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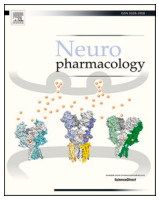

Comparative analysis of the modulation of perineuronal nets in the prefrontal cortex of rats during protracted withdrawal from cocaine, heroin and sucrose self-administration

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ABSTRACT

Relapse into drug use is a significant problem for people recovering from addiction. The ability that conditioned cues have to reinstate and reinvigorate drug-seeking is potentiated over time (incubation of seeking), posing an additional difficulty for maintaining abstinence. While the prefrontal cortex has been involved in the incubation phenomenon and the extracellular matrix, perineuronal nets (PNNs) in particular, may play a vital role in brain plasticity associated to drug relapse, there are no comparative analyses between different drug classes and natural reinforcers. Here, we compare the effects of early (1 day) and protracted (30 days) withdrawal from to cocaine, heroin and sucrose self-administration on the total density and density per intensity range of PNNs of different territories of the prefrontal cortex of male Lewis rats. Our results show that cocaine self-administration increases the density of PNNs in the dorsal prelimbic, infralimbic and ventral orbitofrontal cortices, while protracted withdrawal reversesthis effect in the dorsal prelimbic cortex. Also, heroin self-administration increases the density of PNNs in the infralimbic cortex and ventral orbitofrontal cortices, but this effect is lost after 30 days of withdrawal in the infralimbic cortex. Finally, the self-administration of sucrose-sweetened water or the protracted withdrawal from this powerful reinforcer does not affect any of the PNN parameters analysed. Our results show that two different drugs of abuse (but not a natural reward) with specific pharmacological and physiological actions, differentially modulate PNNs in specific areas of the rodent prefrontal cortex with potential implications for the incubation of seeking phenomenon.

1. Introduction

Relapse is a significant problem in the treatment of addiction (Bossert et al., 2005). Multiple factors contribute to relapse in humans and in animal models, including the exposure to cues and environments that have been consistently paired with the effects of drugs of abuse in the past (O'Brien, 2008). The ability of conditioned cues to elicit drug craving is potentiated (incubated) over time, a phenomenon observed in humans (Bedi et al., 2011; Li et al., 2015a; Parvaz et al., 2016; Wang et al., 2013) and animals, using a wide variety of drugs and other reinforcers (Abdolahi et al., 2010; Aoyama et al., 2014; Bienkowski et al., 2004; Blackwood et al., 2018; Grimm et al., 2002, 2001; Kirschmann et al., 2017; Krasnova et al., 2014; Shalev et al., 2001; Shepard et al., 2004).

During the last decade, several laboratories have suggested that the extracellular matrix (ECM), and in particular perineuronal nets (PNNs), play a vital role in brain plasticity, with an impact on learning and memory processes, as well as in different psychopathologies, including addiction. PNNs are specialised aggregations surrounding the cell bodies and proximal neurites of specific neurons, mostly parvalbumin GABAergic interneurons, that stabilise synapses in the adult brain. It has been shown that the exposure to (either passively or self-administered)

Abbreviations: ECM, extracellular matrix; IL, infralimbic cortex; PL, prelimbic cortex; dPL, dorsal PL; vPL, ventral PL; PNN, perineuronal nets; OFC, orbitofrontal cortex; lOFC, lateral OFC; vOFC, ventral OFC; wd1, withdrawal 1; wd30, withdrawal 30; WFA-FITC, *Wisteria Floribunda* agglutinin conjugated with Fluorescein isothiocyanate.

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and abstinence from drugs of abuse alters the structure of PNNs in various brain regions and, conversely, the manipulation of PNNs affects drug self-administration and extinction learning (Lasek et al., 2018; Slaker et al., 2016b; Sorg et al., 2016).

The prefrontal cortex (PFC) is involved in certain aspects of addiction (Everitt and Robbins, 2016; George and Koob, 2010) and the PNNs located in this region play a role in addictive disorders. For example, continued ethanol consumption (passive administration) or a hypercaloric diet that leads to learning and memory deficits, induce alterations in the PNNs of the PFC (Coleman et al., 2014; Reichelt et al., 2019). Also, the pharmacological manipulation of the ECM in the PFC affects both locomotor sensitisation and the conditioned place preference induced by psychostimulants (Mizoguchi et al., 2007; Slaker et al., 2015). Although the PFC also has been suggested to play a role in the incubation phenomenon (Shin et al., 2018), it is still unknown whether the contingent self-administration of drugs or natural rewards and the subsequent protracted abstinence from them alters PNNs in the different regions of the PFC. To the best of our knowledge, only two studies, Slaker et al. (2016) with sucrose, and Van Den Oever et al. (2010) with heroin have examined this possibility. However, some limitations in the design of these experiments difficult the conclusions that can be drawn from them. More specifically, in the study by Slaker et al. the rats underwent a relapse/extinction test before the analyses, which could have affected the composition of the ECM by itself. On the other hand, in the Van Den Oever report, the components of the ECM were only examined after three weeks of withdrawal (but not acutely, i.e. after 24 h), and only in the medial **(m)** PFC.

In considering all these pieces of evidence, the main goal of this work was to study the modulation of PNNs in different subregions of the PFC, using three rewarding substances with different pharmacological effects –cocaine, heroin and sucrose– at two different abstinence points (1 and 30 days) after a contingent consumption protocol (self-administration) that is known to induce incubation of seeking.

