations, being the majority of them fast-spiking, parvalbumin positive (PV+)

GABAergic interneurons (Hartig et al., 1992; Grimpe and Silver 2002; Soleman et al., 2013; Sorg et al., 2016; Miyata and Kitagawa., 2017; Fawcett et al., 2019). Nevertheless, PNNs have also been found surrounding glutamatergic neurons (Horii-Hayashi et al., 2015), glycinergic output neurons (Blosa et al., 2015), excitatory neurons in DCN (Hirono et al., 2018) and Golgi parvalbumin negative (PV-) GABAergic interneurons in the cerebellar cortex (Seeger et al., 1994; Carulli et al., 2006). PNNs are composed of ECM components such as chondroitin sulfate proteoglycans (CSPGs), TN-R, hyaluronic acid (HA) and mediate protein linkage (Carulli et al., 2006). PNNs have been hypothesized to have a neuroprotective function, defending fast-spiking neurons from oxidative stress (Morawski et al., 2004) by removing the excess of excitatory neurotransmitters, such glutamate, during neuronal firing (Morris and Henderson, 2000). More important, PNNs provide synaptic stabilization through three possible mechanisms: 1) by altering the formation of new synaptic contacts, acting as a physical barrier to synapses (Corvetti and Rossi, 2005; Barritt et al., 2006); 2) by being the binding site for inhibitory molecules to new synapse formation such as oversulphated chondroitin sulphate E (CS-E) (Deepa et al., 2002); or 3) by limiting the mobility of glutamate AMPA neurotransmitter receptors (AMPAR) at synapses (Frischknecht et al., 2009). Several studies have shown how PNN enzymatic digestion or the specific removal of some components of PNNs affects short-term synaptic plasticity and the function of excitatory connections in PV+ interneurons (Frischknecht et al., 2009; Favuzzi et al., 2017). As it is explained in more detail later in this introduction, PNNs are composed of proteoglycans among other molecules. In this thesis, we assess Bcan, which interacts with AMPAR and potassium channels.

AMPAR are involved in synaptic transmission by facilitating Na2+ and Ca2+ currents and the rapid turnover of desensitized AMPA subunits to functional ones near the synaptic contact zone (Heine et al., 2008). The mobility of these transmembrane proteins is restricted in the presence of proteoglycans. Therefore, PNNs are believed to create a microenvironment to support the high activity of PNN-bearing neurons and participate in maintaining the synaptic architecture critical to neural plasticity (Wright and Harding 2009; Fawcett et al., 2019; Brown and Sorg, 2022). Considering the ability of PNNs to limit the formation of new synapses and to regulate feedback inhibition in interneurons, PNNs have been proposed as one of the mechanisms for memory, specifically long-term memory, including drug-induced memory (Dityatev and Schachner 2003; Gogolla et al., 2009; Sorg et al., 2016; Shi et al., 2019). Consequently, PNN digestion with ChABC, an enzyme that targets CSPGs, increases plasticity conditions (Pizzorusso et al., 2002; Corvetti and Rossi, 2005; Lensjø et al., 2017). Both inhibitory and excitatory balance

(Lensjø et al., 2017) and AMPAR mobility (Frischknecht et al., 2009; Favuzzi et al., 2017) are all affected after PNN removal.

PNNs in the cerebellum enwrap specific neuronal populations, large excitatory deep cerebellar nucleus neurons (LEDCN) and Golgi interneurons in the cerebellar cortex (Carulli et al., 2006). PNNs around LEDCN neurons, which mainly receive projections from Purkinje cells, stabilize Purkinje cell-LEDCN neuron synapses, in turn restricting synaptic plasticity (Carulli et al., 2006). In the cerebellar cortex (**Fig. 2**), Golgi interneurons inhibit and synchronize activity in granule cells (Tabuchi et al., 2019). At the same time, its activity is controlled by glutamatergic inputs from granule cells and mossy fibers (Palay and Chan-Palay, 1974), as well as by GABAergic and glycinergic inhibitory interneurons (Sotelo and Llinás, 1972; Dumoulin et al., 2001). In other words, Golgi cells regulate the transmission of neural information from the granule cells and mossy fibers to Purkinje dendrites (Galiano et al., 2010; D'Angelo et al., 2013; Prestori et al., 2019), but at the same time, they receive feedback from parallel fibers, the axons of granule cells. Previous findings from our lab suggest different functions for Golgi and DCN PNNs regarding drug-related plasticity. PNNs around DCN projection neurons would serve as "temporary stickers" to dynamically control the cerebellar output by promoting or restricting plasticity in Purkinje-DCN synapses (Sorg et al., 2016). However, PNNs around Golgi neurons would act as "brain tattoos" (Hustvedt, 2014), stabilizing long-term drug memory encoded in synaptic connections (Sorg et al., 2016).

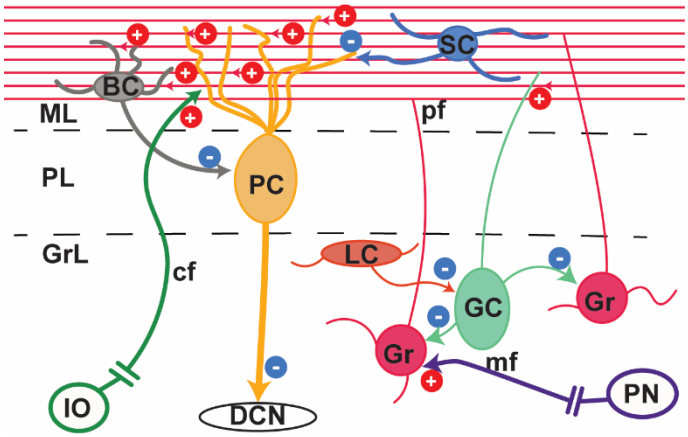

Figure 2. *Schematic representation of the cerebellar circuitry. Mossy fibers (mf) contacts granule (Gr) and Golgi (GC) cells in the granule cell layer (GrL). Gr send their ascending axon to the molecular layer (ML) that bifurcates forming the parallel fibers (pf). In the ML, pf make contact with the dendrites of GC, Purkinje cells (PC), and molecular layer interneurons, Basket (BC) and Stellate (SC) cells. Climbing fibers (cf) from the Inferior Olive (IO) contacts PC dendrites. PC in the Purkinje layer (PL) sends inhibitory projections to the DCN that only sends output signal when PC are inhibited by BC and SC. The cerebellar circuit is organized as a feedforward excitatory network with inhibitory loops.*

Several PNN components can interact with membrane receptors and ion channels and change electrophysiological properties and plasticity events of PNN-bearing neurons (Fawcett et al., 2019; Duncan et al., 2021). The present work focuses on TN-R and Bcan, both with important roles in brain plasticity and neurite outgrowth (Carulli et al., 2007; Dauth et al., 2016). TN-R is a multimeric protein that binds to CSPGs, having an important function as a scaffolding molecule and playing an important role in PNN formation (Weber et al., 1999; Brückner et al., 2000; Lundell et al., 2004; Chiquet-Ehrismann and Tucker, 2011). TN-R has two subunits of 160-180 kDa that can form oligomers of 2 or 3 polypeptide chains. Although it is known that 160 kDa isoform organizes in dimers and the larger isoform in trimers, the functional significance of the two isoforms is not well understood (Pesheva and Probstmeier, 2000). TN-R deficient mice showed enhanced object recognition and faster reversal learning in several behavioral tests, suggesting that TN-R may restrict flexibility in learning a new behavior (Morellini et al., 2010). The other PNN component that is analyzed in this thesis is Bcan, which is localized at the outer surface of neurons, being part of PNNs, where it is particularly enriched at perisynaptic sites being attached with TN-R (Hagihara et al., 1999). Several studies have shown that Bcan mRNA expression is altered after different sensory experiences (Favuzzi et al., 2017). Studies show that TN-R together with Bcan may be up-regulated following self-administration of addictive drugs such as heroin (Van den Oever et al., 2010) or alcohol (Zuo et al., 2012). Bcan has a restrictive role as a

regulator of synaptic plasticity and post-lesion plasticity (Frischknecht and Seidenbecher, 2012). As compared to wild-type Bcan mice (140 kDa), heterozygous Bcan knockout mice exhibited enhanced cocaine conditioned place preference (CPP) after conditioning at 21 days but not at day 1 after conditioning (Lubbers et al., 2016).

The maintenance and remodeling of the ECM components are enzymatic-dependent. The matrix metalloproteinases (MMPs) and disintegrin/metalloproteinase with thrombospondin motifs (ADAMTS) are a family of proteolytic enzymes that cleave ECM proteins, including those in PNNs (Gottschall and Howell, 2015; Lasek et al., 2018), sometimes in a specific way such as the protease ADAMTS4 which cleaves Bcan, located mainly in synapses, generating fragments of approximately 50 kDa (Nakamura et al., 2000; Valenzuela et al., 2014; Gottschall and Howell, 2015). These enzymes usually require serine proteinases like the tissue plasminogen activator (tPA) or other MMPs for activation (Wright and Harding, 2009). For this reason, one can expect increasing levels of tPA when there is a down-regulation of PNNs and thereby a higher MMP activity (Vaquez-Sanroman et al., 2015b) and structural PNN maintenance when tPA levels are not affected (Vaquez-Sanroman et al., 2015a). As a mechanism of plasticity, the expression of these proteins has been related to neural activity, learning and memory (Yuan et al., 2002; Gogolla et al., 2007; Dubey et al., 2017) and, also, to drug-related cues sensitivity, reinstatement and relapse (Brown et al., 2007; Van den Oever et al., 2010).

Many cell-signaling events are regulated through reversible tyrosine phosphorylation of proteins. This phosphorylation is controlled in part by a receptors family of protein tyrosine phosphatases (PTPs) (Lee et al., 2007). PTPσ is a large, highly conserved cell adhesion molecule-like that binds CSPGs (Lee et al., 2007), with several isoforms. In rats, the PCPTP1 ≈70 kDa isoform is the most common, while its shorter isoform (≈60kDa) is PCPTP1-ce (Hendricks et al., 2009). PTPσ has been demonstrated to be essential for the inhibitory effects of CSPGs, as Bcan, on synaptic plasticity (Shen et al., 2009; Duan and Giger, 2010; Coles et al., 2011). In the presence of PNNs or, in general, under reduced plasticity conditions, it interacts with and modulates the activity of tyrosine receptor kinase (TRK) (Faux et al., 2007; Takahashi et al., 2011), inhibiting by dephosphorylation a subtype of TRKR, TRKB receptors (Kurihara and Yamashita, 2012; Lesnikova et al., 2021). When form homo- or heterodimers, PTPσ receptors cannot exert their function, so TRKB signal is not interrupted (Lee et al., 2007). Several cocaine-dependent modifications in plasticity appear to be incubated during withdrawal periods following a repeated experience with the drug. Indeed, Brain-Derived Neurotrophic

Factor (BDNF), a ligand of TRKB, increases after cocaine exposure (Grimm et al., 2003; Boudreau and Wolf, 2005; Boudreau et al., 2007; Ghasemzadeh et al., 2009), thus mediating the incubation of drug-seeking (Li et al., 2013; Loweth et al., 2014). Previous findings of our laboratory indicate that BDNF mechanisms are enhanced in the cerebellum of cocaine-sensitized animals after periods of prolonged withdrawal (Vazquez-Sanroman et al., 2015b).

Drug-related plasticity and metaplasticity involve PNN regulation in different brain regions (Mash et al., 2007; Van den Oever et al., 2010; Xue et al., 2014; Chen et al., 2015; Slaker et al., 2015, 2016; Vazquez-Sanroman et al., 2017; Blacktop et al., 2017; Slaker et al., 2018; Blacktop and Sorg, 2019; Gil-Miravet et al., 2021), including the cerebellum (Vazquez-Sanroman et al., 2015a, b; Carbo-Gas et al., 2017). Drug-induced remodeling of PNNs depends on several factors such as brain region, length of withdrawal period, time course, and the particular behavioral process that is being investigated. Whereas chronic experience with cocaine and alcohol increases PNN expression and, WFA staining in the prelimbic cortex (PL) (Slaker et al., 2018), cerebellum (Vazquez-Sanroman et al., 2015b; Carbo- Gas et al., 2017), and insula (Chen et al., 2015), nicotine self-administration reduces WFA staining in the VTA and orbitofrontal cortex (Vazquez-Sanroman et al., 2017). Removal of PNNs in these brain regions has revealed the functional role of these specialized ECM structures in the acquisition, reinstatement (Slaker et al. 2015; Blacktop et al., 2017) and extinction (Xue et al., 2014) of drug-related behaviors.

In the cerebellar cortex, cocaine-induced conditioned preference has been associated with more intense PNNs around Golgi interneurons (Carbo-Gas et al., 2017; Traver et al., 2018). However, cocaine-dependent regulation of PNNs surrounding output neurons in the DCN was not linked to drug memory (Carbo-Gas et al., 2017) but to neuroplasticity events that occur during abstinence periods in the internal circuits of the cerebellum. Thus, cocaine-induced reinstatement increases PNN expression in the DCN after short withdrawal (Vazquez-Sanroman et al., 2015a) but decreases its expression after protracted abstinence (Vazquez-Sanroman et al., 2015b). Moreover, a neurotoxic lesion of the cerebellum's posterior vermis has shown to facilitate cocaine-induced preference and to increase neural activity and PNN expression in GABAergic PV+ interneurons of the mPFC (Gil-Miravet et al., 2021). Interestingly, previous studies showed that PNN expression in the PL region of mPFC increased after drug-induced conditioned preference and its removal with ChABC impaired acquisition and reconsolidation of this Pavlovian memory (Slaker et al., 2015). Therefore, through PNN regulation, addictive

drugs may affect synaptic plasticity and the conditions for plasticity, creating restrictive or permissive plasticity and metaplasticity (Slaker et al., 2015; Vazquez-Sanroman et al., 2015a; Neuhofer and Kalivas, 2018; Kaushik et al., 2021). In a recent publication of our group (Guarque-Chabrera et al., 2022), we explored the effect of cerebellar PNNs removal, particularly in lobule VIII, on the formation, short-term memory and extinction of drug-induced conditioned preference. In this study, we found that PNNs around Golgi interneurons are essential for short-term cocaine-induced Pavlovian memory and the stabilization of extinction memory. However, the acquisition of preference for cocaine-related cues was not affected after ChABC infusion.

In the present thesis, we first investigated PNN expression around Golgi cells through the medial and lateral cerebellar cortex after short versus long access to cocaine self-administration. Then, we explored which PNN components were affected by cocaine after self-administration and withdrawal. Finally, we removed PNNs during drug abstinence by enzymatic digestion using the bacterial enzyme ChABC to ascertain the role of PNNs on drug seeking (relapse). Our aim was to explore whether cocaine exposure can persistently remodel PNNs after prolonged abstinence and whether ChABC was able to reverse these effects and affect drug seeking.

AIMS AND HYPOTHESIS

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The present thesis is based on the **following premises**:

- The cerebellum plays a crucial role in consolidation of Pavlovian and instrumental conditioned memories.

- The cerebellum has connections with almost all the relevant areas of the addiction circuit.

- Addictive drugs, specifically cocaine, promote persistent neuroplasticity changes in the cerebellum that are revealed under abstinence.

- The dorsal region of the posterior vermis is involved in the inhibitory regulation of cocaine-induced conditioned memory.

- PNNs that surround certain neuronal types are a synaptic stabilization mechanism that favors the formation of persistent memories. In the cerebellar cortex, they are found surrounding Golgi inhibitory interneurons.

- Addictive drugs can dynamically remodel PNNs. Drug-induced PNN remodeling depends on several factor such as brain region, drug type, length of withdrawal period and the behavioral process.

The general aims of the thesis are to investigate the effects of cocaine self-administration on the expression and composition of cerebellar PNNs and study the effects of PNN manipulation during the development of addictive behavior.

Specific aims:

- To study the effects of different levels of exposure to cocaine self-administration on PNN expression and composition in the cerebellar cortex.

- To study the expression of PNN in the cerebellar cortex after different periods of drug withdrawal.

- To explore the effect of PNN removal at lobule VII of the vermis on the incubation of drug-seeking during protracted abstinence.

- To evaluate the effects of PNN removal at lobule VII of the cerebellum in extinction of cocaine-induced instrumental memory.

The present thesis proposes as **a general hypothesis** that the posterior cerebellum is a key contributor in the maintenance of drug-associated memories that promote relapse. In this way, **our predictions were**:

- Cocaine self-administration will increase PNN expression after acquisition and protracted abstinence.

- Cocaine self-administration will increase PNN components parallelly to the increment of its intensity.

- Chondroitinase digestion of cerebellar PNNs, specifically in lobule VII will prevent relapse of drug-seeking after cocaine self-administration.

- Chondroitinase digestion of cerebellar PNNs in lobule VII will improve extinction learning and prevent drug-induced reinstatement of drug-seeking.

CHAPTER 2

MATERIALS AND METHODS

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Animals and housing conditions

Study 1:

Adult male Sprague-Dawley rats (8–10 weeks) (N = 42) (Janvier Labs, France), experimentally naïve at the beginning of the study were housed in a temperature and humidity-controlled environment and maintained on 12-h light/dark cycle (light on at 7 AM) in the animal facility at Université de Poitiers. All experiments were conducted following the European Union directives (2010/63/EU) for the care of laboratory animals and approved by the local ethics committees (COMETHEA). Naïve-control rats (n = 6) remained undisturbed in their home cages and had the same housing conditions as the self-administration groups (n = 36) for the entire duration of the study. Upon arrival, rats were housed two per cage for about 1 week before intra-jugular vein catheterization surgery. After surgery, rats were housed individually for the entire period of self-administration and 3 per cage during the abstinence period.

Studies 2, 3, 4:

Adult male Sprague-Dawley rats (5–6 weeks) (Study 2, N = 39; Study 3, N = 58; Study 4, N = 16) (Janvier Labs, France), experimentally naïve at the beginning of the study were housed in a temperature and humidity-controlled environment and maintained on 12-h light/dark cycle (light on at 8 AM for normal light cycle or 12 PM for inverted light cycle) in the animal facility at Universitat Jaume I. All experiments were conducted following the European Union directive (2010/63/EU), Spanish directive (BOE 34/11370/2013), and local directive (DOGV 26/2010) for the care of laboratory animals and approved by the local ethic committee. Upon arrival, rats were housed individually before intra-jugular vein catheterization surgery. After surgery, rats were housed also individually for the entire period of self-administration and abstinence period.

Drugs and pharmacological agents

Name (commercial name)	CAT#	Provider	Composition	Experimental study
Cocaine hydrochloride (cocaine-HCl)	—	Cooper (Melun Cedex, France)	6 mg/ml dissolved in sterile saline solution (0.9%)	1
Cocaine hydrochloride (cocaine-HCl)	—	Alcaliber S.A. (Madrid, Spain)	3 mg/ml dissolved in sterile saline solution (0.9%)	2, 3, 4
ChABC (Chondroitinase ABC from Proteus vulgaris BSA free)	C3667	Merck Life Science S.L.U. (Madrid, Spain)	25 U/ml dissolved in PBS solution with BSA (0.01%)	3, 4
Enrofloxacin (Syvaquinol 100ml)	055349	Laboratorios Syva S.A. (Leon, Spain)	25 mg/ml	2, 3, 4
Gentamicin, i.v. antibiotic	—	Panpharma (Luitré-Dompierre, France)	0.4% dissolved in sterile saline solution (0.9%)	1
Heparin ((Heparin sodium salt from porcine intestinal mucosa)	H3393	Sigma-Aldrich (Lyon, France)	0.6 mg/ml dissolved in sterile saline solution (0.9%)	1
Heparin ((Heparin sodium salt from porcine intestinal mucosa)	H3393	Merck Life Science S.L.U. (Madrid, Spain)	Catheter maintenance: 7.35 µg/ml dissolved in sterile saline solution (0.9%). Perfusion: 6 µg/ml dissolved in sterile saline solution (0.9%)	2, 3, 4
Isoflurane (Isoflutek 250 ml)	011781	Laboratorios Karizoo S.A. (Barcelona, Spain)	1000 mg/g	1, 2, 3, 4
Meloxicam (Metacam 10 ml)	9993012	Boehringer Ingelheim Vetmedica GmbH (Rhein, Germany)	1.5 mg/ml	2, 3, 4
Sodium pentobarbital (Dolethal 100ml)	015P5502	Vetoquinol Especialidades Veterinarias, S.A.U. (Madrid, Spain)	30 mg/kg	1, 3, 4

Table 1. *Drugs and pharmacological agents used in this thesis.*

Surgeries

Catheterization surgery (Study 1):

This surgery (as well as the subsequent self-administration study) was carried out in the laboratory of Marcello Solinas (Université de Poitiers, INSERM, U-1084, Laboratoire de Neurosciences Expérimentales et Cliniques, Poitiers, France). Rats were implanted with catheters in the jugular vein under isoflurane (**Table 1**) anaesthesia (induction at 2.00 % and maintenance through the whole surgery at 2.50 %) using a Isotec 5 isoflurane anaesthesia vaporizer (Datex-Ohmeda Inc., Madison, WI) as described previously (Thomsen and Caine 2005). Briefly, the catheters, assembled in the laboratory, consisted of a 12-cm-length Silastic tubing (Fischer, France) fitted to a guide cannula (PlasticOne, USA) bent at a 90° angle. The Silastic tubing was fixed to the guide cannula with thermoretractable tubing (Conrad, France) and encased in dental cement anchored with a 2.5 cm^2 durable mesh used as a subcutaneous anchor. The tubing was inserted subcutaneously from the animals back to the right external jugular vein. After surgery, all animals could recover for a minimum of 5 days before given access to cocaine during which catheters were flushed daily with 0.15 ml of a filtered heparinized saline solution 0.6% and gentamicin 4% (**Table 1**).

Catheterization surgery (Studies 2, 3, 4):

After learning the catheterisation surgery described above during the international research internship, it was implemented in our laboratory in Spain. The procedure was the same as described above except for some modifications. Catheters consisted of a 14-cm-length Silastic tubing (Catheter for rat jugular vein, PU 3Fr 14cm, collar @ 2.5cm. sleeve, square tip. Fits 22ga, Cat #I-C30PU-RJV1931, Biogen Científica, Madrid, Spain) fitted to a guide cannula (Vascular Access Button for rat, magnetic, 1 channel 22ga, Cat #I-VABR1B/22, Biogen Científica, Madrid, Spain). Tubing was inserted subcutaneously from the animal back to the right external jugular vein. After surgery, all animals could recover for a minimum of 7 days before given access to cocaine during which catheters were flushed every 12 h with 0.1 ml of a sterile saline solution (0.9%) and 10 mg/kg of antibiotic Syvaquinol and received oral analgesic treatment with meloxicam (**Table 1**) every 24h.

Stereotaxic surgery and enzymatic digestion (Experimental study 3, 4):

The Rat Brain in Stereotaxic Coordinates atlas (Paxinos and Watson, 1998) was used to calculate the coordinates for enzymatic digestions. In both studies, due to weight

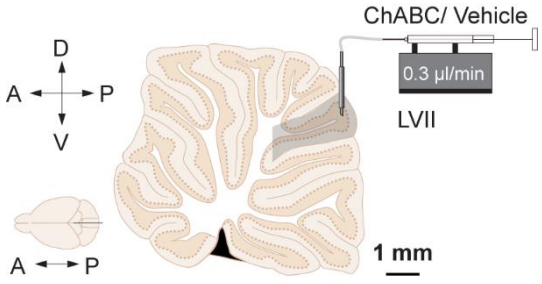

Figure 3. *Schematic diagram of a sagittal section of the vermis depicting the injection site in lobule VII (LVII). Modified from Guarque-Chabrera et al., 2022.*

requirements for catheterization, rats received the surgery weighing ~450 g. Animals were anaesthetised with isoflurane (**Table 1**) (induction at 2.00 % and maintenance through the whole surgery at 2.50 %) using a Isotec 5 isoflurane anaesthesia vaporizer (Datex-Ohmeda Inc., Madison, WI) and placed in a stereotaxic apparatus (Kopf Model 902; David Kopf Instruments, Tujunga, CA, USA). A small craniotomy was performed using a 0.9 mm drill bit (Burrs for Micro Drill; CAT# 19008-09; Fine Science Tools, Heidelberg Germany). Intracranial infusions were performed by placing a stainless-steel guide cannula (23-gauge external diameter) in the posterior vermis (medial cerebellum) (lobule VII; LVII) (AP: −15; ML: 0; DV: −3.5 **(Fig. 3**). Then, a removable stainless-steel injector (30-gauge external diameter) connected to a 2 µl Hamilton syringe (Microliter Syringe Model 7002 N; Cat# 88400; Hamilton Bonaduz AG, Bonaduz, Switzerland) was inserted into the previously placed guide cannula, and 1 µl of bacterial enzyme ChABC for the ChABC groups or vehicle (0.01% BSA dissolved in PBS) for the Sham group were infused (0.3 µl/min) into the targeted area using an infusion pump (Syringe-ONE; Cat# NE-1000; New Era Pump Systems Inc., Farmingdale, NY, USA) (**Table 1**) . After the infusion was completed, the injector remained in place for 3 minutes to allow for proper diffusion and to avoid liquid aspiration. Then, the guide cannula and injector were removed, and the wound was sutured. Rats were left undisturbed for four days after surgery for recovery and received analgesic treatment with meloxicam every 24h for four days (**Table 1**).

Self-administration apparatus and behavioural protocols

Study 1: Regulation of PNNs in the cerebellar cortex during different periods of abstinence from cocaine-self administration

The experimental timeline is schematized in **Fig. 4**. A week after catheter surgery, self-administration training began in operant chambers (Imetronic, Pessac, France) equipped with nose-pokes as operanda and controlled by Imetronic interfaces and software (Imetronic, Pessac, France, www.imetronic.com). Rats were allowed to self-administer cocaine according to a fixed ratio 1 (FR1) schedule of reinforcement. Thus, an active nose-poke response immediately resulted in a single i.v. injection of cocaine for 5 s (0.6

mg/kg), the blinking of house light for 5 s followed by a 5-s time-out period during which the chamber was dark. Further active nose-pokes had no programmed consequences (**Fig. 5**).

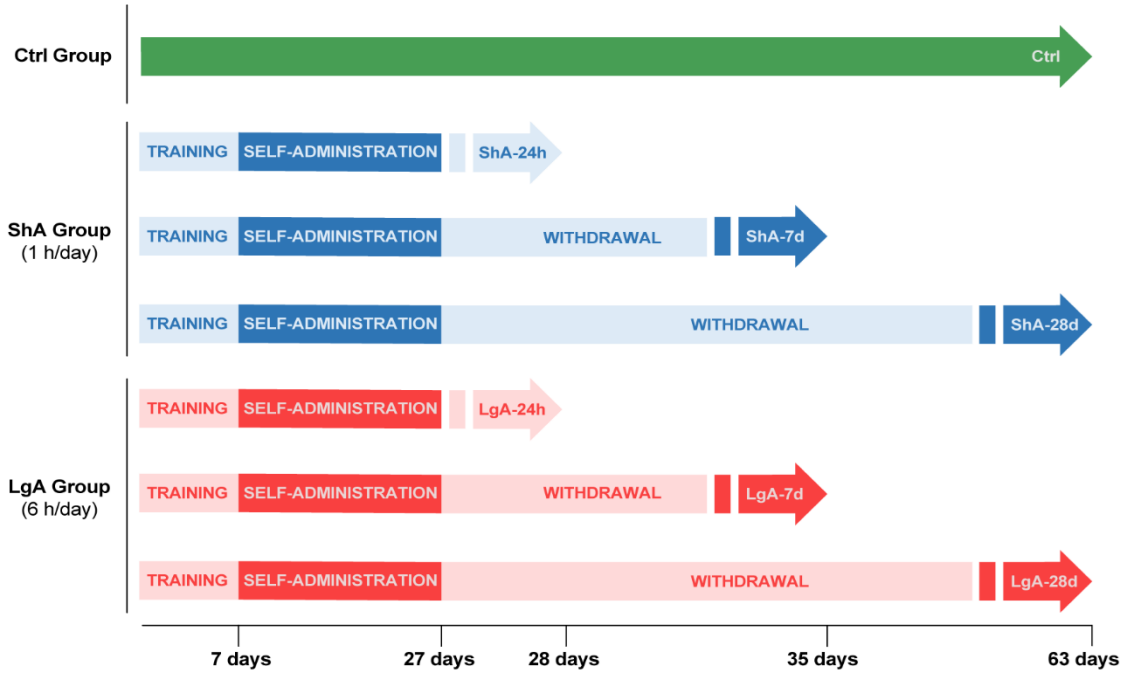

Figure 4. *Study 1 Timeline. After 7 days of cocaine self-administration training (2 h/day), rats were randomly assigned to ShA (1 h/day) and LgA (6 h/day) groups. At the end of 20-days cocaine self-administration period, rats underwent 24 h, 7 days, or 28 days of forced abstinence. Arrowheads mark the points where animals were perfused. Naïve rats (Ctrl) were used as controls and perfused at the end of experiment.*

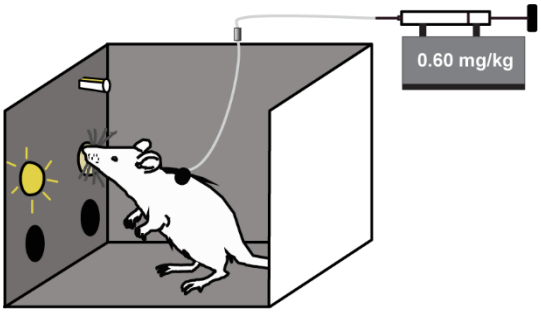

Figure 5. *Schematic representation of the operant chamber used in study 1*

The first 7 sessions lasted 2 h for all rats. Starting from the 8th session, rats were pseudo-randomly divided into two groups after the criterion for the acquisition was reached (at least 15 self-infusions in 2 h): (i) the ShA group, with access to cocaine self-administration for 1 h per day and (ii) the LgA group with access to cocaine self-administration for 6 h/day, for 20 sessions (Ahmed and Koob, 1998).

These two paradigms of drug exposure are designed to mimic recreational versus addictive cocaine intake, respectively (Ahmed and Koob, 1998). Then, ShA and LgA rats were left undisturbed in their home cages for 1, 7, or 28 days before perfusion. A group

of naïve rats (Ctrl group) paired for age and housing conditions did not undergo any behavioural training or exposure to cocaine and served as a control.

Study 2: Assessing the impact of different schedules of cocaine self-administration and abstinence on the expression of PNN-related proteins in the cerebellar cortex

The experimental timeline is schematized in **Fig. 6.** A week after catheter surgery, self-administration training began in Med-Associates operant chambers (Modular Rat Test Chamber, Cat #ENV-007CT, Cibertec, Madrid, Spain) equipped with retractive levers as operandi and controlled by Med-Associates interfaces (Smart Control Panel, #SG-716B, Cibertec, Madrid, Spain) and software (Med-PC IV Software Suite Cibertec, Madrid, Spain). Rats were allowed to self-administer cocaine according to a FR1 schedule of reinforcement. Thus, a response in the active lever immediately resulted in an i.v. injection of cocaine (0.75 mg/kg/injection), activation of the light above the active lever for the duration of the injection, deactivation of the ambient light, and retraction of the levers during 8 seconds of darkness. Presses on the incorrect lever were recorded but have no programmed consequences (**Fig. 7**).

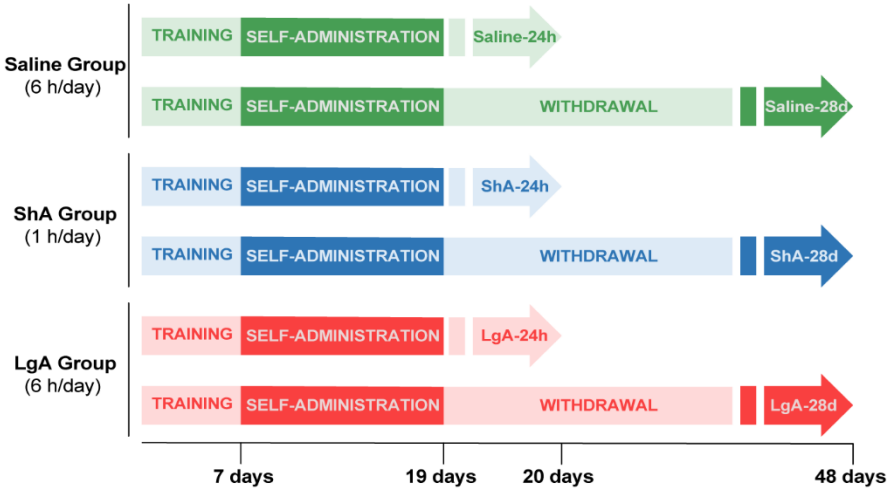

Figure 6. *Study 2 Timeline. After 7 days of cocaine self-administration training (1 h/day), rats were randomly assigned to short access (ShA) (1 h/day) and long access (LgA) (6 h/day) groups. At the end of 12-days cocaine self-administration, rats underwent 24h or 28d of forced abstinence. Arrowheads mark the points where animals were sacrificed. Saline rats (Saline, 6h/day) did not receive cocaine and were sacrificed at the same time points as self-administered groups.*

The protocol began with 7 training sessions of 1h or 20 lever presses for three days, with an automatic programmed cocaine injection if the animal did not pulse within 15 minutes. This was followed by 1 hour (1h) for ShA group and 6h for LgA group of cocaine self-administration, which were conducted one a day for 12 consecutive days. Then, ShA

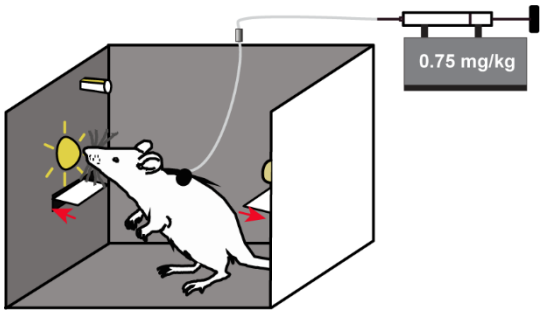

Figure 7. *Schematic representation of the operant chamber used in experimental study 2, 3, 4.*

and LgA rats were left undisturbed in their home cages for 24h, or 28d before euthanasia. A group of saline rats with the same age and housing conditions went through the same procedures and behavioural training but with access to a sterile saline solution (0.9%) for 6 hours (6h) a day and sacrificed at the same time points as the LgA group.

Study 3: Effects of enzymatic digestion of PNNs in the cerebellar cortex on the incubation of drug-seeking

The configuration and programming of operant chambers was the same as in Study 2 (**Fig. 7**). The experimental timeline is schematized in **Fig. 8**. Experimental protocol started with 7 training days followed by 12 sessions of 1 (ShA) or 6h (LgA) a day of cocaine self-administration. The saline group underwent 6h/day of saline self-administration. Twenty-four hours following the cocaine self-administration phase and before protracted abstinence, rats were tested in a drug-seeking test with 3 trials (T1, T2, T3) separated by 10 minutes each, in which rats were returned to their home cages. This procedure was aimed at capturing intrasession changes in operant responding. During each trial, drug-related cues were presented (lever retraction, operant chamber light switch-off, pump noise and switch on of the active lever light) but no cocaine was

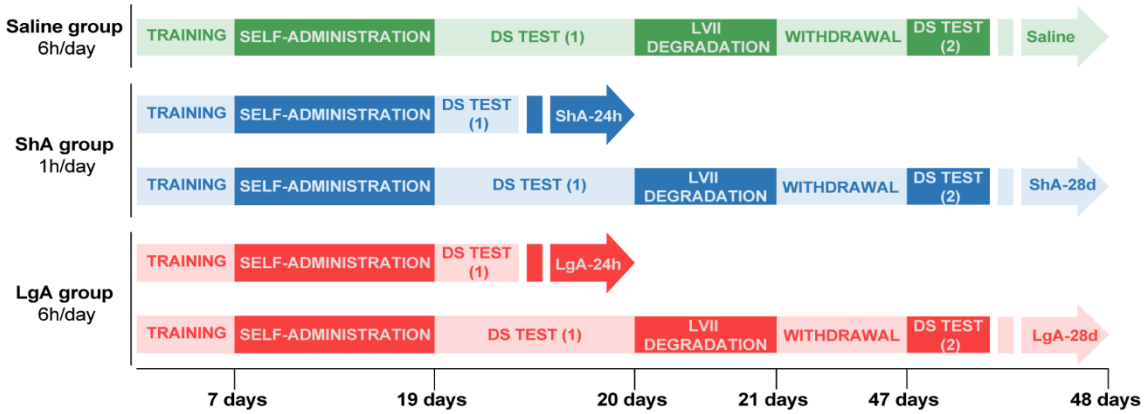

Figure 8. *Study 3 Timeline. After 7 days of cocaine self-administration training (1 h/day), rats were randomly assigned to ShA (1 h/day) or LgA, (6 h/day), saline rats receive the same training but without cocaine. At the end of the 12 days of self-administration, 24h after last self-administration session, rats received the first DS test. Twenty-four hours after the first DS test rats went through stereotaxic surgery and remain 26 days (28 after the last self-administration session) in forced abstinence. At day 28, rats received the second DS test and were perfused.*

injected. The following day, ChABC was infused for enzymatic digestion of PNNs in lobule VII of the vermis through stereotaxic surgery and rats were left undisturbed for 28 days in their home cages for protracted abstinence.

Study 4: Effects of enzymatic digestion of PNNs in the cerebellar cortex on extinction of cocaine self-administration

This study only involved LgA rats. Training, cocaine self-administration and DS tests were identical to previous studies 2 and 3. Stereotaxic surgery for ChABC infusion took place 24h later of the first drug-seeking test (**Fig. 9**), and then animals were allowed to recover for 4 days before they underwent a 10-day extinction phase (1h/day) (**Fig. 9)**. During extinction sessions, the levers were always available, and the operant chambers were programmed with the same cues as during 12 days of self-administration. Active lever press activated the infusion pump, but the catheter was disconnected from it. Active lever presses, inactive lever presses and lever presses made during the time-out period were recorded.

Figure 9. *Study 4 Timeline. After 7 days of cocaine self-administration training (1 h/day), rats start 12 days of cocaine self-administration. Twenty-four hours later, rats received the first drug-seeking test (DS test). Forty-eight hours after the last cocaine self-administration session rats underwent stereotaxic surgery and received 4 days of recovery. After recovery, the extinction phase started and took 10 days (1h/day). At day 17, since the last self- administration session, rats underwent a cue-induced reinstatement test (CR test). At day 18, rats underwent the drug-induced reinstatement test (DR test) and were perfused.*

Twenty-four hours after extinction, rats were tested in a cue-induced reinstatement test (CR test) identical to drug-seeking tests in study 2 and 3. Finally, on the following day, the animals underwent a drug-induced reinstatement test (DR test) and 60 minutes after DR test they were perfused. As in the DS test, the DR test was divided into 3 trials of 1h separated by 10 minutes, in which the rats were returned to their home cages. Immediately before each trial rats received a non-contingent i.v. injection of cocaine (**Fig. 9**) (0.75 mg/kg/injection per test) with no cue connected and levers off (retracted). Levers then came out during the rest of the trial. The presses of the active lever, the inactive lever and the presses made during the time-out period were also recorded. We used the same configuration to assess the effects of cocaine re-exposure on the intrasession decline in operant responding that we observed in DS and CR tests. In addition, we were

interested in investigating whether removal of PNNs in the cerebellar cortex could impact differently on cue- versus drug-induced relapse.

