

The oxytocin receptor blockade in the paraventricular nucleus has anxiolytic effects and contributes to cardiovascular sympathetic/parasympathetic control during restraint stress

Ivaldo Jesus Almeida Belem-Filho¹

ivaldofilho90@hotmail.com

Universidade de São Paulo

Alana Tercino Frias²

Universidade Estadual Paulista (Unesp)

Gabriel Victor Lucena Silva³

Universidade de São Paulo

Helio Zangrossi-Junior¹

Universidade de São Paulo

Fernando Morgan de Aguiar Corrêa

Universidade de São Paulo

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Abstract

Stressful events are commonly linked to psychological and cardiovascular disorders. Restraint stress is a well-established model for studying these changes. While oxytocin (OXT) neurons in the paraventricular nucleus (PVN) are known to be activated by restraint stress, the precise role of OXT in this process is not fully understood. To investigate this, rats were subjected to one or two hours of restraint stress after receiving an OXT receptor antagonist (L-368,899) directly into the PVN (3, 6, or 9 nmol). During the stress, cardiovascular parameters such as mean arterial pressure (MAP) and heart rate (HR) were monitored, and their corticosterone levels were measured. 24 hours later, the animals underwent behavioral tests for anxiety (open field, elevated plus maze, and light/dark box). Restraint stress increased both MAP and HR. The OXT antagonist had complex effects on MAP, reducing it at the 3 nmol dose and increasing it at the 6 nmol dose. The antagonist also reduced the stress-induced heart rate increase at the 3 and 9 nmol doses. Heart rate variability analysis showed that the drug reduced sympathetic activity and increased parasympathetic activity, intensifying bradycardia. In behavioral tests, the antagonist increased anxiety-like responses, except for the 9 nmol dose, which prevented these responses. Corticosterone levels were elevated in all stressed animals, regardless of treatment. These findings suggest that OXT in the PVN plays a crucial role in modulating cardiovascular and behavioral responses to stress. Blocking its receptors altered sympathetic/parasympathetic cardiac regulation and reduced anxiety but did not affect the corticosterone axis.

Highlights

- OXT receptor antagonism increases sympathetic vascular activity, reduces sympathetic cardiac activity, and enhances parasympathetic modulation, leading to reduced tachycardia in response to stress.
- PVN OXT receptors mediate significant cardiovascular effects, particularly enhancing tachycardia during stress.
- OXT receptors are involved in central autonomic control, modulating the balance between sympathetic and parasympathetic activity.
- OXT receptor activation may be crucial for the development of late anxiety behavior, and its blockade can prevent it.

1. Introduction

Stressful situations are common in daily life and involve complex physiological and psychological responses to environmental or internal stimuli, that can lead to adaptive or maladaptive consequences. Acute stressors induce activation of two major neural systems: the hypothalamus-pituitary-adrenal (HPA) axis, increasing stress biomarkers (i.e. cortisol in humans and corticosterone in rodents), and the sympathetic nervous system (SNS), increasing autonomic activity to high values of blood pressure (BP) and heart rate (HR)¹⁻³. Acutely, activation of the stress system triggers a set of behavioral and physiological changes, and these responses are typically adaptive, temporary, and aimed at enhancing the

individual's chances of survival ³. However, if these responses are excessive or inadequate may affect personality, behavior and physiological functions ^{4,5}.

Restraint stress (RS) is a widely used model of stress that evokes instantly autonomic and HPA-axis activation, followed by later behavioral changes, including increased BP, HR, corticosterone release into the blood, and an anxiogenic phenotype observed 24 hours after the restraint session ⁶⁻⁹. Interestingly, many brain areas are involved in the restraint stress responses such as the prefrontal cortex ^{10,11}, insular cortex ¹², lateral septum ¹³, bed nucleus of stria terminalis ¹⁴, dorsal and ventral hippocampus ¹⁵, as well as hypothalamic areas such as the medial preoptic area ¹⁶, dorsomedial hypothalamus ¹⁷, supraoptic nucleus ¹⁸ and the paraventricular nucleus (PVN) ^{19,20}. The PVN serves as a crucial hub for the integration of stress-related information, playing a central role in modulating both the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. Blockade of PVN synapses with cobalt chloride showed that this area is involved with the mediation of the BP increase, tail temperature decrease and plasmatic corticosterone release observed during RS, providing evidence of autonomic and neuroendocrine modulation ¹⁹.

