

# The Oxytocin Analogue Carbetocin Prevents Emotional Impairment and Stress-Induced Reinstatement of Opioid-Seeking in Morphine-Abstinent Mice

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The main challenge in treating opioid addicts is to maintain abstinence due to the affective consequences associated with withdrawal which may trigger relapse. Emerging evidence suggests a role of the neurohypophysial peptide oxytocin (OT) in the modulation of mood disorders as well as drug addiction. However, its involvement in the emotional consequences of drug abstinence remains unclear. We investigated the effect of 7-day opioid abstinence on the oxytocinergic system and assessed the effect of the OT analogue carbetocin (CBT) on the emotional consequences of opioid abstinence, as well as relapse. Male C57BL/6J mice were treated with a chronic escalating-dose morphine regimen (20–100 mg/kg/day, i.p.). Seven days withdrawal from this administration paradigm induced a decrease of hypothalamic OT levels and a concomitant increase of oxytocin receptor (OTR) binding in the lateral septum and amygdala. Although no physical withdrawal symptoms or alterations in the plasma corticosterone levels were observed after 7 days of abstinence, mice exhibited increased anxiety-like and depressive-like behaviors and impaired sociability. CBT (6.4 mg/kg, i.p.) attenuated the observed negative emotional consequences of opioid withdrawal. Furthermore, in the conditioned place preference paradigm with 10 mg/kg morphine conditioning, CBT (6.4 mg/kg, i.p.) was able to prevent the stress-induced reinstatement to morphine-seeking following extinction. Overall, our results suggest that alterations of the oxytocinergic system contribute to the mechanisms underlying anxiety, depression, and social deficits observed during opioid abstinence. This study also highlights the oxytocinergic system as a target for developing pharmacotherapy for the treatment of emotional impairment associated with abstinence and thereby prevention of relapse. *Neuropsychopharmacology* (2014) **39**, 855–865; doi:10.1038/npp.2013.285; published online 4 December 2013

**Keywords:** opioid; oxytocin; abstinence; relapse; depression; sociability

## INTRODUCTION

Opioid addiction is a chronic relapsing disorder characterized by the emergence of negative physical and emotional withdrawal symptoms, which serve as a motivational trigger to re-administer the drug and to relapse (Le Moal and Koob, 2007). The major challenge for opioid addicts who are recovering from their addiction is the maintenance of a drug-free state. Physical symptoms of withdrawal decrease after a short period of time, but symptoms associated with emotional distress and dysphoria such as anxiety, irritability, stress, depression, and anhedonia may persist for several months (Jaffe, 1990; Martin and Jasinski, 1969;

Nunes *et al*, 2004; Peles *et al*, 2007). Moreover, the adverse social consequences of opioid withdrawal in recovering addicts are well recognized and benefits of psychosocial support in maintaining abstinence have been reported (Veilleux *et al*, 2010).

Although some neurotransmitter systems (eg, serotonin and corticotropin-releasing factor, CRF) have been suggested to be involved (Goeldner *et al*, 2011; Koob and Kreek, 2007; Koob, 2008), the mechanisms underlying the emotional impairment associated with opioid abstinence remain largely unclear. Several lines of evidence suggest a role of the 'social' peptide oxytocin (OT) in drug addiction. OT-producing neurons located in the hypothalamus innervate brain regions associated with drug-seeking behavior as well as stress, mood, fear, and emotionality, such as the amygdala, septum, and the bed nucleus of the stria terminalis, where oxytocin receptors (OTRs) are expressed (Gimpl and Fahrenholz, 2001). OT is involved in the regulation of stress via its action on the hypothalamo-pituitary-adrenal (HPA) axis (Windle *et al*, 2004), and has been also implicated in the modulation of emotional and social behaviors (Neumann and Landgraf, 2012). In

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Received 15 August 2013; revised 27 September 2013; accepted 3 October 2013; accepted article preview online 15 October 2013

particular, OT promotes social bonding as well as social memory (Keverne and Curley, 2004) and exerts a potent anxiolytic, antiaggressive, and antidepressant effect in humans (Heinrichs and Domes, 2008; Liu *et al*, 2012), and in animal models (Neumann and Landgraf, 2012).

There is emerging evidence supporting the involvement of OT in the effects of a number of drugs abuse and in preventing relapse to drug-seeking (Broadbear *et al*, 2011; McGregor and Bowen, 2012; McGregor *et al*, 2008; Sarnyai, 2011). With respect to opioids, an inhibitory effect of OT has been demonstrated on morphine tolerance, physical symptoms of naloxone-precipitated morphine withdrawal (Kovacs *et al*, 1985; Kovacs *et al*, 1984), and on heroin self-administration (Kovacs and Van Ree, 1985) in rodents. In addition, marked alterations in OT peptide content and/or synthesis were reported in the forebrain, hippocampus, amygdala, and hypothalamus of rodents following acute and chronic opioid administration (Kovacs *et al*, 1987; You *et al*, 2000). With respect to relapse, OT was shown to facilitate extinction of methamphetamine-induced conditioned place preference (CPP) and to attenuate stress- (Qi *et al*, 2009) and priming- (Carson *et al*, 2010) induced reinstatement of methamphetamine-seeking behavior in rodents. Overall, although the contribution of OT in different stages of drug addiction is recognized, its specific role in the emotional impairment associated with abstinence, which may trigger relapse to drug-seeking, remains largely unknown.

Our aim was to assess whether OT alterations may underlie negative emotional states associated with opioid abstinence. Specifically, we hypothesize that 7-day withdrawal from chronic morphine administration causes alterations of the central OT-ergic system and that an OT analogue may attenuate the emotional consequences of 7-day opioid abstinence and prevent relapse. We used a mouse model of 7-day morphine abstinence to study the effects of withdrawal on OT peptide levels and OTR binding in the brain. The emergence of emotional impairment (ie, anxiety, depressive-like behaviors, and sociability deficits) was also assessed following this 7-day withdrawal period. We then examined the effect of the OT analogue carbetocin (CBT) on the negative emotional consequences of withdrawal, as well as the stress-induced reinstatement of morphine-seeking behavior.

