Environmental enrichment enhances conditioned place preference to ethanol via an oxytocinergic-dependent mechanism in male mice

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Short title: Environmental enrichment enhances alcohol reward

Highlights

Environmental enrichment (EE) and carbetocin enhance ethanol conditioned-place preference (CPP)

EE and carbetocin decrease oxytocin receptor binding (OTR) in the PFC

EE increases oxytocin levels in the hypothalamus

Blockade of OTR during EE prevents EE-induced enhancement of ethanol CPP

Abstract

Environmental conditions, such as stress and environmental enrichment (EE), influence predisposition to alcohol use/abuse; however, the underlying mechanisms remain unknown. To assess the effect of environmental conditions on the initial rewarding effects of alcohol, we examined conditioned place-preference (CPP) to alcohol following exposure to EE in mice. Since social context is a major factor contributing to initial alcohol-drinking, we also assessed the impact of EE on the levels of the "social neuropeptide" oxytocin (OT) and its receptor, OTR. Finally, we assessed the effect of pharmacological manipulations of the oxytocinergic system on EE-induced alcohol CPP. While EE increased sociability and reduced anxiety-like behaviors, it caused a ~3.5-fold increase in alcohol reward compared to controls. EE triggered profound neuroadaptations of the oxytocinergic system; it increased hypothalamic OT levels and decreased OTR binding in the prefrontal cortex and olfactory nuclei of the brain. Repeated administration of the OT analogue carbetocin (6.4 mg/kg/day) mimicked the behavioral effects of EE on ethanol CPP and induced similar brain region-specific alterations of OTR binding as those observed following EE. Conversely, repeated administration of the OTR antagonist L,369-899 (5 mg/kg/day) during EE exposure, but not during the acquisition of alcohol CPP, reversed the pronounced EE-induced ethanol rewarding effect. These results demonstrate for the first time, a stimulatory effect of environmental enrichment exposure on alcohol reward via an oxytocinergic-dependent mechanism, which may predispose to alcohol abuse. This study offers a unique prospective on the neurobiological understanding of the initial stages of alcohol use/misuse driven by complex environmental-social interplay.

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Keywords: environmental enrichment, alcohol, oxytocin, reward, conditioned-place preference, addiction

1. Introduction

Several factors contribute to the initiation of drug-taking, including environment, psychiatric conditions, stress and social factors. Chronic exposure to aversive stressors (foot shock, immobilization) enhances ethanol-induced conditioned-place preference (CPP) and alcohol consumption in rodents (Becker et al., 2011). In contrast, environmental enrichment (EE; model of "eustress") is known to protect stress-induced anxiety (Novaes et al., 2017) and prevent drug addiction progression (Solinas et al., 2010). Indeed, enriched environment prevents cocaine [(Solinas et al., 2008); but see (Green et al., 2010)], heroin (El Rawas et al., 2009) and morphine (Xu et al., 2007) CPP, diminishes amphetamine self-administration (Bardo et al., 2001) and abolishes sensitization to morphine (Xu et al., 2007) and nicotine (Green et al., 2003) in rodents. These effects might be associated with the ability of EE to prevent stress- and drug-induced hypothalamic-pituitary-adrenal (HPA) axis reactivity (Morley-Fletcher et al., 2003) and/or to modulate cognitive processes (van Praag et al., 2000).

Findings with respect to the effects of EE on alcohol-related behaviors are less clear. There is evidence showing that EE prevents alcohol-induced locomotor sensitization (Rueda et al., 2012), decreases alcohol self-administration in alcohol-preferring rats (Deehan et al., 2011), blunts alcohol consumption after stress (Marianno et al., 2017) and reduces ethanol CPP in spontaneously-hypertensive female rats (de Carvalho et al., 2010). Nevertheless, increased ethanol consumption has also been described in EE-raised rats (Rockman et al., 1989). Thus, the exact effects of EE on alcohol rewarding effects and the underlying mechanisms warrant further investigation.

Although the underlying neurobiology is not well understood, it is known that initiation of drug-taking is strongly influenced by several socio-environmental factors (York, 1999). Peer/social-drinking behavior has been linked with increased alcohol use

among young adults, indicating social context as an important factor influencing alcohol-drinking behavior (Cruz et al., 2012; Osgood et al., 2013). In line with this, emerging evidence implicates the "social neuropeptide (Heinrichs and Domes, 2008)" oxytocin (OT), in the regulation of several addiction processes (Bahi, 2015; Georgiou et al., 2015a; Georgiou et al., 2015b; Georgiou et al., 2016a; Georgiou et al., 2016b; King et al., 2017; Zanos et al., 2015a; Zanos et al., 2014a; Zanos et al., 2014b). Whereas OT exerts protective effects in reducing addictive behaviors at later stages of the drug addiction cycle (i.e., chronic use, withdrawal, relapse; McGregor and Bowen, 2012; Zanos et al., 2017), this may not be the case during the initial drug exposure.

Conditioned-place preference is a model used to study the initial rewarding phase of the drug addiction cycle (Koob, 2009). It is worth noting that peripheral OT administration was reported to enhance the expression of morphine-induced CPP (Moaddab et al., 2015), but to reduce place preference for methamphetamine when it is administered directly into the nucleus accumbens core (Baracz et al., 2012). In addition, OT treatment not only increased nicotine intake, but it also alleviated nicotine aversion in rats (Lee et al., 2016), suggesting that elevated oxytocin levels may be a causative factor for the initial smoking behavior, which is also influenced by a variety of socio-environmental factors (Bellatorre et al., 2015; Greenlund et al., 1997).

Based on the aforementioned evidence, we hypothesized that exposure to enriched environmental conditions and/or repeated OT administration, which both exhibit pro-social effects, may augment alcohol reward. We thus examined the effects of EE and repeated carbetocin (OT analogue) administration on alcohol rewarding properties by assessing ethanol CPP in mice. To further shed light into possible interactions between enriched environmental conditions and alterations on the oxytocinergic system, we assessed the effects of EE on central levels of OT and its

receptor (OTR) in the brain. Finally, to determine whether the impact of EE on ethanol CPP is mediated by an oxytocinergic mechanism, we assessed the effect of the selective OTR antagonist L,368-899 during EE exposure on alcohol CPP. Results from this study offer a unique prospective on the neurobiological understanding of the initial stages of alcohol use/misuse driven by a complex environmental-social interplay.

2. Materials and Methods

2.1 Animals

126 male Swiss mice (PND: 70 at the beginning of the experiments; Biomedical Sciences Institute, University of São Paulo) were housed in groups of five/cage, with free access to food and water, in a temperature (21 ± 1°C) and humidity-controlled environment, under a 12/12 h light/dark cycle (lights on: 07:00am). Procedures were approved by the Ethical Committee for Animal Use (CEUA) of the University of São Paulo, registered under protocol nº 132, page 110, book 02. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

2.2 Drugs

Ethanol (20% v/v; Labsynth, Diadema, SP, Brazil) was diluted in 0.9% NaCl solution and administered intraperitoneally (i.p.) at a dose of 2 g/kg. Carbetocin (CBT; Sigma-Aldrich, St Louis, MO, USA) was diluted in 0.9% saline solution and administered i.p. at the dose of 6.4mg/kg, based on previous studies (Georgiou et al., 2015b; Zanos et al., 2014a). CBT was chosen due to its greater stability and higher half-life time, compared to OT itself and due to the fact that it does not activate the vasopressin receptors (Passoni et al., 2016). L-368,899 (Tocris, Mississippi, USA), a blood-brain penetrant

OTR antagonist was diluted in 0,9% saline and administered i.p. at the dose of 5 mg/kg based on previous studies (Lee et al., 2015).

2.3 Housing conditions

Animals were exposed to two different environmental conditions in this study (control and EE). Animals from the control (CT) group were housed in standard polypropylene cages (27.5 x 16.5 x 13 cm) throughout the experiment (see Supplementary Fig. 1).

2.3.1 *Environmental enrichment (EE) protocol*

EE mice were housed in large polycarbonate cages, (42 x 28 x 21.5 cm), where they were exposed to different stimuli, such as toys, tubes, ladders, houses and running wheels (objects were changed/moved three times a week), as previously described by Rueda et al., (2012). Depending on the experimental procedure, animals were maintained under enriched conditions for 31 days (see Supplementary Fig. 1 Ai, E) or 21 days (see Supplementary Fig. 1 Aii, B and C). EE is described as a combination of complex stimuli, including exposure to novelty, voluntary physical exercise and increased social interaction (van Praag et al., 2000). Previous attempts have been made to isolate these factors, including comparison between large spaces and exercise in the running wheel (Bernstein, 1973). Indeed, the variety of stimuli in a cage is more important than the large space in bigger cages to some behavioral responses (Whitaker et al., 2009). In our laboratory, we have observed that mice kept in EE cages did not show similar ethanol-induced behavioral sensitization as that seen in animals kept in large cages without the toys (data not shown). In Marianno et al. (2017), the control mice were kept in cages of the same size as the enriched mice and there were significant differences in ethanol intake between the two groups after stress.

2.4 Experimental Procedures

The study was separated into 5 experiments with different cohorts of animals.

2.4.1 Experiment 1: Behavioral characterization of mice exposed to EE

Mice undergoing EE or control housing conditions were introduced to their respective cage environment for a period of 31 days (Supplementary Fig. 1Ai). Throughout the duration of the study mice were exposed to a series of behavioural tests (Supplementary Fig. 1Ai).

2.4.1.1 Plasma corticosterone levels: In order to evaluate the effects of EE on the hypothalamic-pituitary-adrenal (HPA) axis activity, blood samples were collected from the caudal vein of mice in heparin tubes at the same time (9:00 – 11:00) on Day 1 and Day 21 of the experiment, to avoid circadian changes (Supplementary Fig. 1Ai). Blood samples were immediately spun at 2000 x g (4°C) for 10 minutes and plasma stored at -80°C. The analysis of plasma corticosterone levels was performed using an ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) and following the manufacturer's instructions.

2.4.1.2 Elevated Plus-Maze (EPM)

The EPM was used to assess anxiety-like responses in rodents on day 17 as previously described (Zanos et al., 2015b) (Supplementary Fig. 1Ai; see *Supplementary Information*).

