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Effects of oxytocin receptor ligands on anxiogenic-like effect, social avoidance and changes on medial prefrontal cortex oxytocin receptor expression evoked by chronic social defeat stress in rats

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ABSTRACT

We investigated the effect of systemic administration of the synthetic oxytocin (OXT) analog carbetocin and/or OXT receptor antagonists (atosiban and L-368,899) on social avoidance and anxiogenic-like effect in male rats subjected to chronic social defeat stress (cSDS). Effect of cSDS and pharmacological manipulation of OXT system on expression of OXT receptor within the medial prefrontal cortex (mPFC) subregions [anterior cingulate (Cg), prelimbic (PL) and infralimbic (IL) cortices] was also evaluated. Our behavioral results indicated that cSDS, while not inducing social avoidance in the social interaction test, reliably induced anxiogenic-like effect as measured by the elevated plus maze test. Chronic systemic treatment with either carbetocin or atosiban, but not L-368,899, during cSDS protocol dose-dependently prevented the anxiogenic-like effect. Both atosiban and L-368,899 inhibited the anxiolytic effect of carbetocin in defeated animals, confirming OXT receptor-mediated effect. Also, cSDS increased OXT receptor levels within the Cg, which was inhibited by both atosiban and L-368,899 treatments. Conversely, cSDS did not affect OXT receptor within the PL and IL. However, carbetocin treatment increased OXT receptor expression within the PL and IL of defeated animals, an effect that was blocked by either atosiban or L-368,899. Taken together, our study provides evidence for the critical role of the OXT system and its pharmacological manipulation in modulating anxiogenic-like effects evoked by social stress. Furthermore, the region-specific modulation of OXT receptor expression within the mPFC by stress and OXT system pharmacological manipulation emphasize the complex and dynamic nature of OXT receptor regulation in brain regions crucial for emotional processing.

1. Introduction

Oxytocin (OXT) is a neuropeptide composed of nine amino acids with both central and peripheral actions (Gimpl and Fahrenholz, 2001; Jurek and Neumann, 2018). As a hormone, this peptide is released into the systemic circulation from neurohypophysis by magnocellular neurons located in the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei (Jurek and Neumann, 2018; Swanson and Sawchenko, 1983). The PVN also contains parvocellular oxytocinergic neurons that project throughout the central nervous system (CNS), including the medial prefrontal cortex (mPFC) (Froemke and Young, 2021; Knobloch et al., 2012; Li et al., 2024; Sabihi et al., 2014a, 2014b), where OXT receptors are present (Nakajima et al., 2014b; Sabihi et al., 2017). It is worth mentioning that axon collaterals of magnocellular neurons also project to various forebrain regions (Grinevich and Ludwig, 2021), and OXT immunoreactive neurons were sparsely found in brain areas such as the bed nucleus of the stria terminalis (BNST), dorsomedial hypothalamus, medial amygdala nucleus and *locus coeruleus* (Winter and Jurek, 2019).

Being widely distributed in the CNS, OXT neurotransmission participates in the regulation of various emotion-related behaviors, including those related to anxiety (Benarroch, 2013; Neumann and Landgraf, 2012; Neumann and Slattery, 2016; Sabihi et al., 2017) and social behavior (Bosch and Young, 2017; Calcagnoli et al., 2015; Dumais

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and Veenema, 2016; Froemke and Young, 2021; Rigney et al., 2022; Tan et al., 2019). In this context, exogenous OXT administration has been shown in humans and rodents to typically reduce anxiety, although anxiogenic effects was observed when it was directly microinjected into the septum (Ayers et al., 2011; Cui and Xiao, 2025; Ferrer-Pérez et al., 2021; Huang et al., 2021; Mak et al., 2012; Neumann and Slattery, 2016; Slattery and Neumann, 2010; Winter and Jurek, 2019). Brain OXT also inhibits the expression of stress responses (Jurek and Neumann, 2018; Takayanagi and Onaka, 2021).

The medial prefrontal cortex (mPFC) has been identified as an important region in OXT's neuromodulation of anxiety-related behaviors (Cui and Xiao, 2025; Sabihi et al., 2017, 2014b, 2014a). This cortical region in rodents is dorsally-ventrally divided into three subareas: the anterior cingulate (Cg), the prelimbic (PL), and the infralimbic (IL) cortices (Anastasiades and Carter, 2021; McKlveen et al., 2019, 2015). The mPFC integrates information from different limbic brain regions, including the amygdala, nucleus accumbens (NA), hippocampus, and BNST (Anastasiades and Carter, 2021; Cerqueira et al., 2008; McKlveen et al., 2015). As such, it functionally contributes to the control of emotional and mood states (Faria et al., 2020; Kesner and Churchwell, 2011; Santos-Costa et al., 2021; Tan et al., 2019; Wilson et al., 2010; Yamada et al., 2015), such as behavioral and physiological responses related to stress (Cerqueira et al., 2008; Giannotti et al., 2019; Liu et al., 2021; McKlveen et al., 2019, 2015).

Given the high prevalence of psychosocial stress in contemporary society, robust experimental models are essential for their investigation. The social defeat stress (SDS) paradigm was developed to fulfill this need by simulating this specific type of stressor, entailing the exposure of rodents to a series of intense and inescapable confrontations with an aggressive conspecific (Hollis and Kabbaj, 2014; Miczek, 1979). Crucially, chronic exposure to SDS (cSDS) elicits diverse anxiety and depressive-like phenotypes in these animals. Manifestations include diminished body weight gain, elevated anxiety-like responses in the elevated plus-maze (EPM), cognitive impairment, suppressed novelty-induced feeding, anhedonic states, extended immobility in the forced swim test and social avoidance (Berton et al., 2006; da Costa et al., 2023; Golden et al., 2011; Hollis and Kabbaj, 2014; Jaisinghani and Rosenkranz, 2015; Morais-Silva et al., 2019; Santos-Costa et al., 2021; Venzala et al., 2012).

Neurobiological mechanisms, as well as pharmacological therapeutic approaches to emotional changes related to social stress are not completely established. Specifically regarding OXT system, previous studies documented that cSDS causes a dysfunction in this system in several brain regions, but the consequences in mPFC was never described (Cui and Xiao, 2025; Ferrer-Pérez et al., 2021). Additionally, although reporting that microinjection of OXT into the mPFC inhibited cSDS-evoked social avoidance (Li et al., 2020), a link of this neurochemical mechanism of the mPFC with other behavioral manifestations to cSDS is to be established. Previous studies reported that OXT treatment prevented the anxiogenic-like effect and social avoidance evoked by cSDS in socially monogamic voles (Hale et al., 2021; Hou et al., 2023), but the effect of systemic treatment with OXT receptor agonist and antagonist in rats is still to be described. Based on that, here we investigated the dose-response effects of the systemic administration of the synthetic OXT analog carbetocin and/or the OXT receptor antagonist atosiban or L-368,899 on social avoidance and anxiety-like behaviors induced by cSDS. Subsequently, we evaluated the effect of cSDS and the pharmacological manipulations of OXT system on the expression of OXT receptor within the mPFC.

2. Material and methods

2.1. Animals

Male Wistar rats (230–260 g – used as intruders) and male Long-Evans or Wistar Hannover rats (600–800 g – used as residents) were acquired from the 'Center for Research and Production of Animals – CPPA' at São Paulo State University (Botucatu, São Paulo, Brazil). All animals were housed in the animal facility of the Laboratory of Pharmacology at the School of Pharmaceutical Sciences / São Paulo State University (FCF/UNESP); however, intruders and residents were housed in different rooms. Intruder rats were housed in pairs (38.6 × 25.1 × 24 cm), and resident rats were maintained in a stable colony with a fertile female rat of the same strain (i.e., Long-Evans or Wistar Hannover) (41 × 34 × 27.5 cm cage). The animals had free access to food and water (except during the brief testing periods) and were subjected to an 12-h light/dark cycle (lights on at 7 p.m.) in a temperature-controlled environment (23 ± 1 °C). All intruder rats were *naïve* at the beginning of the experiments and were used only once. Resident rats (6–12 months) were used more than once through a rotation scheme defined by the experimenter. The animal study was conducted following the ARRIVE guideline 2.0 (https://arriveguidelines.org/arrive-guidelines) and all experimental procedures and protocols were reviewed and approved by the Animal Ethics and Use Committee (CEUA) of the FCF/UNESP (approval #: CEUA/FCF/CAr 16/2022).