## MATERIALS AND METHODS

### Animals and Paradigm for Morphine Administration and Withdrawal

Male C57BL/6J mice (7 weeks old, 20–25 g, Charles River Laboratories, Kingston, UK) were housed individually in a temperature-controlled environment with a 12:12-h light/dark cycle (lights on: 0600 hours). Food and water were available *ad libitum*. Mice were left to acclimatize in their new environment for 7 days before the experiments and were handled daily. All procedures received a favorable opinion by the University of Surrey Ethics Committee and were approved by the UK Home Office (Animals Act 1986). Mice were randomly assigned to two treatment groups: control saline-treated groups ( $n = 148$  in total) and chronic morphine-treated groups ( $n = 161$  in total). Mice were

injected (i.p.) either with saline (4 ml/kg) or morphine (Sigma-Aldrich, Poole, UK) with a chronic morphine escalating-dose administration paradigm (20 mg/kg on day 1, 40 mg/kg on days 2–3, 80 mg/kg on days 4–5, and 100 mg/kg on days 6–7), twice per day at 0900 and 1700 hours, as previously described (Goeldner *et al*, 2011; Muller and Unterwald, 2004; Zhou *et al*, 2006), with minor modifications (Supplementary Figure S1A). To induce withdrawal, mice were then left in their home cage for 7 consecutive days without receiving any injection, which was previously shown to induce characteristic enhancement of depressive-like behavior in rats (Anraku *et al*, 2001).

### Effect of Chronic Morphine Treatment and 7-day Abstinence on the Central Oxytocinergic System and Plasma Corticosterone Levels

Mice were killed by decapitation either 1 h after the final treatment injection for the chronic morphine and saline groups or after 7 days of withdrawal. Brains were frozen in isopentane solution ( $-20^{\circ}\text{C}$ ) and sectioned for analysis of autoradiographic OTR binding ( $n = 5$  per group) as previously described (Zanos *et al*, 2013). Alternatively, brain regions were dissected (ie, hypothalamus, septum, amygdala, and hippocampus;  $n = 6-7$  brains per group) for determining OT peptide levels. Peptide extraction was performed according to Szeto *et al* (2011), using a previously described protocol with minor modifications (Christensson-Nylander *et al*, 1985). Measurement of OT levels was performed on tissue extracts using an Enzyme Immunoassay (Enzo Life Sciences (UK) Ltd, Exeter, UK). Trunk blood was also collected for quantification of the plasma corticosterone levels ( $n = 11-12$  per group) using a commercially available radioimmunoassay kit, according to the manufacturer's instructions (MP Biochemical, New York, NY, USA).

### Effect of Morphine Administration and Abstinence on Physical Symptoms of Withdrawal in Mice

Basal locomotor activity, number of withdrawal jumps, fecal boli, body weight, food and water intake were recorded daily, at the same time, throughout the chronic morphine administration paradigm and/or at specific time points during the withdrawal period ( $n = 13-18$  mice per group; Supplementary Methods).

### Effect of Morphine Abstinence and CBT Administration on Anxiety-, Depressive-Like and Sociability Behaviors

A different cohort of mice underwent chronic morphine administration, as described above. Following a 7-day withdrawal period, mice were injected (i.p.) either with saline (4 ml/kg) or CBT (6.4 mg/kg), 15 min before measuring anxiety- and depressive-like behavior using the elevated plus-maze and forced-swim tests, or 5 min before the assessment of sociability and social novelty behavior using the Crawley's three-chambered social approach test (for details, see Supplementary Methods). Each behavioral test was performed on a separate group of animals ( $n = 7-10$  per group). The dose was chosen based on previous studies (Chavarras *et al*, 2010) and a pilot experiment assessing the effects of CBT (i.p.; vehicle  $n = 6$ ; 2 mg/kg,  $n = 3$ ; 6.4 mg/kg,

$n = 10$ , and 20 mg/kg,  $n = 3$ ) on depressive-like behavior in mice withdrawn for 7 days from chronic saline or morphine administration, using the forced-swim test (Supplementary Methods and Supplementary Figure S2). Although CBT did not induce any alterations of parameters characteristic of depressive-like behavior in the saline-withdrawn group, this compound dose-dependently reduced the depressive-like symptoms observed in the morphine-withdrawn mice. We selected the lowest dose able to reverse depressive-like behavior in morphine-abstinent mice, ie, 6.4 mg/kg. This dose did not have any significant effect on locomotor activity in naive mice ( $n = 6$  per group; data not shown). CBT was selected because of its half-life (85–100 min) compared with OT (3–5 min) and based on its selectivity to the OTR in the rat (Engstrom *et al*, 1998).

### Effect of CBT Administration on Stress-Induced Reinstatement of Morphine-Seeking Behavior

We used a CPP apparatus (Opto-Max Activity Meter v2.16, Columbus Instruments, OH, USA) as previously described (Bailey *et al*, 2010). The CPP reinstatement protocol was modified from Mantsch *et al* (2010). Briefly, it consisted of a habituation session, pre-conditioning test, four conditioning sessions (morning saline and 4 h later a 10 mg/kg morphine subcutaneous (s.c.) injections daily), a post-conditioning test, five extinction sessions (ie, morphine injection was replaced by a saline injection), a post-extinction test, and a reinstatement session, each carried out on consecutive days (Figure 5a). During the reinstatement session, mice were pretreated with either saline (4 ml/kg, i.p.;  $n = 14$  mice) or CBT (6.4 mg/kg, i.p.;  $n = 12$  mice) and after 5 min, they were exposed to a 6-min forced-swim session. Following the forced-swim stress, mice were towel-dried and placed in the CPP apparatus for 20 min. Time spent in each compartment was measured during the last 15 min of the session. Mice were killed 30 min after the reinstatement session and trunk blood was collected for quantification of plasma corticosterone levels.

To ascertain that CBT does not have any rewarding or aversive effects on its own when administered chronically, the effects of CBT (6.4 mg/kg, i.p.) were compared with the saline in a CPP paradigm, consisting of one habituation, one pre-conditioning, four conditioning (with saline injection in the morning and CBT administration 4 h later) phases, and one post-conditioning phase. Time spent in each compartment was assessed during the last 15 min of the 20-min post-conditioning session.

### Statistical Analyses

All the values were expressed as mean  $\pm$  SEM. For analyses of OTR binding, OT peptide and corticosterone levels, two-way ANOVA with factors 'treatment' (ie, saline or morphine), and '7-day withdrawal' were performed. The effects of CBT on the elevated plus-maze, forced-swim test, and sociability tests were compared by two-way ANOVA with factors 'treatment' (ie, saline or morphine) and 'CBT' (ie, CBT or saline). Differences in stress-induced reinstatement of morphine-seeking were analyzed using two-way

ANOVA with factor 'CPP phase' (pre-conditioning, post-conditioning, post-extinction, reinstatement) and 'experiment' (ie, CBT or saline). The effects of CBT on CPP and locomotor activity were assessed by repeated measures two-way ANOVA with factors 'CPP phase' and 'CBT' (ie, CBT or saline), and factors 'time' and 'CBT' (ie, CBT or saline), respectively. Differences in weight changes, food and water intake were assessed by two-way repeated measures ANOVA with factors 'treatment' (ie, saline or morphine) and 'days'. Basal locomotor activity was analyzed by repeated measures two-way ANOVA with factors 'treatment' (ie, saline and morphine) and 'days'. ANOVAs were followed by a Holm-Sidak *post hoc* test when significance was reached (ie,  $p < 0.05$ ). All relevant F-values are provided in Table 1. All statistical analyses were performed using SigmaPlot (Systat Software, London, UK).