Brain sampling

Perfusion and dissection protocol (Studies 1, 3, 4):

Animals were deeply anaesthetised with sodium pentobarbital (**Table 1**) and perfused transcardially using first saline with heparin (0.006%) (**Table 1**) and then paraformaldehyde (4%) (Paraformaldehyde, powder, 95%; CAT# 158127; M Sigma-Aldrich, Madrid, Spain). Cerebella were extracted and stored with the same fixative for 24h at room temperature. Then, the tissue was immersed in sucrose solution (30%) with sodium azide (2%) until the brain sank at 4 ºC. Cerebellar tissue was covered them with Neg-50™ (Richard Allan Scientific Neg 50™; Cat# 6502; Thermo Fisher Scientific, Barcelona, Spain) and fast frozen by immersion in liquid nitrogen (N_2). For study 1, five series of coronal sections of the cerebellum vermis were performed at 40 µm with a cryostat microtome (CM3050S, Leica, France) and were stored at −20 °C in cryoprotectant solution. For experimental studies 3 and 4, 6 sagittal sections were performed at 40 µm with a cryostat (Microm HM560, Thermo Fisher Scientific, Barcelona, Spain) and were stored at −20 °C in cryoprotectant solution.

Euthanasia and brain sampling for western-blotting (Study 2):

Animals were first anaesthetised and then sacrificed with CO_2 and decapitated. Brains were extracted, rapidly frozen in cold N_2 and conserved at -80 °C until dissection. Fresh tissue samples from the dorsal region of cerebellar vermis were homogenised in ice-cold RIPA buffer (130 Mm NaCl, 20 Mm Tris-HCl at Ph 8.0, and 1% Nonidet P40) containing protease inhibitors (10 µg/ml of aprotinin, 20 µg/ml of leupetine, and 1 Mm PMSF) and phosphatase inhibitors (10Mm NaF, 1 Mm Na_3VO_4 and 10 Mm DTT). Mechanical tissue lysis was achieved using a sonicator (Hielsher Ultrasound Technology, Teltow, Germany). Homogenates were centrifuged at 13000 rpm for 15 min at 4 °C, supernatants were collected and frozen at -20ºC during western-blot analysis.

Immunolabeling and image analysis

Immunofluorescence:

PNN immunolabeling was performed on free-floating sections as previously published (Vazquez-Sanroman et al., 2015a, b; Carbo-Gas et al., 2017; Guarque-Chabrera et al., 2022). After several rinses with phosphate-buffered saline triton buffer (PBST), cerebellar sections were incubated overnight at 4 °C with biotinylated WFA (**Table 2**). WFA binds to the glycosaminoglycan (GAG) chains of the proteoglycans (CSPGs) and is used to label the PNN structure (Härtig et al., 1992). Tissue samples were incubated for 2 h at room temperature with fluorochrome Cy3-conjugated or FITC- conjugated streptavidin (**Table 2**). The sections were mounted using Mowiol (Calbiochem, Merck Chemicals and Life Science, Madrid, Spain). Imaging and assessment of PNNs were blind to the treatment.

In the studies 1 and 2, to estimate changes in WFA intensity, we analysed all PNNs that showed unquestionable integrity in their structure. A × 40 objective with a × 3.0 zoom was used for a final magnification of × 120. Laser intensity, gain and offset were maintained constant in each acquisition. Brightness intensity of every PNN was estimated by randomly selecting 15 pixels in a single plane of the net pulled from a 40 µm z-stack and calculating their average intensity (Carbo-Gas et al., 2017). That is, we moved in the z-plane until we found the plane in which each PNN showed the highest integrity and marked the position using the "mark and find" function of the equipment to avoid photographing the same PNN twice. This procedure enables one to not underestimate intensity when the PNN appears in a different z-plane (**Fig. 10**). Data were expressed as arbitrary units of intensity (AU of WFA intensity) with a maximum intensity of 255. Then, we selected 30 PNNs randomly per rat to calculate the proportion of strong (more intense) (171–255 AU), medium (86–170 AU) and faint (0–85 AU) PNNs. Pictures of fluorescent-labelled sections were acquired using a Leica SP8 confocal microscope. Image resolution was 1024 × 1024 and scan speed 600 Hz. Laser intensity, gain, and

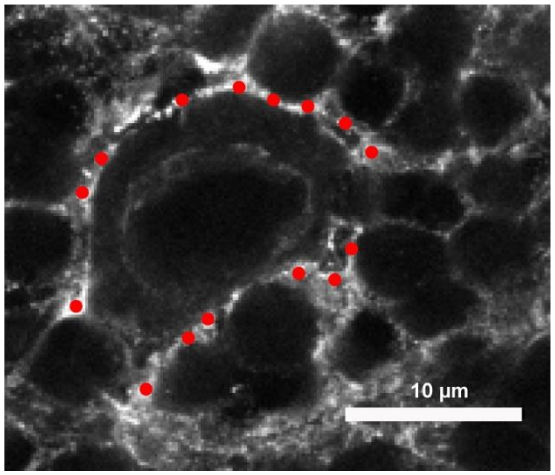

Figure 10. *Distribution of the 15 points for the intensity of the PNN. The PNN was labelled with WFA.*

offset were maintained constant in each acquisition. All quantitative evaluations were made using the FIJI software (Schindelin et al., 2012).

In the study 1, PNNs were labelled and analysed throughout the entire cerebellar cortex, including the vermis and hemispheres. In the studies 3 and 4, the degree of enzymatic digestion using ChABC was also determined by WFA immunolabeling as explained above. Sagittal sections of the cerebellum were selected according to the lateral coordinates from -0.90 to 0.90 mm, comprising the whole vermis (Paxinos and Watson, 1998) in order to determinate ChABC digestion in LVII. In this case, cerebellar sections were incubated with biotinylated WFA and anti-C6S (**Table 2**), which binds to the chondroitin-6-sulphate stubs after ChABC digestion (Chelini et al., 2021) and revealed using FITC-streptavidin and Alexa-647 anti-mouse antibodies (**Table 2**) (**Fig.13**).

To estimate neural activity after PNN digestion, we assessed the expression of the early transcription factor C-Fos which is used as a marker of neuronal activity because it is expressed and transcribed after an action potential (Zapulla et al., 1991). In the cerebellum, the expression peaks between 60 and 90 minutes after the occurrence of external and internal events such as psychostimulant drugs or painful stimulation (Klitenick et al., 1995; Tian and Bishop, 2002; Yin et al., 2010). For this reason, we took brain samples 60 minutes after the last DS test to determine C-Fos expression. Sagittal cerebellar sections were incubated 48h at 4 ºC, with biotinylated WFA and anti- C-Fos (**Table 2)** dissolved in PBST. The next day, and after several rinses, tissue was exposed to FITC-streptavidin and Alexa-647 anti-rabbit (**Table 2**) in PBST for 120 min at room temperature. Once fluorescence reaction occurred, cerebellar sections were rinsed and mounted using Mowiol (Calbiochem, Merck Chemicals and Life Science, Madrid, Spain), and stored at 4 °C until imaging.

To estimate ChABC- dependent degradation of PNNs in the studies 3 and 4, we sampled 15 PNNs from LVII of each animal. Images of PNNs were acquired as was already explained in the first study. However, to assess not only PNN intensity but also integrity, a line was drawn around the perimeter of the net surrounding the soma (**Fig. 11a**). For intensity, we calculated the average intensity of 15 PNNs in LVII of each animal. Then, the proportion of weak (0–85 AU of intensity), medium (86–170 AU of intensity), and strong (171–255 AU of intensity) PNNs was also estimated on the Sham and ChABC groups (Carbo-Gas et al., 2017; Carulli et al., 2020; Foscarin et al., 2011; Guarque-Chabrera et al., 2022; Sanchez-Hernandez et al., 2021; Vazquez-Sanroman et al., 2015a, b). PNN integrity value was estimated using the formula peaks/holes (number of peaks and holes in the line histogram, which range was from the half of the maximum intensity until the maximum intensity, using FIJI software (Schindelin et al., 2012; Tewari et al., 2018; Tewari and Sontheimer, 2019) (**Fig. 11b**).

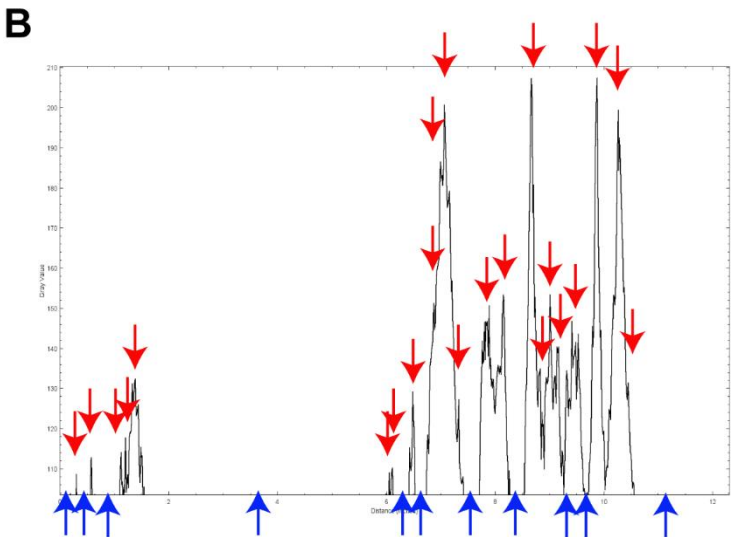

Figure 11. *Procedure to assess PNN intensity and integrity in studies 3 and 4.* ***A)*** *Distribution of the line in the PNN.* ***B)*** *Histogram of intensity in line's layout with peaks (red arrows) and holes (blue arrows).*

Antibody (Host)	**CAT#**	**Provider**	**Concentration (all dissolved in PBST)**	**Experimental study**
Anti-Bcan (Mouse)	MA5-27639	Thermo Fisher Scientific, Barcelona, Spain	1:1000	2
Anti-β-Actin (Mouse)	MA1-744	Merck Life Science S.L.U. (Madrid, Spain)	1:2000	2
Anti-C6S (Mouse)	MAB2035	Merck Life Science S.L.U. (Madrid, Spain)	1:500	3, 4
Anti- C-Fos (Rabbit)	226008	Synaptic Systems, Goettingen, Germany	1:2000	3
Anti-Mouse HRP (Goat)	10799354	Thermo Fisher Scientific, Barcelona, Spain	1:2000	2
Anti-TN-R (Mouse)	SC-100419	Santa Cruz Biotechnoloy, (Santa Cruz, USA)	1:200	2
Anti-TN-R (Mouse)	217011	Synaptic Systems, Goettingen, Germany	1:1000	2
Anti-PTP-σ	SC-100419	Santa Cruz Biotechnoloy, (Santa Cruz, USA)	1:200	2
Alexa Fluor-647 anti-rabbit (Donkey)	A31573	Thermo Fisher Scientific, Barcelona, Spain	1:250	3, 4
Alexa Fluor-647 anti-mouse (Donkey)	715-605-151	Jackson Immunoresearch Europe Ltd., Suffolk, UK	1:250	3, 4
FITC-streptavidin	016-010-084	Jackson Immunoresearch Europe Ltd., Suffolk, UK	1:200	3, 4
Cy3-streptavidin	016-160-084	Jackson Immunoresearch Europe Ltd., Suffolk, UK	1:200	1
WFA-biotin	L1516	Merck Life Science S.L.U. (Madrid, Spain)	1:200	1

Table 2. *Primary and secondary antibodies used in this work.*

C-Fos expression was analysed in three sagittal sections per rat. For Purkinje cells, C-Fos was estimated in a ROI of 250 µm x 500 µm in order to adjust the ROI to differences between lobule sizes among subjects in particular, when lobule VII becomes double (**Fig. 12a**). C-Fos expression was assessed in 3 ROIs of 140 µm x 140 µm in the granule cell layer at the dorsal region of lobule VII, and two ROIs of 140 µm x 140 µm ventrally in the same layer (**Fig. 12b**). Every C-Fos+ cell within ROIs was tagged and the number quantified using the cell-counter plug-in from FIJI software (Schindelin et al., 2012).

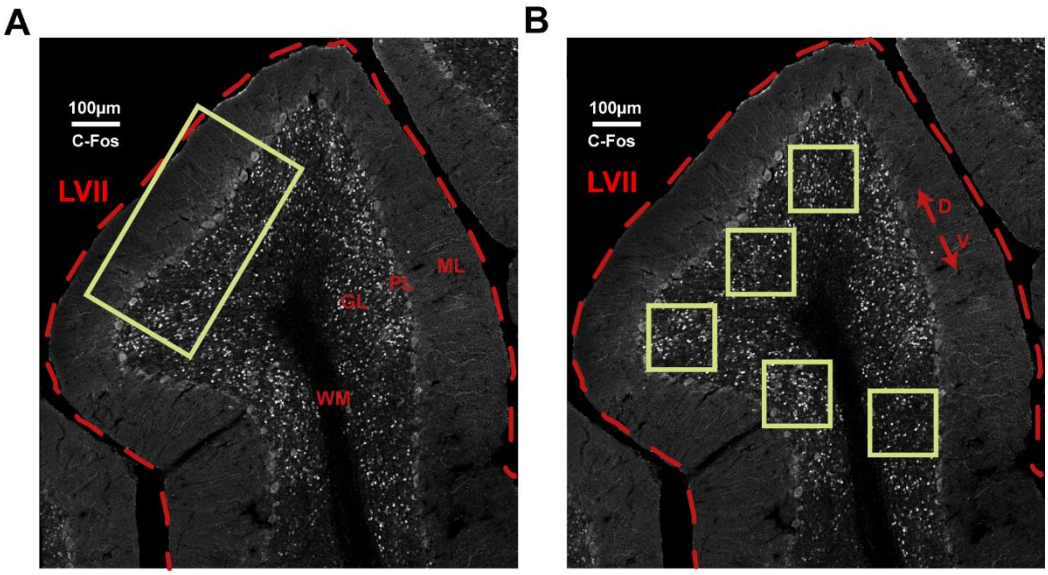

Figure 12. *Distribution of the ROIs (yellow) in the two different regions of the lobule VII (LVII), dorsal (red arrow, D) and ventral (red arrow, V). Molecular layer (ML), Purkinje layer (PL), Granular Layer (GL) and white matter (WM).* ***A)*** *ROI to measure C-Fos expression (white) in Purkinje cells.* ***B)*** *Distribution of ROIs to measure C-Fos expression (white) in granule cells.*

In study 3 and 4, the effects of ChABC on loose ECM and PNNs were assessed with tile-scan Z-stack images (25 steps) acquired using a confocal microscope (Leica DMi8, Leica Microsystems CMS GmbH, Wetzlar, Germany), with a 20x objective, a zoom of 2, and 1024 x 1024 px. Laser intensity, gain, and offset were maintained constant in each acquisition. Leica Application Suite X (LAS X, Leica Microsystems CMS GmbH, Wetzlar, Germany) was used to perform a maximal projection of the tile-scan Z-stacks in order to determinate the maximal extension of ChABC digestion (**Fig. 11**). Animals with digestion misplacement were not included in the statistical analysis.

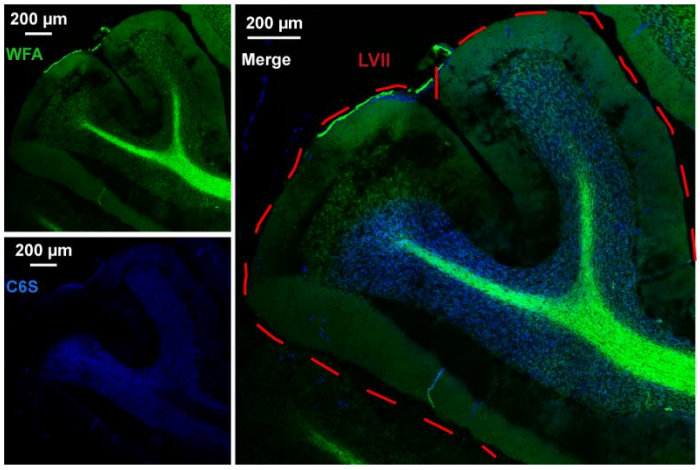

Figure 13. *Effect of ChABC on the ECM of LVII.* ***Left-Up*** *ECM labelled with WFA (green).* ***Left-Down*** *C6S residues from digestion with ChABC (blue).* ***Right*** *Merged image.*

Immunoblotting (Experimental study 2):

Aliquots of supernatants were collected and used BCA quantification of total protein. Every sample was heated at 95 °C for 5 min for protein denaturation. Equal amounts (20 µg) of dorsal region of cerebellar vermis protein samples were separated by 10% or 12% SDS-PAGE and transferred to nitrocellulose membrane (Global Life Sciences Solutions Operations UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire, UK). Filtering membranes were incubated in 5% not-fat milk dissolved in Tris buffered saline-tween20 (TBS-T), and then incubated overnight at 4 °C with primary antibodies: anti-TN-R (half of the samples should be labelled with a different anti-TN-R antibody from Synaptic Systems due to a problem with the batch acquired from Santa Cruz Biotechnology and a loss of stock 2 months before the deposit of this work), anti-Bcan, anti-PTP-σ and anti-β-Actin (**Table 2**). After several washes with TBS-T buffer, the membranes were incubated for 1h at RT with peroxidase-conjugated secondary antibodies goat anti-mouse (**Table 2**). Staining was developed using “Chemiluminescence reagent for Horseradish Peroxidase” (Cat # 42582.01, Quimigen, Madrid, Spain), and digital images were captured with a charge-coupled device imager (IMAGEQUANT LAS 4000, GE Healthcare Litle Chalfont, UK). Every sample was replicated at least twice to ensure the reproducibility of the method. Immunoreactive bands were quantified with FIJI software and normalised to the β-Actin signal, this is, by dividing the intensity of the protein of interest by the intensity of its corresponding actin, in each sample.

Statistics

Study 1:

Results of cocaine self-administration were analysed by two-way repeated-measures ANOVA with sessions as the within-subject factor and cocaine access (ShA/LgA) as the between-subject factor. One-way (group) and two-way (group x abstinence days) ANOVAs were used for the analysis of PNN intensity. Differences between means were analysed through Tukey's HDS and Sidak's tests. We also classified PNNs in three categories, strong, medium, and weak, and compared the percentage of PNNs in each category using the $\chi 2$ test. Kruskal-Wallis test was used for the analysis of the number of PNNs. Data is presented as mean ± SD and 95% confidence intervals (CI). All the statistical analyses were performed using GraphPad Prism software 8 (San Diego, CA, USA). To calculate linear correlations between lever pressing on the last session of cocaine self-administration session and PNN expression at different timepoints of withdrawal, we used Spearman r correlation coefficients (rho).

Experimental study 2:

Results of cocaine self-administration were analysed by two-way repeated-measures ANOVA with sessions as the within-subject factor and cocaine access (ShA/LgA/Saline) as the between-subject factor. Due to the low number of observations, it was not possible to assume a normal standard distribution, so WB data were analysed using non parametric statistics. Kruskal-Wallis test was used for three or more group comparisons (ShA/LgA/Saline) and Mann-Whitney test for two groups comparisons (24h/28d). Data is presented as medians ± range and 95% confidence intervals (CI). All the statistical analyses were performed using GraphPad Prism software 8 (San Diego, CA, USA).

Experimental study 3:

Results of cocaine self-administration were analysed by two-way repeated-measures ANOVA with sessions as the within-subject factor and cocaine access (ShA/LgA) as the between-subject factor. One-way (group) and two-way (group x abstinence days) ANOVAs were used for the analysis of PNN intensity and integrity, Purkinje and granule cell C-Fos expression. Two-way (ChABC x DS test) ANOVAs were used for the analysis of DS tests. Differences between means were analysed through Tukey's HDS and Sidak's tests. Data is presented as mean ± SD and 95% confidence intervals (CI). We also classified PNNs in three categories, strong, medium, and weak, and compared the percentage of PNNs in each category using the $\chi 2$ test. To calculate linear correlations,

we used Spearman r correlation coefficients (rho). All the statistical analyses were performed using GraphPad Prism software 8 (San Diego, CA, USA).

Experimental study 4:

Results of cocaine self-administration were analysed by one-way repeated-measures ANOVA. Two-way repeated-measures ANOVA with sessions as the within-subject factor and enzymatic digestion of PNNs (ChABC/Sham) as the between-subject factor used for the analysis of extinction. Differences between means were analysed through Tukey's HDS and Sidak's tests. T-Test analyses were used for test comparisons (DSxCR, DSxDR, CRxDR). Data is presented as mean ± SD and 95% confidence intervals (CI). All the statistical analyses were performed using GraphPad Prism software 8 (San Diego, CA, USA).

CHAPTER 3

EXPERIMENTAL STUDIES

Study 1

Time-dependent regulation of perineuronal nets in the cerebellar cortex during abstinence of cocaine-self administration

Study 1: Time-dependent regulation of perineuronal nets in the cerebellar cortex during abstinence of cocaine-self administration

The present experiment evaluated the effect of different schedules of cocaine self-administration on cerebellar PNNs over different periods of withdrawal. Male Sprague Dawley rats were randomly assigned to the naïve group (Ctrl), which had no access to cocaine throughout the experiment; the restricted access or short access group (ShA) to cocaine for 1 h/day and the extended access or long access group (LgA) to cocaine for 6 h/day. Subsequently, we assessed the expression of cerebellar PNNs by analysing their intensity by WFA labelling. For a visual representation of the experimental timeline, please see **Fig. 4**, Materials and Methods.

Cocaine intake escalated in extended access rats

Voluntary cocaine self-administration training under a FR1 schedule lasted for seven sessions (2 h/daily). After this initial training, rats self-administered cocaine for 1 h (ShA) (n =18) or 6 h (LgA) (n = 18) for 20 sessions (**Fig. 14**). Data are shown as mean ± SD with 95% confidence interval of mean for all groups. ShA rats did 66.73 ± 7.01, 95% CI [63.45, 70.01] active nose-pokes in 1-h session, whereas LgA rats did 241.70 ± 31.03, 95% CI [227.2, 256.2] active nose-pokes in 6 h. A two-way ANOVA for active nose-pokes (cocaine access × sessions) showed an effect of cocaine access ($F (1, 34) = 153.10$, $P < 0.0001$), sessions ($F (19, 646) = 2.00$, $P = 0.0004$) and cocaine × session interaction ($F (19, 646) = 2.01$, $P = 0.0066$). The analysis of the number of cocaine injections yielded similar results. ShA rats received 41.11 ± 4.51, 95%CI [39.00, 43.22] cocaine infusions, while LgA rats received 158.8 ± 15.43, 95% CI [151.60, 166.0]. A two-way ANOVA showed an effect of cocaine access ($F (1, 34) = 276.70$, $P < 0.0001$), sessions ($F (19, 646) = 2.90$, $P < 0.0001$) and cocaine × session interaction ($F (19, 646) = 1.717$, $P = 0.0052$). In the ShA group, the number of cocaine injections for 20 sessions was stable and did not escalate, whereas in the LgA group, it escalated significantly from the 12th session, which was confirmed by a Dunnett's post hoc test (**Fig. 14a, b**). Finally, ShA rats responded with an average of 23.33 ± 4.5, 95% CI [21.22, 25.44] inactive nose-pokes, and LgA rats did 17.34 ± 6.78, 95% CI [14.17, 20.52]. An ANOVA showed no effect of cocaine access ($F (1, 34) = 1.84$, $P = 0.1843$), session ($F (19, 646) = 1.58$, $P = 0.1654$), nor access × session interaction ($F (19, 646) = 1.563$, $P = 0.0602$) (**Fig. 14c**).

Protracted abstinence increases PNN expression in the cerebellar cortex

To characterize the effects of short (n = 18) versus extended access (n = 18) to cocaine self-administration on cerebellar PNN expression during abstinence, we compared WFA intensity around Golgi interneurons 24 h, 7 days, or 28 days after the last cocaine self-administration session (**Fig. 15**). A two-way ANOVA (cocaine access × withdrawal time) yielded significant differences for cocaine access ($F(1, 30) = 7.15$, $P = 0.012$) and abstinence time ($F(2, 30) = 14.20$, $P < 0.0001$) but not for the interaction between these two factors ($F(2, 30) = 0.44$, $P = 0.6513$). The lack of a significant interaction indicated that PNN upregulation through abstinence occurred in both cocaine groups. Nevertheless, as can be seen in **Fig. 15**, the pattern was more evident in the LgA group. These are the mean ± SD and 95% CI for the different groups: 24 h (ShA: 59.86 ± 16.28, 95% CI [43.58, 76.14]; LgA: 78.57 ± 21.02, 95% CI [57.55, 99.6]), 7 days (ShA: 103.44 ± 20.44, 95% CI [83, 123.88]; LgA: 118.31 ± 32.20, 95% CI [86.11, 150.51]), and 28 days (ShA: 103.46 ± 33.76, 95% CI [69.7, 137.22]; LgA: 136.29 ± 20.03, 95% CI [116.26, 156.33]) (n = 6).

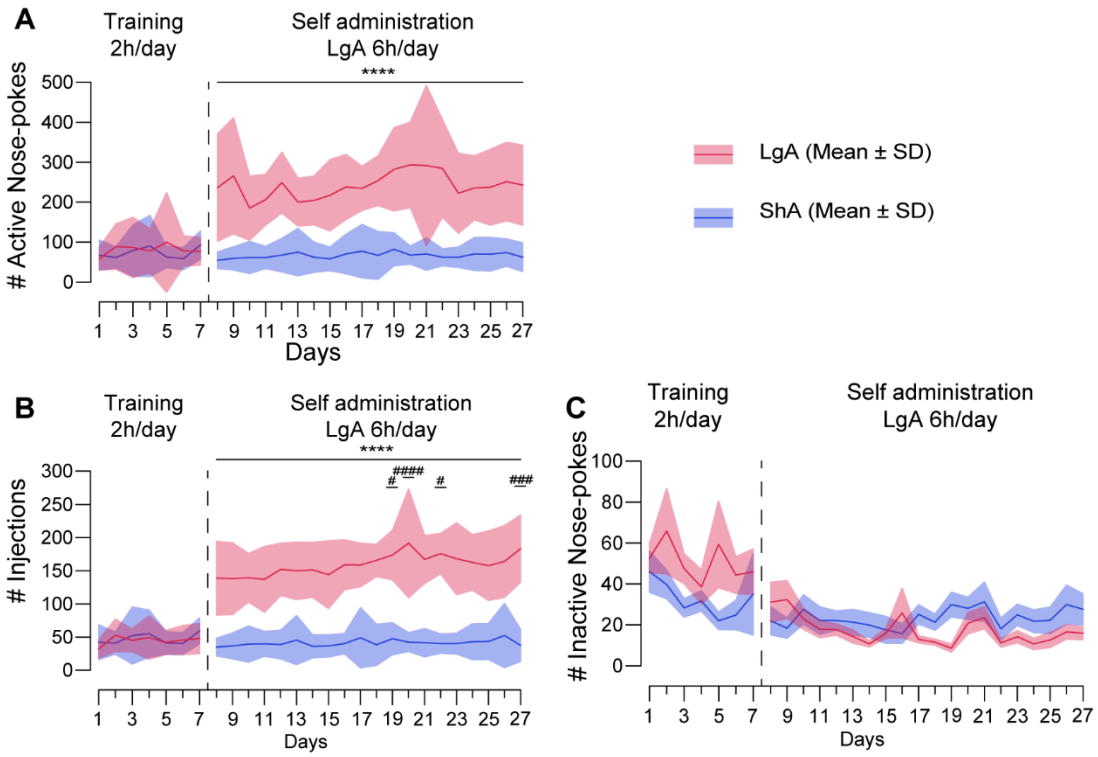

Figure 14. *Cocaine self-administration.* ***A)*** *Number of active nose-pokes during the escalation phase in which animals had access to cocaine for 1 h (ShA, n = 18) or 6 h (LgA n = 18).* ***B)*** *Number of cocaine injections in ShA and LgA rats.* ***C)*** *Number of inactive nose-pokes. ****P < 0.0001 LgA compared to ShA group. ####P < 0.0001, ###P < 0.001, #P < 0.05 for within-group comparisons against the first day of cocaine self-administration (day 8).*

In addition, the distribution of strong and faint PNNs in the cerebellar cortex was different among groups ($\chi 2$ (10) = 158.80, $P < 0.0001$). ShA rats expressed 81% of faint and 1.6% of more intense PNNs 24 h after cocaine self-administration. After 28 days of abstinence, the percentage of faint PNNs decreased to 52% while strong PNNs increased to 22%. In the LgA group, 67% of PNNs were faint and 4% were strong 24 h after extended access to cocaine. Protracted abstinence reduced the proportion of faint PNNs up to 27% but enhanced the proportion of strong PNNs (38%) in LgA rats (**Fig. 15c**).

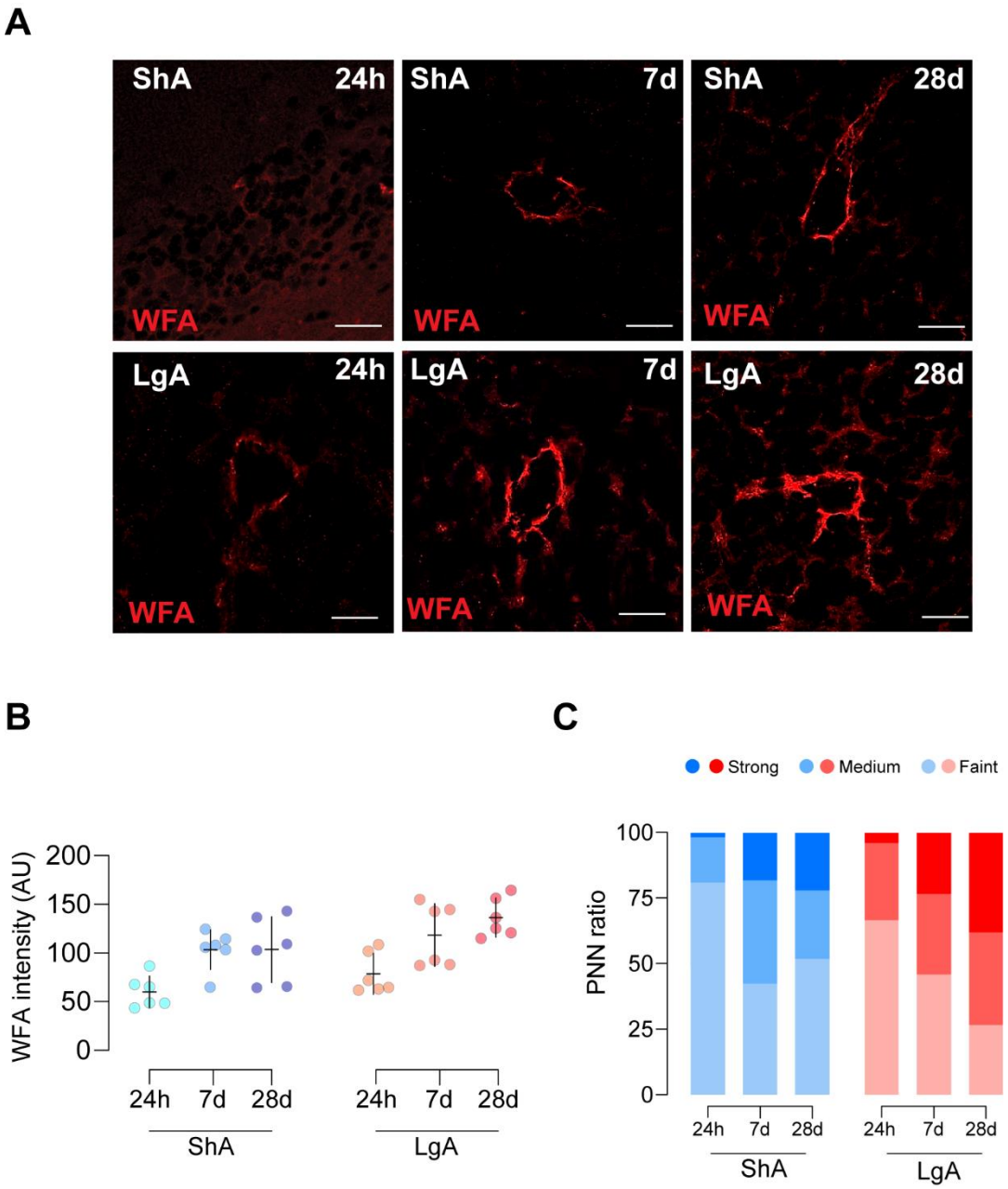

Figure 15. *Expression of PNNs in the cerebellar cortex at different time points of cocaine abstinence in ShA versus LgA rats. A) Representative confocal images (× 120) of PNNs in each group. Protracted abstinence from cocaine increased PNN expression around Golgi interneurons in the cerebellar cortex. Scale bar 20 μm. B) Scatter plots of Individual scores of WFA intensity from PNNs throughout different periods (24 h, 7 days, and 28 days) of force abstinence in ShA and LgA rats. 24 h ShA (n = 6), 7 days ShA (n = 6), 28 days ShA (n = 6), 24 h LgA (n = 6), 7 days LgA (n = 6), and 28 days LgA (n = 6). Plots indicate mean and SD. The Y-axis represents WFA intensity expressed in arbitrary units (AU). C) Proportion of Golgi neurons expressing a faint, medium, or strong PNNs in the cerebellar cortex.*

Cocaine self-administration reduces PNN expression in the cerebellar cortex

To determine whether different history of cocaine self-administration can change PNN expression in the cerebellum, ShA and LgA rats were compared with naïve rats 24 h after the last cocaine self-administration session (Ctrl; n = 6, 83.44 ± 4.22, 95% CI [79.01, 87.87]) (**Fig. 16**). Cocaine self-administration under short access conditions reduced WFA intensity in Golgi cells bearing a PNN ($F(2,15) = 3.85$, $P = 0.0447$). The reduction was clear in the ShA group so that the majority of subjects exhibited WFA intensity below control levels ($P = 0.0472$, DF: 15) (**Fig. 16a**). However, WFA intensity in the LgA group after 24 h did not differ from naïve rats ($P = 0.8515$, DF: 15) (**Fig. 16b**). We also found a different distribution of strong (> 170 AU of WFA) and faint (< 85 AU of WFA) PNNs among groups. While naïve rats expressed 63% of faint and 12% of strong PNNs, the ShA group exhibited 81% of faint PNNs but only 1.6% of strong ones ($\chi2(4) = 28.76$, $P < 0.0001$). LgA rats showed 67% of faint and 4% of strong PNNs, and they were not different from naïve-control rats (**Fig. 16c**).

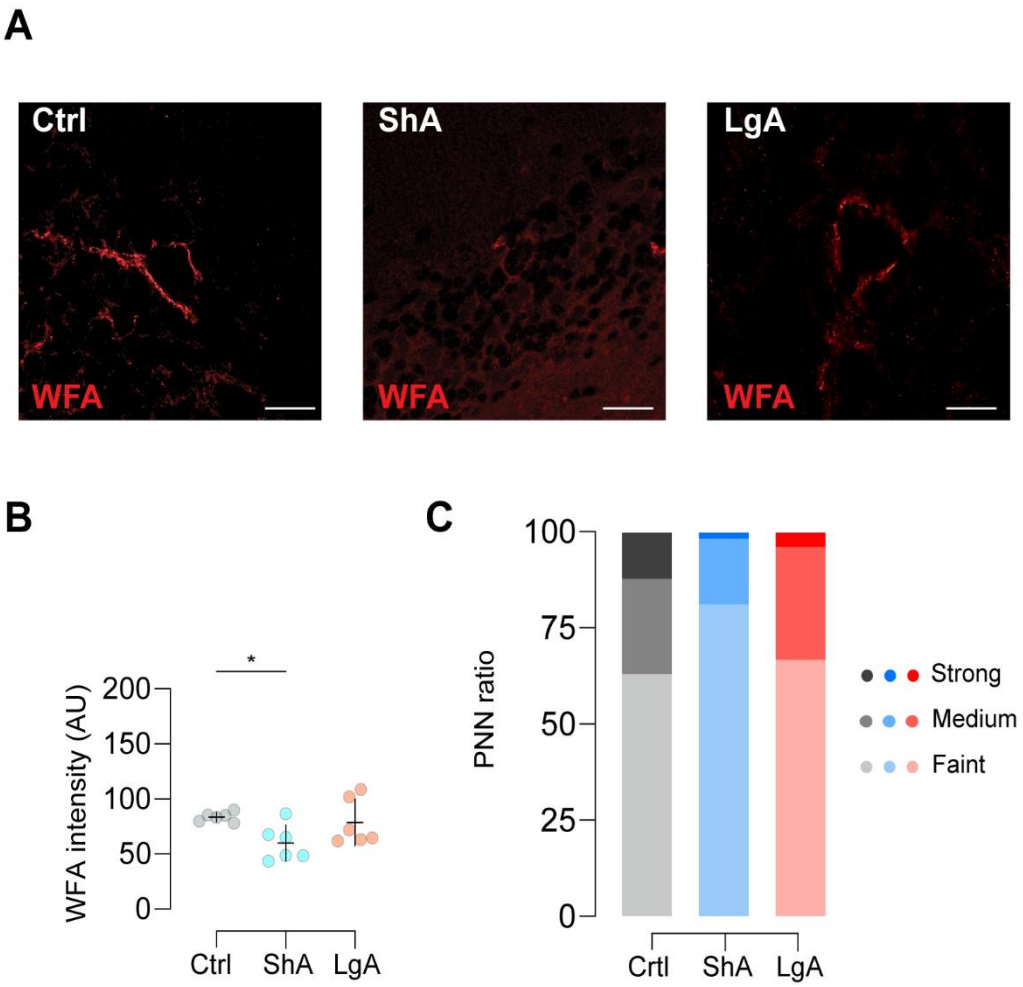

Figure 16. *PNN expression in the cerebellar cortex 24 h after the last cocaine self-administration session.* ***A)*** *Representative confocal PNN images (× 120) of naïve (Ctrl), short-access (ShA), and extended-access (LgA) groups. Scale bar 20 µm.* ***B)*** *Scatter plots of individual scores show the effects of cocaine self-administration on WFA intensity in PNNs around Golgi interneurons of the cerebellar cortex after 24 h of abstinence. Plots indicate mean and SD. The Y-axis represents WFA intensity expressed in arbitrary units (AU): ShA (n=6) and LgA (n=6). Ctrl (n = 6) had no access to cocaine. *P < 0.05 compared with the control group.* ***C)*** *Proportion of Golgi neurons expressing a faint, medium, or strong PNNs in the cerebellar cortex. Cocaine self-administration decreased PNN expression in the cerebellar cortex after ShA to cocaine.*

PNN expression did not return to control levels after protracted abstinence

To assess whether PNN expression returns to control levels after protracted abstinence, we compared ShA and LgA rats with naïve controls after 28 days of abstinence. Extended access to cocaine self-administration caused a significant increase in WFA intensity around Golgi cells that did not return to control levels after 28 days of abstinence

(F (2, 15) = 8.22, P = 0.0039) (LgA: P = 0.0030; DF: 15). This effect was not evident in ShA rats (P = 0.3096, DF: 15) (**Fig. 17**).

