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Pharmacological inactivation of the prelimbic cortex emulates compulsive reward seeking in rats

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ABSTRACT

Drug addiction is a chronic, relapsing brain disorder characterized by compulsive drug use. Contemporary addiction theories state that loss of control over drug use is mediated by a combination of several processes, including a transition from goal-directed to habitual forms of drug seeking and taking, and a breakdown of the prefrontally-mediated cognitive control over drug intake. In recent years, substantial progress has been made in the modelling of loss of control over drug use in animal models, but the neural substrates of compulsive drug use remain largely unknown. On the basis of their involvement in goal-directed behaviour, value-based decision making, impulse control and drug seeking behaviour, we identified the prelimbic cortex (PrL) and orbitofrontal cortex (OFC) as candidate regions to be involved in compulsive drug seeking. Using a conditioned suppression model, we have previously shown that prolonged cocaine self-administration reduces the ability of a conditioned aversive stimulus to reduce drug seeking, which may reflect the unflagging pursuit of drugs in human addicts. Therefore, we tested the hypothesis that dysfunction of the PrL and OFC underlies loss of control over drug seeking behaviour, apparent as reduced conditioned suppression. Pharmacological inactivation of the PrL, using the GABA receptor agonists baclofen and muscimol, reduced conditioned suppression of cocaine and sucrose seeking in animals with limited self-administration experience. Inactivation of the OFC did not influence conditioned suppression, however. These data indicate that reduced neural activity in the PrL promotes persistent seeking behaviour, which may underlie compulsive aspects of drug use in addiction.

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1. Introduction

Drug addiction is a chronic relapsing brain disorder, characterized by persistent drug-directed behaviour even with explicit knowledge of its negative consequences (American Psychiatric Association, 2000, 2013; Leshner, 1997; O'Brien and McLellan, 1996; Volkow and Li, 2004). Addiction is an enormous public health problem with major socio-economic and legal consequences. Indeed, drug addiction has been calculated to account for more than 40% of the financial cost to society of all major neuropsychiatric disorders (Uhl and Grow, 2004). However, despite its high prevalence and costs to society, treatment options for addiction are limited in number and efficacy (Koob et al., 2009; O'Brien, 2008; Pierce et al., 2012; van den Brink, 2012) and only a minority of addicts receives any form of treatment. Since loss of control over drug use is considered to be a core feature of addiction, understanding its neural underpinnings may greatly aid the development of innovative treatments for this disorder.

Contemporary addiction theories hypothesize that loss of control over drug use is mediated by a combination of several processes, including a transition from goal-directed to habitual use, and breakdown of the cognitive control over drug intake mediated by the prefrontal cortex (PFC) (Jentsch and Taylor, 1999; Everitt and Robbins, 2005; Koob and Volkow, 2010; Pierce and Vanderschuren, 2010; Goldstein and Volkow, 2011). In order to test these hypotheses, we and others have developed animal models that explicitly capture compulsive aspects of addictive behaviour, in the form of insensitivity to adversity after prolonged drug taking experience (Deroche-Gamonet et al., 2004; Dickinson et al., 2002; Hopf et al., 2010; Lesscher et al., 2010; Pelloux et al., 2007; Vanderschuren and Everitt, 2004; Wolffgramm, 1991, for reviews see Hopf and Lesscher, 2014; Lesscher and Vanderschuren, 2012; Vanderschuren and Ahmed, 2013). Although important progress has been made in our understanding of the neural mechanisms underlying loss of control over drug seeking and taking in recent years (Chen et al., 2013; Corbit et al., 2012; Jonkman et al., 2012; Kasanetz et al., 2010, 2013; Lesscher et al., 2012; Seif et al., 2013; Zapata et al., 2010), we are only beginning to understand how compulsive aspects of addiction occur in the brain.

In the present study, we tested the involvement of two PFC subregions, i.e. the prelimbic cortex (PrL) and the orbitofrontal cortex (OFC) in compulsive cocaine and sucrose seeking in rats. We chose to investigate these regions, because of their possible involvement in cognitive control processes that may serve to limit drug use. Thus, the PrL has been implicated in response inhibition (Chudasama and Muir, 2001; Bari et al., 2011), and lesions of the PrL have been shown to facilitate the development of rigid stimulus-response habits (Killcross and Coutureau, 2003), that have been hypothesised to contribute to the development of compulsive drug seeking (Everitt and Robbins, 2005; Pierce and Vanderschuren, 2010). In the context of addictive behaviour, an important role for PrL function has been demonstrated in the reinstatement of drug seeking (Martín-García et al., 2014; McLaughlin and See, 2003; Pelloux et al., 2013; Peters et al., 2008, for review see Bossert et al., 2013). Interestingly, using setups to study compulsive aspects of drug seeking, recent studies have revealed a role for the PrL in control over cocaine seeking (Chen et al., 2013; Kasanetz et al., 2013; Mihindou et al., 2013, but see Pelloux et al., 2013). The OFC has been ascribed an important role in value-based decision making (Schoenbaum et al., 2009), and in various aspects of impulse control (Chudasama et al., 2003; Eagle et al., 2008; Mar et al., 2011), processes which, if impaired, may contribute to compulsive aspects of drug use. Disrupting OFC function has been shown to reduce the influence of drug-associated cues on behaviour (Fuchs et al., 2004; Hutcheson and Everitt, 2003; Lasseter et al., 2009), and to disinhibit cocaine seeking and taking (Fuchs et al., 2004; Grakalic et al., 2010; Lasseter et al., 2009).