2. Materials and methods

Animals. Tissue for PNNs analyses was derived from 84 Lewis male rats purchased from Harlan International Ibérica weighing 300–320 g at the beginning of the experiments. Upon arrival, the animals were housed in groups of three in the vivarium at a constant temperature (20 ± 2 °C) and on a 12h:12h light:dark cycle (lights on at 08:00 a.m.), with food (Panlab, commercial diet for rodents A04/A03) and water available ad libitum. Animals were maintained and handled in accordance with European Union Laboratory Animal Care Standards (2010/63/EU). In the spirit of reducing the number of animals used in the experiments, these rats belonged to a subset of animals of a study previously published by us, focused on the incubation of seeking of cocaine, heroin or sucrose and examining the glutamatergic, GABAergic and endocannabinoid systems (Roura-Martínez et al., 2020). Of note, these animals showed a self-administration pattern that was indistinguishable from the behaviour of the rats that underwent extinction testing and that served us to verify that we would indeed observe the incubation phenomenon under our conditions. Detailed methodological information and behavioural results can be found in this previous work (Roura-Martínez et al., 2020).

Experimental design. Two batches of rats were used, one submitted to jugular catheter surgery (for cocaine, heroin or saline self-administration) and the other left intact (for sucrose or water self-administration). After ten self-administration sessions, the rats underwent 1 (wd1) or 30 days (wd30) of forced withdrawal with regular handling without extinction testing. The animals that received intravenous administration were segregated into six groups (n = 8 rats per group, three substances –heroin, cocaine, saline– × two withdrawal periods), while the rats that consumed the substance orally were distributed in four groups (n = 9 rats per group, two substances –sucrose-sweetened tap water, tap water– × two withdrawal periods).

Surgical procedures. An intravenous (i.v.) polyvinylchloride tubing (0.064 mm i.d.) catheter was implanted into the right jugular vein at approximately the level of the atrium and passed subcutaneously to exit the midscapular region. Surgical procedures were performed under isoflurane gas anaesthesia (5% for induction and 2% for maintenance) and buprenorphine analgesia (0.05 mg/kg s.c.). After surgery, the rats were allowed to recover for seven days, and a nonsteroidal anti-inflammatory drug (meloxicam, Metacam™: 15 drops of a 1.5 g/mL solution per 500 mL of water) was added to the drinking water. The catheters were flushed daily with 0.5 mL of gentamicin (40 mg/mL) dissolved in heparinised (100 IU/mL) saline in order to prevent infection and to maintain patency.

Self-administration. All the self-administration sessions were performed in Skinner boxes (Coulbourn Instruments or Med-Associates), and they were monitored with Med-PC software. The house light was off during the sessions, although we allowed some environmental light from the room (the door of the sound-attenuating cubicle was left ajar) in order to keep the light:dark cycle. Two levers were used, an active (L1) and inactive lever (L2). Each time L1 was pressed by the animal (L1 presses, LP1; fixed-ratio 1), a pump outside the box was switched on for 5 s and either 100 μL of the drug or saline solution was infused through the catheter, or 100 μL of sucrose solution or water was dispensed into a receptacle placed in between the levers. A cue-light over the active lever also switched on for 10 s at the same time. Subsequently, there was a time-out period of 40 s in which there were no programmed consequences, although the responses at each lever were recorded. Cocaine, heroin or saline self-administration sessions lasted 6 h each day (Airavaara et al., 2011; Conrad et al., 2008). Rats orally self-administering sucrose or water were subjected to 2 h daily sessions, a session length that is sufficient to induce incubation of sucrose seeking in rats (Harkness et al., 2010). The doses per injection used in the experiments were 0.075 mg/kg of heroin; 0.75 mg/kg of cocaine-HCl; and 10% w/v sucrose (Sigma-Aldrich S1888), diluted in 100 μL of saline (0.9% NaCl physiological saline Vitulia-ERN, for the intravenous infusions) or tap water. In the first two self-administration sessions, two sucrose pellets were placed on the active lever to facilitate the acquisition of self-administration behaviour.

Animal sacrifice. One or thirty days after the last self-administration session, the animals were weighed and sacrificed by decapitation between 11:00 and 13:00. The brain of the rats was quickly extracted and submerged in isopentane chilled on dry ice for 10 s and then stored at −70 °C.

Tissue Processing. Each brain was embedded in TissueTek (Sakura, 4583) and kept at −20 °C in a cryostat chamber (Microm, Cryostat HM 500O). After 1 h, 40 μm coronal slices (two per animal) of the PFC (+3.7 mm from Bregma) were obtained and placed on adhesive slides (Thermo Scientific, SuperFrost® Plus, Menzel Gläser). After air-drying, the slides were stored at −30 °C until further use. Six brains were lost due to storage problems.

Fluorescent staining of PNNs. The samples were thawed at 4 °C for 30 min, kept at room temperature (RT) for another 30 min, and then incubated in cold (−20 °C) post-fixation solution (4% w/v formaldehyde in PBS 0.1 M pH 7.4) for 15 min. They were allowed to air dry at RT for 20 min and washed 3 × 15 min in PB. The samples were then incubated overnight with *Wisteria floribunda* agglutinin (WFA)-FITC (Vector FL1351) 1:250 in PB with 0.25% v/v Triton X-100. The incubation was carried out in a humid chamber in the dark, at 4 °C on a flat surface. The next day, three 15-min washes were performed in PB. Each slice was incubated in 100 μL of DAPI (Sigma-Aldrich D9542) 1:1500 in PB at RT in the dark for 5 min and then two 15 min washes were performed in PB. The slices were covered with ProLong Gold antifade reagent (Thermo Fischer P36930) and coverslips and left to dry 24 h in the dark.