Finally, we did not find significant effects on the number of PNNs as a Kruskal-Wallis test showed (H (7) = 10.03, P = 0.1235; n = 3). We show mean ± SD with 95% confidence interval of mean for all groups: (Ctrl: 153.67 ± 131.17, 95% CI [-172.2, 479.5]; ShA 24 h: 247 ± 68.46 95%, CI [76.93, 417.1]; ShA 7 days: 434.67 ± 44.02, 95% CI [325.3, 544]; ShA 28 days: 446 ± 201.20, 95% CI [-53.82, 945.8]; LgA 24 h: 330.33 ± 53.67, 95% CI [197, 463.7]; LgA 7 days: 365.67 ± 120.95, 95% CI [65.21, 666.1]; LgA 28 days: 404.67 ± 73.19, 95% CI [222.9, 586.5]).

Overall, the results of this study indicate the existence of a dynamic regulation of plasticity conditions in the cerebellum during abstinence after cocaine self-administration. Shortly after drug consumption, we found a reduction in the expression of PNNs around Golgi interneurons in animals with restricted access to cocaine. Nevertheless, over the course of prolonged abstinence, the expression of cerebellar PNNs increased, and a higher proportion of intense PNNs formed throughout the first month. The intensity of PNNs across time was not different in ShA rats but it was enhanced during abstinence after an escalated cocaine intake (LgA). These findings suggest that cocaine intake increases conditions for plasticity. However, a history of cocaine abuse causes restrictive conditions for synaptic remodelling in Golgi interneurons over abstinence that may contribute to the incubation of drug seeking. In the next chapters, we investigate what components of PNN structure could be upregulated and what is the role of PNNs around Golgi cells in the incubation of drug seeking.

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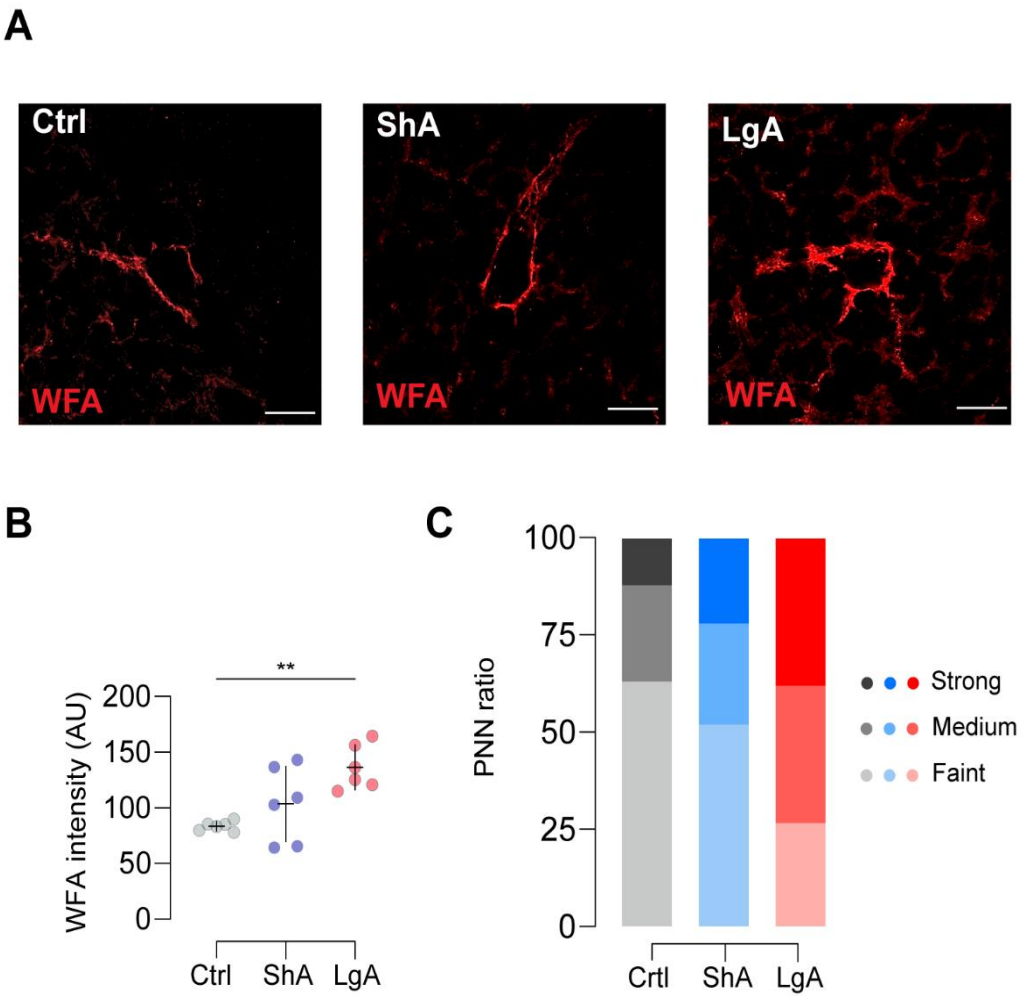

Figure 17. *PNN expression in the cerebellar cortex 28 d after the last cocaine self-administration session. A) Representative confocal PNN images (× 120) of naïve (Ctrl), short-access (ShA), and extended-access (LgA) groups. Scale bar 20 μm. B) Scatter plots of individual scores show the effects of cocaine self-administration on WFA intensity in PNNs around Golgi interneurons of the cerebellar cortex after 28 d of abstinence. Plots indicate mean and SD. The Y-axis represents WFA intensity expressed in arbitrary units (AU): ShA (n=6) and LgA (n=6). Ctrl (n = 6) had no access to cocaine. **P < 0.01 compared with the control group. C) Proportion of Golgi neurons expressing a faint, medium, or strong PNNs in the cerebellar cortex. Cocaine self-administration increased PNN expression in the cerebellar cortex after LgA to cocaine.*

Study 2

Assessing the impact of different schedules of cocaine self-administration and abstinence on the expression of PNN-related proteins in the cerebellar cortex

Study 2: Assessing the impact of cocaine self-administration and withdrawal on the expression of PNN-related proteins in the cerebellar cortex

This study assessed the effect of different schedules of access to cocaine self-administration and withdrawal periods on the levels of two PNN components: TN-R and Bcan. Both have shown to play an important role as scaffolding molecules in PNNs and to contribute to restricting synaptic plasticity. In addition, we included the analysis of PTPσ levels, a member of the protein tyrosine phosphatase family (PTPs), that has been demonstrated to be essential for the inhibitory effects of CSPGs such as Bcan on synaptic plasticity. All protein determinations were carried out by WB analysis.

Male Sprague Dawley rats were randomly assigned to the saline group (Saline), which had access to saline 6 h/day, or to the two schedules of cocaine access previously described, ShA and LgA. Brain samples were obtained 24 h or 28 days after the last session of cocaine self-administration for WB analysis. We determined the relative amounts of each protein by analysing their bands intensity and normalising against the load control (β-actin). For a visual representation of the experimental timeline, please see **Fig. 6**, Materials and Methods.

Only extended access rats escalated cocaine intake

Restricted (ShA) and extended (LgA) access rats could self-administer cocaine under a FR1 schedule for seven sessions (1 h/daily) and Saline rats receive saline at 0.9% (**Fig. 18**). After this initial training, rats self-administer saline for 6h (n=12), cocaine for 1 h (ShA) (n =14) or cocaine for 6 h (LgA) (n = 12) for 12 sessions (**Fig. 19**). We show mean ± SD with 95% confidence interval of mean for all groups: Saline rats did 2.96 ± 0.83, 95% CI [1.08, 4.25] active lever-presses in 6h; ShA rats emitted 7.93 ± 1.56, 95% CI [5.64, 10.14] active lever-presses in 1-h session, whereas LgA rats did 63.77 ± 9.73, 95% CI [42.33, 75.00] active lever-presses in 6 h.

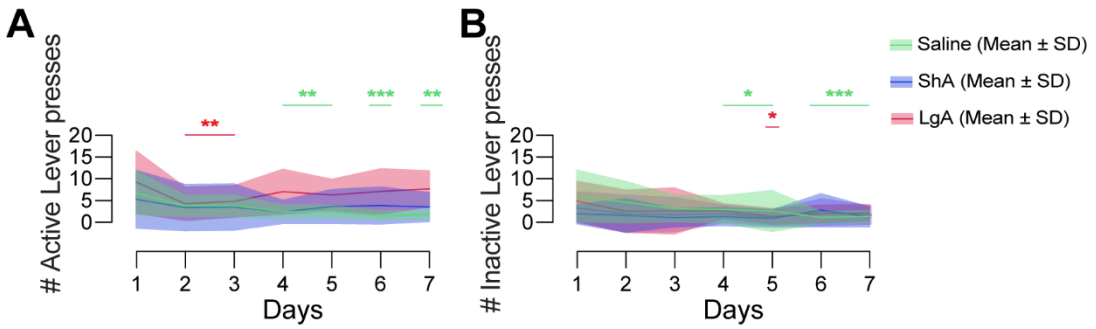

Figure 18. *Training of cocaine/saline self-administration.* ***A)*** *Number of active nose-pokes during the training phase in which animals had access to saline (Saline, n=12) for 1 h or cocaine (ShA, n = 14, and LgA, n = 12)* ***B)*** *Number of inactive lever-presses during the training phase. ***P < 0.001, **P < 0.01, *P < 0.05 for within-group comparisons against first day of training.*

A two-way ANOVA for active lever-presses during 1h/6h sessions (cocaine access × sessions) showed an effect of cocaine access ($F(2, 35) = 380.3$, $P < 0.0001$), sessions ($F(11, 385) = 15.16$, $P < 0.0001$), and cocaine access × session interaction ($F(22, 385) = 11.19$, $P < 0.0001$). The analysis of the active lever-presses during the first hour of self-administration over the 12 self-administration sessions (**Fig. 20**) showed similar results. Saline rats emitted 2.09 ± 0.63. 95% CI [1.00, 2.92] active lever in the first hour, ShA rats did 7.93 ± 1.56, 95% CI [5.64, 10.14], while LgA rats emitted 14.42 ± 3.53, 95% CI [7.75, 20.83]. A two-way ANOVA showed an effect of cocaine access ($F(2, 35) = 57.88$, $P < 0.0001$), sessions ($F(11, 385) = 8.51$, $P < 0.0001$) and cocaine access × session interaction ($F(22, 385) = 4.648$, $P < 0.0001$). In the Saline and ShA groups, the number of injections for 12 sessions were stable and did not escalate, whereas in the LgA group, it escalated significantly from the 2nd session as confirmed by Dunnett's post hoc test (**Fig. 19d**). Finally, Saline rats responded with an average of 2.18 ± 1.28, 95% CI [0.67, 4.17] inactive lever-presses, ShA rats with 1.09 ± 0.48, 95% CI [0.29, 2.14] inactive lever-presses, and LgA rats did 1.03 ± 0.74, 95% CI [0.08, 2.58]. A two-way ANOVA showed effect of cocaine access ($F(2, 35) = 3.913$, $P = 0.0293$) and session ($F(11, 385) = 2.141$, $P = 0.017$), but no for access × session interaction ($F(22, 385) = 1.466$, $P = 0.0815$) (**Fig. 19e**). This suggest that both ShA and LgA rats learn throughout the 12 sessions that the inactive lever does not have any consequence. However Saline rats, which does not receive cocaine, were not able to learn about contingencies.

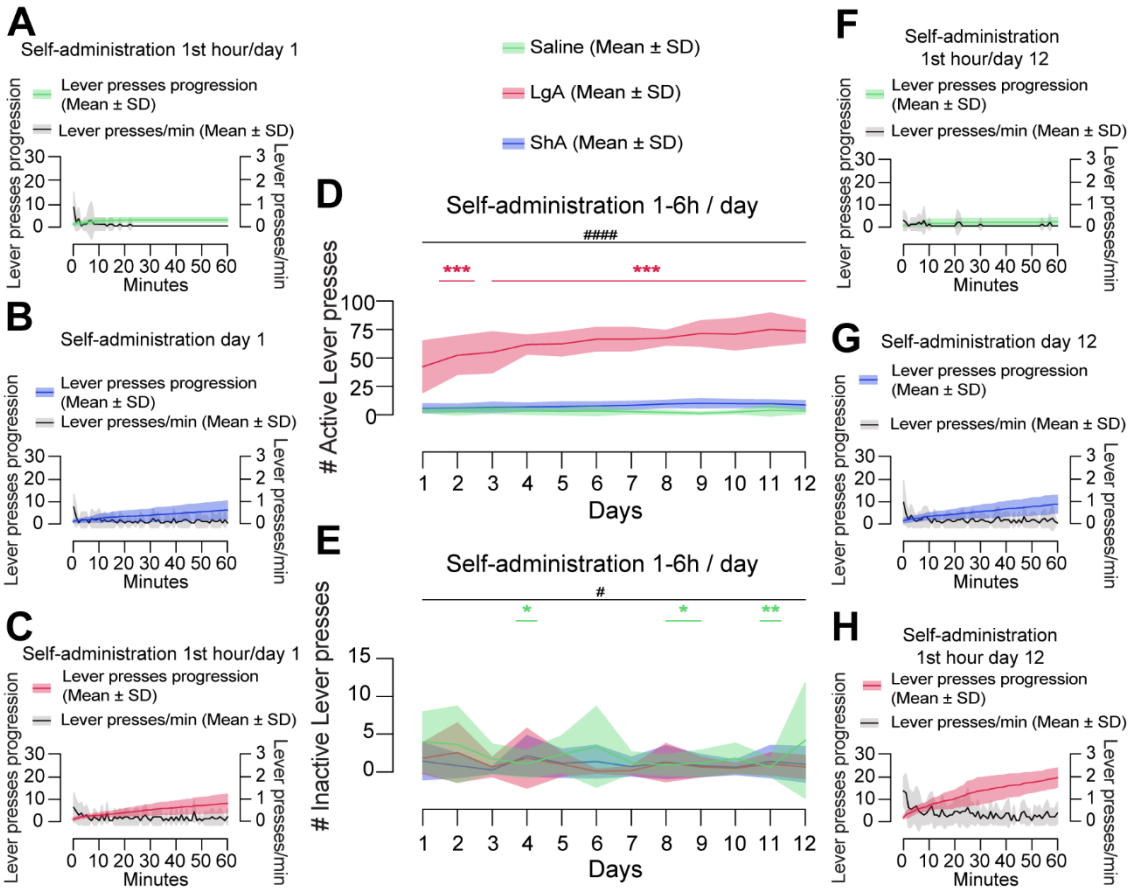

Figure 19. *Cocaine/saline self-administration phase.* ***A)*** *Lever presses progression and Lever presses/min of Saline rats (n=12) during the first hour of the first day of self-administration.* ***B)*** *Lever presses progression and Lever presses/min of LgA rats (n=12) during the first hour of the first day of self-administration.* ***C)*** *Lever presses progression and Lever presses/min of ShA rats (n=14) during first day of self-administration.* ***D)*** *Number of active nose-pokes during the self-administration phase in which animals had access to saline (Saline) or cocaine (LgA) for 6 h or cocaine for 1h (ShA).* ***E)*** *Number of inactive lever-presses during the self-administration phase.* ***F)*** *Lever presses progression and Lever presses/min of Saline rats during the first hour of the last day of self-administration.* ***G)*** *Lever presses progression and Lever presses/min of LgA rats during the first hour of the last day of self-administration.* ***C)*** *Lever presses progression and Lever presses/min of ShA rats during the last day of self-administration. ***P < 0.001, **P < 0.01, *P < 0.05 for within-group*

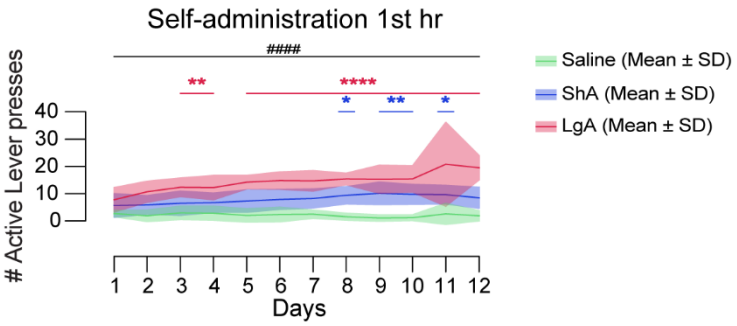

Figure 20. *1st hour active-lever presses during self-administration phase for Saline (n=12), ShA (n=14) and LgA (n=12) rats. ****P < 0.0001, **P < 0.01, *P < 0.05 for within-group comparisons against first day of self-administration. ####P < 0.0001, Saline compared to ShA and LgA groups.*

Effect of cocaine self-administration on Bcan 55 kDa-fragment levels in the posterior vermis

Following the self-administration phase, rats were sacrificed after short-term (24h) or long-term (28d) withdrawal and the posterior cerebellum was dissected and analysed by WB as is explained in Chapter 2. Briefly, after CO_2 euthanasia, cerebellums were rapidly frozen in liquid N_2 and lysate in RIPA lysis buffer. Homogenates were quantified to use 20µg of total protein per well. Here, data are presented as median ± range with 95% confidence interval of arbitrary units of relative intensity.

Twenty-four hours after the last self-administration session, the levels of **Bcan 55kDa-fragment in** the posterior vermis were for Saline rats (n = 4) 0.702 ± 1.64, 95% CI [0.20, 1.84] AU; for ShA-24h (n = 3) rats 0.2917 ± 0.32, 95% CI [0.22, 0.54] AU; and for LgA-24h (n = 3) rats 0.54 ± 0.13, 95% CI [0.54, 0.67] AU. Kruskal-Wallis test did not show a significant effect of cocaine self-administration at 24h on Bcan 55kDa-fragment levels (H (3) = 1.47, p=0.5233) (**Fig. 21**). Twenty-eight days after cocaine self-administration, Bcan 55kDa-fragmen levels of Saline rats (n = 4) were 0.1515 ± 0.46, 95% CI [0.11, 0.56]; of ShA-28d (n = 4) rats were 0.5171 ± 0.51, 95% CI [0.43, 0.94]; and of LgA-28d rats (n =3) were 0.1922 ± 0.06, 95% CI [0.18, 0.23]**.** Kruskal-Wallis analysis did not yield significant effects of cocaine self-administration after 28 days of withdrawal on Bcan 55kDa-fragment (H (3) = 4.48, p=0.1022) (**Fig. 21**). Thus, cocaine self-administration did not alter Bcan 55kDa-fragment levels at any time of abstinence, although it is clear that there is a small number of observations and variability in Saline animals was too high to confirm the tendencies observed in the results.

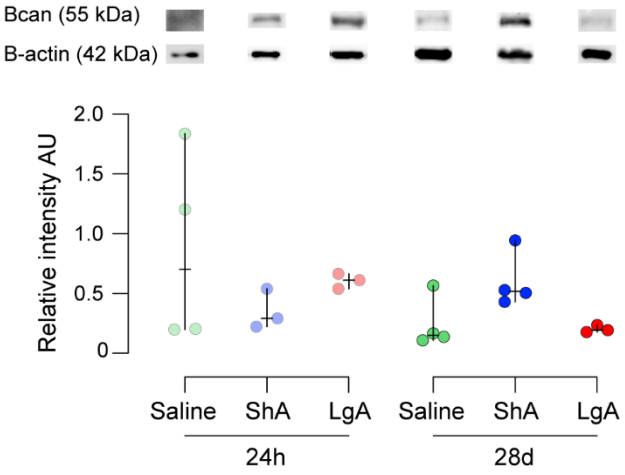

Figure 21. *Effect of cocaine self-administration, 24h and 28 days (28d) after last self-administration session on Bcan 55kDa-fragment protein level in posterior cerebellum. Upper part: representative WB showing two bands corresponding to Bcan 55kDa-fragment (molecular weight of 55kDa) and actin (molecular weight of 42 kDa). Each line contains 20ug of posterior vermis homogenates*.

Effect of cocaine self-administration on Bcan 50 kDa-fragment levels in the posterior vermis

At 24h, the levels of **Bcan 50kDa-fragment** in the posterior vermis from Saline rats (n = 4) were 0.3080 ± 0.77, 95% CI [0.03, 0.81]; from ShA-24h (n = 3) rats 0.08094 ± 0.16, 95% CI [0.07, 0.24]; and from LgA-24h (n = 4) rats were 0.3287 ± 0.30, 95% CI [0.14, 0.44]. Kruskal-Wallis test did not show a significant effect of cocaine self-administration on Bcan 50kDa-fragment at 24h (H (3) = 2.05, p=0.3879) (**Fig. 22**). However, 28 days after the last self-administration session, Bcan 50kDa-fragment was upregulated after extended access to cocaine self-administration as Kruskal-Wallis test showed (H (3) = 6.41, p=0.0234). Levels of **Bcan 50kDa-fragment from** Saline rats (n = 4) were 0.1919 ± 0.22, 95% CI [0.08, 0.30]; from ShA-28d (n = 4) rats 0.1481 ± 0.27, 95% CI [0.03, 0.30]; and from LgA-28d rats (n =3) 0.3722 ± 0.07, 95% CI [0.32, 0.38] (**Fig. 22**). Bcan 50kDa-fragment levels increased in LgA-28d group as confirmed by Dunn's post hoc tests (p=0.041).

These results parallel PNN increased intensity observed for LgA group after protracted abstinence (28d) showed in study 1 and suggested that Bcan 50kDa-fragment may be one of the PNN components upregulated during incubation (**page 43**).

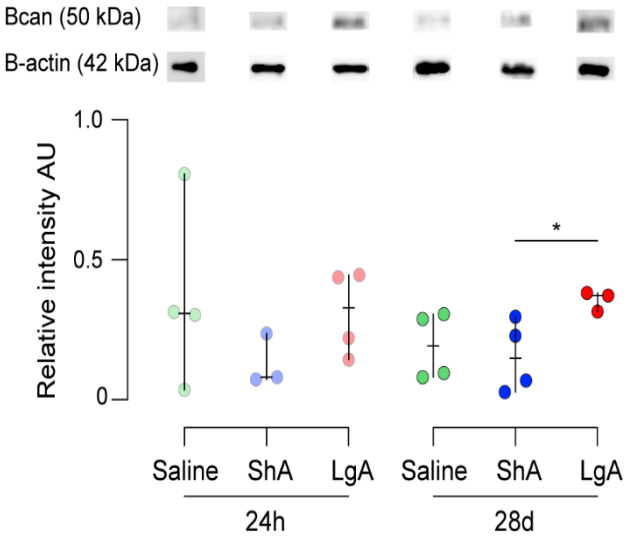

Figure 22. *Effect of cocaine self-administration, 24h and 28 days (28d) after last self-administration session on Bcan 50kDa-fragment protein level in the posterior cerebellum. *P < 0.05 for within-group comparisons. Upper part: representative WB showing two bands corresponding Bcan 50kDa-fragment (molecular weight of 50kDa) and actin (molecular weight of 42 kDa). Each line contains 20ug of posterior vermis homogenate.*

Effect of cocaine self-administration on TN-R 180 kDa levels in the posterior vermis

Kruskal-Wallis test showed a reduction in TN-R 180 kDa isoform levels 24h after cocaine self-administration in both cocaine groups (H (3) = 5.80, p=0.0491) (**Fig. 21).** Levels for Saline rats (n = 6) were 0.4557 ± 0.32, 95% CI [0.20, 0.53]; for ShA-24h (n = 6) rats were 0.2695 ± 0.31, 95% CI [0.16, 0.48] and for LgA-24h (n = 6) rats were 0.1663 ± 0.41, 95% CI [0.14, 0.44]. Dunn's post hoc tests did not reach statistical significance for any of the comparisons. However, 28 days after the last self-administration session no changes were observed on TN-R 180 kDa isoform as Kruskal-Wallis test showed (H (3) = 2.211, p=0.3527) (**Fig. 23**). TN-R 180 kDa isoform levels in Saline rats (n = 6) were 0.4437 ± 0.25, 95% CI [0.24, 0.49]; in ShA-28d (n = 6) rats were 0.3595 ± 0.36, 95% CI [0.14, 0.49]; and in LgA-28d rats (n =6) were 0.3029 ± 0.13, 95% CI [0.24, 0.36]. In conclusion, cocaine self-administration reduces TN-R 180 kDa isoform levels at 24h in the posterior vermis, replicating the reduction in PNN intensity observed in study 1 (**page 42**).

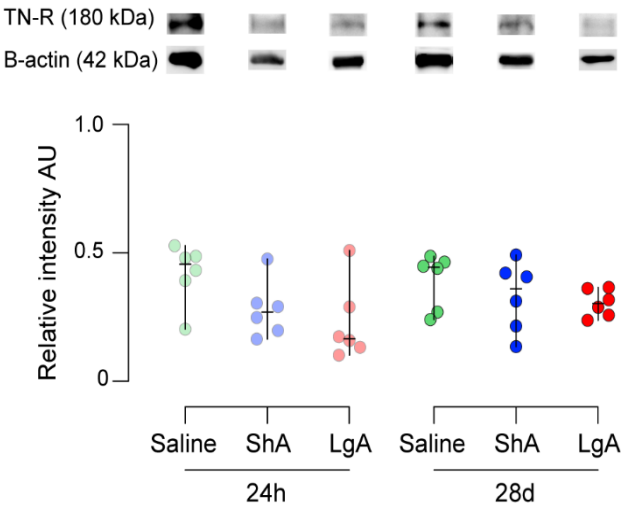

Figure 23. *Effect of cocaine self-administration, 24h and 28 days (28d) after last self-administration session on TN-R 180 kDa isoform level in the posterior cerebellum. Upper part: representative WB showing two bands corresponding TN-R 180 kDa isoform (molecular weight of 180kDa) and actin (molecular weight of 42 kDa). Each line contains 20ug of posterior cerebellum homogenates.*

Effect of cocaine self-administration on PCPTP1-ce levels in the posterior vermis

Twenty-four hours after the last session of self-administration, there was an increase in PCPTP1-ce levels as Kruskal-Wallis test yield (H (3) = 6.41, p=0.0216). PCPTP1-ce levels increased in the ShA-24h (0.5123 ± 0.33, 95% CI [0.36, 0.69]) group as compared to LgA-24h (0.2533 ± 0.13, 95% CI [0.16, 0.29] rats (p=0.034), but not to Saline-24h (0.3218 ± 0.17, 95% CI [0.20, 0.37]) as confirmed by Dunn's post hoc test (**Fig. 22).** However**,** protracted abstinence reduced PCPTP1-ce levels in the posterior vermis of both cocaine groups although Dunn's comparisons were only significant for the LgA group (H (3) = 7.212, p=0.0090): ShA-28d (n = 4) rats, 0.3336 ± 0.07, 95% CI [0.31, 0.37] (p=0.099); LgA-28d rats (n =3), 0.2933 ± 0.11, 95% CI [0.28, 0.40] (p=0.045); and Saline rats (n = 4), 0.8522 ± 0.35, 95% CI [0.62, 0.97] (**Fig. 24**). The present results may also indicate an increase in PCPTP1-ce expression with age that could be prevented by cocaine exposure as revealed when Saline-24h and Saline-28d groups were compared by Mann-Whitney U test (p = 0.0286).

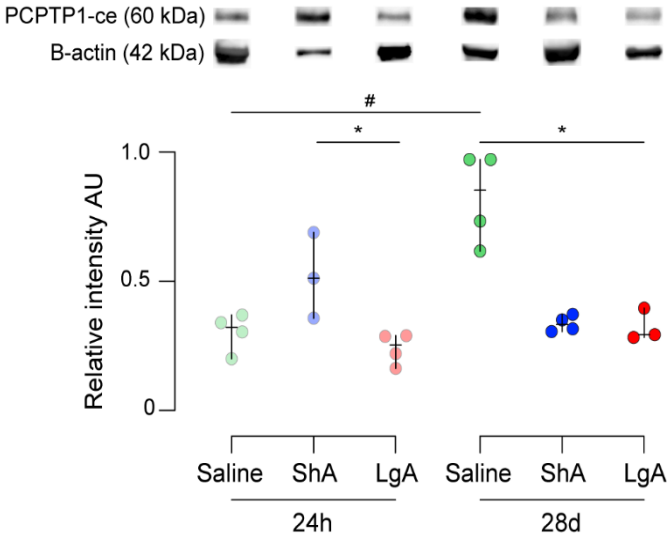

Figure 24. *Effect of cocaine self-administration, 24h and 28 days (28d) after last self-administration session on PCPTP1-ce levels in the posterior cerebellum. Upper part: representative WB showing two bands corresponding to PCPTP1-ce isoform (molecular weight of 60kDa) and actin (molecular weight of 42 kDa). Each line contains 20ug of posterior cerebellum homogenates.*

Effect of cocaine self-administration on PCPTP1 levels in the posterior vermis

No effect of cocaine self-administration was observed on PCPTP1 levels in the posterior vermis at 24h (H (3) = 1.68, p=0.4700). PCPTP1 levels were 0.3868 ± 0.11, 95% CI [0.33, 0.44] for Saline rats (n = 4); 0.6466 ± 0.39, 95% CI [0.34, 0.74] for ShA-24h (n = 3) rats; and 0.3641 ± 0.22, 95% CI [0.32, 0.53] for LgA-24h (n = 4) rats (**Fig. 23).** Neither cocaine self-administration changed PCPTP1 levels following 28 days of abstinence as revealed by Kruskal-Wallis test (H (3) = 4.89, p=0.0859). PCPTP1 levels after 28 days of abstinence were 0.2227 ± 0.24, 95% CI [0.13, 0.36] for Saline rats (n = 4); 0.5726 ± 0.51, 95% CI [0.32, 0.83] for ShA-28d (n = 4) rats; and 0.4430 ± 0.27, 95% CI [0.33, 0.60] for LgA-28d rats (n =4) (**Fig. 25**). Nevertheless, a tendency to increasing levels over abstinence could be seen in cocaine groups although it could not be confirmed with this small sample.

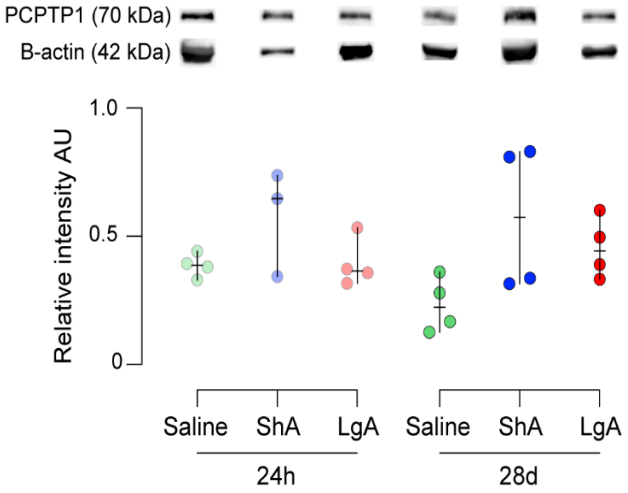

Figure 25. *Effect of cocaine self-administration, 24h and 28 days (28d) after last self-administration session on PCPTP1 levels in the posterior cerebellum. Upper part: representative WB showing two bands corresponding to PCPTP1 isoform (molecular weight of 70kDa) and actin (molecular weight of 42 kDa). Each line contains 20ug of posterior cerebellum homogenates.*

Effect of cocaine self-administration on PTP-PTP dimers levels in the posterior vermis

PTP-PTP dimers increased 24h after cocaine self-administration (H (3) = 6.04, p=0.0365). The increase was observed only in ShA-24h (n = 4) rats, 0.3509 ± 0.57, 95% CI [0.21, 0.77] (p=0.043) as compared to the LgA-24h group (n = 4) (0.1016 ± 0.16, 95% CI [0.05, 0.21]) as Dunnet test showed. PTP-PTP dimers levels for Saline rats (n = 4) were 0.2364 ± 0.16, 95% CI [0.13, 0.29] (**Fig. 26**). After 28 days of abstinence, we could not see consistent effects on PTP-PTP dimers levels as Kruskal-Wallis test showed (H (3) = 3.50, p=0.1965). Levels of PTP-PTP dimers were 0.06133 ± 0.15, 95% CI [0.01, 0.16] for Saline rats (n = 4); 0.3076 ± 0.49, 95% CI [0.09, 0.58] for ShA-28d (n = 4) rats; and 0.1169 ± 0.09, 95% CI [0.09, 0.18] for LgA-28d rats (n =4) (**Fig. 26**).

These results showed an increase in PTP-PTP dimers levels in ShA group at 24h after short-access cocaine self-administration that confirm what we described for PCPTP1-ce levels in the same group.

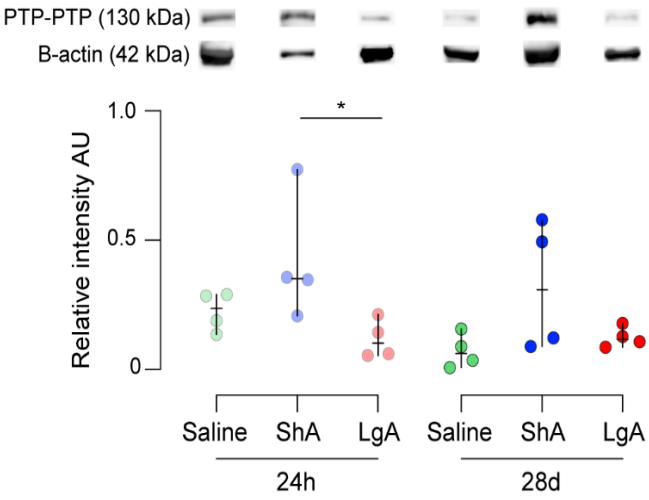

Figure 26. *Effect of cocaine self-administration, 24h and 28 days (28d) after last self-administration session on PTP-PTP dimer levels in the posterior vermis. Upper part: representative WB showing two bands corresponding to PTP-PTP dimers (molecular weight of 130kDa) and actin (molecular weight of 42 kDa). Each line contains 20ug of posterior cerebellum homogenates.*

In summary, the results of this study indicate that the dynamic regulation of PNNs shown in study 1 affects proteoglycans such as Bcan and other components as TN-R although at different time points of abstinence. Increased Bcan levels could reduce probability of synaptic remodelling during abstinence and contribute to the incubation of drug-seeking. Additionally, the changes observed in PCPTP1 family levels could affect downstream signalling pathways, since this family of phosphatases exerts an inhibitory action on TRKb that are the receptors of the BDNF mature form. However, these results are preliminary, given the small sample size. Further experiments will be necessary to expand the sample and confirm these findings.

Study 3

Effects of enzymatic digestion of PNNs in the cerebellar cortex on incubation of drug-seeking

Study 3: Effects of enzymatic digestion of PNNs in the cerebellar cortex on incubation of drug-seeking

The present study investigated the effect of PNN digestion on lobule VII of the vermis on incubation of drug seeking. Male Sprague Dawley rats were randomly assigned to the saline, ShA or LgA groups as previously explained. After self-administration, animals were tested in a drug-seeking test as described in Materials and Methods, and then ChABC was infused into the dorsal region of Lobule VII. Rats were left undisturbed in their home-cages for 24h or 28d. Rats were then retested for drug seeking on day 28 of abstinence.

In this study, we also assessed PNN expression by analysing WFA intensity and PNN integrity. To evaluate neuronal activity in Purkinje and granule cell layer in lobule VII after PNN degradation we used C-Fos expression. For a visual representation of the experimental timeline, please see **Fig. 8**, Materials and Methods.

Cocaine intake escalates after extended access cocaine self-administration

ShA and LgA rats could self-administer cocaine under a FR1 schedule for seven sessions (1 h/daily) and Saline rats receive saline at 0.9% (**Fig. 27**). After this initial training, rats self-administer saline for 6h (n=7), cocaine for 1 h (ShA) (n =25) or cocaine for 6 h (LgA) (n = 26) for 12 sessions (**Fig. 28**). Data are presented as mean ± SD with 95% confidence interval of mean for all groups. Saline rats did 3.2 ± 1.29, 95% CI [2.38, 4.02] active lever-presses in 6h, ShA rats emitted 7.47 ± 1.56, 95% CI [6.47, 8.46] active lever-presses in 1-h session, whereas LgA rats did 53.57 ± 14.10, 95% CI [44.61, 62.53] active lever-presses in 6 h. A two-way ANOVA for active lever-presses (cocaine access × sessions) showed an effect of cocaine access ($F(2, 55) = 127.80$, $P < 0.0001$), sessions ($F(11, 605) = 10.20$, $P < 0.0001$), and cocaine access × session interaction ($F(22, 605) = 13.38$, $P < 0.0001$). The analysis of the active lever-presses during the first hour of self-administration (**Fig. 30**) showed similar results. Saline rats emitted 2.05 ± 0.91. 95% CI [1.47, 2.63] active lever-presses in the first hour, ShA rats did 7.47 ± 1.56, 95% CI [6.47, 8.46] active lever-presses, while LgA rats did 11.66 ± 3.29, 95% CI [9.57, 13.75]. A two-way ANOVA showed an effect of cocaine access ($F(2, 55) = 18.77$, $P < 0.0001$), sessions ($F(11, 605) = 7.99$, $P < 0.0001$) and cocaine access × session interaction ($F(22, 605) = 6.13$, $P < 0.0001$). In the Saline and ShA groups, the number

of injections for 12 sessions were stable and did not escalate, whereas in the LgA group, it escalated significantly from the 2nd session as confirmed by Dunnett's post hoc test (**Fig. 29d**). Finally, Saline rats responded with an average of 2.16 ± 1.16, 95% CI [1.42, 2.89] inactive lever-presses, ShA rats with 0.67 ± 0.24, 95% CI [0.52, 0.82] inactive lever-presses, and LgA rats did 1.76 ± 1.45, 95% CI [0.84, 2.68]. A two-way ANOVA showed effect of cocaine access ($F(2, 55) = 6.88$, $P = 0.0022$), session ($F(11, 605) = 2.92$, $P = 0.0009$), and access × session interaction ($F(22, 605) = 2.25$, $P = 0.0010$) (**Fig. 29e**). These results indicate that both ShA and LgA rats can learn over the 12 sessions that the inactive lever does not have any consequence. However, Saline rats, which do not receive cocaine, did not learn about contingencies. The results of this study replicated the findings on cocaine self-administration presented in the previous chapters.