2.4.1.3 Novel Object Recognition (NOR)

The NOR test was used to assess short- and long-term recognition memory, as previously described (Bevins and Besheer, 2006), with minor modifications (Supplementary Fig. 1Ai; see *Supplementary Information*). The test was carried out between day 19 and 20.

2.4.1.4 Locomotor activity (Day 19): In order to assess the effects of EE on exploration activity in a novel environment, locomotor activity was measured during the first session of the habituation phase of the NOR on day 19 using TopScan automated system (CleverSys, Inc, Reston, VA, USA), as previously described (Zanos et al., 2015b).

2.4.1.5 Conditioned-place preference (CPP)

The CPP experiment was performed on days 22-31 to assess the effects of EE on alcohol reward, as previously described by Pildervasser et al. (2014), with minor modifications (Supplementary Fig. 1Ai; see *Supplementary Information*). The sessions were recorded by an overhead camera and manually analyzed.

2.4.1.6 Three-chamber social approach test

The three-chamber social approach test was used in a different cohort of animals on day 21 to measure animal's sociability behaviors, as previously described (Zanos et al., 2016) (Supplementary Fig. 1Aii; see *Supplementary Information*).

2.4.2 Experiment 2: Effect of EE exposure on brain OT and OTR levels in mice

A separate cohort of animals underwent the same EE paradigm, but were not tested in any behavioral protocols to avoid influences on the biochemical measurements (Supplementary Fig. 1B). On Day 21, mice were euthanized by cervical dislocation and brains were excised, frozen and stored at -80°C. Half of the brains (n=5/group; randomly selected) were sectioned for OTR binding analysis using a cryostat. Autoradiographic binding of OTR was performed as previously described (Zanos et al., 2014b) (see *Supplementary Information*). The other half of the brains (n=5/group) were used to measure OT peptide levels in the brain. Olfactory nuclei (bregma 2.46 mm),

prefrontal cortex (bregma 2.46 mm), amygdala (bregma -2.06 mm) and hypothalamus (bregma -2.06 mm) were dissected using mouse brain matrix on ice and immediately preserved in dry ice and stored in -80°C. Peptides were extracted as previously described (Zanos et al., 2014a). OT content was measured by Prof. Landgraf's laboratory (Max Planck Institute of Psychiatry, Munich, Germany), as previously described (Landgraf, 1981) and is reported as fold change from the control group.

2.4.3 Experiment 3: Effect of EE on brain oxytocin receptor gene (*Oxtr*) expression and DNA methylation

To better understand the effects of EE on OTR regulation, another cohort of animals raised in the same EE conditions was used, and *Oxtr* mRNA levels, as well as DNA methylation were evaluated in the prefrontal cortex (see Supplementary Fig. 1C). The prefrontal cortex was targeted since (a) it is a region profoundly involved in drug addiction and emotional regulation and (b) it is one of the main regions where we observed significant alterations on the OTR binding following EE (see Fig. 4A,B). Mice were euthanized via cervical dislocation following 21 days of EE or standard housing exposure and the brains were rapidly excised, prefrontal cortex dissected and frozen in liquid nitrogen and stored in -80 °C until use. See *Supplementary Information* for details on DNA/RNA isolation, *Oxtr* gene expression analysis and *Oxtr* DNA methylation.

2.4.4 Experiment 4: Effect of chronic administration of the oxytocin analogue carbetocin on ethanol CPP and OTR binding

Since EE was found to increase OT levels in the brain (see Fig 4J), we assessed whether repeated activation of OTR by administration of the OT analogue carbetocin, mimics the effect of EE alcohol CPP and brain OTR.

2.4.4.1 Effect of 5-day carbetocin administration on OTR binding in the brain: To assess the effect of repeated activation of the OTR on the receptor binding, we administered CBT (6.4 mg/kg, i.p.; once daily) for 5 days, and 24 hours after the last injection, mice were euthanized by cervical dislocation. The brains were sectioned for OTR binding analysis, as described above (see *Supplementary information*).

2.4.4.2 Effect of chronic administration of the OT analogue carbetocin on ethanol CPP: Mice were treated with CBT (6.4 mg/kg, i.p., once daily) or saline (i.p.) for 4 days prior to the habituation phase of the CPP and every other day during the CPP protocol (also see Supplementary Fig. 1E). This protocol was chosen as we demonstrated it induced similar brain-specific alterations in OTR density with the aforementioned EE protocol (see Fig. 5A) and the intermittent administration was used to minimize tolerance effects. CBT was administered at 4:00 PM and CPP was conducted at 9:00 AM of the following day to avoid any effects of CBT on CPP performance. In order to guarantee a non-biased protocol, half of the animals received ethanol and the other half received saline on the days following CBT injection. The CPP protocol was the same as described above.

2.4.5 Experiment 5: Effect of chronic intermittent administration of an OTR antagonist on ethanol CPP in mice housed under EE conditions

To determine if the effects of EE on alcohol CPP is mediated by the oxytocinergic system, we used the selective OTR antagonist L-368,899 and assessed its effect on EE-induced ethanol CPP. Three groups of mice were used and housed in control or EE housing conditions, as described above. The first group of mice (n = 16; 8 CT and 8 EE mice) was treated with saline every other day during their housing phase, as well as during the CPP period. The second group of mice (n = 18; 10 CT and 8 EE mice) was

treated with saline every other day during their housing phase followed by the OTR antagonist, L-368,899 (5 mg/kg, i.p.; n = 18; 10 CT and 8 EE mice), 15 minutes before alcohol conditioning during the CPP phase (Days 22-30). The third group of mice was treated with L-368,899 (5 mg/kg, i.p.; n = 14; 8 CT and 6 EE mice) every other day during their housing period, as well as during the CPP protocol (Supplementary Fig. 1E). During the CPP phase, saline or L-368,899 were administered at 16:00 and CPP was conducted at 9:00 of the following day to avoid any direct effects of L-368,899 on CPP performance. All mice were tested for alcohol CPP on Day 31. The CPP protocol was the same as described above.

2.5 Statistical analyses

All statistical analyses were performed using Statistica *v7*. ANOVAs were followed by Holm-Sidak *post-hoc* analysis, when significance was reached (i.e., $p<0.05$). All data are represented as mean ± S.E.M. Details of the statistical tests and sample size for each experiment are reported in the Supplementary Table S2 (*Supplementary Information*).

3. Results

3.1 Experiment 1: EE suppresses anxiety and explorative behavior but enhances sociability and alcohol CPP

3.1.1 Anxiety-like behaviors: To evaluate the effects of EE on anxiety–like behaviors, latency to enter, number of entries and time spent on the open arms, as well as number of total crosses were measured in the EPM test (Fig. 1A, B, C, D). EE mice showed significantly lower latency to enter the open arm compared to controls ($p<0.05$), which has been associated with decreased anxiety-like behaviors in rodents (Fernandez et al., 2003). There were no differences between the experimental groups for the open arms entries ($p>0.05$; Fig. 1C), or total crosses ($p>0.05$; Fig. 1D).

3.1.2 Locomotor activity: We assessed the locomotor activity of EE mice in an open field environment during the habituation period of the NOR test. EE mice displayed decreased locomotor activity compared to the controls ($p<0.001$; Fig. 1E).

3.1.3 Novel object recognition – time interacting with objects: During all the test phases (acquisition, 1 hr retention and 24 hrs retention), EE animals showed a significant decreased time exploring the objects compared to CT group ($p<0.001$; Fig. 1F).

3.1.4 Novel object recognition – discrimination ratio: EE did not alter 1- or 24hrs object recognition memory compared to controls ($p>0.05$; Fig. 1G) indicating a lack of effect on short and long-term object recognition memory. However, we observed decreased discrimination ratio at 24 hours compared to 1-hour retention, irrespective of the environmental conditions ($p<0.001$; Fig. 1G).

3.1.5 Ethanol CPP: Ethanol conditioned preference (Post-Cond/Pre-Cond time in the drug-paired compartment) in control and EE animals is shown in Fig. 1H. While all groups successfully conditioned to ethanol (One-sample *t*-test with the hypothetical value=1, followed by Wilcoxon Signed-Rank Test; CT: $p<0.05$; EE: $p<0.01$), EE animals had a 3.5-fold higher ethanol conditioning ratio compared to CT mice ($p<0.01$), suggesting a profound enhancement of the reinforcing effect of alcohol in the EE group compared to control.

3.1.6 Sociability behaviors: EE mice exhibited a significantly higher social interaction ratio compared to control mice ($p<0.05$; Fig.2A). Similar to the decreased exploration manifested by the EE animals during the habituation phase of the novel object recognition test, we also observed decreased locomotor activity of these mice during the social interaction paradigm ($p<0.001$; Fig.2B).

3.2 Experiment 2: Region specific alterations of OT and OTR in the brain of mice exposed to EE

3.2.1 OTR autoradiographic binding: EE decreased OTR binding in the olfactory nuclei, prefrontal cortex, cingulate cortex and piriform cortex compared to controls ($p<0.001$) (Fig. 3A-F). No significant ANOVA effects were observed for the other brain regions analyzed.

3.2.2 OT peptide levels: No significant differences in OT peptide levels caused by EE were observed in the olfactory nuclei, prefrontal cortex, or amygdala compared to controls ($p>0.05$; Fig. 3G-I). However, EE increased hypothalamic OT levels compared to controls ($p=0.05$; Fig. 3J).

3.2.3 Plasma corticosterone levels: EE did not alter plasma corticosterone levels compared to controls ($p>0.05$; Fig. 3K) and corticosterone levels following 21 days exposure to EE or CT conditions were comparable to baseline.

3.3 Experiment 3: EE does not cause significant alterations in *Oxtr* gene expression and DNA methylation

3.3.1 Oxtr gene expression: EE did not induce any difference in the mRNA levels in the prefrontal cortex ($p>0.05$; Supplementary Fig. 2A). There was no difference in GAPDH levels between the two groups (data not shown).

3.3.2 OTR DNA methylation: From the 10 CpG positions of the *Oxtr* gene analysed, EE caused only a subtle decrease in *Oxtr* DNA methylation in CpG position 4 within the prefrontal cortex of mice ($p<0.05$; Supplementary Fig. 2B).

