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Oxytocin treatment in the prelimbic cortex reduces relapse to methamphetamine-seeking and is associated with reduced activity in the rostral nucleus accumbens core

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ABSTRACT

Addiction to the psychostimulant Methamphetamine (METH) is characterised by high rates of relapse. Currently there are no approved effective pharmacotherapies for METH dependence. The neuropeptide oxytocin (OXY) potently reduces METH-seeking behaviours in rodent models of relapse and is now being used in clinical trials to treat drug-dependent individuals. However, OXY administration in humans may be impeded by its poor penetration of the brain. Therefore, identification of the neural mechanisms by which OXY reduces METH relapse may guide the development of improved OXY-based therapies for METH addiction. Systemic OXY administration is associated with attenuated METH-induced activity in the prelimbic cortex (PrL); a key brain region which exerts control over much of the reward and addiction circuitry. However, it is not known whether OXY acts directly in the PrL to cause reductions in drug-seeking and downstream brain activity. Therefore, the present study sought to determine whether OXY infused into the PrL reduces cue-induced and METH-primed reinstatement and METH-induced neuronal activity in the downstream nucleus accumbens core (NAcc). Male Sprague Dawley rats underwent intravenous METH self-administration, extinction, and subsequent reinstatement tests. OXY was infused bilaterally into the PrL prior to cue-induced (0, 1 μg/side) and METH-primed reinstatement (0, 0.33, 1.0, 3.0 μg/side). Finally, we quantified cFos immunofluorescence in the NAcc as a proxy for downstream neuronal activity following a PrL infusion of OXY (0, 1 μg/side) prior to METH-primed reinstatement. OXY in the PrL significantly reduced both cue-induced and METH-primed reinstatement. Additionally, intra-PrL OXY reduced METH-induced cFos expression in the rostral but not caudal pole of the NAcc. These findings demonstrate OXY action in the PrL in reducing METH-seeking behaviours and METH-induced activity in the reward circuit. Furthermore, these results suggest that the therapeutic effects of systemically administered OXY on reducing METH-seeking behaviours may involve the PrL-NAc pathway.

1. Introduction

Methamphetamine (METH) is a powerfully addictive psychostimulant drug. Current treatment approaches are largely ineffective at preventing relapse to promote enduring abstinence (Morley et al., 2017). Research into the neuropeptide oxytocin (OXY) continues to demonstrate preclinical efficacy in reducing various types of METH-seeking behaviours in rodents (Qi et al., 2009; Carson et al., 2010a; Qi et al., 2012; Baracz et al., 2012; Han et al., 2014; Baracz et al., 2015, 2016a, 2016b; Ferland et al., 2016; Hicks et al., 2016; Cox et al., 2017; Westenbroek et al., 2019; Everett et al., 2018; for reviews see Baracz and Cornish, 2016; McGinty et al., 2019). Demonstrated by Carson et al. (2010a) in an intravenous model of METH self-administration, systemic OXY treatment reduced motivation to self-administer METH, and METH-primed reinstatement to METH-seeking behaviours in rats. Furthermore, OXY treatment reduced METH-induced neuronal activity in the nucleus accumbens core (NAcc) and subthalamic nucleus (STh; Carson et al., 2010b) implicating these regions as likely targets of systemic OXY treatment in reducing METH-seeking behaviours. Microinjection studies by Baracz et al. (2015, 2016a, 2016b) confirmed that OXY acts directly in the NAcc and STh to reduce METH-primed reinstatement, although the effect size was modest compared to the profound inhibitory effects of systemic OXY administration on METH-seeking behaviour (Carson et al., 2010a; Cox et al., 2017; Everett et al., 2018). This suggests that brain regions other than what were initially identified by Carson et al. (2010b) may be involved in the inhibitory effects of OXY on METH addiction. Interestingly, Carson et al. (2010b) did find that after acute METH administration, activity in the prelimbic

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(PrL) subregion of the medial prefrontal cortex was modestly, albeit not significantly attenuated by OXY pre-treatment. However, following acute METH exposure the PrL is only being moderately activated (Carson et al., 2010b), whereas after chronic METH intake, the PrL becomes sensitized to METH (Wearne et al., 2017). It is therefore plausible that in the chronic METH exposed animal, the PrL may be more sensitive to the inhibitory effect of OXY administration on METH-seeking behaviours.

In the context of addictive behaviours, the PrL receives input from dopaminergic neurons of the ventral tegmental area (VTA; Morales and Margolis, 2017), and sends glutamatergic projections to various subcortical targets including the NAcc (Kalivas et al., 2005). This excitatory pathway from the PrL to the NAc undergoes substantial plasticity following long term psychostimulant exposure and has been implicated as a key driver of reinstated METH-seeking after a period of abstinence (Rocha and Kalivas, 2010). Indeed, optogenetic inactivation of this pathway reduces psychostimulant-seeking behaviours (Stefanik et al., 2013). Furthermore, pharmacological manipulation of glutamatergic signaling in the NAcc modulates psychostimulant relapse behaviour (Cornish et al., 1999; Cornish and Kalivas, 2000; McFarland et al., 2003), supporting the role of glutamate input to the NAcc in driving this response. In a similar way to the optical approaches mentioned above, pharmacological inhibition of the PrL through local infusion of the GABA-A agonist muscimol results in reduced reinstatement of psychostimulant-seeking behaviour, an effect not shared with reinstatement to food-seeking behaviour (McFarland et al., 2003). This suggests that targeting the PrL-NAcc pathway by increasing GABA activity in the PrL or reducing glutamate activity to the NAcc may be useful treatments for reducing addictive behaviours without altering natural reward seeking behaviours. In line with this, intracerebroventricular administration of OXY increased basal GABA levels, inhibited METH-induced glutamate dialysates in the PrL (Qi et al., 2012), and upregulated expression of the glutamate reuptake transporter GLT1 (Han et al., 2014). Furthermore, local infusion of OXY to the PrL increased activity of GAD67 positive cells, a marker of GABA neurons (Sabihi et al., 2017).