Fluorescence microscopy. Images from each of the seven studied regions of the PFC (both hemispheres) were obtained from each slice with an Olympus U-RFL-T fluorescence microscope (Olympus DP70 camera). These regions were the anterior cingulate cortex, the dorsal prelimbic cortex (dPL), the ventral prelimbic cortex (vPL), the

infralimbic cortex (IL), the insular cortex, the ventral orbitofrontal cortex (vOFC) and lateral orbitofrontal cortex (lOFC), according to the atlas of Paxinos and Watson (2007) (see Fig. 1). Two micrographs per region were taken comprising all the cortical layers, except for the case of the orbitofrontal cortices, where one micrograph was sufficient to cover the desired area.

Analysis of PNNs. The images were analysed with the free software Fiji (ImageJ; Schindelin et al., 2012). An approximate area of 1 mm^2 was analysed in each image (0.21 μm/pixel). We extracted the information from the green channel and then converted it to grayscale. The regions of interest (ROIs), each containing PNNs and areas with no evident PNNs (average blank), were delineated by an experimented researcher blind to the experimental groups. The background of the entire image was then subtracted using the sliding paraboloid algorithm with a radius of 20 pixels, and then the integrated density (area*mean grey value) of each ROI was calculated. Subsequently, we subtracted the average integrated blank density, corrected by area, to each PNN. We classified each individual PNN according to its intensity, normalised by the average intensity of the PNNs of the corresponding control group (saline, water) at wd1, and allocated them to ranges of intensity of 25% per range (for example, the 100% interval corresponded to an intensity range between 75 and 100% of the average). Then, we calculated the density of PNNs per interval (number per mm^2) and computed the average of these values for each technical replicate to be included in the statistical analysis. Finally, for each animal, slice (technical replicate), region and hemisphere, we measured the total density of PNNs (i.e. the sum of the densities across all the intensity intervals) and the total intensity per mm^2.

Statistical analyses. We used unpaired t-tests to analyse the potential differences in the cumulative infusions (equivalent to the cumulative active lever presses) between groups self-administering the same substance (drug/saline or sucrose/water) and later subjected to different withdrawal times. The effects on the PNN parameters measured (total density, total intensity and density by range of intensity) were analysed by factorial ANOVA in each area, using *Treatment* (cocaine/saline, heroin/saline or sucrose/water) and *Withdrawal day* (wd1/wd30) as between-subject factors and *Hemisphere* (left/right) and *Intensity* (in the case of the analysis by intensity interval) as the within-subject factors (see SPSS syntax in Supplementary Materials for further details). Statistical significance was set to $\alpha = 0.05$.

3. Results

3.1. Behavioral results

A detailed analysis of the self-administration behaviour of the rats from which the tissue was obtained can be found in our previous report (Roura-Martínez et al., 2020). Briefly, rats undergoing 1 or 30 days of withdrawal displayed similar self-administration behaviour within each of the rewards tested ($p > 0.30$, unpaired *t*-test), with the following values (mean ± SD) of cumulative active lever presses: saline wd1 36.9 ± 17.6, wd30 28.9 ± 13.5; cocaine wd1 166.4 ± 90.5, wd30 180.5 ± 70.3; heroin wd1 545.5 ± 368.5, wd30 668.4 ± 440.6; water wd1 23.0 ± 8.7, wd30 23.6 ± 10.7; sucrose wd1 394.8 ± 141.7, wd30 377.6 ± 129.2.