3.4 Experiment 4: Repeated administration of carbetocin induces stimulatory effects on ethanol CPP and OTR binding, comparable to the effects of EE

To further explore the involvement of the oxytocinergic system in the enhancing effect of EE on alcohol CPP, we assessed the effect of chronic administration of the oxytocin analogue carbetocin on alcohol CPP and OTR binding in the brain.

3.4.1 OTR autoradiographic binding: CBT decreased OTR binding in the anterior olfactory nucleus-medial ($p<0.01$), anterior olfactory nucleus-ventral (statistical trend: $p=0.06$), prefrontal cortex ($p<0.05$), piriform cortex ($p<0.05$) and lateral septum ($p<0.05$). No significant effects of CBT were observed in any other brain region analyzed (Fig. 4A).

3.4.2 Ethanol CPP: While both saline- and carbetocin-treated mice developed ethanol-induced conditioned-place preference (One-sample *t*-test; hypothetical value=1, followed by Wilcoxon Signed-Rank Test; SAL: $p=0.05$; CBT: $p<0.01$), carbetocin-treated mice showed an enhanced response to the ethanol conditioned place preference, as indicated by the higher Post-conditioning/Pre-conditioning ratio compared to controls ($p=0.05$; Fig. 4B), similar to the effect of EE.

3.5 Experiment 5: Chronic intermittent administration of L-368,899 during EE exposure reverses the EE-induced enhancement of alcohol CPP

All mice of this study developed ethanol-induced CPP (One-sample *t*-test; hypothetical value=1, followed by Wilcoxon Signed-Rank Test; $p<0.05$). EE enhanced ethanol CPP in the SAL-treated mice ($p<0.05$ *vs* SAL-CT; Fig. 6). L-368,899 administration during the acquisition of ethanol CPP, did not prevent the enhanced EE-induced ethanol CPP ($p<0.05$ *vs* CT mice administered L-368,899 only during CPP; Fig. 5). In contrast, administration of L-368,899 during EE housing conditions and during ethanol CPP prevented the enhancement effect of EE on ethanol CPP ($p<0.05$ *vs* EE mice administered L-368,899 only during CPP; Fig. 5). This is also supported by the lack of

difference in CPP ratio between the control animals receiving L-368,899 and the corresponding EE group ($p>0.05$).

4. Discussion

We demonstrated that exposure to EE profoundly enhances the rewarding effects of alcohol. EE increases OT levels in the hypothalamus and decreases OTR binding in olfactory and key cortical areas of the brain. Notably, repeated activation of the central OTR system by administration of carbetocin yielded similar behavioral and neurochemical effects as EE. Specifically, it induced an augmentation of ethanol CPP and decreased OTR binding in almost the same brain regions as EE did. Blockade of the OTR along the duration of EE housing, but not solely during CPP acquisition, prevented the EE-induced augmentation of ethanol reward. Together these findings clearly demonstrate that EE enhances the rewarding effect of alcohol via a novel, previously unidentified, oxytocinergic mechanism.

Despite the evidence indicating a protective role of EE (Stairs and Bardo, 2009) and OT administration in drug addiction (King et al., 2017; McGregor and Bowen, 2012; Peters et al., 2013; Zanos et al., 2017), we demonstrated a clear augmentation of ethanol-seeking behavior following EE exposure, via a chronic activation of the central oxytocinergic system. Indeed, not only did we demonstrate that EE increases OT peptide levels in the hypothalamus, where OT is produced, and that repeated administration of the OTR agonist carbetocin, similar to the EE, enhances ethanol CPP, we also showed that administration of the OTR antagonist L-368,860 during the EE period prevented the pro-alcohol seeking properties of EE. Given the anxiolytic properties of OT (Gimpl and Fahrenholz, 2001), it is tempting to speculate that chronic activation of the oxytocinergic system by EE exposure, or by repeated CBT

administration, exerted an anxiolytic effect that could drive the observed augmentation of alcohol CPP via reducing the initial aversive properties of the drug. Indeed, there is well-documented evidence of aversive properties of initial alcohol use (Chester et al., 2003; Stewart and Grupp, 1989). In agreement, OT administration in rats was shown to increase the initial nicotine intake, and to also alleviate the aversive properties of nicotine (Lee et al., 2016). Together, these findings suggest that enhanced oxytocinergic activity might be a causative factor for the initial drug use and that oxytocinergic-based pharmacotherapies should be considered with caution for at least the initial stages of alcohol use.

In contrast to the increased OT levels, we observed a downregulation of OTR binding in the olfactory nuclei, prefrontal cortex, cingulate cortex and piriform cortex following chronic exposure to EE. This OTR downregulation could have also directly contributed to the enhancement of ethanol CPP, since overexpression of OTRs in the brain of mice has been previously shown to reduce ethanol-primed CPP (Bahi, 2015). It is highly plausible that this brain region-specific decrease in OTR levels to be the result of compensatory neuroadaptations driven by a persistent EE-induced increase of OT levels in the brain, which is consistent with other studies showing concomitant opposite central changes in OT peptide and OTR levels (Zanos et al., 2014a). In fact, OTRs undergo rapid desensitization, clathrin-dependent internalization and subsequently down-regulation following persistent agonist stimulation (Evans et al., 1997). Accordingly, we showed that chronic activation of OTR by repeated administration of CBT decreases OTR binding in very similar brain regions as in EE-exposed mice. It is highly unlikely that EE-induced changes in OTR protein levels are driven by alterations in OTR mRNA synthesis, since we showed that EE does not alter OTR mRNA levels and does not induce significant epigenetic changes in the *Oxtr* gene in the prefrontal

cortex; thus, supporting the concept that EE-induced changes in OTR are occurring at the receptor trafficking level (i.e., internalization, recycling). OT neurotransmission within the olfactory brain regions, where we observed the OTR changes, regulates social recognition responses in rats (Dluzen et al., 2000) and plays a critical role in social cue-processing (Oettl et al., 2016). Thus, activation of the OTR by enhanced oxytocinergic neurotransmission within the olfactory nuclei, might be involved in the observed increase in sociability behavior induced by EE. Social alcohol drinking has been associated with increased drinking behavior (Cruz et al., 2012; Osgood et al., 2013), suggesting that the pro-social effects of EE (likely via activation of the OT system) may also contribute to the alcohol reward enhancing effect observed in this study, although this hypothesis needs further investigation.

Interestingly, exposure to EE decreased locomotor activity in a novel environment and reduced exploration of novel objects, as demonstrated in our NOR experiments. Reduced locomotor activity in EE-exposed mice has been associated with faster habituation to a novel environment and faster sensory/memory processing (Amaral et al., 2008). However, no differences were detected in memory index among groups, as measured by the NOR test, suggesting that increased ethanol CPP in EE mice is not due to learning/memory improvement. Therefore, the results of our study suggest a distinct and differential effect of EE exposure on different types of motivational behaviors. While EE seems to suppress exploration of novel objects and novel environment, it increases the reinforcing properties of social interaction and alcohol, thus shifting motivational drive from environmental/contextual cue-induced process towards social and drug related cues/rewards. This supports our hypothesis that molecular markers associated with social and drug reward interplay, such as OT, are involved in the behavioral consequences of EE.

5. Conclusions

The present study demonstrates that exposure to EE, via an oxytocin-dependent mechanism, or repeated exogenous OT-based pharmacotherapy may constitute important risk factors for alcohol use/misuse, at least during the initial stages of alcohol consumption experience. The fact that both EE and OT exhibit a strong pro-social effect may at least partially shed light on the mechanism underlying the importance of social factors in the initiation of drug use. This study offers a unique prospective on the neurobiological understanding of the initial stages of alcohol use/misuse driven by a complex environmental-social interplay.

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Conflict of interest

The authors declare no conflict of interest.

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Position: 87455

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Table/Figure Legends

Table 1. *Oxtr* mRNA expression primer sets

Table 2. Statistical analyses

Figure 1. Effects of environmental enrichment on anxiety-like behaviors, locomotion, object exploration and novel object recognition memory and ethanol-induced conditioned-place preference. **(A)** Latency to enter the open arms, **(B)** time spent in the open arms, **(C)** number of entries in the open arms and **(D)** number of total crosses were measured in the elevated plus-maze test (n=20/group). **(E)** Locomotor activity during the first session of the novel object recognition habituation phase (10 min). **(F)** Time exploring both objects during the acquisition (5 min) and retention (5 min) phases of the NOR test and **(G)** Discrimination ratio (time exploring new object / time exploring both objects) 1 and 24 hours after first exposure to objects were measured in the NOR (n=18-21/group). **(H)** Conditioning ratio (time spent on drug-paired compartment during post-conditioning day/time spent on that compartment during pre-conditioning day) was measured in the alcohol CPP paradigm (n=9-11/group). Data are the mean ± SEM. * $p<0,05$; ** $p<0.01$; *** $p<0,001$. *Abbreviations*: CT, control; EE, environmental enrichment.