The PVN, where the hormones vasopressin and oxytocin (OXT) are produced, has been reported to be activated during either acute ²¹ or chronic RS with increase of OXT secretion within the PVN ²². OXT is also secreted to the periphery ^{23,24}. As well, PVN oxytocin-producing neurons are known to have close anatomical and functional interactions with corticotropin-releasing hormone (CRH) neurons. This intricate relationship suggests that PVN-derived oxytocin is a key component of the body's stress-coping mechanisms. Under acute and chronic stress conditions, locally released oxytocin may act as a potent stress buffer. Centrally, PVN OXT is believed to inhibit CRH release and prevent HPA axis overactivation either by activating PVN GABAergic interneurons or by directly binding to oxytocin receptors (OXTRs) on CRH neurons, thereby contributing to anxiolytic effects ²⁵⁻²⁷. Our group showed that OXT has peripheral effects on HR during RS modulating tachycardiac responses, thus preventing the overload of the cardiovascular system ²⁸.

However, further studies are necessary to clarify the role played by central OXT in the PVN during restraint stress. Intracerebroventricular OXT injection attenuated c-FOS expression in the PVN, as well the HPA-axis activity induced by RS ²⁶ and stress-induced anxiety-related behaviors in rats ²⁹. Moreover, PVN OXT neurons prevents hypoxia-induced hypertension by activating cardiac vagal neurons in dorsal motor nucleus ³⁰, and are coupled to sympathetic and parasympathetic areas that control autonomic responses to stress ³¹. It has been reported that OXT has anxiolytic effects ^{32,33}; in chronic models of stress, intraperitoneal OXT or PVN stimulation reversed the anxiogenic effects of stress in males and females voles ³⁴. Injection of OXT, either centrally or peripherally decreased the expression of anxiety-related behaviors in male mice, and such effects were blocked by intracerebral injections of an OXT antagonist, providing evidence that OXT's anxiolytic properties are mediated by central OXT receptors ³⁵. Furthermore, OXT knockout mice expressed increased levels of anxiety ³⁶. The PVN serves as a key autonomic integration center, modulating blood pressure and heart rate through direct projections to brainstem and

spinal cord regions. These projections are critical for orchestrating both sympathetic and parasympathetic outflow. Specifically, PVN neurons project to the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMNV) to modulate parasympathetic activity, and to the rostral ventrolateral medulla (RVLM) and the intermediolateral cell column (IML) of the spinal cord to regulate sympathetic tone³¹.

Regardless of the growing evidence supporting the role of OXT in modulating stress responses, its role in the PVN is not fully understood. Therefore, this study aimed to explore the central role of OXT receptors in the PVN in the responses evoked by acute RS, addressing the lack of information on their specific contributions to autonomic, behavioral and neuroendocrine regulation under stressful conditions.

2. Methods

All procedures followed the ARRIVE guidelines and adhered to ethical standards for animal studies. The procedures were approved by Animal Ethics Committee of the Medical School of Ribeirão Preto, University of São Paulo (CEUA 079/2017).

2.1. Animals

Male Sprague Dawley (SD) rats, 7–8 week old, weighing 250–290 grams, were used. The animals were provided by the University of São Paulo animal facility and housed in plastic cages (5 animals per cage) at room temperature of 22–24°C, with a 12 hours light-dark cycle (lights on 6:00-lights off 18:00) in the Animal Care Unit of the Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo. They were maintained with water and food *ad libitum* throughout the study, except during the experiment period. All protocols are described below and followed the schematic diagram (Fig. 1).

2.2. Treatments

Ten minutes before the RS session, animals were treated intra-PVN with either the OXT antagonist L-368,899 hydrochloride (Tocris Bioscience, UK) at doses 3, 6 and 9 nmol / 100 nL or 100 nL of vehicle (NaCl 90 g/L; NaCl 100 mmol/L, Na₃PO₄ 2 mmol/L, KCl 2.5 mmol/L, MgCl₂ 1 mmol/L, NaHCO₃ 27 mmol/L, CaCl₂ 2.5 mmol/L at pH7.4), per brain side. Bilateral injections were performed in a volume of 100 nL^{37,38}, using a 1 µL syringe (7001KH; Hamilton, Reno, Nevada, United States) connected to a microinjection needle (gauge 33—Small Parts, Miami Lakes, FL, United States) through a piece of PE-10 polyethylene tubing. Animals were anesthetized with tribromoethanol (250 mg/kg, i.p. Sigma Aldrich, USA) for stereotaxic surgeries. For the artery cannulation, animals were anesthetized with ketamine (50 mg/kg, i.p. Dopalen®, CEVA, Brazil) + xylazine (5 mg/kg i.p. Dopaser®, CEVA, Brazil). After all surgical procedures, animals received a poly-antibiotic (Pentabiotico®, 0.27 mg/kg, i.m. Fort Dodge, Brazil) to prevent infection, and a non-steroidal anti-inflammatory (Banamine®, Flunixin Meglumine, 0.025 mg/kg, i.m., Schering Plough, Brazil) for postoperative analgesia. For histological procedures, animals were anesthetized with urethane (1.25 g/kg i.p., Sigma Aldrich, USA).