## RESULTS

### Effect of Chronic Morphine Administration and 7-day Withdrawal on the Central Oxytocinergic System and Plasma Corticosterone Levels

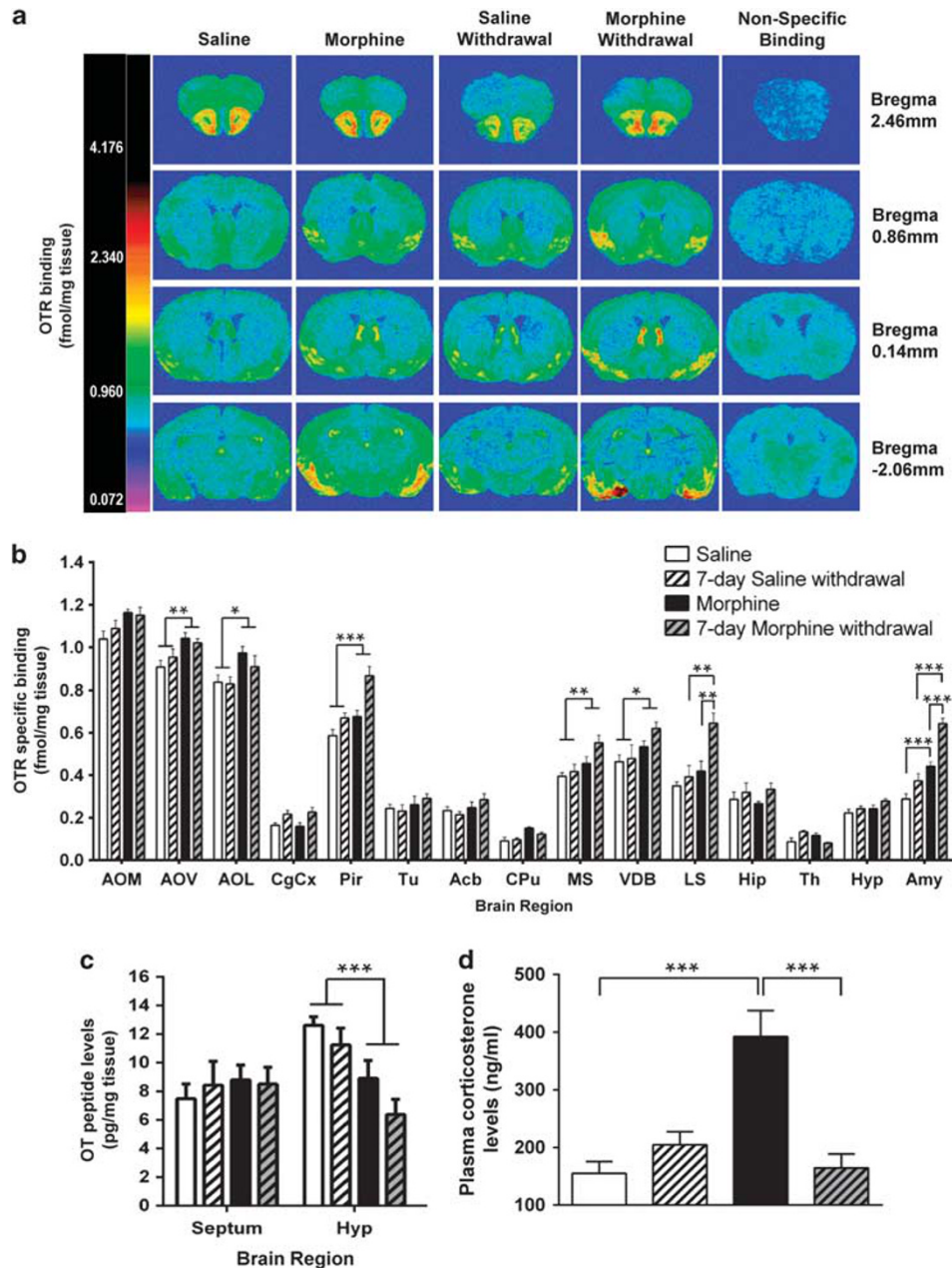
We first investigated the effects of chronic, escalating-dose morphine administration, and 7-day withdrawal on OTR binding in the brain (Figure 1a and b; Table 1). A significant effect of the 7-day morphine treatment was observed in several brain regions (Table 1). The morphine-treated groups showed an increase in OTR binding in the piriform cortex, medial septum, vertical limb of the diagonal band of Broca, as well as the anterior olfactory nucleus-ventral and -lateral, compared with the control saline-treated groups (Table 1). In the lateral septum and amygdala, a significant 'treatment'  $\times$  'withdrawal' interaction was identified (Table 1). Although chronic morphine administration did not alter the OTR binding in the lateral septum, we observed a significant increase in the 7-day morphine-withdrawn group compared with the saline-withdrawn and the chronic morphine-treated groups (Figure 1a and b). In the amygdala, the OTR binding was increased both following chronic morphine administration and after 7-day morphine withdrawal compared to control groups. Moreover, the OTR binding showed a significant 1.5-fold increase following the 7-day withdrawal period compared to the morphine administration group (Figure 1a and b).

We also assessed OT peptide levels in several brain regions. A significant 'treatment' effect (Figure 1c; Table 1) was observed in the hypothalamus, with a significant decrease in OT levels in the morphine-treated groups compared with the control groups, whereas no changes were observed in the septum. OT content in the hippocampus and amygdala was below the detection level (data not shown).

To assess the effect of chronic morphine treatment and withdrawal on HPA axis activity, we measured plasma corticosterone levels at the end of the chronic administration paradigm and after a 7-day withdrawal period. Chronic morphine treatment increased plasma corticosterone levels, whereas levels were comparable to the control group following a 7-day morphine withdrawal period (Figure 1d; Table 1).