2.2. Drugs and solutions

The synthetic OXT analog carbetocin (Sigma-Aldrich, SML0748) at doses of 0.01, 0.1, and 1 mg/kg (MacFadyen et al., 2016; Ramos et al., 2013); the OXT receptor antagonist atosiban (Sigma-Aldrich, A3480) at doses of 0.01, 0.1, and 1 mg/kg (da Cruz et al., 2019; Vera Klenerova et al., 2009) and the OXT receptor antagonist L-368,899 (Cayman Chemical, 29868) at doses of 0.01, 0.1, and 1 mg/kg (Blitzer et al., 2017; Boccia et al., 2007) were administered intraperitoneally (i.p.). These drugs were prepared in sterile saline (0.9 % NaCl), which served as the control solution (2 ml/kg). The investigator was blinded to the experimental groups throughout the drug administration. Urethane, at a dose of 1.2 g/kg (i.p.) was used as an anesthetic for transcardiac perfusion procedure.

2.3. Chronic social defeat stress (cSDS)

The intruder-resident protocol for cSDS was performed as previously described (Morais-Silva et al., 2019). Briefly, one h prior to each aggressive encounter, the females and their pups were temporarily removed from the resident's home cage (resident: male Long-Evans or Wistar Hannover rats) and remained undisturbed in the animal facility with *ad libitum* access to food and water. Resident males were then transferred to the defeat room at least one h before the scheduled aggressive encounters. Intruder animals were exposed to the home cage of an aggressive, unfamiliar conspecific resident. Each stress episode lasted 25 min, divided into three phases: initially, the intruder was placed in the aggressor's cage for 5 min, protected from the resident's attacks by a protective wire mesh cage (20.7 × 11 × 10.5 cm). Subsequently, the intruder was removed from the protective cage and exposed to the agonistic encounters for 10 min. In the third phase, the intruder returned to the protected compartment and remained in the resident's cage for 10 min. In absence of agonistic interaction, the intruder was then subjected to a new resident. Finally, the intruder was returned to its home cage. Episodes were conducted at 48-h intervals over 7 days. Thus, each intruder underwent four stress sessions, each with an unfamiliar resident. The control group was subjected to a similar procedure, except that the animals underwent non-aggressive interaction (NAI) through contact with a non-aggressive familiar rat.

2.4. Social interaction test (SIT)

Twenty-four h after the last SDS session, intruder rats were tested in a social interaction arena. The arena consisted of an opaque acrylic box with grey walls and black floor (80 × 80 × 25 cm), featuring an acrylic containment box (target, 20 × 15 × 24 cm) centered on one of its walls,

where the unfamiliar resident animal was placed. The arena was divided into an interaction zone (IZ: projected 15 cm around the containment box) and two corner zones (CZ: more distant areas, measuring 15 × 15 cm each, located in the corners opposite the containment box). To assess basal exploration, subjects (intruders) were individually placed in the center of the arena, facing away from the empty target (containment box without the resident), and allowed to freely explore the entire environment for 150 s. Subsequently, the intruder rat was removed from the arena and placed into a holding cage, and an unfamiliar resident (Long-Evans or Hannover rats) was placed in the containment box (target). Then, the intruder was individually placed back in the arena for another 150 s to evaluate social interaction behavior. All sessions were recorded under red light illumination (5 lx on the arena floor) with a vertically mounted camera. Exploration time (in seconds) in the IZ and CZ was recorded in the presence and absence of the target. Social avoidance behavior was quantified using a social interaction ratio, obtained by dividing the time spent in the IZ when the target was present by the time spent in the interaction zone when the target was absent. The arena and containment box were thoroughly cleaned with 20 % alcohol between subjects (Golden et al., 2011; Morais-Silva et al., 2019). Behavioral analyses were performed by using the ANY-maze software (Stoelting Co., Wood Dale-IL, USA).

2.5. Elevated plus-maze (EPM)

Twenty-four h after the SIT, animals were tested in the EPM, which is used to evoke defensive responses related to anxiety and is pharmacologically validated (Carobrez and Bertoglio, 2005; Pellow et al., 1985). The EPM consists of two platforms, elevated 50 cm from the floor, positioned perpendicularly and comprising two enclosed arms (50 × 10 × 40 cm) and two open arms (50 × 10 × 0.25 cm) joined by a central platform (10 × 10 cm). Briefly, each animal was placed in the center of the EPM and allowed freely explore the apparatus for a 5-min period. The maze was cleaned with 20 % alcohol between subjects. Experiments occurred under red light illumination (5 lx on the central platform). Sessions were recorded with a vertically mounted camera linked to a monitor. Behavioral analysis, conducted by a trained observer blinded using the "X-Plo-rat 2005" software (University of São Paulo) (Tejada et al., 2018), included spatiotemporal measures such as frequencies of enclosed-arm entries (EAE) and open-arm entries (OAE) (an arm entry was defined as all four paws entering the arm), as well as the time spent (in seconds) in the open arms (OAT). Data was used to calculate the percentage of open-arm entries [%OAE: OAE/(OAE + EAE) × 100] and the percentage of open-arm time [%OAT: (OAT/300) × 100].

2.6. Immunofluorescence (IF)

Twenty-four h after the EPM test, rats were anesthetized and transcardially perfused with phosphate-buffered saline (PBS; 3.2 g KH2PO4; 7.1 g K2HPO4; 9.1 g NaCl in 100 ml distilled water), followed by 4 % paraformaldehyde (4 g NaOH; 4 g paraformaldehyde; 16.58 g NaH2PO4). Brains were then post-fixed in paraformaldehyde for 1 h and transferred to 30 % sucrose solution in PBS at 4°C. After two days, brains were frozen and stored at −80°C until sectioning on a cryostat into 35 μm coronal sections (CM1900, Leica, Germany).

The brain structure analyzed was the mPFC, evaluating individual labeling in each one of its subareas (i.e., Cg, PL, and IL cortices). The immunofluorescence histochemistry technique was performed after obtaining sections of the regions of interest. Coronal slices were washed 5 times with PBS and then incubated with blocking solution (10 % goat serum; 0.3 % Triton X-100 dissolved in PBS) for one h at room temperature. After blocking, slices were incubated with the primary rabbit anti-Oxytocin Receptor antibody (1:750; cat. # AVR-013, Alomone Labs) in vehicle solution for 24 h at 4°C. To confirm antibody specificity, control sections were processed identically, except that the primary antibody was omitted. Following this incubation, slices were washed 5 times with PBS and incubated with a secondary antibody (goat anti-rabbit IgG, Alexa Fluor 488; 1:1000; cat. # ab150077, Abcam) diluted in vehicle solution for 3 h at room temperature. Subsequently, slices were washed 5 times with PBS and transferred to PBS solution, then mounted on gelatin-coated slides. Next, 2–3 drops of Fluoroshield Mounting Medium (Sigma-Aldrich) were added, and the slides were sealed with coverslips. For quantification of fluorescence images were acquired at 20X magnification (representative images of the PL was obtained at 40 and 100X) using a fluorescence microscope (Axio Imager. D2, Carl Zeiss Microscopy, LLC, Thornwood-NY, USA) connected to a computer and digitized by Zen Pro 2.0 software (Carl Zeiss Microscopy, LLC, Thornwood-NY, USA). Quantification of fluorescence was performed using ImageJ software (NIH), applying the Measurement of Mean Fluorescence Intensity (MFI) in a Region of Interest (ROI) method (MFI = MFI of an ROI – MFI of Background) (Shihan et al., 2021). The ROIs (800 × 700 μm) across mPFC subareas (bregma from +2.70 to +3.20 mm) were based on the rat brain atlas (Paxinos and Watson, 2006). Values from 3 images per brain area/hemisphere/rat were averaged, and these values were used to calculate the group means and variance. The MFI of the background from 3 different points of each image was obtained, and the average was used to subtract from the MFI of the ROI. The investigator was blinded to the experimental groups throughout microscopy and analysis.