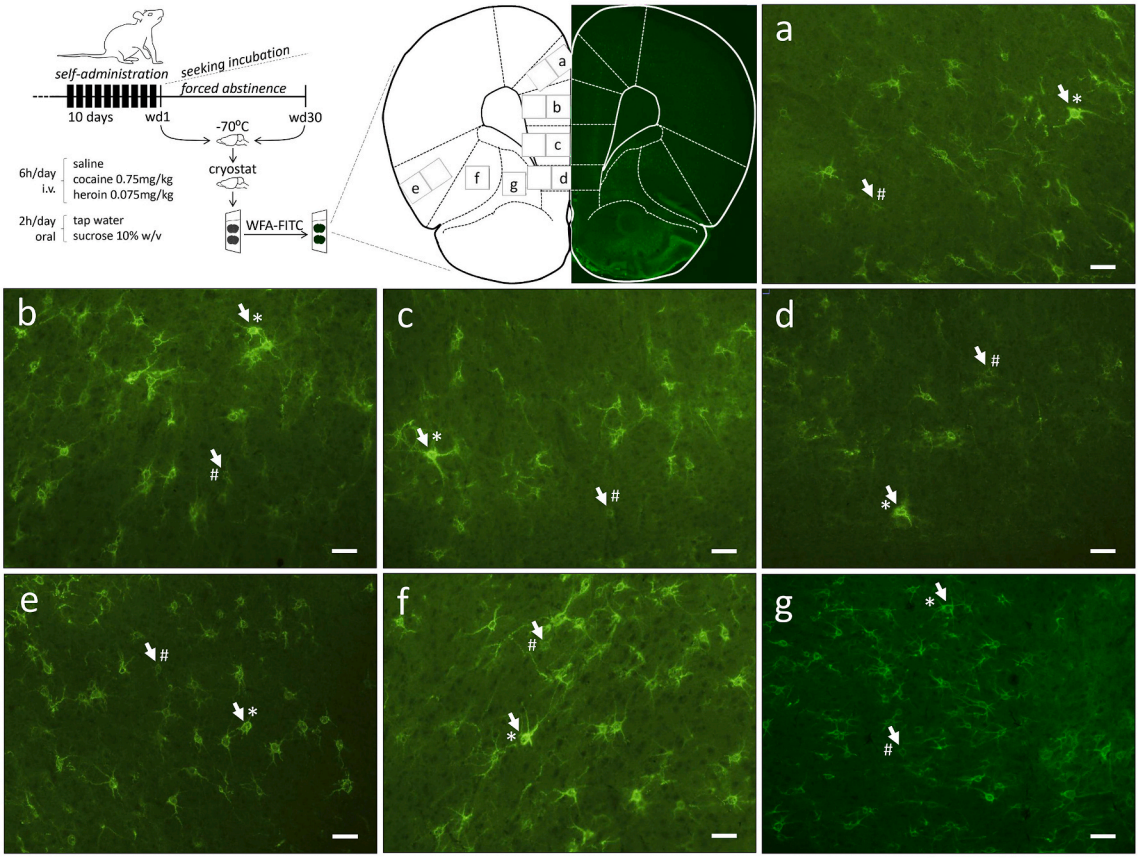

Fig. 1. Outline of the experiment. Rats underwent 10 sessions (1 per day) of intravenous (saline, heroin, cocaine) or oral (tap water, sucrose-sweetened water) self-administration and were sacrificed after 1 day (wd1) or 30 days (wd30) of withdrawal. The brains were removed and stored at -70 ° C. We obtained 40 μm slices for staining with *Wisteria floribunda* agglutinin (WFA) labelled with fluorescein isothiocyanate (FITC). We took 2 micrographs of different prefrontal cortical regions according to Paxinos and Watson (2007) including: a) anterior cingulate cortex, b) dorsal prelimbic cortex, c) ventral prelimbic cortex, d) infralimbic cortex, e) insula, and 1 micrograph of f) lateral orbitofrontal cortex, and g) ventral orbitofrontal cortex. Arrows point to examples of low (#) and high (*) intensity PNNs. Scale: 50 μm.

3.2. PNNs analysis

As regards the general effects of *Intensity*, *Hemisphere* and *Withdrawal day* on the PNN parameters analysed, in all the cases we found an effect of *Intensity*, obtaining a distribution of intensities equivalent to that reported in previous works of other laboratories (Dingess et al., 2018; Slaker et al., 2018). We found no main effects of *Withdrawal day* or *Hemisphere*. The values of the different PNNs parameters analysed by substance, withdrawal day, hemisphere and intensity are presented in the Supplementary Material. No significant effects were observed in the sucrose/water experiment.

3.2.1. Heroin self-administration increased the number of PNNs in the IL and vOFC while protracted withdrawal reversed this effect in the IL

The analysis by range of intensity in the IL revealed a significant *Treatment* × *Hemisphere* × *Intensity* interaction effect (F(6.94,194.33) = 2.57, p = 0.015, η^2_p = 0.084, Huynh-Feldt correction; Fig.2A,D). The simple effects analysis showed a significant increase in the density of medium-high intensity PNNs (range of ~200%) and a decrease in the density of PNNs in the very low intensity range (~25%), in the right hemisphere (Sidak's test, p < 0.05; Fig. 2E) in rats that self-administered heroin. We also obtained a significant *Treatment* × *Withdrawal day* interaction effect in the total density of PNNs (F(1,28) = 5.06, p = 0.032, η^2_p = 0.153). The simple effects analysis revealed a significant increase at wd1 in the rats that self-administered heroin, that was normalised at wd30 (Sidak's test, p < 0.05; Fig. 2C,F).

The analysis of the total density of PNNs in the vOFC revealed a significant *Treatment* effect, showing an increase after heroin consumption (F(1,27) = 5.78, p = 0.023, η^2_p = 0.176; Fig. 4). This effect was also observed in the analysis of the total intensity (F(1,27) = 4.65, p = 0.040, η^2_p = 0.147; data not shown).

There were no significant effects in the rest of the regions analysed.

3.2.2. Cocaine self-administration increased the number of PNNs in the dPL, vPL, IL and vOFC, and protracted withdrawal partially reversed this effect in the d PL

The analysis by range of intensity in the dPL revealed an almost significant *Treatment* × *Withdrawal day* × *Hemisphere* × *Intensity* interaction effect (F(6.57,177.29) = 2.08, p = 0.052, η^2_p = 0.072, Huynh-Feldt correction; Fig.3A,E). We observed a significant increase at wd1 in the density of PNNs in some ranges of intensity that reversed at wd30: more specifically, PNNs of low to medium intensity (~50–100%) increased their density in the left hemisphere (Sidak's test, p < 0.05; Fig. 3B and C), and medium-high (~100–200%) and high (~300–400%) intensity PNNs also increased their density values in the right hemisphere (Sidak's test, p < 0.05; Fig. 3F and G). We also detected a significant *Treatment* × *Hemisphere* interaction effect in the total density of PNNs (F(1,27) = 7.90, p = 0.009, η^2_p = 0.226, Huynh-Feldt correction). The simple effects analysis revealed a higher density of PNNs in the right hemisphere than in the left hemisphere after cocaine self-administration as compared to wd30 rats (Sidak's test, p < 0.05; Fig. 3D,H).