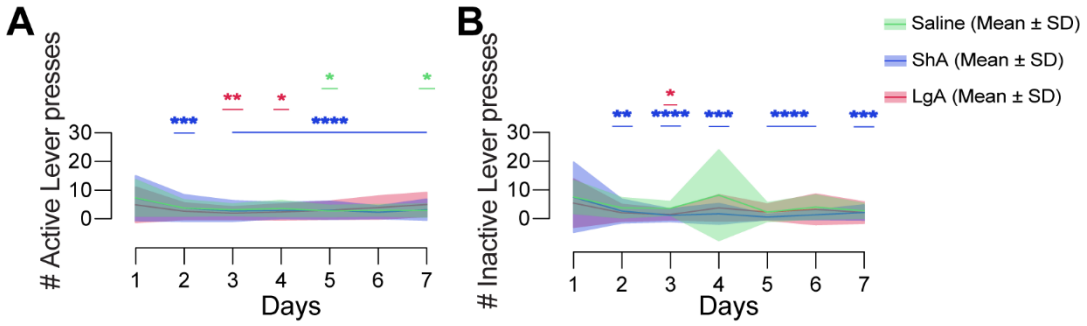

Figure 28. *Training of cocaine/saline self-administration.* ***A)*** *Number of active lever-presses during the training phase in which animals had access to saline (Saline, n = 7) or cocaine (ShA and LgA) for 1 h* ***B)*** *Number of inactive lever-presses during the training phase. ****P < 0.001, ***P < 0.001, **P < 0.01, *P < 0.05 for within-group comparisons against first day of training.*

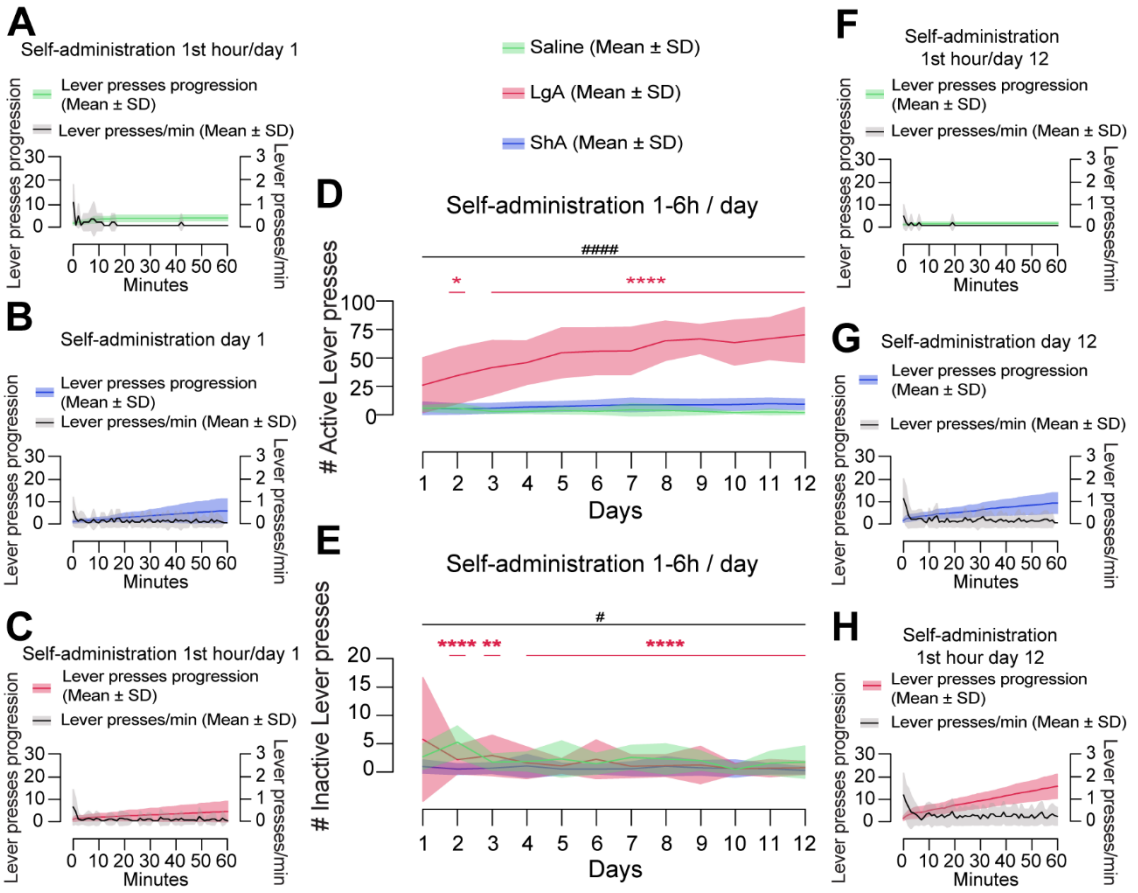

Figure 29. *Cocaine/saline self-administration phase.* ***A)*** *Lever presses progression and Lever presses/min of Saline rats (n=7) during the first hour of the first day of self-administration.* ***B)*** *Lever presses progression and Lever presses/min of ShA rats (n=25) during first day of self-administration.* ***C)*** *Lever presses progression and Lever presses/min LgA rats (n=26) during the first hour of the first day of self-administration.* ***D)*** *Number of active lever-presses during the self-administration phase in which animals had access to saline (Saline) or cocaine (LgA) for 6 h or cocaine for 1h (ShA).* ***E)*** *Number of inactive lever-presses during the self-administration phase.* ***F)*** *Lever presses progression and Lever presses/min of Saline rats during the first hour of the last day of self-administration.* ***G)*** *Lever presses progression and Lever presses/min of ShA rats during the last day of self-administration.* ***H)*** *Lever presses progression and Lever presses/min of LgA rats during the first hour of the last day of self-administration.* *****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 for within-group comparisons against first day of self-administration. ####P < 0.0001, Saline compared to ShA and LgA groups, #P < 0.05 Saline compared to ShA group.*

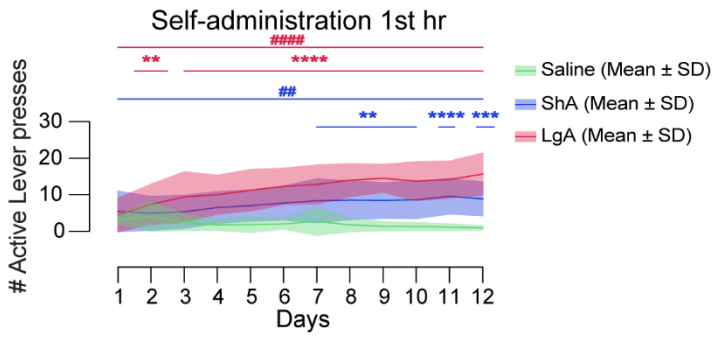

Figure 30. *1st hour active-lever presses during self-administration phase for Saline (n=7), ShA (n=25) and LgA (n=26) rats. ****P < 0.0001, **P < 0.01, *P < 0.05 for within-group comparisons against first day of self-administration. ####P < 0.0001, Saline compared to ShA and LgA groups.*

Enzymatic digestion of PNNs affects incubation of drug-seeking after extended access to cocaine self-administration

Twenty-four hours after the last self-administration session, and before the stereotaxic surgery, rats underwent the first DS-test. In this test, Sham and ChABC groups were identical. Nevertheless, to simplify ulterior comparisons, we present the results of the two groups on this DS test separately. Data are shown as mean ± SD with 95% confidence interval of mean. We are aware that the number of observations for saline groups is small to be compared with the other groups. Thus, we have eliminated saline rats from the global analysis of variance (**Fig. 31**) and present the results in separate figures (**Fig. 32**).

Regarding the average number of active lever presses during trials of the first DS test, Saline-Sham rats (n = 3) did not (0.00 ± 0.00, 95% CI [0.00, 0.00]) emitted any active lever-press on the first (T1) and second (T2) trials, and 0.33 ± 0.58, 95% CI [-1.10, 1.77] on the third trial (T3). Saline-ChABC rats (n = 4) did 2.75 ± 3.59, 95% CI [-2.97, 8.47] active lever-presses on T1, 1.00 ± 1.16, 95% CI [-0.84, 2.84] on T2 and 0.75 ± 0.96, 95% CI [-0.77, 2.27] on T3. ShA-Sham rats (n = 9) did 27.56 ± 10.67, 95% CI [19.71, 35.41] active lever-presses on T1, 10.67 ± 5.81, 95% CI [6.20, 15.13] on T2 and 4.22 ± 3.03, 95% CI [1.89, 6.55] on T3. ShA-ChABC rats (n = 8) did 23.00 ± 15.74, 95% CI [9.84, 36.16] active lever-presses on T1, 10.50 ± 5.13, 95% CI [6.21, 14.79] on T2 and 7.00 ± 6.41, 95% CI [1.64, 12.36] on T3. LgA-Sham rats (n = 9) emitted 9.11 ± 4.94, 95% CI [5.32, 12.91] active lever-presses on T1, 5.11 ± 3.44, 95% CI [2.46, 7.76] T2 and 4.00 ± 4.27, 95% CI [0.72, 7.28] on T3, while LgA-ChABC rats (n = 9) did 9.11 ± 7.39, 95% CI [3.43, 14.79] active lever-presses on T1, 2.89 ± 3.26, 95% CI [0.39, 5.39] on T2 and 2.44 ± 2.13, 95% CI [0.81, 4.08] on T3. (**Fig. 31a-32**).

Therefore, on the first DS test, both ShA and LgA rats exhibited a drug-seeking response over the two first trials although the magnitude of the response seems to be higher in the ShA groups. A three-way ANOVA of repeated measures supports these conclusions and

showed an effect of the trial (F (2, 62) = 48.30 P<0.0001), cocaine access (F (1, 31) = 24.11 P<0.0001) but no effect of ChABC (F (1, 31) = 0.312 P=0.580). Only trial x cocaine access interaction showed a significant effect (F (2, 62) = 13.40 P<0.0001). Neither trial x ChABC interaction F (2, 62) = 0.5724 P=0.567, cocaine access x ChABC (F (1, 31) = 0.0321 P=0.859) nor trial x cocaine schedule x ChABC (F (2, 62) = 1.432 P=0.247) were significant.

Following PNN digestion and 28 abstinence days, rats undergo the second DS-test. Saline-Sham rats (n = 3) did 3.00 ± 1.00, 95% CI [0.52, 5.48] active lever-presses on T1, 1.33 ± 1.53, 95% CI [-2.46, 5.128] on T2 and 0.67 ± 0.58, 95% CI [-0.77, 2.10] on T3. Saline-ChABC rats (n = 4) did 6.75 ± 7.54, 95% CI [-5.26, 18.76] active lever-presses on T1, 1.75 ± 1.71, 95% CI [-0.97, 4.47] on T2 and 1.00 ± 0.82, 95% CI [-0.30, 2.30] on T3. ShA-Sham rats (n = 9) did 23.67 ± 8.93, 95% CI [16.80, 30.53] active lever-presses on T1, 12.22 ± 8.56, 95% CI [5.65, 18.80] on T2 and 6.78 ± 5.81, 95% CI [2.32, 11.24] on T3. ShA-ChABC rats (n = 8) did 26.63 ± 14.61, 95% CI [14.41, 38.84] active lever-presses on T1, 14.25 ± 9.48, 95% CI [6.32, 22.18] on T2 and 10.25 ± 8.70, 95% CI [2.98, 17.52] on T3. LgA-Sham rats (n = 9) did 33.33 ± 13.86, 95% CI [22.68, 43.98] active lever-presses on T1, 23.22 ± 11.12, 95% CI [14.67, 31.77] on T2 and 13.11 ± 7.34, 95% CI [7.46, 18.77] on T3. LgA-ChABC rats (n = 9) did 29.56 ± 10.00, 95% CI [21.87, 37.24] active lever-presses on T1, 12.44 ± 10.11, 95% CI [4.67, 20.22] on T2 and 7.22 ± 4.71, 95% CI [3.60, 10.84] on T3 (**Fig. 31b-32**).

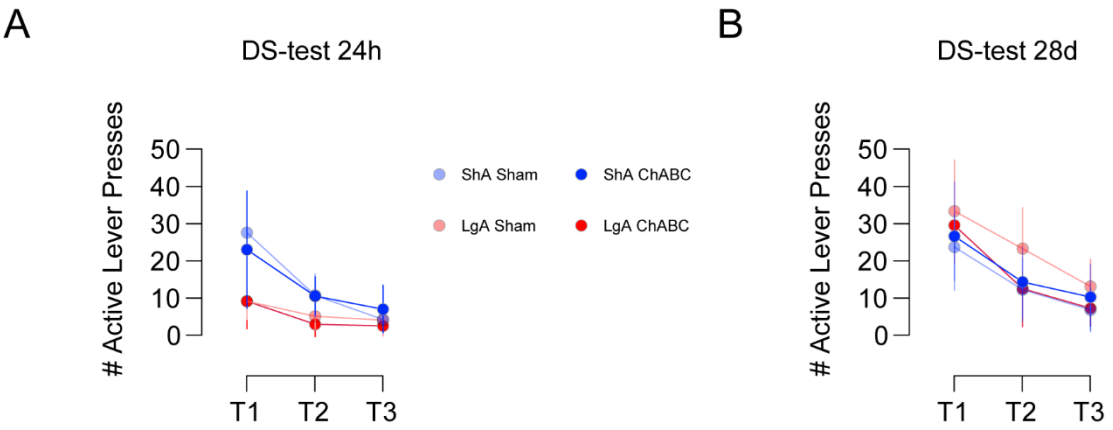

Figure 31. *DS-Test active lever presses (mean ± SD). ShA-Sham (n=9, ShA-ChABC (n=8) rats, LgA-Sham (n=9) and LgA-ChABC (n=9).* ***A)*** *Active-lever presses for first DS-test (24h).* ***B)*** *Active-lever presses for second DS-test (28d).*

After protracted abstinence, both ShA and LgA rats exhibited a clear drug-seeking response that decreased over the trials. A three-way ANOVA of repeated measures for active lever presses yield only an effect of the trial (F (2, 62) = 84.02 P<0.0001). No any

other factor or interaction was significant: cocaine schedule (F (1, 31) = 2.187 P=0.149); ChABC (F (1, 31) = 0.499 P=0.485); trial x cocaine schedule interaction (F (2, 62) = 1.242 P=0.296); trial x ChABC interaction (F (2, 62) = 0.989 P=0.378); cocaine schedule x ChABC (F (1, 31) = 2.900 P=0.099); trial x cocaine schedule x ChABC (F (2, 62) = 0.521 P=0.597). Thus, when all the trials are taken together, it seems that both cocaine groups exhibited incubation of drug seeking, and PNN degradation did not affect drug seeking significantly.

However, since we appreciated that average responses of ShA and LgA rats were different before and after PNN degradation, we decided to further explore these effects and compare both DS tests (24 h/28 days) in each group separately (**Fig. 32**). A two-way ANOVA for the first trial (T1) (ChABC x withdrawal time) in the Saline group showed an effect of withdrawal (F (1, 5) = 8.08, P = 0.0362), but no effect of ChABC (F (1, 5) = 0.98, P = 0.3685), or ChABC x withdrawal interaction (F (1, 5) = 0.16, P = 0.7015) (**Fig. 32a**). As expected, Saline rats did not incubate and PNN removal did not have any impact on behaviour**.** In ShA rats, an ANOVA showed no effect of ChABC (F (1, 15) = 0.02, P = 0.8782), withdrawal (F (1, 15) = 0.00, P = 0.9684), or ChABC x withdrawal interaction (F (1, 15) = 1.32, P = 0.2694) (**Fig. 32d**). Interestingly, although ShA rats showed a strong drug-seeking response, they did not incubate given that drug seeking was very similar across the tests. Finally, in LgA group, the analysis showed no effect of ChABC (F (1, 16) = 0.44, P = 0.5171), a strong effect of withdrawal (F (1, 16) = 61.34, P < 0.0001), but no ChABC infusion x withdrawal interaction (F (1, 16) = 0.44, P = 0.5171) in T1. Sidack post-hoc tests showed significant differences between DS-24h and DS-28d tests bot in LgA-Sham (p < 0.0001) and LgA-ChABC (p = 0.0002) groups (**Fig. 32g**). Extended access to cocaine, which mimics some of the features of drug addiction, caused the animals to incubate drug seeking during protracted abstinence.

For the second trial (T2), a two-way ANOVA showed no effect of ChABC (F (1, 5) = 0.63, P = 0.4642), withdrawal (F (1, 5) = 5.02, P = 0.0752), and for ChABC x withdrawal interaction (F (1, 5) = 0.39, P = 0.5581) in the Saline group (**Fig. 32b**). In the ShA group, the analysis yields no effect of ChABC (F (1, 15) = 0.10, P = 0.7598), withdrawal (F (1, 15) = 1.68, P = 0.2152), or ChABC x withdrawal interaction (F (1, 15) = 0.2865, P = 0.6003) (**Fig. 32e**). Finally, in LgA group, the analysis showed effect of ChABC (F (1, 16) = 5.028, P = 0.0395), an effect of withdrawal (F (1, 16) = 35.44, P < 0.0001), but no ChABC x withdrawal interaction (F (1, 16) = 3.39, P = 0.0843) in T2. Sidack post-hoc tests showed significative differences between DS-24h and DS-28d tests bot in LgA-Sham (p < 0.0001) and LgA-ChABC (p = 0.0204) groups, and between LgA-Sham and

ChABC in DS-28d test (p = 0.0133) but no, as expected, in DS-24h test (p = 0.8010) (**Fig. 32h**). Saline rats continues without showing drug seeking or an incubation effect as expected. In both cocaine groups drug seeking decreased in this second trial. However, ShA rats did not exhibit either incubation or an effect of PNN removal. Only drug seeking in LgA rats demonstrated to incubate and was reduced by the digestion of PNNs.

In the third trial (T3), a two-way ANOVA showed no effect of ChABC ($F(1, 5) = 1.02$, $P = 0.3599$), withdrawal ($F(1, 5) = 0.39$, $P = 0.5581$), or ChABC x withdrawal interaction ($F(1, 5) = 0.01$, $P = 0.9321$) in the Saline group (**Fig. 32c**). In ShA rats, the analysis indicated no effect of ChABC ($F(1, 15) = 1.33$, $P = 0.2671$), but an effect of withdrawal ($F(1, 15) = 4.72$, $P = 0.0463$), however there was no effect of ChABC x withdrawal interaction ($F(1, 15) = 0.07$, $P = 0.7985$) (**Fig. 32f**). Finally, in the LgA group, the analysis yields an effect of ChABC ($F(1, 16) = 4.77$, $P = 0.0442$), an effect of withdrawal ($F(1, 16) = 18.56$, $P = 0.0005$), but no ChABC x withdrawal interaction ($F(1, 16) = 1.81$, $P = 0.1977$) in T3. Sidack pot-hoc test showed significative differences between DS-24h and DS-28d tests in LgA-Sham (p = 0.0021) but no in LgA-ChABC (p = 0.1020) groups, and again between LgA-Sham and ChABC Sidack post-hoc test showed significative differences in DS-28d test (p = 0.0344) but no, as expected, in DS-24h test (p = 0.7619) (**Fig. 32i**). Sham-LgA rats still exhibited incubation of drug seeking at the last DS trial. Importantly, PNN digestion reduced drug-seeking response.

These results indicate that incubation of drug seeking is only seen after extended access to cocaine, and that ChABC infusion, although unable to prevent drug-seeking response, made it decline faster. Remarkably, PNN removal only affected the LgA group, suggesting that PNNs are important for drug-related memory strongly established and may have a role in its consolidation and stability.

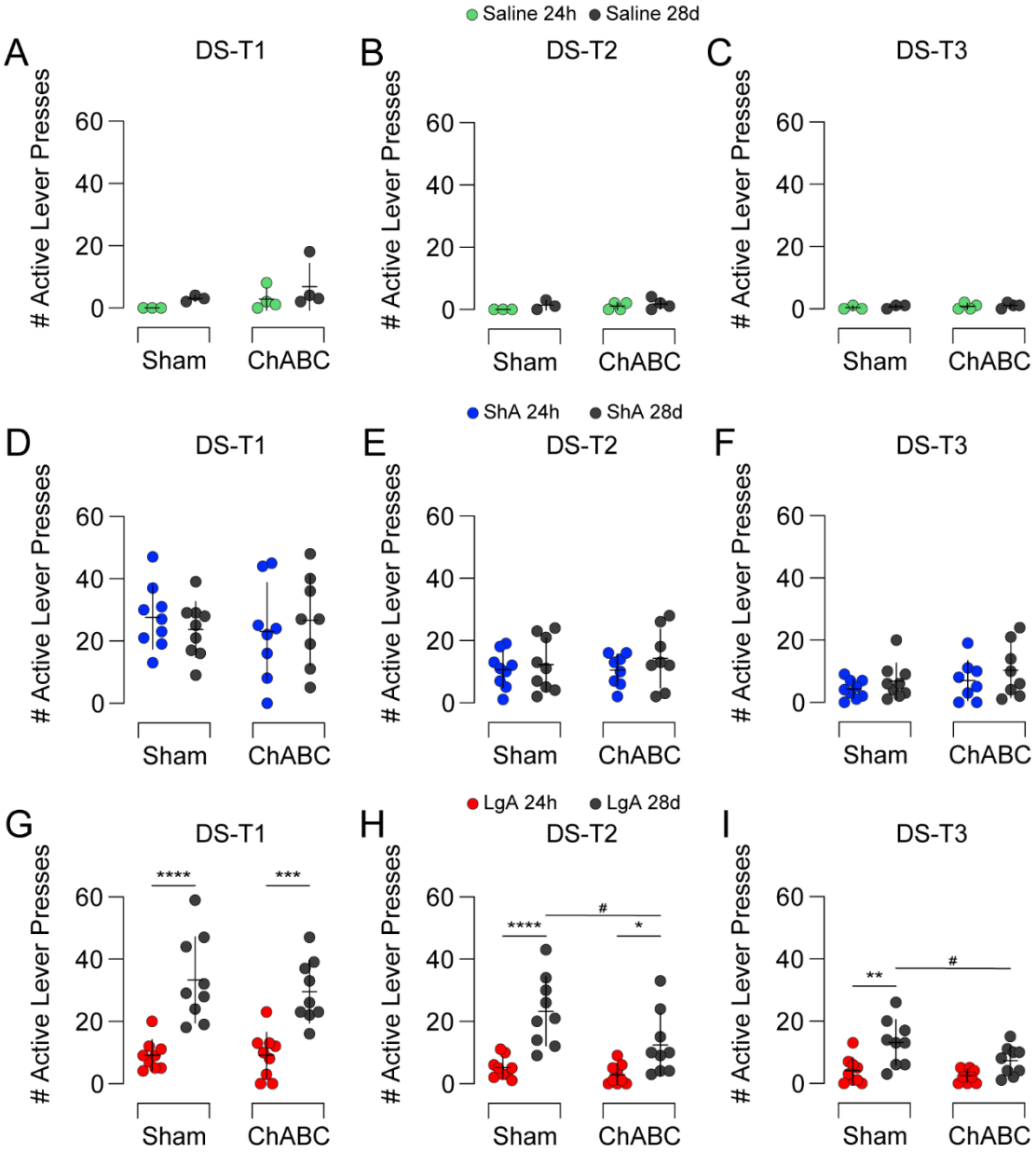

Figure 32. *Dot plots of active-lever presses in DS-test for Saline-Sham (n=3), Saline-ChABC (n=4), ShA-Sham (n=9), ShA-ChABC (n=8), LgA-Sham (n=9) and LgA-ChABC (n=9) rats.* ***A)*** *Saline DS-T1.* ***B)*** *Saline DS-T2.* ***C)*** *Saline DS-T3.* ***D)*** *ShA DS-T1.* ***E)*** *ShA DS-T2.* ***F)*** *ShA DS-T3.* ***G)*** *LgA DS-T1.* ***H)*** *LgA DS-T2.* ***I)*** *LgA DS-T3. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 for within-group comparisons (DS-test day 1 vs day 30). #P < 0.05 Sham compared to ChABC.*

A three-way ANOVA of repeated measures for inactive lever presses in the first DS test showed an effect of the trial (F (2, 62) = 8.65 P=0.0005), cocaine schedule (F (1, 31) = 6.31 P=0.0172) but no effect of ChABC (F (1, 31) = 2.45 P=0.1279). As for active lever comparisons, only trial x cocaine schedule interaction showed a significant effect (F (2,

62) = 3.50 P=0.0362). Neither trial x ChABC interaction F (2, 62) = 1.16 P=0.3216, cocaine schedule x ChABC (F (1, 31) = 2.17 P=0.1511) nor trial x cocaine schedule x ChABC (F (2, 62) = 0.10 P=0.9008) were significant.

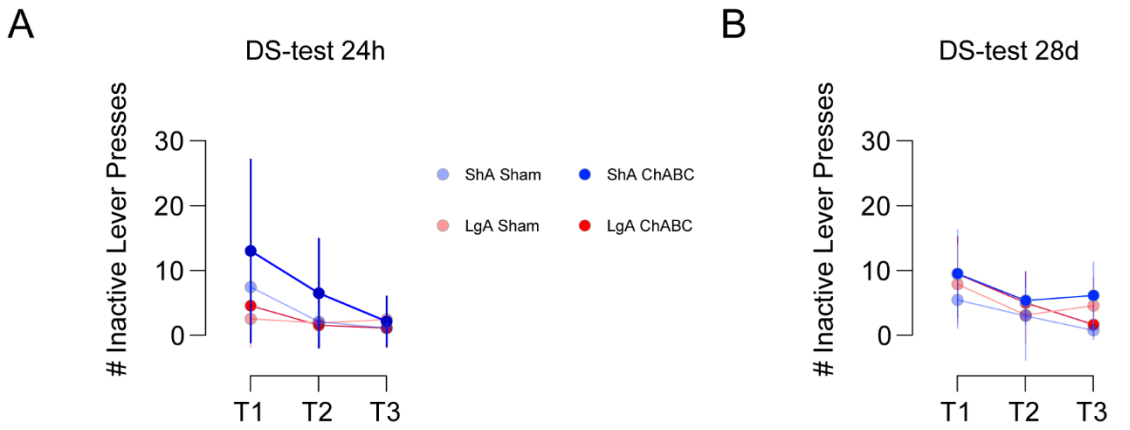

Figure 33. *DS-Test inactive lever presses (mean ± SD). ShA-Sham (n=9), ShA-ChABC (n=8), LgA-Sham (n=9) and LgA-ChABC (n=9) rats.* ***A)*** *Inactive-lever presses for DS-test day 1.* ***B)*** *Inactive-lever presses for DS-test day 30.*

In the second test after 28 withdrawal days, a three-way ANOVA of repeated measures for inactive lever presses showed an effect of the trial (F (2, 62) = 15.51 P<0.0001), but no of cocaine schedule (F (1, 31) = 0.04 P=0.8505) or ChABC (F (1, 31) = 2.63 P=0.1148). Neither trial x cocaine schedule interaction (F (2, 62) = 0.4112 P=0.6646), trial x ChABC interaction F (2, 62) = 0.37 P=0.6914, cocaine schedule x ChABC (F (1, 31) = 2.18 P=0.15) nor trial x cocaine schedule x ChABC (F (2, 62) = 2.40 P=0.0992) were significant.

A two-way ANOVA for inactive lever presses during T1 (ChABC x withdrawal) showed no effect of ChABC (F (1, 5) = 0.92, P = 0.3827), withdrawal (F (1, 5) = 2.14, P = 0.2031), or ChABC x withdrawal interaction (F (1, 5) = 0.95, P = 0.3739) in the Saline group (**Fig. 34a**). In ShA rats, neither main factors nor the interaction were significant: ChABC (F (1, 15) = 2.13, P = 0.1648), withdrawal (F (1, 15) = 1.10, P = 0.3102), or ChABC x withdrawal interaction (F (1, 15) = 0.08, P = 0.7784) (**Fig. 34d**). Finally, in LgA rats, the analysis showed no effect of ChABC (F (1, 16) = 1.04, P = 0.3238), or ChABC x withdrawal interaction (F (1, 16) = 0.015, P = 0.9031), but an effect of withdrawal (F (1, 16) = 8.10, P = 0.0117) (**Fig. 34g**). These results suggest that all cocaine groups but not saline rats discriminate the inactive lever and that PNN removal did not affect the discrimination.

For T2, two-way ANOVA showed no effect of ChABC ($F(1, 5) = 1.75$, $P = 0.2428$), withdrawal ($F(1, 5) = 0.11$, $P = 0.7506$), or for ChABC x withdrawal interaction ($F(1, 5) = 0.11$, $P = 0.7506$) in the Saline group (**Fig. 34b**). In the ShA group, there was no effect of ChABC ($F(1, 15) = 2.15$, $P = 0.1629$), withdrawal ($F(1, 15) = 0.01$, $P = 0.9436$) or ChABC x withdrawal interaction ($F(1, 15) = 0.38$, $P = 0.5485$) (**Fig. 34e**). Finally, no effect of ChABC ($F(1, 16) = 0.3819$, $P = 0.5453$), withdrawal ($F(1, 16) = 3.96$, $P = 0.0639$), or ChABC x withdrawal interaction ($F(1, 16) = 0.8984$, $P = 0.3573$) were observed in the LgA group (**Fig. 34h**).

Finally, in T3, Saline rats did not show an effect of ChABC ($F(1, 5) = 0.02$, $P = 0.8814$), withdrawal ($F(1, 5) = 3.57$, $P = 0.1174$), or ChABC x withdrawal interaction ($F(1, 5) = 0.14$, $P = 0.7210$) (**Fig. 34c**). In the ShA group the analysis showed an effect of ChABC ($F(1, 15) = 8.23$, $P = 0.0117$), but no effect of withdrawal ($F(1, 15) = 2.321$, $P = 0.1484$) or ChABC x withdrawal interaction ($F(1, 15) = 3.24$, $P = 0.0919$) (**Fig. 34f**). In the LgA group, an ANOVA indicated no effect of ChABC ($F(1, 16) = 2.65$, $P = 0.1228$) or ChABC x withdrawal interaction ($F(1, 16) = 2.32$, $P = 0.1473$) but an effect of withdrawal ($F(1, 16) = 6.82$, $P = 0.0189$), (**Fig. 34i**).

At the firs DS-test, Saline-Sham rats (n = 3) did 0.33 ± 0.58, 95% CI [-1.10, 1.77] inactive lever-presses, no responses (0.00 ± 0.00, 95% CI [0.00, 0.00]) in T2, and 1.33 ± 1.57, 95% CI [-1.54, 4.20] in T3. Saline-ChABC rats (n = 4) did 0.25 ± 0.50, 95% CI [--0.55, 1.05] inactive lever-presses in T1, 0.25 ± 0.50, 95% CI [-0.55, 1.05] in T2 and 1.50 ± 1.73, 95% CI [-1.26, 4.26] in T3. ShA-Sham rats (n = 9) did 7.44 ± 6.89, 95% CI [2.15, 12.74] inactive lever-presses in T1, 2.11 ± 2.42, 95% CI [0.25, 3.97] in T2 and 1.11 ± 2.09, 95% CI [-0.49, 2.72] in T3. ShA-ChABC rats (n = 8) did 13.00 ± 14.10, 95% CI [1.21, 24.79] inactive lever-presses in T1, 6.50 ± 8.42, 95% CI [-0.54, 13.54] in T2 and 2.13 ± 3.87, 95% CI [-1.11, 4.36] in T3. LgA-Sham rats (n = 9) emitted 2.56 ± 4.48, 95% CI [-0.88, 6.00] inactive lever-presses in T1, 1.89 ± 2.80, 95% CI [-0.27, 4.04] in T2 and 2.44 ± 3.36, 95% CI [-0.14, 5.03] in T3. LgA-ChABC rats (n = 9) did 4.56 ± 4.77, 95% CI [0.89, 8.22] inactive lever-presses in T1, 1.56 ± 2.07, 95% CI [-0.03, 3.15] in T2 and 1.11 ± 1.27, 95% CI [0.14, 2.09] in T3. (**Fig. 33a-34**).

In the second DS-test, Saline-Sham rats (n = 3) did an average of 1.33 ± 1.53, 95% CI [-2.46, 5.13] inactive lever-presses in T1, 0.00 ± 0.00, 95% CI [0.00, 0.00] in T2 and 0.33 ± 0.58, 95% CI [-1.10, 1.77] in T3. Saline-ChABC rats (n = 4) did 5.25 ± 6.7, 95% CI [-5.41, 15.91] inactive lever-presses in T1, 0.50 ± 1.00, 95% CI [-1.09, 2.09] in T2 and 0.00 ± 0.00, 95% CI [0.00, 0.00] in T3. ShA-Sham rats (n = 9) did 5.44 ± 4.36, 95% CI

[2.09, 8.80] inactive lever-presses in T1, 3.00 ± 6.87, 95% CI [-2.28, 8.28] in T2 and 0.78 ± 1.39, 95% CI [-0.29, 1.85] in T3. ShA-ChABC rats (n = 8) did 9.50 ± 6.78, 95% CI [3.83, 15.17] inactive lever-presses in T1, 5.38 ± 3.70, 95% CI [2.28, 8.47] in T2 and 6.13 ± 5.14, 95% CI [1.83, 10.42] in T3. LgA-Sham rats (n = 9) did 7.89 ± 6.07, 95% CI [3.22, 12.56] inactive lever-presses in T1, 3.11 ± 4.31, 95% CI [-0.21, 6.43] in T2 and 4.56 ± 4.48, 95% CI [1.12, 8.00] in T3. LgA-ChABC rats (n = 9) did 9.44 ± 5.77, 95% CI [5.01, 13.88] inactive lever-presses in T1, 5.00 ± 4.74, 95% CI [1.35, 8.65] in T2 and 1.67 ± 1.41, 95% CI [0.58, 2.75] in T3 (**Fig. 33b-34**).

These results indicate that LgA rats emitted more responses during the first DS test than after 28 days of abstinence, but incubation did not affect inactive lever pressing.

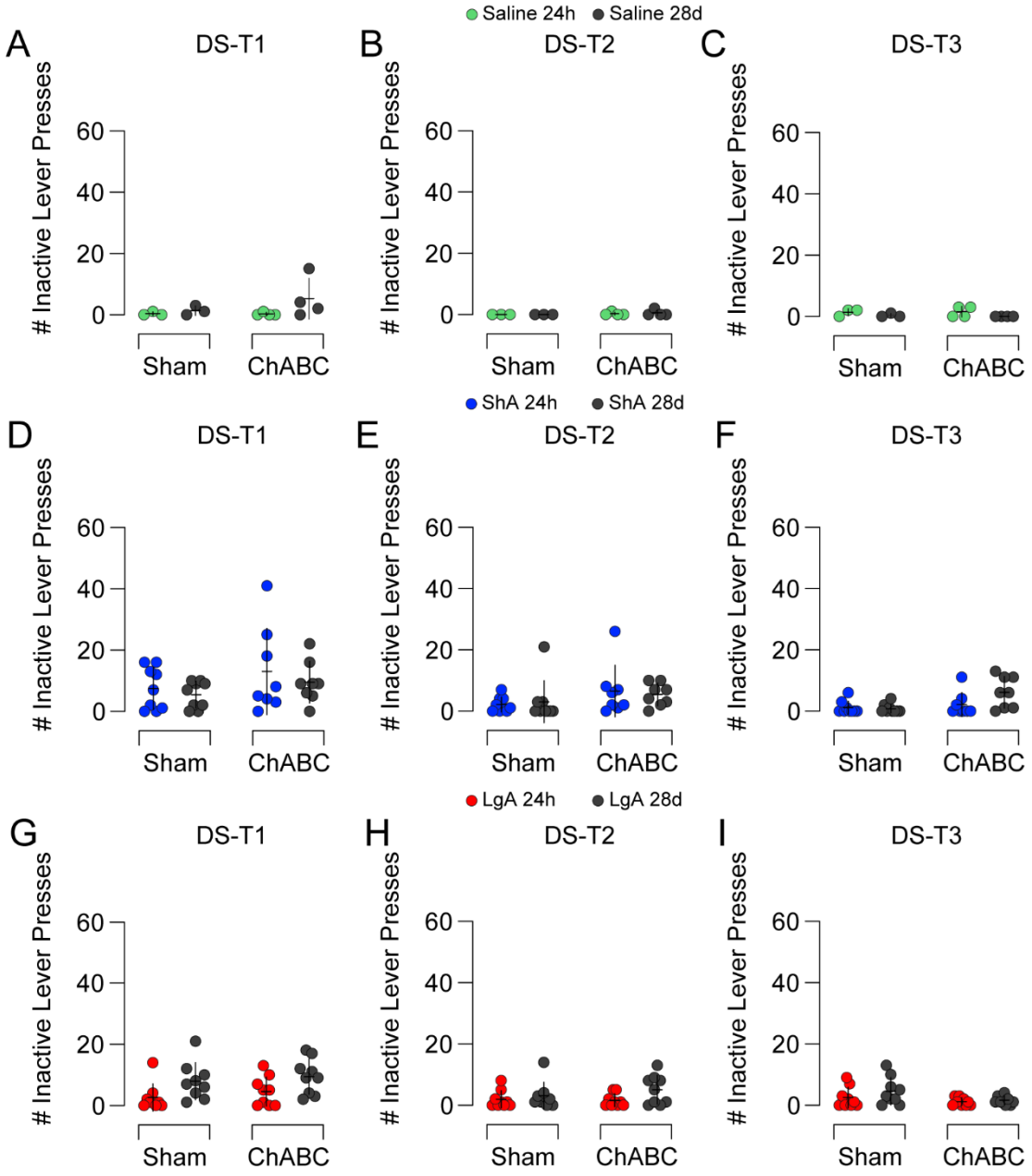

Figure 34. *Dot plots of inactive lever presses in DS-test for Saline-Sham (n=3), Saline-ChABC (n=4), ShA-Sham (n=9) and ShA-ChABC (n=8), LgA-Sham (n=9) and LgA-ChABC (n=9) rats.* ***A)*** *Saline DS-T1.* ***B)*** *Saline DS-T2.* ***C)*** *Saline DS-T3.* ***D)*** *ShA DS-T1.* ***E)*** *ShA DS-T2.* ***F)*** *ShA DS-T3.* ***G)*** *LgA DS-T1.* ***H)*** *LgA DS-T2.* ***I)*** *LgA DS-T3. *P < 0.05 for within-group comparisons (DS-test day 1 vs day 30). ##P < 0.01, Sham compared to ChABC.*

ChABC enzymatic digestion effectively reduced PNN expression during protracted abstinence in the cerebellar cortex

To characterise the effects of ChABC infusion in LVII of the cerebellum during protracted abstinence, we compared WFA intensity and integrity around Golgi interneurons before (24h) and after digestion (28d) for each group. Data are presented as mean± SD and 95% of confidence intervals of the mean.

