Received Date : 03-May-2016
Revised Date : 15-Jul-2016
Accepted Date : 18-Jul-2016
Article type : Short Communication

Cocaine abstinence induces emotional impairment and brain region-specific upregulation of the oxytocin receptor binding.

Authors: Polymnia Georgiou[1,2] (Ph.D.), Panos Zanos[1,2] (Ph.D.), Susanna Hourani[1] (Ph.D.), Ian Kitchen[1] (Ph.D.), Alexis Bailey[1,3,*] (Ph.D.)

[1] School of Biosciences and Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, GU2 7XH, Surrey, UK

[2] Department of Psychiatry, University of Maryland School of Medicine, Baltimore, MD, 21209, USA

[3] Institute of Medical and Biomedical Education, St George's University of London, London SW17 0R

Correspondence:

*Alexis Bailey
Institute of Medical and Biomedical Education
St George's University of London
London SW17 0RE
UK
Tel: +44 (0) 20 8672 9944
Email: abailey@sgul.ac.uk

Running title: Cocaine abstinence alters the oxytocin system

Keywords: cocaine withdrawal, anxiety, anhedonia, corticosterone, mice

Abstract

The key problem in treating cocaine addiction is the maintenance of a drug-free state since negative emotional symptoms during abstinence often trigger relapse. The mechanisms underpinning the emotional dysregulation during abstinence are currently not well-understood. There is evidence suggesting a role of the neuropeptide oxytocin in the modulation of drug addiction processes. However, its involvement during long-term abstinence from cocaine use remains unclear. In this study, we aimed to behaviourally characterize a mouse model of long-term cocaine withdrawal and assess the effect of chronic cocaine administration and long-term cocaine abstinence on the central oxytocinergic system and the Hypothalamic-Pituitary-Adrenal-axis. Fourteen-day escalating-dose cocaine administration (3 x 15-30 mg/kg/day) and 14-day withdrawal increased plasma corticosterone levels and oxytocin receptor (OTR) binding in piriform cortex, lateral septum and amygdala. A specific cocaine withdrawal-induced increase in OTR binding was observed in the medial septum. These biochemical alterations occurred concomitantly with the emergence of memory impairment, contextual psychomotor sensitization and an anhedonic and anxiogenic phenotype during withdrawal. Our study established a clear relationship between cocaine abstinence and emotional impairment in a novel translationally-relevant model of cocaine withdrawal and demonstrated for the first time brain region-specific neuroadaptations of the oxytocin system, which may contribute to abstinence-induced negative emotional state.

Introduction

Cocaine addiction is a chronic relapsing disorder characterized by negative emotional symptoms during long-term abstinence. Former cocaine addicts experience emotional impairment, which can trigger relapse to drug use, and there is currently no effective pharmacotherapy for the treatment of cocaine addiction (Soyka & Mutschler, 2016).

While the acute and chronic consequences of cocaine use have been broadly investigated, the mechanisms underlying abstinence-induced emotional impairment remain to be elucidated. There is evidence to suggest that the oxytocin (OT) system, which is implicated in emotional regulation due to

its anxiolytic, prosocial and antidepressant properties (see Neumann & Landgraf, 2012; Neumann & Slattery, 2016) may have a role in cocaine addiction/withdrawal. Indeed, central and peripheral OT administration decreases cocaine-induced hyper-locomotion, stereotypies and self-administration in rodents (see Sarnyai & Kovacs, 1994; McGregor & Bowen, 2012; Zhou *et al.*, 2015). Moreover, i.c.v. administration of OT attenuates cue-induced reinstatement of cocaine-seeking (Morales-Rivera *et al.*, 2014). Chronic cocaine administration decreases plasma OT levels and OT immunoreactivity within the hypothalamus and hippocampus (Sarnyai *et al.*, 1992; Johns *et al.*, 1997). Moreover, increased OTR binding was observed in the amygdala following cocaine reinstatement in mice (Georgiou *et al.*, 2015a). With regards to OT's role during drug withdrawal, we have previously demonstrated a dysregulation of the OT system following opioid abstinence and that the oxytocin analogue carbetocin prevents opioid abstinence-induced emotional impairment in mice, suggesting a key role of this system in emotional regulation during abstinence (Zanos *et al.*, 2014a); however the role of oxytocin during cocaine abstinence remains unclear.

Here, we behaviourally characterized a mouse model of prolonged cocaine abstinence and assessed its effect on the OTR system. We also examined the effect of chronic cocaine administration and withdrawal on the hypothalamic-pituitary-adrenal (HPA) axis activity, since cocaine-abuser pregnant women showed lower plasma OT levels and a concomitant blunted cortisol response (Light *et al.*, 2004). Our findings clearly demonstrate the emergence of an emotional impairment during prolonged cocaine abstinence and brain region-specific neuroadaptations of the oxytocin system, which may contribute to these affective behaviors. Given the impact of the abstinence-induced negative emotional state towards triggering relapse and the lack of effective pharmacotherapies, our model may assist in the development of novel drug targets for the treatment of cocaine addiction.

Material and Methods

Animals

Male, 7-week old, C57BL/6J mice (Charles River, UK), were singly-housed in a temperature-controlled environment with a 12:12 hour light/dark cycle and access to food/water *ad libitum*.

Ethical standards: All experimental procedures were approved by the U.K. Animal Scientific Procedures Act (1986).