2.7. Experimental design

To investigate the effects of different pharmacological manipulations on social avoidance and anxiety following cSDS, four distinct experimental series were conducted (Fig. 1). In all experiments, animals were subjected to the cSDS protocol (as detailed in Section 2.3). Following the last SDS session, rats in all groups underwent behavioral testing in the social interaction test (SIT) and elevated plus maze (EPM) (as described in Sections 2.4 and 2.5), at 24 and 48 h after the last SDS session, respectively. The primary distinction among the experiments lay in the specific drug administered and its timing relative to the SDS sessions.

2.7.1. Experiment 1: modulation of social avoidance and anxiety by carbetocin during cSDS

A dose-response study of intruder rats treated with carbetocin at doses of 0.01, 0.1, and 1 mg/kg during the SDS period was performed. Carbetocin was administered intraperitoneally (i.p.) 30 min before each SDS session (MacFadyen et al., 2016; Ramos et al., 2013). As a control, the study included a group that underwent non-aggressive interaction (NAI) (through contact with a non-aggressive familiar rat) and was treated with saline (NAI+S) 30 min before each interaction.

2.7.2. Experiment 2: modulation of social avoidance and anxiety by atosiban during cSDS

In this study intruder rats received i.p. injections of atosiban at doses of 0.01, 0.1, and 1 mg/kg before each SDS session. Atosiban was administered 60 min prior to each SDS session (da Cruz et al., 2019; Vera Klenerova et al., 2009). This study also included a NAI+S group as detailed in Experiment 1, which saline was administrated 60 min before the non-aggressive interaction.

2.7.3. Experiment 3: modulation of social avoidance and anxiety by L-368,899 during cSDS

This experiment involved the i.p. administration of L-368,899 at doses of 0.01, 0.1, and 1 mg/kg to intruder rats. Consistent with Experiment 2, L-368,899 was injected 60 min before each SDS session (Boccia et al., 2007). This study also included a NAI+S group as detailed in Experiment 1, which saline was administrated 60 min before the non-aggressive interaction.

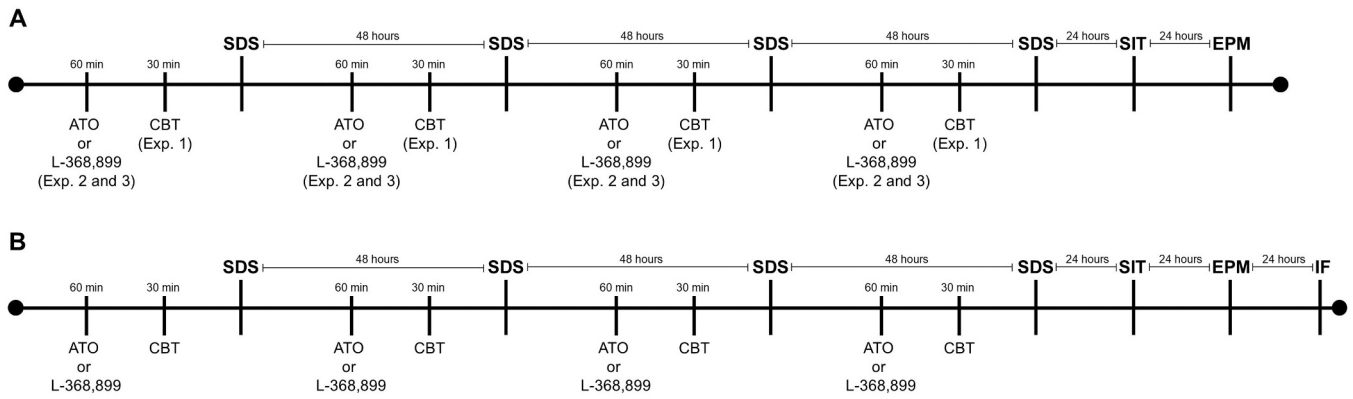

Fig. 1. Experimental Design. (A) Experiments 1, 2 and 3: single i.p. injections of 3 doses of the OXT receptor agonist or antagonists or saline were administrated 30 min [Experiment 1; drug: CBT] or 60 min [Experiments 2 and 3; drugs: ATO and L-368–899, respectively] before each SDS session. Rats were tested in the SIT and EPM 24 and 48 h after the last SDS session, respectively. As a control, each study also included a group underwent non-aggressive interaction (NAI) (through contact with a non-aggressive familiar rat) that received saline at the same time as the animals subjected to pharmacological manipulation of OXT system (i.e., 30 or 60 min before each SDS session). (B) Experiment 4: combined i.p. injections of ATO or L-368,899 and CBT were administrated 60 min and 30 min, respectively, before each SDS session. Rats were tested in the SIT and EPM 24 and 48 h after the last SDS session, respectively. The investigator was blinded to the experimental groups throughout the drug administration. Brain collection for IF protocol was performed 24 h after EPM test. This study also included a NAI group treated with vehicle 60 min and 30 min before each SDS session. ATO, atosiban. CBT, carbetocin. SDS, social defeat stress. SIT, social interaction test. EPM, elevated plus-maze. IF, immunofluorescence.

2.7.4. Experiment 4: modulation of carbetocin-induced effects on OXT receptor expression, social avoidance, and anxiety by OXT antagonists during cSDS

In this experiment, the ineffective doses of atosiban and L-368–899 obtained, respectively, in Experiments 2 and 3 were used to block the effect of the effective dose of carbetocin (Experiment 1). For this purpose, the antagonists were administered 60 min before each SDS session, whereas treatment with carbetocin was performed 30 min later (i.e., 30 min before the SDS session). After the cSDS protocol, animals were tested in the SIT and EPM 24 and 48 h after the last SDS session, respectively. Twenty-four h after the EPM test, intruder rats were then euthanized for transcardiac perfusion and brain collected for IF processing (Fig. 1B). As a control, this study included a NAI group treated with vehicle 60 min and 30 min before each SDS session.

2.8. Statistical analysis

Results of the SIT were subjected to two-way repeated measures analysis of variance (ANOVA) (Factor 1: treatment; Factor 2: target). Results from the EPM and IF analyses were subjected to one-way ANOVA. In cases of significance, analyses were followed by Newman-Keuls multiple comparisons test. A p-value less than or equal to 0.05 was considered statistically significant.