The analysis by range of intensity in the vPL cortex revealed an almost significant *Treatment* × *Intensity* interaction effect (F(5.17,139.65) = 2.24, p = 0.051, η^2_p = 0.077, Huynh-Feldt correction; Fig. 3I). We detected a significant increase in the density of medium-high intensity PNNs (~200%) at the expense of those PNNs in the lowest intensity range (~25%) (Sidak's test, p < 0.05; Fig. 3J).

The analysis by range of intensity in IL revealed a significant *Treatment* × *Intensity* interaction effect F(6.61,178.35) = 3.51, p = 0.002, η^2_p = 0.115, Huynh-Feldt correction; Fig. 3L). The simple effects analysis revealed significant increases in the densities of PNNs of a wide range of intensities (~100–400%) at the expense of those PNNs in the lowest intensity range (~25%) (Sidak's test, p < 0.05; Fig. 3M). An effect of *Treatment* (an increase) was also observed in the analysis of the total intensity (F(1,27) = 6.39, p = 0.018, η^2_p = 0.191; data not shown).

Finally, the analysis of the total density of PNNs in the vOFC revealed a trend for a *Treatment* effect (F(1,26) = 3.67, p = 0.066, η^2_p = 0.124), in the same direction (an increase) as the effect observed after heroin self-administration (Fig. 4).

There were no significant effects in the rest of the regions analysed.

4. Discussion

In this work, we have performed the first analysis of the effects of early and late withdrawal from three rewarding substances (a natural reward and two drugs of abuse with different pharmacological profiles) in the density of PNNs of specific subregions of the PFC of the rat. Our results suggest that heroin self-administration increases the number of PNNs in the IL and vOFC (depending on the hemisphere and intensity range), while protracted withdrawal reverses this effect in the IL. We also found that cocaine self-administration increased the density of PNNs in the dPL, vPL, IL and vOFC (also depending on the hemisphere and intensity range), while protracted withdrawal normalised PNN density in the dPL. Finally, sucrose self-administration or withdrawal

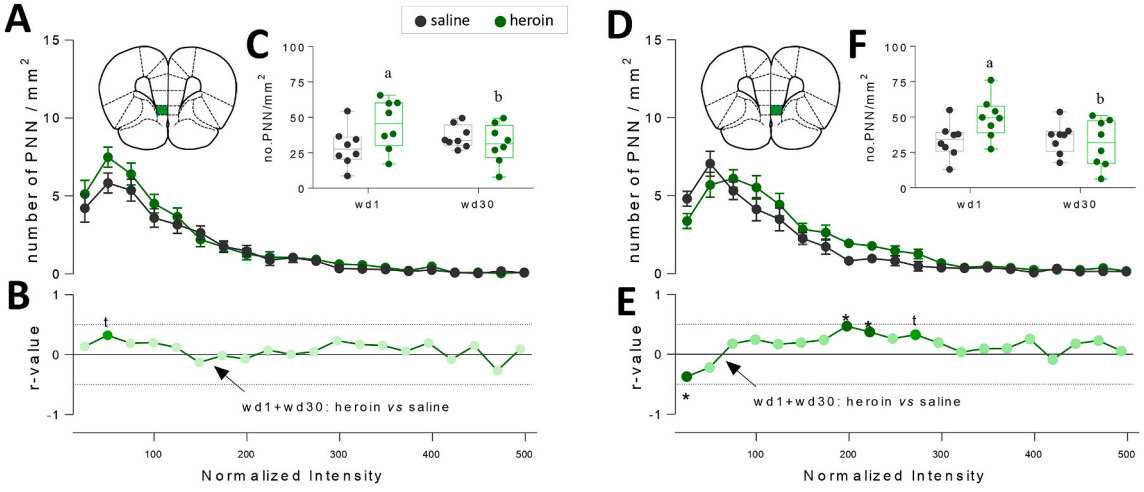

Fig. 2. Changes in PNNs after heroin self-administration in the infralimbic cortex in the left (A–C) and right (D–F) hemispheres. *Black*: saline groups; *Green*: heroin groups. A,D: Distribution of PNNs by range of intensity (mean±SEM). B,E: Effect size (r-value; see Supplementary Methods) of the heroin versus saline self-administration comparison by range of intensity (*p<0.05; t p<0.10). C,F: Total density of PNNs by substance and withdrawal day (a, p<0.05 heroin vs saline at wd1, both hemispheres; b, p<0.05 heroin wd1 vs heroin wd30, both hemispheres). n = 8 rats per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