Chronic escalating-dose cocaine administration paradigm

Mice were randomly divided into four treatment groups: chronic saline or cocaine and saline or cocaine withdrawal. Mice were injected (i.p.) with saline (4ml/kg) or escalating-dose cocaine (Fig. 1A; Sigma-Aldrich, Poole, UK), as previously described (Bailey *et al.*, 2008). Cocaine-treated mice received 3 cocaine injections 1-hour apart (3x15 mg/kg/day on days 1-4, 3x20 mg/kg/day on days 5-8, 3x25 mg/kg/day on days 9-12, and 3x30 m**g**/kg/day on days 13 and 14; Fig. 1A). Body weights were measured daily before the injections and 1, 6, 12 and 14 days following the last treatment injection. For biochemical measurements, animals were euthanized by a 30-sec exposure to CO_2 followed by decapitation 1 hour (chronic group) and 14 days (withdrawal group) after their last treatment injection. Brains were stored at -80^{o}C until use.

Experiment 1: Behavioral characterization

Following cocaine withdrawal, mice were assessed for sociability, memory, anxiety, motor coordination, anhedonia and depressive-like behaviors (timeline: Figure 1A).

Three-chambered social approach test (3-CB)

The 3-CB test was used to assess for sociability behaviors and was carried out as previously described (Zanos *et al.*, 2014a). Briefly, the test mouse was placed into the middle chamber of an interconnected three-chambered box. During the habituation phase, the test animal had free access to all the chambers

for 10 min. Then, two small cages were placed in each of the end-chambers and an unfamiliar male C57BL/6J mouse (same age as the test mouse) was placed into one of these smaller cages (social chamber), while the other cage remained empty (empty chamber). Then, the test mouse was allowed to explore the apparatus for another 10 min. Time spent in each chamber of the 3-CB apparatus was automatically measured by an automated tracking system (EthoVision v.3.0, Noldus Information Technology, Netherlands). Sociability behaviour was determined by calculating the time each animal spent in the social *vs* empty chamber.

Novel object recognition (NOR)

Mice were assessed for short-term recognition memory in the NOR test, as previously described (Georgiou *et al.*, 2016). Briefly, mice were habituated to the NOR arena (30x20x20cm) for 20 minutes. During the acquisition phase two identical objects (either dice or marble attached on a clear plastic square block with dimensions 4.5x4.5x4.0cm) were placed in the arena and mice allowed to explore both objects for 20 minutes. Then, mice were returned to their home cages for a retention time of 35 minutes. During the testing phase one of the "familiar" objects was replaced by a novel object and the mouse was re-introduced into the NOR arena and allowed to explore for 2 minutes. Objects were placed in the same spatial location every time and the object chosen to be the novel object was counterbalanced. The sessions were recorded using a video-camera and the time spent interacting with each object (when the mouse's nose was in contact with the object or directed at the object within ≤2 cm) was scored by an observer blind to the treatment groups.

Light/Dark box

Anxiety-like behaviors were assessed using the Light/Dark box (L/D box), as previously described (Crawley *et al.*, 1997). Briefly, mice were placed in the illuminated compartment of the L/D box (40x20x20cm) and allowed to explore for 5 minutes. The sessions were recorded using a video-camera and the time spent in the illuminated and dark compartment was scored. Anxiety-like

behaviour was determined by calculating the amount of time spent in the illuminated *vs* the dark compartment.

Rota-rod

Motor coordination was assessed using the accelerated rota-rod (Ugo Basile, Milan, Italy) paradigm, which consisted of 3 trials (60-120 sec inter-trial interval) during the same day. The rotarod was set to accelerate at a rate of 20 rpm with a staring speed of 4rpm. The latency to fall was scored live during the experimental session by an observer blind to treatment groups. The average latency for the three trials was calculated.

Sucrose preference - anhedonia

The sucrose preference test was carried out as previously described (Zanos *et al.*, 2016). Briefly, on day 1, two bottles containing tap water were introduced on the two opposite sides of the home-cages. On day 2, water consumption from each bottle was measured and another bottle containing 1% sucrose solution was placed at the least preferred side for another 24 hours. Sucrose preference was calculated as [sucrose consumption/(total liquid consumption) x100] for the assessment of anhedonia.

Forced-swim test (FST)

The FST was carried out as previously described (Zanos *et al.*, 2015b). Briefly, mice were individually placed into clear glass cylinders (25cm height x 17cm diameter) filled with 2.5 litres of water at room temperature (24±1^{o}C) for 6 minutes. The test session was recorded using a digital video-camera (Sony Handycam CX-250, Sony, Japan). Immobility time, defined as passive floating with no additional activity other than that needed to keep the mouse head above water, was scored by an observer blind to the treatment groups for the last 4 minutes of the 6-min test.

Experiment 2: Locomotion/Biochemical alterations

Locomotor activity

Locomotor activity was measured daily during the chronic administration paradigm described above, as well as 14 days following drug cessation in locomotor chambers (40cm x 20cm x 20cm; Linton Instrumentation, U.K.), according with Wright *et al.*, (2016). During the administration period, mice were habituated daily in locomotor chambers for 60 minutes prior to saline/cocaine injections in order to assess basal activity. Then, mice received an i.p. injection of saline or cocaine (escalating dose as described above) and were returned immediately to the locomotor chambers. Mice received 3 daily injections (saline or cocaine) at 1-hour interval and horizontal and vertical (i.e., rearing) activities were measured after each injection (3hrs in total). Horizontal and vertical activities were recorded as the number of sequential infrared beam breaks every 5 mins. The average activity, during both basal and stimulated activities, was calculated.