WFA intensity for ShA rats was: At 24h: 66.53 ± 8.59, 95% CI [59.35, 73.71] (n = 8); and at 28 days: Sham: 79.56 ± 9.46, 95% CI [72.28, 86.83] (n = 9); ChABC: 49.00 ± 10.31, 95% CI [40.38, 57.62]) (n = 8). WFA intensity for LgA rats at 24h: 69.25 ± 9.05, 95% CI [61.68, 76.82] (n = 8); and at 28 days: Sham: 119.11 ± 22.05, 95% CI [102.16, 136.06] (n = 9); ChABC: 70.11 ± 15.43, 95% CI [58.25, 81.97]) (n = 9). Cerebella from Saline rats were only processed at 28 withdrawal days: (Sham: 89.33 ± 8.08, 95% CI [69.25, 109.41] (n = 3); ChABC: 70.50 ± 19.23, 95% CI [39.91, 101.09]) (n = 4).

Saline rats did not show effect of ChABC infusion as Mann-Whitney indicated ($U= 2$, $P = 0.229$). Both ShA and LgA groups showed an effect of ChABC infusion: ShA ($F (2, 22) = 22.06$, $P < 0.0001$), LgA ($F (2, 23) = 25.94$, $P < 0.0001$) (**Fig. 35b**) (**Fig. 36b**). In ShA groups, Tukey post-hoc analysis showed significant differences between ShA-24h and ShA-28d (Sham: $p = 0.0255$) and (ChABC: $p = 0.0034$), and between Sham and ChABC ($p < 0.0001$) in the ShA-28d rats (**Fig. 35b**). In LgA groups, Tukey post-hoc analysis showed differences between LgA-24h and LgA-28d Sham ($p < 0.0001$), however not between LgA-24h and LgA-28d ChABC ($p = 0.9938$), and yes between LgA-28d Sham and ChABC ($p < 0.0001$) (**Fig. 36b**). Thus, PNNs were effectively digested during abstinence and PNN degradation was still evident at the end of the abstinence period.

We also wondered whether cocaine access might interact with ChABC effects. A two-way ANOVA (cocaine access × ChABC) yields differences for cocaine access ($F (2, 36) = 17.01$, $P < 0.0001$) and ChABC infusion ($F (1, 36) = 39.38$, $P < 0.0001$) but not for the interaction between these two factors ($F (2, 36) = 2.94$, $P = 0.0656$). The absence of a significant interaction indicates that PNN structure was remodelled through abstinence in all groups.

In addition, the distribution of strong, medium and faint PNNs in the cerebellar cortex was different between groups. PNN proportions in each category was different in ShA-Sham ($\chi2 (1) = 5.07$, $P = 0.0244$) and ShA-ChABC ($\chi2 (1) = 18.59$, $P < 0.0001$) rats (**Fig. 35c**). Analysis of PNN proportions showed differences in LgA-Sham ($\chi2 (1) = 73.11$, $P < 0.0001$) but no in LgA-ChABC ($\chi2 (1) = 0.48$, $P = 0.4896$) (**Fig. 36c**).

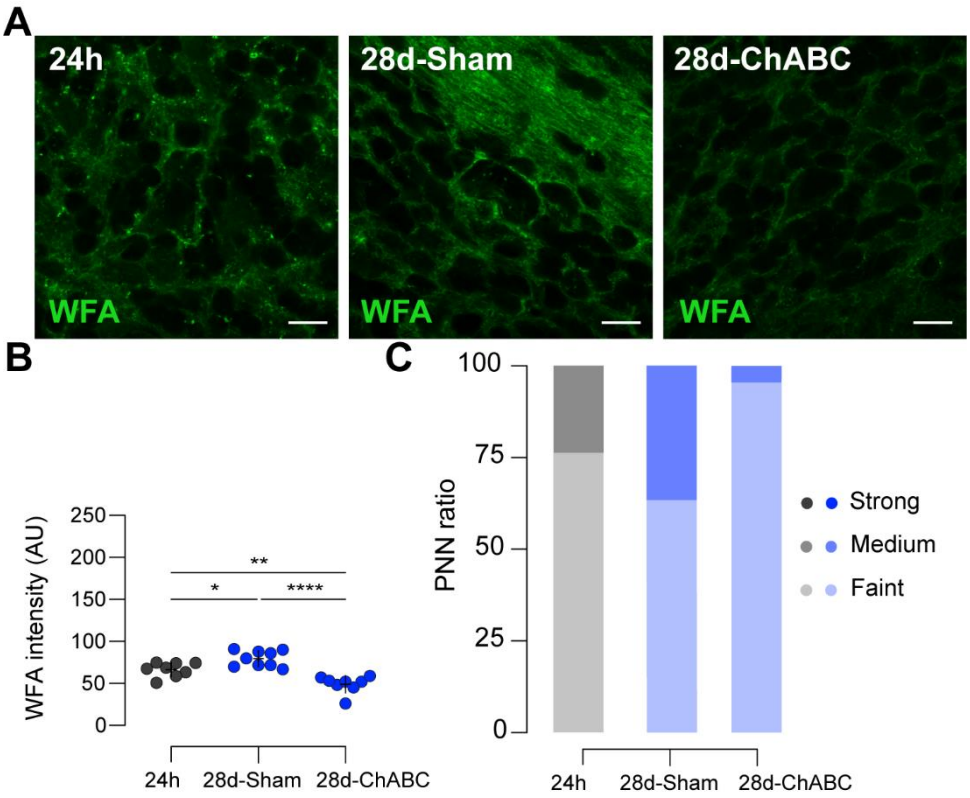

Figure 35. *PNN expression in the cerebellar cortex for the ShA group before (24h) and after digestion of PNNs (28d) in Sham or ChABC groups* ***A)*** *Representative confocal PNN images (× 120) of ShA-24h (24h) (n = 8), ShA-Sham (28d-Sham) (n = 9), ShA-ChABC (28d-ChABC) (n = 8) groups. Scale bar 10 µm.* ***B)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on WFA intensity in PNNs around Golgi interneurons of the cerebellar cortex for ShA-24h (24h), ShA-Sham (28d-Sham) and ShA-ChABC (28d-ChABC). Plots indicate mean and SD. The Y-axis represents WFA intensity expressed in arbitrary units (AU). P < 0.05, **P < 0.01, ***P < 0.001 for within group comparisons.* ***C)*** *Proportion of Golgi neurons expressing a faint, medium, or strong PNNs in the cerebellar cortex. ChABC infusion decreased PNN expression in the cerebellar cortex after 28d of cocaine abstinence.*

24h after cocaine self-administration ShA rats expressed 76.7% of faint and 23.3% of medium PNNs, while LgA rats expressed 73% of faint and 27% medium PNNs. After protracted abstinence, Saline-Sham rats expressed 51.1% of faint and 48.9% of medium PNNs, and Saline-ChABC rats expressed 66.7% of faint and 33.3% of medium PNNs. In ShA-Sham rats, the percentage of faint PNNs decreased to 63.7% while medium PNNs increased to 36.3%. In the ShA-ChABC group, faint PNNs increased to 95.8 % and medium PNNs decreased to 4.2 %. In cocaine extended access groups, abstinence reduced the proportion of faint PNNs up to 16% but enhanced the proportion of medium

(72%) and strong PNNs (12%) in LgA-Sham rats. However, in LgA-ChABC rats, faint PNNs represented 69% and the rest of PNNs were of medium intensity (31%).

In addition, we assessed **PNN integrity** before and after ChABC infusion (**Fig. 37**). We found that ChABC infusions decrease PNN integrity in all groups, independently of saline or cocaine self-administration. A two-way ANOVA (cocaine access × ChABC) did not show differences for cocaine access ($F(2, 36) = 2.95$, $P = 0.0652$), there was an effect of ChABC infusion ($F(1, 36) = 14.93$, $P = 0.0004$) but no effect of interaction between these two factors ($F(2, 36) = 2.18$, $P = 0.1276$).

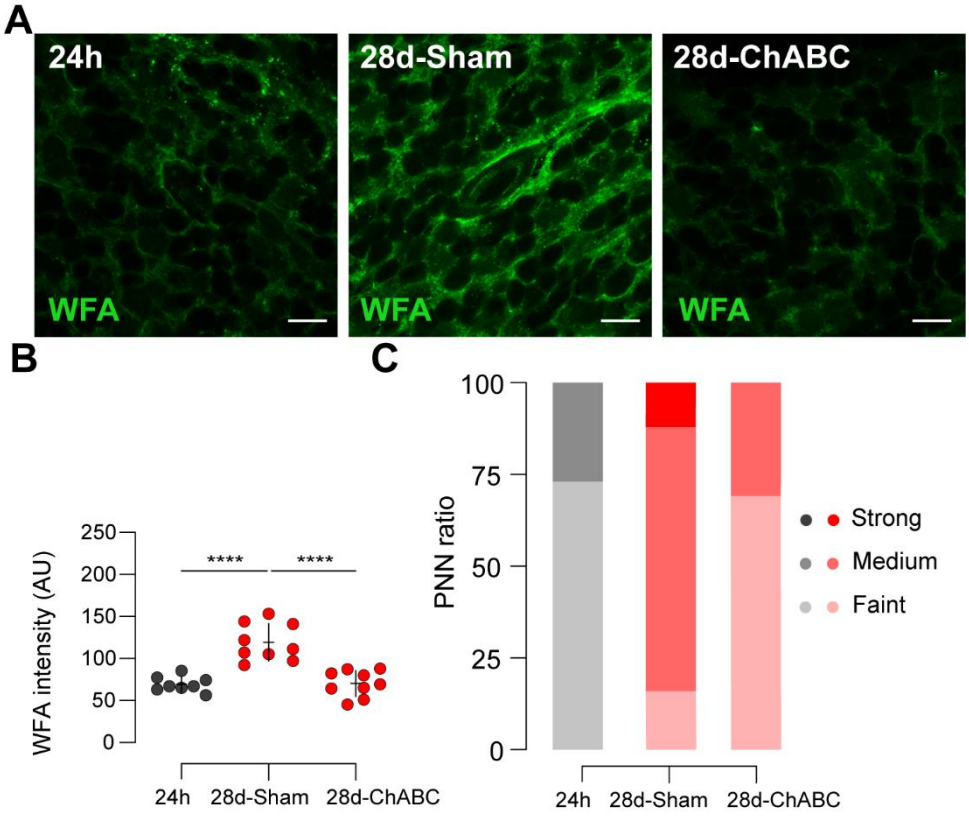

Figure 36. *PNN expression in the cerebellar cortex for the LgA group before (24h) and after digestion of PNNs (28 days) in Sham or ChABC groups* ***A)*** *Representative confocal PNN images (× 120) of LgA-24h (24h) (n = 8), LgA-Sham (28d-Sham) (n = 9), LgA-ChABC (28d-ChABC) (n = 8) groups. Scale bar 10 µm.* ***B)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on WFA intensity in PNNs around Golgi interneurons of the cerebellar cortex for LgA-24h (24h), LgA-Sham (28d-Sham) and LgA-ChABC (28d-ChABC). Plots indicate mean and SD The Y-axis represents WFA intensity expressed in arbitrary units (AU). ****P < 0.0001 for within group comparisons.* ***C)*** *Proportion of Golgi neurons expressing a faint, medium, or strong PNNs in the cerebellar cortex. ChABC infusion decreased PNN expression in the cerebellar cortex after 28d of cocaine abstinence.*

One-way ANOVA showed an effect of ChABC infusion in ShA ($F(2, 22) = 12.71$, $P = 0.0002$) and LgA ($F(2, 23) = 10.16$, $P = 0.0007$) rats (**Fig. 37b, c**). In ShA groups, Tukey post-hoc analysis did not show differences between ShA-24h and ShA-28d in Sham rats ($p = 0.1967$), but it showed differences between ShA-24h and ShA-28d in the ChABC group ($p = 0.0132$), and between Sham and ChABC in ShA-28d ($p = 0.0002$) (**Fig. 37b**). Tukey post-hoc analysis on PNN integrity in LgA groups revealed differences between LgA-24h and LgA-28d in Sham animals ($p = 0.0021$), but not between LgA-24h and LgA-28d in the ChABC rats ($p = 0.9941$), although ChABC reduced PNN integrity as compared to Sham animals at 28 days ($p = 0.0020$). Again, the effect of ChABC was more evident in the ShA group as confirmed by Tukey post-hoc tests (**Fig. 37c**).

Data are show as mean ± SD and 95% CI: Saline (Sham: 2.57 ± 0.21, 95% CI [2.04, 3.09] (n = 3); ChABC: 2.37 ± 0.58, 95% CI [2.04, 3.09]) (n = 4). ShA (24h: 2.42 ± 0.33, 95% CI [2.14, 2.70] (n = 8); Sham: 2.72 ± 0.40, 95% CI [2.41, 3.02] (n = 9); ChABC: 1.89 ± 0.28, 95% CI [1.65, 2.12]) (n = 8). LgA groups (24h: 2.10 ± 0.49, 95% CI [1.69, 2.52] (n = 8); Sham: 3.42 ± 1.07, 95% CI [2.60, 4.24] (n = 9); ChABC: 2.14 ± 0.22, 95% CI [1.97, 2.31]) (n = 9).

These results indicate that ChABC infusion successfully reduced PNN expression in LVII throughout the whole abstinence period. Nevertheless, given that PNN expression increased over withdrawal in LgA rats, ChABC only was able to partially reduce PNNs.

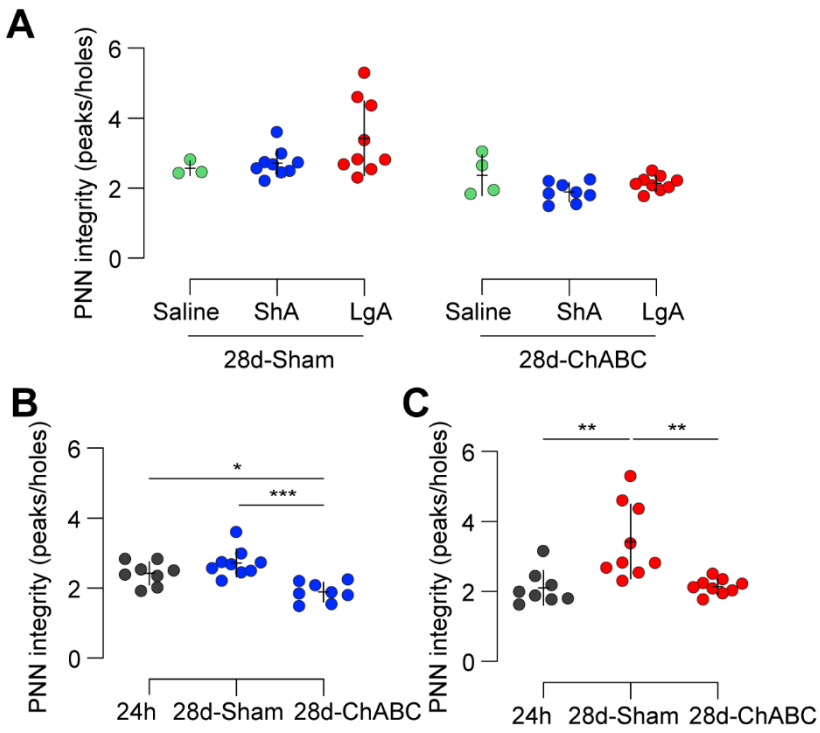

Figure 37. *PNN integrity in the cerebellar cortex.* ***A)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on PNN integrity around Golgi interneurons of the cerebellar cortex after 28d of saline or cocaine (ShA and LgA) abstinence in Sham (28d-Sham) versus ChABC (28d-ChABC) rats.* ***B)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on PNN integrity in ShA rats after 24h and 28d (Sham and ChABC) abstinence periods.* ***C)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on PNN integrity in LgA rats after 24h and 28d (Sham and ChABC) abstinence periods. Saline Sham (n = 3), Saline ChABC (n = 4), 24h ShA (n = 8), ShA Sham (n = 9), ChABC Sham (n = 8), 24 h LgA (n = 8), LgA Sham (n = 9), and LgA ChABC (n = 9). Plots indicate mean and SD. The Y-axis represents PNN integrity expressed in peaks/holes. *P < 0.05, **P < 0.01, ***P < 0.001 for within group comparisons.*

Purkinje C-Fos expression in LVII was not affected after ChABC enzymatic digestion of PNNs

To estimate the effect of ChABC infusion on neural activity, we assessed C-Fos expression in the Purkinje cell layer (C-Fos+ cells/mm^2) in all our rats. A two-way ANOVA (cocaine access × ChABC) did not show significant differences for cocaine access ($F(2, 36) = 0.49$, $P = 0.6157$), ChABC infusion ($F(1, 36) = 1.63$, $P = 0.2092$) or the interaction between these two factors ($F(2, 36) = 0.32$, $P = 0.7311$) (**Fig. 38a**). Then, we compared C-Fos expression in Purkinje cells before and after ChABC infusion in each group. A one-way ANOVA did not reveal an effect of ChABC infusion either in ShA ($F(2, 22) = 0.96$, $P = 0.3993$) or LgA ($F(2, 23) = 1.67$, $P = 0.2102$) rats (**Fig. 38b, c**).

Data are shown as mean ± SD and 95% CI: Saline (Sham: 56.00 ± 20.13, 95% CI [5.98, 106.01] (n = 3); ChABC: 80.00 ± 15.40, 95% CI [55.50, 104.50]) (n = 4). ShA (24h: 73.33 ± 22.45, 95% CI [54.57, 92.10] (n = 8); Sham: 52.44 ± 33.54, 95% CI [26.66, 78.22] (n = 9); ChABC: 62.33 ± 35.26, 95% CI [32.86, 91.81]) (n = 8). LgA groups (24h: 82.67 ± 13.30, 95% CI [71.55, 93.78] (n = 8); Sham: 63.70 ± 26.07, 95% CI [43.66, 83.75] (n = 9); ChABC: 67.26 ± 24.94, 95% CI [48.09, 86.43]) (n = 9).

These results suggest that the removal of PNNs in LVII does not affect Purkinje cell activity.

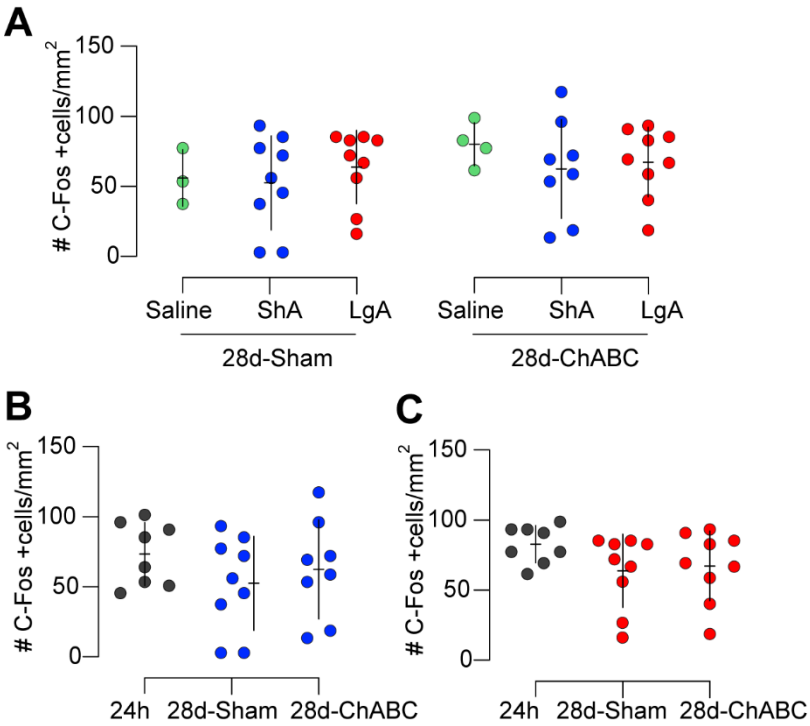

Figure 38. *Purkinje C-Fos+ cells/mm² in lobule VII.* ***A)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on C-Fos+ cells/mm² after 28d of saline or cocaine (ShA and LgA) abstinence in Sham (28d-Sham) versus ChABC (28d-ChABC) rats.* ***B)*** *Scatter plots of individual scores show C-Fos+ cells/mm² in Purkinje cells of ShA rats before (24h) and after (28 days) PNN digestion* ***C)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on C-Fos+ cells/mm² in LgA rats before (24h) and after (28 days) PNN digestion. Saline Sham (n = 3), Saline ChABC (n = 4), 24h ShA (n = 8), ShA Sham (n = 9), ChABC Sham (n = 8), 24 h LgA (n = 8), LgA Sham (n = 9), and LgA ChABC (n = 9). Plots indicate mean and SD. The Y-axis represents neural activity expressed as C-Fos+ cells/mm².*

C-Fos expression in the granule cell layer increased after protracted abstinence but it was not affected by PNN enzymatic digestion

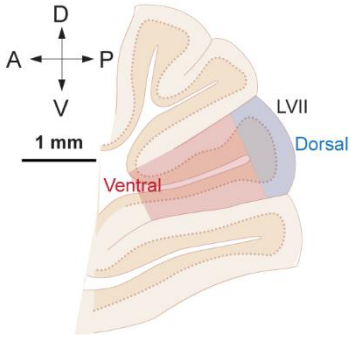

Figure 39. *Schematic diagram of a sagittal section of the vermis depicting the regions of lobule VII (LVII), dorsal (blue) and ventral (red).*

To further understand the effects of ChABC infusion on the neural activity, we analysed C-Fos expression in the granule cell layer at the dorsal and ventral regions of LVII (**Fig. 39**). A two-way ANOVA for the dorsal region of lobule VII (cocaine access × ChABC) did not show significant differences for cocaine access ($F(2, 36) = 1.67$, $P = 0.2024$), ChABC infusion ($F(1, 36) = 0.63$, $P = 0.4312$) or the interaction between these two factors ($F(2, 36) = 2.51$, $P = 0.0957$) (**Fig. 40a**). A one-way ANOVA for the dorsal region showed a tendency to increase neural activity in ShA rats after PNN digestion ($F(2, 22) = 3.17$, $P = 0.0619$) (**Fig. 40b**). Extended access to cocaine self-administration increased C-Fos expression in the granule cell layer independently of PNN degradation ($F(2, 23) = 14.32$, $P < 0.0001$). This was confirmed by Tukey post-hoc analysis which showed significative differences between LgA-24h and LgA-28d in Sham rats ($p < 0.0001$), and between LgA-24h and LgA-28d in ChABC animals ($p < 0.0083$), and no differences between Sham and ChABC in LgA-28d rats ($p = 0.1180$) (**Fig. 40c**).

Data from the dorsal region of LVII are presented as mean ± SD and 95% CI of the mean for: Saline rats (Sham: 2477.32 ± 1541.56, 95% CI [-1352.13, 6306.78] (n = 3); ChABC: 3456.63 ± 993.63, 95% CI [1875.55, 5037.71]) (n = 4). ShA rats (24h: 2262.00 ± 1058.00, 95% CI [1377.00, 3147.00] (n = 8); Sham: 3069.41 ± 1587.71, 95% CI [1848.99, 4289.83] (n = 9); ChABC: 3883.22 ± 1103.39, 95% CI [2960.76, 4828.68]) (n = 8). LgA groups (24h: 987.80 ± 250.30, 95% CI [778.50, 1197.00] (n = 8); Sham: 3148.14 ± 814.69, 95% CI [2521.92, 3774.37] (n = 9); ChABC: 2331.82 ± 1135.79, 95% CI [1458.78, 3204.87]) (n = 9).

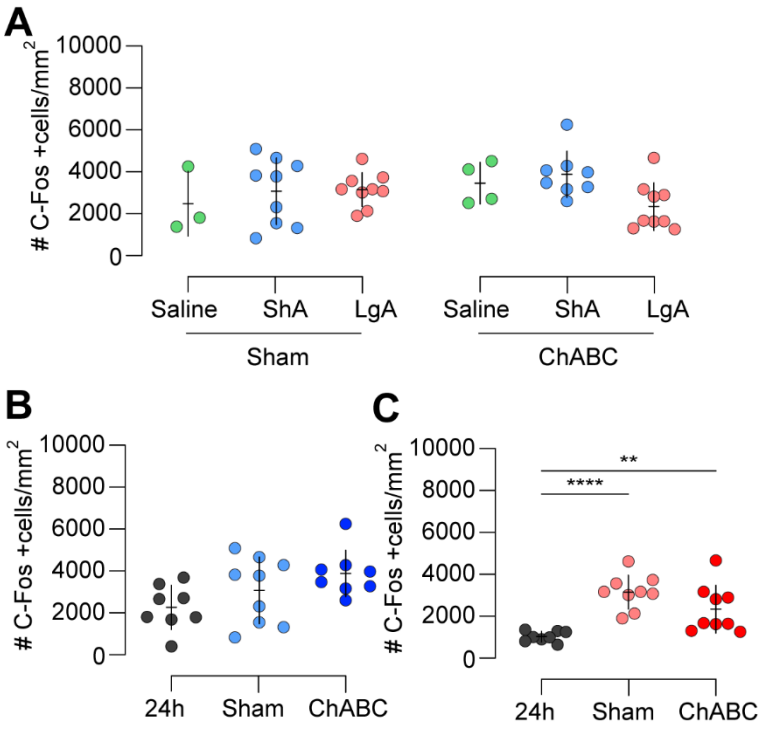

Figure 40. *C-Fos+ cells/mm² in the granule cell layer of lobule VII (Dorsal)* ***A)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on C-Fos+ cells/mm² after 28d of abstinence* ***B)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on C-Fos+ cells/mm² in ShA rats before and after PNN digestion* ***C)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on C-Fos+ cells/mm² in LgA rats before and after PNN digestion. Saline Sham (n = 3), Saline ChABC (n = 4), 24h ShA (n = 8), ShA Sham (n = 9), ChABC Sham (n = 8), 24 h LgA (n = 8), LgA Sham (n = 9), and LgA ChABC (n = 9). Plots indicate mean and SD. The Y-axis represents neural activity expressed as C-Fos+ cells/mm². **P < 0.01, ****P < 0.0001 for within group comparisons.*

In the ventral region, a two-way ANOVA (cocaine access × ChABC) showed a significant effect of cocaine access (F (2, 36) = 3.71, P = 0.0343), but it did not show any effect of ChABC infusion (F (1, 36) = 1.05, P = 0.3132) or interaction between these two factors (F (2, 36) = 0.52, P = 0.6006) (**Fig. 41a**). One-way ANOVA for ventral granule C-Fos+ cells expression indicated a significant effect for the ShA (F (2, 22) = 3.76, P = 0.0395) (**Fig. 41b**) and LgA (F (2, 23) = 7.23, P = 0.0037) groups that was independent of PNN digestion. This was confirmed by Tukey post-hoc analysis which showed significative differences between LgA-24h and LgA-28d in Sham rats (p = 0.0491), and between LgA-24h and LgA-28d in ChABC rats (p < 0.0029). Sham and ChABC were no different in LgA-28d rats (p = 0.4228) (**Fig. 41c**).

Data is presented as mean ± SD and 95% CI of mean for: Saline rats (Sham: 1088.44 ± 1003.15, 95% CI [-1403.53, 3580.40] (n = 3); ChABC: 1594.39 ± 761.00, 95% CI [383.47, 2805.305]) (n = 4). ShA rats (24h: 1419.00 ± 662.40, 95% CI [865.30, 1973.00] (n = 8); Sham: 2346.22 ± 839.69, 95% CI [1700.78, 2991.66] (n = 9); ChABC: 2274.66 ± 763.06, 95% CI [1636.73, 2912.60]) (n = 8). LgA groups (24h: 1127.00 ± 276.40, 95% CI [895.30, 1358.00] (n = 8); Sham: 2149.47 ± 554.68, 95% CI [1723.11, 2575.83] (n = 9); ChABC: 2653.06 ± 1281.56, 95% CI [1667.97, 3638.15]) (n = 9).

These results suggest that neural activity increased over abstinence in granule cells after cocaine self-administration and that removal of PNNs had no effect.

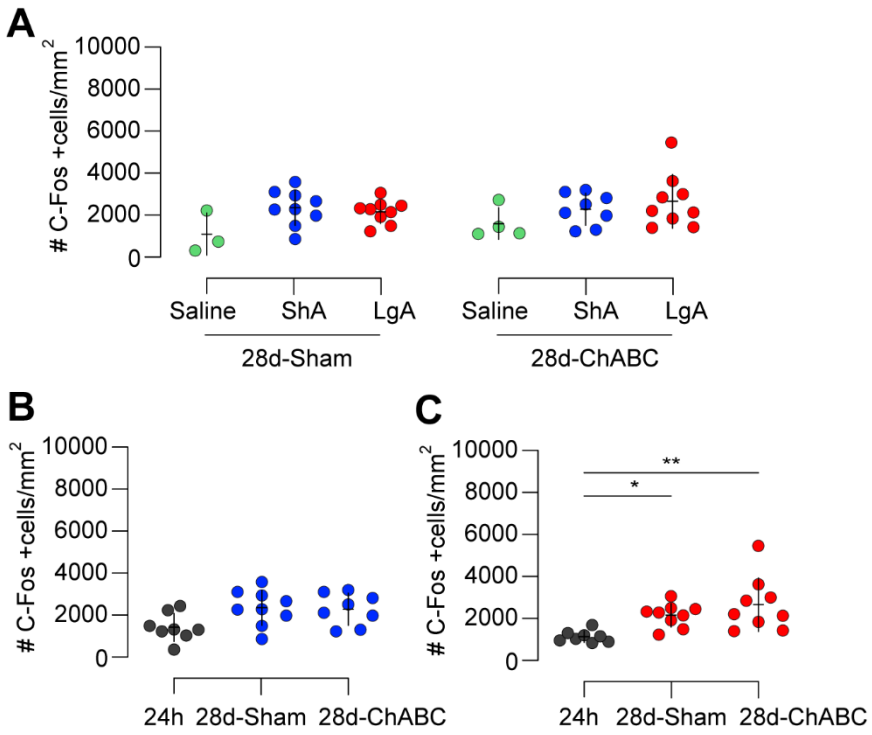

Figure 41. *C-Fos+ cells/mm² in the granule cell layer of lobule VII (Ventral)* ***A)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on C-Fos+ cells/mm² after 28d of abstinence* ***B)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on C-Fos+ cells/mm² in ShA rats before and after PNN digestion* ***C)*** *Scatter plots of individual scores show the effects of chondroitinase infusion on C-Fos+ cells/mm² in LgA rats before and after PNN digestion. Saline Sham (n = 3), Saline ChABC (n = 4), 24h ShA (n = 8), ShA Sham (n = 9), ChABC Sham (n = 8), 24 h LgA (n = 8), LgA Sham (n = 9), and LgA ChABC (n = 9). Plots indicate mean and SD. The Y-axis represents neural activity expressed as C-Fos+ cells/mm². *P < 0.05, **P < 0.001 for within group comparisons.*

Intensity and integrity of PNNs in the cerebellar cortex positively correlated

For correlations we used Spearman r correlation coefficient (rho) (**Fig. 42**). First, we pulled all the animals perfused 24h after the last cocaine self-administration (**Fig. 42a**). Then, we tested this relationship after protracted abstinence (**Fig. 42b, c**). As expected, we observed a positive relationship between PNN intensity and integrity except for the LgA-24h group (rho = 0.40 [-0.51, 1.32], P = 0.3268) (**Fig. 42a**), in which there was a decorrelation between both variables in some animals.

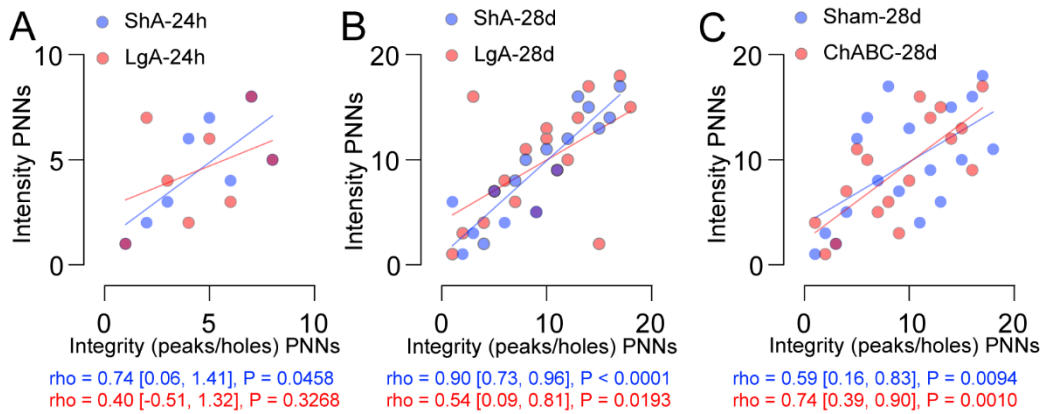

Figure 42. *Intensity PNNs Vs integrity of PNNs and Spearman r correlation coefficients (rho) with line of best fit* ***A)*** *PNN expression and integrity in LVII before PNN degradation (24h) in ShA (n = 8) and LgA (n=8) groups.* ***B)*** *PNN intensity and integrity in lobule VII after 28-days abstinence in ShA (n = 17) and LgA (n=18) groups.* ***C)*** *PNN intensity and integrity in LVII after 28d abstinence (28d) in Sham (n = 18) and ChABC (n=17) groups.*

PNN intensity correlated with active lever presses in DS-test 24 hours after extended access to cocaine self-administration

To determine whether there is a relationship between the active lever presses performed in the DS-24h and the intensity of PNNs, we assessed the correlation between intensity of PNNs and DS (24h) in T1 and in T3 (**Fig. 43a, b**). PNN intensity correlated with lever pressing in the trial 3 of the first tests after extended access to cocaine (LgA-24h) (rho = 0.86 [0.34, 1.37], P = 0.0107).

Then, we correlated PNN intensity with lever pressing during the DS test after PNN removal and long-term abstinence in T1 and T3 (**Fig. 44**) We did not see any correlation between these two variables.

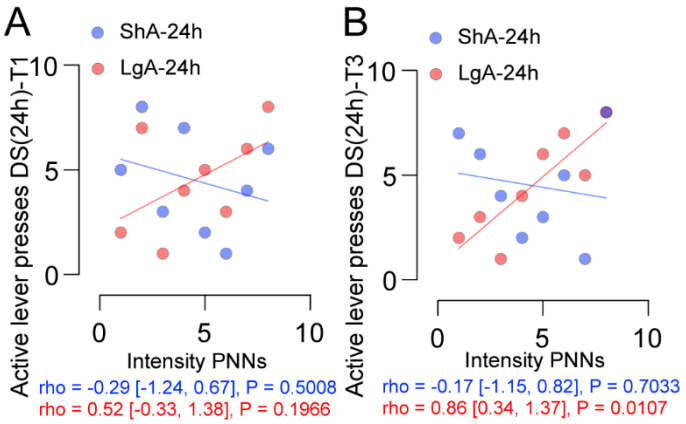

Figure 43. *Active lever presses in DS-test at 24h (T1-T3) performed for ShA and LgA Vs PNN expression (Intensity PNNs) scatterplots and Spearman r correlation coefficients (rho) with line of best fit* ***A)*** *DS (24h)-T1 Vs PNN intensity in lobule VII after short-term abstinence (24h) in ShA-24h (n = 8) and LgA-24h (n=8) groups.* ***B)*** *DS (24h)-T3 Vs PNN expression in LVII after short-term abstinence (24h) in ShA-24h (n = 8) and LgA-24h (n=8) groups.*

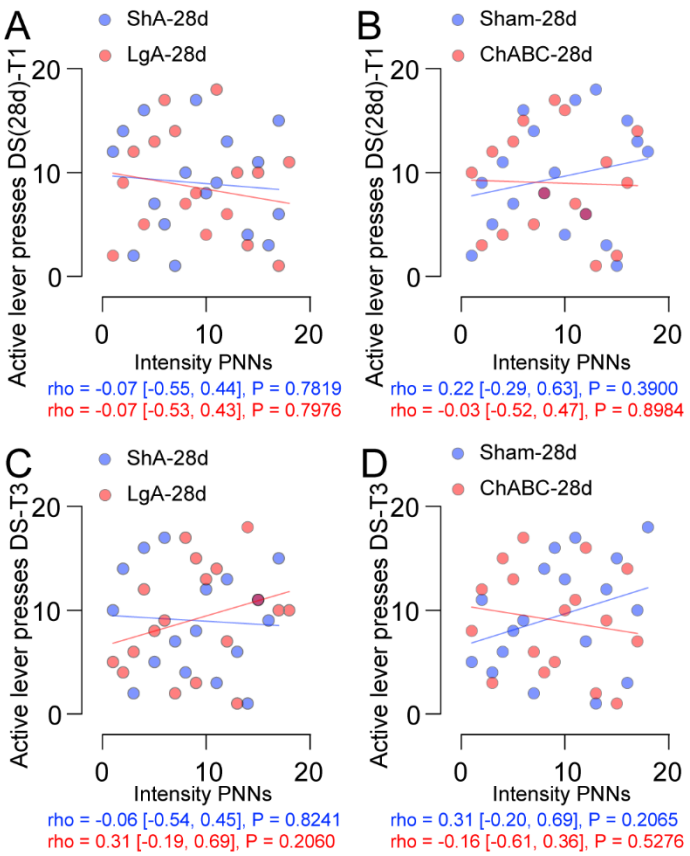

Figure 44. *Active lever presses in DS-test at 28d (T1-T3) performed for ShA and LgA Vs PNN expression (Intensity PNNs) scatterplots and Spearman r correlation coefficients (rho) with line of best fit* ***A)*** *DS (28d)-T1 Vs PNN intensity in LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***B)*** *DS (28d)-T1 Vs PNN expression in LVII long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17) rats.* ***C)*** *DS (28d)-T3 Vs PNN intensity in LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***D)*** *DS (28d)-T3 Vs PNN expression in lobule VII long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17) rats.*

PNN integrity does not correlate with active lever presses in DS-test

Regarding the correlation between PNN integrity and active lever pressing lever pressing, we did not see any relationship at any time explored. (**Fig. 45-46**).