**Table 1** Relevant Effects for Biochemical and Behavioral Data (two-way ANOVA)

	Treatment effect	Interaction effect
<i>Overall effects for Figure 1</i>		
OTR autoradiography	Factor 'treatment'	Factor 'treatment' × '7-day withdrawal'
AOM	$F_{[1,16]} = 0.323; p = 0.32$	$F_{[1,16]} = 0.83; p = 0.38$
AOV	$F_{[1,16]} = 11.40; p < 0.01$	$F_{[1,16]} = 1.20; p = 0.29$
AOL	$F_{[1,16]} = 8.14; p < 0.05$	$F_{[1,16]} = 0.49; p = 0.49$
CgCx	$F_{[1,16]} = 0.02; p = 0.89$	$F_{[1,16]} = 0.17; p = 0.69$
Pir	$F_{[1,16]} = 19.88; p < 0.001$	$F_{[1,16]} = 2.80; p = 0.11$
Tu	$F_{[1,16]} = 1.88; p = 0.19$	$F_{[1,16]} = 0.56; p = 0.46$
Acb	$F_{[1,16]} = 3.34; p = 0.09$	$F_{[1,16]} = 1.55; p = 0.23$
CPu	$F_{[1,16]} = 0.94; p = 0.35$	$F_{[1,16]} = 0.21; p = 0.65$
MS	$F_{[1,16]} = 10.06; p < 0.01$	$F_{[1,16]} = 1.48; p = 0.24$
VDB	$F_{[1,16]} = 6.40; p < 0.05$	$F_{[1,16]} = 0.76; p = 0.40$
LS	$F_{[1,16]} = 13.72; p < 0.01$	$F_{[1,16]} = 4.46; p = 0.05$
Hip	$F_{[1,16]} = 0.01; p = 0.93$	$F_{[1,16]} = 0.31; p = 0.59$
Th	$F_{[1,16]} = 0.04; p = 0.84$	$F_{[1,16]} = 3.10; p = 0.10$
Amy	$F_{[1,16]} = 64.55; p < 0.001$	$F_{[1,16]} = 4.98; p < 0.05$
OT peptide levels	Factor 'treatment'	Factor 'treatment' × '7-day withdrawal'
Septum	$F_{[1,22]} = 0.32; p = 0.58$	$F_{[1,22]} = 0.25; p = 0.63$
Hyp	$F_{[1,22]} = 15.81; p < 0.001$	$F_{[1,22]} = 0.29; p = 0.60$
Corticosterone	$F_{[1,43]} = 10.81; p < 0.01$	$F_{[1,43]} = 21.34; p < 0.001$
<i>Overall effects for Figure 2</i>		
Anxiety-like behavior	Factor 'treatment'	Factor 'treatment' × 'CBT'
Open-arm time	$F_{[1,29]} = 6.80; p < 0.05$	$F_{[1,29]} = 5.67; p < 0.05$
Open-arm entries (%)	$F_{[1,29]} = 0.78; p = 0.39$	$F_{[1,29]} = 2.36; p = 0.14$
<i>Overall effects for Figure 3</i>		
Depressive-like behavior	Factor 'treatment'	Factor 'treatment' × 'CBT'
Immobility time	$F_{[1,28]} = 24.67; p < 0.001$	$F_{[1,28]} = 19.11; p < 0.001$
Latency to first immobility	$F_{[1,28]} = 17.36; p < 0.001$	$F_{[1,28]} = 5.37; p < 0.05$
Faecal boli	$F_{[1,28]} = 17.30; p < 0.001$	$F_{[1,28]} = 8.66; p < 0.01$
<i>Overall effects for Figure 4</i>		
Social approach behavior	Factor 'treatment'	Factor 'treatment' × 'CBT'
Sociability	$F_{[1,25]} = 0.003; p = 0.95$	$F_{[1,25]} = 10.19; p < 0.01$
Preference for social novelty	$F_{[1,25]} = 11.72; p < 0.01$	$F_{[1,25]} = 4.04; p = 0.06$
<i>Overall effects for Figure 5</i>		
Conditioned place preference	Factor 'experiment'	Factor 'experiment' × 'CPP phase'
Morphine-induced reinstatement	$F_{[1,96]} = 10.52; p < 0.01$	$F_{[3,96]} = 3.26; p < 0.05$
	Factor 'CBT'	Factor 'CBT' × 'CPP phase'
CBT-induced CPP	$F_{[1,10]} = 0.05; p = 0.83$	$F_{[1,10]} < 0.001; p = 1.00$
<i>Overall effects for Supplementary Figure 1</i>		
Physical dependence	Factor 'treatment'	Factor 'treatment' × 'days'
Weight	$F_{[1,34]} = 17.17; p = 0.22$	$F_{[1,4,476]} = 23.70; p < 0.001$
Food consumption	$F_{[1,34]} = 2.32; p = 0.14$	$F_{[1,4,466]} = 107.04; p < 0.001$
Water consumption	$F_{[1,34]} < 0.001; p = 9.76$	$F_{[1,4,446]} = 23.85; p < 0.001$
<i>Overall effects for Supplementary Figure 2</i>		
Depressive-like behaviour	Factor 'treatment'	Factor 'treatment' × 'CBT'
Immobility time	$F_{[1,36]} = 15.63; p < 0.001$	$F_{[3,36]} = 4.88; p < 0.01$
Latency to first immobility	$F_{[1,36]} = 25.58; p < 0.001$	$F_{[3,36]} = 3.86; p < 0.05$
Faecal boli	$F_{[1,36]} = 9.38; p < 0.01$	$F_{[1,28]} = 3.69; p < 0.01$
<i>Overall effects for Supplementary Figure 4</i>		
Effect of withdrawal on basal locomotion	Factor 'treatment'	Factor 'treatment' × 'days'
Horizontal activity	$F_{[1,16]} = 27.77; p < 0.001$	$F_{[7,112]} = 5.89; p < 0.001$
Vertical activity	$F_{[1,16]} = 0.40; p = 0.39$	$F_{[7,112]} = 0.39; p = 0.91$