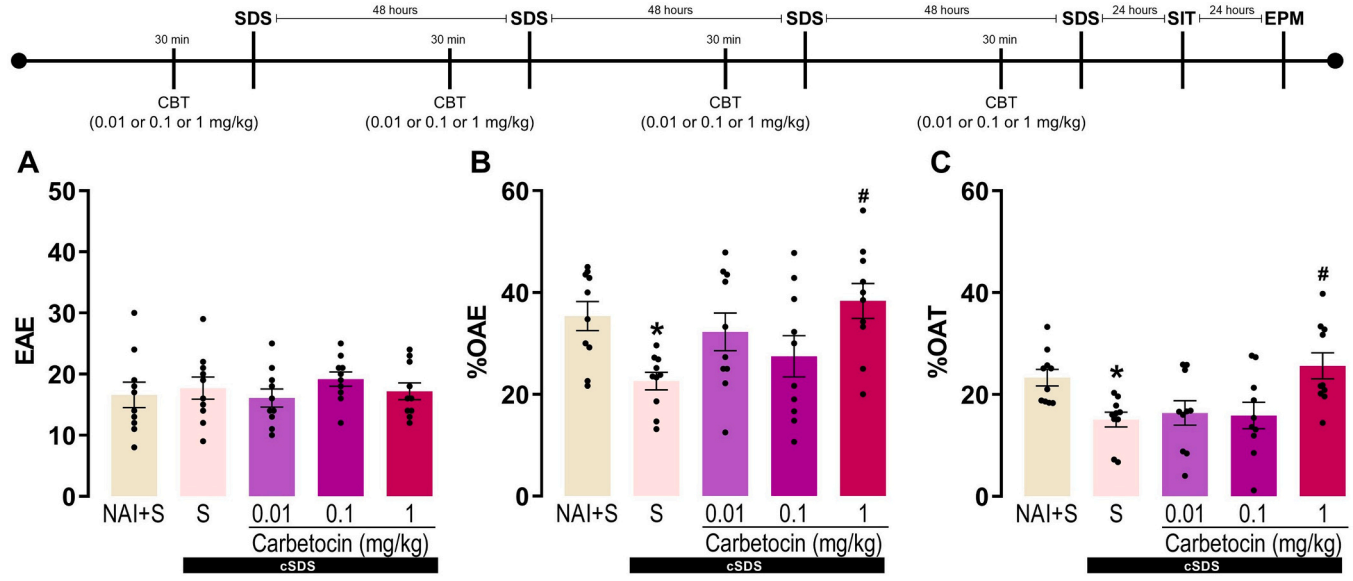

Fig. 2. Carbetocin (CBT) Prevents cSDS-Induced Anxiogenic-like Behavior. Bars with scatter dot plot represent mean (±SEM) from NAI+S (n = 10), Saline (n = 10), CBT 0.01 mg/kg (n = 10), CBT 0.1 mg/kg (n = 10) and CBT 1 mg/kg (n = 10) subjected to Elevated Plus Maze test (EPM). (A) EAE: Entries into enclosed arms. (B) % OAE: Percentage of entries into the open arms. (C) %OAT: Percentage of time spent in the open arms. NAI+S, Non-Aggressive Interaction + saline; S, animals subjected to cSDS and treated with saline; Carbetocin, animals subjected to cSDS and treated with carbetocin. Black bar represents groups subjected to cSDS. *p ≤ 0.05 Saline group *versus* NAI+S group. #p ≤ 0.05 CBT 1 *versus* Saline group.

3. Results

3.1. Experiment 1: carbetocin reverses the anxiogenic-like effect induced by cSDS

To determine the dose-dependent effect of the oxytocin receptor agonist carbetocinduring the cSDS protocol, we evaluated its impact on social interaction test (SIT) and anxiety-like behavior (EPM). Figures SM1 and 2 represent the results of rats tested in the SIT (Fig. SM1A, SM1B, and SM1C) and EPM (Figs. 2A, 2B, and 2C) tests that were treated with carbetocin and exposed to cSDS protocol. Regarding the time spent in the IZ (Fig. SM1A), a two-way repeated measures ANOVA indicated a significant effect for both the treatment [$F_{(4,45)} = 2.60$, $p \leq 0.05$] and target [$F_{(1,45)} = 59.16$, $p \leq 0.005$] factors, but without interaction between factors [$F_{(4,45)} = 1.20$, $p = 0.32$]. A *post hoc* test revealed that the group treated with the intermediate dose showed less exploration of the IZ compared to the saline group, independently of the presence of the target ($p \leq 0.05$). Moreover, the highest dose of carbetocin presented higher values in relation to animals treated with 0.01 mg/kg of carbetocin ($p \leq 0.05$). Regarding the time spent in the CZ, a two-way ANOVA indicated a significant effect for the target factor [$F_{(1,45)} = 12.75$, $p \leq 0.005$], independently of the treatment [$F_{(1,45)} = 0.90$, $p = 0.47$] and interaction between factors [$F_{(1,45)} = 0.22$, $p = 0.93$]. Regarding the social interaction ratio, ANOVA indicated no significant difference between groups [$F_{(4,45)} = 0.66$, $p = 0.62$].

Regarding the EPM data, ANOVA showed a significant difference between groups concerning %OAE [$F_{(4,45)} = 3.74$, $p = 0.01$] and %OAT [$F_{(4,45)} = 4.92$, $p = 0.002$], but not for the number of EAE [$F_{(4,45)} = 0.54$, $p = 0.70$]. Newman-Keuls test revealed that saline-treated cSDS rats showed a decrease in open arm exploration of the EPM compared to the NAI+SAL group (%OAE, $p = 0.04$; %OAT, $p = 0.05$). Moreover, the highest dose of carbetocin (1 mg/kg) reversed the effect of cSDS ($p \leq 0.01$).

3.2. Experiment 2: atosiban reverses the anxiogenic-like effect induced by cSDS

In this experiment, we examined the dose-dependent effect of the oxytocin receptor antagonist atosiban administered during the cSDS protocol on subsequent social and anxiety-like behaviors. Figures SM2 and 3 illustrate the data of the SIT (Fig. SM2A, SM2B, and SM2C) and EPM (Figs. 3A, 3B, and 3C) tests. Regarding the SIT, two-way repeated measure ANOVA indicated no significant difference in the interaction zone (IZ) and corner zone (CZ) (Fig. SM2A and 2B) for the treatment factor [IZ: $F_{(4,47)} = 2.39$, $p = 0.06$; CZ: $F_{(4,47)} = 2.01$, $p = 0.11$] nor any interaction between factors [IZ: $F_{(4,47)} = 0.89$, $p = 0.48$; CZ: $F_{(4,47)} = 1.33$, $p = 0.27$]. However, the two-way ANOVA showed a significant difference for the target factor [IZ: $F_{(1,47)} = 71.47$, $p \leq 0.005$; AZ: $F_{(1,47)} = 18.35$, $p \leq 0.005$]. Regarding the social interaction ratio, ANOVA revealed no significant difference between groups [$F_{(4,47)} = 0.67$, $p = 0.62$]. These results suggest that neither stress nor different doses of atosiban interfered with social avoidance behavior as assessed in the SIT.

Concerning the EPM data, ANOVA showed a significant difference between groups regarding the %OAE [$F_{(4,47)} = 3.65$, $p = 0.01$] and % OAT [$F_{(4,47)} = 3.92$, $p = 0.008$], but not for the number of EAE [$F_{(4,47)} = 0.78$, $p = 0.54$]. Newman-Keuls *post hoc* test revealed that animals subjected to cSDS and administered saline showed a decrease in open arm exploration of the EPM compared to the NAI+SAL group (%OAE, $p = 0.02$; %OAT, $p = 0.01$). Furthermore, atosiban at a dose of 1 mg/kg reversed the stress effect ($p \leq 0.02$).

3.3. Experiment 3: L-368,899 treatment does not alter the anxiogenic-like effect induced by cSDS

To compare the behavioral outcome of atosiban with a potent/pure OXT receptor antagonist (*for details, see Discussion section*), we evaluated the dose-dependent effect of L-368,899 during the cSDS protocol. Figures SM3 and 4 display the data of the SIT (Fig. SM3A, SM3B, and SM3C) and EPM (Figs. 4A, 4B, and 4C) tests. For the SIT, the two-way repeated measure ANOVA for the IZ indicated a significant effect for the treatment [IZ: $F_{(4,37)} = 2.75$, $p = 0.04$] and target [IZ: $F_{(1,37)} = 274.39$, $p < 0.001$] factors, as well as an interaction between factors [IZ: $F_{(4,37)} = 8.87$, $p < 0.001$]. Despite factor interaction, the *post hoc* test revealed that the difference only occurred between the presence or absence of the target, independent of the condition and treatment

