

TITLE PAGE

The oxytocin analogue carbetocin prevents priming-induced reinstatement of morphine-seeking: involvement of dopaminergic, noradrenergic and MOPr systems.

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ABSTRACT

Relapse to illicit drug-seeking following abstinence is a major challenge for the treatment of addiction as no effective pharmacotherapy is currently available. We have recently shown that activating the central oxytocinergic system prevents emotional impairment and stress-induced reinstatement associated with opioid withdrawal. Here, we investigated whether the oxytocin analogue carbetocin (CBT) is able to reverse *morphine-primed reinstatement conditioned place preference (CPP)* in mice. The neurochemical mechanism underlining the behavioural effect of CBT was investigated by assessing the involvement of the striatal noradrenergic and dopaminergic systems in CBT reversal of priming- and stress-induced reinstatement of opioid *CPP*. In addition, given recent evidence suggesting the presence of OTR- μ -opioid receptor (MOPr) interactions in the brain, we further explored these interactions by carrying out OTR autoradiographic binding in brain of mice lacking MOPr. CBT administration prevented priming-induced reinstatement of morphine *CPP*. While an acute effect of CBT in enhancing dopamine turnover was observed following stress- and priming-induced reinstatement, CBT significantly decreased striatal noradrenaline turnover only following priming-induced reinstatement. Moreover, a significant increase in OTR binding was observed in the nucleus accumbens, septum and amygdala of MOPr knockout mice, indicating the presence of a possible OTR-MOPr interaction which may be involved in the modulation of opioid addiction and relapse. These results support the oxytocinergic system as a promising target for the prevention of relapse to opioid use and highlight the differential involvement of striatal noradrenergic and dopaminergic systems on the effects of OTR stimulation in preventing stress- and priming-induced reinstatement of opioid *CPP* behaviour.

INTRODUCTION

Relapse to opioid use is the major challenge for the treatment of opioid addiction and can be elicited by exposure to the drug itself, by drug-associated cues and/or by stress during abstinence. Several lines of evidence suggest a role of the neuropeptide oxytocin in opioid addiction. It has been shown that peripherally administered OT attenuated heroin self-administration and the development of tolerance to morphine and blocked naloxone-precipitated morphine withdrawal in rodents (see Sarnyai and Kovacs, 1994). Additionally, acute and chronic opioid administration, as well as withdrawal altered oxytocin peptide content and/or synthesis in the brain of rodents (see McGregor and Bowen, 2012; Sarnyai and Kovacs, 1994). We have recently demonstrated that chronic morphine administration and withdrawal induces a hypo-oxytocinergic state in the hypothalamus and an increase in OTR binding in the amygdala, which was associated with a negative emotional state during withdrawal (Zanos et al., 2014). In the same study, we showed that administration of the oxytocin analogue, carbetocin (CBT), was able to reverse stress-induced reinstatement of morphine *CPP*.

A possible mechanism underlying the effects of OT in the prevention of relapse to opioid-seeking might involve its direct effects on the dopaminergic and noradrenergic systems in the brain. Early evidence by Kovacs and Telegdy (1983), showed that peripherally administered OT increases striatal dopamine turnover in rats. More recently, Qi et al. (2008) demonstrated that OT inhibits methamphetamine-induced decrease in dopamine turnover in the striatum. Moreover, it has been shown that OT administration enhances noradrenaline release in the supraoptic nucleus of the hypothalamus, which in turn activates hypothalamic OT neurons (Onaka et al., 2003). The striatal noradrenergic system has been also implicated in the modulation of negative emotional state during opioid withdrawal, since naloxone-induced aversion in morphine-dependent mice

enhanced noradrenaline transmission in the nucleus accumbens (Acb) (Gomez-Milanes et al., 2012).

Another mechanism which might underlie CBT's effects on the prevention of opioid reinstatement might involve its effects on the hypothalamic-pituitary-adrenal (HPA) axis. Indeed, intracerebroventricular (i.c.v) administration of an OTR antagonist increased plasma corticosterone levels (Neumann et al., 2000), whereas i.c.v OT administration decreased stress-induced corticosterone release (Windle et al., 1997) in rats.