Altogether, these data suggest that exogenous OXY administration may increase inhibitory signaling in the PrL to decrease METH-associated glutamate output to the NAcc, causing a reduction in reinstatement to METH-seeking behaviours. In order to investigate this possibility, the present study will investigate the effects of local infusion of OXY into the PrL on cue-induced and METH-primed reinstatement to METH-seeking behaviours, and will determine this effect of OXY administration on METH-induced neuronal activity in the NAcc.

2. Methods

2.1. Animals

Male Sprague Dawley rats (Animal Resource Centre, WA, Australia) were pair-housed in a humidity and temperature controlled colony room (23 ± 1 °C) on a 12:12 h light:dark cycle, with lights on at 7 am. All experiments occurred during the light phase. Rats had ad libitum access to water and food in their home cage. All procedures were approved by the Macquarie University Animal Ethics Committee and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013).

2.2. Drugs

METH was purchased from the Australian Government Analytical Laboratories (Pymble, NSW, Australia). OXY was synthesised by ChinaPeptide (Shanghai, China). All drugs were dissolved in saline (0.9%) for intravenous (i.v., 0.05 mL/infusion) or intraperitoneal (i.p., 1 mL/kg/injection) injection purposes. Vehicle (VEH) administration was 0.9% saline solution.

2.3. Apparatus

All testing occurred in standard operant conditioning chambers within sound-attenuating boxes (Med Associates, VT, USA), which were equipped with a fan, two retractable levers (one active, one inactive), two cue-lights, one house-light, infrared locomotor activity detectors (4 horizontal beams), and an infusion pump. The infusion pump was located on top of the operant chamber, so that the distinctive sound of infusion acted as a cue for classical conditioning purposes. Each chamber also contained an adjustable metal arm and spring connector, which were attached to a swivel. Polyethylene tubing threaded through the spring connector was attached to a 10 mL syringe driven by the infusion pump (Med Associates). The tubing which extended from the spring connector was connected to the back-mounted intravenous catheter for drug self-administration.

2.4. Surgery

Rats were implanted with a chronic indwelling catheter in the right jugular vein for intravenous self-administration, followed by bilateral implantation of intracranial cannula into the PrL for micro infusions. Except for stereotaxic coordinates, all surgical and post-operative procedures were conducted identically to Baracz et al. (2016a, 2016b). In brief, rats were anaesthetised with isoflurane gas (3% in 2 L/min oxygen) and following standard catheter implantation procedures, rats were placed in a stereotaxic apparatus. Bilateral stainless-steel guide cannulae (26 gauge; 10 mm) were lowered to 1 mm above the PRL (with nosebar = −3.3 mm, measured from bregma: anterior/posterior, +3.2 mm; medial/lateral, ± 0.7 mm; dorsal/ventral, −2.2 mm). Coordinates were adapted from the rat brain atlas of Paxinos and Watson (2007). Guide cannulae were secured in place with dental cement, and 10 mm stainless steel stylets were inserted into each cannulae to maintain patency during self-administration procedures, until testing began. Following 7 days of post-operative care (Baracz et al., 2016a, 2016b), self-administration procedures commenced.

2.5. Self-administration and extinction

Rats acquired METH self-administration during 4-hour fixed ratio-1 sessions conducted 5 days per week for three weeks, for a total of 15 sessions. For each session, catheters were flushed with heparinized saline (10 IU in 0.1 mL), and the rats were connected to the infusion lines, and placed in the operant chambers. Initiation of the session was marked by extension of the levers and illumination of the house light. Levers were randomly allocated as active or inactive. Depression of the active lever delivered an infusion of METH (0.1 mg/kg/0.05 mL infusion). Each infusion was associated with 3 s of illumination of the cue-light above the active lever, the sound of the infusion pump, as well as the house light extinguishing for 20 s to demarcate a time-out period during which depression of the active lever was recorded yet had no programmed consequences. Engagement of the inactive lever had no programmed consequences at any time. Each rat was limited to a maximum of 100 infusions per session or 4 h, at which point the session ended, as indicated by retraction of the levers, and extinguishing of the house light. Active and inactive lever presses, and infusions were recorded, and locomotor activity in the operant chamber was assessed by infrared beam breaks. At the end of each session, catheters were flushed with the antibiotic cephazolin sodium in heparinized saline solution (60 IU in 0.2 mL).

Following 15 days of intravenous self-administration (IVSA) of METH, rats underwent daily extinction training sessions. During extinction sessions, the house light remained extinguished, and depression of the active lever resulted in no programmed consequences. This preserved the pavlovian associations between environmental and METH delivery, for cue-induced reinstatement purposes. The duration of extinction sessions was progressively tapered down, whereby

extinction sessions 1–3 were run for 4 h, sessions 4–6 for 3 h, 7–9 for 2 h, and 10–15 for 1 h. All rats made < 10 active lever presses per session for the final three sessions. Our criterion of < 10 active lever presses/1 h indicated successful extinction of operant responding on the METH-paired lever. Rats received sham microinjections into the PrL on extinction sessions 14 and 15 (stylet removed and replaced), to habituate them to the reinstatement procedures.

2.6. Reinstatement and microinjections

For cue-induced reinstatement, rats received bilateral microinjection of VEH or OXY (1 μg/side) into the PrL. Infusions of 500 nL/hemisphere were delivered over 1 min by stainless steel microinjectors (33 gauge; 11 mm) attached to a 1 μL Hamilton syringe via polyethylene tubing, and driven by a microinjection pump (Harvard Apparatus, Holliston, MA, USA). Microinjectors extended 1 mm beyond the implanted cannulae and remained in place for 60 s after completion of the microinjection, and were then returned to their home cage. Five minutes following, rats were placed in the self-administration chamber for 1 h. At the beginning of the session, the levers extended, and the house light illuminated. At 30 s into the session, a non-contingent compound cue was presented, whereby the cue light was illuminated, the houselight was turned off, and the infusion pump sounded for 3 s. Thereafter, depression of the active lever resulted in the compound cue presenting for 3 s, with no programmed time-out period. Microinjection treatments were counterbalanced across two test days, separated by three extinction sessions. Following the final cue-induced reinstatement test, rats underwent three days of extinction, during which they received i.p. injections of saline (1 mL/kg) prior to the session to habituate them to the METH-priming procedures.