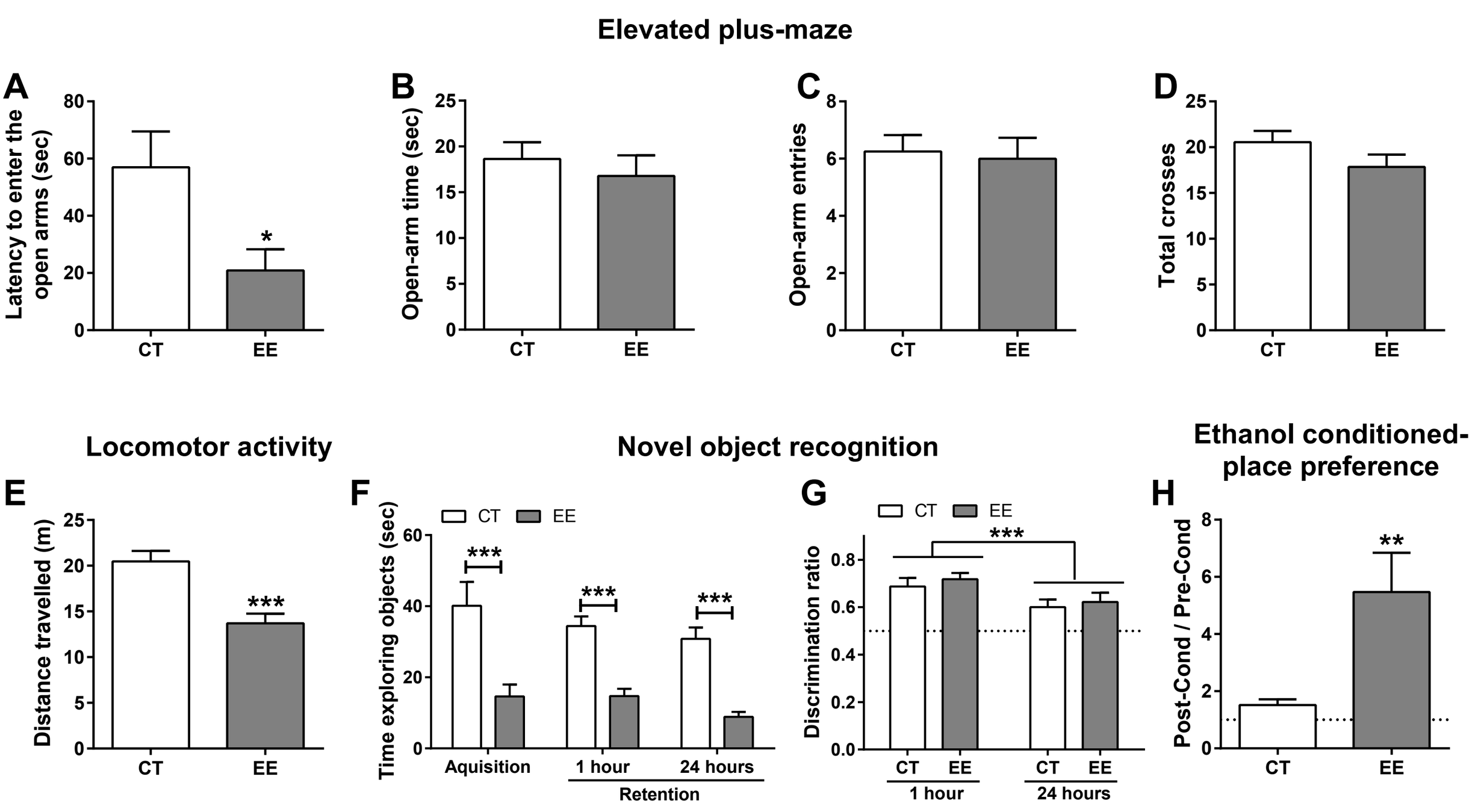
Figure 2. Effect of environmental enrichment on sociability behaviors. **(A)** Social interaction ratio, defined as the time spent by the test mouse interacting with the stranger mouse divided by the time interacting with an empty cage was calculated as an indicator of sociability behavior in the social approach test. **(B)** Locomotor activity during the social approach test. Data are the mean ± SEM. * $p<0,05$; *** $p<0.001$ (n=8-10/group). *Abbreviations*: CT, control; EE, environmental enrichment.

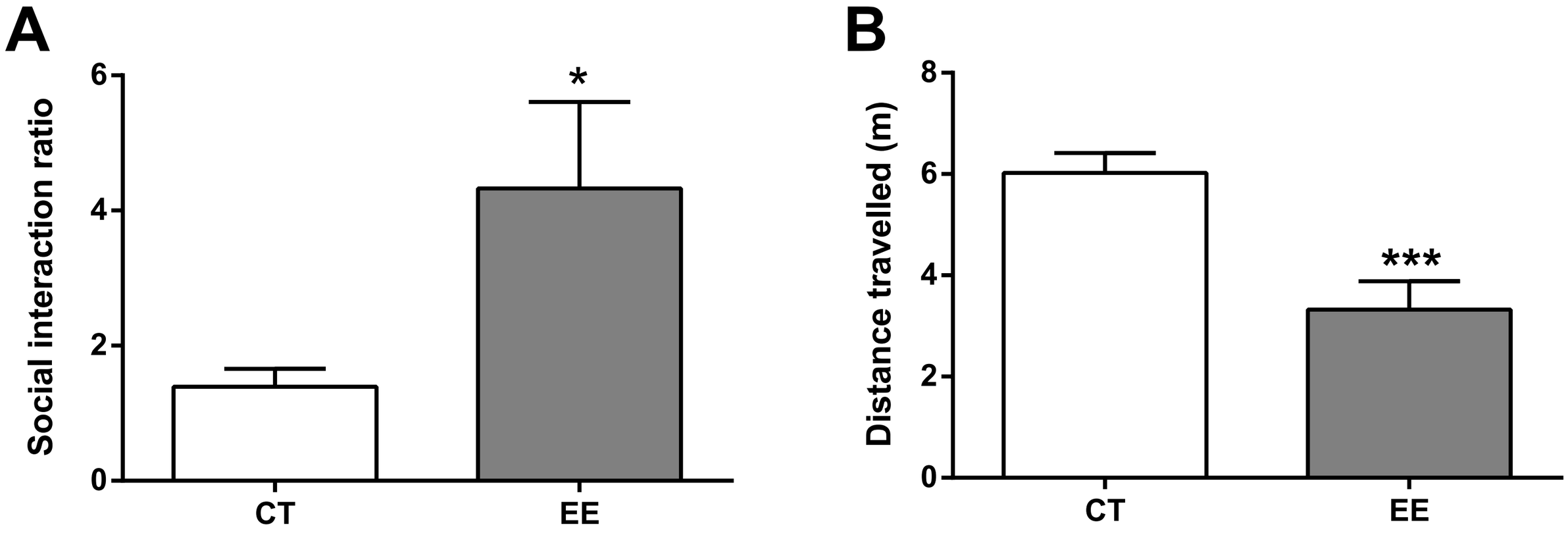
Figure 3. Effects of environmental enrichment on brain OTR binding, OT peptide and plasma corticosterone levels. **(A)** Computer-enhanced representative OTR autoradiograms of adjacent coronal brain sections OTRs were labelled with [^{125}I]-OVTA (50 pM) and shown at the level of the olfactory nuclei (Bregma 2.46, row 1), at the level of the CPu (Bregma 0.86, row 2), at the level of the globus pallidus (Bregma 0.14, row 3) and at the level of the thalamus (Bregma -2.06, row 4). The color bar illustrates a pseudo-color interpretation of black and white film images in fmol/mg tissue equivalent. Representative images for the non-specific binding (50pM [^{125}I]-OVTA in the presence of 50μM unlabeled oxytocin) are shown. Quantitative oxytocin receptor autoradiographic binding in the (B) olfactory nuclei, (C) cortex, (D) striatum, (E) septum and (F) forebrain of mice. Oxytocin levels in the **(G)** olfactory nuclei, **(H)** prefrontal cortex, **(I)** amygdala and **(J)** hypothalamus. **(K)** Plasma corticosterone levels at baseline and following 21 day exposure to EE or CT. Data are the mean ± SEM. *p<0,05; *** p<0.001 (n=5/group). *Abbreviations*: AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Amy, amygdala; AOL, anterior olfactory nucleus-lateral; AOM, anterior olfactory nucleus-medial; AOV, anterior olfactory nucleus-ventral; CgCx, cingulate cortex; CPu, caudate-putamen; CT, control; EE, environmental enrichment; Hip, hippocampus; Hyp, hypothalamus; LS, lateral septum; MS, medial septum; OT, oxytocin; OTR, oxytocin receptor; PFC, prefrontal cortex; PirCx, piriform cortex; Th, thalamus; VDB, vertical limb of the diagonal band of Broca.

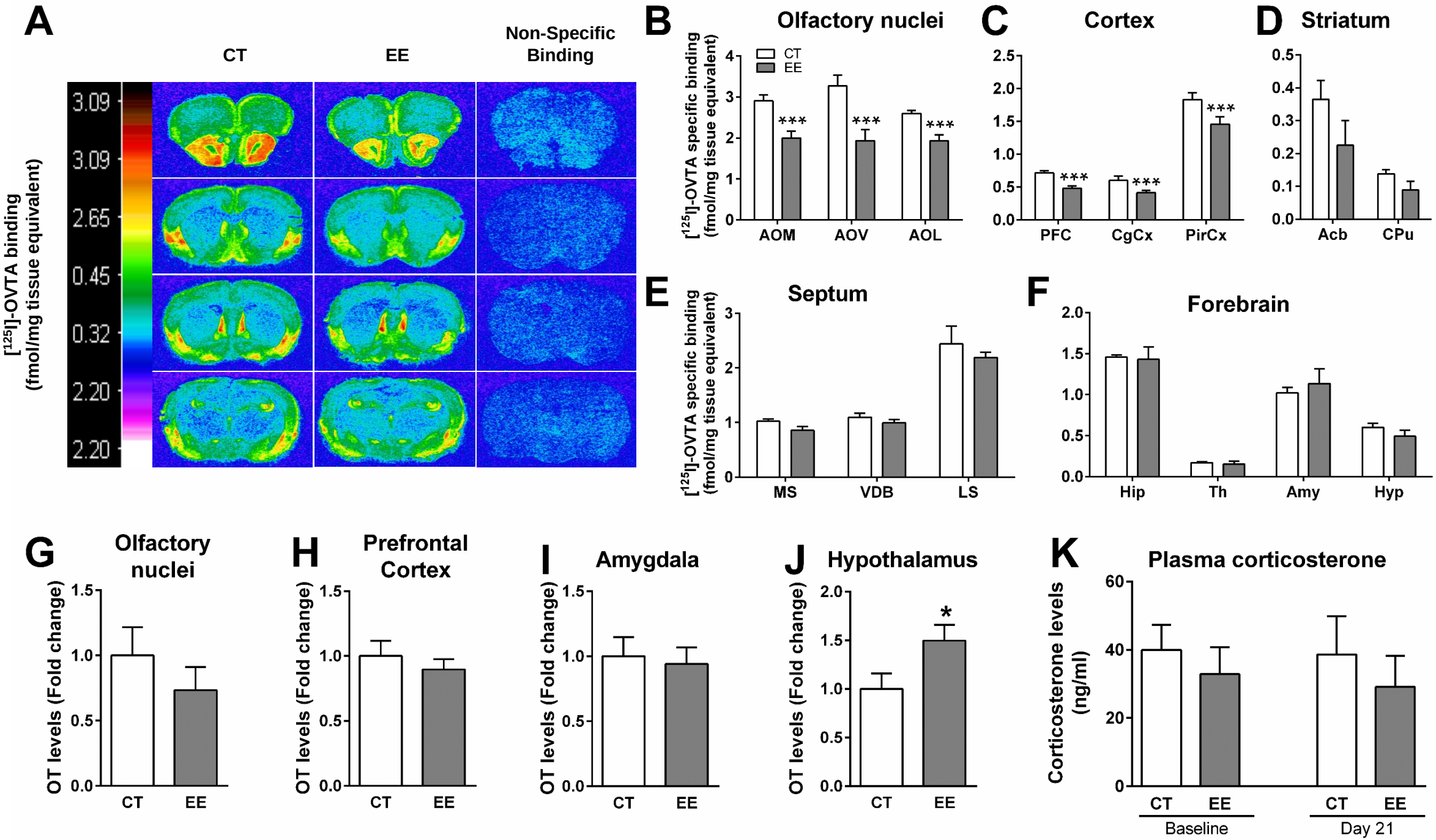
Figure 4. Effect of repeated activation of OTR on ethanol-induced conditioned-place preference and OTR levels (A) Quantitative oxytocin receptor autoradiographic binding in brain regions of mice following a 5-day saline or CBT administration period (n=6/group). **(B)** Mice were treated with CBT (6.4 mg/kg, i.p., once daily) or saline (i.p.) for 4 days prior to the habituation phase of the CPP and every other day during the