We therefore investigated whether CBT prevents priming-induced reinstatement of morphine *CPP* in mice. The neurochemical mechanism underlying the behavioural effect of CBT was also investigated by comparing the involvement of the striatal noradrenergic and dopaminergic systems in CBT reversal of priming- and stress-induced reinstatement of opioid *CPP* a. In addition, given the evidence suggesting the presence of OTR-MOPr interactions in the brain (Becker et al., 2014; Gigliucci et al., 2014), which might also be involved in the modulation of opioid addiction and relapse, we further explored these receptor interactions by carrying out OTR autoradiographic binding in brains of MOPr knockout mice.

EXPERIMENTAL PROCEDURES

Male C57BL/6J mice (7-week old, Charles River, Kingston, UK), were housed individually in a temperature-controlled environment (12-hour light/dark cycle;06:00-18:00). Food and water were available *ad-libitum*. All procedures were approved by the UK Home Office under The Animals (Scientific Procedures) Act 1986.

Reinstatement of conditioned place preference in mice

We used a CPP apparatus (Opto-Max Activity Meter v2.16, Columbus Instruments, OH, USA), as previously described (Zanos et al. 2014). The CPP reinstatement protocol consisted of a habituation session, a pre-conditioning test, 4 conditioning sessions (morning saline and 4 hours later a 10 mg/kg, s.c., morphine injections), a post-conditioning test, 4 extinction sessions, a post-extinction test and a reinstatement session, each carried out on consecutive days. During the reinstatement session, mice were pre-treated with either saline (4 ml/kg, i.p.; n=10) or CBT (6.4 mg/kg, i.p.; n=11) and after 5 minutes, they received a morphine priming injection (2 mg/kg, i.p.). Ten minutes post-morphine injection, mice were placed in the CPP apparatus for 20 minutes. Time spent in each compartment was measured during the last 15 minutes of the session. Locomotor activity of the animals during all CPP sessions was scored by an automated program (Opto-Max Activity Meter v2.16, Columbus Instruments, OH, USA). Stress-induced reinstatement protocol is detailed in our previous publication (Zanos et al. 2014). Mice from both stress- and priming-induced reinstatement experiments were euthanized 30 minutes after the reinstatement sessions and brains were preserved in isopentane (-20°C) and stored in -80°C until use. Trunk blood was collected in EDTA-containing tubes.

Dopamine and Noradrenaline measurements

Since there is evidence for the involvement of dorsal and ventral striatal dopaminergic system during both stress- and priming-induced reinstatement (Shaham et al., 2003; Xi et al., 2004; Cruz et al., 2010; Vidal-Infer et al., 2012), and given the well-characterized regulatory effect of oxytocin on modulating monoamine turnover in the striatum (Baskerville and Douglas, 2010), we sought to assess the effects of CBT on the striatal dopaminergic turnover following priming- and stress- induced reinstatement of morphine CPP. Brains of saline- and CBT-treated animals (n=6/group; randomly selected) from the priming- and stress-induced reinstatement experiments were dissected using a mouse matrix. The Acb and caudate putamen (i.e., striatum) were homogenized in 0.1 M perchloric acid, 0.02% ethylenediaminetetraacetic acid (EDTA), 0.02% sodium metabisulfite using an ultrasonic cell disrupter (Cole-Parmer, Vernon Hills, IL, USA). Homogenates were centrifuged (45 min, 4°C, 15300 x g.) and supernatants filtered through a 0.22 µm syringe filter. Dopamine, noradrenaline and their principal metabolites DOPAC and MHPG respectively, were measured using High performance liquid chromatography (HPLC), as previously described (Gomez-Milanes et al., 2012).

Plasma corticosterone levels

Trunk blood from mice (n=10-11/group) was spun for 15 min at 2000xg at 4°C. Plasma was collected and corticosterone levels were measured using a rat/mouse corticosterone [¹²⁵I] kit (MP Biomedicals, New York, NY, USA), according to the manufacturer's instructions.