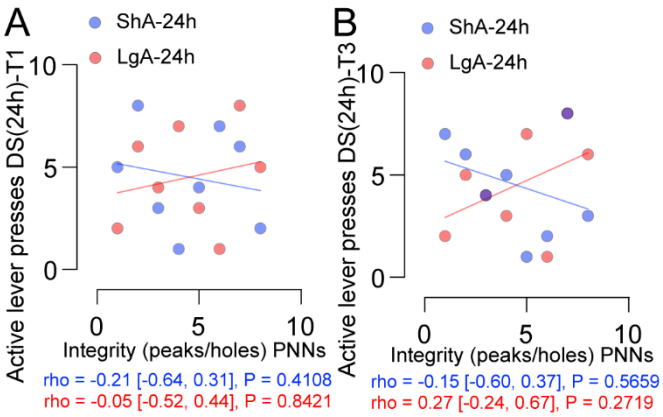

Figure 45 *Active lever presses in DS-test at 24h (T1-T3) performed for ShA and LgA Vs PNN integrity (Integrity (peaks/holes) PNNs) scatterplots and Spearman r correlation coefficients (rho) with line of best fit* ***A)*** *DS (24h)-T1 Vs PNN integrity (Integrity (peaks/holes) PNNs) in LVII after short-term abstinence (24h) in ShA-24h (n = 8) and LgA-24h (n=8) groups.* ***B)*** *DS (24h)-T Vs PNN integrity (Integrity (peaks/holes) PNNs) in LVII after short-term abstinence (24h) in ShA-24h (n = 8) and LgA-24h (n=8) groups.*

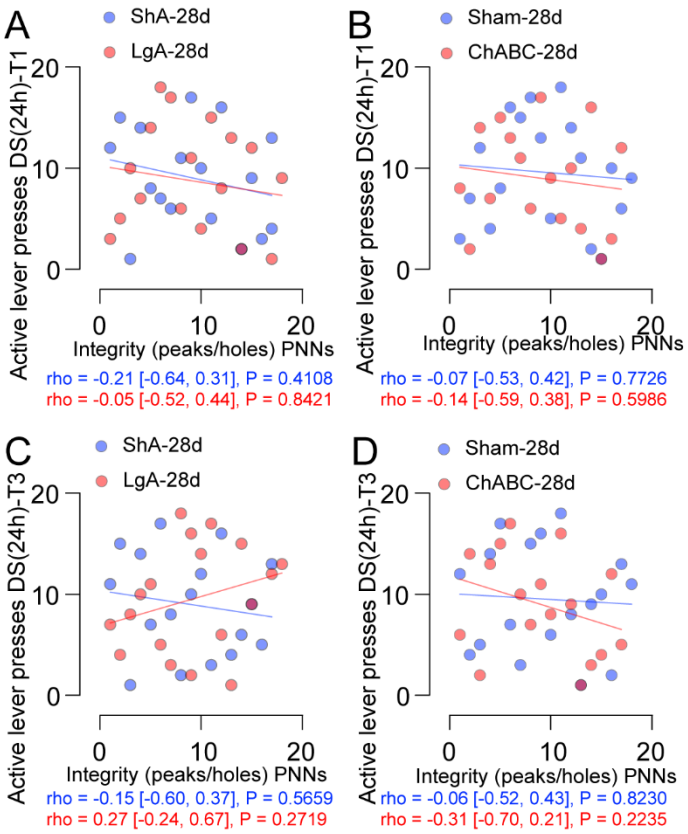

Figure 46. *Active lever presses in DS-test at 28d (T1-T3) performed for ShA and LgA Vs PNN integrity (Integrity (peaks/holes) PNNs) scatterplots and scatterplots and Spearman r correlation coefficients (rho) with line of best fit* ***A)*** *DS (28d)-T1 Vs PNN integrity (Integrity (peaks/holes) PNNs) in LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***B)*** *DS (28d)-T1 Vs PNN integrity (Integrity (peaks/holes) PNNs) in LVII long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17) rats.* ***C)*** *DS (28d)-T3 Vs PNN integrity (Integrity (peaks/holes) PNNs) in LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***D)*** *DS (28d)-T3 Vs PNN integrity (Integrity (peaks/holes) PNNs) in LVII long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17) rats.*

<u>Active lever presses in the DS-test after abstinence strongly correlated with granule cell activity in the dorsal LVII after extended access to cocaine</u>

Spearman correlation coefficient revealed a positive relationship between active lever presses in the DS test after abstinence and C-Fos expression in granule cells of the dorsal region for the LgA-28d group in T1 (rho = 0.60 [0.16, 0.84], P = 0.0091) (**Fig. 47a**) and T3 (rho = 0.74 [0.40, 0.90], P = 0.0005) (**Fig. 47c**). We also found a positive relationship between both variables for ChABC-28d group in T1 (rho = 0.55 [0.08, 0.82], P = 0.0244) (**Fig. 47b**) and T3 (rho = 0.68 [0.28, 0.88], P = 0.0035) (**Fig. 47d**).

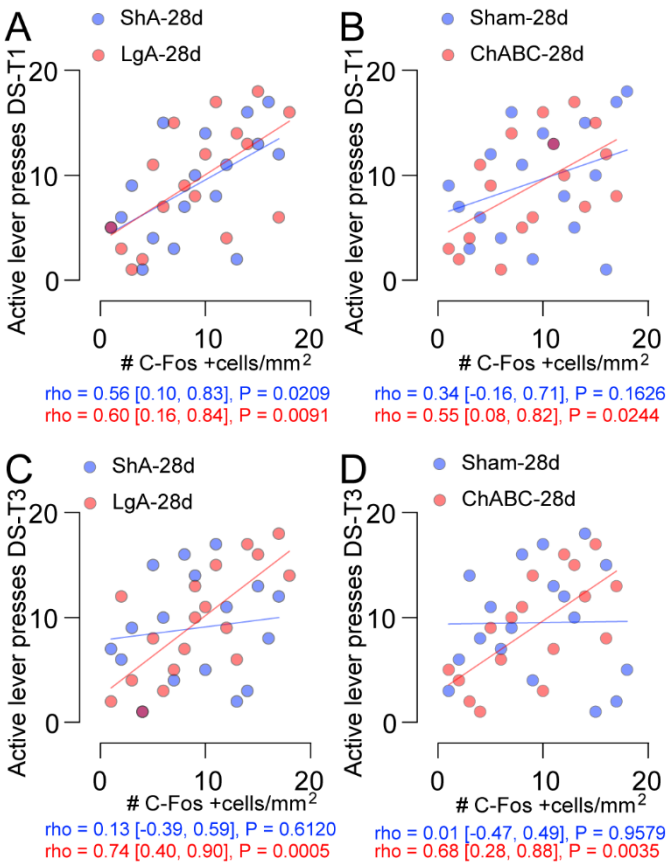

Figure 47. *Active lever presses in the DS-test (trials 1 and 3) Vs C-Fos expression in the dorsal region of LVII (C-Fos +cells/mm²) scatterplots Spearman r correlation coefficients (rho) with line of best fit* ***A)*** *DS-T1 Vs C-Fos expression in the dorsal region of LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***B)*** *DS-T1 Vs C-Fos expression in dorsal region of LVII after long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17) rats.* ***C)*** *DS-T3 Vs C-Fos expression in the dorsal region of LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***D)*** *DS-T3 Vs C-Fos expression in dorsal region of LVII after long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17) rats.*

However, no relationship could be demonstrated when we analysed the neural activity in the ventral region of LVII (**Fig. 48**). These results point to a functional regionalization in LVII in drug-seeking.

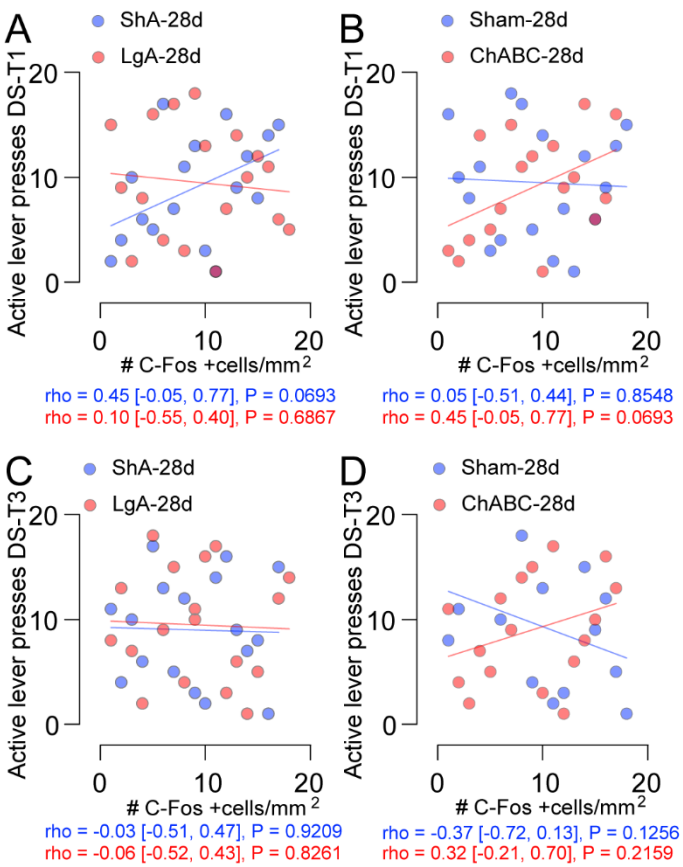

Figure 48. *Active lever presses in the last DS-test (trials 1 and 3) performed for each group before its perfusion Vs C-Fos expression in ventral region of LVII (C-Fos +cells/mm²) scatterplots and Spearman r correlation coefficients (rho) with line of best fit* ***A)*** *DS-T1 Vs C-Fos expression in ventral region of LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***B)*** *DS-T1 Vs C-Fos expression in ventral region of LVII after long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17).* ***C)*** *DS-T3 Vs C-Fos expression in ventral region of LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***D)*** *DS-T3 Vs C-Fos expression in ventral region of LVII after long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17).*

C-Fos expression in granule cell layer was not related to either PNN intensity or integrity

We did not see any correlation between PNN intensity or integrity and neural activity in the dorsal or ventral regions of LVII (**Fig. 49-50**).

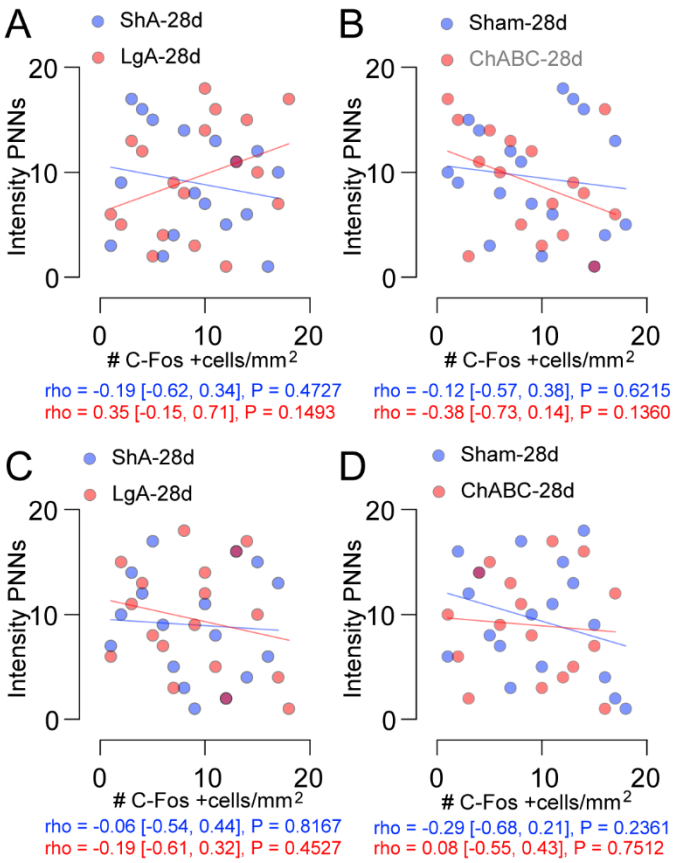

Figure 49. *PNN expression (Intensity PNNs) Vs C-Fos expression in dorsal and ventral regions of LVII (C-Fos +cells/mm²) scatterplots and Spearman r correlation coefficients (rho) with line of best fit* ***A)*** *PNN expression Vs C-Fos expression in dorsal region of LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***B)*** *PNN expression Vs C-Fos expression in dorsal of LVII after long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17).* ***C)*** *PNN expression Vs C-Fos expression in ventral region of LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***D)*** *PNN expression Vs C-Fos expression in ventral region of LVII after long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17).*

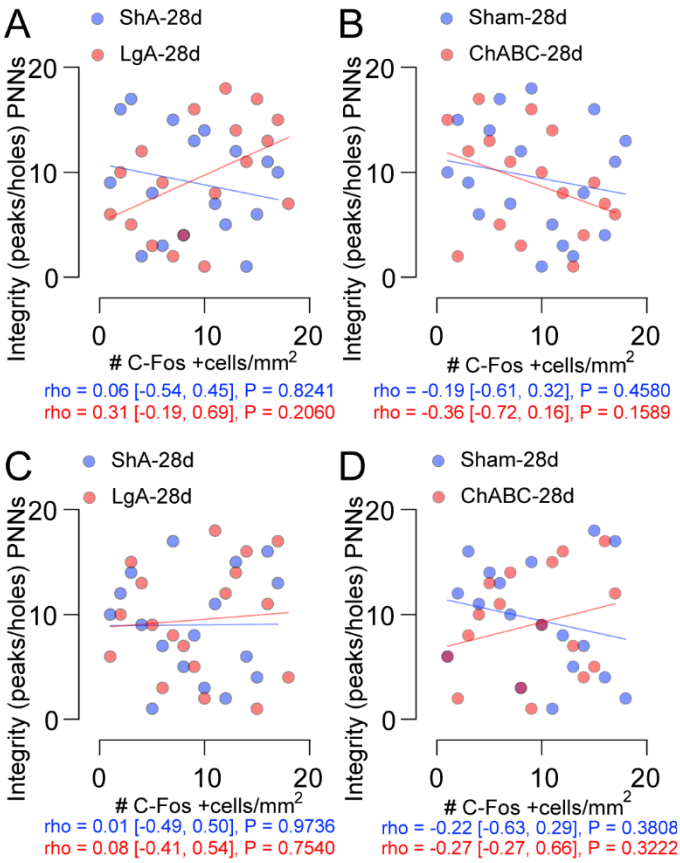

Figure 50. *PNN integrity (Integrity (peaks/holes) PNNs) Vs C-Fos expression in dorsal and ventral regions of LVII (C-Fos +cells/mm²) scatterplots and Spearman r correlation coefficients (rho) with line of best fit* ***A)*** *PNN integrity Vs C-Fos expression in dorsal region of LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***B)*** *PNN integrity Vs C-Fos expression in dorsal of LVII after long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17).* ***C)*** *PNN integrity Vs C-Fos expression in ventral region of LVII after long-term abstinence (28d) in ShA (n = 17) and LgA (n=18) groups.* ***D)*** *PNN integrity Vs C-Fos expression in ventral region of LVII after long-term abstinence (28d) in Sham (n = 18) and ChABC (n=17).*

Overall, the results of this study indicate a faster decline in drug seeking after PNN digestion in LVII when degradation covered the entire incubation period after extended cocaine access. These findings support the results shown in study 1, which suggested that cerebellar PNNs are required to stabilise synaptic changes underlying incubation of craving during protracted abstinence. Moreover, these findings involve LVII of the vermis in drug seeking, considering the positive relationship between LVII neural activity (measured as C-Fos expression) and active lever presses in the DS test after protracted abstinence. Nevertheless, given that the correlation was observed in both ShA and LgA rats, the involvement of this lobule in the incubation effect is not so evident. Finally, we did not see clear effects of PNN digestion in neural activity of LVII. In the next experiment, we elucidate whether extinction of drug seeking could be facilitated after PNN digestion in LVII.

Study 4

Effects of enzymatic digestion of PNNs in the cerebellar cortex on extinction of cocaine self-administration

Study 4: Effects of enzymatic digestion of PNNs in the cerebellar cortex on extinction of cocaine self-administration

This experiment explores the effect of the degradation of PNNs in LVII on the formation of extinction memories after extended access (LgA) cocaine self-administration. To investigate whether DS response was effectively extinguished, we tested animals for cue and drug-induced reinstatement. Male Sprague Dawley rats were randomly assigned to Sham or ChABC group, which underwent PNNs digestion in lobule VII after extended access to cocaine self-administration. For a visual representation of the experimental timeline, please see **Fig. 9**, Materials and Methods.

Rats escalated cocaine intake during long access to cocaine self-administration

Rats (N = 16) self-administered cocaine under a FR1 schedule for seven sessions (1 h/daily) (**Fig. 51**). After this initial training, rats underwent cocaine self-administration for 6 h for 12 sessions (**Fig. 52**). Data are shown as mean ± SD with 95% confidence interval of mean. Rats did 59.64 ± 25.91, 95% CI [55.95, 63.32] active lever presses and 1.16 ± 5.75, 95% CI [0.34, 1.98] inactive lever presses in 6 h. One-way ANOVA for repeated measures showed an effect of sessions in active lever presses ($F (11, 165) = 37.35$, $P < 0.0001$) (**Fig. 52c**) but no in inactive lever presses $F (11, 165) = 1.17$, $P = 0.3635$) (**Fig. 52d**). These results demonstrate that rats escalated significantly from the 2nd session as confirmed by Dunnett's post hoc test (**52c, d**).

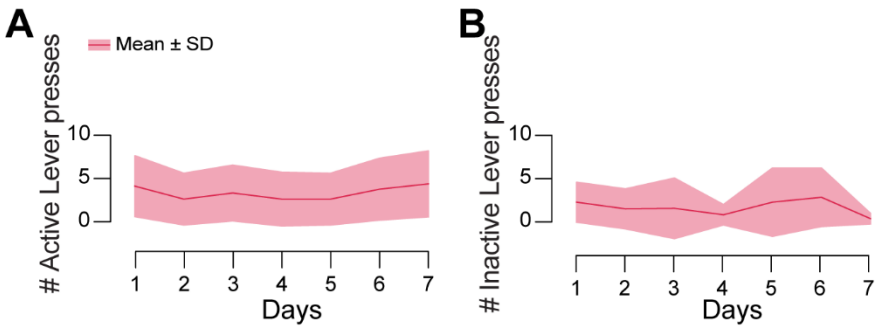

Figure 51. *Training of cocaine self-administration.* ***A)*** *Number of active lever presses during the training phase in which animals (N = 16) had access to cocaine for 1 h* ***B)*** *Number of inactive lever presses during the training phase.*

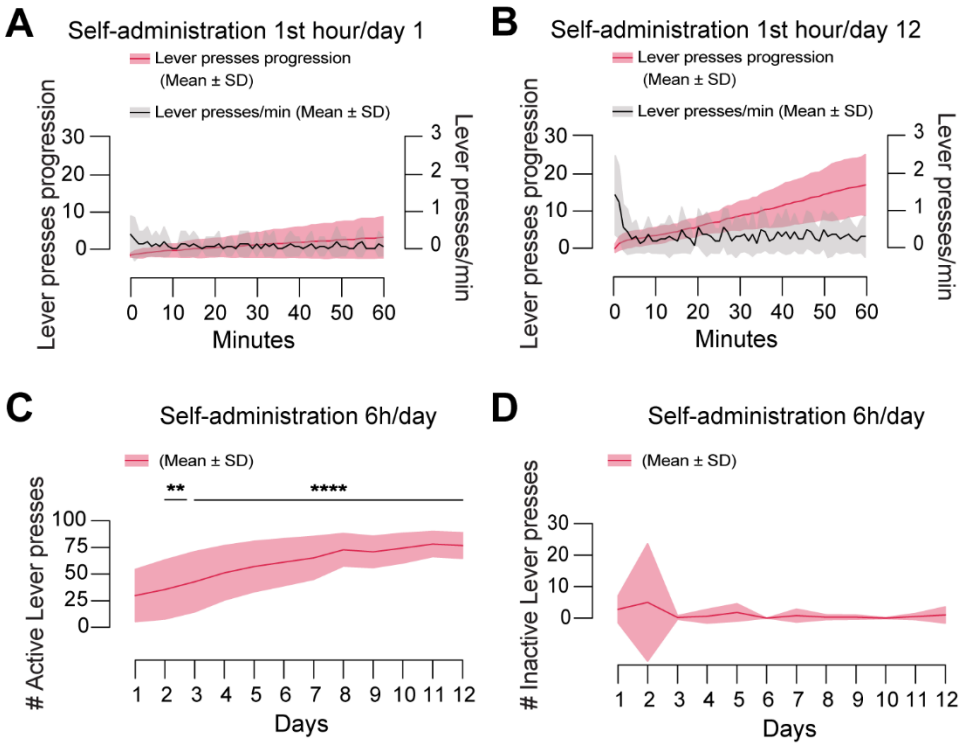

Figure 52. *Cocaine/saline self-administration phase.* ***A)*** *Lever presses progression and Lever presses/min during the first hour of the first day of self-administration.* ***B)*** *Lever presses progression and Lever presses/min during the first hour of the last day of self-administration.* ***C)*** *Number of active lever presses during the self-administration phase in which animals had access to cocaine (LgA) for 6 h.* ***D)*** *Number of inactive lever presses during the self-administration phase.* ***E)*** *Number of inactive lever-presses during the self-administration phase. ****P < 0.0001, **P < 0.01, *P < 0.05 for within-group comparisons against first day of self-administration.*

<u>Extinction learning is not affected by PNN removal in LVII</u>

With the purpose of studying the effects of degradation of LVII of the cerebellum, 8 rats were randomly selected for PNN digestion by infusion of ChABC (ChABC group, n = 8) into LVII of the cerebellum in a stereotaxic surgery (**Chapter 2 page 23**). Sham rats also

received the same surgery but were infused with the vehicle (Sham group, n = 8). After four days of recovery (6 days from the last cocaine self-administration) all animals underwent extinction phase of cocaine self-administration for 10 days (**Fig. 9**, Materials and Methods**).** All data are expressed as mean ± SD with 95% confidence interval of mean. Sham rats (n = 8) did 5.58 ± 4.08, 95% CI [4.67, 6.48], whereas ChABC rats (n = 8) did 6.88 ± 4.80, 95% CI [5.81, 7.94] active lever presses in extinction phase. These data are represented in **Figure 53.** In this figure, we also show average values of active lever presses in the three trials of DS test before extinction (**Fig. 53a**), CR **(Fig. 53c**) and DR test (**Fig. 53d**). Also, in DS test we differentiate between Sham and ChABC groups even when this test was performed 24h before stereotaxic surgery (**Fig. 53**) to facilitate comparisons. Two-way ANOVA analysis for active lever presses (ChABC × sessions) did no show effect of ChABC ($F(1, 14) = 1.64$, $P = 0.2211$) or ChABC × session interaction ($F(9, 126) = 0.92$, $P = 0.5076$) but a clear effect of session ($F(9, 126) = 6.67$, $P < 0.0001$) (**Fig. 53b**).

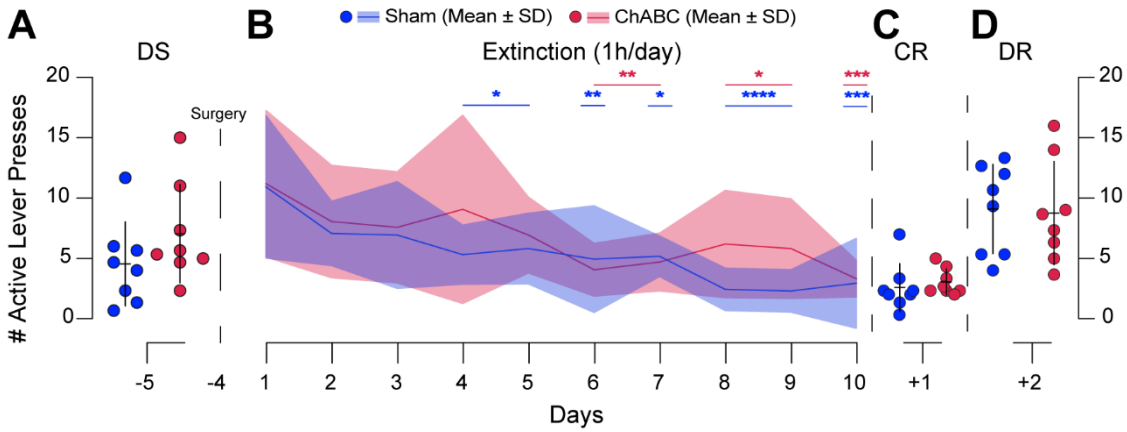

Figure 53*. Extinction phase (Active lever presses) in Sham (n = 8) and ChABC (n = 8) groups.* ***A)*** *Average number of active lever presses during DS test (5 days before extinction)* ***B)*** *Number of active lever presses lever presses during the extinction phase in which animals had no access to cocaine for 1 h.* ***C*** *Average number of active lever presses during CR test (1 day after extinction).* ***D.*** *Average number of active lever presses during DR test (2 days after extinction). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 against first day of extinction.*

In addition, we studied the differences between both groups (Sham and ChABC) in extinction by a survival analysis (Log-rank [Mantel-Cox] test), we chose as extinction criterion two consecutive days with a median score lower than the sham group (5.00). This analysis did not show differences between both survival curves ($\chi2(1) = 1.27$, $P = 0.2591$) (**Fig. 54**).

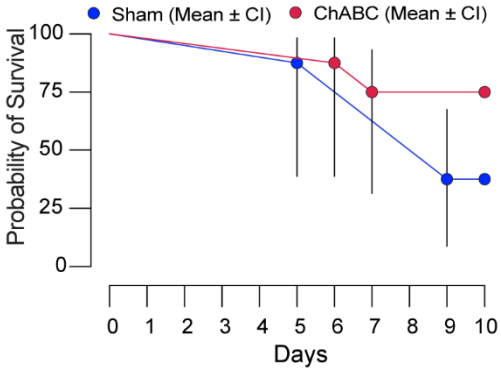

***Figure 54**. Survival curves for active lever presses in extinction phase. Data are represented as mean ± CI of the mean Sham (n=8) ChABC (n=8).*

Regarding inactive lever presses during extinction days, Sham rats (n = 8) did 2.36 ± 2.93, 95% CI [1.71, 3.02], whereas ChABC rats (n = 8) did 2.90 ± 3.66, 95% CI [2.09, 3.72] inactive lever presses during extinction phase. These data are represented in **Figure 55**, in this figure, again we show average values of DS (**Fig. 55a**), CR (**Fig. 55c**) and DR test (**Fig.55d**) inactive lever presses. Average values are also calculated only with the purpose of making more understandable the representation of data. A two-way ANOVA for inactive lever presses (ChABC × sessions) did not show an effect of ChABC ($F(1, 14) = 0.30$, $P = 0.5935$), session ($F(9, 126) = 1.77$, $P = 0.0802$) or ChABC × session interaction ($F(9, 126) = 0.98$, $P = 0.4560$) (**Fig. 55b**). Looking at these results, we determine that removal of PNNs in LVII of the cerebellar vermis does not affect the acquisition of a new extinction memory.

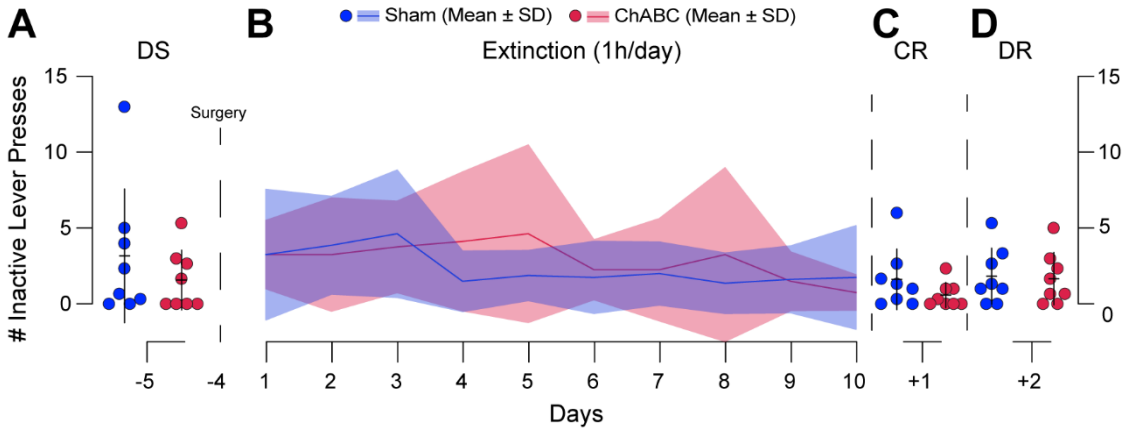

***Figure 55**. Extinction phase (Inactive lever presses) in Sham (n = 8) and ChABC (n = 8) groups. **A)** Average number of inactive lever presses during DS test (5 days before extinction) **B)** Number of inactive lever presses lever presses during the extinction phase in which animals had no access to cocaine for 1 h. **C** Average number of inactive lever presses during CR test (1 day after extinction). **D.** Average number of inactive lever presses during DR test (2 days after extinction).*

The effect of extinction learning in cue-induced reinstatement is not affected by PNN removal on LVII

Following the extinction phase, rats were tested in a cue-induced reinstatement test (CR test) identical to drug seeking test explained in Chapter 2 (**page 27**). Briefly, Sham and ChABC rats under extinction underwent three 1 hour-drug seeking trials in the operant chambers separated by 10 min each in which rats were left undisturbed in their home cages. To see the effect of LVII PNN removal and extinction we compare the active lever presses on each trial of the DS test (before extinction) with the corresponding trial of the CR test (after extinction) both in Sham and ChABC groups (**Fig. 56**). Data are presented as mean ± SD with 95% confidence interval of mean. In DS test, Sham rats (n = 8) did 8.25 ± 6.34, 95% CI [2.95, 13.55] active lever presses on T1, 3.25 ± 3.41, 95% CI [0.40, 6.10] on T2, and 2.13 ± 2.17, 95% CI [0.31, 3.94] on T3, meanwhile ChABC rats (n = 8) did 14.63 ± 8.82, 95% CI [7.26, 21.99] active lever presses on T1, 3.62 ± 3.42, 95% CI [0.77, 6.48] on T2 and 2.88 ± 2.80, 95% CI [0.53, 5.22] on T3. On the other hand, in CR test, Sham rats (n = 8) did 1.75 ± 1.28, 95% CI [0.68, 2.82] active lever presses on T1, 3.50 ± 4.07, 95% CI [0.10, 6.90] on T2, and 2.50 ± 2.07, 95% CI [0.77, 4.23] on T3, meanwhile ChABC rats (n = 8) did 4.50 ± 2.56, 95% CI [2.36, 6.64] active lever presses on T1, 2.88 ± 1.46, 95% CI [1.66, 4.09] on T2, and 1.75 ± 2.61, 95% CI [-0.43, 3.93] on T3.

First, we compared the first trial (T1) of both DS and CR tests. A two-way ANOVA for active lever presses (ChABC x test) showed effect of ChABC ($F(1, 14) = 4.86$, $P = 0.0447$) and test ($F(1, 14) = 19.18$, $P = 0.0006$), but there was no effect of ChABC x test interaction ($F(1, 14) = 0.91$, $P = 0.3558$). This suggests that both Sham and ChABC rats have extinguished equally and that drug seeking response did not reinstate after presentation of drug-related cues. However, the difference showed by Sidak post-hoc test between DS-T1 and CR-T1 on the ChABC group ($p=0.004$) (**Fig. 56**) could be explained by variability within the group, which despite being randomly assigned (**Fig. 9**, Materials and Methods), showed a subject that pressed much more the active lever than the mean of the group. However, the ROUT test for identification of outliers did not exclude this value.

For the second trial (T2), two-way ANOVA for comparison between DS and CR test showed no effect of ChABC ($F(1, 14) = 0.01$, $P = 0.9157$), test ($F(1, 14) = 0.05$, $P = 0.8284$), or ChABC x test interaction ($F(1, 14) = 0.20$, $P = 0.6654$) (**Fig. 56b**). Neither in the third trial (T3), confirmed by two-way ANOVA which did not show an effect of ChABC

(F (1, 14) = 0.00, P > 0.9999), test (F (1, 14) = 0.21, P = 0.6503), or ChABC infusion x test interaction (F (1, 14) = 0.86, P = 0.3698) (**Fig. 56c**).

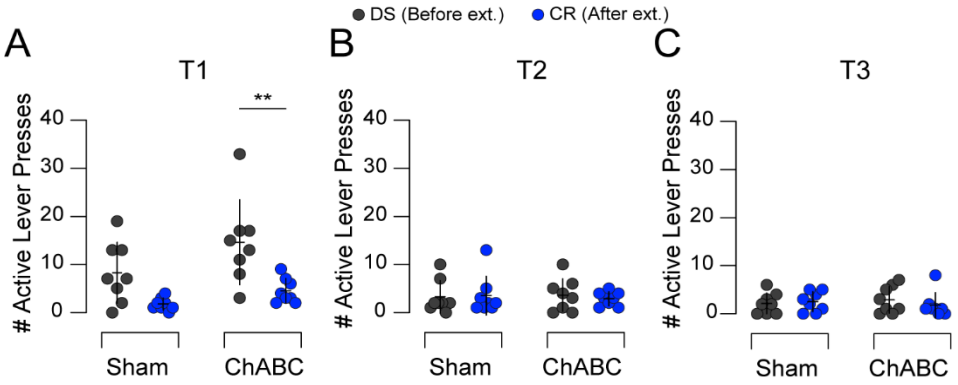

***Figure 56**. Dot plots of active lever presses in DS, before extinction (ext.), and CR tests for Sham (n=8) and ChABC (n=8) groups. **A)** T1 active lever presses. **B)** T2 active lever presses. **C)** T3 active lever presses. **P < 0.01 for within-group comparisons (DS vs CR test).*

With respect to the inactive lever presses in DS test, Sham rats (n = 8) performed 2.38 ± 3.07, 95% CI [-0.19, 4.94] inactive lever presses on T1, 1.25 ± 2.77, 95% CI [-1.06, 3.56] on T2, and 5.85 ± 12.73, 95% CI [-4.77, 16.52] on T3. While ChABC rats (n = 8) emitted 3.88 ± 5.57, 95% CI [-0.78, 8.53] inactive lever presses on T1, 0.375 ± 1.06, 95% CI [-0.51, 1.26] on T2 and 0.50 ± 0.76, 95% CI [-0.13, 1.13] on T3. On the other hand, in CR test, Sham rats (n = 8) did 1.25 ± 1.75, 95% CI [-0.22, 2.72] inactive lever presses on T1, 2.88 ± 3.68, 95% CI [-0.20, 5.96] on T2, and 0.75 ± 1.75, 95% CI [-0.72, 2.22] on T3. While ChABC rats (n = 8) did 0.88 ± 1.46, 95% CI [-0.34, 2.09] inactive lever presses on T1, 0.25 ± 0.71, 95% CI [-0.34, 0.84] on T2, and 0.63 ± 1.06, 95% CI [-0.26, 1.51] on T3.

A two-way ANOVA (ChABC x extinction) for T1 inactive lever presses on DS and CR tests did not find a significative effect of ChABC (F (1, 14) = 0.24, P = 0.6341), test (F (1, 14) = 2.81, P = 0.1157), or ChABC x test interaction (F (1, 14) = 0.58, P = 0.4586) (**Fig. 57a**). For the second trial (T2), two-way ANOVA showed no effect but a strong tendence of ChABC (F (1, 14) = 4.55, P = 0.0511), no effect for test (F (1, 14) = 0.75, P = 0.4021) or ChABC infusion x test interaction (F (1, 14) = 1.02, P = 0.3305) (**Fig. 57b**). In the third trial (T3), two-way ANOVA showed no effect of ChABC (F (1, 14) = 1.51, P = 0.2388), test (F (1, 14) = 1.15, P = 0.3018), or ChABC x test interaction (F (1, 14) = 1.27, P = 0.2792) (**Fig. 57c**).

These results show that ChABC infusion in LVII before extinction learning does not affect cue-induced relapse.

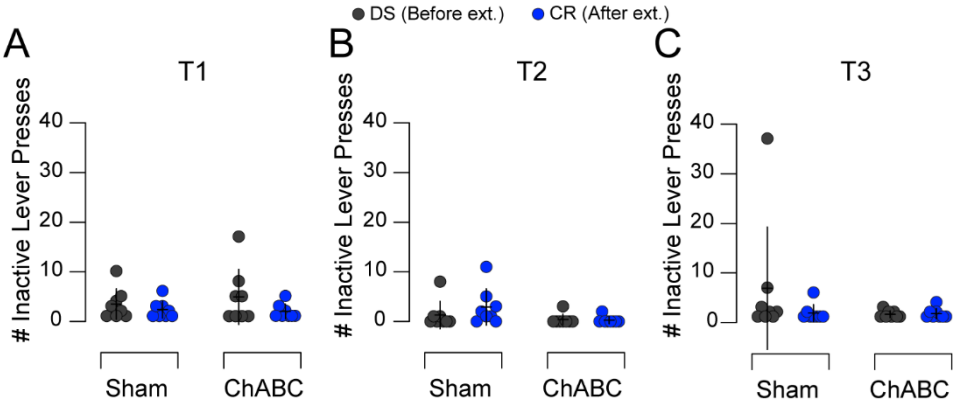

Figure 57*. Dot plots of inactive lever presses in DS, before extinction (ext.), and CR tests for Sham (n=8) and ChABC (n=8) groups.* ***A)*** *T1 inactive lever presses.* ***B)*** *T2 inactive lever presses.* ***C)*** *T3 inactive lever presses.*

Removal of PNNs in LVII does not affect drug-induced reinstatement after extinction

Twenty-four hours after CR test, rats were tested in a drug-induced reinstatement test (DR test) identical to DS test explained in Chapter 2 (**page 27**) but the rats received a non-contingent i.v. injection of cocaine right before each trial (0.75 mg/kg/injection per test) with no cue switch on and levers retracted. We compared both active and inactive lever presses in DS (before extinction) with those realized in DR for Sham and ChABC groups.

Mean ± SD with 95% confidence interval of mean for active and inactive lever presses in the DS test are detailed in the previous section. Regarding DR active lever presses Sham rats (n = 8) did 12.13 ± 5.22, 95% CI [7.76, 16.49] active lever presses on T1, 7.25 ± 3.20, 95% CI [4.58, 9.92] on T2, and 7.88 ± 4.82, 95% CI [3.84, 11.91] on T3. ChABC rats (n = 8) did 12.75 ± 5.78, 95% CI [7.92, 17.58] active lever presses on T1, 7.88 ± 4.19, 95% CI [4.37, 11.38] on T2, and 5.63 ± 4.72, 95% CI [1.68, 9.57] on T3.

A two-way ANOVA for T1 active lever presses comparing DS and DR test (ChABC x drug reinstatement) did not show an effect of ChABC infusion ($F(1, 28) = 2.20$, $P = 0.1496$), test ($F(1, 28) = 0.18$, $P = 0.6753$) or ChABC x drug test interaction ($F(1, 28) = 1.48$, $P = 0.2337$) (**Fig. 58a**). Both Sham and ChABC groups reinstated equally the drug-seeking response after receiving a new cocaine infusion. For T2, a two-way ANOVA showed no effect of ChABC ($F(1, 28) = 0.17$, $P = 0.6954$), but there was a significative effect of test ($F(1, 28) = 10.65$, $P = 0.0029$) but no ChABC x test interaction ($F(1, 28) = 0.01$, $P = 0.9219$). Sham and ChABC groups decreased drug seeking regarding the first trial (**Fig. 58b**). In T3, a two-way ANOVA showed no effect of ChABC ($F(1, 14) = 0.31$,

P = 0.5821), a significant effect of test (F (1, 28) = 9.95, P = 0.0038) but no ChABC x test interaction (F (1, 28) = 1.24, P = 0.2750) (**Fig. 58c**).