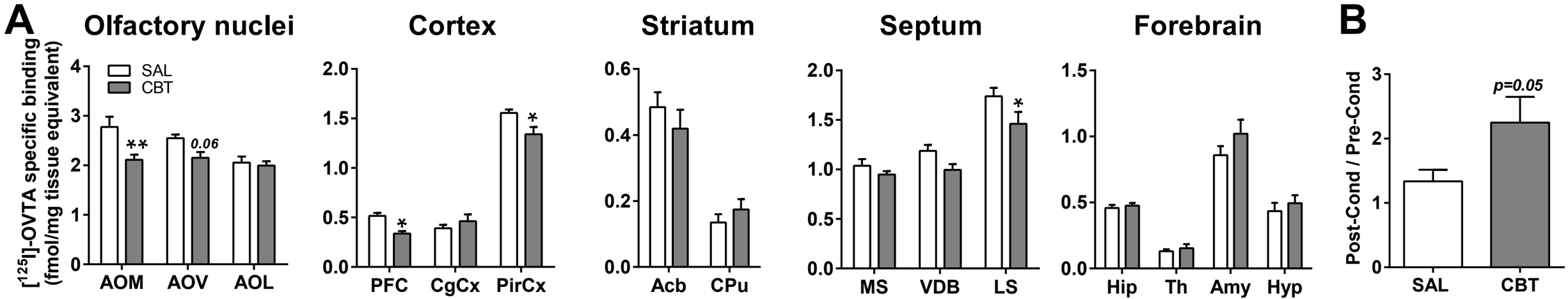
CPP protocol. Conditioning ratio (time spent in ethanol-paired compared during post-conditioning day/time spent on drug-paired compartment during pre-conditioning day) of mice treated with saline (SAL) or carbetocin (CBT) (n=9-10/group). Data are the mean ± SEM. * $p<0.05$; ** $p<0.01$. *Abbreviations:* AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Amy, amygdala; AOL, anterior olfactory nucleus-lateral; AOM, anterior olfactory nucleus-medial; AOV, anterior olfactory nucleus-ventral; CBT, carbetocin; CgCx, cingulate cortex; CPu, caudate-putamen; CT, control; EE, environmental enrichment; Hip, hippocampus; Hyp, hypothalamus; LS, lateral septum; MS, medial septum; PFC, prefrontal cortex; PirCx, piriform cortex; SAL, saline; Th, thalamus; VDB, vertical limb of the diagonal band of Broca.

Figure 5. Effect of OTR blockade in EE-induced alcohol CPP. Mice were treated with the OTR antagonist, L-368,899 (5 mg/kg, i.p.;) or saline, every other day during the EE or CT housing period and during the ethanol CPP protocol or during the CPP period alone. Conditioning ratio of CT and EE mice treated with SAL or the OTR antagonist L-368,899 (n=6-10/group). Conditioning ratio was calculated as the time spent in ethanol-paired compared during post-conditioning day divided by the time spent on drug-paired compartment during pre-conditioning day. Data are the mean ± SEM. * $p<0.05$; ** $p<0.01$. *Abbreviations:* CPP, conditioned-place preference; CT, control; EE, environmental enrichment; SAL, saline.


Elevated plus-maze
A
Latency to enter the open arms (sec)
80
60
40
20
0
*
CT
EE
B
Open-arm time (sec)
25
20
15
10
5
0
CT
EE
C
Open-arm entries
8
6
4
2
0
CT
EE
D
Total crosses
25
20
15
10
5
0
CT
EE
Locomotor activity
E
Distance travelled (m)
25
20
15
10
5
0
***
CT
EE
Novel object recognition
F
CT
EE
Time exploring objects (sec)
60
40
20
0
***
***
***
Aquisition
1 hour
24 hours
Retention
G
CT
EE
***
Discrimination ratio
0.8
0.6
0.4
0.2
0.0
CT
EE
CT
EE
1 hour
24 hours
Ethanol conditioned-place preference
H
Post-Cond / Pre-Cond
8
6
4
2
0
**
CT
EE


A
Social interaction ratio
6
4
2
0
*
CT
EE
B
Distance travelled (m)
8
6
4
2
0
***
CT
EE


A
CT
EE
Non-Specific Binding
[125I]-OVTA binding (fmol/mg tissue equivalent)
3.09
3.09
2.65
0.45
0.32
2.20
2.20
B
Olfactory nuclei
[125I]-OVTA specific binding (fmol/mg tissue equivalent)
CT
EE
***
AOM
AOV
AOL
C
Cortex
PFC
CgCx
PirCx
D
Striatum
Acb
CPu
E
Septum
MS
VDB
LS
F
Forebrain
Hip
Th
Amy
Hyp
G
Olfactory nuclei
OT levels (Fold change)
H
Prefrontal Cortex
I
Amygdala
J
Hypothalamus
*
K
Plasma corticosterone
Corticosterone levels (ng/ml)
Baseline
Day 21


A
Olfactory nuclei
Cortex
Striatum
Septum
Forebrain
[125I]-OVTA specific binding (fmol/mg tissue equivalent)
SAL
CBT
**
0.06
*
*
*
*
AOM
AOV
AOL
PFC
CgCx
PirCx
Acb
CPu
MS
VDB
LS
Hip
Th
Amy
Hyp
B
Post-Cond / Pre-Cond
p=0.05
SAL
CBT

Controls
EE

Post-Cond / Pre-Cond

4
3
2
1
0

*
*
*

SAL - all time
during CT/EE
and CPP

SAL - for L
during CT/EE
and **L-368,899**
during CPP

L-368,899 all
during EE
and CPP

Supplementary Materials and Methods

Elevated Plus-Maze (EPM)

The maze consisted of four arms; two of them were open (33.5 cm length x 7 cm width) and the other two were enclosed (33.5 cm length x 7 cm width, enclosed by 19-cm walls). The apparatus was elevated 50 cm above the floor. Each animal was placed in the center of the EPM apparatus with its head facing the open arm. Mice were allowed to explore the apparatus for a period of 5 min. Experiment was performed in the afternoon (14:00 – 16:00) under low lighting conditions. Between each session, the maze was cleaned with an alcohol solution of 5% to avoid possible olfactory interferences. All sessions were recorded by an overhead digital video-camera and were analyzed by a trained observer blind to the experimental groups. Latency to enter, time spent in the open arms and total open- and closed-arm entries (total crosses) were measured for the total period of 5 minutes.

Novel Object Recognition (NOR)

The NOR apparatus consisted of a white open-filed chamber (35 x 29 x 16 cm) and all sessions were performed during the afternoon (13:00 – 16:00), under dim light conditions. During the habituation phase (Day1), the animals were placed in the NOR chamber and explored the apparatus for 10 min three times with 1 hour interval. Twenty-four hours later, during the acquisition phase, animals were placed in the NOR chamber containing two identical objects (A+A), placed equally distant from the walls of the box, and were allowed to explore the apparatus for 10 minutes. After a retention period of 1(short-term memory) and 24 (long-term memory) hours following the acquisition phase, one of the objects was replaced with a new object (A+B and A+C, respectively) and animals were allowed to explore the apparatus for 5 minutes. All the objects were equal-sized (object A: glass bottle; object B: Lego© toy; object C pencil sharpener) and were attached to the floor of the NOR chamber, so that the animals were unable to move them. All phases of the test were recorded by a digital video-camera and the amount of time animals spent exploring both objects was evaluated by a trained observer. Interaction with an object was considered if the animal touched

the objects with their front paws or nose (sniffing), or faced the object in an equal or less than 1 cm distance. When an animal climbed and/or sat on top of the objects, it was not considered as object exploration. After each session, the box and both objects were cleaned with a 70% alcohol solution to prevent possible odor cues. The discrimination ratio (DR) was calculated as the time spent exploring the novel object divided by the time spent exploring the new and the familiar object ($DR = t_{novel}/[t_{novel} + t_{familiar}]$) on each test session.

Conditioned-place preference (CPP)

The CPP apparatus consisted of a rectangular box (44 x 14 x 14cm) divided into three chambers: two equal-sized (17.5 x 13 x 14cm) end-chambers; one with black walls and bar floor and the other one with white walls and grid floor. These end-chambers were separated by a smaller middle chamber (7 x 13 x 14cm) with grey walls and two removable doors.

The CPP protocol consisted of a pre-conditioning phase, 8 conditioning sessions and a post-conditioning test phase. On Day 1 (pre-conditioning phase) each test mouse was placed in the center chamber with the guillotine doors open allowing them to explore all compartments for a period of 15 minutes. During the conditioning phase, ethanol (2 g/kg, i.p.) was administered and mice were placed in their least-preferred side (CS+; measured during the pre-conditioning phase) on alternating days (i.e., days 2, 4, 6 and 8) for 5 minutes. On days 3, 5, 7 and 9 mice received saline injections and were paired with their pre-conditioning preferred compartments (CS-) for 5 minutes.