For METH-primed reinstatement, rats received bilateral PrL microinjections of VEH or OXY (0.33, 1, 3 μg/side), and were returned to their home cage. Five minutes later, rats received METH (1 mg/kg, i.p.) and were then placed in the operant chamber for 1 h. Reinstatement conditions were identical to extinction, where depression of the active lever resulted in no programmed consequences. Each reinstatement session was separated by at least 3 extinction sessions to ensure the behavioural effects of reinstatement or PrL drug infusion had dissipated.

Doses of OXY for intra-PrL microinjection procedures were primarily informed by the work of Sabihi et al. (2014, 2017) who demonstrated that 1 μg but not 0.1 μg/side OXY in the PrL reduced unconditioned anxiety in rats, likely through activating GABAergic cells of the PrL and reducing glutamatergic output. As METH exposure has been shown to dysregulate OXY (Zanos et al., 2014; Georgiou et al., 2016; Baracz et al., 2016b; Krasnova et al., 2017) and vasopressin systems (Cadet et al., 2014; Jayanthi et al., 2018), the efficacy of 1 μg/side OXY in METH animals may differ from that in Sabihi et al. (2017). Therefore, 1 μg/side was selected as the middle dose.

2.7. Tissue collection and immunofluorescence

Following three days of extinction after the final METH-primed reinstatement test, rats were randomly allocated to one of three groups (1) i.c. VEH + i.p. VEH; (2) i.c. VEH + i.p. METH (1 mg/kg) (3) i.c. OXY (1 μg/side) + i.p. METH (1 mg/kg). Five minutes after intra-PrL infusion, rats received i.p. treatment and then underwent a 90-minute extinction session. Ninety-minutes after i.p. treatment, rats were euthanized with an overdose of pentobarbitone sodium (2 mL Lethabarb, diluted 1:1 with saline; Clifford Hallam Healthcare, Roselands, NSW, Australia) and perfused via intracardiac puncture with 300 mL ice-cold Dulbeco's Modified Eagle Medium (Sigma-Aldrich, Castle Hill, NSW, Australia) followed by 300 mL ice-cold paraformaldehyde (PFA; 4%). Brains were removed and post-fixed in 4% PFA for 24 h at 4 °C, then placed in escalating concentrations of sucrose (10, 20, 30%) for 24 h per solution. Brains were then placed in cryoprotectant solution until they sunk to the bottom of the specimen jar and were then stored in a −20 °C freezer until processed. Whole brains were sliced (50 μm) on a vibrating microtome (VT1200 S; Leica Microsystems, North Ryde, NSW, Australia) and sections containing the nucleus accumbens (+1.56 mm to +2.28 mm from bregma) were sequentially captured in three 25-mL pots containing phosphate buffered saline (PBS). For the immunofluorescence procedure, slices were initially washed 3 times for 15 min in PBS with tween (PBT) at room temperature. Next, tissue was incubated with a primary antibody to detect cFos (rabbit anti-cfos, dilution 1:1000, Catalogue #ABE457, Lot #2905394 Merck), in a solution of Tris PBS (Tris-HCL 10 mM + sodium phosphate buffer 0.1 M + 0.9% NaCl) with 0.05% merthiolate (TPBSm) and 10% normal horse serum (NHS) for 16 h at room temperature. After 3 15-minute washes in PBS, with the final wash containing PBT, tissue was then incubated with a secondary antibody (dilution 1:500, Cy5-conjugated donkey anti-rabbit, Catalogue #711-175-152, Lot #132485, Jackson Immunoresearch) in TPBSm and NHS for 4 h at room temperature. After washing the tissue 3 times for 15 min in PBS, slices were mounted on glass slides and cover slipped with Fluoroshield with DAPI (F6057, Sigma Aldrich), for nuclear identification.

2.8. Image acquisition and quantification of cFos immunoreactivity in the NAcc

Coronal sections of the NAcc were imaged at 10× magnification using ZenPRO software (Carl Zeiss, Germany). Exposure times were held constant across slices. For each rat, images from the rostral (2.16 to 2.28 mm) and caudal (1.56 to 1.68 mm) aspects of the NAcc were selected based on the rat brain atlas of Paxinos and Watson (YEAR). The number of cFos immuno-reactive cells was quantified in an identical manner as follows using ImageJ software (National Institutes of Health, Bethesda, USA). Each image was converted to an 8-bit binary image, background noise was subtracted, and the MaxEntropy threshold was applied. A standardized region of interest was then created (1000 × 1000 pixels) and positioned over the NAcc, dorso-medially to the anterior commissure. This ensured the same area size, and same positioning was repeated for each slice, and is broadly consistent with the approach taken by Sabihi et al. (2017) which also assessed downstream cFos following intra-PrL OXY. The analyze particle function was then used to automatically count all black circles within this region of interest. Two experimenters hand counted select slices to ensure that this quantification approach was consistent with human scoring.

2.9. Statistical analysis

Daily rates of active and inactive lever pressing, infusions, and locomotor activity during IVSA and extinction were analysed using repeated measures ANOVAs. To demonstrate that rats reinstated to METH-paired lever pressing, active lever pressing during VEH + CUE and VEH + METH were compared to the extinction session prior to the respective test session, using paired sample *t*-tests. All reinstatement analyses (lever presses and locomotor counts) were conducted within one-factor repeated measures ANOVAs. For cue-induced reinstatement, the within-subjects variable of PrL infusion had two levels (VEH, OXY 1 μg), and for METH-primed reinstatement, 4 levels (VEH, OXY 0.33 μg, 1 μg, 3.0 μg). Planned contrasts comparing each OXY dose to VEH were conducted within this model, with α set at 0.05 for cue-induced reinstatement, and α = 0.017 for Bonferonni adjusted non-orthogonal contrasts of METH-primed reinstatement (Field, 2009). Analysis of METH-induced cFos counts in the NAcc was conducted in a two-factor mixed-model ANOVA, with the between-subjects variable of intra-PrL infusion (VEH, OXY) and the within-subjects variable of NAcc pole (Rostral, Caudal) as the interacting factors (α = 0.05). Statistical analyses were performed using SPSS 20 Graduate Student Version for Windows (SPSS Inc., Chicago, IL, USA). Graphs were created using GraphPad (Prism, Version 7.04). Data are displayed as mean ± SEM.