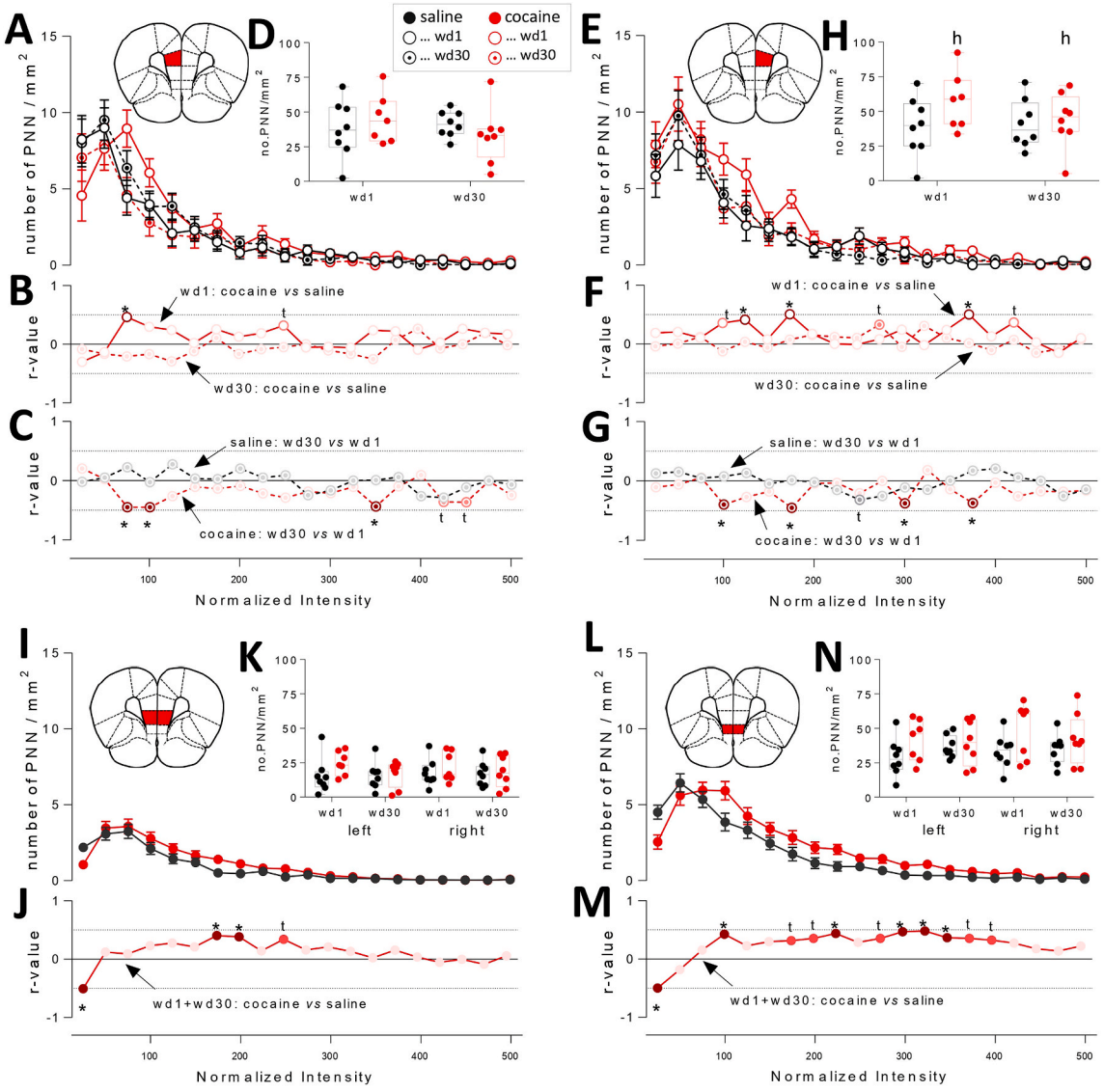

Fig. 3. Changes in PNNs after cocaine self-administration in the dorsal prelimbic cortex, left (A–D) and right (E–H) hemispheres, ventral prelimbic cortex (I–K) and infralimbic cortex (L–N). *Black*: saline groups; *Red*: cocaine groups; ○, wd1 groups; □, wd30 groups; ●, wd1+wd30 groups (except for D,H,K,N, where dots depict individual rats). A,E,I,L: Distribution of PNNs by range of intensity (mean±SEM). B,F,J,M: Effect size (r-value; see Supplementary Methods) of the cocaine versus saline self-administration comparison by range of intensity (*$p<0.05$; t $p<0.10$). C,G: Effect size of the wd30 versus wd1 comparison by range of intensity. D,H,K,N: Total density of PNNs by substance and withdrawal day (h, $p<0.05$ right vs left hemisphere in cocaine groups, at both withdrawal days). n = 7–8 rats per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

had no effects on the PNN parameters analysed.

Considering that PNNs play a role in synaptic plasticity, the functional consequences of the reported changes are likely to be related to this vital property of neuronal circuits. The modulation of brain plasticity by PNNs may be attained by means of their known effects on neurotransmitter receptors. For example, PNNs have a role in the lateral diffusion of glutamatergic AMPA receptors (Frischknecht et al., 2009; Saroja et al., 2014), which are vital mediators of plastic processes in the brain (Diering and Huganir, 2018; Kano and Kato, 1987). In our previous report, we detected some variations in the gene expression of the GluA1 subunit of the AMPA receptor and the GluA1/GluA2 ratio that, in the dorsomedial (dm) PFC, follow a similar time course as the one described here for PNNs in cocaine-exposed animals (Roura-Martínez et al., 2020). In addition, we also observed some changes in the gene expression of other subunits of different receptors (like $GABA_A$ or NMDA receptors) after one month of withdrawal from heroin or cocaine (Roura-Martínez et al., 2020). Of note, these receptors are also involved in synaptic plasticity (Jay et al., 1995; Shi et al., 2019) and their presence in the synapse may be modulated by PNNs, as we will further discuss in the subsequent sections of this discussion.