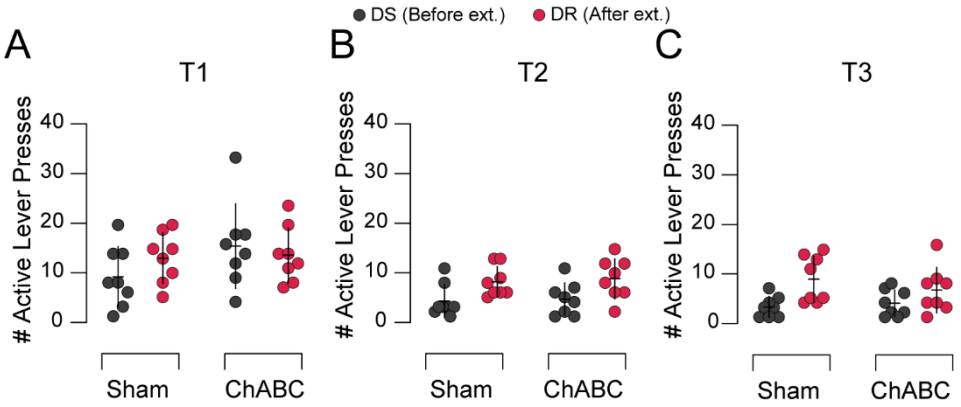

***Figure 58**. Dot plots of active lever presses in DS before extinction (ext.) and DR tests for Sham (n=8) and ChABC (n=8) groups. **A)** T1 active lever presses. **B)** T2 active lever presses. **C)** T3 active lever presses. *P < 0.05 for within-group comparisons (DS vs DR test).*

Regarding the effect of PNNs removal on inactive lever-presses, in DR-T1 Sham rats (n = 8) did 3.13 ± 2.80, 95% CI [0.78, 5.47] inactive lever presses, 1.00 ± 1.07, 95% CI [0.11, 1.89] on T2, and 1.38 ± 2.72, 95% CI [-0.90, 3.65] on T3. ChABC rats (n = 8) did 2.63 ± 2.39, 95% CI [0.63, 4.62] inactive lever presses on T1, 1.63 ± 3.07, 95% CI [-0.94, 4.19] on T2 and 0.75 ± 1.67, 95% CI [-0.22, 1.72] on T3. A two-way ANOVA for T1 inactive lever presses comparing DS and DR tests (ChABC x test) did not show effect of ChABC (F (1, 28) = 0.15, P = 0.7030), test (F (1, 28) = 0.04, P = 0.8487) or ChABC x test interaction (F (1, 28) = 0.59, P = 0.4476) (**Fig. 59a**). For T2, a two-way ANOVA showed no effect of ChABC (F (1, 28) = 0.03, P = 0.8734), no effect of test (F (1, 28) = 0.41, P = 0.5252) or ChABC x test interaction (F (1, 28) = 0.93, P = 0.3427 (**Fig. 59b**). Finally, in T3, a two-way ANOVA showed no effect of ChABC (F (1, 28) = 1.68, P = 0.2056) test (F (1, 28) = 0.84, P = 0.3665), or ChABC x test interaction (F (1, 28) = 1.05, P = 0.3137) (**Fig. 59c**). Removal of PNNs from LVII did not have any impact on active or inactive lever presses during drug reinstatement test.

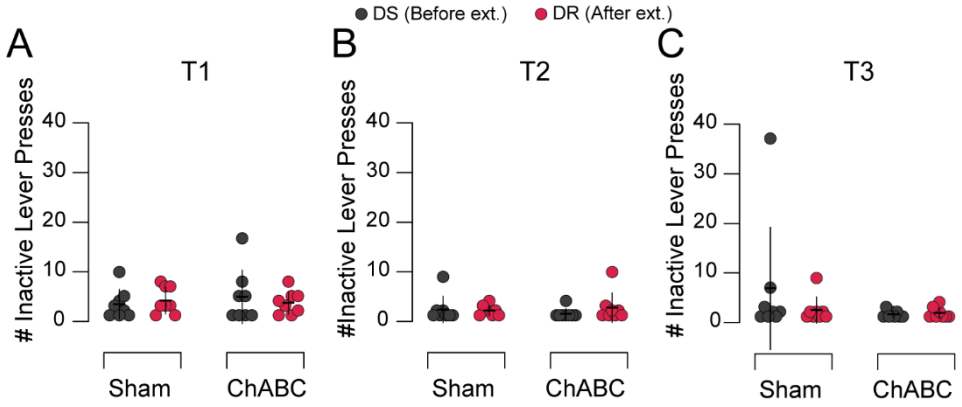

Figure 59*. Dot plots of inactive lever presses in DS, before extinction (ext.), and DR tests for Sham (n=8) and ChABC (n=8) groups.* ***A)*** *T1 active lever presses.* ***B)*** *T2 active lever presses.* ***C)*** *T3 active lever presses.*

In summary, these results show that animals with PNN removal by ChABC infusion in LVII can establish new memories like extinction in the same way that animals with intact PNNs. Also, PNN degradation does not impact cue- or drug-induced reinstatement.

CHAPTER 4

DISCUSSION

DISCUSSION

This chapter will conclude the present doctoral thesis by summarising the key research findings in relation to the research aims, as well as the value and contribution thereof. It will also review the limitations of the study and propose opportunities for future research.

As we have already discussed, addiction is a disorder that can be attributed to the ability of certain substances such as cocaine to hijack natural learning and memory mechanisms (Hyman, 2005; Kalivas et al., 2005; Robbins et al., 2008). These long-lasting changes in brain circuits are associated with frequent relapses even several years after quitting drug consumption (Koob and Volkow, 2016). One of the most widely used paradigms to model substance abuse in animal research is the escalation model of cocaine intake, which implies extended access to intravenous cocaine self-administration. This paradigm emulates more accurately than traditional drug self-administration models the transition from recreational cocaine use to the abuse in cocaine intake that is developed during addiction in humans (Ahmed and Koob, 1998). It also includes the canonical characteristics of drug self-administration models such as the voluntariness of consumption as well as the flexibility in manipulating variables such as dose, frequency or time of exposure, and ability to incorporate behavioural procedures such as extinction or reinstatement to model drug seeking and relapse (Watterson and Olive, 2016; Belin et al., 2018; Schwendt and Knackstedt, 2021). The present research focuses on unravelling the effects of extended access to cocaine self-administration on PNNs around Golgi interneurons in the cerebellar cortex and the implications of their manipulation by ChABC on drug-seeking relapse.

It is well-known that addictive drugs activate the prefrontal-striatal-limbic circuit, modulated by the dopamine mesolimbic system (Everitt and Robbins, 2005). Typically, the cerebellum has been relegated to sensorimotor functions, automatism, and motor learning. However, in recent years, growing number of studies have contributed to broaden the role of the cerebellum in reward, cognition, and emotion (Stoodley et al., 2012; Wagner et al., 2017; Stoodley and Schmahmann, 2018; Carta et al., 2019). This new focus on cerebellar functions is due in part to the more than demonstrated connections of the cerebellum with areas such as the striatum, mPFC and VTA (Bostan and Strick, 2018; Chen et al., 2014; Carta et al., 2019; Gil-Miravet et al., 2021).

We have previously proposed that changes in synaptic plasticity in the cerebellum would be key to the development of drug compulsive behaviour (Miquel et al., 2009; Miquel et al., 2019). One of the plasticity regulation mechanisms are PNNs, which stabilise synaptic connections and thereby have been involved in learning and memory (Dityatev and Schachner 2003; Gogolla et al., 2009; Sorg et al., 2016; Shi et al., 2019), including drug-induced memory (Chen et al., 2015; Slaker et al., 2015, 2016, 2018; Vazquez-Sanroman et al., 2015b; Sorg et al., 2016; De Luca and Papa, 2016; Carbo- Gas et al., 2017; Blacktop et al., 2017; Dong et al., 2017; Blacktop and Sorg, 2019; Roura-Martinez et al., 2020; Brown and Sorg, 2022). PNNs are structures composed by ECM molecules such as hyaluronic acid, proteoglycans and link proteins (Carulli et al., 2006) that involve the soma and proximal neurites of several neuronal populations, most of them fast-spiking PV+ GABAergic interneurons (Härtig et al., 1992; Sorg et al., 2016; Fawcett et al., 2019), but also other neuronal types such as PV- Golgi GABAergic interneurons in the cerebellar cortex (Carulli et al., 2006).

PNNs enable synaptic stability by acting as a physical barrier to the formation of new synapses (Corvetti and Rossi, 2005), facilitating the binding of synaptogenesis inhibitory molecules (Deepa et al., 2002) or limiting the mobility of AMPA receptors at synapses (Frischknecht et al., 2009). The regulation of these perineuronal structures depends on proteases such as the MMPs and ADAMTs family (Gottschall and Howell, 2015; Lasek et al., 2018) whose enzymatic activity is also affected in drug addiction both in the brain (Brown et al., 2007) and cerebellum (Vaquez-Sanroman et al., 2015a, b). However, the regulation of PNNs may also be due to changes in their own components. In this investigation, we focused on TN-R (a link-protein) and Bcan (a CSPG) both playing important roles in plasticity (Carulli et al., 2007; Dauth et al., 2016) and are up-regulated in drug addiction (Van den Oever et al., 2010). Another protein to consider is the PTPσ receptor, essential for the inhibitory function in synaptic plasticity of CSPGs such as Bcan (Shen et al., 2009). PTPσ inhibits by dephosphorylation the TRKB receptor (Kurihara and Yamashita, 2012; Lesnikova et al., 2021), which is crucial for the role of BDNF in synaptic plasticity and it has been found to be increased in the cerebellum of cocaine-sensitised animals during the incubation period that involved synaptic modifications under prolonged withdrawal (Vazquez-Sanroman et al., 2015b).

The role of PNNs in cerebellar plasticity has been poorly studied (Corvetti and Rossi, 2005; Deepa et al., 2006; Carulli et al., 2006, 2007, 2020; Fawcett et al., 2019). Digestion of PNNs in the cerebellar cortex using the bacterial enzyme ChABC generates sprouting of Purkinje axons that in the absence of specific external signals is not able to foster

stable synaptic connections (Corvetti and Rossi, 2005; Deepa et al., 2006; Carulli et al., 2006, 2007, 2020; Fawcett et al., 2019). This finding indicates that PNN removal creates permissive conditions for plasticity that have to be driven by external information. Degradation of PNNs in the interposed nucleus of the cerebellum (DCN) improved eye blink conditioning but disrupted retention of the motor memory that declined over time (Carulli et al., 2020).

Our lab has been investigating for years how cocaine exposure remodels PNN in the cerebellum (Vazquez-Sanroman et al., 2015a, b; Carbo-Gas et al., 2017). Cocaine-induced Pavlovian memory increases PNN expression in the cerebellar cortex (Carbo-Gas et al., 2017). However, PNN expression in the DCN is regulated by cocaine exposure but not for drug learning (Vazquez-Sanroman et al., 2015a; Carbo-Gas et al., 2017). Recently, we have explored the role of cerebellar PNNs in drug-induced Pavlovian memory (Guarque-Chabrera et al., 2022). We degraded PNNs specifically in LVIII at various stages of cocaine-induced learning and showed that PNNs surrounding Golgi interneurons are essential for cocaine-induced Pavlovian short-term memory and stabilisation of extinction memory but not for the acquisition of cocaine-induced preference conditioning. Degradation of PNNs in DCN did not have any impact on cocaine-induced memory.

In the present work, we have gone further to explore whether cocaine self-administration can persistently remodel PNNs and what is and what is the impact of abstinence on PNN expression in the cerebellar cortex. Moreover, we wanted to investigate whether PNN enzymatic digestion by ChABC was able to affect drug seeking. For this reason, we analysed PNN expression around Golgi interneurons through the medial and lateral cerebellar cortex after short versus long access cocaine self-administration. Then, we explored which PNN-related proteins were affected by cocaine after self-administration and withdrawal. Finally, we degraded PNNs over abstinence in LVII using ChABC to ascertain the role of PNNs on drug seeking (relapse) and the consequences of PNN removal for neural activity in the cerebellar cortex.

The present findings suggest that extended access cocaine self-administration, which parallels some of the features of drug addiction, may regulate dynamically plasticity conditions in the cerebellum throughout abstinence and increase PNN expression during the period when the incubation of drug seeking has been described (Grimm et al., 2001; Lu et al., 2004; Pickens et al., 2011). One of CSPG components that can be upregulated during abstinence from cocaine abuse is Bcan as our **second study** revealed. Finally,

and against our hypothesis, PNN removal during protracted abstinence does not prevent the incubation of drug seeking but generates a faster decline of the drug-seeking response that does not seem to be caused by a facilitation of extinction.

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

Cocaine self-administration induced time-dependent regulation of PNNs in the cerebellar cortex

Overall, the results of this study indicate the existence of a dynamic regulation of PNNs in the cerebellar cortex during abstinence after cocaine self-administration. Shortly after the cessation of drug consumption, we found a reduction in the expression of PNNs around Golgi interneurons in animals with restricted access to cocaine. Decreased PNN expression can facilitate synaptic plasticity and enable synaptic remodelling as demonstrated by enzymatic degradation studies in different areas of the brain (Pizzorusso et al., 2002; Lensjø et al., 2017), cerebellar DCNs (Carulli et al., 2020) and the cerebellar cortex (Corvetti and Rossi, 2005). The regulation of PNNs during the first days of abstinence after cocaine is not the same in all brain regions. However, any possible comparison among studies should be taken carefully because they involve distinct behavioural protocols for cocaine administration and diverse drug-induced memory processes. Cocaine self-administration increased density of high intensity PNNs in the right PL, IL, and ventral orbitofrontal cortex 24 h after cocaine consumption (Roura-Martinez et al., 2020). Nevertheless, in the same study and under the same conditions, the density of low- and medium-intensity PNNs was higher in the left hemisphere. Non-contingent i.p. cocaine administration decreased PNN expression in the IL, but it increased in the PL 5 days after injections (Slaker et al., 2018) Also, our group found increased PNN expression in the DCN 24 h after reinstatement following 7 days of withdrawal (Vazquez-Sanroman, et al., 2015a).

In **Study 1,** we showed that over the course of protracted abstinence the expression of PNNs surrounding Golgi interneurons did not change in rats that had a restricted access to cocaine self-administration. However, it enhances after an escalated cocaine intake. These findings suggest that cocaine self-administration increases permissive conditions for plasticity in the cerebellar cortex at short-term by downregulating PNN expression. However, a history of cocaine abuse causes restrictive conditions for synaptic remodelling in Golgi interneurons over abstinence that may contribute to the incubation

of drug seeking. As is explained at the beginning of this chapter, endogenous enzymatic remodelling of PNNs relays on several families of proteases. The activity of these enzymes is dynamically regulated during cocaine abstinence and reinstatement in several regions (Van den Oever et al., 2010; Smith et al., 2014; Vazquez-Sanroman et al., 2015b). Both MMP 2 and 9, and ADAMTS4, have been detected in the cerebellum (Westling et al., 2004; Stamenkovic et al., 2017), although their role in drug-induced plasticity and memory is unknown. Stronger PNNs, could stabilise synaptic modifications related to drug-cue associations induced by extended cocaine access, and enable their persistence during long-term abstinence by preventing synaptic remodelling (Sorg, et al., 2016; Lasek et al., 2018). According to this hypothesis, its removal in areas such as the anterior hypothalamic area prevents the reinstatement of cocaine self-administration (Blacktop et al., 2017). We tested this hypothesis in **studies 3** and **4**.

Golgi interneurons are known to be an essential element of the cerebellar glomeruli, a functional unit of the cerebellar cortex (D'Angelo et al., 2013). Their axons inhibit nearby granule cells (Tabuchi et al., 2019) and synchronise their excitatory activity exerted through parallel fibers to Purkinje neurons. Previous work from our group showed that cocaine-induced preference conditioning increased granule and Golgi cells activity along with PNN expression around Golgi interneurons (Carbo-Gas et al., 2017).

Levels of PNN-related proteins which the main role is to reduce permissive conditions for synaptic plasticity increase in the cerebellar cortex during protracted abstinence after extended cocaine self-administration

In general, the results of this study indicate that cocaine self-administration access and the length of abstinence interact to dynamically regulate PNN-related proteins. This dynamic regulation of PNNs shown in **study 1** affects proteoglycans such as Bcan, other components such as TN-R, and downstream signalling pathways linked to BDNF receptors.

Bcan together with versican, neurocan and aggrecan, also known as lecticans, form the group of hyalectans of CSPGs and participate in the organization of the ECM. In CNS, CSPGs and their interacting partners contribute to the PNN structure (Yamaguchi, 2000). Bcan is localized at the outer surface of neurons where it is particularly enriched at perisynaptic sites being attached with TN-R (Hagihara et al., 1999). It has a restrictive role as a regulator of synaptic plasticity and post-lesion plasticity (Frischknecht and Seidenbecher, 2012).

In our WB analysis, we did not detect the band corresponding to 140 KDa Bcan, but two bands located at 55-50 kDa that may correspond to products of the enzymatic action of protease families such as MMP and ADAMTS, specifically ADAMTS4 (Nakamura et al., 2000; Valenzuela et al., 2014; Gottschall and Howell, 2015). ADAMTS4 can be activated, at least in vitro, by MMP9 (Tortorella et al., 2005), a metalloprotease located in the cerebellum, and known for its role in the regulation of PNN expression. The lack of signal of the full Bcan isoform observed in all experimental groups could be due to problems in the transfer of high-molecular weight proteins. Nevertheless, given the lack of time and the possibility of running out of brain samples, we decided to analyse these two bands found at 55-50 kDa. Our results indicate that cocaine self-administration did not impact on Bcan 55kDa-fragment but on Bcan 50kDa-fragment levels, although more samples are required to confirm the present results. However, it is evident that Bcan 50kDa-fragment levels were upregulated during abstinence after extended access to cocaine self-administration.

These results parallel the increased intensity in PNNs observed for LgA group after protracted abstinence (28d) shown in **study 1,** and suggest that Bcan 50kDa-fragment may be one of the PNN components upregulated during incubation. Then, increased Bcan levels could reduce the probability of synaptic remodelling during abstinence and contribute to the incubation of drug-seeking. Bcan has been implicated in the control of cellular and synaptic plasticity by regulating the localization of potassium channels and AMPAR, respectively (Frischknecht and Seidenbecher, 2012; Favuzzi et al., 2017). Like PNNs expression, the observed effects on Bcan levels vary depending on the brain region and the addictive drug studied. Earlier work reported reductions in the expression of Bcan levels and other PNNs-related proteins (TN-R) in the PV+ GABAergic neurons of the mPFC following heroin self-administration (Van den Oever et al., 2010). Bcan expression in the dorsal hippocampus, on the other hand, appears to be necessary for long-term incubation of preference in cocaine-induced CPP (Lubbers et al., 2016). Nevertheless, it is necessary to be careful when interpreting these results, given the impossibility of measuring the full Bcan isoform.

Previous studies showed that TN-R together with Bcan may be up-regulated following self-administration of addictive drugs such as heroin (Van den Oever et al., 2010) or alcohol (Zuo et al., 2012). TN-R is a multimeric protein that links CSPGs between them and thus, has an important role as a scaffolding PNN molecule (Weber et al., 1999; Brückner et al., 2000; Lundell er al., 2004; Chiquet-Ehrismann and Tucker, 2011). TN-R contains two subunits of 160-180 kDa that can form oligomers of 2 or 3 polypeptide

chains. Although it is known that 160 kDa isoform organizes in dimers and the larger isoform in trimers, the functional significance of the two isoforms is not well understood (Pesheva and Probstmeier, 2000). Our results indicate that cocaine self-administration reduces TN-R 180 kDa isoform levels at 24h in the posterior vermis. Importantly, these findings replicate the reduction in PNN intensity observed in study 1. It has been previously discussed how the expression of PNNs may be regulated by the activity of MMP and ADMTS family proteases (Huntley, 2012; Gottschall and Howell, 2015; Lasek et al., 2018) and how, an increase in this activity following cocaine administration may be related to a reduction in the intensity of cerebellar DCN PNNs (Vazquez-Sanroman et al., 2015b). Our results seem to support the hypothesis that addictive drugs initially create a hyper-plasticity state that favours the acquisition of memories related to the drug-stimulus association that could subsequently be fixed during abstinence (Hyman et al., 2006).

We did not observe changes in TN-R levels at 28 days of abstinence. Nonetheless, we had to change TN-R antiserum due to a supply disruption by the company, thus further experiments should confirm this lack of effect. Alternatively, if these results were confirmed it would mean that TN-R is not necessary for strengthening PNNs during the withdrawal period, leaving this function to CSPGs such as Bcan or other tenascins, such as Tenascin-C (TN-C) (Stamenkovic et al., 2017), which is also widely expressed in the brain and cerebellum (Dauth et al., 2016).

BDNF is a neurotrophin implicated in neuronal differentiation and synaptic growth that not only regulates neuronal activity during brain development, but also during adulthood (Rossi et al., 2006). Both the production and actions of neurotrophins have been linked to mechanisms of long-term synaptic plasticity by promoting the recovery of affected brain connections in various disorders (Smith et al., 1995; Gobbo and O'Mara, 2004). This neurotrophic factor exerts it effects through its union with TRKB receptor and increases after cocaine exposure (Grimm et al., 2003; Boudreau and Wolf, 2005; Boudreau et al., 2007; Ghasemzadeh et al., 2009), thus mediating the incubation of drug-seeking (Li et al., 2013; Loweth et al., 2014). In previous works from our laboratory, we found that BDNF mechanisms are enhanced in the cerebellar cortex after prolonged withdrawal from cocaine (Vazquez-Sanroman et al., 2015b). PTPσ is a large, highly conserved cell adhesion molecule-like that binds CSPGs (Lee et al., 2007) and interacts with and modulates, by dephosphorylation, the activity of TRKB (Kurihara and Yamashita, 2012; Lesnikova et al., 2021). The PTP family member most common in rats is the PCPTP1 isoform followed by PCPTP1-ce (Hendricks et al., 2009). When it forms

homo- or heterodimers (PTP-PTP), these proteins cannot exert their function, so the TRKB signal is not interrupted (Lee et al., 2007). Our data show that PCPTP1-ce levels increase with age (**Fig. 24**) and that cocaine exposure prevents this age-dependent effect. However, PTP-PTP dimers appear to be increased in both cocaine groups following protracted abstinence, suggesting that during abstinence TRKB-BDNF mechanisms may be fuelled (Lee et al., 2007). Given the small sample size, these results are preliminary, even when they support previous findings. Further experiments will be necessary to enhance the sample and confirm these findings with transcripts analysis by RT-PCR.

Enzymatic digestion of PNNs in the cerebellar cortex during protracted abstinence interfered with stability of the drug-seeking response

The results from **study 1** demonstrated an enhanced expression of PNNs around Golgi inhibitory interneurons during protracted abstinence; this effect was only observed in those rats under extended access to cocaine self-administration. In humans, it has been described that craving and drug seeking in animals incubate throughout abstinence (Grimm et al., 2001; Pickens et al., 2011). In rodents, incubation of drug seeking picks around one month after drug abuse (Grimm et al., 2000; Lu et al., 2004). Therefore, in study 3, we hypothesised that by removing PNNs in the cerebellar cortex we would be able to prevent or reduce the drug-seeking response after one month of abstinence. Also, we wondered whether PNN digestion would have an impact on neural activity in the cerebellar cortex.

In this study, we estimated incubation of drug seeking by comparing the two drug-seeking tests at two specific time-points, 24h after the last self-administration session (before PNN removal) and after 28 days of abstinence (after PNN removal).

It seems that drug-associated contexts are more likely to be selected in a drug-free state, which may reactivate the prediction of drug availability. The perception of drug-related cues under abstinence could generate the mismatch between the prediction and the sensorial experience and may lead to drug seeking. In the first DS test, both the ShA and LgA groups show a drug-seeking response, although drug seeking was higher after restricted cocaine access. The increase in the ShA group has also been observed in other studies that assessed drug seeking immediately after drug self-administration (Kerstetter et al., 2008; Thorn et al., 2014). Restricted cocaine self-administration is highly rewarding although this schedule is unable to reproduce other features of drug addiction. As expected, incubation of drug seeking at the end of 28 abstinence days

could be only seen after extended cocaine access. PNN removal in LVII did not prevent neither the drug-seeking response nor its incubation, but it made it decline faster. Remarkably, PNN digestion only affected the LgA group. These results support the hypothesis that PNNs are important for strongly established drug-related memories and play a key role in its stabilization during abstinence as shown in previous studies (Sorg, et al., 2016; Lasek et al., 2018; Guarque-Chabrera et al., 2022). The observed fast decline in drug seeking after PNN degradation points to LVII as a part of the mechanisms for persistence of drug-related memory.

ChABC infusion successfully reduced PNN expression in lobule VII throughout the whole abstinence period. Nevertheless, the enzymatic digestion was less effective than that found in our previous studies (Guarque-Chabrera et al., 2022), particularly in the cocaine extend access group in which PNNs were upregulated throughout abstinence. One plausible explanation is that mechanisms for PNN remodelling counterbalanced the effects of ChABC during abstinence. Maybe this is the reason why we could not demonstrate a larger behavioural effect after degradation.

Finally, in this doctoral thesis, we addressed activity changes in the cerebellar cortex after PNN degradation. An early study demonstrated that cocaine-induced preference conditioning increases neuronal activity in granule cells and Golgi interneurons (Carbo-Gas et al., 2017). Here, we assessed C-Fos expression in Purkinje and granule cell neurons after the last drug seeking test. Therefore, C-Fos expression resulted from neuronal events during the test that could be modulated by cocaine exposure and removal of PNNs. We were unable to demonstrate any change in neural activity as an effect of PNN degradation. However, a recent study has found a reduction in C-Fos expression of PNNs-bearing neurons in DCNs after digestion with ChABC (Carulli et al., 2020). Only granule cell activity increased after drug seeking test at 28 days of abstinence. Interestingly, granule cell activity correlates with lever pressing during this test in ShA and LgA groups which supports the function of granule cells in encoding reward processes proposed by other groups (Wagner et al., 2017). This finding indicates that LVII in the vermis is involved in drug seeking but it would not be so evident its involvement in the incubation effect. The consequence of increased granule cell activity is a higher excitation of inhibitory interneurons of molecular layer (basket and stellate cells) through parallel fiber synapses that, in turn, could enhance their inhibitory action on Purkinje activity and encourage cerebellar output signals (Gao et al., 2016; Albergaria et al., 2018).

Enzymatic digestion of PNNs in the cerebellar cortex does not affect extinction of cocaine self-administration

To better understand the results obtained in study 3 and to clarify the role of LVII in drug seeking, in this last section, we explored the effect of PNN enzymatic digestion in lobule VII on the formation of extinction memories after extended access cocaine self-administration. Subsequently, we tested extinction using two tests, cue- and drug-induced reinstatement. Given that DS tests are conducted under extinction, and they include three trials, we wondered whether the faster decline found in the third trial of the DS-test might be related to the facilitation of extinction learning. We digested PNNs in LVII before extinction training and did not find any effect of PNN removal in this lobule. These findings disagree with previous findings in the cerebellum and other brain regions. A recent study from our lab showed that PNN removal in LVIII facilitates the formation of extinction memory (Guarque-Chabrera et al., 2022). PNN removal in the amygdala followed by extinction training improved extinction learning and prevented relapse of morphine self-administration (Xue et al., 2014). However, the elimination of PNNs in this area did not affect the consolidation of drug-related cues. Thus, when incubation of drug seeking occurs without LVII PNNs the stability of instrumental memories that trigger and drive the drug-seeking response are impaired.

Conclusions

Overall, this thesis presents a working model for the function of PNNs in LVII. Our results show that cocaine self-administration, in an extended access schedule, dynamically regulates synaptic plasticity conditions in the cerebellum during abstinence and increases the expression of PNNs after prolonged abstinence. This is consistent with the observed expression levels of Bcan and TN-R, both essential components of PNNs, and with the elevated expression levels of PTP-PTP dimers (inactive form of PTP receptors, responsible for down-regulation of TRKB receptor function in synaptic plasticity). Moreover, the present findings support that PNNs around Golgi interneurons are mechanisms required for the persistence of drug-induced memories and their upregulation through abstinence may be linked to stabilisation of parts of the engrams that sustained these memories. On the contrary, synaptic plasticity modifications that underlie the incubation of craving do not appear to require intact cerebellar PNNs.

STRENGTHS AND LIMITATIONS

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Strengths

- The principal contribution of this thesis is to propose a working model for the function of PNNs in LVII of cerebellum in drug-addiction. To our best knowledge, this is the first investigation to study cerebellar functions in drug-seeking after cocaine self-administration.

- In this thesis, we use the intravenous self-administration paradigm in extended access that not only emphasises the voluntariness of drug intake but mimics some of the features of drug addiction in humans.

- The present thesis represents the first attempt at addressing the behavioural role of PNNs in the cerebellar cortex. Previous studies described their role in neuronal plasticity during development as well as PNN components and their functions. Preceding research on behavioural functions of cerebellar PNNs aimed exclusively at PNNs in DCN.

Limitations

- Sample size in study 2 is small and there is a considerable dispersion in WB data, which makes them inconsistent and difficult to interpret. An improvement of the WB procedure is needed as well as an increase in the sample size to allow us to correctly elucidate the effect of cocaine self-administration on the proteins of interest.
- Although WB is a widely extended technique to determine the relative amounts of proteins of interest, it does not provide information about the origin of their changes. Future experiments should address mRNA transcripts of the proteins of interest by PCR, and enzymatic activity analysis of MMPs and ADAMTS.
- The expression of C-Fos is widely used as a marker of neuronal activity. However, electrophysiology would be a better approach to demonstrate cocaine-induced neuronal activity changes after removal of PNNs.
- In this investigation, we have only focused on the effect of PNN degradation on LVII of the vermis. However, it would be interesting to explore the role of PNNs and neuronal activity in other parts of the cerebellum. Likewise, it is crucial to

know the consequences of PNN degradation in LVII for the rest of the addiction circuit. This will be part of the Master's final work of one of our Master's students who has already performed some experiments in his final degree work and is shown in Annex II.

- Since all our research has been conducted in males, it is necessary to explore the role of cerebellar PNNs, the effect of cocaine self-administration on PNN activity, as well as the effect of its degradation on drug-seeking using female rodents.

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ANNEX I

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ORIGINAL INVESTIGATION

Time-dependent regulation of perineuronal nets in the cerebellar cortex during abstinence of cocaine-self administration

Aitor Sanchez-Hernandez[1] · Celine Nicolas[2] · Isis Gil-Miravet[1] · Julian Guarque-Chabrera[1] · Marcello Solinas[2] · Marta Miquel[1]

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Abstract

Rationale The probability of structural remodeling in brain circuits may be modulated by molecules of perineuronal nets (PNNs) that restrict neuronal plasticity to stabilize circuits. Animal research demonstrates that addictive drugs can remodel PNNs in different brain regions, including the cerebellum.

Objective This study aimed to investigate the effects of short versus extended access to cocaine self-administration on PNN expression around Golgi interneurons in the cerebellar cortex after different periods of abstinence.

Methods After 1 week of training (2 h/day), Sprague-Dawley rats self-administered cocaine daily for 20 days under short (ShA) or extended (LgA) access. PNN expression in the cerebellum was assessed after 1 day, 7 days, and 28 days of forced abstinence. PNNs were immunolabeled using *Wisteria floribunda* agglutinin (WFA) and captured by confocal microscopy.

Results WFA intensity increased in PNN-bearing Golgi neurons over the abstinence period and a higher proportion of more intense PNNs were formed throughout the first month of abstinence. After the first 24 h of cocaine abstinence, however, we found a reduction in WFA intensity in the cerebellar cortex of rats with ShA to cocaine as compared to naïve animals. When comparing with naïve rats, LgA rats showed consistent PNN upregulation at 28 days of cocaine abstinence.

Conclusions Our results suggest that cocaine self-administration produces modifications in PNN that enhance conditions for synaptic plasticity in the cerebellar cortex. These modifications are revealed shortly after the cessation of drug intake but PNNs become more intense during protracted abstinence in the LgA group, pointing to the stabilization of drug-induced synaptic changes. These findings indicate that extended access to cocaine self-administration dynamically regulates conditions for plasticity in the cerebellum during abstinence.

Keywords Cerebellum · Cocaine-extended access · Perineuronal nets · Rats · WFA · Golgi interneurons

Introduction

The probability of structural remodeling in brain circuits may be modulated by the activity of regulatory molecules that restrict neuronal plasticity to stabilize circuits (Foscarin et al. 2011). This regulatory activity occurs in lattice cartilage-like structures called perineuronal net (PNN) consisting of molecules of extracellular matrix (ECM) that wrap several neuronal populations, the majority of them fast-spiking GABAergic interneurons (Grimpe and Silver 2002; Carulli et al. 2006; Soleman et al. 2013; Sorg et al. 2016; Miyata and Kitagawa 2017; Fawcett et al. 2019).

Several PNN components can interact with membrane receptors and ion channels, and change electrophysiological properties and plasticity events of PNN-bearing neurons (Fawcett et al. 2019). In particular, PNNs appear to funnel or restrict the number of possible future plastic events and to represent a form of cellular memory (Dityatev and Schachner 2003). Consequently, PNN digestion with chondroitinase ABC (ChABC), an enzyme that targets the chondroitin sulfate proteoglycans (CSPGs), causes the reversion to an immature brain stage, thus increasing plasticity conditions (Pizzorusso et al. 2002; Lensjø et al. 2017). Both inhibitory and excitatory balance (Lensjø et al. 2017), and

✉ Marta Miquel
miquel@uji.es

[1] Área de Psicobiología, Universitat Jaume I, Avenida Vicente Sos Baynat sn, 12071 Castellón de la Plana, Spain

[2] INSERM, U-1084, Laboratoire de Neurosciences Expérimentales et Cliniques, Université de Poitiers, Poitiers, France

AMPA receptor mobility (Frischknecht et al. 2009; Favuzzi et al. 2017) are all affected after PNN removal. Therefore, PNNs participate in maintaining the synaptic architecture critical to neural plasticity, which is believed to mediate learning and memory, and thereby, it could underlie drug addiction (Wright and Harding 2009; Fawcett et al. 2019).

Findings from animal and human research evidence the ability of addictive drugs to remodel PNNs dynamically. Drug-related plasticity and metaplasticity involve PNN regulation in different brain regions (Mash et al. 2007; Van den Oever et al. 2010; Xue et al. 2014; Chen et al. 2015; Slaker et al. 2015, 2016; Vazquez-Sanroman et al. 2017; Blacktop et al. 2017; Slaker et al. 2018; Blacktop and Sorg 2019; Gil-Miravet et al. 2019), including the cerebellum (Vazquez-Sanroman et al. 2015a, b; Carbo-Gas et al. 2017). Drug-induced remodeling of PNNs depends on several factors such as the brain region, the length of the withdrawal period, the time course, and the behavioral process investigated. Indeed, whereas chronic experience with cocaine and alcohol increases PNN expression and, thus, *Wisteria floribunda* agglutinin (WFA) staining in the prelimbic cortex (Slaker et al. 2018), cerebellum (Vazquez-Sanroman et al. 2015b; Carbo-Gas et al. 2017), and insula (Chen et al. 2015), nicotine self-administration reduces WFA staining in the VTA and orbitofrontal cortex (Vazquez-Sanroman et al. 2017). Removal of PNNs in these brain regions has revealed the functional role of this specialized ECM in the acquisition, reinstatement (Slaker et al. 2015; Blacktop et al. 2017), and extinction (Xue et al. 2014) of drug-related behaviors.

A neurotoxic lesion of the cerebellum's posterior vermis has shown to facilitate cocaine-induced preference conditioning and to increase neural activity and PNN expression in GABAergic parvalbumin + interneurons of the medial prefrontal cortex (Gil-Miravet et al. 2019). In the cerebellar cortex, cocaine-induced conditioned preference has been associated with more intense PNNs around Golgi interneurons (Carbo-Gas et al. 2017; Traver et al. 2018). However, cocaine-dependent regulation of PNNs surrounding output neurons in the deep cerebellar nuclei (DCN) was not linked to drug memory (Carbo-Gas et al. 2017) but to neuroplasticity events that occur during abstinence periods. Cocaine-induced reinstatement increases PNN expression after short abstinence (Vazquez-Sanroman et al. 2015a) but decreases its expression after protracted abstinence (Vazquez-Sanroman et al. 2015b) in the DCN. Therefore, through PNN regulation, addictive drugs may affect synaptic plasticity and the conditions for plasticity, creating restrictive or permissive metaplasticity (Slaker et al. 2015; Vazquez-Sanroman et al. 2015a; Neuhofer and Kalivas 2018).

In the present research, we investigated the effects of short versus extended access to cocaine self-administration, a model of development of addiction (Ahmed and Koob 1998), on PNN expression in the cerebellum. PNN expression was evaluated after 1 day, 7 days, or 28 days of abstinence from cocaine self-administration. We aimed at exploring whether long-term cocaine exposure can remodel PNNs around Golgi inhibitory interneurons and whether PNN regulation persists after protracted abstinence.

Material and methods

Subjects and housing conditions

Our materials and methods are the same described in Nicolas et al. (2017) for all behavioral experiments. Adult male Sprague-Dawley rats (8–10 weeks) ($N = 40$) (Janvier Labs, France), experimentally naïve at the beginning of the study, were housed in a temperature and humidity-controlled environment and maintained on 12-h light/dark cycle (light on at 7 AM). All experiments were conducted following the European Union directives (2010/63/EU) for the care of laboratory animals and approved by the local ethics committees (COMETHEA). Upon arrival, rats were housed two per cage for about 1 week before intra-jugular vein catheterization surgery. After surgery, rats were housed individually for the entire period of self-administration and 3 per cage during the abstinence period. Naïve-control rats had the same housing conditions. We chose these housing conditions because social isolation is a well-known stressors in rodents that influences addiction-related processes and social housing appears a better control for the effects of environmental enrichment (see Solinas et al. 2010 for discussion). Both our group and other groups have used social housing during abstinence and found long-lasting cocaine seeking and incubation of cocaine craving (Chauvet et al. 2009; Chauvet et al. 2012; Thiel et al. 2009a, b, 2011).

Drugs

Cocaine HCl was obtained from Cooper (Melun Cedex, France) and dissolved in sterile saline solution (0.9%). Heparin (0.6%) was obtained from Sigma-Aldrich (Sigma-Aldrich Chimie S.a.r.l. Lyon, France) and dissolved in sterile saline solution. Gentamicin (4%) was obtained from Panpharma (Luitré-Dompierre, France).