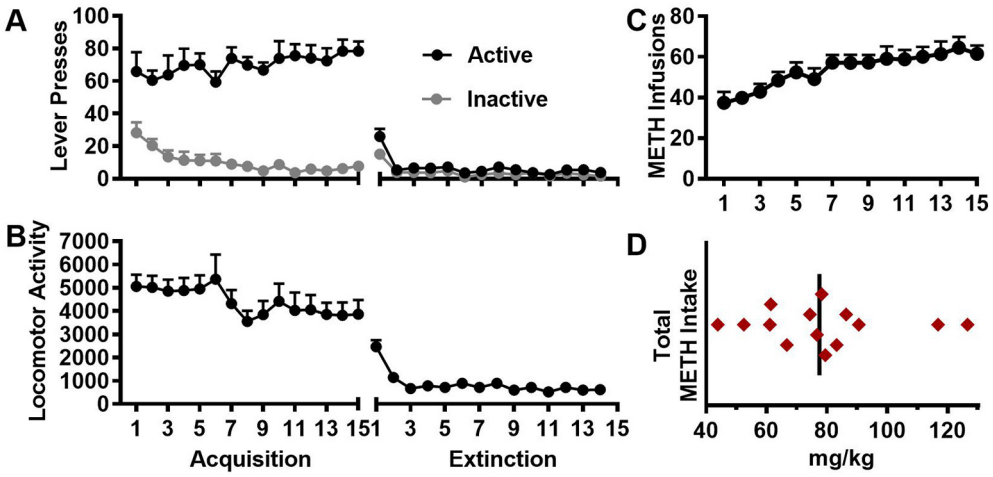

Fig. 1. Acquisition and extinction of METH intravenous self-administration (0.1 mg/kg/infusion). (A) Active and inactive lever presses, (B) locomotor activity, (C) infusions over 15 days of acquisition (4 h/day) and 15 days of extinction. (D) Lifetime METH intake. n = 14. Data is displayed as mean + SEM.

3. Results

3.1. Excluded animals

One rat did not finish IVSA training, and one rat failed to reinstate to cue- and METH-primed reinstatement, defined as achieving at least 15 active lever presses in the respective VEH session. As such, 14 rats were included in the reinstatement analyses. For cFos analyses, two rats in the VEH + METH condition did not reinstate to this final test, and thus were excluded. One rat from the OXY + METH condition did not have patent bilateral cannula, so was excluded. This resulted in 4 rats per condition.

3.2. METH self-administration and extinction

All rats acquired METH self-administration, as demonstrated by significant discrimination between active and inactive lever presses at day 15 ($t(1,13) = 14.220$, $P = 0.000$; Fig. 1A), and escalation of METH intake over the 15-day self-administration period (Day 1 vs 15 infusions: $t(1,13) = 4.542$, $P = 0.001$; Fig. 1C). Behavioural extinction produced a rapid decrease in lever pressing, evidenced on day 15 of extinction as there was no significant difference in active and inactive lever pressing ($t(1,13) = 2.154$, $P = 0.051$). Surprisingly, these rats did not demonstrate the typical extinction burst on the first day of extinction. Although the reasons for this are unknown, this may be due to the longer access paradigm used presently, as others have found a similar lack of extinction burst in 6 h/day trained rats (Shepard et al., 2004). The mean lifetime METH intake was 77.66 mg/kg (SEM = 5.68), although there was substantial inter-rat variability (Fig. 1D).

3.3. Effect of OXY in the PrL on cue-induced reinstatement

The VEH + CUE session resulted in significantly higher active lever pressing than during extinction ($t(1, 13) = 4.784$, $P = 0.000$; Fig. 2A and D). Compared with VEH, OXY (1 μg/side) infused into the PrL resulted in significantly lower active lever pressing ($F(1, 13) = 7.649$, $P = 0.016$; $\alpha = 0.05$), although this was still significantly higher than during extinction ($F(1, 13) = 16.704$, $P = 0.001$). Reinstatement following VEH + CUE on day 1 and day 2 did not significantly differ ($t(12) = 0.380$; $P = 0.711$; data not shown).

Inactive lever pressing did not significantly differ between extinction and VEH + CUE ($t(1,13) = 1.636$, $P = 0.126$; Fig. 2B), or VEH + CUE and OXY + CUE ($F(1, 13) = 1.579$, $P = 0.231$). Compared with extinction, the VEH + CUE session resulted in significantly higher locomotor activity ($t(1, 13) = 4.218$, $P = 0.001$; Fig. 2C). Compared with VEH pre-treatment, OXY (1 μg) infused into the PrL resulted in significantly lower METH-induced locomotor activity ($F(1, 13) = 7.076$, $P = 0.020$), although only to a level not significantly different from extinction activity ($F(1, 13) = 4.113$, $P = 0.064$).

Despite the large inter-rat variability in cue-induced reinstatement (Fig. 2E) and lifetime METH intake, no correlation between these variables was found ($r = 0.145$; $P = 0.6061$). Correct injection locations within the prelimbic cortex are depicted in Fig. 2F.

3.4. Effects of OXY in the PrL on METH-primed reinstatement

The VEH + METH session resulted in significantly higher active lever pressing than during extinction ($t(1, 13) = 7.495$, $P = 0.000$; Fig. 3A and C). Compared with VEH, 1 and 3 μg/side OXY in the PrL prior to METH-priming resulted in significantly lower active lever pressing, whilst 0.33 μg/side did not (0.33 μg: $F(1, 13) = 5.642$, $P = 0.034$; 1 μg: $F(1, 13) = 10.826$, $P = 0.006$; 3 μg: $F(1, 14) = 8.920$, $P = 0.011$; $\alpha = 0.017$). A one-way between-subjects ANOVA assessing active lever presses during reinstatement to VEH + METH tests across the four counterbalanced days was not significant ($F(3, 13) = 2.476$; $P = 0.121$; data not shown).

Compared with extinction, VEH + METH significantly increased inactive lever pressing ($t(1,13) = 2.342$, $P = 0.036$; Fig. 3B), which was not affected by intra-PrL OXY administration (0.33 μg: $F(1, 13) = 0.060$, $P = 0.810$; 1 μg: $F(1, 13) = 3.449$, $P = 0.086$; 3 μg: $F(1, 13) = 0.000$, $P = 1.000$).