In order to investigate the involvement of the PrL and the OFC in compulsive seeking behaviour, we used a conditioned suppression setup, in which cocaine or sucrose seeking is reduced by presentation of a footshock-associated conditioned stimulus (CS) (Kearns et al., 2002; Vanderschuren and Everitt, 2004; Limpens et al., 2014). We have previously shown that the ability of conditioned aversive stimuli to suppress cocaine seeking is diminished after an extended cocaine self-administration history (Vanderschuren and Everitt, 2004; Limpens et al., 2014), which is thought to reflect the unflagging pursuit of drugs observed in human addicts (American Psychiatric Association, 2000, 2013; Volkow and Li, 2004). Thus, assuming that hypofunction of the PFC contributes to loss of control over drug use in addiction, we hypothesised that temporary, pharmacological inactivation of the PrL and the OFC would inhibit conditioned suppression in animals with limited self-administration experience.

2. Results

2.1. Histology

Infusion sites are presented in Fig. 1. Infusion sites in the OFC were within the lateral and ventrolateral subregions of the OFC.

2.2. Pharmacological inactivation of the PrL reduces conditioned suppression of sucrose and cocaine seeking

The effect of PrL inactivation on conditioned suppression of sucrose seeking is presented in Fig. 2A and B. There was a main effect on suppression ratio [$H(3)=12.6$, $p<0.05$]. Post-hoc analysis showed that the suppression ratio in the CS-shock-saline group was significantly higher than in the control-saline group [$U=4.5$, $p<0.05$]. Infusion of B&M into the PrL had no effect on suppression ratio in the control group (control-saline vs. control-B&M: [$U=6.5$, n.s.]). In the CS-shock group, however, B&M infusion reduced suppression ratio (CS-shock-B&M vs. CS-shock-saline [$U=2.0$, $p<0.05$]). Suppression ratio did not differ between the CS-shock-B&M group and the control-B&M [$U=12.5$, n.s.] (Fig. 2A). Although a visual impression of the seeking latency data yields a comparable pattern of effects as the suppression ratio data, there was no main effect on latency to first response [$H(3)=5.3$, n.s.]) (Fig. 2B). Fig. 2C and D shows the effect of inactivation of the PrL on conditioned suppression of cocaine seeking. There was a main effect on suppression ratio [$H(3)=10.4$, $p<0.05$]) (Fig. 2C). Post-hoc analysis revealed that, compared