Oxytocin autoradiographic binding

Brains from MOPr knockout mice and their wild-type littermates (n=6/group) were provided by Prof. Brigitte Kieffer (IGBMC, Strasbourg, France). The methodology for the generation of MOPr knockout mice has been previously described (Matthes et al., 1996). OTR autoradiography on coronal brain sections was carried out in accordance with Georgiou et al., (2014). For the determination of total binding, slides were incubated in 50 pM [¹²⁵I]-ornithine vasotocin (OVTA) (PerkinElmer, 81.4 TBq/mmol) for 60 minutes. For non-specific binding, 50 μM unlabelled (Thr4, Gly7)-oxytocin (Bachem, Germany) was used.

Slides were apposed for 3 days on films (Kodak BioMax MR-1; Sigma-Aldrich, Gillingham, UK) along with appropriate ¹⁴C micro-scale standards (Amersham Pharmacia Biotech, Bucks, U.K.). All structures were identified by reference to the mouse brain atlas of Franklin & Paxinos (2007), and analyzed using an image analyzer (MCID; Image Research, Linton, UK).

Statistical analysis

Values are expressed as the mean ± SEM. Differences in priming-induced reinstatement of morphine *CPP* were analyzed using two-way ANOVA with factors ‘CPP phase’ and ‘treatment’ (saline/CBT). The locomotor data were analyzed using two-way repeated measures ANOVA with factors ‘treatment’ and ‘time (days)’. The effects of CBT administration on locomotion during priming-induced reinstatement, as well as plasma corticosterone were analyzed by unpaired Student’s *t*-test. The effect of CBT on striatal dopamine and noradrenaline turnover was assessed by two-way ANOVA with factors ‘treatment’ (saline/CBT) and ‘experiment’ (priming-/stress-induced reinstatement). For analysis of OTR autoradiographic binding, unpaired Student’s *t*-test in each individual region was used, as previously described (Gigliucci et al.,

2014). ANOVAs were followed by a Holm-Sidak *post-hoc* test when significance was reached ($p < 0.05$). All statistical analyses were performed using SigmaPlot (Systat Software, London, UK).

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RESULTS

Carbetocin administration reversed priming-induced reinstatement of morphine *place preference*

Two-way ANOVA revealed a significant effect of ‘CPP phase’ ($F_{[3,57]}=11.32$; $p<0.001$) and ‘treatment’ x ‘CPP phase’ interaction ($F_{[3,57]}=4.43$; $p<0.01$). Morphine administration increased time spent in the drug-paired compartment in the post-conditioning phase compared to the pre-conditioning phase ($p<0.01$). The 4-day extinction period led to a significant decrease in the time spent in the drug-paired compartment (post-extinction phase vs post-conditioning phase; $p<0.01$; Figure 1A). A priming injection of morphine reinstated CPP in mice pre-treated with saline, (reinstatement vs post-extinction phase; $p<0.01$; Figure 1A). In contrast, mice pre-injected with CBT did not manifest morphine reinstatement behaviour and the time spent in morphine-paired compartment was significantly lower compared to the saline group ($p<0.001$; Figure 1A).

Pre-treatment with CBT prior to the morphine-priming injection did not induce any changes on the ambulatory activity of the animals compared to saline pre-treatment (Figure 1C).

Acquisition of locomotor sensitisation following steady-dose morphine administration

Repeated measures two-way ANOVA revealed a significant effect of ‘treatment’ ($F_{[1,20]}=960.32$; $p<0.001$), ‘time’ ($F_{[3,60]}=3.391$; $p<0.05$) and ‘treatment’ x ‘time’ interaction ($F_{[3,60]}=27.127$; $p<0.001$). Morphine treatment increased ambulatory activity during the 4-day morphine administration ($p<0.001$; Figure 1B). Behavioural sensitisation to the acute motor-enhancing properties of morphine was observed from day 2 of the conditioning paradigm and persisted through day 4 (compared to day 1; $p<0.001$; Figure 1B).

Carbetocin administration increased dopamine turnover and decreased noradrenaline turnover in the striatum of mice undergoing priming-induced reinstatement of morphine place preference

CBT pre-treatment increased dopamine turnover following stress- and priming-induced reinstatement of morphine **CPP** (treatment effect: $F_{[1,20]} = 4.851$; $p < 0.05$; experiment effect: $F_{[1,20]} = 3.229$; $p = 0.09$; 'treatment' x 'experiment' interaction: $F_{[1,20]} = 0.063$; $p = 0.81$; Figure 1D).