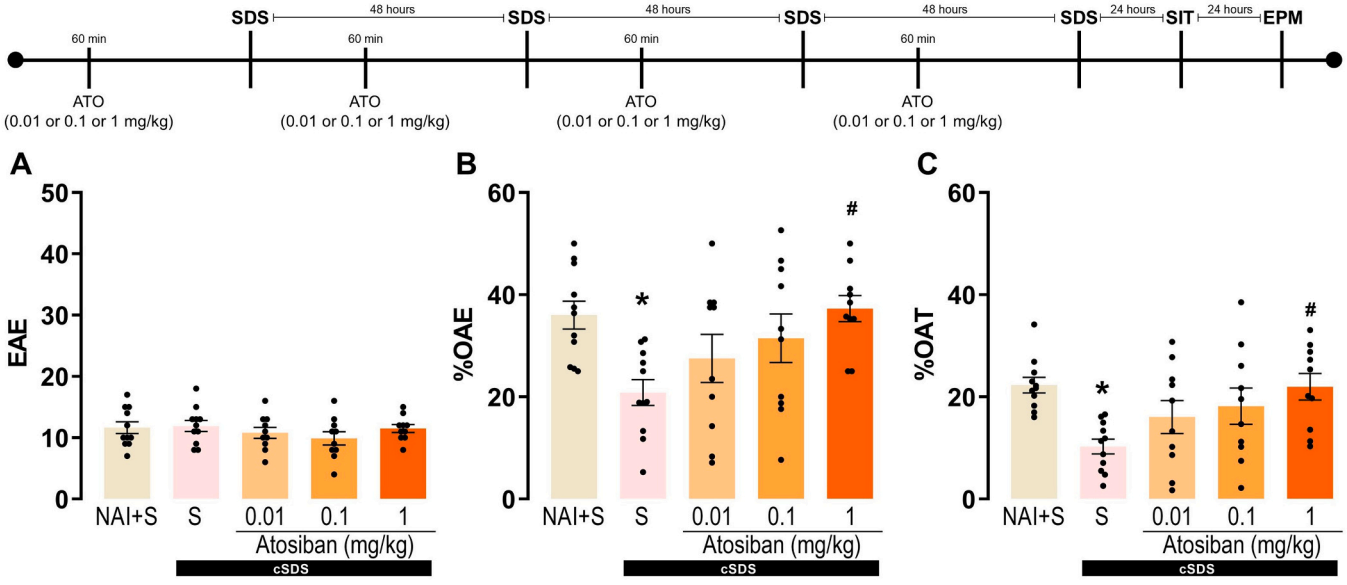

Fig. 3. Atosiban (ATO) treatment prevents cSDS-Induced Anxiogenic-like Behavior. Bars with scatter dot plot represent mean (±SEM) from NAI+S (n = 11), Saline (n = 11), ATO 0.01 mg/kg (n = 10), ATO 0.1 mg/kg (n = 10) and ATO 1 mg/kg (n = 10) subjected to Elevated Plus Maze test (EPM). (A) EAE: Entries into enclosed arms. (B) %OAE: Percentage of entries into the open arms. (C) %OAT: Percentage of time spent in the open arms. NAI+S, Non-Aggressive Interaction + saline; S, animals subjected to cSDS and treated with saline; Atosiban, animals subjected to cSDS and treated with atosiban. Black bar represents groups subjected to cSDS. *$p \leq 0.05$ Saline group *versus* NAI+S group. #$p \leq 0.05$ ATO 1 *versus* Saline group.

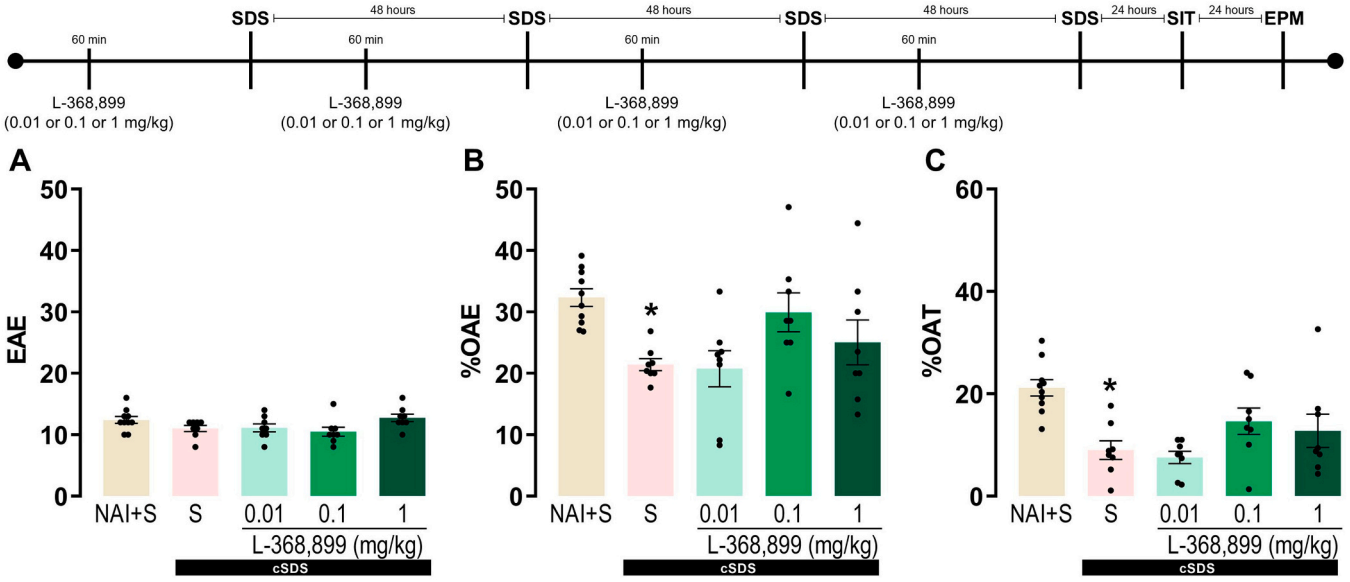

Fig. 4. Lack of Effect of L-368,899 on cSDS-Induced Anxiogenic-like Behavior. Bars with scatter dot plot represent mean (±SEM) of NAI+S (n = 10), Saline (n = 8), L-368,899 0.01 (n = 8), L-368,899 0.1 (n = 8) and L-368,899 1 mg/kg (n = 8) subjected to Elevated Plus Maze test (EPM). (A) EAE: Entries into enclosed arms. (B) % OAE: Percentage of entries into the open arms. (C) %OAT: Percentage of time spent in the open arms. NAI+S, Non-Aggressive Interaction + saline; S, animals subjected to cSDS and treated with saline; L-368,899, animals subjected to cSDS and treated with L-368,899. Black bar represents groups subjected to cSDS. *$p \leq 0.05$ Saline group *versus* NAI+S group.

($p < 0.05$). Regarding the CZ, statistical analysis indicated a main effect only for the target factor [Treatment: $F_{(4,37)} = 1.58$, $p = 0.20$; Target: $F_{(1,37)} = 9.44$, $p \leq 0.004$; Treatment vs. target: $F_{(4,37)} = 1.38$, $p = 0.26$]. Regarding the social interaction ratio, ANOVA revealed no significant difference between groups [$F_{(4,37)} = 2.10$, $p = 0.10$]. These results suggest that neither stress nor different doses of L-368,899 interfered with social avoidance behavior as assessed in the SIT.

With respect to the EPM data, ANOVA indicated a significant difference between groups regarding %OAE [$F_{(4,37)} = 4.20$, $p = 0.007$] and %OAT [$F_{(4,37)} = 6.65$, $p < 0.001$], but not for the number of EAE [$F_{(4,37)} = 2.44$, $p = 0.06$]. Newman-Keuls *post hoc* test revealed that animals subjected to cSDS and administered saline showed a decrease in open arm exploration of the EPM compared to the NAI+SAL group (%OAE, $p = 0.02$; %OAT, $p = 0.01$). However, none of the doses of L-368–899 were statistically different of the saline group.

3.4. Experiment 4: combined treatment with atosiban or L-368,899 blocks the effect of carbetocin in the EPM and on OXT receptor expression in the mPFC

This experiment aimed to confirm the OXT receptor-mediated nature of carbetocin's anxiolytic effect by co-administering it with sub-effective doses of the OXT receptor antagonists atosiban and L-368,899. Impact of OXT system pharmacological manipulation and/or cSDS on OXT receptor expression in the mPFC was also assessed in this protocol. Results of rats subjected to cSDS and tested in the SIT and EPM are presented in Figures SM4 and 5. These animals received a combined treatment consisting of previously determined ineffective doses of atosiban (0.01 mg/kg; Experiment 2) or L-368,899 (0.01 mg/kg; Experiment 3), administered concurrently with the highest effective dose of carbetocin (1 mg/kg; Experiment 1). Regarding the time spent in the IZ (Fig. SM4A), a two-way repeated measures ANOVA indicated a significant effect for the target factor [$F_{(1,71)} = 87.17$, $p \leq 0.05$], but no effect for the treatment factor or interaction between factors [Treatment, $F_{(6,71)} = 0.49$, $p = 0.82$; Target × Treatment, $F_{(6,71)} = 1.23$, $p = 0.30$]. Regarding the time spent in the CZ, there was no significant difference for the evaluated factors [Target, $F_{(1,71)} = 0.65$, $p = 0.42$; Treatment, $F_{(6,71)} = 1.86$, $p = 0.10$; Target × Treatment, $F_{(6,71)} = 0.35$, $p = 0.91$]. Regarding the social interaction ratio (Fig. SM4C), ANOVA revealed no significant difference between groups [$F_{(6,71)} = 0.74$, $p = 0.62$]. These results suggest that neither stress nor pharmacological treatments interfered with social avoidance behavior as assessed in the SIT.