During the post-conditioning test phase (Day 10), mice were placed in the CPP middle chamber with the doors open and allowed to freely explore the whole apparatus for 15 min. Sessions were recorded using a digital video-camera. Time spent in each compartment was measured and CPP was reported as a ratio between the time the mice spent in the drug-paired compartment during the post-conditioning phase and the pre-conditioning phase (CPP ratio = Post-Cond/Pre-Cond).

Three-chamber social approach test

The three-chamber social approach test was used to measure animal's sociability behaviours as previously described (Zanos *et al*, 2016), with minor modifications (see Fig. 1Aii for timeline; also see *Supplementary Information*). The apparatus consisted of a rectangular box (48 cm length, 34 cm width and 23 cm height) divided in three chambers: two end-chambers (17 cm length) and a central smaller chamber (15 cm length). Separate cohort of animals was used for this experiment.

The protocol consisted of a habituation and a social interaction phase. During the habituation phase, each animal was placed in the central compartment and allowed to freely explore the empty apparatus for 10 minutes. Immediately after, a novel conspecific animal was placed inside a cylindrical cage (9 cm height, 9 cm diameter) into one of the end-chambers. An exact same cage was placed on the other end-chamber of the apparatus, but this cage remained empty. Animals were allowed to explore the apparatus for another 10 minutes.

Both phases were recorded and the time each mouse spent interacting (nose-sniffing contact) with the novel animal and the empty cage were assessed using TopScan automated system (CleverSys, Inc, Reston, VA, USA).

Oxytocin receptor autoradiography

Tissue preparation: Brains were excised, immediately frozen in -25ºC isopentane solution and stored at -80ºC. Prior to sectioning, brains were removed from -80ºC and placed into a -21˚C cryostat apparatus (Zeiss Microm 505E, Hertfordshire, U.K.) and coronally aligned by fixing the cerebellum onto a mounting stage using a plastic embedding liquid (O.C.T. compound, BDH chemicals, Dorset, U.K.).

Adjacent 20 µm coronal brain sections were cut at 300µm distances using a cryostat apparatus (Zeiss Microm 505E, Hertfordshire, U.K.). Slices were thaw-mounted onto gelatine-coated ice-cold microscope slides.

OTR binding: Sections were rinsed two times in a rinse buffer solution (50mM Tris-HCl, pH 7.4, room temperature) for 10 min each to remove endogenous OT. Total binding was determined by incubating sections with 50pM [^{125}I]-ornithine vasotocin (OVTA) in an incubation buffer containing: 50mM Tris-HCl, 10mM $MgCl_2$, 1mM ethylenediaminetetraacetic acid (EDTA), 0.1 % w/v bovine serum albumin, 0.05 % w/v bacitracin; Sigma-Aldrich, Poole, UK (pH 7.4 at room temperature). To determine non-specific binding, adjacent sections were incubated with [^{125}I]-OVTA (50 pM) in the presence of 50µM unlabelled (Thr^4,Gly^7)-oxytocin (Bachem, Germany),. Following an incubation period of 60 min, slides were rinsed for 3 x 5 min in ice-cold rinse buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$ (pH 7.4), followed by a 30-min wash in the ice-cold rinse buffer and then a 2-sec wash in ice-cold distilled water. Slides were then dried and stored in sealed containers with anhydrous calcium sulphate (Drierite-BDH Chemicals, Dorset, U.K.) for 2 days.

Autoradiographic film apposition: Slides were apposed to Kodak MR-1 films (Sigma-Aldrich, UK) in Hypercassettes with autoradiographic [^{14}C] microscales of known radioactive concentrations (GE Healthcare Life Sciences, Amersham, U.K.) for 3 days.

Film development: The films were developed in a 50% Kodak D19 developer solution (Sigma-Aldrich, Poole, UK) for 3 minutes, following by a 30-sec wash distilled water cotaiining glacial acetic acid. Then films were fixed by a 5-minwash in a Kodak rapid fix solution (Sigma-Aldrich, Poole, UK).

Image analysis (Quantitative autoradiography): Quantitative autoradiographic analysis of all structures were carried out by reference to the mouse brain atlas of Frankin and Paxinos (1997) and binding was analysed as previously described (Kitchen et al., 1997), using MCID image analyser (Image Research, Ontario, Canada). Specific binding was determined by subtracting the non-sepcific binding from the total binding in the images of the brain sections.

DNA/RNA isolation

DNA/RNA was simultaneously isolated from frontal cortex tissue frozen in RNALater. DNA and RNA were isolated using Qiagen's AllPrep DNA/RNA mini kit (Qiagen, UK) following the manufacturer's protocol. Quantification was performed using a NanoDrop 2000 UV-Vis spectrophotometer (Thermoscientific, UK) and quality was confirmed by gel electrophoresis. Harvested DNA was stored at 4°C for immediate use and at -20°C for long-term storage. RNA was stored at -80°C.

Gene Expression

Bisulphate treatment of DNA was performed and quantitative Real Time Reverse Transcriptase PCR was used to quantify *Oxtr* gene expression. A set of primers was designed for *Oxtr* and one housekeeping gene *Gapdh* using web-based Primer 3 software (see Supplementary Table 1), blasted using UCSC Genome Browser blast program for sequence similarity and custom-synthesized by Eurofins Genomics, Germany. The experiment was performed in triplicates.

DNA methylation

All the reagents and buffers used were obtained from Qiagen, UK, and the procedure followed as previously described by Coley *et al.*,(2012), with minor modifications, as described below.

Bisulfite treatment was performed on 500 ng DNA/sample using Zymo EZ Gold Kit (Cambridge Biosciences, Cambridge, UK), according to the manufacture's protocol. The bisulfite-converted DNA was than eluted with 20μL of EB buffer per column and stored at -20°C. For methylation analysis, the target sequence was identified in CpG Island 89 within the promoter region using UCSC Genome Browser Mouse NCBI 37/mm9 assembly. Primers were designed for pyrosequencing using PyroMark Assay Design 2.0.0 software taking into consideration the number of CpGs per target and filtering for an overall score for a particular primer set (*i.e.* GC content, melting temperature, loop formation, base-pair mis-match, among others). A score above 80% was considered as suitable. The selected primer set targeting 10 CpG sites and with an overall score of 87% was as follows:

Forward primer: $^{5'}$GATATTAGTAATAGTAGGTAGGTGGAGG$^{3'}$

Reverse primer: Bio-$^{5'}$ATCCTATATCTCATACTATTCCTAACTC$^{3'}$

Sequencing Primer:$^{5'}$GGTGTTTTATGAAAAAGAAGAG$^{3'}$

Sequence to analyze: GYGYGAGTGT TTGTGGYGYG TYGTAYGTAG YGTTAGTAGT AYGTAYGYGT TGTTATTTAG AGTTAGGAAT AGT

HotStart Taq polymerase (Qiagen, UK) was used for PCR amplification of the target sequence. The final PCR reaction volume was 50μl/sample, with 2μl of bisulfite-converted DNA per reaction. The PCR cycle used was: 15 minutes at 95°C, followed by 50 cycles of denaturation at 95°C, annealing at 60°C and extension at 72°C for 30 seconds each, followed by final extension at 72°C for 5 minutes. The amplification product (269 bp) was confirmed by gel electrophoresis. All PCR runs included a non-template control. PSQ MD pyrosequencer (Biotage) was used for determining the level of DNA methylation.

Statistical analyses

For the analyses of the elevated plus-maze, locomotor activity, ethanol CPP conditioning ratio, social interaction ratio, distance travelled during the three-chamber social approach test, brain OT peptide levels and Oxtr mRNA expression levels Student's unpaired t-test was performed. The NOR discrimination ratio and plasma corticosterone levels were analyzed by two-way repeated

measures ANOVA with factors "experimental group" and "phase" for the NOR, and "experimental group" and "day" for plasma corticosterone levels. Two-way ANOVA for factors "experimental group" and "CpG site" was used to analyze the OTR methylation data, followed by individual non-parametric *t*-tests for confirmation of the ANOVA results. To analyze the effects of EE and CBT on OTR binding two-way ANOVA was applied in each brain sub-region with factors "brain region" and "treatment or experimental group". Two-way ANOVA, with factors "experimental group" and "treatment" was used to analyze the effects of the OTR antagonist L-368,899 on ethanol CPP. ANOVA's were followed by Duncan's *post-hoc* test where appropriate.

Legends for Supplementary Figures

Supplementary Figure 1. Diagrammatical representation of experimental design. (A) Behavioural characterization of mice exposed to EE (experiment 1). Experimental timeline for the **(Ai)** anxiety, memory and ethanol CPP tests, (**Aii**) sociability behaviors; **(B)** Brain oxytocin receptor autoradiography and OT brain levels of mice exposed to EE (experiment 2); **(C)** Brain *Oxtr* mRNA expression and DNA methylation in mice exposed to EE (experiment 3); **(E)** Effect of intermittent carbetocin administration on OTR binding and ethanol CPP (experiment 4); **(D)** Effect of intermittent administration of the OTR antagonist (OTRA) L-368,899 on ethanol CPP of mice houseed under EE conditions (experiment 5). *Abbreviations*: CBT, carbetocin; CPP, conditioned-place preference; CORT, corticosterone; CT, control; EE, environmental enrichment; EPM, elevated plus-maze; NOR, novel object recognition; OT, oxytocin; OTR, oxytocin receptor; *Oxtr*, oxytocin receptor gene; SAL, saline.

Supplementary Figure 2. Effects of environmental enrichment on frontal cortex *Oxtr* gene expression and DNA methylation. (A) *Oxtr* mRNA expression levels were measured using Quantitative Real Time Reverse Transcriptase PCR, using three different primer sets for confirmation purposes (n=9-10 mice per group). **(B)** PSQ MD pyrosequencer was used for determining the level of DNA methylation by pyrosequencing the bisulfute-converted DNA (n=8-9 per group). Data are the mean ± SEM. * $p<0.05$. *Abbreviations:* CT, control; EE, environmental enrichment groups; *Oxtr*, oxytocin receptor gene.