Two-way ANOVA revealed a significant effect of 'treatment' ($F_{[1,20]} = 12.75$; $p < 0.01$) and a 'treatment' x 'experiment' interaction ($F_{[1,20]} = 6.268$; $p < 0.05$) on noradrenaline turnover. CBT pre-treatment decreased striatal noradrenaline turnover compared to saline pre-treatment during morphine-primed reinstatement, but it did not alter noradrenaline turnover of animals undergoing stress-induced reinstatement of morphine **CPP** ($p < 0.01$; Figure 1E). Importantly, morphine priming injection induced significantly higher striatal noradrenaline turnover in saline pre-treated animals compared to saline pre-treated animals subjected to stress ($p < 0.001$; Figure 1E).

Carbetocin administration had no effect on plasma corticosterone levels

No changes in plasma corticosterone levels were observed in the CBT-treated group compared with the saline-control group following priming-induced reinstatement ($p > 0.05$; Figure 1F).

Brain region-specific up-regulation of oxytocin receptors in the μ -opioid receptor knockout mouse brains

The pattern of distribution of OTR on MOPr knockout mice brains was identical to the wild-type (Figure 1G). OTR binding in MOPr knockout mouse brains was significantly higher in the Acb ($p < 0.05$), septum ($p < 0.05$) and amygdala ($p < 0.001$) compared to wild-type mice (Figure 1G,H).

Regression analysis was carried out to determine if there was a correlation between the MOPr binding levels in regions of wild-type mouse brains and % change in OTR binding in brain regions from MOPr knockout mice compared to wild-type mice. Pearson correlation coefficient revealed a significant correlation ($r=0.79$, $n=11$, $p<0.01$; Figure 11).

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DISCUSSION

This is the first study, to our knowledge, to report beneficial effect of the oxytocin analogue CBT in preventing priming-induced reinstatement of morphine *CPP* following extinction. Moreover, we show that CBT administration increases striatal dopamine turnover following both stress- and priming-induced reinstatement, whilst it decreases striatal noradrenaline turnover specifically following morphine-primed reinstatement. These effects of CBT were not associated with alterations in locomotion or HPA-axis activity. Moreover, this study provided evidence for the presence of an OTR-MOPr interaction in the Acb, septum and amygdala, brain regions important in drug addiction and emotional regulation.

A possible mechanism underlying CBT's ability to prevent reinstatement of morphine *CPP* was suggested to involve modulation of the striatal dopaminergic system. It has been shown that OT administration facilitates dopamine turnover in the striatum of treatment-naïve rats (Kovacs and Telegdy, 1983) and MAP-treated mice (Qi et al., 2008). In the present study we demonstrated a CBT-induced increase in striatal DOPAC/DA ratio following both priming- and stress-induced reinstatement of morphine *CPP*, suggesting that CBT might exert its effects in reversing morphine priming- and stress-induced reinstatement by modulating the striatal dopaminergic neurotransmission.

In addition, we observed a CBT-induced decrease in striatal noradrenaline turnover following morphine priming- but not stress-induced reinstatement, indicating a possible noradrenaline-dependent mechanism underlying the effect of CBT in preventing specifically morphine-primed reinstatement. It has been previously shown that peripheral administration of OT facilitates the disappearance of noradrenaline in the mesencephalon of rats (Kovacs and Telegdy, 1983).

Therefore, the decrease in noradrenaline turnover observed in the present study might be due to an indirect effect of CBT in preventing morphine-induced increases in noradrenaline release. While this is the first evidence for a direct effect of an oxytocinergic intervention on the striatal noradrenergic system to prevent priming-induced reinstatement of opioid *CPP*, the exact mechanism underlying this effect remains to be elucidated.

Even though another possible mechanism that might account for the observed effects of CBT during morphine reinstatement may involve the modulation of the HPA-axis activity, in the present study CBT pre-treatment did not induce any alterations in plasma corticosterone levels. While this finding is suggestive of an HPA-axis independent effect of CBT, our data do not exclude CBT's effects on the extra-hypothalamic CRF system. In agreement, Zanos et al., (2014) showed no effect of CBT on plasma corticosterone levels following stress-induced reinstatement of morphine *CPP* in mice.