Concerning the EPM data, ANOVA indicated a significant difference between groups regarding the EAE [$F_{(6,71)} = 2.55$, $p = 0.03$]. A *post hoc* test revealed that the group treated with L-368,899 + carbetocin (L + C) showed a higher number of entries into enclosed arms compared to the control group (S+S, $p = 0.04$) (Fig. 5A). Regarding the %OAE and % OAT measures, statistical analysis indicated a significant difference between groups [%OAE: $F_{(6,71)} = 6.02$, $p \leq 0.001$; %OAT: $F_{(6,71)} = 6.66$, $p \leq 0.001$]. A *post hoc* test revealed that cSDS rats treated with saline (S+S group) showed a decrease in open arm exploration compared to the NAI+SAL group (%OAE, $p = 0.059$; %OAT, $p \leq 0.001$), and that animals treated with carbetocin (S+C) showed an increase in open arm exploration in the EPM for both spatial-temporal measures compared to the S+S group ($p \leq 0.003$). This carbetocin-induced effect was abolished by co-administration of either atosiban (A+C group; $p \leq 0.002$) or L-368,899 (L+C group; $p \leq 0.008$) (Figs. 5B and 5C).

Representative photomicrographs illustrating OXT receptor immunoreactivity in the mPFC are presented in Fig. 6A-I. Fig. 6C specifically depicts an OXT receptor-labeled pyramidal neuron located within the PL cortex. Fig. 6D-I represent OXT receptor expression at magnification used to calculate the Mean Fluorescence Intensity (MFI).

Given the absence of detected hemispheric differences (data not shown), the MFI score was assessed across both hemispheres. Regarding the OXT receptor expression pattern, in the Cg subarea, ANOVA indicated a significant difference between experimental groups [$F_{(6,31)} = 13.30$, $p \leq 0.001$]. A *post hoc* test revealed that stress resulted in increase of OXT receptor expression compared to the NAI+S group ($p \leq 0.001$), an effect reversed by treatment with either atosiban or L-368,899 ($p \leq 0.001$), independently of the combination of the treatment with carbetocin.

Regarding the PL and IL subregions, ANOVA indicated a significant difference between the experimental groups in both subareas [PL: $F_{(6,31)} = 7.01$, $p \leq 0.001$; IL: $F_{(6,31)} = 10.46$, $p \leq 0.001$]. However, a *post hoc* test revealed that in both structures, cSDS did not result in changes of OXT receptor expression. Nevertheless, carbetocin treatment increased

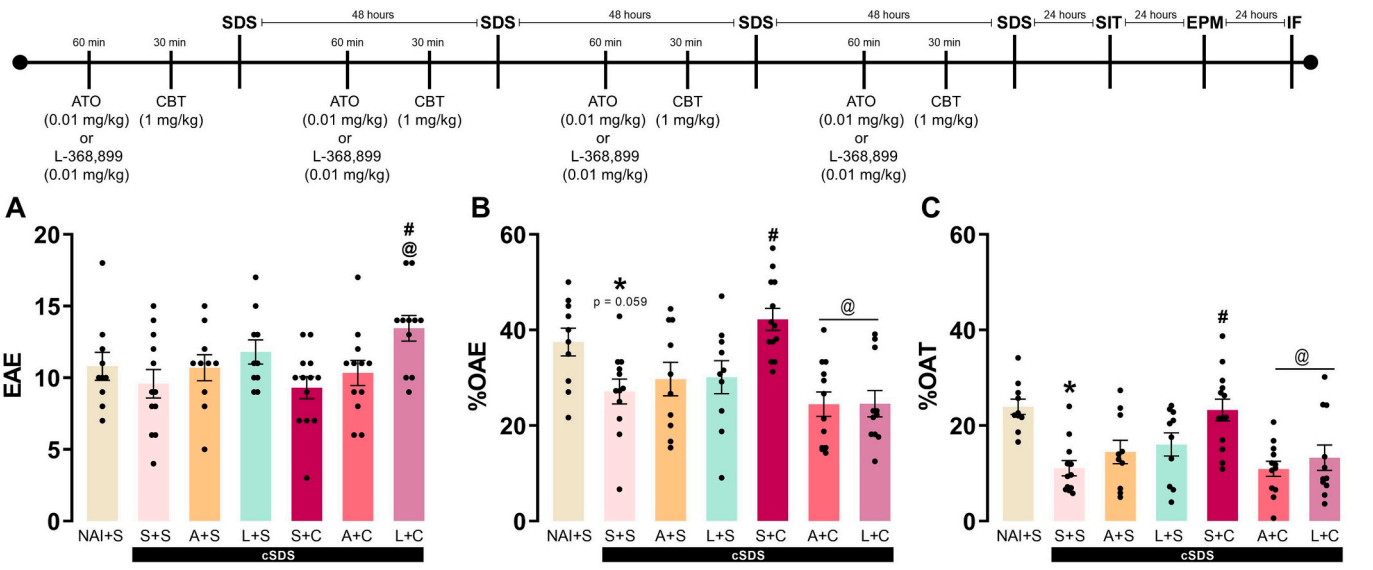

Fig. 5. Combined Effects of atosiban (ATO) or L-368,899 and Carbetocin on Anxiety-like Behavior in the Elevated Plus-Maze. Bars with scatter dot plot represent mean (±SEM) of NAI+S (n = 10), S+S (n = 12), A+S (n = 10), L+S (n = 10), S+C (n = 13), A+C (n = 12), and L+C (n = 11) subjected to EPM. (A) EAE: Entries into enclosed arms. (B) %OAE: Percentage of entries into the open arms. (C) %OAT: Percentage of time spent in the open arms. NAI+S, Non-Aggressive Interaction + saline. S, animals subjected to cSDS protocol and treated with saline. A, animals subjected to cSDS and treated with atosiban. L, animals subjected to cSDS and treated with L-368899. C, animals subjected to cSDS and treated with carbetocin. Black bar represents groups subjected to cSDS. *p ≤ 0.05 S+S group *versus* NAI+S group. #p ≤ 0.05 L+C or S+C *versus* S+S group. @p ≤ 0.05 A+C or L+C *versus* S+C group.

OXT receptor expression in both PL and IL (p ≤ 0.001), and this effect was blocked by either atosiban or L-368,899 (p ≤ 0.001) (Figs. 6H and 6I).

4. Discussion

The present study aimed to investigate the systemic effects of OXT receptor modulators—carbetocin, atosiban, and L-368,899—on social behavior and anxiety in rats exposed to cSDS. Additionally, we examined the influence of cSDS and OXT system pharmacological manipulation on OXT receptor expression within the mPFC. Our findings reveal distinct modulatory roles for these compounds in cSDS-induced behavioral alterations and OXT receptor quantitative expression, shedding light on the intricate neurobiological mechanisms underlying these processes.

Our behavioral results indicate that cSDS, while not inducing social avoidance in the SIT, reliably induced anxiety-like behavior as measured by the EPM test. This aligns with previous literature demonstrating that psychosocial stressors can lead to persistent anxiety-related impairments in rodents (da Costa et al., 2023; Jianhua et al., 2017; Macedo et al., 2018; Patki et al., 2014), even if direct social interaction patterns remain unchanged.

Extensive clinical and preclinical studies demonstrate the anxiolytic effects of OXT (Ayers et al., 2011; Bale et al., 2001; Blume et al., 2008; de Oliveira et al., 2012; Naja and Aoun, 2017) and its analog, carbetocin (V Klenerova et al., 2009; Mak et al., 2012; Zanos et al., 2014). OXT's anxiolytic properties in rodents have been demonstrated across various anxiety models including EPM, black-white box, or paradigms employing punished crossings (Vera Klenerova et al., 2009; Zanos et al., 2014). Given this, the observation that carbetocin reversed the anxiogenic-like effect of cSDS in the EPM was anticipated. However, it is worth highlighting that although evidence that OXT treatment prevented cSDS-evoked anxiogenic-like effect in socially monogamic mandarin voles (Hou et al., 2023), findings reported here are the first describing this effect in rats. Taken together with previous evidence, the new data reported here suggest that effect of OXT receptor agonism on anxiogenic response evoked by social stress is not species-specific. It is important to acknowledge that the current findings are restricted to a prophylactic context, as the drugs were administered prior to each stress session, potentially modifying the animals' subjective experience of the stressor itself. This design does not allow us to distinguish a preventative effect from a therapeutic one, a critical area for future post-hoc intervention studies.