Legends for Supplementary Tables

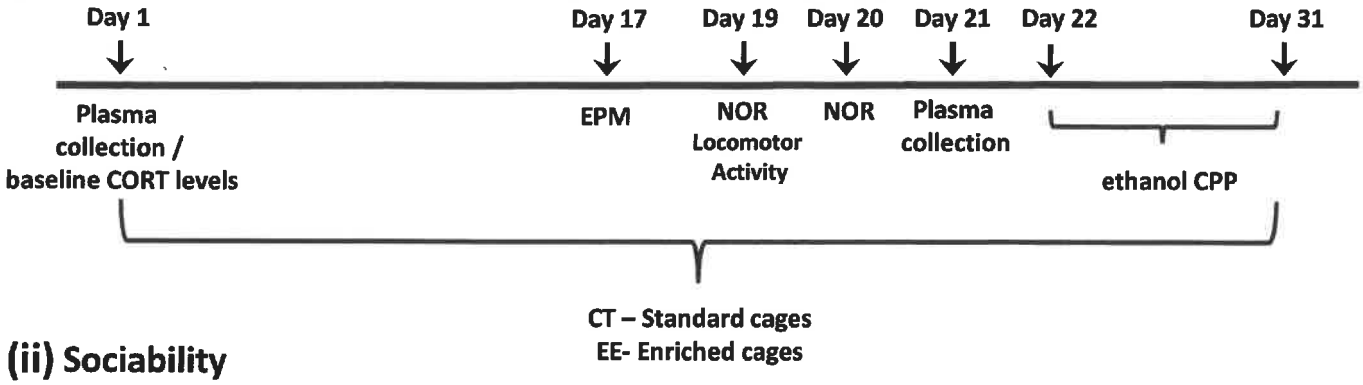
Supplementary Table S1. *Oxtr* mRNA primer sequences. *Oxtr* mRNA expression levels were measured using Quantitative Real Time Reverse Transcriptase PCR, using three different primer

sets for confirmation purposes. *Abbreviations:* GC, guanine-cytosine; Nt, nucleotide; *Oxtr*, oxytocin receptor gene; Tm, melting temperature.

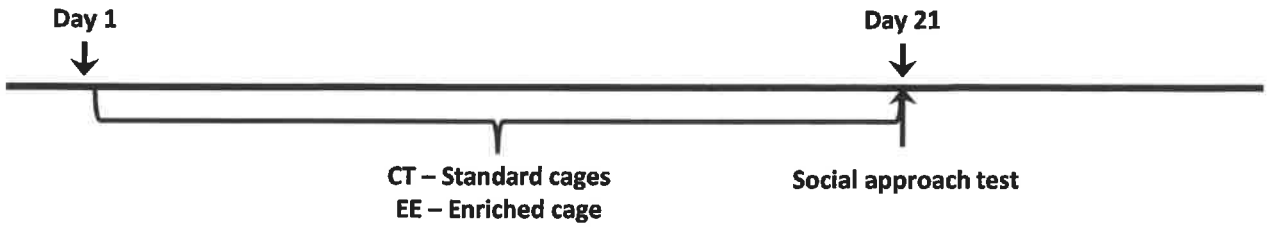
Supplementary Table S2. ***Relevant statistical analyses.*** *Abbreviations:* AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Amy, amygdala; AOL, anterior olfactory nucleus-lateral; AOM, anterior olfactory nucleus-medial; AOV, anterior olfactory nucleus-ventral; CBT, carbetocin; CgCx, cingulate cortex; CPP, conditioned-place preference; CPu, caudate-putamen; CT, control; EE, environmental enrichment; Hip, hippocampus; Hyp, hypothalamus; LS, lateral septum; MS, medial septum; OT, oxytocin; OTR, oxytocin receptor; *Oxtr*, oxytocin receptor gene; PFC, prefrontal cortex; PirCx, piriform cortex; RM, repeated measures; Th, thalamus; VDB, vertical limb of the diagonal band of Broca.

A *Experiment 1:* Behavioural characterisation

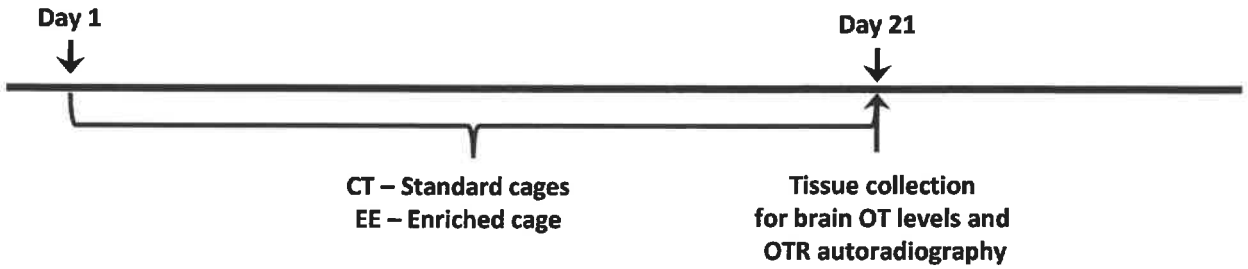
(i) Anxiety, memory, ethanol CPP



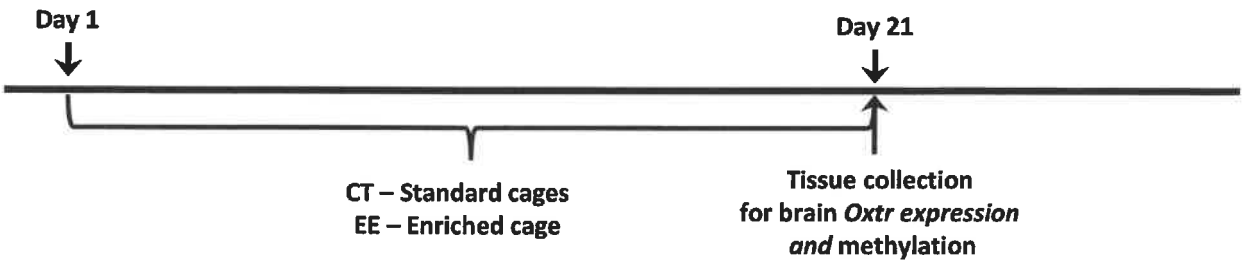
(ii) Sociability



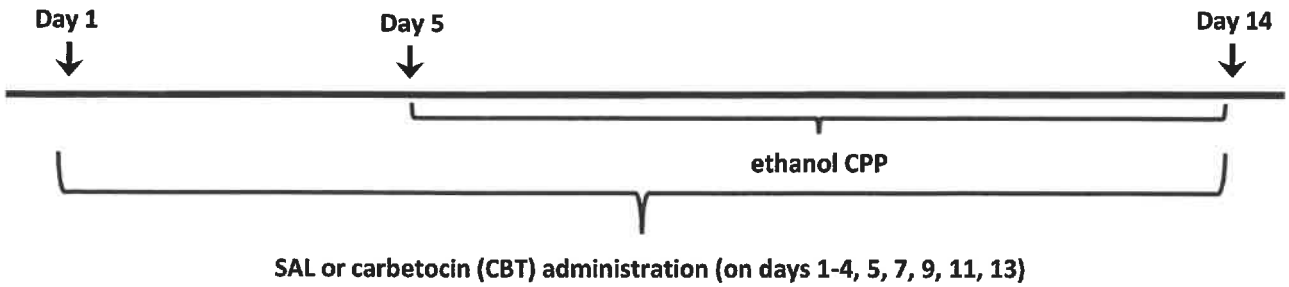
B *Experiment 2: Brain OT levels and OTR binding autoradiography*



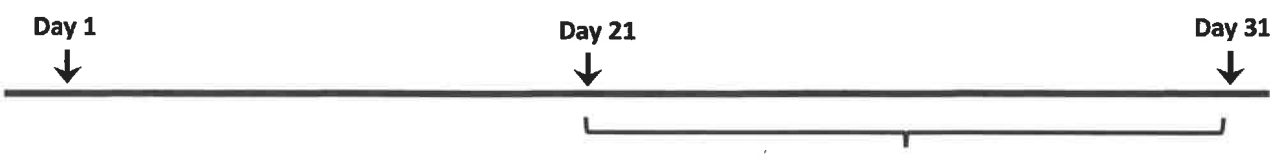
C *Experiment 3: Brain Oxtr expression and DNA methylation*