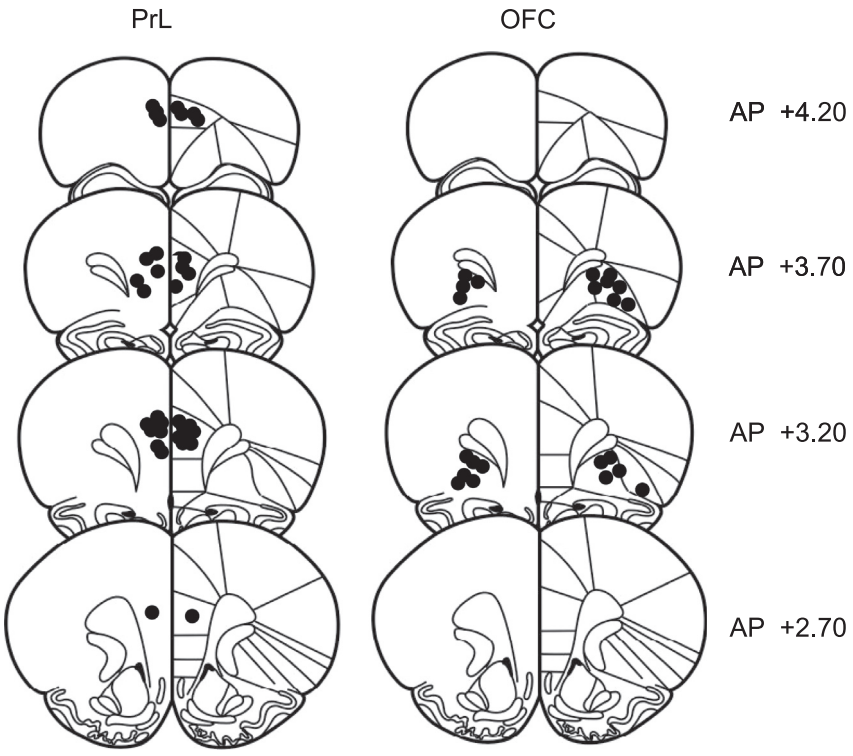

Fig. 1 – Schematic representation of the bilateral injection sites in the prelimbic cortex (PrL, left) and orbitofrontal cortex (OFC, right). AP = anterior-posterior level in mm from bregma. Adapted from Paxinos and Watson (1998).

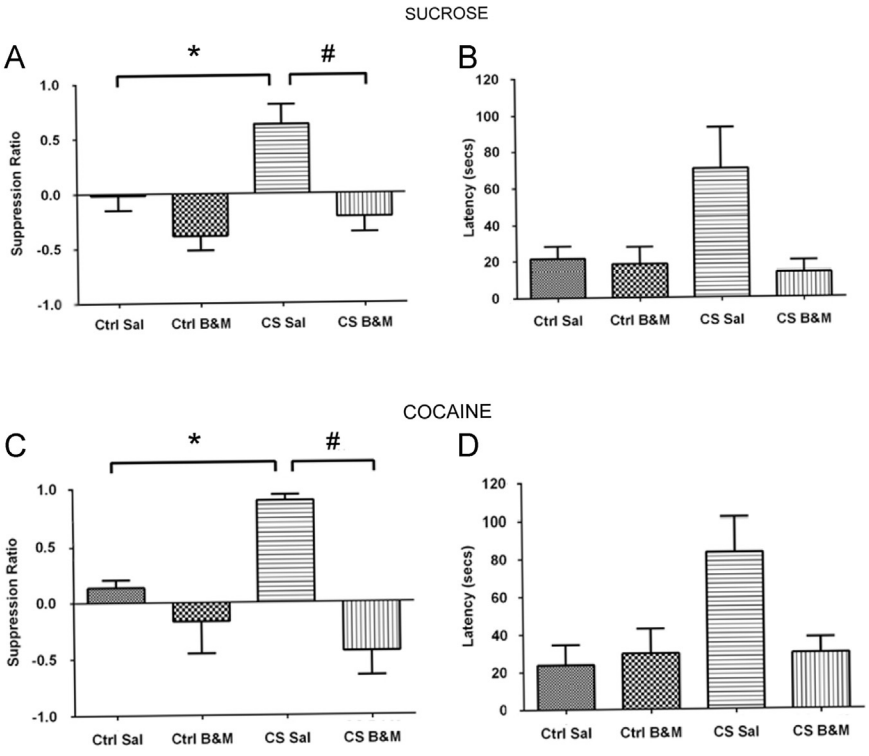

Fig. 2 – Effect of inactivation of the PrL on conditioned suppression of sucrose (A and B) and cocaine seeking (C and D). Graphs depict the suppression ratio (A and C) and latency to first response (B and D) after infusion of saline (sal) or baclofen/muscimol (B&M). Ctrl: control conditioned rats; CS: CS-shock conditioned rats. Cocaine Crtl B&M $n=5$; Cocaine CS B&M $n=5$, all other groups $n=6$. Data are presented as mean +SEM. $^{*}p<0.05$, Crtl Sal different from CS Sal; $^{\#}p<0.05$, CS Sal different from CS B&M.

to the control-saline group, the suppression ratio of the CS-shock-saline was significantly increased [$U=6.5$, $p<0.05$]. Intra-PrL infusion of B&M had no effect on the suppression ratio in the control group (control-saline vs. control-B&M: [$U=10.0$, n.s.]). In contrast, B&M infusion caused a reduction in the suppression ratio in the CS-shock group (CS-shock-saline vs. CS-shock-B&M: [$U=2.0$, $p<0.05$]). There was no main effect on latency to first response [$H(3)=5.1$, n.s.], although the patterns of effects was comparable to the suppression ratio data (Fig. 2D).