Compared with extinction, the VEH + METH session resulted in significantly higher locomotor activity ($t(1, 13) = 8.912$, $P = 0.000$; Fig. 3D). There was no effect of intra-PrL OXY doses on locomotor activity induced by METH priming when compared to VEH + METH treatment (0.33 μg: $F(1, 13) = 1.177$, $P = 0.298$; 1 μg: $F(1, 13) = 0.037$, $P = 0.851$; 3 μg: $F(1, 13) = 2.172$, $P = 0.164$).

To determine whether the doses of OXY affected METH-primed reinstatement differentially across the 60-minute test period, active lever presses were divided into three 20-min bins (Fig. 3C). Overall, there was no significant main effect of time ($F(2, 26) = 0.855$; $P = 0.437$), although the time * treatment interaction was trending toward significance ($F(6, 78) = 1.932$; $P = 0.086$). Follow-up 2 × 2 interaction tests were conducted between doses in ascending order and each timepoint, which revealed that when compared with the 1 μg or 3 μg dose, the 0.33 μg dose resulted in higher active lever pressing at the 21–40 min time point, compared to the first 20 min (0.33 vs 1.0 μg: $F(1, 13) = 6.445$; $P = 0.025$; 0.33 vs 3.0 μg: $F(1, 13) = 5.759$; $P = 0.032$), however after Bonferroni corrections for repeated comparisons, neither of these interactions were statistically significant.

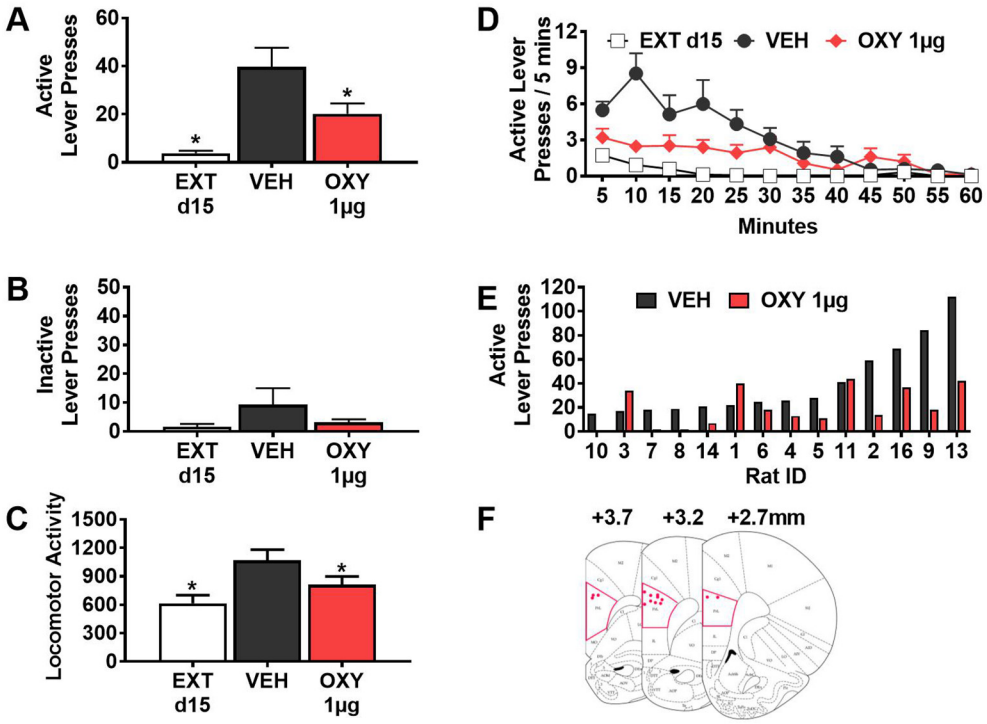

Fig. 2. Effects of intra-PrL OXY on cue-induced reinstatement to METH-seeking behaviours. (A) Active lever pressing, (B) inactive lever pressing and (C) locomotor activity for the 60-minute reinstatement test. (D) Time-course of cue-induced reinstatement to drug-paired lever pressing, presented as active lever pressing in 5-minute buckets. (E) Active lever presses from individual rats in response to intra-PrL OXY, presented in ascending order by VEH + Cue response. (F) Mapping of PrL infusion locations. The extinction data depicted is from the final day of extinction training prior to the commencement of cue-induced reinstatement tests, although statistical comparisons were conducted on the extinction day prior to each respective test. Infusions were bilateral but are represented unilaterally in the figure. Each circle indicates an individual rat. *P < 0.05 compared to VEH + METH. n = 14.

There was substantial inter-rat variability in the magnitude of METH-primed reinstatement (Fig. E), however this did not correlate with lifetime METH intake ($r = 0.350$; P = 0.201), or active lever presses during cue-induced reinstatement ($r = 0.184$; P = 0.513). Paired *t*-tests confirmed that all four METH-primed reinstatement tests produced significantly higher active lever presses, inactive lever presses, and locomotor activity than their respective extinction day prior (all tests P < 0.05; data not shown).