The dose used was calculated based on the work of Olson and colleagues³⁹, who demonstrated that a dose of 9 nmol injected ICV was effective in blocking central anorexigenic effects, yet showed no effect, *per*

se. Additionally, Mullis and colleagues⁴⁰ utilized doses of 1 and 5 micrograms intra-VTA, showing an effect with the 5 microgram dose. The conversion of these doses to nanomoles corresponds to: 1 µg (1.59 nmol), and 5 µg (7.97 nmol). We used approximate doses to generate an equidistant logarithmic inhibition-response curve (3, 6, and 9 nmol).

2.3. Stereotaxic surgery

Surgery was performed according to Busnardo et al⁴¹. Five days before experiments, animals were anesthetized with tribromoethanol (250 mg/kg, i.p. Sigma Aldrich, USA) and their head were fixed to a stereotaxis apparatus (Stoelting, Wood Dale, Illinois, USA). The skull was exposed and trepanned with a dental drill to insert stainless steel bilateral guide cannulas (24G and 15 mm-long) aimed at the PVN, according coordinates: anteroposterior = + 6.9 mm, lateral = 2.1 mm, ventral = -7.0 mm, from the interaural line, and angled 10°⁴². The guide cannulas were positioned 1 mm above the intended stimulation sites and fixed to the skull with a metal screw and dental cement. After surgery, animals received poly-antibiotic (Pentabiotico®, 0.27 mg/kg, i.m. Fort Dodge, Brazil) to prevent infection and a non-steroidal anti-inflammatory (Banamine®, Flunixin Meglumine, 0.025 mg/kg, twice/day for 3 days, i.m., Schering Plough, Brazil) for postoperative analgesia.

2.4. Artery cannulation

Five days after stereotaxic surgery for intra-PVN guide cannula implantation, the animals underwent femoral artery cannulation surgery. The animals were anesthetized with ketamine (50 mg/kg, i.p. Dopalen®, CEVA, Brazil) + xylazine (5 mg/kg i.p. Dopaser®, CEVA, Brazil), and a polyethylene catheter was implanted into the femoral artery for cardiovascular recording. The catheter was exposed on the animal's dorsum and attached to the skin. After surgical procedures, animals were injected with the non-steroidal anti-inflammatory (Banamine®, Flunixin Meglumine, 0.025 mg/kg, i.m., Schering Plough, Brazil). The following day, animals were connected to the cardiovascular parameter acquisition system, and the experiment was conducted

2.5. Restraint stress paradigm

Animals were subjected to RS as previously reported^{8,38}. In detail, animals were placed in a plastic cylindrical tube (6.5 x 15 cm, diameter x length) with holes (1 cm) that cover 20% of tube surface to allow ventilation and restrained for 1 hour for cardiovascular recordings and corticosterone measurement. Another group of animals, used for behavioral analyses, was restrained for 2 hours in a metal tube (6.3 x 19.3 cm, diameter x length, with an adjustable roof and ventilation holes), then returned to their home cage, where they were kept isolated for 24 hours.

2.6. Behavioral analysis

Behavioral tests were performed 24 hours after the acute RS session. Behavioral testing was initiated with the elevated plus maze, followed sequentially by the open field test and the light-dark box, with a one-hour interval between each assessment. Another set of animals was used to behavioral analysis and in this protocol, we added naïve group (non-treated and non-stressed group, n = 13).

2.6.1. Elevated plus maze test

The elevated plus maze test was performed according to Padovan and Guimarães⁴³. The apparatus is made of wood and consists of two open arms (50 cm length x 10 cm width), crossed at a right angle by two arms with the same dimension; the closed arms have 40 cm-high walls enclosing the sides, with no roof. The maze is located 50 cm above the floor. After each session, the apparatus was cleaned with 10% ethanol solution (V/V) to eliminate odors and allowed to dry before introducing a new animal. The total duration of test was 5 minutes, and behavior was recorded using a video camera and analyzed with software (AnyMaze Software, version 4.7, Stoelting). The percentage of entries in the open arm were calculated according this: % entries in open arms = (entries in open arms/(entries in open arms + entries in closed arms)*100. The percentage of time in the open arm were calculated according this To % time in open arms = (time in open arms/(time in open arms + time in closed arms)*100.

2.6.2. Open field test

The open field test was carried out a acrylic plastic arena (60x60 cm, length x width, that were divided into 16 minor squares 15x15 cm) in a sound-attenuated and temperature-controlled (24°C) room. Animals were placed in center and allowed to freely move for 5 minutes⁴⁴. After each session, the apparatus was cleaned with 10% ethanol solution (V/V) to eliminate odors and allowed to dry before introducing a new animal. The peripheral area consists of the 4 minor squares that are surrounded by walls (60x45 cm) and the central area consists of the 4 minor squares in the center, for from the wall (30x30 cm). Behavior was recorded through a video camera and analyzed with software (AnyMaze Software, version 4.7, Stoelting).