2.3. *Pharmacological inactivation of the OFC does not alter conditioned suppression of sucrose and cocaine seeking*

The effect of inactivation of the OFC on conditioned suppression of sucrose seeking is shown in Fig. 3A and B. There was a main effect on suppression ratio [$H(3)=17.0$, $p<0.05$] (Fig. 3A) and on latency to first response [$H(3)=17.3$, $p<0.05$] (Fig. 3B). Post-hoc analysis revealed profound suppression of seeking behaviour in the CS-shock-saline group compared to the control-saline group (suppression ratio: [$U=0.0$, $p<0.05$]; latency to first response: [$U=1.0$, $p<0.05$]). Infusion of B&M into the OFC did not alter conditioned suppression in the CS-shock group (CS-shock-saline vs. CS-shock-B&M: suppression ratio: [$U=0.0$, n.s.]; latency to first response: [$U=1.0$, n.s.]). Furthermore, B&M infusion had no effect on seeking behaviour in the control group (control-saline vs. control-B&M: suppression ratio: [$U=10.0$, n.s.]; latency to first response: [$U=10.0$, n.s.]). The lack of effect on conditioned suppression was further supported by the increase in suppression ratio [$U=0.0$, $p<0.05$] and seeking latency [$U=0.0$, $p<0.05$] in the CS-shock-B&M group compared to the control-B&M group.

Fig. 3C and D shows the effect of inactivation of the OFC on conditioned suppression of cocaine seeking behaviour. There was a main effect on suppression ratio [$H(3)=8.9$, $p<0.05$] (Fig. 3C) and on latency to first response [$H(3)=8.2$, $p<0.05$] (Fig. 3D). Post-hoc analysis revealed a significant suppression of seeking in the saline treated rats (control-saline vs. CS-shock-saline: suppression ratio: [$U=6.5$, $p<0.05$]; latency to first response: [$U=1.0$, $p<0.05$]). Infusion of B&M into the OFC did not alter conditioned suppression, neither in the CS-shock group (CS-shock-saline vs. CS-shock-B&M: suppression ratio: [$U=22.5$, n.s.]; latency to first response: [$U=24.0$, n.s.]), nor in the control group (control-saline vs. control-B&M: (suppression ratio: [$U=11.5$, n.s.]; latency to first response: [$U=9.0$, n.s.]).

3. Discussion

In the present study, we tested the hypothesis that dysfunction of two discrete PFC subregions, i.e. the PrL and the OFC, contributes to the compulsive aspects of drug seeking that characterize addictive behaviour. To that aim, we investigated the effect of pharmacological inactivation of these areas, using a mixture of the GABA receptor agonists baclofen and muscimol (McFarland and Kalivas, 2001; Van Kerkhof et al., 2013) on conditioned suppression of cocaine and sucrose seeking. We, and others, have previously shown that presentation of a footshock-associated CS inhibits cocaine and sucrose seeking in rats with limited self-administration experience (Kearns et al., 2002; Limpens et al., 2014; Vanderschuren and Everitt, 2004). After an extended drug taking history, cocaine, but not sucrose seeking becomes impervious to presentation of the aversive CS (Limpens et al., 2014; Vanderschuren and Everitt, 2004), which we interpret as a reflection of persistent drug seeking despite the anticipation of aversive events, a defining characteristic of addictive behaviour (American Psychiatric Association,

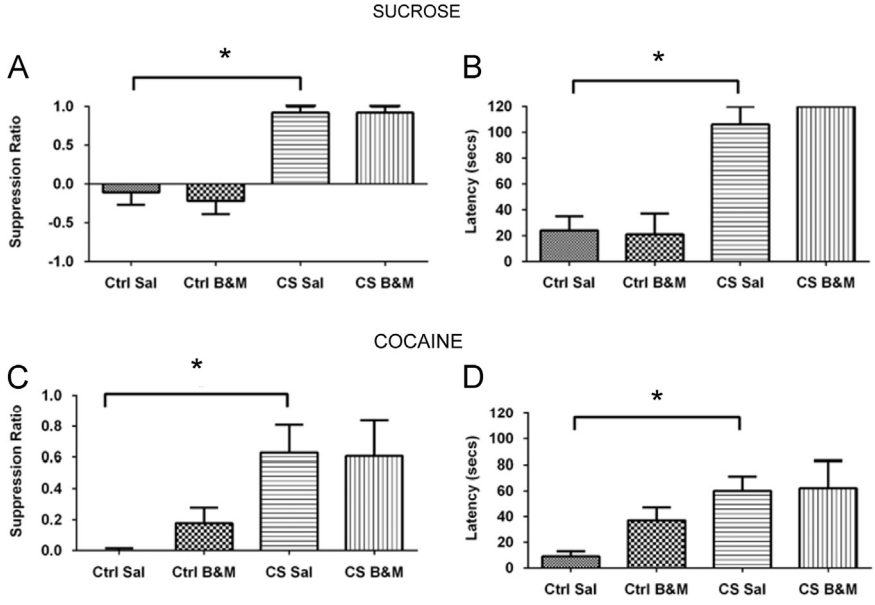

Fig. 3 – Effect of inactivation of the OFC on conditioned suppression of sucrose (A and B) and cocaine seeking (C and D). Graphs depict the suppression ratio (A and C) and latency to first response (B and D) after infusion of saline (sal) or baclofen/muscimol (B&M). Ctrl: control conditioned rats; CS: CS-shock conditioned rats. Cocaine Crtl B&M $n=8$; Cocaine CS Sal $n=8$, all other groups $n=6$. Data are presented as mean +SEM. *$p<0.05$, Crtl Sal different from CS Sal.