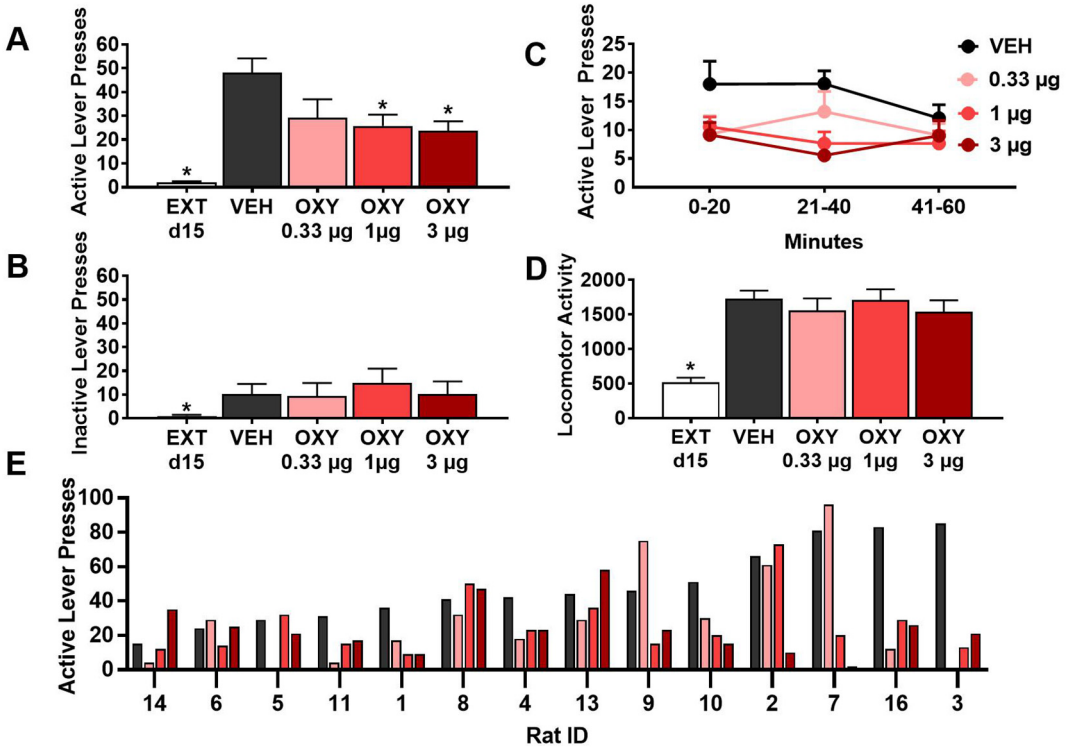

Fig. 3. Effects of intra-PrL OXY on METH-primed reinstatement to METH-seeking behaviours. (A) Active lever pressing, (B) inactive lever pressing, (C) time-course of active lever pressing, and (D) locomotor activity for the 60-minute reinstatement test. (E) Active lever presses from individual rats in response to each dose of intra-PrL OXY, presented in ascending order by VEH + METH response. The extinction data depicted is from the final day of extinction training prior to the commencement of cue-induced reinstatement tests, although statistical comparisons were conducted on the extinction day prior to each respective test.*P < 0.017 compared to VEH + METH. n = 14.

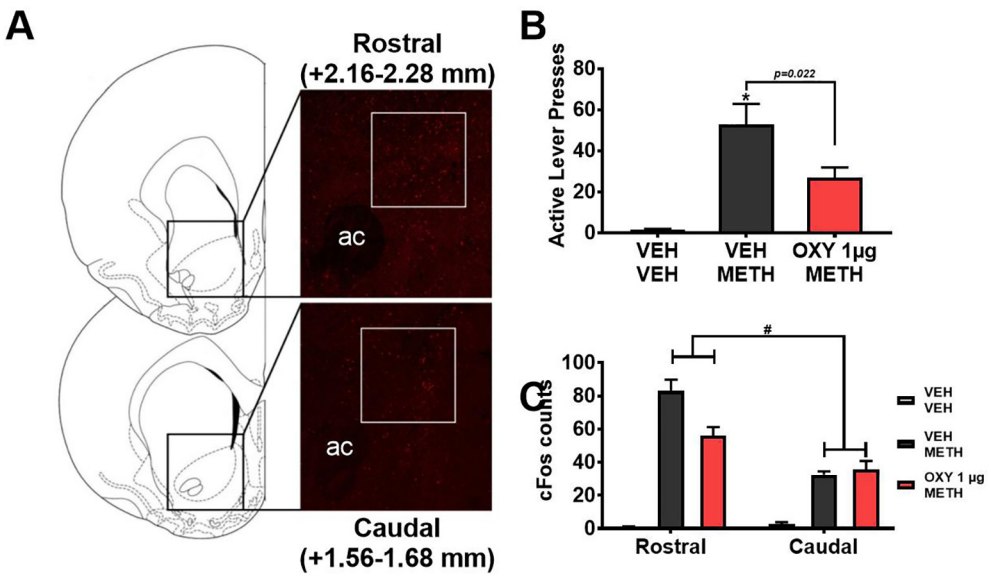

Fig. 4. Quantification of cFos+ cells in the rostral (+2.16 to 2.28 mm from bregma) and caudal (1.56 to 1.68 mm) NAcc after PrL infusion with OXY or VEH, prior to METH-primed reinstatement. (A) Schematic representation of rostral and caudal NAcc in which cFos counts were taken. (B) Active lever pressing during METH-primed reinstatement. (C) Analysis of effect of intra-PrL OXY on cFos counts across the rostral and caudal NAcc. ac = anterior commissure. White box represents the standardized region of interest for automated counting. *P < 0.05 compared to VEH + VEH. #P < 0.05 interaction test of bregma level (rostral, caudal) by PrL infusion (VEH, OXY). n = 4/group.

3.5. Effects of OXY in the PrL on METH-induced cFos in the NAcc

cFos+ cells were counted in the rostral and caudal NAcc poles (Fig. 4A), from a control group (intra-PrL VEH + i.p. VEH; n = 4), a METH-primed reinstatement group (intra-PrL VEH + i.p. METH; n = 4), and a group which received intra-PrL OXY (1 µg/side) prior to METH-primed reinstatement. The suppressant effect of intra-PrL OXY on METH-primed reinstatement previously observed in Fig. 3 was replicated in this sub-sample. There was a significant main effect of group allocation on active lever presses ($F(2, 11) = 15.575$; $P = 0.001$), and follow-up paired contrasts revealed that: compared with VEH + VEH, VEH + METH resulted in higher active lever presses ($t(1,9) = 5.581$; $P < 0.000$). Compared with VEH + METH, OXY 1 µg + METH resulted in lower active lever presses ($t(1,9) = 2.777$; $P = 0.022$), and when compared with VEH + VEH, OXY 1 µg + METH also resulted in higher active lever presses ($t(1,9) = 2.804$; $P = 0.021$). It should be noted that these two comparisons did not meet statistical significance cutoffs after Bonferroni corrections ($\alpha = 0.017$ for these contrasts).