The discovery of the same effect in atosiban- and carbetocin-treated rats was unexpected. This suggests a complex involvement of the OXT system in modulating anxiety-like responses to chronic stress. In this sense, the OXT receptor, a member of the G-protein coupled receptor family, is known to engage with various G-protein subtypes (including Gq, Gi, and Gs), thus potentially leading to a range of diverse effects (Busnelli et al., 2012; Busnelli and Chini, 2017; Thakur et al., 2019). In particular, the OXT receptor has demonstrated a preferential association with Gq and Gi protein subunits (Brighton et al., 2020; Strakova and Soloff, 1997). Within neuronal cells, these associations can result in antagonistic actions (Gravati et al., 2010; Williams et al., 2020). Atosiban has been reclassified as a "biased agonist" for the OXT receptor coupled to Gi-protein (Busnelli et al., 2012; Reversi et al., 2005). This selective activation can trigger various functional effects, such as the release of GABA in the brain (Thakur et al., 2019), which is generally associated with anxiolytic effects (Arora et al., 2024). Interestingly, atosiban does not behave as a partial agonist for Gq-coupled OXT receptor (Reversi et al., 2005). Instead, it acts as a competitive antagonist, preventing OXT from binding to the OXT receptor/Gq complex. This action promotes or stabilizes a receptor conformation that favors Gi- over Gq-coupled receptor (Busnelli et al., 2012; Reversi et al., 2005). Therefore, the atosiban's anxiolytic action could be mediated by this unique property of activating Gi-coupled OXT receptor, rather than a classical antagonistic blockade. Additionally, it has been demonstrated that atosiban is not selective for OXT receptor in relation to V_{1A} receptor (Manning et al., 2001, 1995), in some conditions presenting even higher affinity for the vasopressinergic receptor (Manning et al., 2005). Contrary to OXT, endogenous vasopressin presents anxiogenic effects (Morales-Medina et al., 2016; Neumann and Landgraf, 2012), and it has been proposed that anxiety status is linked to balanced OXT *versus* vasopressin levels in the brain (Neumann and Landgraf, 2012). Therefore, other possibility is that anxiolytic-like effect of atosiban might the related to a shift in OXT/vasopressin balance toward the former as

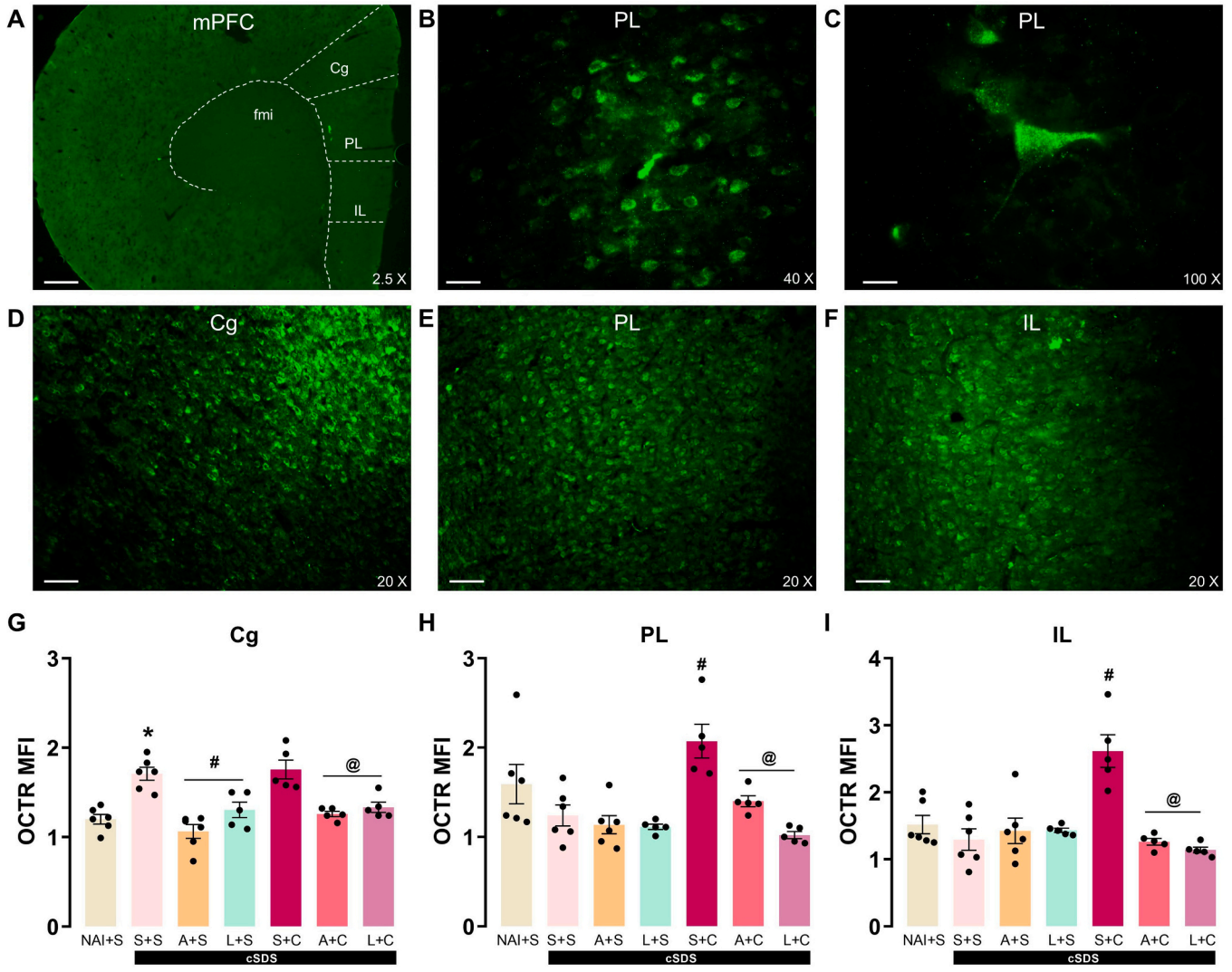

Fig. 6. Effects of cSDS and OXT system pharmacological manipulation on OXT receptor expression within the Medial Prefrontal Cortex Subregions. (A) Representative image (2.5 X) of the left mPFC and subareas (Cg, PL and IL) (scale bar = 500 μm). (B and C) Representative images (40 and 100 X, respectively) of the left PL showing immunoreactivity for OXT receptor (scale bars = 50 and 10 μm, respectively). (D, E and F) Representative images (20 X) of the left Cg, PL and IL showing immunoreactivity for OXT receptor at magnification used to calculate the Mean Fluorescence Intensity (MFI) (scale bar = 100 μm). MFI for OXT receptor positive neurons in the Cg (G), PL (H) and IL (I). Bars with scatter dot plot represent mean (±SEM) of NAI+S (n = 6), S+S (n = 6), A+S (n = 6), L+S (n = 5), S+C (n = 5), A+C (n = 5), and L+C (n = 5). mPFC, medial prefrontal cortex; Cg, anterior cingulate cortex; PL, prelimbic cortex; IL, infralimbic cortex; fmi, forceps minor of the corpus callosum. NAI+S, Non-Aggressive Interaction + saline. S, animals subjected to cSDS and treated with saline. A, animals subjected to cSDS and treated with atosiban. L, animals subjected to cSDS and treated with L-368,899. C, animals subjected to cSDS and treated with carbetocin. Black bar represents groups subjected to cSDS. *p ≤ 0.05 S+S group *versus* NAI+S group. #p ≤ 0.05 A+S or L+S or S+C *versus* S+S group. @p ≤ 0.05 A+C or L+C *versus* S+C group.

resulted of the vasopressin receptor antagonism.