2000, 2013). We reasoned that if dysfunction of the PrL or OFC is involved in compulsive drug seeking, then inactivation of these regions inhibits conditioned suppression in animals with limited self-administration experience. Indeed, after inactivation of the PrL, conditioned suppression of cocaine and sucrose seeking was markedly reduced, whereas OFC inactivation did not influence conditioned suppression, however. Thus, our findings directly implicate the PrL in loss of control over drug seeking.

The finding that inactivation of the PrL emulates an addicted phenotype adds to an emerging literature that implicates this PFC subregion in compulsive aspects of addictive behaviour (Chen et al., 2013; Kasanetz et al., 2013; Mihindou et al., 2013; Seif et al., 2013). Thus, using a setup in which cocaine seeking was punished by probabilistic footshock (Pelloux et al., 2007), Chen et al. (2013) identified a subpopulation of rats that was insensitive to punishment. In these rats, excitability of PrL neurons was decreased. Using optogenetic manipulation of PrL neuronal activity, it was observed that PrL stimulation reduced punished cocaine seeking in resistant rats, whereas PrL inhibition increased punished seeking behaviour in rats that were sensitive to punishment (Chen et al., 2013). Comparable findings were reported for alcohol intake punished by footshock or quinine adulteration (Seif et al., 2013). In this study, the involvement of a projection from the medial PFC (whereby an explicit distinction between medial PFC subregions was not made) to the nucleus accumbens core in punished alcohol seeking was identified (Seif et al., 2013). In addition, functional activity of the PrL has been shown to be necessary for the successful inhibition of responding for cocaine during signalled unavailability of the drug (Mihindou et al., 2013). Taken together with the present findings, these data broadly implicate the PrL in inhibitory control over seeking behaviour, whether it is imposed by direct punishment (Chen et al., 2013; Seif et al., 2013), punishment-associated CSs (present study) or stimuli signalling the absence of drug (Mihindou et al., 2013). In this regard, it is worth noting that PrL inactivation inhibited conditioned suppression of both cocaine and sucrose seeking, indicating that this effect is not specific for a drug reinforcer. This suggests that the PrL imposes control over instrumental responding in general, and that dysfunction of the PrL induced by prolonged intake of addictive substances, perhaps in combination with a pre-existing, addiction-prone phenotype (Chen et al., 2013; Kasanetz et al., 2013; Martín-García et al., 2014) results in uncontrolled drug seeking that is insensitive to punishment.

The psychological mechanisms by which the PrL governs control over drug seeking remain to be identified, but several candidate mechanisms can be mentioned. First, PrL activity has been implicated in goal-directed behaviour (Balleine and O'Doherty, 2010), as opposed to rigid habitual behaviour that is thought to facilitate the descent from casual drug use to addictive behaviour (Everitt and Robbins, 2005; Pierce and Vanderschuren, 2010). That is, in animals with lesions of the PrL, instrumental responding is insensitive to the value of the outcome (Killcross and Coutureau, 2003). In the context of addictive behaviour, this means that in the absence of a functional PrL, devaluation of drug seeking by impending punishment no longer affects the pursuit of the drug. This interpretation resonates well with the observation that PrL function is necessary for the ability of aversive CSs to influence behaviour (Sierra-Mercado et al., 2011). However, Pelloux et al. (2013) did not find an effect of PrL lesions on punished cocaine seeking. Given the dissociation between conditioned suppression of cocaine seeking and conditioned freezing (Vanderschuren and Everitt, 2004), it is therefore unlikely that the effect of PrL inactivation on conditioned suppression is a result of the mere inability to express conditioned fear. Alongside an involvement of rigid stimulus-response habits, it is also possible that PrL inactivation facilitates compulsive drug seeking through the impairment of impulse control. Lesioning or inactivation of the PrL has been shown to result in perseverative responding in the 5-choice serial reaction time task (Chudasama and Muir, 2001) and to slow response inhibition in the stop task (Bari et al., 2011). Thus, pharmacologically-induced dysfunction of the PrL could therefore impair the rats' ability to withhold responding for cocaine when presentation of the footshock-associated CS causes them to anticipate punishment. In contrast to the possible involvement of impaired impulse control and stimulus–response habits, it is not likely that PrL inactivation inhibited conditioned suppression by increasing the rewarding properties of cocaine, so that inflated cocaine value would outweigh the threat of impending punishment. That is, our own data (Limpens et al., unpublished findings) show that pharmacological inactivation of the PrL actually reduces responding for cocaine under both fixed-ratio 1 and progressive ratio schedules of reinforcement, whereas responding for sucrose is not affected (see also Capriles et al., 2003). These data indicate a dissociation between instrumental responding for cocaine (reduced) and sucrose (not affected), and conditioned suppression of cocaine and sucrose seeking (both inhibited) after pharmacological inactivation of the PrL, suggesting that an increased incentive value of a reinforcer cannot account for the insensitivity to punishment (see also Limpens et al., 2014; Vanderschuren and Everitt, 2004, who showed no change in the incentive value of cocaine in rats insensitive to conditioned suppression).