In regards to cFos+ counts, there was a significant 2 × 3 interaction of the within-subjects factor of NAcc pole (rostral, caudal), and the between-subjects factor of group (VEH + VEH, VEH + METH, OXY + METH; $F(2, 9) = 28.889$; $P < 0.000$). Follow-up comparisons revealed that when comparing between the two METH-treated groups (VEH + METH, OXY + METH), there was a significant interaction of bregma level and PrL treatment ($F(1, 6) = 13.107$; $P = 0.11$), whereby the effect of intra-PrL OXY to reduce NAcc cFos counts was greater in the rostral pole than the caudal pole.

4. Discussion

The present study demonstrates that OXY infused into the PrL reduced cue-induced and METH-primed reinstatement of METH-seeking behaviours in male rats. Additionally, OXY in the PrL attenuated downstream METH-induced cFos immunofluorescence in the rostral NAcc, a major recipient of PrL projections. Together, these findings suggest that the PrL is a key neural site where exogenous OXY treatment acts to produce anti-addiction effects, and to modulate addiction-related activity in the NAcc.

Cue-induced reinstatement of drug-paired lever pressing peaked in the first 10 min of the relapse session and endured for approximately 30 min. Intra-PrL OXY infusion blunted this initial peak, and attenuated reinstated lever pressing for the entire session. Accompanying this suppressant effect of OXY on drug-seeking was a modest but non-significant reduction in locomotor activity. However, the resulting locomotor activity was similar to extinction levels, and is likely a by-product of the reduction in lever pressing behaviour. Additionally, inspection of individual animals' responses to OXY treatment indicates that the highest relapsing animals received similar, if not heightened benefit from intra-PrL OXY infusion. Although the present sample size does not enable a statistical comparison by phenotype, it does point to some specificity of OXY acting in PrL to reduce METH-seeking behaviours. Indeed, optogenetic inhibition of the PrL and PrL-NAc pathway has been shown to reduce cue-induced reinstatement of psychostimulant-seeking, but not food-seeking behaviour (Stefanik et al., 2013), suggesting that modulation of the PrL may specifically influence psychostimulant but not natural reward seeking. However, examination of OXY mechanisms in this pathway in a sucrose-seeking task would be required to confirm the specificity of OXY in the PrL on reducing addictive behaviours and should be explored in future studies.

Recently, seemingly conflicting findings to the present data set were published by Weber et al. (2018), which found that a much lower dose of OXY in the PrL increased cue-induced reinstatement of cocaine-seeking. Although these disparate findings could be reflective of the neurobiological differences between cocaine and METH relapse (e.g. Peters et al., 2008; Rocha and Kalivas, 2010), it may be the case that OXY exhibits crucial dose-dependent effects at its receptors, and therefore on behavioural outcomes (e.g. Boccia and Baratti, 2000). The authors hypothesise that this enhancement of cocaine reinstatement is through action at OTRs on excitatory projection neurons terminating in the NAcc. However, at the doses used in the current study (e.g. 1 µg), but not lower (0.1 µg), OXY activates GABA cells of the PrL (Sabihi et al., 2017), an effect which would be expected to reduce METH-seeking behaviours. Indeed, agonism of GABA-A/B receptors in the PrL reduce METH-seeking behaviours (Rocha and Kalivas, 2010), and our group has unpublished data indicating that pharmacologically increasing tonic GABA activity in the PrL also reduces METH-reinstatement. Additionally, OXY administration increased expression of glutamate reuptake transporters (Han et al., 2014), which is significant given that drugs which increase glutamate reuptake in the NAcc, a major recipient of prefrontal glutamate, reduce psychostimulant relapse (Reissner et al., 2015). Further, OXY treatment may also increase activity of astrocytes, which maintain glutamate homeostasis (Mahmoud et al., 2019), and which have been shown to express OTRs (Wang et al., 2017). This potential interaction with astrocytes is promising, as increasing activity of astrocytes using selective chemogenetic strategies has shown decreased cue-induced cocaine seeking (Scofield et al.,

2015). Therefore, exogenous OXY likely acts on various glial and neuronal systems expressing its receptors, depending upon the administered dose.

Beyond explaining the difference in results between the present study and Weber et al. (2018), this disparity should also motivate careful consideration of the utility of OXY-based therapies. For example, systemic OXY doses resulting in brain concentrations sufficient to reduce METH-related activity in the PrL may be magnitudes greater than the doses required to modulate activity of neurons in the NAc (e.g. Baracz et al., 2016a, 2016b). Similarly, brain concentrations of OXY which achieve suppression of METH-related NAc activity may counterproductively increase METH-related PrL activity (Weber et al., 2018). Additionally, chemogenetic or pharmacological strategies which promote endogenous OXY secretion may be sufficient to recapitulate the relatively low OXY concentrations required to modulate NAc activity, but are unlikely to achieve levels sufficient to recreate the present effects in the PrL.

METH-priming resulted in reinstatement of lever pressing which remained constant for approximately 40 min. All three doses of intra-PrL OXY reduced lever pressing in a very similar manner, and for most of this time period. However, the lowest dose appeared to lose efficacy by 30 min, with lever pressing climbing upwards toward vehicle-like levels after this time. Other than this modest time-dependent effect, there were no apparent effects of dose. Interestingly, OXY had no effect on METH-induced hyperactivity. This strongly supports the conclusion that OXY reduced motivated drug-seeking, and not just the locomotor activity required to enact the motivated behaviour. However, if we are proposing that OXY is interacting with GABA, the effect on locomotor activity is somewhat surprising, increasing tonic GABA activity in the PrL reduces METH-hyperactivity (unpublished). Furthermore, our group has unpublished data indicating that a systemic injection with OXY reduces METH-hyperactivity primarily in rats with a history of METH self-administration, and less so in rats exposed to acute METH injection. Numerous other studies have also demonstrated the suppressing effects of OXY on psychostimulant hyperactivity when administered systemically (Carson et al., 2010a; Cox et al., 2017; Everett et al., 2018), but not locally in the NAcc (Baracz et al., 2016a, 2016b; Cox et al., 2017) or STh (Baracz et al., 2015). Therefore, it appears that OXY administration into the PrL has highly specific effects on relapse-like behaviours, whilst reducing METH-hyperactivity may involve OXY activity at other presently unstudied regions.