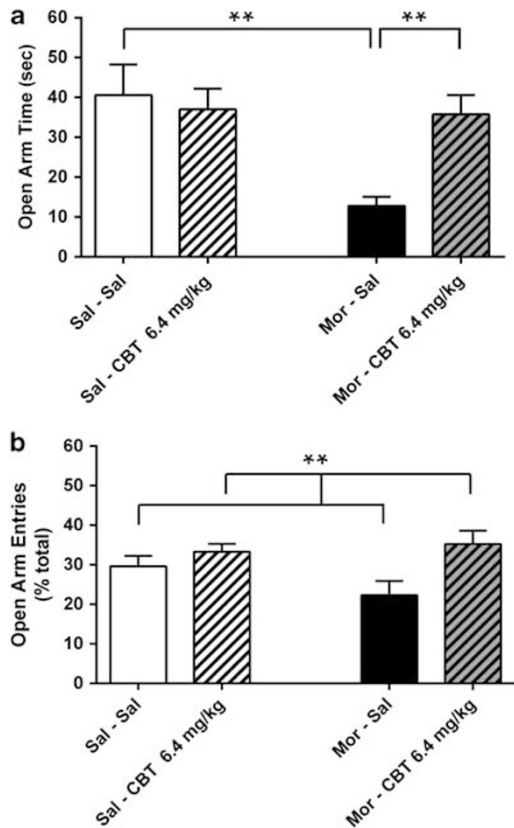


**Figure 1** Oxytocin receptor (OTR) binding, oxytocin (OT) peptide levels in the brain and plasma corticosterone levels following 7-day morphine abstinence. Male C57BL/6J mice were treated for 7 days with either saline or chronic morphine followed by a withdrawal period of 7 days. (a) Representative autoradiograms of 50 pM [<sup>125</sup>I]-ornithine vasotocin analogue binding to OTRs in coronal brain sections at the level of the olfactory nuclei (row 1), striatum (row 2), septum (row 3), and thalamus (row 4). Binding levels are represented using a pseudo-color interpretation of black and white film images in fmol/mg of tissue equivalent. (b) Quantitative OTR binding levels in the brain regions where OTRs are expressed. (c) OT peptide levels in the septum and hypothalamus. (d) Corticosterone levels in the plasma. Data are expressed as mean ± SEM (*n* = 5–7 per group). \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001 (two-way ANOVA followed by the Holm-Sidak *post hoc* test when appropriate). Acb, nucleus accumbens; Amy, amygdala; AOL, anterior olfactory nucleus-lateral; AOM, anterior olfactory nucleus-medial; AOV, anterior olfactory nucleus-ventral; CgCx, cingulate cortex; CPu, caudate putamen; Hip, hippocampus; Hyp, hypothalamus; LS, lateral septum; MS, medial septum; Pir, piriform cortex; PV Th, paraventricular nucleus of thalamus; Th, thalamus; Tu, olfactory tubercle; and VDB, vertical limb of diagonal band of Broca.

### Effect of Chronic Morphine Treatment and Subsequent Abstinence on Physical and Behavioral Withdrawal Symptoms

Acute, 1-day withdrawal from chronic morphine treatment induced withdrawal jumping ( $40 \pm 13$  vs  $0 \pm 0$  jumps; *p* < 0.001; *n* = 13–18 per group) and increased fecal boli production ( $12 \pm 1$  vs  $3 \pm 1$ ; *p* < 0.001; *n* = 13–18 per group),

compared with the saline-control group. In contrast, no significant differences between the morphine- and saline-treated groups were observed in either jumping behavior ( $0 \pm 0$  vs  $0 \pm 0$  jumps; *p* > 0.05) or defecation ( $4 \pm 1$  vs  $4 \pm 1$ ; *p* > 0.05) following 7-day withdrawal. Chronic morphine administration induced a significant decrease in food and water consumption, associated with a reduction in body mass (Supplementary Figure 1S; Table 1). The drug



**Figure 2** Anxiety-like behavior induced by 7-day morphine withdrawal is reversed by the oxytocin analogue carbetocin (CBT). (a) Time spent in the open arms and (b) percentage of entries in the open arms of the elevated plus-maze. Seven-day withdrawal from chronic morphine treatment induced an increase in anxiety-like behavior, which was reversed by CBT (6.4 mg/kg, i.p.). Data are expressed as mean  $\pm$  SEM ( $n = 6-10$  per group).  $^{***}p < 0.01$  (two-way ANOVA followed by the Holm-Sidak *post hoc* test when significance was reached); Mor, morphine; Sal, saline.

withdrawal period was characterized initially by an increase in body mass as well as food and water intake. Water consumption and body weight normalized to levels similar to saline-treated animals at the end of the 7-day withdrawal period (Supplementary Figure S1). Moreover, morphine treatment reduced basal horizontal activity from day 3 to 7 of the administration paradigm (Supplementary Figure S3A; Table 1). This reduction persisted following the 7-day withdrawal period (Supplementary Figure S3A). No alteration of basal vertical activity was observed between saline- and morphine-treated mice (Supplementary Figure S3B).

### Effect of CBT, an OT Analogue, on Anxiety-Like, Depressive-Like and Sociability Behaviors Associated with 7-day Morphine Withdrawal

We assessed anxiety-like behavior following the 7-day withdrawal from morphine using the elevated plus-maze. Seven-day withdrawal from morphine decreased the time spent in the open arms and the percentage of open-arm entries compared with the control group (Figure 2a and b), indicating anxiety-like behavior. Administration of CBT before the behavioral test increased the time spent in the open arms in morphine-withdrawn mice to levels similar to the control group (Figure 2a). In addition, CBT increased

the number of entries in the open arms (Figure 2b). CBT treatment did not induce any changes in the time spent and number of entries in the open arms for the control group (Figure 2a and b).

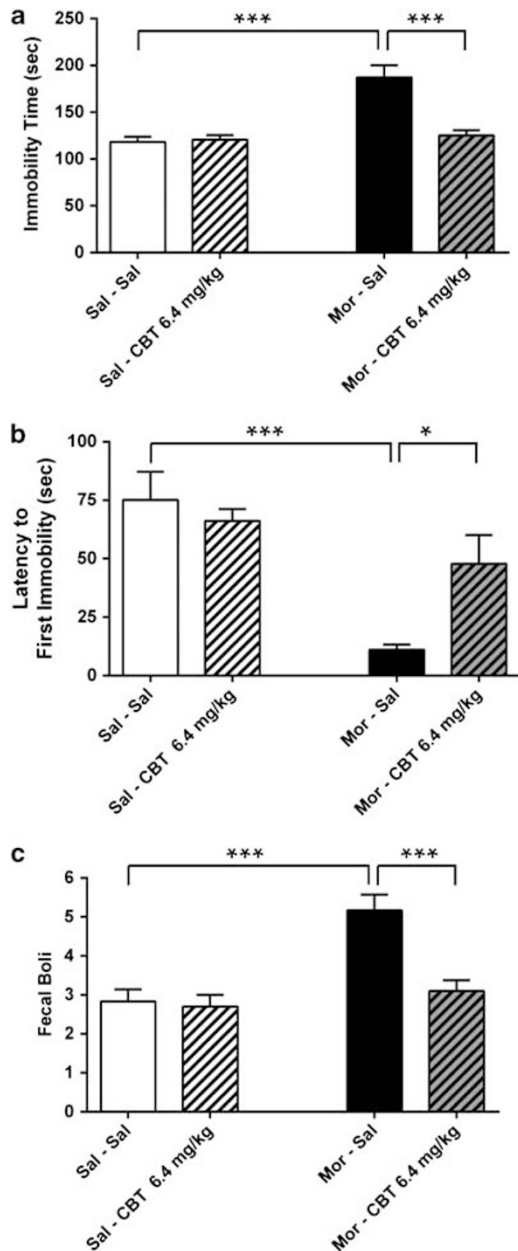
The forced-swim test was used to assess depressive-like behavior following 7-day withdrawal from morphine. Withdrawal increased immobility time (Figure 3a) and reduced the latency to the first episode of immobility (Figure 3b). In addition, an increase in fecal boli production was observed (Figure 3c), also indicative of depressive-like behavior (Craft *et al*, 2010). CBT reduced the time spent immobile during the forced-swim test, as well as the production of fecal boli, and increased the latency to the first immobility episode, in the morphine-withdrawn group to levels similar to the control group (Figure 3a-c). Saline-withdrawn mice administered with CBT showed no significant changes in immobility time, latency to first immobility, and fecal boli production compared with controls (Figure 3a-c).