In view of the important role in drug addiction that has been ascribed to the OFC (Ersche et al., 2012; Goldstein and Volkow, 2011; Lucantonio et al., 2012), it is surprising that we did not observe an effect of OFC inactivation on conditioned suppression. Indeed, the OFC has been implicated in impulsive behaviour (Chudasama et al., 2003; Eagle et al., 2008; Mar et al., 2011), albeit in different aspects of impulse control than the PrL (for reviews see Eagle and Baunez, 2010; Pattij and Vanderschuren, 2008). Moreover, functional inactivation of the OFC has been found to result in disinhibited forms of drug seeking (Fuchs et al., 2004; Grakalic et al., 2010; Lasseter et al., 2009), and OFC dysfunction has recently been reported to underlie the impaired representation of the value of instrumental outcomes after cocaine exposure (Lucantonio et al., 2014). Combined, these findings support the notion that impaired OFC function, either as a premorbid risk factor or as a result of chronic drug abuse may confer a set of behavioural alterations that propagate addictive behaviour and hamper rehabilitation (Ersche et al., 2012; Goldstein and Volkow, 2011; Lucantonio et al., 2012). However, OFC dysfunction does not appear to play a primary role in persistent drug seeking that is insensitive to adversity.

A number of limitations of this study need to be acknowledged. First, the study used a relatively small number of animals. In our recent study on conditioned suppression of

cocaine and sucrose seeking (Limpens et al., 2014) we found that robust and reliable conditioned suppression could be demonstrated using sample size of $n=6$. However, in the present PrL inactivation experiment, we did not find a statistically significant effect on the latency to make the first lever press, even though the pattern of effects strongly resembled that of the suppression ratio. With this caveat in mind, we are confident that our results are valid, but replication using a larger sample size may be helpful to support our finding that PrL dysfunction is involved in compulsive drug seeking behaviour. Second, on the basis of the available literature, we hypothesized that the PrL and the OFC would be involved in conditioned suppression of seeking behaviour. This does, of course, not preclude a potential involvement of other PFC subregions in compulsive drug seeking. Given the functional heterogeneity of the PFC (see e.g. Chudasama et al., 2003; Killcross and Coutureau, 2003; Mar et al., 2011), which has also been found in the context of drug seeking (e.g. Fuchs et al., 2004; Peters et al., 2008), the possibility that other (prefrontal) cortical subregions play a role in the loss of control that characterizes addictive behaviour needs to be investigated in future studies. For example, the recent demonstration that projections from the insular cortex to the nucleus accumbens core contribute to aversion-resistant alcohol intake (Seif et al., 2013) calls for an investigation of the insular cortex in conditioned suppression of drug seeking.

In conclusion, the present study shows that pharmacological inactivation of the PrL, but not the OFC, reduces conditioned suppression of cocaine and sucrose seeking in rats. These data suggest that dysfunction of the PrL underlies the unflagging pursuit of drugs that characterizes addiction. Remediation of PrL dysfunction, using, for example, deep brain stimulation or transcranial magnetic stimulation (Luigjes et al., 2012; Li et al., 2013) may therefore be a viable treatment strategy for drug addiction.