Again, further inspection of individual responding indicates that the rats which relapsed the most to METH-priming received substantial, if not heightened benefit from intra-PrL OXY. As no correlation between lifetime METH-intake and METH-primed reinstatement was found, it is unlikely that OXY is just interacting with METH-induced neuroadaptations. Rather, it is interesting to speculate that OXY treatment may preferentially modulate PrL activity in animals with a pre-existing neural vulnerability to addictive behaviour (Buisman-Pijlman et al., 2014). As such, an investigation of OXY efficacy across addicted phenotypes is warranted. Interestingly, no correlation between cue-induced and METH-primed reinstatement was found. That is, the highest responders to cues, were not the highest responders to METH-priming. This was unexpected, although not surprising given the distinct brain circuitry and chemistry which drives these two forms of relapse (Kalivas and McFarland, 2003). It is plausible that the PrL exhibits control over drug-cues and METH priming via distinct glutamate projections to regions beyond the NAcc, such as to the basolateral amygdala (Mashhoon et al., 2010); and that OXY is capable of inhibiting several of these relapse-associated outputs. To address this question however, progress in the basic understanding of dissociated prefrontal control over relapse is needed.

As the present doses of OXY likely increased activity of GABA cells in the PrL (Sabihi et al., 2017) or increased glutamate reuptake (Qi et al., 2012; Han et al., 2014), it is possible that the crucial relapse-driving excitatory projections from the PrL were inhibited, attenuating relapse-induced PrL input to downstream regions, such as to the NAcc. Indeed, the present study indicates that OXY in the PrL was associated with reduced METH-activation of the rostral but not caudal compartment of the NAcc. The NAcc is a major target of glutamatergic projections from the PrL (Kalivas et al., 2005), and inactivation of this pathway prevents reinstatement of psychostimulant seeking behaviours (Rocha and Kalivas, 2010). Furthermore, retrograde and anterograde tracing methods have identified that the PrL, and particularly the rostral PrL, more densely innervate the rostral NAcc than the caudal NAcc (Gorelova and Yang, 1996; Vertes, 2004). Therefore, impaired METH-associated activity in the rostral NAcc is consistent with reduced excitatory output from the PrL, and directly supports other studies showing reduced NAcc activity after systemic OXY treatment (Carson et al., 2010b; Leong et al., 2017; Cox et al., 2017).

Whilst we did not explore the receptor mechanisms by which OXY modulates PrL activity during reinstated METH-seeking, our doses were based on those of Sabihi et al. (2017), who demonstrated that OXY in the PrL via OTR and GABA-A receptors reduced unconditioned anxiety. Therefore, we can speculate that the present findings may involve OXY activity at OTRs and/or GABA-A receptors. Indeed, as previously mentioned, GABA-A receptor agonism, albeit used as a reversible pharmacological lesion, similarly reduced psychostimulant-seeking behaviours (McFarland et al., 2003). Although our findings are in keeping with Sabihi et al. (2017), it needs to be recognized that OXY administration may interact with different receptor mechanisms in METH-naïve and METH-experienced animals. For example, chronic psychostimulant self-administration dysregulated the expression of OTR's in the NAc and STh (Baracz et al., 2015) and amygdala (Zanos et al., 2014), and so may also affect PrL OTR expression. Additionally, we have shown that the vasopressin V1A receptor mediates the effects of OXY in the NAc on METH-seeking (Everett et al., 2018), and mRNA encoding for V1A receptors is upregulated 8-fold in the NAc 30 days following a single METH injection (Jayanthi et al., 2018). Whilst Sabihi et al. (2017) demonstrated a V1A independent effect of intra-PrL OXY on anxiety, it is not yet known how chronic METH exposure changes the expression of OTR, V1A and GABA-A receptors in the PrL, necessitating follow-up experiments to examine the receptor mechanisms behind the present findings. Clearly then, greater understanding of the receptor- and dose-dependent effects of OXY on the addiction circuitry is needed to help translate this growing body of preclinical anti-addiction OXY research into targeted addiction therapies for the clinic.

Several limitations of this study should be noted. Firstly, although the primary behavioural dataset is based on a sample of 14 rats, the immunohistochemical dataset was limited to just 4 rats per treatment group. This was evidently sufficient to detect the large effect of intra-PrL OXY on METH-induced cFos expression in the rostral NAcc, however would not be sufficient to detect modest or small effect sizes. Due to this low statistical power, we cannot confidently rule out the lack of an effect at the caudal NAcc as a false-negative. Additionally, the present study only investigated the effects of intra-PrL OXY in male rodents, which may limit the generality of these findings given multiple sources of evidence indicating sex-dependent interactions of OXY and METH-seeking (Cox et al., 2013). Furthermore, chronic OXY treatment has been shown to produce modest yet enduring suppression of METH-seeking behaviours (Hicks et al., 2016; Ferland et al., 2016). In the present study, each rat received 4 intra-PrL OXY infusions across a two-week period, suggesting the possibility of carryover effects on METH-seeking from repeated OXY exposure. Although the present methods do not enable us to rule out this carryover effect entirely, the use of a latin-square design reduces the likelihood of these findings being due to treatment order. Indeed, several rats received all four PrL infusions of OXY before their final VEH + METH session, and inspection of their data indicates robust reinstatement despite this (< 40 active lever presses). Lastly, a near-significant interaction of the OXY 0.33 μg dose and time emerged, indicating that the inhibitory effects of the 0.33 μg dose on METH-primed reinstatement dissipated faster than following

the 1.0 or 3.0 μg doses. This time-dependent effect of OXY doses is unlikely to have emerged if the order of treatments was the primary driver of these effects, giving confidence to the suggestion of a modest dose-dependent effect of intra-PrL OXY.

In summary, these findings demonstrate that OXY in the PrL reduces relapse to METH-seeking, and that this might be associated with blunted METH-induced neuronal activity downstream in the rostral NAcc. As such, we identify a novel brain site where exogenous OXY, which is currently in use in clinical trials, acts to reduce relapse to METH-seeking. Future studies should identify the receptor, and post-synaptic mechanisms by which OXY modulates PrL-driven METH-seeking behaviour to uncover the crucial dose-dependent actions of OXY as an addiction therapy.

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