We also assessed mouse sociability using the three-chambered social approach test. Sociability was measured by the time the subject mouse spent in the 'social chamber' containing a novel conspecific compared with the 'empty chamber'. We found a reduction of sociability behavior in morphine-withdrawn mice compared with the saline-withdrawn group (Figure 4a). Administration of CBT before the behavioral test increased the time spent by morphine-withdrawn mice in the chamber containing the novel mouse compared with the empty chamber (Figure 4a), indicating a restoration of sociability behavior. CBT had no effect on sociability in control mice undergoing withdrawal from saline (Figure 4a).

Furthermore, we assessed the preference of the subject mouse for either the chamber with the former conspecific ('familiar chamber') or the chamber with a second 'novel' mouse ('novel chamber') as indicative of preference for social novelty. Two-way ANOVA revealed an effect of morphine treatment (Figure 4b), whereas the interaction 'treatment' (morphine *vs* saline) and 'CBT' was close to significance ( $p = 0.06$ ).

### Effect of CBT on Stress-Induced Reinstatement of Morphine-Seeking Behavior

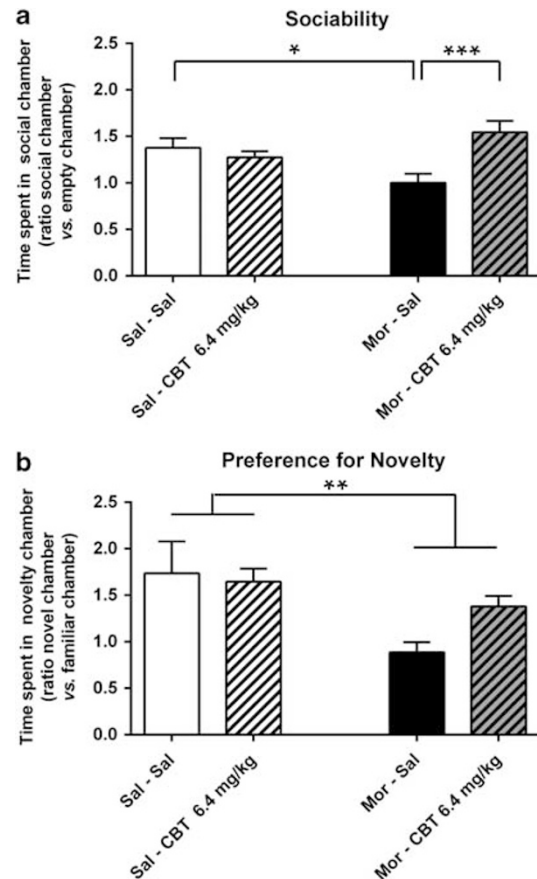
The CPP reinstatement paradigm is commonly used to assess relapse to drug-seeking (Cordery *et al*, 2012; Redila and Chavkin, 2008). Morphine administration induced a place preference in the CPP paradigm with an increase in the time spent in the drug-paired compartment in the post-conditioning phase compared with the pre-conditioning phase. The 5-day extinction period led to a significant decrease in the time spent in the drug-paired compartment (post-extinction phase *vs* post-conditioning phase) (Figure 5b; Table 1). A forced-swim stress induced reinstatement of the CPP in mice treated with saline, as shown by the increased time spent in the drug-paired compartment compared with the post-extinction phase (Figure 5b). In contrast, mice pre-injected with CBT did not show this increase in preference for the morphine-paired compartment during the reinstatement phase, compared with the saline pretreated group (Figure 5b). Furthermore, no changes in plasma corticosterone levels were observed in



**Figure 3** Depressive-like behavior induced by 7-day morphine withdrawal is prevented by the oxytocin analogue carbetocin (CBT). (a) Immobility time, (b) latency to the first episode of immobility, and (c) number of fecal boli were measured during the 5-min forced-swim test. In 7-day morphine-withdrawn animals, immobility time and production of fecal boli were increased, whereas the latency to the first episode of immobility was decreased in the forced-swim test, indicative of enhanced depressive-like behaviors. Administration of CBT (6.4 mg/kg, i.p.) reversed these effects of 7-day morphine withdrawal to levels comparable to the saline-control group. Data are expressed as mean  $\pm$  SEM ( $n = 6-10$  per group). \* $p < 0.05$ ; \*\*\* $p < 0.001$  (two-way ANOVA followed by the Holm-Sidak *post hoc* test when significance was reached). Sal, saline; Mor, morphine.

the CBT-treated group ( $244.3 \pm 30.0$  ng/ml;  $n = 14$ ) compared with the saline-control group ( $252.6 \pm 16.7$  ng/ml;  $n = 12$ ) following stress-induced reinstatement.

A possible mechanism by which CBT modulates the emotional impairment associated with 7-day morphine withdrawal and the stress-induced reinstatement of