There is evidence to suggest the existence of functional association between the central MOPr and oxytocin systems (see Vuong et al., 2010) which may also constitute a mechanism underlining the marked effect of the oxytocin analogue in preventing reinstatement of morphine CPP following a period of extinction. More specifically we postulate that acute administration of carbetocin prevents priming reinstatement of morphine seeking by restoring the oxytocinergic tone evident in the brains of morphine-treated mice undergoing extinction. There is indeed ample amount of evidence suggesting such a hypo-oxytocinergic state in opioid abstinent mice undergoing reinstatement (Clarke and Patrick, 1983; Hartman et al., 1986) (Arnauld et al., 1983; Wakerley et al., 1983; Pumford et al., 1991) (You et al., 2000) (Lindow et al., 1992). The strongest evidence arises

from our recent study which demonstrated profound oxytocin deficit in the hypothalamus of the same strain of mice treated with a similar chronic morphine administration protocol which persisted following 7 days withdrawal (Zanos et al., 2014). This decrease in OT levels was found to be concomitant with a marked upregulation in the OTR binding in the highly MOPr expressing regions such as the lateral septum and amygdala (Zanos et al., 2014) of morphine-abstinent mice pointing towards brain specific OTR-MOPr receptor interactions. The exact nature of these possible MOPr-OTR interactions is unclear and is indeed a topic of intense investigation in our laboratory. Possible suggestions include the presence of functional OTR-MOPr interactions in neuronal populations where these receptors are co-localized. Indeed there is a direct evidence for the co-localization of OTR and MOPr in neurons within the supraoptic nucleus, as assessed using double-labeled in situ hybridization combined with immunohistochemistry (Li et al., 2001). Another possibility may include physical interactions between OTR and MOPr to form dimers and although OTR-MOPr hetero-dimerisation has not been studied per se yet, there is evidence demonstrating that OTR can form heterodimers with the dopamine D₂ receptor in the striatum (Romero-Fernandez et al., 2013), which also makes this suggestion rather appealing (also see below). Recent reports from the Nestler laboratory have revealed that chronic morphine treatment induces epigenetic changes leading to marked alterations in gene expression of a whole range of genes (Koo et al., 2015). Certainly this exciting new research does not preclude the possibility that the interaction between MOPr and OTR would involve epigenetic mechanisms, especially considering the evidence supporting epigenetic modification of the OTR by a range of factors (Kumsta et al., 2013). Nonetheless, our evidence indicates that in a situation where MOPr are desensitized following chronic

morphine administration/extinction (Sim et al., 1996), OTRs are profoundly upregulated in regions of high MOPr expression such as the amygdala and septum. To further examine the nature of OTR-MOPr interactions, we sought to investigate the effect of another opioid receptor manipulation, such as the MOPr knockout mouse, on the OTR system. In accordance to our morphine/withdrawal findings, in a model where MOPr are genetically deleted, upregulation of OTRs were identified in the same brain regions where we found upregulation of OTR following chronic morphine/withdrawal (i.e., septum and amygdala; also see Figure 1B above). This was concomitant with reduced levels of OT transcript in these mice (Becker et al., 2014), further supporting the presence of an MOP-OTR interaction. Thus, we can postulate that functional inactivation/desensitization of MOPr triggers an upregulation of OTR in high MOPr-expressing brain regions, such as the amygdala or alternatively that endogenous MOPr tone negatively modulates OTR binding.

Given the evidence demonstrating learning and memory deficits effect of oxytocin in animal models (Ferrier et al., 1980; Kovacs and Telegdy, 1982; Bruins et al., 1992; Kovacs and De Wied, 1994) and considering that the conditioned-place preference paradigm involves a contextual/spatial memory aspect (Tzschentke, 2007), we cannot completely preclude the possibility of CBT to have affected the associate memory related to the morphine-paired environment during reinstatement. However, this is unlikely, since we have previously assessed the exploratory activity of male C57BL/6J mice following a sub-chronic (4-day) administration of CBT (6.4 mg/kg, i.p.) in the CPP chambers and we showed that CBT did not affect contextual habituation with the pre-exposed environment (Zanos et al 2014). These data support that CBT does not affect retrieval of spatial memory, at least at the dose used.