Stereotypy scoring

All mice were scored for stereotypy behaviour 30 min after each daily injection for a period of 30 seconds. The rating was based on a slight modification of the scale used elsewhere (Bailey *et al.*, 2007; Metaxas *et al.*, 2012) and consists of a graded scale of behaviours; 1: asleep, inactive; 2: alert, actively grooming; 3: increased sniffing; 4: intermittent rearing and sniffing; 5: increased locomotion; 6: intense sniffing in one location; 7: continuous pivoting and sniffing/hyperlocomotion; 8: intermittent rearing and sniffing with locomotion; 9: maintained rearing and sniffing; 10: splayed limbs. Stereotypy behaviour of each mouse was recorded during the saline/cocaine administration period (i.e., days 1-14), as well as following 14 days of withdrawal (i.e., day 28). The median stereotypy score of the three injections on each day was calculated for each treatment group. Scoring was performed during locomotor testing by two experimenters blind to the treatment groups.

OTR autoradiography

OTR autoradiography in brains of chronically-treated and 14–day withdrawn mice was carried out as previously described (Zanos *et al.*, 2014b; Zanos *et al.*, 2015a). For the determination of total binding, slides were incubated in 50pM [^{125}I]-ornithine vasotocin (OVTA) (PerkinElmer, 81.4 TBq/mmol) for 60 minutes. For the determination of NSB, adjacent sections were incubated with [^{125}I]-OVTA (50pM) in the presence of 50μM unlabelled (Thr4, Gly7)-oxytocin (Bachem, Germany).

Plasma corticosterone levels

Trunk blood was collected in EDTA-containing tubes and spun at 2000xg at 4^{o}C for 15 minutes. Plasma corticosterone measurements were performed as previously described (Georgiou *et al.*, 2015b).

Statistical Analysis

All the values are expressed as the mean ± SEM. All statistical analyses were performed using GraphPad *v6* (GraphPad software Inc., La Jolla, CA, USA). For the analysis of locomotor activity, stereotypy scores and body weights, two-way repeated measures ANOVA was performed with factors, “treatment” and “day (repeated factor)”. Since the expression of behavioural stereotypy was measured with a behavioural rating scale, non-parametric Mann Whitney U-test was used on individual test days as a supplementary analysis to confirm ANOVA results. Two-way repeated measures ANOVA was performed for the 3-CB with factors “treatment” and “side (repeated factor)”. For the NOR test, one-sample *t*-test was performed and the reference value was set at 0.5 discrimination ratio. Student’s unpaired *t*-test was performed for the light/dark box, rotarod, FST and sucrose preference test. Differences in OTR binding were assessed with a two-way ANOVA with factors “brain region” and “treatment”. Plasma corticosterone levels were analysed by a two-way ANOVA with factors “treatment” and “withdrawal”. ANOVAs were followed by Holm-Sidak *post-hoc* comparison when significance was reached ($p<0.05$).

Results

Effect of cocaine withdrawal on sociability, memory, anxiety-like, motor coordination, anhedonia and depressive-like behaviors.

Three-chambered social approach test. Both saline- and cocaine-withdrawn animals spent more time in the social chamber compared to the empty cage ('side' $F_{[1,8]}$=10.47, *p<0.05;* 'treatment' $F_{[1,8]}$=1.599, *p>0.05;* 'side'x'treatment' $F_{[1,8]}$=1.431, *p>0.05;* Figure 1B)

Novel object recognition. Saline-withdrawn animals manifested normal object recognition memory (p<0.05), whereas cocaine-withdrawn animals showed object recognition memory impairment (p>0.05; Figure 1C).

Light/Dark Box. Cocaine-withdrawn animals spent less time in the illuminated area showing higher anxiety-like behavior compared to saline-withdrawn animals (p<0.05; Figure 1D).

Rota-rod. There was no difference between saline- and cocaine-withdrawn animals in the latency to fall from the rotating rod (p>0.05; Figure 1E).

Sucrose-preference. Cocaine-withdrawn mice had decreased sucrose preference compared to controls, indicative of anhedonic phenotype (p<0.05; Figure 1F).

Forced-swim test. There was no statistically significant difference in immobility time of cocaine- and saline-withdrawn animals (p>0.05; Figure 1G).

Effects of chronic cocaine administration and withdrawal on stimulated and basal horizontal and vertical activity

Basal locomotor activity: A significant effect of 'treatment' ($F_{[1,10]}$=8.677, *p<0.05*), 'day' ($F_{[14,140]}$=23.68, *p<0.001*) and 'treatment' x 'days' interaction ($F_{[14,140]}$=3.672, *p<0.001*) was also observed in basal horizontal activity. Cocaine-treated animals increased their basal horizontal activity on days 7, 9-14 during chronic administration, as well as following 14 days of withdrawal (day 28)

compared to controls (Figure 2B). A significant main effect of 'treatment' ($F_{[1,10]}$=18.43; *p<0.01*) was observed in basal vertical activity (Figure 2C).

Stimulated locomotor activity: A significant main effect of 'treatment' ($F_{[1,10]}$=108.0, *p<0.001*), 'day' ($F_{[13,130]}$=3.887, *p<0.001*) and 'treatment' x 'days' interaction ($F_{[13,130]}$=6.915, *p<0.001*) was revealed following cocaine administration (Figure 2D). Cocaine-treated animals showed increased horizontal locomotor activity compared to controls (*p<0.001*). Following 3 days of cocaine administration, there was an increase in cocaine-stimulated horizontal activity compared to Day 1 (*p<0.001*), indicating the acquisition of behavioural sensitization. A significant main effect of 'treatment' ($F_{[1,10]}$=13.45, *p<0.01*) was observed in stimulated vertical activity following chronic cocaine administration (Figure 2E).