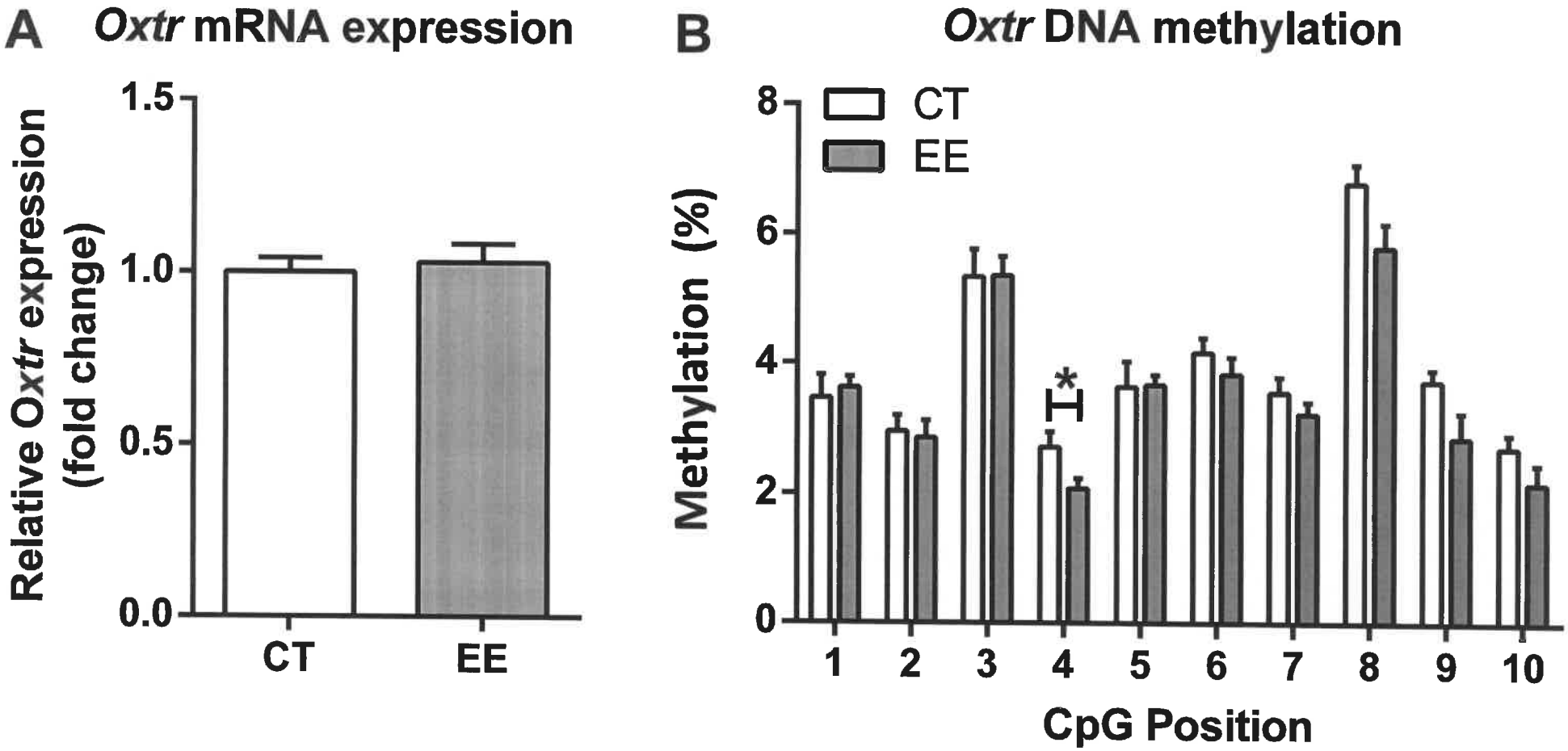


D *Experiment 4:* Effect of repeated carbetocin administration on ethanol CPP



E *Experiment 5:* Effect of L-368,899 administration during EE and CPP on ethanol CPP




A
Oxtr mRNA expression
Relative Oxtr expression (fold change)
1.5
1.0
0.5
0.0
CT
EE
B
Oxtr DNA methylation
Methylation (%)
8
6
4
2
0
CT
EE
*
1
2
3
4
5
6
7
8
9
10
CpG Position

Supplementary Table 1: *Oxtr* mRNA expression primer sets

Gene	Primer ID	Sequence (5'-3')	Nt	Tm	GC%
Oxtr	*Oxtr* _1	Forward: AAGCTTCTGCCTTCATCATTGC	22	58.4	45.5
		Reverse: GGCCCGTGAAGAGCATGTAG	20	61.4	60
Gadph	*Gadph_1*	Forward: CCAATGTGTCCGTCGTGGATCT	22	64	58
		Reverse: GTTGAAGTCGCAGGAGACAACC	22	60	52

Amplicon size
84
149

	Statistical analysis	Sample size	Factorial effect		Interaction effect	
Overall effects for Figure 1						
Elevated Plus Maze						
			Experimental groups			
Latency to enter the open arms (s)	Student's unpaired *t*-test	n = 20	*p < 0.05**			
Open-arm time (s)	Student's unpaired *t*-test	n = 20	*p = 0.52*			
Open-arm entries	Student's unpaired *t*-test	n = 20	*p = 0.78*			
Total crosses	Student's unpaired *t*-test	n = 20	*p = 0.14*			
Locomotor activity						
			Experimental groups			
Distance travelled (m)	Student's unpaired *t*-test	n= 19	*p < 0.001****			
Novel object recognition						
			Experimental groups	***Phase***	***Experimental groups x Phase***	
Time exploring objects (s)	Two-way RM ANOVA	n= 18-21	$F_{(1,35)} = 36.80$ *p < 0.001****	$F_{(2,70)} = 3.21$ *p < 0.05**	$F_{(2,70)} = 0.46$	*p = 0.62*
Discrimination ratio	Two-way RM ANOVA	n= 18-21	$F_{(1,34)} = 0.40$ *p = 0.52*	$F_{(1,34)} = 14.90$ *p < 0.001****	$F_{(1,34)} = 0.03$	*p = 0.84*
Ethanol conditioned place preference						
			Experimental groups			
Conditioning ratio	Student's unpaired *t*-test	n= 9-11	*p < 0.001****			
Overall effects for Figure 2						
Three-chamber social approach test						
			Experimental groups	***Social side***	***Social side x Treatment***	
Social interaction ratio	Student's unpaired *t*-test	n= 8-10	*p < 0.05**			
Distance travelled (m)	Student's unpaired *t*-test	n= 8-10	*p < 0.001****			
Overall effects for Figure 3						
OTR autoradiography						
			Experimental groups	***Brain Region***	***Experimental groups x Brain Region***	
Olfatory Nuclei: AOM, AOV, AOL	Two-way ANOVA	n= 5	$F_{(1,24)} = 38.40$ *p < 0.001****	$F_{(2,24)} = 1.56$ *p = 0.22*	$F_{(2,24)} = 1.52$	*p = 0.23*
Cortex: PFC, CgCx, PirCx	Two-way ANOVA	n= 5	$F_{(1,24)} = 19.79$ *p < 0.001****	$F_{(2,24)} = 149.0$ *p < 0.001****	$F_{(2,24)} = 0.87$	*p = 0.42*
Striatum: Acb, CPu	Two-way ANOVA	n= 5	$F_{(1,16)} = 3.69$ *p = 0.072*	$F_{(1,16)} = 13.54$ *p < 0.01***	$F_{(1,16)} = 0.82$	*p = 0.37*
Septum: MS, VDB, LS, Hip	Two-way ANOVA	n= 5	$F_{(1,24)} = 2.11$ *p = 0.15*	$F_{(2,24)} = 53.85$ *p <0.001****	$F_{(2,24)} = 0.12$	*p = 0.88*

Forebrain	Th Amy Hyp	Two-way ANOVA	n= 5	$F_{(1,32)}$ =0.02	*p = 0.88*	$F_{(3,32)}$ =72.50	*p <0.001****	$F_{(3,32)}$ =0.48	*p = 0.69*

OT peptide levels

			Experimental groups	
Prefrontal cortex	Student's unpaired *t* -test	n=5	*p = 0.48*	
Olfactory Nuclei	Student's unpaired *t* -test	n=5	*p = 0.37*	
Amygdala	Student's unpaired *t* -test	n=5	*p = 0.76*	
Hypothalamus	Student's unpaired *t* -test	n=5	*p =0.05**	

Plasma Corticosterone

			Experimental groups		***Day***		***Experimental groups x Day***		
Corticosterone levels (%)	Two-way RM ANOVA	n=6	$F_{(1, 10)}$ =0.0005	*p = 0.98*	$F_{(1, 10)}$ =0.35	*p = 0.57*	$F_{(1, 10)}$ =0.0006	*p = 0.98*	

Overall effects for Figure 4

OTR autoradiography - CBT

			Treatment Experimental groups		***Brain Region***		***Experimental groups x Brain Region***		
Olfatory Nuclei	AOM AOV AOL	Two-way ANOVA	n= 6	$F_{(1,30)}$ = 13.17	*p < 0.01***	$F_{(2,30)}$ = 6.09	*p < 0.01***	$F_{(2,30)}$ = 2.90	*p = 0.07*
Cortex	PFC CgCx PirCx	Two-way ANOVA	n= 6	$F_{(1,31)}$ = 7.67	*p < 0.01***	$F_{(2,31)}$ = 306.2	*p < 0.001****	$F_{(2,31)}$ = 5.30	*p < 0.05**
Striatum	Acb CPu	Two-way ANOVA	n= 6	$F_{(1,20)}$ = 0.09	*p = 0.76*	$F_{(1,20)}$ = 51.23	*p < 0.001****	$F_{(1,20)}$ = 1.59	*p = 0.22*
Septum	MS VDB LS	Two-way ANOVA	n= 6	$F_{(1,30)}$ = 9.07	*p < 0.01***	$F_{(2,30)}$ = 37.12	*p <0.001****	$F_{(2,30)}$ = 0.78	*p = 0.46*
Forebrain	Hip Th Amy Hyp	Two-way ANOVA	n= 6	$F_{(1,40)}$ =2.74	*p = 0.10*	$F_{(3,40)}$ =67.04	*p <0.001****	$F_{(3,40)}$ =0.69	*p = 0.55*

Ethanol conditioned place preference - CBT

			Treatment	
Conditioning ratio	Student's unpaired *t* -test	n=9	*p = 0.05**	

Overall effects for Figure 5

Ethanol conditioned place preference - L-368,899

			Treatment		***Experimental groups***		***Experimental groups x Treatment***		
Conditioning ratio	Two-way ANOVA	n= 6-8	$F_{(2, 42)}$ =1.32	*p = 0.28*	$F_{(1, 42)}$ =1.32	*p < 0.01***	$F_{(2, 42)}$ =2.80	*p = 0.07*	

Overall effects for Supplementary Figure 2

Oxtr mRNA expression

			Experimental groups		
Oxtr mRNA	Student's unpaired *t*-test	n= 9-10	*p = 0.69*		
Oxtr DNA methylation					
			Experimental groups	***CpG position***	***Experimental groups x CpG***
Methylation (%)	Two-way ANOVA		$F_{(1, 150)}$ =8.18 *p < 0.01***	$F_{(9, 150)}$ =39.08 *p < 0.001****	$F_{(9, 150)}$ =0.99 *p = 0.45*
CpG 1	Mann-Whitney *t*-test	n= 8-9	*p=0.72*		
CpG 2	Mann-Whitney *t*-test	n= 8-9	*p=0.94*		
CpG 3	Mann-Whitney *t*-test	n= 8-9	*p=0.79*		
CpG 4	Mann-Whitney *t*-test	n= 8-9	*p < 0.05**		
CpG 5	Mann-Whitney *t*-test	n= 8-9	*p=0.76*		
CpG 6	Mann-Whitney *t*-test	n= 8-9	*p=0.59*		
CpG 7	Mann-Whitney *t*-test	n= 8-9	*p=0.37*		
CpG 8	Mann-Whitney *t*-test	n= 8-9	*p = 0.11*		
CpG 9	Mann-Whitney *t*-test	n= 8-9	*p=0.07*		
CpG 10	Mann-Whitney *t*-test	n= 8-9	*p=0.23*		