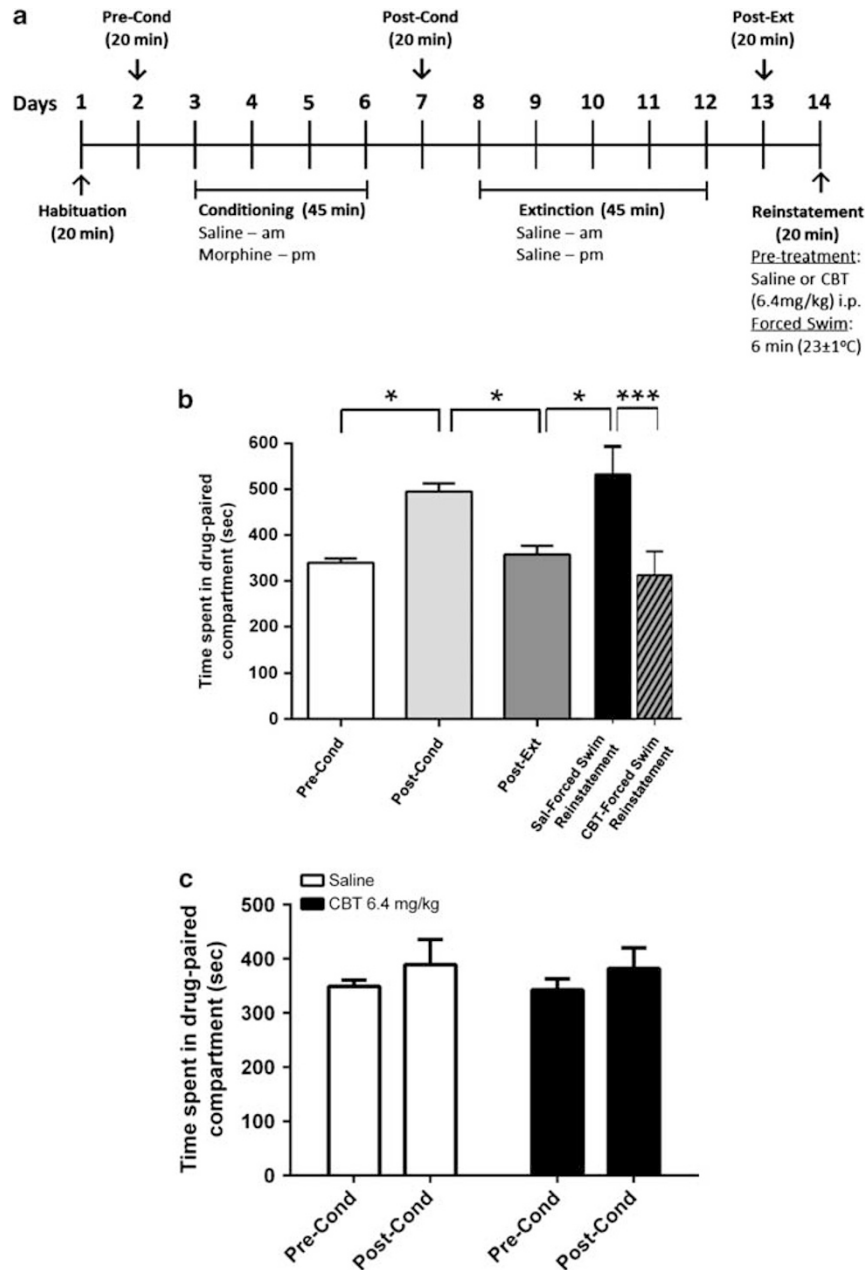


**Figure 4** Carbetocin (CBT) restores sociability behavior following 7-day morphine withdrawal using the three-chambered social approach test. (a) Sociability defined by the ratio of the time spent in the chamber containing the novel conspecific ('social chamber') vs the empty chamber during the 10-min 'sociability' phase. Mice were then tested for (b) preference for social novelty as defined by the ratio of the time spent in the chamber containing a novel unfamiliar mouse ('novelty chamber') vs the chamber containing the former conspecific ('familiar chamber') during the 10-min 'preference for social novelty' phase. Although mice withdrawn from chronic morphine treatment displayed a reduction in sociability compared with the saline-withdrawn group, CBT (6.4 mg/kg, i.p.) restored sociability. Preference for social novelty was decreased in the chronic morphine-treated groups. Data are expressed as mean  $\pm$  SEM ( $n = 6-10$  per group). \* $p < 0.05$ , \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  (two-way ANOVA followed by the Holm-Sidak *post hoc* test when significance was reached); Mor, morphine; Sal, saline.

morphine CPP is by inducing reward. Thus, we assessed the rewarding or aversive properties of CBT (6.4 mg/kg; i.p.) using the CPP paradigm. CBT did not induce any CPP or aversion (Figure 5c; Table 1) compared with the saline-control group.

## DISCUSSION

This study demonstrated the protective effect of the OT analogue, CBT, on anxiety-, depressive-like behaviors and sociability impairment associated with abstinence from opioid administration, as well as on drug relapse, in a mouse model. In addition, we observed alterations of the OT-ergic system in the brain, which are likely to contribute to the emotional impairment observed following a 7-day



**Figure 5** Effect of carbetocin (CBT) on stress-induced reinstatement of morphine-seeking behavior and conditioned place preference (CPP). (a) Experimental protocol with the different phases of the CPP paradigm. Twenty-six mice underwent the following protocol: habituation phase; pre-conditioning (Pre-Cond) phase: assessment of spontaneous place preference; conditioning phase: 4 consecutive days with saline injection (10 ml/kg, s.c., first half of light period) in the preferred compartment and administration of morphine (10 mg/kg, s.c. 4 h after the saline injection) in the least-preferred compartment; post-conditioning (Post-Cond) session: assessment of conditioning with no injection; extinction phase: 5 consecutive days with saline injection (10 ml/kg, s.c. first half of light period) and then, administration of saline (10 ml/kg, s.c. 4 h after the first daily injection) in the drug-paired compartment; post-extinction (Post-Ext) test: assessment of extinguished behavior—no injection; for the reinstatement test, mice were then subdivided into two experimental groups to receive either saline (4 ml/kg, i.p.;  $n = 14$ ) or CBT (6.4 mg/kg, i.p.;  $n = 12$ ), followed by exposure to a forced-swim stress 5 min later ( $23 \pm 1^\circ\text{C}$ ; 6 min). Mice were then tested for reinstatement of morphine-seeking behavior during a 20-min session. (b) Time spent in the morphine-paired compartment for each phase of the CPP paradigm (analysis of the last 15 min of the 20-min session). Data are expressed as mean  $\pm$  SEM ( $n = 12$ –14 mice per group); \* $p < 0.05$ ; \*\*\* $p < 0.001$ . (c) Time spent in the CBT- or saline-paired compartment (analysis of the last 15 min of the 20-min session) during the pre-conditioning (Pre-Cond) and post-conditioning (Post-Cond) phases of the CPP paradigm. Data are expressed as mean  $\pm$  SEM ( $n = 6$  mice per group).

withdrawal period from morphine. Altogether these findings highlight a key role of the OT-ergic system in opioid addiction and relapse, suggesting this system as a novel target for the treatment of opioid addiction, and in particular for preventing relapse.

We first established and characterized a mouse model of 7-day opioid abstinence that mimics the physical and emotional symptoms usually displayed by human opioid-withdrawn addicts (Jaffe, 1990; Martin and Jasinski, 1969). Although acute physical withdrawal symptoms (ie, with-