Effects of chronic cocaine administration and withdrawal on stereotypy scores

A significant effect of 'treatment' ($F_{[1,10]}$=1751.0, *p<0.001*), 'day' ($F_{[14,140]}$=10.00, *p<0.001*) and 'treatment' x 'days' interaction ($F_{[14,140]}$=13.16, *p<0.001*) was observed in stereotypy behavior. Cocaine-treated animals increased their stereotypy behavior throughout the cocaine administration paradigm (days 1-14; Figure 2F). Following 14 days of withdrawal (day 28), no significant difference in the stereotypy behaviors was observed in cocaine- *vs* saline-treated mice (Figure 2F).

Effects of chronic cocaine administration and withdrawal on body weight

A significant effect of 'treatment' ($F_{[1,10]}$=19.06, *p<0.01*), 'day' ($F_{[17,170]}$=2.854, *p<0.001*) and 'treatment' x 'days' interaction ($F_{[17,170]}$=3.372, *p<0.001*) was observed on body weights. Body weight of cocaine-treated animals was decreased reaching significance on day 4 compared to saline-treated mice (Figure 2G). This decrease was persistent throughout the administration paradigm and up to day 22 (8-days withdrawal). Following 12- and 14-days withdrawal from cocaine, no statistically significant difference was observed in the body weights compared to controls (Figure 2G).

Effects of chronic cocaine administration and 14-day withdrawal on the oxytocinergic system.

Two-way ANOVA did not reveal significant 'brain region', 'treatment' (olfactory nuclei: $F_{[3,53]}$=0.3721; *p>0.05;* striatum: $F_{[3,48]}$=2.785; *p>0.05*), or an interaction (olfactory nuclei: $F_{[6,53]}$=0.5148; *p>0.05;* striatum: $F_{[6,48]}$=0.639; *p>0.05*) effect in the olfactory nuclei or the striatum of chronically cocaine-treated and -withdrawn mice (Figure 3A,B). A significant effect of 'brain region' ($F_{[1,36]}$=365.5; *p<0.001*) and 'brain region' x 'treatment' interaction ($F_{[3,36]}$=3.869; *p<0.05*) was observed in the cortical regions analyzed. *Post-hoc* analysis showed a significant increase in OTR binding in the piriform cortex following cocaine administration and withdrawal (Figure 3A,C). A significant 'treatment' ($F_{[3,52]}$=16.94; *p<0.001*) effect and a close to significance 'brain region' x 'treatment' interaction ($F_{[6,52]}$=2.118; *p=0.06*) was observed in the septum. *Post-hoc* analysis revealed a significant increase in OTR binding following the 14-day withdrawal in the medial septum (MS) and ventral limb of diagonal band (VDB) compared to control (Figure 3A,E) and chronic cocaine groups (Figure 3A,E). In the lateral septum (LS), OTR binding was increased following cocaine administration and withdrawal compared to controls (*p<0.01*; Figure 3A,E). In the forebrain, a significant effect of 'brain region' ($F_{[2,54]}$=192.7; *p<0.001*), 'treatment' ($F_{[3,54]}$=4.505; *p<0.01*) and an interaction ($F_{[6,54]}$=2.640; *p<0.05*) was observed. *Post-hoc* analysis showed an increase in amygdalar OTR binding following cocaine administration and withdrawal compared to the respective controls (Figure 3A,F).

Effects of chronic cocaine administration and 14-day withdrawal on plasma corticosterone levels

Two-way ANOVA showed a significant main effect of chronic cocaine treatment ($F_{[1,35]}$=15.88, *p<0.001*; Figure 3G) on plasma corticosterone levels. Corticosterone levels were persistently increased following cocaine treatment/withdrawal.

Discussion

We demonstrated that chronic cocaine administration increases OTR binding in the piriform cortex, amygdala and LS; an increase that persists following withdrawal. A withdrawal-specific increase in OTR binding was observed in the MS and VDB. These receptor changes were accompanied by the emergence of an anxiogenic-like phenotype, anhedonia and memory impairment. Increased plasma corticosterone levels were also observed following chronic cocaine administration and abstinence.

Although cocaine withdrawal did not induce social avoidance, motor incoordination or behavioral despair, we observed memory impairments, anxiety-like behavior and anhedonia. These findings are in line with clinical observations from abstaining cocaine addicts (Gawin & Kleber, 1986; Weddington et al., 1990; Morton, 1999; Kelley et al., 2005; Woicik et al., 2009; Leventhal et al., 2010), supporting the translational value of our model. Moreover, we observed increased locomotor activity 14 days after drug cessation. Increased locomotor activity in the drug-paired environment has been linked with contextual sensitization, which has been associated with persistent molecular neuroadaptations that might contribute to drug craving following abstinence (see Robinson & Berridge, 2008). Drug craving is common among cocaine abstinent individuals, and it is often triggered by environmental cues previously associated with cocaine use (see Wolf, 2016), which further highlights the validity of our model. Interestingly, increased activity has been also associated with anxiety-like behaviours in mice (Lever et al., 2006), which is in line with the observed cocaine withdrawal-induced anxiogenic phenotype.