Since atosiban treatment reversed the cSDS effect in the EPM, we sought to investigate the effects of a potent full OXT receptor antagonist, the L-368,899. This drug is described as a non-peptide OXT receptor antagonist without biased agonistic effects (Boccia et al., 2007). However, the three doses tested revealed that L-368,899 did not significantly alter SDS-induced anxiety or social avoidance behaviors. The difference in behavioral outcome between atosiban and L-368,899 further support the idea of atosiban acting as a biased agonist, leading to a functional outcome distinct of a pure antagonist. Previous research indicates that L-368,899 penetrates the central nervous system (CNS) and accumulates in limbic brain areas implicated in social behavior (Boccia et al., 2007), suggesting it can reach relevant brain regions. However, its lack of effect here, unlike atosiban, points to differential pharmacological profiles or downstream signaling pathways, suggesting that atosiban effect is related to biased agonistic action on Gi-coupled OXT receptor.

The investigation of the combined effects of the ineffective doses of atosiban and L-368,899 with the effective dose of carbetocin provided critical insights. The lower doses of either atosiban or L-368,899 were able to block the anxiolytic effect of carbetocin in the EPM. This confirms their antagonistic properties against the anxiolytic actions of exogenous carbetocin, at least at these specific doses and experimental conditions. This "blockade" effect, even with low doses of the antagonists, highlights the sensitivity of OXT receptor-mediated anxiety regulation and suggests that the anxiolytic effect of carbetocin is indeed mediated by OXT receptor activation. Furthermore, this finding further reinforces the idea that atosiban anxiolytic effects are mediated by signaling pathway distinct of that recruited by carbetocin. Considering evidence stated above that atosiban act as antagonist of Gq-coupled OXT receptor (Busnelli et al., 2012; Reversi et al., 2005), we can assume that carbetocin anxiolytic effect is mediated by this signaling pathway. Taken together, our findings indicate that activation of either Gi- or Gs-coupled OXT receptor can prevent anxiogenic effect caused by cSDS. This raises the conceptual question: how can the Gq-mediated effect of

carbetocin and the Gi-mediated effect of atosiban both produce the same anxiolytic outcome? We propose that this may reflect a mechanism of functional convergence at the circuit level. Ultimately, the anti-anxiety effect likely depends on the net inhibitory influence exerted on anxiety-promoting neural circuits. Within the mPFC, OXT receptor expression is predominantly localized to GABAergic interneurons (Nakajima et al., 2014a; Schimmer et al., 2024), and OXT activates these inhibitory neurons (Sabihi et al., 2017). In this context, the Gq signaling induced by carbetocin may exert its anxiolytic-like effect by preferentially exciting local GABAergic interneurons, thereby producing a net suppression of principal neuron output (Sabihi et al., 2017). Conversely, although previous studies have demonstrated limited expression of OXT receptor in mPFC pyramidal neurons relative to local interneurons (Schimmer et al., 2024), as shown in Fig. 6C, we identified detectable expression of this receptor in pyramidal neurons. This suggests that atosiban's Gi signaling may exert its anxiolytic-like effect by directly inhibiting these anxiety-driving neurons. Alternatively, the effect of atosiban may also involve inhibition of anxiety-promoting principal neurons in other brain regions, such as the hippocampus (Eyring et al., 2020; Liu et al., 2022). Thus, while the intracellular pathways are distinct, both mechanisms may converge functionally to inhibit neurons of anxiety circuit.

Regarding OXT receptor quantitative expression in the mPFC subareas, our immunofluorescence data yielded complex results. In the Cg, cSDS increased OXT receptor expression, and both atosiban and L-368,899, but not carbetocin, treatments prevented this effect. This suggests that cSDS might influence OXT receptor expression in the Cg as consequence of the own OXT receptor activation. Decrease in OXT receptor mRNA levels in the hypothalamus following chronic atosiban treatment has been previously reported (Babic et al., 2015), which aligns with our findings. Our results are also supported by previous evidence obtained in voles that OXT treatment prevented decrease in OXT receptor levels within the nucleus accumbens following cSDS (Hou et al., 2023) and increased mesolimbic OXT receptor binding in defeated animals (Hale et al., 2021). This suggests a regulatory mechanism that OXT receptor activation might lead to receptor upregulation.

In contrast, the PL and IL data indicated that cSDS did not affect OXT receptor expression. However, carbetocin treatment did increase OXT receptor expression in these regions, and this effect was subsequently blocked by the antagonists. This suggests that while cSDS itself might not directly upregulate OXT receptor in PL and IL, exogenous OXT (via carbetocin) can induce such an upregulation, and this process is OXT receptor-dependent. These findings underscore regional specificities in OXT receptor regulation within the mPFC in response to stress and pharmacological interventions. Differential expression and modulation of OXT receptor across mPFC subregions could contribute to their distinct roles in anxiety and social behavior, as these subregions are known to participate in diverse aspects of emotional and behavioral control (Faria et al., 2020; Giannotti et al., 2019; Kesner and Churchwell, 2011; Liu et al., 2021; McKlveen et al., 2019, 2015; Oliveira et al., 2022, 2021; Santos-Costa et al., 2021; Tan et al., 2019; Wilson et al., 2010; Yamada et al., 2015).

The results obtained for social avoidance in the SIT, where neither stress nor any of the drug treatments (carbetocin, atosiban, or L-368,899) significantly altered social interaction, contrast with our EPM findings. This suggests a dissociation between anxiety-like behavior (EPM) and specific social avoidance (SIT) in this cSDS model. It is possible that the SDS model used, while effective in inducing a generalized anxious state, does not robustly induce social avoidance in this rat strain or under our testing conditions. Different social stress paradigms, or variations in the social interaction test, might be necessary to consistently elicit social avoidance.

Given previous reports demonstrating sex differences in the effects of OXT on stress responses (Love, 2018), it is important to acknowledge the limitation of the present study, which exclusively utilized male rats. This decision was primarily driven by the absence of a widely validated and standardized SDS model specifically designed for female rodents at the time of study design. The male SDS model relies on inter-male territorial aggression, which is not typically observed in female-to-female interactions under standard laboratory conditions. Male-to-female aggression is also rare or complex to induce. However, growing evidence highlights significant sex differences in responses to stress and in the role of the oxytocin system in mediating social behavior and anxiety. Stress-related disorders, such as anxiety, are more prevalent in women than in men (McLean et al., 2011), yet preclinical research has historically underrepresented studies in females (Gualtierotti, 2025). Recent advancements in developing and validating female social stress models (Harris et al., 2018), including those utilizing chemogenetic activation to induce male aggression towards females or inter-female aggression paradigms (Kuske and Trainor, 2021), underscore the feasibility and importance of such investigations. Therefore, a critical next step for future research is to investigate the effects of OXT receptor modulation with carbetocin, atosiban, and L-368,899 on anxiety-like behavior and OXT receptor expression in female rats exposed to cSDS. Such studies are essential to elucidate potential sex-specific mechanisms and identify novel therapeutic targets for stress-related disorders that are relevant to both sexes.

In summary, our study provides evidence for the critical role of the OXT system in modulating anxiety-like behavior following cSDS. The differential effects of atosiban (anxiolytic-like, possibly due to biased agonism) and L-368,899 (pure antagonism) highlight the importance of considering the specific pharmacological profile of OXT receptor ligands. The ability of both antagonists to block carbetocin's anxiolytic effects confirms OXT receptor mediation. Furthermore, the region-specific modulation of OXT receptor expression in the mPFC by stress, carbetocin, and OXT receptor antagonists emphasize the complex and dynamic nature of OXT receptor regulation in brain regions crucial for emotional processing. These findings contribute to a deeper understanding of OXT's therapeutic potential in stress-related neuropsychiatric disorders.

Disclosures

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CRediT authorship contribution statement

Carlos C. Crestani: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Cristiane Busnardo:** Writing – review & editing, Funding acquisition. **Daniela Baptista-de-Souza:** Writing – review & editing. **Lucas Canto-de-Souza:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lucas Canto-de-Souza reports financial support was provided by National Council for Scientific and Technological Development - CNPq. Daniela Baptista-de-Souza reports financial support was provided by São Paulo Research Foundation (FAPESP). Carlos C Crestani reports financial support was provided by São Paulo Research Foundation (FAPESP). Cristiane Busnardo reports financial support was provided by São Paulo

Research Foundation (FAPESP). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pneurobio.2025.102853.

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