4. Experimental procedures

4.1. Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) weighing 260–280 g at the time of arrival were individually housed in Macrolon cages ($40 \times 25 \times 18$ cm; $l \times w \times h$) in climate-controlled rooms (temperature 20–21 °C, 55±15% relative humidity) under a reversed 12 h light–dark cycle (lights on at 19.00 h). Animals were allowed to habituate to the housing conditions for at least 9 days before surgery. Rats received 20 g chow (SDS, UK) per day, which is sufficient to maintain body weight and growth. Water was available ad libitum. Self-administration sessions were carried out between 9 AM-6 PM, for 5–7 days a week. Experiments were approved by the Animal Ethics Committee of Utrecht University, and were conducted in agreement with Dutch legislation (Wet op de dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

4.2. Apparatus

All subjects were trained and tested in operant conditioning chambers ($29.5 \times 24 \times 25$ cm; $l \times w \times h$; Med Associates, Georgia, VT, USA). The chambers were placed in light- and sound-attenuating cubicles equipped with a ventilation fan. Each chamber was equipped with two 4.8 cm wide retractable levers, placed 11.7 cm apart and 6.0 cm from the grid floor. The assignment of the left and right lever as seeking and taking lever (see below) was counterbalanced across rats. A cue light (28 V, 100 mA) was present above each lever and a house light (28 V, 100 mA) was located on the opposite wall. A liquid dipper (0.04 ml) delivered sucrose solution to a recessed magazine situated between the levers. Cocaine infusions were controlled by a syringe pump placed on top of the cubicles. During the cocaine self-administration sessions, polyethylene tubing ran from the syringe placed in the syringe pump via a swivel to the cannula on the subjects' back; in the operant chamber tubing was shielded with a metal spring. Experimental events and data recording were controlled by procedures written in MedState Notation using MED-PC for Windows.

4.3. Surgery

Rats were anaesthetised with ketamine hydrochloride (Narketan, 75 mg/kg i.m.) and medetomidine hydrochloride (Cepetor, 0.4 mg/kg s.c.), supplemented with ketamine if needed. A single intravenous catheter was implanted into the right jugular vein aimed at the left vena cava. Catheters (Camcaths, Cambridge, UK) consisted of a 22 g cannula attached to silastic tubing (0.012 ID) fixed to nylon mesh. The mesh end of the catheter was sutured subcutaneously on the dorsum. Next, the animals were placed in a stereotaxic apparatus and 26 G guide cannulas (Plastics One, Roanoke, VA, USA) were implanted bilaterally, 1 mm above target structures. Coordinates relative to bregma (Paxinos and Watson, 1998) were as follows: PrL: anteroposterior (AP) +3.0 mm, mediolateral (ML) ±0.6 mm, dorsoventral (DV) −2.8 mm; OFC: +3.0 mm AP, 3.5 mm ML, −4.2 mm DV at an angle of 10°. Cannulas were fixed to the skull with stainless steel screws and dental acrylic and a stylet was inserted into each cannula. Carprofen (50 mg/kg, s.c.) was administrated once before and twice after surgery. Gentamycin (5 mg/kg, s.c.) was administered before surgery and for 5 days post-surgery. Animals were allowed 7–9 days to recover from surgery.

4.4. Behavioural procedures

4.4.1. Cocaine self-administration

Rats were trained to lever press for cocaine under a heterogeneous seeking–taking (ST) chain schedule of reinforcement (Limpens et al., 2014; Olmstead et al., 2000; Vanderschuren and Everitt, 2004; Veeneman et al., 2012a) with a random interval (RI) of 120 s on the seeking link (ST(RI-120)). Self-administration training started with the acquisition of the taking response under a fixed-ratio 1 (FR-1) schedule of reinforcement. During acquisition sessions, only the taking lever was present. Pressing this lever resulted in the infusion of 0.25 mg cocaine in 0.1 ml saline delivered over 5.6 s, the illumination of the cue light above the taking lever for 5.6 s, the retraction of the lever, and the switching off of the house light. After a 20 s time-out period, the taking lever was reintroduced and the house light illuminated, signalling the start of a new cycle. Once animals had acquired cocaine self-administration, they were gradually introduced to the ST

chain schedule, starting with a schedule with a RI requirement of 2 s on the seeking link. ST(RI)-sessions started with the introduction of the seeking lever and the illumination of the house light. The first press on the seeking lever initiated the RI and pressing this lever was without consequences until the RI had elapsed. When the RI had elapsed, pressing the seeking lever resulted in retraction of the seeking lever and insertion of the taking lever. Next, responding on the taking lever (under the FR-1 schedule of reinforcement) resulted in an infusion with cocaine, illumination of the cue light, retraction of the taking lever and the switching off of the house-light. This was followed by a 10 min time-out period to minimize the influence of cocaine-induced psychomotor effects on responding for the next infusion. After the time-out period, a new cycle started with the reintroduction of the seeking lever and the illumination of the house-light. When the rats had acquired the task under a RI of 2 s, the RI was progressively increased between sessions until animals had acquired the task under an RI of 120 s. The program automatically ended after 2 h or if animals had obtained 10 rewards, whichever occurred first. After each self-administration session, intravenous catheters were flushed with a gentamycin–heparin–saline solution to maintain the patency of the catheters. Priming infusions of cocaine to stimulate self-administration were never given.