2.6.3. Ligh-dark box test

The light-dark box test was carried out according to Matthiesen and collaborators⁴⁵. Animals were placed into an arena made of plastic acrylic that consists of one lightened chamber (24 cm x 27 cm, length x width, 40 lux) and one dark chamber (24 cm x 27 cm, length x width, not illuminated) for 5 minutes, and were allowed to move freely throughout the arena. After each session, the apparatus was cleaned with 10% ethanol solution (V/V) to eliminate odors and allowed to dry before introducing a new animal. Behavior was recorded using a video camera and analyzed with software (AnyMaze Software, version 4.7, Stoelting).

2.7. Cardiovascular recording in unanesthetized rats

On the day of experiments, awake animals were connected via the artery cannula to a pressure transducer to record pulsatile arterial pressure (PAP, mmHg) for 10 minutes before intra-PVN treatment, 10 minutes after treatment, and 60 minutes during restraint stress. PAP was recorded using an amplifier (Model AECAD 04, AVS projects, São Paulo, SP, Brazil) coupled to a computerized acquisition system (MP100A, Biopac, Santa Barbara, CA, USA). Mean arterial pressure (MAP) and heart rate (HR) data were calculated from PAP records using the Acknowledge III software (Biopac, Santa Barbara, CA, USA), and PAP peak interval were integrated every 6 seconds⁴¹.

2.7.1. Blood pressure and heart rate variability

PAP recordings from animals were processed using a computer program (LabChart 7 Pro, ADInstruments, Bella Vista, Australia) to determine systolic arterial pressure (SAP) and cardiac interval (CI). The analysis of frequency domain was performed using online software (CardioSeries v2.4, available on www.danielpenteado.com) and the fast Fourier transform method. Recordings were visually inspected, and segments with artifacts or nonstationary data were excluded from the analysis. The spectra were integrated into very low (0.0–0.2 Hz), low (0.2–0.75 Hz) and high frequency (0.75–3.0 Hz) bands. The sampling was fixed as 5 min recordings at 4 different times: basal, post-treatment, and restraint point 1 and 2. For the analyses during the restraint, the time sampling was fixed between 15–20 and 45–50 min of restraint for heart rate variability. The power of low frequency band is considered to reflect both sympathetic and parasympathetic influences, whereas the high-frequency component is linked to parasympathetic activity⁴⁶. The sympathovagal balance measurement was calculated as the ratio between low and high frequency (LF/HF); a higher LF/HF ratio is associated with a relative increase in the sympathetic drive^{47,48}. For SAP, results were represented in absolute units (mmHg²), and CI were represented in absolute units (ms²) and normalized units (nu).

2.7.2. Spontaneous baroreflex analysis

Using the Cardioseries v2.4 software, we performed a sequencing method to determine spontaneous baroreflex⁴⁹. In this method signals are considered in the time domain, and successive spontaneous increases or decreases (ramps) in the arterial pressure might elicit baroreflex-mediated responses in either HR or sympathetic nerve activity. In summary, SAP time series were scanned to identify SAP elevations of three or more beats accompanied by a progressive increase in the R-R interval, characterizing up ramps. For down ramps, we considered the opposite, SAP reductions of three or more beats accompanied by a progressive decrease in the R-R interval. The correlation coefficient (*r*) for inclusion should be higher than 0.80. The parameters measured were mean gain and baroreflex effectiveness index (BEI). The gain of a sequence is represented by the slope of the linear regression of SAP versus CI (R-R interval), for a correlation coefficient greater than the threshold *r*. The final gain is the average of the slopes obtained from all the sequences considered, for up, down, and all ramps. BEI was calculated as the number of sequences divided by the number of baroreflex ramps, for up, down, and all ramps⁵⁰.

2.8. Brain and blood collection

Blood samples of 1 mL were collected directly from the femoral cannula at the end of the RS. The samples (~ 1 mL) were transferred to Falcon tubes with heparin, centrifugated, and the plasma was collected and stored at -20°C. Thereafter, animals were anesthetized with urethane (1.25 g/kg, i.p.) and 100 nL of 1% Evans's Blue dye was injected bilaterally into the PVN to label injections sites, and their brains were perfused. In detail, the thoracic cavity was opened to assess the heart, and a formaldehyde solution (10%) was perfused through the left ventricle. Brains were collected and postfixed for 24 hours at 4°C. Animals that underwent behavioral tests were perfused at the end of behavioral experiments.

2.8.1. Histological procedures

Brains sections (40 μm -thick) were cut using a cryostat (CM-1900, Leica, Germany). Sections were stained with 1% neutral blue and propylene glycol for light microscopy analysis. Each injection site was meticulously examined under a light microscope (Eclipse E-600 microscope, Nikon, Japan) to confirm its location within PVN and the actual placement of the injection needles was determined by analyzing serial sections and identified them according to a rat brain atlas ⁴².