We then assessed the effect of chronic cocaine administration and withdrawal on the oxytocinergic system in order to identify possible mechanisms underlying the observed emotional impairment. We found a persistent cocaine-induced upregulation of OTR in the amygdala and LS, and a specific withdrawal-induced upregulation in the MS and VDB. Considering the role of the amygdala and septum in the antidepressant/anxiolytic actions of OT (Curley *et al.*, 2012; Zanos *et al.*, 2014a), the

upregulation of the OTR binding in these regions may represent a neuroadaptive response of the oxytocinergic system to counteract the negative consequences of cocaine administration/withdrawal. Indeed, OT administration has been shown to reduce anhedonia in schizophrenic patients (see Rich & Caldwell, 2015) and induce a hedonic state in rodents (see Slattery & Neumann, 2010). Therefore, dysregulation of the OTR system might contribute to the development of anhedonia observed in the present study.

Oxytocin administration has also been shown to decrease anxiety-like behaviors in rodents (see Neumann & Slattery, 2016), and humans (Guastella *et al.*, 2009) and to decrease amygdala reactivity to fear in patients with geneneralized social anxiety disorder (Labuschagne *et al.*, 2010). Notably, amygdala is one of the brain regions where we have observed an increase in OTR binding following cocaine administration/withdrawal, suggesting that this dysregulation may underlie the development of anxiety-like behaviors during withdrawal. In agreement, a similar dysregulation of the OTR system was previously observed in the amygdala following chronic morphine administration/withdrawal, which was associated with an anxiogenic phenotype (Zanos *et al.*, 2014a). Although in the present study we have not assessed whether emotional regulation occurs during chronic cocaine administration, the fact that the OTR dysregulation in the LS and amygdala occurred both following cocaine administration and withdrawal suggests that behavioral deficits would have been also present during chronic cocaine exposure. Indeed, earlier studies have identified emotional deficits during chronic cocaine exposure in both animals (Morales-Rivera *et al.*, 2014) and humans (Morton, 1999; Zubaran *et al.*, 2013).

A plausible mechanism underlying these neuroadaptations in the OTR system is that there might be a compensatory mechanism to counteract a hypo-oxytocinergic state caused by chronic cocaine administration/withdrawal. Indeed, Sarnyai *et al*., (1992) reported decreased hypothalamic OT content and reduced plasma OT levels in rats chronically-treated with cocaine. In addition, Baracz *et al.*,

(2016) found decreased plasma OT levels following methamphetamine self-administration/extinction. Taken together, it can be postulated that cocaine withdrawal induces a hypo-oxytocinergic tone and a subsequent neuroadaptation of the OTR system to counteract the negative behavioural consequences of abstinence. Therefore, it would be of great interest for future studies to investigate the effect of oxytocin administration in preventing cocaine withdrawal-induced behavioral impairments. However, it is important to mention that OT also binds and activates vasopressin receptors (Busnelli *et al.*, 2013), which might induce anxiogenic and pro-stress effects (see Neumann & Landgraf, 2012). Therefore, studies assessing the effects of exogenous administration of OT should be cautious on the dosing regimen.

We have also observed increased corticosterone levels following cocaine administration, which persisted following withdrawal, suggesting that the cocaine effect on HPA axis activity does not normalize even after prolonged abstinence. Elevated cortisol secretion is evident in abstinent cocaine abusers, lasting for at least 3 weeks following cessation of cocaine-taking (Contoreggi *et al.*, 2003), further supporting the translational validity of our model. In line with our findings, it has been previously demonstrated that cocaine-treated rats, have increased plasma corticosterone levels following a 14-day withdrawal (Zhou *et al*., 2011). The persistent cocaine-induced hypersensitivity of the HPA axis may be the cause or consequence of the observed alterations on the oxytocinergic system. Indeed, there is evidence supporting both these hypotheses. One of the mechanisms underlying the anti-stress effect of oxytocin involves the inhibition of hypothalamic CRF release (Neumann *et al.*, 2000). In addition, a 5-day oxytocin administration decreased plasma corticosterone levels in rats (Petersson *et al.*, 1999). Moreover, dexamethasone administration increased OTR binding in the septum and amygdala of rats (Patchev *et al.*, 1993). Therefore, it is possible that alterations of the oxytocinergic system may lead to a dysregulated HPA axis activity and consequently to the emotional impairment, or a persistent dysregulation of the HPA axis to have caused the changes observed in the oxytocinergic system. These hypotheses require further investigation.

In conclusion, we have demonstrated profound alterations in the oxytocinergic system following chronic cocaine administration and withdrawal in brain regions associated with the observed behavioural impairment, confirming a key role of the OT system during cocaine drug withdrawal.

Acknowledgements

This study was supported by grant a Royal Society grant (RG120556; P.I. Alexis Bailey) and European commission grant (contact number: LSHM-CT2004-005166). The sponsors had no involvement in the design of study and in the collection, analysis and interpretation of the data, nor in the writing of the manuscript and the decision to submit this article for publication. The authors would like to thank Mrs. Hannah Hobson for her help in brain dissection.

Financial disclosures

The authors declare no conflict of interest and derive no financial interests from this research.

References

Bailey, A., Metaxas, A., Yoo, J.H., McGee, T. & Kitchen, I. (2008) Decrease of D2 receptor binding but increase in D2-stimulated G-protein activation, dopamine transporter binding and behavioural sensitization in brains of mice treated with a chronic escalating dose 'binge' cocaine administration paradigm. *Eur J Neurosci*, **28**, 759-770.