4.1. A perdurable increase of PNN density in the PFC after cocaine or heroin self-administration

We found long-lasting increases in the density of PNNs in the PFC of the rats after drugs self-administration, with some differences in the affected regions, depending on the drug. Thus, both cocaine and heroin caused increases in PNN density the IL and vOFC, and cocaine also affected the dPL and vPL.

Previous studies on the effect of drugs over the level of PNNs in the PFC show contradictory results. Intragastric injections of alcohol during adolescence increased the level of PNNs in the orbitofrontal cortex, observed once the animals reached adulthood (Coleman et al., 2014). These results are in the same line as our observations of increases in vmPFC after heroin and cocaine self-administration. In another study, 6 weeks, but not 1 week of free access to ethanol (4h/day x 4 days/week)

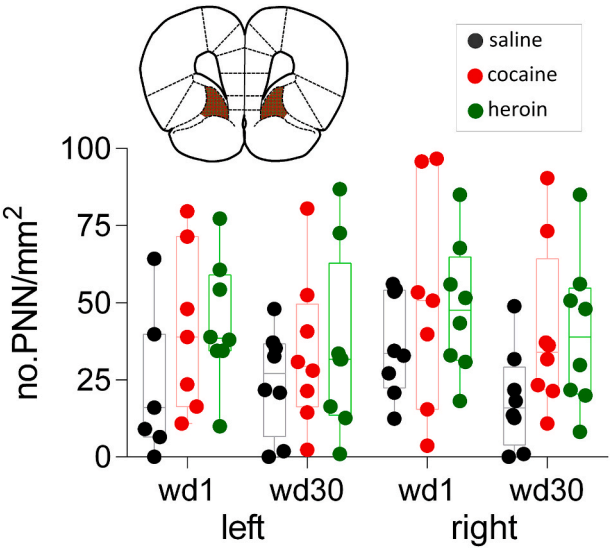

Fig. 4. Changes in PNNs after cocaine and heroin self-administration in the ventral orbitofrontal cortex. We observed an increase (in total density) in heroin-exposed rats ($p<0.05$; *green*) and a trend for an increase in cocaine-exposed animals ($p<0.066$; *red*). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

caused an increase in the level of PNNs in the left granular insula after early withdrawal (1 day; Chen et al., 2015). In another study, intraperitoneal cocaine injections (1 per day for 5 days) did not change the levels of PNNs in prelimbic or infralimbic cortices early in withdrawal (1 day). Of note, these mentioned studies used non-contingent administration or free access to the drug, which do not always have the same effects in the PFC as contingent administration does (Radley et al., 2015).

To the best of our knowledge, only two studies have focused on the effect on PNN parameters of a drug withdrawal time after drug self-administration (of at least one day, so that the potential acute effects of the self-administered drug are circumvented). Van Den Oever et al. (2010) observed that heroin self-administration (3h/day x 16 days) followed by protracted withdrawal (21 days) decreased the level of some of the components of PNNs in the mPFC. Although we observed a decrease in PNNs density when we compared wd30 vs wd1 groups, we did not observe a decrease in the density of PNNs after protracted withdrawal from heroin as compared to the saline group; actually, we observed a general increase. This discrepancy between our results and those previously reported by Van der Oever, could be due to the fact that in their study, Van den Oever and colleagues did not quantify PNNs directly, but instead they focused on their neurochemical constituents. In another study, Vazquez-Sanroman et al. (2017) observed that nicotine self-administration (1h/day x 21 days) followed by a short withdrawal (3 days) did not modify the level of PNNs in PL, IL or vOFC. This contrasts with our observations of the increases in the density of PNNs in the IL and vOFC in the case of cocaine and heroin, and also in the PL in the case of cocaine. It could be argued that nicotine has different pharmacological targets from those of cocaine or heroin. Another potential explanation points to the length and number of self-administration sessions used in each experiment (6h/day x 10 days in our case). Indeed, extended access (>3h/day) protocols are typically associated to more potent effects (Ahmed, 2012). It is then feasible that the extended access to a drug causes long-lasting increases, as those reported here, during withdrawal in the level of PNNs in several cortices, causing an impeded plasticity, as discussed by Sorg et al. (2016).

The alterations in PNNs here reported were also accompanied by perdurable changes in the expression of some receptors in the same animals (see Roura-Martínez et al. (2020) for a detailed account of these alterations). Concomitant to the changes of PNNs in the dPL after cocaine self-administration, we also observed a decrease in the expression of cannabinoid receptor CB_1 in the dmPFC (a region that comprises the anterior cingulate cortex and dPL). In addition, the increases in PNNs in ventromedial (vm) PFC (which comprises vPL and IL) after cocaine and heroin self-administration, were paralleled by an observed increase in the gene expression of extrasynaptic subunits of $GABA_A$ receptors and, in the case of heroin, increases in the gene expression of the endocannabinoid degrading enzyme fatty acid amidohydrolase. Lastly, there were also decreases of subunits of NMDA and $GABA_A$ receptors and of β-actin (Roura-Martínez et al., 2020). While the relationship between PNN structural changes and gene expression is unclear, it could be argued that some of these gene expression modulations could translate into an augmentation of the GABAergic tone and a subsequent hypofunction of the mPFC. Two facts support this claim: first, extrasynaptic $GABA_A$ receptors mediate tonic inhibition (Brickley and Mody, 2012) and second, CB_1 receptors located at GABAergic interneurons have been shown to reduce the local inhibitory tone (Hill and Tasker, 2012).