The locomotor sensitization data presented in the manuscript support the validity of our morphine administration paradigm for the induction of opioid CPP; behavioral sensitization following repeated administration of morphine has been also described elsewhere (Volpicelli et al., 1999; Zarrindast et al., 2007). The significance of sensitization in drug addiction has been well-characterized with repeated use of addictive drugs and the subsequent behavioral sensitization leading to neuroadaptive changes (Robinson and Kolb, 1997; Robinson and Kolb, 1999), which might persist during withdrawal and may even be involved in the propensity of relapse. Although not assessed in the present study, there is evidence demonstrating that OT is involved in drug induced sensitization and tolerance (Kovacs et al., 1985; Kovacs and Telegdy, 1987; Kovacs et al., 1998) (Sarnyai et al., 1992c)

Taken together, we demonstrated that the oxytocin analogue CBT prevents priming-induced reinstatement of morphine *CPP*, possibly via modulation of the dopaminergic and noradrenergic neurotransmission in the striatum. The present study also highlights a differential regulatory effect of CBT on the striatal noradrenergic system during stress- vs priming-induced reinstatement of morphine *CPP*. Moreover, our data strongly suggest the existence of an OTR-MOPr interaction in regions associated with drug addiction, reward and emotions. Overall, this study supports the OT system as a potential target for the treatment of opioid addiction and prevention of relapse.

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Author contributors

PG, PZ, JAGC and AB designed the study; PG, PZ and JAGC performed the experiment; PG and PZ analysed the data; PG, PZ, JAGC, SH, IK, BLK, MLL and AB wrote the paper and provided critical revision. All authors contributed to and approved the final version of the paper.

Conflict of interest

The authors declare no conflict of interest.

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FIGURE LEGEND

Effects of carbetocin pre-treatment on priming- and stress-induced reinstatement of morphine CPP behaviour in mice; involvement of the dopaminergic, noradrenergic and MOPr systems. (A) Time spent in morphine-paired compartment for each phase of the conditioned-place preference paradigm; $**p < 0.01$; $***p < 0.001$. (B) Locomotor activity during morphine-conditioning (10 mg/kg) was measured daily. The average activity of the 5-minute bins of the total 45 minutes was calculated; $***p < 0.001$ vs Saline; $##p < 0.01$ vs Morphine Day 1. (C) Ambulatory activity was measured during priming-induced reinstatement of morphine CPP in 5-min bins for 20 minutes for both saline- and CBT- pre-treated mice. Male C57BL/6J mice were pre-treated with saline or CBT (6.4 mg/kg, i.p.) and they were either subjected to a forced-swim stress or received a priming injection of morphine to induce reinstatement. Striatal (D) DOPAC/DA and (E) MHPG/NA ratio was calculated following both priming- and stress-induced reinstatement of morphine CPP. (F) Plasma corticosterone levels were also measured in saline- and CBT- pre-injected animals. (G) Representative autoradiograms of 50pM [125 I]OVTA binding to OTR in coronal brain sections of MOPr knockout and wild-type mice. Autoradiograms of brain sections were taken at the level striatum (Bregma: 0.62mm; first row), septum (Bregma: 0.14mm; second row) and amygdala (Bregma: -1.82mm; third row). Binding levels are represented using a pseudocolour interpretation of black and white film images in fmol/mg of tissue equivalent. (H) Quantitative OTR binding in the brain of MOPr knockout and wild-type mice. $*p < 0.05$, $***p < 0.001$ vs wild-type. (I) Correlation analysis between percentage change in OTR binding in MOPr knockout mice and MOPr binding in wild-type mice. Data are the mean \pm SEM. *Abbreviations:* Acb, nucleus accumbens; Amy, amygdala; CBT, carbetocin; CgCx, cingulate cortex; CPu, caudate putamen; DA, dopamine; Hip, hippocampus; Hyp, hypothalamus; MOPr, μ -opioid receptor; NA, noradrenaline; NSB, non-specific binding; OTR, oxytocin receptor; Pir, piriform cortex; Th, thalamus; WT, wild-type.