Bailey, A., Yoo, J.H., Racz, I., Zimmer, A. & Kitchen, I. (2007) Preprodynorphin mediates locomotion and D2 dopamine and mu-opioid receptor changes induced by chronic 'binge' cocaine administration. *J Neurochem*, **102**, 1817-1830.

Baracz, S.J., Parker, L.M., Suraev, A.S., Everett, N.A., Goodchild, A.K., McGregor, I.S. & Cornish, J.L. (2016) Chronic Methamphetamine Self-Administration Dysregulates Oxytocin Plasma Levels and Oxytocin Receptor Fibre Density in the Nucleus Accumbens Core and Subthalamic Nucleus of the Rat. *J Neuroendocrinol*, **28**.

Busnelli, M., Bulgheroni, E., Manning, M., Kleinau, G. & Chini, B. (2013) Selective and potent agonists and antagonists for investigating the role of mouse oxytocin receptors. *J Pharmacol Exp Ther*, **346**, 318-327.

Contoreggi, C., Herning, R.I., Koeppl, B., Simpson, P.M., Negro, P.J., Jr., Fortner-Burton, C. & Hess, J.

(2003) Treatment-seeking inpatient cocaine abusers show hypothalamic dysregulation of both basal prolactin and cortisol secretion. *Neuroendocrinology*, **78**, 154-162.

Crawley, J.N., Belknap, J.K., Collins, A., Crabbe, J.C., Frankel, W., Henderson, N., Hitzemann, R.J., Maxson, S.C., Miner, L.L., Silva, A.J., Wehner, J.M., Wynshaw-Boris, A. & Paylor, R. (1997) Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. *Psychopharmacology (Berl)*, **132**, 107-124.

Curley, J.P., Jensen, C.L., Franks, B. & Champagne, F.A. (2012) Variation in maternal and anxiety-like behavior associated with discrete patterns of oxytocin and vasopressin 1a receptor density in the lateral septum. *Horm Behav*, **61**, 454-461.

Gawin, F.H. & Kleber, H.D. (1986) Abstinence symptomatology and psychiatric diagnosis in cocaine abusers. Clinical observations. *Arch Gen Psychiatry*, **43**, 107-113.

Georgiou, P., Zanos, P., Ehteramyan, M., Hourani, S., Kitchen, I., Maldonado, R. & Bailey, A. (2015a) Differential regulation of mGlu5 R and MuOPr by priming- and cue-induced reinstatement of cocaine-seeking behaviour in mice. *Addict Biol*, **20**, 902-912.

Georgiou, P., Zanos, P., Garcia-Carmona, J.A., Hourani, S., Kitchen, I., Kieffer, B.L., Laorden, M.L. & Bailey, A. (2015b) The oxytocin analogue carbetocin prevents priming-induced reinstatement of morphine-seeking: Involvement of dopaminergic, noradrenergic and MOPr systems. *Eur Neuropsychopharmacol*, **25**, 2459-2464.

Georgiou, P., Zanos, P., Garcia-Carmona, J.A., Hourani, S., Kitchen, I., Laorden, M.L. & Bailey, A. (2016) Methamphetamine abstinence induces changes in mu-opioid receptor, oxytocin and CRF systems: Association with an anxiogenic phenotype. *Neuropharmacology*, **105**, 520-532.

Guastella, A.J., Howard, A.L., Dadds, M.R., Mitchell, P. & Carson, D.S. (2009) A randomized controlled trial of intranasal oxytocin as an adjunct to exposure therapy for social anxiety disorder. *Psychoneuroendocrinology*, **34**, 917-923.

Johns, J.M., Lubin, D.A., Walker, C.H., Meter, K.E. & Mason, G.A. (1997) Chronic gestational cocaine treatment decreases oxytocin levels in the medial preoptic area, ventral tegmental area and hippocampus in Sprague-Dawley rats. *Neuropeptides*, **31**, 439-443.

Kelley, B.J., Yeager, K.R., Pepper, T.H. & Beversdorf, D.Q. (2005) Cognitive impairment in acute cocaine withdrawal. *Cogn Behav Neurol*, **18**, 108-112.

Labuschagne, I., Phan, K.L., Wood, A., Angstadt, M., Chua, P., Heinrichs, M., Stout, J.C. & Nathan, P.J. (2010) Oxytocin attenuates amygdala reactivity to fear in generalized social anxiety disorder. *Neuropsychopharmacology*, **35**, 2403-2413.

Leventhal, A.M., Brightman, M., Ameringer, K.J., Greenberg, J., Mickens, L., Ray, L.A., Sun, P. & Sussman, S. (2010) Anhedonia Associated With Stimulant Use and Dependence in a Population-Based Sample of American Adults. *Experimental and Clinical Psychopharmacology*, **18**, 562-569.

Lever, C., Burton, S. & O'Keefe, J. (2006) Rearing on hind legs, environmental novelty, and the hippocampal formation. *Rev Neurosci*, **17**, 111-133.

Light, K.C., Grewen, K.M., Amico, J.A., Boccia, M., Brownley, K.A. & Johns, J.M. (2004) Deficits in plasma oxytocin responses and increased negative affect, stress, and blood pressure in mothers with cocaine exposure during pregnancy. *Addict Behav*, **29**, 1541-1564.

McGregor, I.S. & Bowen, M.T. (2012) Breaking the loop: oxytocin as a potential treatment for drug addiction. *Horm Behav*, **61**, 331-339.