drawing jumping, defecation, as well as changes in body mass) had disappeared after 7 days of morphine abstinence, anxiety-, depressive-like behaviors were increased and sociability reduced, highlighting the translational value of this model and the direct relationship between opioid abstinence and the emergence of negative emotional state. Consistent with the work of Zhou *et al* (2006), although chronic morphine treatment enhanced the HPA axis activity, corticosterone levels returned to levels similar to those of controls after the 7-day withdrawal period, demonstrating a normalization of the HPA axis. We also demonstrated that chronic morphine administration and/or withdrawal was associated with alterations of the central OT-ergic system. We not only observed reduced levels of OT in the hypothalamus, the main site of OT synthesis in the brain, but also an increase in OTR binding in several regions where OT-producing neurons project (ie, olfactory nuclei, piriform cortex, septum, and amygdala) (Gimpl and Fahrenholz, 2001), suggestive of compensatory mechanisms to the reduced OT-ergic tone. Similar neuroadaptive changes in the oxytocinergic system were previously observed following an acute morphine tolerance paradigm (Sarnyai *et al*, 1988). Using a specific OTR antagonist, Sarnyai *et al* (1988) showed a key role of OTRs located in the posterior olfactory nucleus and central nucleus of the amygdala in acute tolerance to morphine. Our findings are also consistent with decreased OT contents in several brain regions following chronic morphine administration in rodents (Kovacs *et al*, 1987; You *et al*, 2000). In addition, our data demonstrate that these OT-ergic alterations not only persisted following the 7-day withdrawal period but was further heightened with respect to OTR binding in the septum and amygdala, regions playing a key role in the regulation of stress and emotions (Phelps and LeDoux, 2005; Singewald *et al*, 2011). This is in line with a previous study in a rodent model of social deficit in schizophrenia showing that, although hypothalamic OT mRNA levels were significantly decreased, OTR binding was increased in the central nucleus of the amygdala (Lee *et al*, 2005). The fact that both the septum and amygdala have been implicated in the stress-regulating and social-enhancing properties of OT (Domes *et al*, 2007; Lukas *et al* 2013) is further suggestive of the OT-ergic system involvement in the negative emotional consequences of opioid withdrawal.

In this translational model, we demonstrated that CBT reversed not only anxiety- and depressive-like behaviors but also restored sociability in morphine-withdrawn mice to levels similar to the control saline-withdrawn groups. Importantly, CBT did not affect the anxiety level, depressive-like state, or sociability in control saline-withdrawn mice. In addition, locomotor activity showed no difference after CBT administration in naive mice compared with saline-treated mice (data not shown). These findings strongly support the specificity of the anxiolytic, antidepressant, and pro-social effects induced by a relatively low dose of CBT to counteract the negative consequences of withdrawal. Although CBT shows a high affinity for both OTR and vasopressin V1 receptors in rat myometrial homogenates (Engstrom *et al*, 1998), its effects are likely mediated by OTR, as a recent study demonstrated antidepressant-like effects of the same dose via specific activation of the central OTR and not vasopressin V1A

receptors (Chavarras *et al*, 2010). Addressing emotional impairment associated with opioid abstinence is essential given the high comorbidity with depression along with the ambiguous efficacy of classic antidepressant treatment in this patient population (Nunes *et al*, 2004). In addition, the pro-social effect of CBT in morphine-withdrawn mice is consistent with previous reports demonstrating the key role of OT in the positive modulation of social behaviors (Lukas *et al*, 2011; Neumann and Landgraf, 2012). This is of particular interest as it represents the first attempt to modulate sociability deficits associated with opioid addiction. Although understudied, prolonged use of addictive substances often results in disintegration of the social lives due to social isolation and poor decision making at the expense of compulsive preoccupation with the drug and its related cues (Dawe *et al*, 2009; Volkow *et al*, 2011). Considering the therapeutic success of social support programs as well as the benefits of social rehabilitation and reintegration in keeping former addicts in a drug-free state (McGregor and Bowen, 2012), the use of OT-ergic compounds in combination with psychosocial support may prove to be effective in preventing relapse by promoting positive social behavior. The pro-social effect of OT-ergic pharmacotherapy is being assessed in clinical trials for the treatment of social deficit in autistic spectrum disorders and schizophrenia (Carter, 2007; Heinrichs and Gaab, 2007). Interestingly, a decrease in the hypothalamic OT mRNA expression, as well as an increase in OTR binding in the amygdala, was observed in a mouse model of schizophrenia displaying social behavior deficits (Lee *et al*, 2005). Overall, these findings further support a causal relationship between the alterations observed within the OT peptidergic system and impaired social behavior.

In addition, CBT prevented stress-induced reinstatement of morphine CPP. Our findings are consistent with Qi *et al* (2009) who showed a beneficial effect of OT on attenuating reinstatement of methamphetamine CPP induced by restraint stress. This modulatory effect of CBT may involve regulation of drug reward as shown by the ability of OT to inhibit both methamphetamine- and cocaine-induced increase in dopamine utilization in the striatum (Kovacs *et al*, 1990; Qi *et al*, 2008). However, in our study, CBT, at 6.4 mg/kg (i.p.), was neither rewarding nor aversive in the CPP paradigm. An alternative mechanism underlying the effects of CBT may involve modulation of the HPA axis activity (Windle *et al*, 2004). However, in our study, CBT did not induce any differences in plasma corticosterone levels following stress-induced reinstatement. Although these results suggest that the peripheral part of the HPA axis is not altered by CBT, its potential action via extra-hypothalamic brain stress systems (eg, CRF) cannot be ruled out. In particular, the CRF system in the amygdala has been shown to have a key role in the aversive/emotional consequences of drug withdrawal (Erb, 2010; Heinrichs *et al*, 1995; Koob, 2009; Stinus *et al*, 2005) and stress-induced reinstatement of drug-seeking (Erb *et al*, 2001; Erb *et al*, 1998; Koob, 2009; Wang *et al*, 2006).

Taken together, our findings showed that the OT analogue CBT can prevent the negative emotional impairment, encompassing anxiety-, depressive-like behavior, reduction of sociability, as well as reinstatement of drug-seeking in a translational mouse model of opioid withdrawal. Future studies using opiate self-administration paradigms could be used to

validate further these findings. Overall, our results highlight the alterations of the OT-ergic system as one of the underlying mechanisms of emotional impairment associated with long-term abstinence in opioid addicts. Thus, OT-ergic pharmacotherapy might constitute a novel potential target to assist opioid addicts with relapse prevention by treating mood disorders and social impairment associated with drug withdrawal. The effects of chronic OT-ergic pharmacotherapy on the alleviation of negative emotional symptoms remain to be characterized to validate its progress toward clinical development.

## FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We thank Prof Ingrid Nylander for valuable advice on OT peptide measurements, Dr Sheryl Moy and Ms Pamela Farshim for advice given on the behavioral tests, as well as Dr van der Veen and Helen Keyworth for advice on statistical analyses. This study was supported by Spyroula and Soteris Zanos, Lilia and Charalambos Georgiou and by a Royal Society grant (RG120556; P.I. Alexis Bailey). We declare that over the past 3 years PZ has received financial support from Mr and Mrs Zanos and PG has received financial support from Mr and Mrs Georgiou. The sponsors had no involvement in the design of the study and in the collection, analyses, and interpretation of the data, nor in the writing of the report and the decision to submit this article for publication. PZ, PG, SRW, SMH, IK, RWS, AB wrote the paper; PZ, PG performed the experiments; PZ, PG, RWS analysed the data; AB, PZ, PG designed the experiments; AB conceived the idea for the experiments and was PI for the study.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)