We defined as a successful injection when the tip of the cannula track and the visible diffusion of the injected dye marker were clearly confined to the PVN. We achieved a success rate of approximately 50% for bilateral PVN injections. Only data from animals with confirmed bilateral injections ($n = 71$) were included in the final analysis. The data from animals with misplaced injections ($n = 71$) were used for a separate control analysis to confirm the site-specific effects of treatment.

2.9. Corticosterone plasmatic dosage

Corticosterone samples were extracted with absolute ethanol, centrifuged, lyophilized and stored. The samples were reconstituted, and plasmatic concentrations were measured by an enzymatic immunoassay method (Enzo Life Sciences, Farmingdale, NY, EUA) according to manufactory instructions.

2.10. Statistical analysis

Statistical analysis and graphical representation were performed using GraphPad Prism version 9.0.2 (San Diego, California, USA). Data are expressed as mean $\pm$ standard error deviation (SEM). Basal values of MAP and HR were compared using values on 1 minute before injection and 1 minute before restraint session using a paired Student's t test (Before vs after). Repeated measurements (Time) two-way ANOVA (Time and treatment) were used to compare temporal curves of ΔMAP , ΔHR , blood pressure variability, heart rate variability and spontaneous baroreflex. To behavior analysis and corticosterone were made compared using one-way ANOVA. Were used Fischer's LSD post hoc test.

3. Results

The distribution of the microinjection sites throughout the rostro-caudal extension of the PVN of all animals from both protocols (cardiovascular and behavioral) is illustrated in Fig. 1B-F.

3.1. Basal values of mean arterial pressure (MAP) and heart rate (HR) of unanesthetized animals

The effects of all treatments on basal MAP and HR parameters are presented in Table 1. In this protocol animals were divided into: Vehicle ($n = 7$), L-368,899 3 nmol ($n = 8$), L-368,899 6 nmol ($n = 7$) and L-368,899 9 nmol ($n = 8$). The paired t test reveals that neither injections of vehicle nor L-368,899 (all three doses) affected MAP values compared to basal values recorded before the injections ($P > 0.05$). No effects on basal HR were observed after the treatments with vehicle or L-368,899 at the doses of 3 nmol 9 nmol ($P > 0.05$). However, a baseline HR increase was observed in the group treated with L-368,899 at 6 nmol ($P < 0.05$).

3.2. Cardiovascular and endocrine effects of oxytocin receptor blockade in the PVN on RS-induced cardiovascular responses

The two-way ANOVA of the temporal curves of the cardiovascular response to restraint stress showed effect only in the time factor for MAP ($F_{26,261}=6.975$; $P < 0.0001$, Fig. 2A) and HR ($F_{26,261}=6.240$; $P < 0.0001$, Fig. 2B). No significant effect was observed in the treatment factor (vehicle or the three doses of L-368,899) for MAP ($F_{3,26}=0.6758$; $P > 0.05$) or HR ($F_{3,26}=1.803$; $P > 0.05$). Nonetheless, the Fischer's LSD post-hoc test indicated that animals treated with L-368,899 at a dose of 3 and 9 nmol had a reduced RS-evoked tachycardia response compared to vehicle-treated group ($P < 0.05$, Fig. 2D and 2H, respectively).

An one-way ANOVA of corticosterone measurement showed that animals subjected to RS had an increase in hormone levels compared to naïve controls (animals not stressed) independent of treatment ($F_{4,27} = 3,056$; $P = 0.0337$). Post-hoc analyses revealed that vehicle ($P = 0.01$, $n = 6$), L-368,899 3 nmol ($P = 0.0206$, $n = 7$), 6 nmol ($P = 0.03540$, $n = 5$) and 9 nmol ($P = 0.0071$, $n = 6$) were different from naïve ($n = 8$), with no differences between them (Fig. 2I).

3.3 Spectral analysis and spontaneous baroreflex activity after oxytocin receptor blockade in the PVN under basal conditions and during restraint stress

The two-way ANOVA of the spectral analysis of blood pressure data showed effect of time factor ($F_{26,78}=1.887$; $P = 0.0177$) and no effect of treatment ($F_{3,26}=0.7820$; $P > 0.05$). Post-hoc analyses indicated that injections of L-368,899 6 and 9 nmol increased the VLF component compared to the vehicle group ($P < 0.05$; Fig. 3A). The LF component showed effect of the time factor ($F_{26,78}=2,131$; $P = 0.0056$) and no effect of treatment ($F_{3,26}=0.9924$; $P > 0.05$). Interestingly, post-hoc analyses revealed that L-368,899 9 nmol increased the LF component during RS (point 1) compared to the vehicle group ($P < 0.05$; Fig. 3B).