Metaxas, A., Keyworth, H., Yoo, J., Chen, Y., Kitchen, I. & Bailey, A. (2012) The stereotypy-inducing and OCD-like effects of chronic 'binge' cocaine are modulated by distinct subtypes of nicotinic acetylcholine receptors. *Br J Pharmacol*, **167**, 450-464.

Morales-Rivera, A., Hernandez-Burgos, M.M., Martinez-Rivera, A., Perez-Colon, J., Rivera, R., Montalvo, J., Rodriguez-Borrero, E. & Maldonado-Vlaar, C.S. (2014) Anxiolytic effects of oxytocin in cue-induced cocaine seeking behavior in rats. *Psychopharmacology (Berl)*, **231**, 4145-4155.

Morton, W.A. (1999) Cocaine and Psychiatric Symptoms. *Prim Care Companion J Clin Psychiatry*, **1**, 109-113.

Neumann, I.D. & Landgraf, R. (2012) Balance of brain oxytocin and vasopressin: implications for anxiety, depression, and social behaviors. *Trends Neurosci*, **35**, 649-659.

Neumann, I.D. & Slattery, D.A. (2016) Oxytocin in General Anxiety and Social Fear: A Translational Approach. *Biol Psychiatry*, **79**, 213-221.

Neumann, I.D., Wigger, A., Torner, L., Holsboer, F. & Landgraf, R. (2000) Brain oxytocin inhibits basal and stress-induced activity of the hypothalamo-pituitary-adrenal axis in male and female rats: partial action within the paraventricular nucleus. *J Neuroendocrinol*, **12**, 235-243.

Patchev, V.K., Schlosser, S.F., Hassan, A.H. & Almeida, O.F. (1993) Oxytocin binding sites in rat limbic and hypothalamic structures: site-specific modulation by adrenal and gonadal steroids. *Neuroscience*, **57**, 537-543.

Petersson, M., Hulting, A.L. & Uvnas-Moberg, K. (1999) Oxytocin causes a sustained decrease in plasma levels of corticosterone in rats. *Neurosci Lett*, **264**, 41-44.

Rich, M.E. & Caldwell, H.K. (2015) A Role for Oxytocin in the Etiology and Treatment of Schizophrenia. *Front Endocrinol (Lausanne)*, **6**, 90.

Robinson, T.E. & Berridge, K.C. (2008) Review. The incentive sensitization theory of addiction: some current issues. *Philos Trans R Soc Lond B Biol Sci*, **363**, 3137-3146.

Sarnyai, Z. & Kovacs, G.L. (1994) Role of oxytocin in the neuroadaptation to drugs of abuse. *Psychoneuroendocrinology*, **19**, 85-117.

Sarnyai, Z., Vecsernyes, M., Laczi, F., Biro, E., Szabo, G. & Kovacs, G.L. (1992) Effects of cocaine on the contents of neurohypophyseal hormones in the plasma and in different brain structures in rats. *Neuropeptides*, **23**, 27-31.

Slattery, D.A. & Neumann, I.D. (2010) Oxytocin and Major Depressive Disorder: Experimental and Clinical Evidence for Links to Aetiology and Possible Treatment. *Pharmaceuticals*, **3**, 702-724.

Soyka, M. & Mutschler, J. (2016) Treatment-refractory substance use disorder: Focus on alcohol, opioids, and cocaine. *Prog Neuropsychopharmacol Biol Psychiatry*, **70**, 148-161.

Weddington, W.W., Brown, B.S., Haertzen, C.A., Cone, E.J., Dax, E.M., Herning, R.I. & Michaelson, B.S. (1990) Changes in mood, craving, and sleep during short-term abstinence reported by male cocaine addicts. A controlled, residential study. *Arch Gen Psychiatry*, **47**, 861-868.

Woicik, P.A., Moeller, S.J., Alia-Klein, N., Maloney, T., Lukasik, T.M., Yeliosof, O., Wang, G.J., Volkow, N.D. & Goldstein, R.Z. (2009) The neuropsychology of cocaine addiction: recent cocaine use masks impairment. *Neuropsychopharmacology*, **34**, 1112-1122.

Wolf, M.E. (2016) Synaptic mechanisms underlying persistent cocaine craving. *Nat Rev Neurosci*, **17**, 351-365.

Wright, S.R., Zanos, P., Georgiou, P., Yoo, J.H., Ledent, C., Hourani, S.M., Kitchen, I., Winsky-Sommerer, R. & Bailey, A. (2016) A critical role of striatal A2A R-mGlu5 R interactions in modulating the psychomotor and drug-seeking effects of methamphetamine. *Addict Biol*, **21**, 811-825.

Zanos, P., Georgiou, P., Metaxas, A., Kitchen, I., Winsky-Sommerer, R. & Bailey, A. (2015a) Region-specific up-regulation of oxytocin receptor binding in the brain of mice following chronic nicotine administration. *Neurosci Lett*, **600**, 33-37.

Zanos, P., Georgiou, P., Wright, S.R., Hourani, S.M., Kitchen, I., Winsky-Sommerer, R. & Bailey, A. (2014a) The oxytocin analogue carbetocin prevents emotional impairment and stress-induced reinstatement of opioid-seeking in morphine-abstinent mice.

Neuropsychopharmacology, **39**, 855-865.

Zanos, P., Moaddel, R., Morris, P.J., Georgiou, P., Fischell, J., Elmer, G.I., Alkondon, M., Yuan, P., Pribut, H.J., Singh, N.S., Dossou, K.S., Fang, Y., Huang, X.P., Mayo, C.L., Wainer, I.W., Albuquerque, E.X., Thompson, S.M., Thomas, C.J., Zarate, C.A., Jr. & Gould, T.D. (2016) NMDAR inhibition-independent antidepressant actions of ketamine metabolites. *Nature*, **533**, 481-486.