4.2. Decrease of PNN density in the medial prefrontal cortex after protracted withdrawal from cocaine or heroin self-administration

We found transient changes in the density of PNNs in the mPFC after the self-administration of both drugs. Specifically, the density of PNNs increased in the IL after heroin self-administration (total density) and in dPL after cocaine self-administration (low-intensity ranges in the left hemisphere and of high-intensity ranges in the right hemisphere). Importantly, in both cases, the density values returned to control levels after protracted withdrawal. This time course is similar to that of the incubation of seeking (Roura-Martínez et al., 2020), suggesting that these two processes could be related.

The implication of the individual prefrontal cortical subregions in the incubation phenomenon is not clear, as highlighted by the contradictory results of the pharmacological manipulations performed in the different subdomains of the mPFC during the incubation of seeking of psychostimulants (Ben-Shahar et al., 2013; Gould et al., 2015; Koya et al., 2009; Li et al., 2015b; Miller et al., 2017; Müller Ewald et al., 2018; Shin et al., 2018; Swinford-Jackson et al., 2016; Szumlinski et al., 2018). In spite of the controversy, PNNs in the mPFC have been occasionally associated with a decreased seeking response. For example, by pharmacologically interfering with the degradation of the ECM (with an i.c.v. injection of an inhibitor of metalloproteinases), Van Den Oever et al. (2010) showed that the levels of some components of ECM in the mPFC increased, along with a concomitant decrease in heroin seeking. In the same line, another study showed that environmental enrichment increased the intensity of PNNs in the prelimbic and infralimbic cortices and decreased sucrose seeking (Slaker et al., 2016a). Thus, the results of these studies suggest that the decrease in PNNs observed during the incubation of heroin and cocaine seeking could be associated with the increase in seeking responses.

The decreases in PNNs that we observe after one month of withdrawal were always accompanied by changes in the expression of some receptors in the same animals (see Roura-Martínez et al. (2020)). Concomitant to the decrease of perineuronal nets in the dorsal prelimbic cortex after cocaine protracted withdrawal, we observed an increase of the gene expression of the synaptic subunits of NMDA receptors in the dmPFC (Roura-Martínez et al., 2020), similar to the results in the mPFC reported by Ben-Shahar et al. (2009). In addition, the decreases in perineuronal nets in the IL after protracted withdrawal from heroin were accompanied by a pronounced increase of the extrasynaptic subunits of $GABA_A$ receptors in the vmPFC (Roura-Martínez et al., 2020). As in the case of the perdurable changes in PNN parameters discussed above, the relationship between the modifications of perineuronal nets and the aforementioned changes in neurotransmitter receptors is unclear at the moment, but one potential explanation is that they occur in specific

ensembles that promote the incubation of seeking. More research is needed to test this possibility.

4.3. Lack of changes in PNN densities after sucrose self-administration

We have not found any PNN alterations after sucrose self-administration or withdrawal. This is surprising considering the previous data available in the literature. To the best of our knowledge, three studies have measured the effect of natural oral rewards in PNN parameters in the PFC. Dingess et al. (2018) observed that a high-fat diet decreased the intensity and density of PNNs in prelimbic and ventral orbitofrontal (but not infralimbic) cortices while Reichelt et al. (2019) observed that a high-fat and high-sugar diet decreased the density of PNNs in infralimbic cortex (but not anterior cingulate or prelimbic cortices). Using a sucrose self-administration paradigm similar to the one used by us, Slaker et al. (2016a) found that the levels of PNNs did not change after short (1 day) or protracted (30 days) withdrawal in the PL, IL or vOFC. Of note, the authors performed an extinction test before sacrificing the animals –which may have reversed a decrease in the content of PNNs in the mPFC after protracted abstinence (Van den Oever et al., 2010)–; however, our study confirms the lack of effects of sucrose self-administration withdrawal in these animals.

5. Conclusions

The problem of relapse in addictive disorders remains unsolved. This is due, in part, to the lack of a complete understanding of the general mechanisms that govern relapse across the different types of addictions, which likely involve learning and memory processes. PNNs are essential structures that regulate synaptic plasticity and neuronal function and as such, are potential actors in relapse. Here, we have provided a much-needed comparative analysis of PNNs dynamics in a self-administration model that allows the examination of the neurobiological alterations across the time course of abstinence. The up-regulation of PNNs appeared as a common effect after drug self-administration, as well as their down-regulation during abstinence but, in certain regions, there were drug-specific effects that call for a more detailed analysis. With regard to the hemisphere specificity of our data, there is not enough data available in the literature to provide a convincing explanation for these effects. However, the existence of neurochemical laterality after neurochemical manipulations has been previously reported (for example, Paldy et al., 2013) and we would like to recommend that future work take this variable into consideration. Finally, we wish to suggest that if we are to fully understand the intricate network of mechanisms that operate in the relapse phenomenon, this sort of comparative analysis using self-administration models with different drugs and natural rewards should be widely adopted in the future.

CRediT authorship contribution statement

David Roura-Martínez: Investigation, Data curation, Visualization, Formal analysis, Writing - original draft. **Paula Díaz-Bejarano:** Investigation, Visualization. **Marcos Ucha:** Data curation, Writing - review & editing. **Raquel R. Paiva:** Data curation. **Emilio Ambrosio:** Funding acquisition, Project administration. **Alejandro Higuera-Matas:** Conceptualization, Methodology, Supervision, Writing - review & editing, Visualization, Project administration.

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Appendix A. Supplementary data

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