4.4.2. *Sucrose self-administration*

Rats were trained to lever press for sucrose under a ST(RI-120) schedule of reinforcement. This procedure was similar to the ST (RI-120) with cocaine as the reward, with the following exceptions. After a response on the taking lever, 0.2 ml of a 20% sucrose solution was delivered by presenting the dipper five times for 5 s at a rate of one presentation per second. In addition, the session was terminated when 30 min had passed or when a maximum of 30 rewards had been obtained during the FR-1 training, or after 10 rewards during the RI training sessions.

4.4.3. *Acquisition of the CS-shock association*

Acquisition and expression of conditioned suppression was performed as previously reported (Limpens et al., 2014; Vanderschuren and Everitt, 2004). Thus, once stable responding under the ST(RI-120) schedule was achieved (i.e. when the mean number of seeking responses per minute of the last three training sessions of an individual rat did not exceed a difference of 10% of the overall mean of those three sessions), rats were assigned to groups that either underwent conditioning with CS-footshock pairings (CS-shock group) or underwent control conditioning (control group). Assignment to the groups was based on the mean seeking responses per minute and seeking latency of the three last training sessions prior to conditioning, so that CS-shock and control groups had equal mean response rates and seeking latencies.

Acquisition of the CS-shock association was established in operant chambers different from those where the rats had received training for self-administration of sucrose of cocaine. To facilitate CS-shock, rather than context-shock association, the animals were pre-exposed to the shock boxes for 30 min for 2 days. The CS-shock conditioning session comprised a lead-in period of 5 min followed by two periods of 10 min with a 85 dB, 2900 Hz tone (with an intertrial-interval of 10 min) during which 10 unpredictable, scrambled footshocks (1 s duration) were delivered (i.e. 20 shocks in total). The tone-shock association session ended with a lead-out period of 5 min. On the basis of our parametric analysis of conditioned suppression of reward seeking (Limpens et al., 2014), the animals were conditioned with the lowest shock intensities that produce reliable conditioned suppression, i.e. 0.40 mA (for cocaine self-administering rats) and 0.35 mA (for sucrose self-administering rats). Rats in the control group were subjected to the same procedure but without the delivery of footshocks.

4.4.4. *Conditioned suppression of sucrose- and cocaine-seeking behaviour*

After conditioning, rats received 4 additional ST(RI-120) training sessions. Subsequently, a test session for conditioned suppression of sucrose- or cocaine-seeking behaviour was performed. This conditioned suppression test was conducted in the same operant chambers where rats received self-administration training. After a lead-in period of 2 min, the seeking lever was inserted for 14 min with the house light illuminated. Two-minute intervals in which the tone CS was presented (CS-ON interval) were alternated with two-minute intervals where the tone CS was absent (CS-OFF interval).

Prior to testing, the rats received an infusion of saline or a mixture of the GABA receptor agonists baclofen (1.0 nmol) and muscimol (0.1 nmol).

4.5. *Drugs and infusion procedures*

Cocaine-HCl (Bufa BV, The Netherlands), baclofen (Tocris Bioscience, UK), and muscimol (Tocris Bioscience, UK) were dissolved in sterile physiological saline (0.9% NaCl).

After responding under the ST(RI-120) schedule had stabilized (see above), the rats received a habituation infusion with saline into the PrL or OFC. Infusion procedures were as previously described (Trezza et al., 2011; Van Kerkhof et al., 2013; Veeneman et al., 2012b). Microinfusions were made through 33 G injector cannulas (Plastics One, USA) that extended 1.0 mm below the guide cannulas. Using a syringe pump (Harvard Apparatus, USA), bilateral infusions (0.3 μl/side/60 s) were made, and the injectors were left in place for another 60 s to allow for diffusion. After the infusion, the stylets were replaced. The test for conditioned suppression began 7 min after the start of the microinfusion.

4.6. *Histology*

Rats were sacrificed using an overdose of pentobarbital. The brains were removed, immediately fresh-frozen on dry-ice and stored at −80 °C. Coronal sections (20 μm) were sliced on a cryostat and stained with cresyl violet. Cannula placements were assessed under a light microscope. Data obtained from rats with cannula placements outside the target regions were discarded from the analysis.

4.7. *Statistical analysis*

The following parameters were analysed to test for conditioned suppression: 1. Suppression ratio, which was calculated as [(number of responses during CS-OFF—number

of responses during CS-ON)/(number of responses during CS-OFF+number of responses during CS-ON)]. A suppression ratio of 0 or lower means no suppression, a suppression ratio of 1 means complete suppression of responding during CS-ON periods. 2. The latency to make the first response on the seeking lever. Main effects were analysed with a Kruskal–Wallis *H* test followed by a post-hoc Mann–Whitney *U* test when appropriate.

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