Zanos, P., Piantadosi, S.C., Wu, H.Q., Pribut, H.J., Dell, M.J., Can, A., Snodgrass, H.R., Zarate, C.A., Jr., Schwarcz, R. & Gould, T.D. (2015b) The Prodrug 4-Chlorokynurenine Causes Ketamine-Like Antidepressant Effects, but Not Side Effects, by NMDA/GlycineB-Site Inhibition. *J Pharmacol Exp Ther*, **355**, 76-85.

Zanos, P., Wright, S.R., Georgiou, P., Yoo, J.H., Ledent, C., Hourani, S.M., Kitchen, I., Winsky-Sommerer, R. & Bailey, A. (2014b) Chronic methamphetamine treatment induces oxytocin receptor up-regulation in the amygdala and hypothalamus via an adenosine A2A receptor-independent mechanism. *Pharmacol Biochem Behav*, **119**, 72-79.

Zhou, L., Sun, W.L., Young, A.B., Lee, K., McGinty, J.F. & See, R.E. (2015) Oxytocin reduces cocaine seeking and reverses chronic cocaine-induced changes in glutamate receptor function. *Int J Neuropsychopharmacol*, **18**.

Zhou, Y., Litvin, Y., Piras, A.P., Pfaff, D.W. & Kreek, M.J. (2011) Persistent increase in hypothalamic arginine vasopressin gene expression during protracted withdrawal from chronic escalating-dose cocaine in rodents. *Neuropsychopharmacology*, **36**, 2062-2075.

Zubaran, C., Foresti, K., Thorell, M.R. & Franceschini, P.R. (2013) Anxiety symptoms in crack cocaine and inhalant users admitted to a psychiatric hospital in southern Brazil. *Rev Assoc Med Bras*, **59**, 360-367.

Figure Legends

Figure 1. Behavioral effects of cocaine withdrawal. ***Figure 1. Behavioral effects of cocaine withdrawal.*** **(A)** Experimental timeline. While cocaine withdrawal did not induce **(B)** sociability deficits it induced **(C)** short-term objectect recognition impairment and **(D)** an anxiogenic phenotype in the light/dark box task. **(E)** Cocaine-withdrawan mice did not show motor-coordination deficits in the rota-rod test. In addition, cocaine withdrawal **(F)** decreased sucrose preference compared to saline-witdrawan mice, whereas it did not significantly affect **(G)** immobility time in the forced-swim test. Data are expressed as mean ± SEM (n=5-6/group); *$p<0.05$. *Abbreviations*: *3-CB, three-*

chambered social approach test; COC, cocaine; FST, forced-swim test; Inj, injection; L/D, light/dark box; NOR, novel-object recognition; RR, rotarod; SAL, saline; sec, seconds; Sucr. Pref., sucrose preference.

Figure 2. Effects of cocaine administration and withdrawal on locomotor activity, stereotypy and body weight. **(A)** Experimental timeline. Chronic cocaine administration and 14-day withdrawal increased basal (non-stimulated) **(B)** horizontal and, **(C)** vertical activity. Chronic cocaine administration increased stimulated **(D)** horizontal and, **(E)** vertical activity. **(F)** Chronic cocaine administration increased stereotypy behaviors. **(G)** Cocaine treatment decreased the body weight of mice during administration and following 8 days of withdrawal. No difference in body weight was observed following 12 and 14 days withdrawal. Data are expressed as mean ± SEM (n=6/group); *p<0.05, **p<0.01, ***p<0.001 *vs* saline, $^{\#\#\#}p$<0.001 *vs* cocaine Day 1. *Abbreviations*: *COC, cocaine; CORT, corticosterone; Inj, injection; OTR, oxytocin receptor; SAL, saline.*

Figure 3. Effects of chronic cocaine administration and withdrawal on brain oxytocin receptor binding and plasma corticosterone levels. **A)** Representative OTR autoradiograms of adjacent coronal brain sections at the level of the olfactory nuclei (Bregma 2.46mm, first row), cortex/striatum (Bregma 0.86mm, second row), septum (Bregma 0.14mm, third row) and the forebrain (Bregma -2.06mm, second row). The color bar illustrates a pseudo-color interpretation of black and white film images in fmol/mg tissue equivalent. Quantitative oxytocin receptor autoradiographic binding in **(B)** olfactory nuclei (n=5-6/group), **(C)** cortex (n=6/group), **(D)** (n=4-6/group) striatum, **(E)** septum (n=4-6/group) and **(F)** forebrain (n=6/group) of mice treated with cocaine or underwent cocaine withdrawal. **(G)** Chronic cocaine administration and 14-day withdrawal increased plasma corticosterone levels (n=6-12/group). Data are expressed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 *vs* saline or saline withdrawal. *Abbreviations*: *Acb, nucleus accumbens; Amy, amygdala; AOL, anterior olfactory nucleus-lateral; AOM, anterior olfactory nucleus-medial;*

AOV, anterior olfactory nucleus-ventral; CgCx, cingulate cortex; COC, cocaine; CORT, corticosterone; CPu, caudate putamen; Hip, hippocampus; LS, lateral septum; MS, medial septum; NSB, non-specific binding; OTR, oxytocin receptor; SAL, saline; Th, thalamus; Tu, olfactory tubercle; VDB, vertical limb of diagonal band of Broca.