Catheter surgery

Rats were implanted with catheters in the jugular vein under isoflurane (2%) anesthesia (Belamont ®, PiRAMAL, London, UK) as described previously (Thomsen and Caine 2005). Briefly, the catheters, assembled in the laboratory, consisted of a 12-cm-length Silastic tubing (Fischer, France) fitted to a guide cannula (PlasticOne, USA) bent at a 90° angle. The Silastic tubing

was fixed to the guide cannula with thermoretractable tubing (Conrad, France) and encased in dental cement anchored with a 2.5-cm^2 durable mesh used as a subcutaneous anchor. The tubing was inserted subcutaneously from the animals back to the right external jugular vein. After surgery, all animals could recover for a minimum of 5 days before given access to cocaine during which catheters were flushed daily with 0.15 ml of a filtered heparinized saline solution 0.6% (Sigma, St. Quentin Fallavier, France) and gentamicin 4% (Panpharma, Luitré, France).

Self-administration apparatus and procedure

The experimental timeline is schematized in Fig. 1. A week after catheter surgery, self-administration training began in operant chambers (Imetronic, Pessac, France) equipped with nose-pokes as operanda and controlled by Imetronic interfaces and software (Imetronic, Pessac, France, www.imetronic.com). Rats were allowed to self-administer cocaine according to a fixed ratio 1 (FR1) schedule of reinforcement. Thus, an active nose-poke response immediately resulted in an IV injection of cocaine for 5 s (0.3 mg/injection), the blinking of house light for 5 s followed by a 5-s time-out period during which the chamber was dark, and further active nose-pokes had no programmed consequences. The first 7 sessions lasted 2 h for all rats. Starting from the 8th session, rats were pseudo-randomly divided into two groups after the criterion for the acquisition was reached (at least 15 self-infusions in 2 h) (Ahmed and Koob 1998): (i) short-access group (ShA), with access to cocaine self-administration for 1 h per day and (ii) long-access group (LgA) with access to cocaine self-administration for 6 h/day, for 20 sessions. These two paradigms of drug exposure are designed to mimic recreational versus addictive cocaine intake, respectively (Ahmed and Koob 1998). Then, ShA and LgA rats were left undisturbed in their home cages for 1, 7, or 28 days before perfusion. A group of naïve rats paired for age and housing conditions did not undergo any behavioral training and did not have exposure to cocaine and served as a control.

Perfusion and dissection protocol

Animals were deeply anesthetized with sodium pentobarbital (30 mg/kg) and perfused transcardially, first with 0.9% saline solution and 4% paraformaldehyde. After perfusion, the brain was immediately dissected and placed in a container with 4% paraformaldehyde for 24 h. After this time, tissue was cryoprotected in 30% sucrose solution until complete immersion. Brain tissue was rapidly frozen by immersion in liquid nitrogen, and coronal sections were performed at 40 μm with a cryostat (CM3050S, Leica, France). Five series of tissue sections were collected and stored at − 80 °C in a cryoprotectant solution. Coronal sections of the cerebellum were selected for immunoanalysis.

Immunolabeling and image analysis

PNN immunolabeling was performed on free-floating sections as previously published (Vazquez-Sanroman et al. 2015a, b; Carbo-Gas et al. 2017). After several rinses with PBS 0.1 M Triton X-100 (0.1%), cerebellar sections were incubated overnight at 4 °C with biotinylated *Wisteria floribunda* agglutinin (WFA) (1:200; Sigma-Aldrich, Madrid, Spain) dissolved in PBS 0.1 M Triton X-100. WFA binds to the glycosaminoglycan (GAG) chains of the proteoglycans (CSPGs) and was used to label the PNN structure (Härtig et al. 1992). Tissue samples were incubated for 2 h at room temperature with fluorochrome Cy3-conjugated streptavidin (1:200 Jackson Immunoresearch Europe Ltd., Suffolk, UK). The sections were mounted using Mowiol (Calbiochem, Merck Chemicals and Life Science, Madrid, Spain).

PNNs were labeled and analyzed throughout the entire cerebellar cortex, including the vermis and hemispheres. Imaging and assessment of PNNs were blind to the treatment. In the cerebellar cortex, only Golgi inhibitory interneurons express a PNN (Corvetti and Rossi 2005; Carulli et al. 2006). To estimate changes in WFA intensity, we analyzed all PNNs that showed unquestionable integrity in their structure. A × 40 objective with a × 3.0 zoom was used for a final magnification of

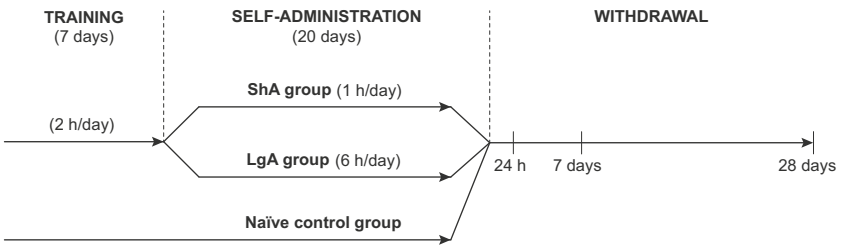

Fig. 1 Experimental timeline. After 7 days of cocaine self-administration training (2 h/day), rats were randomly assigned to short access (ShA, 1 h/day) and long access (LgA, 6 h/day). At the end of the 20 days of cocaine self-administration, rats underwent 1 day, 7 days, or 28 days of force abstinence. Naïve rats were used as controls

× 5120. Brightness intensity of every PNN was estimated by randomly selecting 15 pixels in a single plane of the net pulled from a 40-μm *z*-stack and calculating their average intensity (Carbo-Gas et al. 2017). That is, we moved in the *z*-plane until we found the plane in which each PNN showed the highest integrity. This procedure enables to not underestimate intensity when the PNN appears in a different *z*-plane. Data were expressed as arbitrary units of intensity (AU of WFA intensity) with a maximum intensity of 255. Then, we selected 30 PNNs randomly per rat to calculate the proportion of strong (more intense) (171–255 AU), medium (86–170 AU) and faint (0–85 AU) PNNs. Pictures of fluorescent-labeled sections were acquired using a Leica SP8 confocal microscope. Image resolution was 1024 × 1024 and scan speed 600 Hz. Laser intensity, gain, and offset were maintained constant in each acquisition. All quantitative evaluations were made using the FIJI software (Schindelin et al. 2012).

Statistics

Results of cocaine self-administration were analyzed by two-way repeated-measures ANOVA with sessions as a within-subject factor and cocaine access (ShA/LgA) as a between-subject factor. One-way (group) and two-way (group x abstinence days) ANOVAs were used for the analysis of PNN intensity. Differences between means were analyzed through Tukey's HDS and Sidak's tests. We also classified PNNs in three categories, strong, medium, and weak, and compared the percentage of PNNs in each category using the χ^2 test. Data is presented as mean ± SD and 95% confidence intervals (CI). All the statistical analyses were performed using GraphPad Prism software 8 (San Diego, CA, USA).

Results

Cocaine intake escalated in extended access rats

All rats could self-administer cocaine under a FR1 schedule for seven sessions (2 h/daily). After this initial training, rats self-administer cocaine for 1 h (ShA) ($n = 17$) or 6 h (LgA) ($n = 17$) for 20 sessions (Fig. 2.). ShA rats emitted 68.42 ± 9.52, 95% CI [64.66, 72.19], active nose-pokes in 1-h session, whereas LgA rats emitted 200.00 ± 76.79, 95% CI LgA [169.7, 230.4] active nose-pokes in the first of the 6 h. A two-way ANOVA for active nose-pokes (cocaine access × sessions) showed an effect of cocaine access ($F(1, 31) = 110.10$, $P < 0.0001$), sessions ($F(26, 806) = 10.81$, $P = 0.0001$), and cocaine × session interaction ($F(26, 806) = 12.16$, $P < 0.0001$). The analysis of the number of cocaine injections yielded similar results. ShA rats received 42.71 ± 6.11, 95% CI [40.29, 45.13] cocaine infusions, while LgA rats received 129.00 ± 53.15, 95% CI [107.90, 150.0]. A two-way

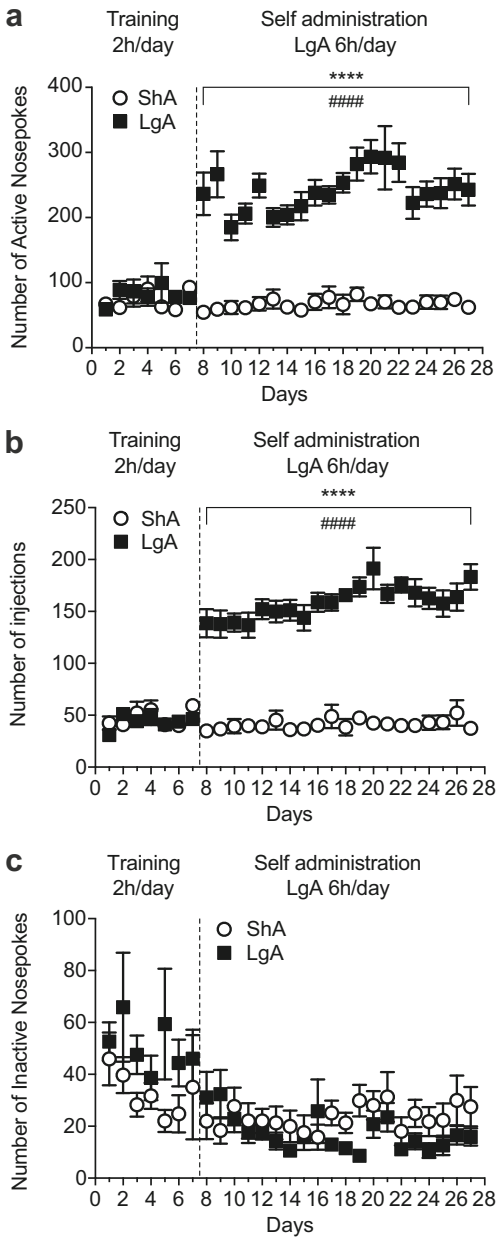

Fig. 2 Cocaine self-administration. **a** Number of active nose-pokes during the escalation phase in which animals had access to cocaine for 1 h (ShA, $n = 17$) or 6 h (LgA $n = 17$). **b** Number of cocaine injections in ShA and LgA rats. **c** Number of inactive nose-pokes. $^{****}P < 0.0001$ LgA compared to ShA group. $^{\#\#\#}P < 0.0001$, $^{\#}P < 0.05$ for within-group comparisons

ANOVA showed an effect of cocaine access ($F(1, 31) = 195.10$, $P < 0.0001$), sessions ($F(26, 806) = 22.27$, $P < 0.0001$) and cocaine × session interaction ($F(26, 806) = 26.42$, $P < 0.0001$). In the ShA group, the number of cocaine injections for 20 sessions were stable and did not escalate, whereas in the LgA group, it escalated significantly from the 8th session as confirmed by Sidak's post hoc test (Fig. 2a, b). Therefore, escalation occurred for cocaine intake but not for the number of nose-pokes. Finally, ShA rats responded with

an average of 26.70 ± 6.93, 95% CI [22.96, 28.44] inactive nose-pokes, and LgA rats did 25 ± 16.58, 95% CI [19.41, 32.53]. An ANOVA showed no effect of cocaine access ($F(1, 31) = 0.003$, $P = 0.9500$) but a significant effect for the session ($F(26, 806) = 4.07$, $P < 0.0010$), and access × session interaction ($F(26, 806) = 1.79$, $P = 0.0090$) (Fig. 2c).

Protracted abstinence increases PNN expression in the cerebellar cortex

To characterize the effects of short ($n = 18$) versus extended access ($n = 18$) to cocaine self-administration on cerebellar PNN expression during abstinence, we compared WFA intensity around Golgi interneurons 1, 7, or 28 days after the last cocaine self-administration session (Fig. 3). A two-way ANOVA (cocaine access × withdrawal time) yields significant differences for cocaine access ($F(1, 10) = 13.71$, $P = 0.0041$) and abstinence time ($F(2, 20) = 11.46$, $P = 0.0005$) but not for the interaction between these two factors ($F(2, 20) = 0.3510$, $P = 0.7083$). The lack of a significant interaction indicates that PNN upregulation through abstinence occurred in both cocaine groups. Nevertheless, as can be seen in Fig. 3a, b, the pattern was more evident in the LgA group. We calculated mean ± SD and 95% CI for 1 day (ShA: 59.86 ± 16.22, 95% CI [42.78, 76.94]; LgA: 78 ± 21.02, 95% CI [56.51, 100.6]), 7 days (ShA: 103.43 ± 22.84, 95% CI [81.90, 124.88]; LgA: 118.31 ± 32.20, 95% CI [84.51, 152.10]), and 28 days (ShA: 103.50 ± 37.74, 95% CI [68.02, 138.88]; LgA: 136.29 ± 22.39, 95% CI [115.27, 157.31]) ($n = 6$).

In addition, the distribution of strong and faint PNNs in the cerebellar cortex was different among groups ($\chi^2(10) = 158.30$, $P < 0.0001$). ShA rats expressed 81% of faint and 1.6% of more intense PNNs at 1 day after cocaine self-administration. After 28 days of abstinence, the percentage of faint PNNs decreased to 53% while strong PNNs increased to 22%. Moreover, 66% of PNNs were faint and 4% were strong 1 day after extended accesses to cocaine. Protracted abstinence reduced the proportion of faint PNNs (27%) but enhanced the proportion of strong (38%) (Fig. 3c).

Cocaine self-administration reduces PNN expression in the cerebellar cortex

To determine whether different history of cocaine self-administration change PNN expression in the cerebellum, ShA and LgA rats were compared with naïve rats 1 day after the last cocaine self-administration session (Ctrl; $n = 6$, 83.44 ± 4.22, 95% CI [79.01, 87.87]) (Fig. 4). Cocaine self-administration under short-access conditions reduced WFA intensity in Golgi cells bearing a PNN ($F(2,15) = 3.85$, $P = 0.0450$). The reduction was clear in the ShA group so that the majority of subjects exhibited WFA intensity below control levels ($P = 0.0470$, DF: 15) (Fig. 4a). However, WFA intensity in the LgA group after 24 h did not differ from naïve rats ($P = 0.8520$, DF: 15) (Fig. 4b). We also found a different distribution of strong (> 170 AU of WFA) and faint (< 85 AU of WFA) PNNs among groups. While naïve rats expressed 63% of faint and 12% of strong PNNs, the ShA group exhibited 81% of faint PNNs but only 1.6% of strong

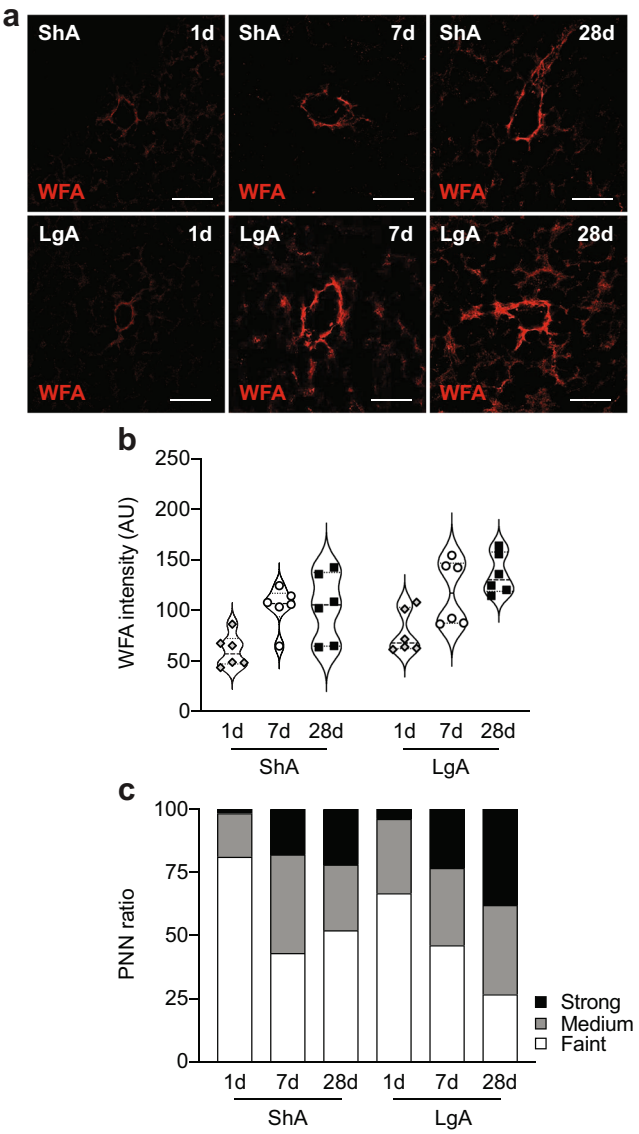

Fig. 3 Expression of PNNs in the cerebellar cortex at different time points of cocaine abstinence in ShA versus LgA rats. a Violin plots with individual scores of WFA intensity from PNNs throughout different periods (1, 7, and 28 days) of force abstinence in ShA and LgA rats. 1 day ShA (n = 6), 7 days ShA (n = 6), 28 days ShA (n = 6), 1 day LgA (n = 6), 7 days LgA (n = 6), and 28 days LgA (n = 6). Plots indicate data range, quartiles, and median. The Y-axis represents WFA intensity expressed in arbitrary units (AU). b Representative confocal images (× 120) of PNNs in each group. Protracted abstinence from cocaine increased PNN expression around Golgi interneurons in the cerebellar cortex. Scale bar 20 μm. c Proportion of Golgi neurons expressing a faint, medium, or strong PNNs in the cerebellar cortex

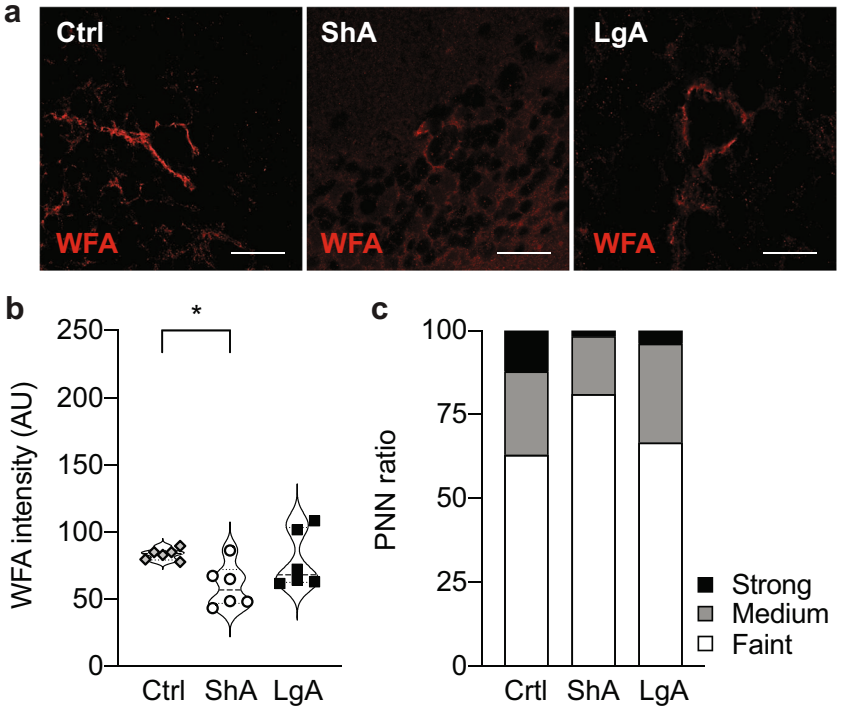

Fig. 4 PNN expression in the cerebellar cortex 24 h after the last cocaine self-administration session. **a** Representative confocal PNN images (× 120) of naïve (Ctrl), short-access (ShA), and extended-access (LgA) groups. Scale bar 20 μm. **b** Violin plots with individual scores show the effects of cocaine self-administration on WFA intensity in PNNs around Golgi interneurons of the cerebellar cortex after 24 h of abstinence. Plots indicate data range, quartiles, and median. The *Y*-axis represents WFA intensity expressed in arbitrary units (AU): ShA ($n = 6$) and LgA ($n = 6$). Ctrl ($n = 6$) had no access to cocaine. $^{**}P < 0.01$ compared with the control group. **b**, **c** Proportion of Golgi neurons expressing a faint, medium, or strong PNNs in the cerebellar cortex. Cocaine self-administration decreased PNN expression in the cerebellar cortex after ShA to cocaine

ones ($\chi^2(4) = 28.76$, $P < 0.0001$). LgA rats showed 66% of faint and 4% of strong PNNs, and they were not different from naïve-control rats (Fig. 4c).

PNN expression did not return to control levels after protracted abstinence

To assess whether PNN expression returns to control levels after protracted abstinence, we compared ShA and LgA rats with naïve controls after 28 days of abstinence. Extended access to cocaine self-administration caused a significant increase in WFA intensity around Golgi cells that did not return to control levels after 28 days of abstinence ($F(2, 15) = 8.220$, $P = 0.0039$) (LgA: $P = 0.0030$; DF: 15). This effect was not evident in ShA rats ($P = 0.3096$, DF: 15) (Fig. 5).

Finally, we did not find significant effects in the number of PNNs as a Kruskal-Wallis test showed ($H(7) = 10.03$, $P = 0.1235$; $n = 3$). We show mean ± SD and median with 95% confidence interval of median for all groups: (Crtl: 153.66 ± 131.16; ShA 24 h: 247 ± 68.46; ShA 7 days: 434.66 ± 44.01; ShA 28 days: 446 ± 201.20; LgA 24 h: 330.33 ± 53.66; LgA 7 days: 365.66 ± 120.95; LgA 28 days: 404.66 ± 73.18); (median and 95% CI of median; (Ctrl: 106, 95% CI [53, 302]); (ShA 24 h: 248, 95% CI [168, 289]); ShA 7 days: 436, 95% CI [309, 478]; (ShA 28 days: 540, CI 95% [215, 583]); (LgA 24 h: 315, 95% CI [286, 309]); LgA 7 days: 335, 95% CI [263, 499]; LgA 28 days: 380, 95% CI [347–487]).

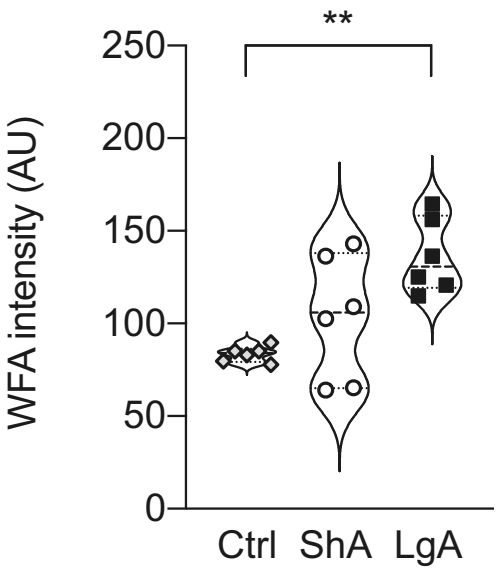

Fig. 5 PNN expression in ShA and LgA rats after 28 days of abstinence as compare to naïve rats. Violin plots with individual scores show the effects of protracted cocaine abstinence on WFA intensity in the cerebellar cortex. Ctrl ($n = 6$); 28 days ($n = 6$). Plots indicate data range, quartiles, and median. $^{**}P < 0.01$; $^{*}P < 0.05$. Proportion of Golgi neurons expressing faint, medium, or strong PNNs in ShA rats

Discussion

In a previous investigation, we showed that the formation of cocaine-induced conditioned preference is associated with increases in PNN expression surrounding Golgi interneurons of the cerebellar cortex (Carbo-Gas et al. 2017). More intense PNNs correlated with a higher preference for cocaine-related cues (Carbo-Gas et al. 2017). In the present study, our results show a dynamic regulation of PNNs during abstinence in the cerebellar cortex. After the first 24 h of abstinence, we found a reduction in the expression of PNNs around these cerebellar GABAergic interneurons in animals with short access to cocaine. Over the course of prolonged abstinence, however, the expression of cerebellar PNNs increased, and a higher proportion of intense PNNs formed throughout the first month. The intensity of PNNs across time was not different in ShA rats, but it enhanced during abstinence after an escalated cocaine intake (LgA).

It has been hypothesized that PNNs play a role in regulating neural plasticity via three possible mechanisms: (1) altering the formation of new neuronal contacts (Corvetti and Rossi 2005; Barritt et al. 2006); (2) acting as a scaffold for molecules that can inhibit synaptic formation (Deepa et al. 2006); and (3) limiting receptor motility at synapses (Frischknecht et al. 2009). It is clear that reduction in the expression of PNNs can facilitate synaptic plasticity and enable synaptic remodeling (Pizzorusso et al. 2002; Lensjø et al. 2017; Carulli et al. 2020). Accordingly, degradation of PNNs around Golgi interneurons by the enzyme chondroitinase ABC increases structural plasticity in the cerebellar cortex (Corvetti and Rossi 2005). Our findings suggest that cocaine self-administration might enhance conditions for synaptic plasticity in this region that were revealed shortly after the cessation of drug intake (24 h) in ShA rats. The regulation of PNNs during this first 24 h after chronic cocaine is not the same in all brain regions. Nonetheless, any possible comparison among studies should be taken with caution because they involve distinct behavioral protocols for cocaine administration and diverse drug-induced memory processes. Density of high intensity PNNs increases in the right prelimbic, infralimbic, and ventral orbitofrontal cortex 24 h after cocaine self-administration (Roura-Martínez et al. 2020). Under the same conditions, the density of low- and medium-intensity PNNs was higher in the left hemisphere (Roura-Martínez et al. 2020). Moreover, PNN expression decreased in the infralimbic cortex, but it increased in the prelimbic cortex after 5 days of i.p. cocaine administration (Slaker et al. 2018). Endogenous enzymatic remodeling of PNNs relays on several families of proteases, including matrix metalloproteinases (MMPs) (Huntley 2012) and proteases that belong to the plasminogen-activating system such as the tissue plasminogen activator (tPA) (De Luca and Papa 2016). The activity of these enzymes is dynamically regulated during cocaine abstinence and reinstatement in several regions (Van den Oever et al. 2010; Smith et al. 2014; Vazquez-Sanroman et al. 2015b). Both MMP 2 and 9 have been detected in the cerebellum (Stamenkovic et al. 2017), although their role in drug-induced plasticity and memory is unknown. In our previous research, we found that 24 h following a new i.p cocaine challenge administered after protracted abstinence (1 month), tPA activity enhanced and PNN expression was downregulated in the deep cerebellar nuclei (Vazquez-Sanroman et al. 2015b). No changes in tPA levels were detected following a shorter period of abstinence (1 week) (Vazquez-Sanroman et al. 2015a).

The present results also indicate that PNNs around Golgi interneurons increased their expression progressively during abstinence. More intense PNNs may stabilize synaptic modifications induced by the extended access to cocaine and enable their persistence during abstinence. Several of constituent molecules in a PNN such as brevican, neurocan, and tenascin-R are involved in the maintenance of short- and long-term plasticity (Brakebusch et al. 2002; see Fawcett et al. 2019 for a recent review). Strong PNNs might represent a common mechanism to maintain drug-related memories and, therefore, contribute to plasticity leading to relapse. In agreement with this hypothesis, the digestion of PNNs in the anterior hypothalamic area prevented reinstatement of cocaine self-administration, but it did not affect sucrose self-administration (Blacktop et al. 2017). Moreover, degradation of PNNs into the amygdala during extinction training blocked reinstatement of cocaine- and morphine-induced conditioned place preference (Xue et al. 2014). In other words, strong PNNs might "stamp in" synaptic arrangements that represent drug-cue associations and contribute to long-lasting drug memories by preventing synaptic remodeling (Sorg et al. 2016; Lasek et al. 2018).

Golgi neurons are crucial elements of local circuits in the cerebellar cortex (see D'Angelo and De Zeeuw 2009; D'Angelo et al. 2013 for reviews). Their axons inhibit neighboring granule cells (GC) (Tabuchi et al. 2019) and play a crucial role in the synchronization of activity in GC clusters (Eccles et al. 1964). Golgi cell activity is controlled by glutamatergic inputs from GC and mossy fibers (Palay and Chan-Palay 1974), as well as by GABAergic and glycinergic inhibitory signals from other populations of cerebellar interneurons (Sotelo and Llinás 1972; Dumoulin et al. 2001). Golgi cells thus appear to be essential regulators of plasticity in the cerebellar cortex (D'Angelo et al. 1999; Armano et al. 2000). The present study is the first to involve Golgi cells bearing a PNN in plasticity linked to abstinence after cocaine self-administration. Our previous findings indicated that cocaine memory increases activity of GC and Golgi neurons, and generates strong and fully condense PNNs around these inhibitory interneurons after 48 h (Carbo-Gas et al. 2017). The expected consequence of increasing activity in GC is an enhanced

inhibitory effect of molecular interneurons over Purkinje activity, since parallel fibers stimulate dendrites of molecular interneurons (Gao et al. 2016; Albergaria et al. 2018). Although in the present work, we did not evaluate activity changes in the cerebellar cortex, it is conceivable that the cerebellar cortex would present a similar pattern after protracted abstinence. Namely, stronger PNNs around Golgi neurons being associated with increased activity in GC. If this were the case, protracted cocaine abstinence would decrease Purkinje activity and facilitate the cerebellar output to other brain regions. It is now clear that the cerebellum may control the activity of dopaminergic and non-dopaminergic neurons within the VTA (Carta et al. 2019). Moreover, we have recently shown that a permanent perturbation of Purkinje activity by a neurotoxic lesion in the posterior cerebellar cortex facilitates the acquisition of cocaine-induced conditioned memory and increases neural activity in the DCN, VTA, ventral and dorsal striatum, and the medial prefrontal cortex (Gil-Miravet et al. 2019). Time-dependent increases in the ability of drug-related cues to trigger relapse during abstinence is known as the incubation effect (Grimm et al. 2001; Dong et al. 2017). The circuit involved in such effect includes regions of the VTA, the amygdala, the nucleus accumbens, and the medial prefrontal cortex (Dong et al. 2017). Overall, the most recent findings strongly point to a cerebellar modulation of these brain regions (Carta et al. 2019; Gil-Miravet et al. 2019). Further investigation will elucidate the particular role of cerebellar PNNs in the incubation of craving and relapse.

Concluding remarks

Even though several findings indicated the cerebellum's involvement in the functional alterations observed after prolonged drug use (Miquel et al. 2009; Moulton et al. 2014; Miquel et al. 2016), this brain structure has been traditionally ignored in the addiction field. Previous findings from our lab demonstrate the substantial ability of cocaine to induce molecular and structural changes in the cerebellum (Carbo-Gas et al. 2014a, b; Vazquez-Sanroman et al. 2015a, b). Moreover, we have proposed a cerebellum role in cocaine-induced conditioned memory (Gil-Miravet et al. 2018, 2019). Our present findings indicate that extended access to cocaine self-administration may dynamically regulate conditions for plasticity in the cerebellum during abstinence. Further research is required to determine whether the degradation of these cerebellar PNNs can prevent relapse after protracted abstinence.

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Author's contribution All authors made a notable contribution to the manuscript, and they were involved in critically revising the present version. CN conducted cocaine self-administration experiments that were coordinated by MS. ASH, IGM, and JGCH were all involved in immunofluorescence and image analysis. MM designed the study and was involved in data analysis. ASH, MM, and MS drafted the manuscript. All authors approved the present version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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ANNEX II

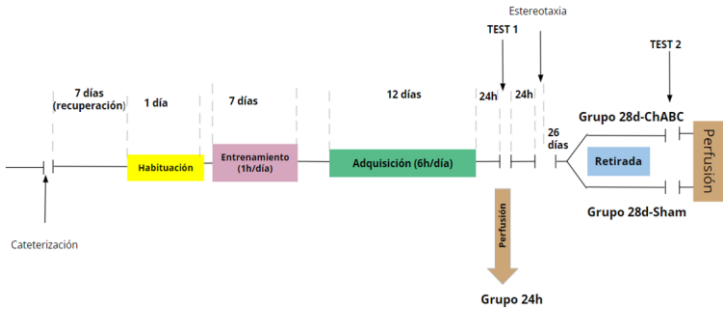
Effect of degradation of dorsal cerebellar perineuronal nets on the addiction circuitry

Abel Fábrega Leal (Tutora: Marta Miquel Salgado-Araujo)

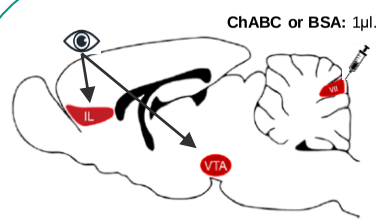
INTRODUCTION

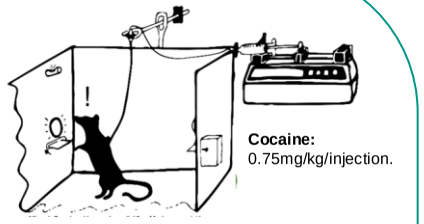
Perineuronal nets (PNNs), specializations of the extracellular matrix that surround the soma and proximal dendrites of some types of neurons, have been proposed as a mechanism of synaptic stabilization. In particular, PNNs appear to funnel or restrict the number of possible future plastic events and to represent a form of cellular memory. Consequently, degradation of the extracellular matrix (ECM), and thus the PNNs with the enzyme chondroitinase ABC (ChABC), reverses this restrictive state to an immature stage. Therefore, PNNs are involved in maintaining the synaptic architecture critical to neuronal plasticity, which is thought to mediate learning and memory, and thus may underlie drug addiction. Findings from animal and human research evidence the ability of addictive drugs to remodel PNNs dynamically. Specifically, the degradation of PNNs has been done in the dorsal cerebellum, which has already been shown to be part of the addiction circuitry in previous studies, being related to areas of the addiction circuitry such as the infralimbic area (IL) and the ventral tegmental area (VTA). The aim of the study is to test whether the degradation of the PNNs in lobule VII of the cerebellum alters the neuronal activity of several regions of the addiction circuitry such as the IL and VTA, by assessing cFos levels after a 24-hour or 28-day of abstinence. Our hypothesis is that the activity of these areas is modified after the degradation of PNNs in the cerebellar cortex (LVII) since the cerebellum exerts a modulatory control on these regions as our previous researches shown.

TIME-LINE

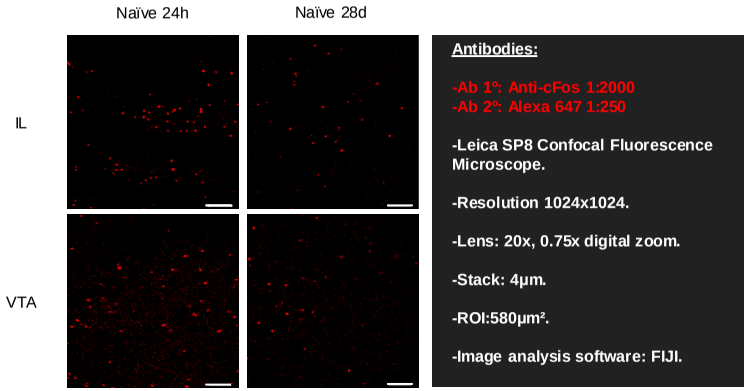


METHODOLOGY





Modificado de Miquel, Sánchez-Hernández e Ibáñez-Marín (2021)



Antibodies:

-Ab 1º: Anti-cFos 1:2000
-Ab 2º: Alexa 647 1:250

-Leica SP8 Confocal Fluorescence Microscope.

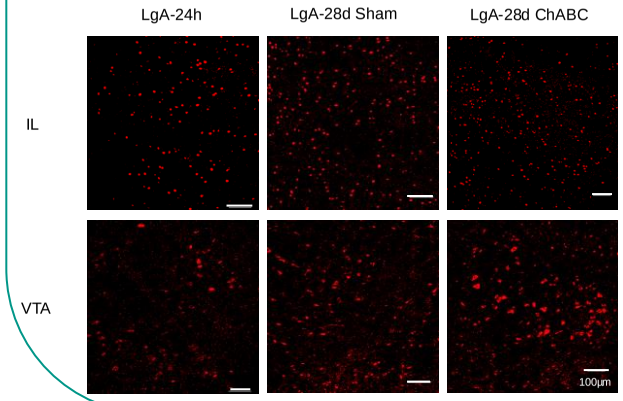
-Resolution 1024x1024.

-Lens: 20x, 0.75x digital zoom.

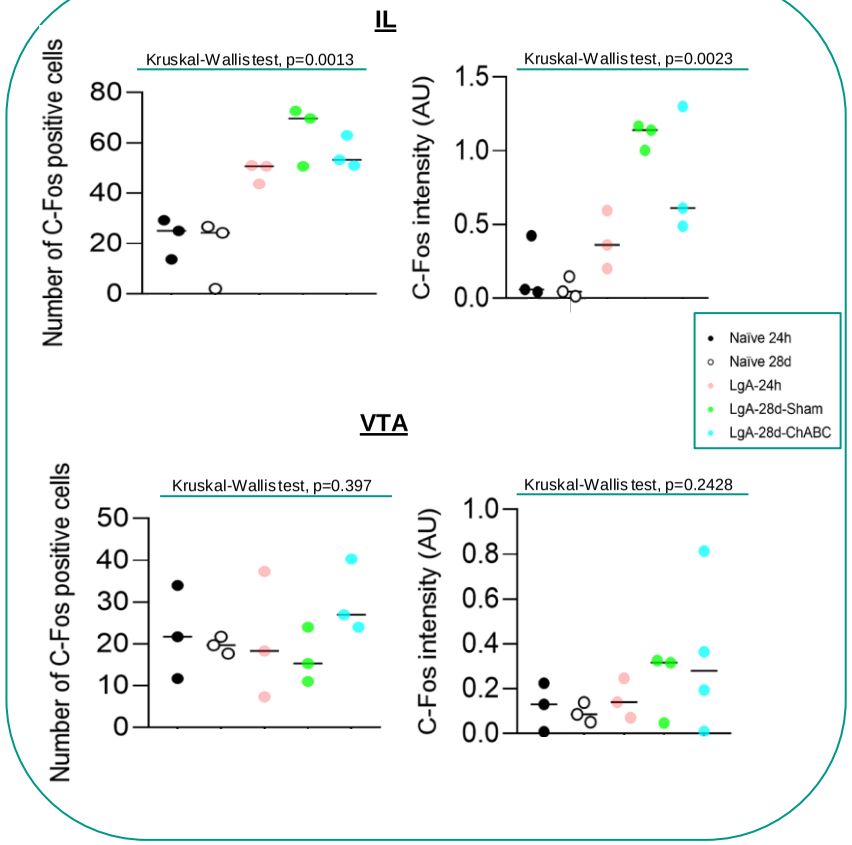
-Stack: 4µm.

-ROI:580µm².

-Image analysis software: FIJI.



RESULTS



CONCLUSIONS

Looking at these results, it seems that (1) the infralimbic cortex is involved in the incubation of drug seeking and (2) increased IL activity during protracted abstinence may be reversed by PNN disruption in the cerebellar cortex. Therefore, our findings point to an important role of the PNNs around Golgi interneurons of the cerebellum in restricting plasticity linked to the incubation effect.