Two-way ANOVA of HR variability showed a time factor effect in all parameters measured (VLF: $F_{26,78}=6.250$; $P < 0.0001$; Fig. 3C, LF: $F_{26,78}=2.346$; Fig. 3D, $P = 0.0021$, HF: $F_{26,78}=2.537$; $P = 0.0009$; Fig. 3E, LF normalized units: $F_{26,78}=3.253$; $P < 0.0001$; Fig. 3F, HF normalized units: $F_{26,78}=3.253$; $P < 0.0001$; Fig. 3G and LF/HF: $F_{26,78}=2.329$; $P = 0.0023$; Fig. 3H). No effect of the treatment factor of any parameter measured (VLF: $F_{3,26}=0.3483$; $P > 0.05$; Fig. 3C LF: $F_{3,26}=1.083$; $P > 0.05$; Fig. 3D, HF: $F_{3,26}=0.5843$; $P > 0.05$; Fig. 3E, LF normalized units: $F_{26,78}=0.5838$; $P > 0.05$; Fig. 3F, HF normalized units: $F_{26,78}=0.5838$; $P > 0.05$; Fig. 3G and LF/HF: $F_{3,26}=0.7268$; $P > 0.05$; Fig. 3H).

Post-hoc analyses showed difference in basal conditions of group L-368,899 6 nmol before injection compared to all 3 other groups ($P < 0.05$) in the LF component (Fig. 3D). HF components increased after treatment of L-368,899 6 nmol compared to low doses (3nmol, $P < 0.05$; Fig. 3E). During RS L-368,899, at three doses, showed a decrease of LF component in normalized units, 6 nmol at point 1 ($P < 0.05$)

compared to ASCF and high dose 9 nmol, 3 and 9 nmol showed at point 2 ($P < 0.05$) compared to vehicle group (Fig. 3F). In an opposite manner, HF normalized units showed that 6 nmol at point 1 ($P < 0.05$) was different compared to ASCF and high dose 9 nmol, 3 and 9 nmol shown at point 2 ($P < 0.05$) compared to vehicle group (Fig. 3G). The sympathovagal balance (LF/HF) showed a decrease in all three doses of L-368,899, 6 nmol at point 1 ($P < 0.05$) compared to ASCF and high dose 9 nmol; 3 and 9 nmol shown at point 2 ($P < 0.05$), compared to vehicle group (Fig. 3H).

Analysis of spontaneous baroreflex effectiveness index (BEI) showed an effect of the time factor in all parameters measured (Up: $F_{26,78}=2.189$; $P = 0.0043$, Fig. 4A; Down: $F_{26,78}=2.831$; $P = 0.0002$, Fig. 4B; and All: $F_{26,78}=2.858$; $P = 0.0002$, Fig. 4C), and no effect of treatment factor of any parameter measured (Up: $F_{3,26}=1.441$; $P > 0.05$, Fig. 4A; Down: $F_{3,26}=0.4962$; $P > 0.05$, Fig. 4B and All: $F_{3,26}=1.001$; $P > 0.05$; Fig. 4C). The gain of baroreflex was measured and showed effect in all parameters (Gain Up: $F_{26,78}=3.772$; $P < 0.0001$, Fig. 4D; Gain Down: $F_{26,78}=3.264$; $P < 0.0001$, Fig. 4E and Gain All: $F_{26,78}=3.853$; $P < 0.0001$, Fig. 4F). Also, no effect of treatment was observed for all parameters (Gain Up: $F_{3,26}=0.02541$; $P > 0.05$, Fig. 4D, Gain Down: $F_{3,26}=0.1560$; $P > 0.05$, Fig. 4E and Gain All: $F_{3,26}=0.09409$; $P > 0.05$; Fig. 4F).

Post-hoc analyses revealed that L-368,899 9 nmol increased BEI Up and BEI All during restraint point 1 compared to vehicle ($P < 0.05$, Fig. 4A and 4C), and L-368,899 6 nmol showed an increase in BEI Up and BEI All at point 2, compared to L-368,899 9 nmol (Fig. 4A and 4C).

3.4 Behavioral effects of oxytocin receptor blockade into PVN over locomotion and anxiety-related behaviors 24-hs after restraint stress

In this protocol we used naïve group ($n = 13$) vehicle ($n = 12$), L-368,899 3 nmol ($n = 13$), L-368,899 6 nmol ($n = 9$) and L-368,899 9 nmol ($n = 9$). First, we evaluated animals in elevated plus maze test, one-way ANOVA showed an effect on entries and time in the open arms (% entries: $F_{4,50} = 3.620$, $p = 0.0115$, Fig. 5A; time: $F_{4,50} = 3.855$, $p = 0.0082$, Fig. 5B). Post-hoc analyses showed effect of stress inducing anxiogenic effect in the vehicle group compared to naïve in entries and time ($P < 0.05$). Oxytocin receptor antagonism showed divergent dose-dependent effects: 6 nmol caused anxiogenic effect compared to naïve and L-368,899 9 nmol groups ($p < 0.05$). L-368,899 9 nmol showed an anxiolytic effect compared to vehicle group ($p < 0.05$, Fig. 5A) and no effect in the numbers of entries into closed arms ($F_{4,50} = 0.5166$; $p = 0.7238$; Fig. 5C). The post-hoc analysis of time into open arms showed again effect of stress inducing anxiogenic behavior in vehicle group compared to naïve ($p < 0.05$), and again L-368,899 9 nmol group showed an anxiolytic behavior compared to vehicle and L-368,899 6 nmol groups ($p < 0.05$, Fig. 5B).

Following, we used open field arena. Animals were evaluated in open field arena; one-way ANOVA showed effect in locomotor activity ($F_{4,51} = 3.067$, $p = 0.0244$; Fig. 5D), post-hoc analyses showed that L-368,899 6 nmol induced hyperlocomotion in the animals compared to naïve, vehicle and L-368,899 3 nmol groups ($p < 0.05$, Fig. 5D). We analyzed the anxiety-associated behavior on open field arena, and for this we evaluated time spent in center and peripheral zones on the arena; one way ANOVA did not show effect in these

parameters (time center: $F_{4, 51} = 2.063$, $p = 0.0994$, Fig. 5E; time periphery: $F_{4, 51} = 1.966$, $p = 0.1138$, Fig. 5F), the post-hoc analyses showed effect of restraint stress in animals treated with vehicle and stressed to staying less time in the center ($p = 0.0082$, Fig. 5E) and more in the periphery zone ($p = 0.0106$, Fig. 5F) compared to naïve group.

Sequentially, animals were tested in the light/dark box (Fig. 5). One-way ANOVA evaluation of entries in the light side of the box showed a borderline effect ($F_{4, 50} = 2.454$, $p = 0.0578$, Fig. 5H). Post-hoc analyses showed anxiogenic effect with a reduction on entries in the light side, of treatment with L-368,899 3 nmol compared to naïve, vehicle and L-368,899 9 nmol groups ($p < 0.05$, Fig. 4H). Regarding the time spent on the light side of the box, one-way ANOVA showed no effect ($F_{4, 50} = 2.166$; $p = 0.0864$, Fig. 5I). Post hoc analysis showed effect of L-368,899 3 and 6 nmol inducing anxiogenic behavior with reduction on time spent in light area compared to Naïve group ($p < 0.05$, Fig. 5I). The last parameter analyzed was transition between light and dark areas (Fig. 5J), one-way ANOVA showed no effect in this parameter ($F_{4, 50} = 2.158$, $p = 0.0874$). Post-hoc analysis showed that L-368,899 3 nmol presents a reduction of transitions compared to naïve and vehicle groups ($p < 0.05$, Fig. 5J).

4. Discussion

The present study indicates that OXT receptors in PVN are related to autonomic activation, primarily tachycardic responses during restraint stress, by increasing sympathetic activity and/or reducing parasympathetic activation. Moreover, activation of oxytocin receptors during restraint stress is necessary to avoid the development of late anxiety behavior observed in this stress model. OXT is widely known by the effects on parturition and milk ejection, social bond and reproductive roles^{51–53}. However, it is also implicated as a cardiovascular hormone with effects on BP and HR regulation^{54–56} and also has anxiolytic effects^{57,58}.

The PVN is a central structures divided in three magnocellular and five parvocellular subdivisions⁵⁹. Magnocellular neurons project mainly to the posterior hypophysis, while parvocellular neurons project to many areas of the brain, including limbic areas⁵³ and brain stem and spinal cord, which are involved in sympathetic activation and cardiovascular control⁶⁰.

Although there is growing interest in the effects of neurotransmitters on cardiovascular central regulation, both under basal conditions and during stress, there is a lack of literature about PVN OXT cardiovascular effects in the restraint stress model. It is well documented that OXT neurons in the PVN depolarize sympathetic preganglionic neurons⁶¹, which abundantly express OXT receptors⁶². Peripheral OXT administration induces neural activation in the PVN, which is positively correlated with activation in brainstem cardio regulatory areas, specifically the dorsal vagal complex, nucleus ambiguous, and rostral ventrolateral medulla (RVLM) in prairie voles⁶³. This suggests that OXT may indirectly regulate cardiovascular responses by the PVN, modulating brainstem regions involved in cardiovascular responses. Previous work showed that 38% of PVN neurons that innervate the spinal cord express mRNA OXT⁶⁴. These PVN neurons also extend projections to the nucleus of the solitary tract, dorsal motor nucleus of the

vagus, rostral ventrolateral medulla, and intermediolateral column of the spinal cord^{60,65–68}, regions involved in regulating vagal and sympathetic outflow to the heart and blood vessels.

OXT intrathecal injection or PVN elicits increase in BP and HR in anesthetized animals, and treatment with L-368,899 was able to block the HR increase⁶⁹. In the present study, we report that injection of increasing doses of L-368,899 into the PVN caused different effects on the hypertensive response evoked by restraint stress. The dose of 3nmol reduced the BP increase, 6 nmol potentiated BP increase while 9 nmol did not affect this parameter. In contrast, the effects of the antagonist on the HR response were consistent with the findings of Yang and collaborators⁶⁹, with a reduction in RS-evoked tachycardia observed after injection of 3 and 9 nmol, whereas the 6 nmol dose had no effect.

The interpretation of our findings hinges on a critical distinction in the neuroanatomy of the PVN. While the activity of oxytocin-producing neurons is essential for stress coping, our results demonstrate that the integrity of the PVN OXTR system is essential for regulating stress-induced cardiovascular and behavioral responses. The administration of an OXTR antagonist directly into the PVN provided clear evidence that oxytocin signaling in this specific brain region plays a critical role in modulating the body's reaction to stress. The specificity of our approach is further supported by recent molecular studies, which indicate that oxytocin-producing neurons in the PVN have low expression of classic neurotransmitter markers like glutamate and dopamine⁷⁰. This suggests that these cells primarily exert their influence through their hormonal product, making the local blockade of OXTRs a highly effective strategy to disrupt this system output. This pharmacological blockade not only reduced the cardiovascular responses evoked by the stressor but also resulted in distinct alterations in behavioral outcomes.

OXT knockout mice showed an increase in basal BP and no effect in HR, however, during baroreflex tests, animals presented an increased tachycardic response compared to wild-type animals⁷¹, suggesting that OXT can exert tonic modulation on the BP. There is evidence in the literature indicating that OXT can modulate BP in behavioral situations. Under basal conditions, SD rats that received OXT antagonist by intracerebroventricular (i.c.v.) infusion displayed increased HR and a more prominent increase BP in sham animals or animals subjected to a heart failure model⁷². In the same study it was shown that treatment with an OXT antagonist, but not with OXT, was capable of increasing BP and HR responses observed when using air-jet stress. However, OXT injection reduced BP and HR in the heart failure group to values similar to those similar in the sham group⁷². In spontaneously hypertensive rats (SHR), central administration of OXT reduces stress-evoked hypertension and OXT antagonists potentiate this response, with no effect on tachycardic responses⁷³. Our results showed that OXT antagonism in the PVN reduced the stress-evoked hypertension at the dose of 3 nmol of L-368,899 and increased it at 6 nmol. Spectral analyses showed an increase in the SAP LF component, which related to vessel sympathetic activity, after the dose of 9 nmol. Taken together, these results suggest that oxytocin in the PVN exerts a modulatory effect in reducing the hypertensive response to stress, whereas higher doses of the antagonist lead to an increased BP response or enhanced sympathetic vascular activity.

In agreement with this idea, SHR showed decreased expression of OXT and increased OXT receptor mRNA in the PVN ⁷⁴, which may represent a compensatory effect for reduction in OXT. The authors also reported that training sessions induce plasticity from PVN to the dorsal brain stem, and that the main effect of this change is on HR control, increasing OXT density in NTS in both normotensive and hypertensive individuals ⁷⁴. Consistently, our results indicated a greater impact of the PVN OXT receptor modulation on the HR response to RS.

Regarding HR tachycardic control by central OXT, Braga and collaborators ⁷⁵ showed that OXT administration in the NTS/DMV reduces tachycardia evoked by exercise in trained rats. Furthermore, the use of OXT antagonist potentiated the tachycardic response during treadmill exercise. OXT antagonism had no effect in sedentary animals. In contrast, Morris et al. ⁵⁴ showed that OXT projections from the PVN are essential to tachycardic response to stress. Using a shaker stress model, the authors injected antisense oligomers targeting OXT into the PVN, which blunted cardiac responsiveness during stress. It is important to note that the cardiac central control of OXT is mediated by situation (basal, exercise or stress) and area (DMV/NTS or PVN). However, our results corroborate the findings of Morris and collaborators ⁵⁴ as the injection of an OXT antagonist into the PVN decreased the tachycardia evoked by RS.

In the present study, HR variability analyses indicated an increase in HF band, which is more related to parasympathetic activity, and an increase in the Up ramps of the spontaneous baroreflex analyses, favoring bradycardic reflex. In agreement, we have shown previously that the parasympathetic branch is activated during restraint stress ^{6,28}, favoring the idea that OXT in the PVN modulates this effect. Previous work showed that administration of OXT in PVN increases LF, HF and LF/HF bands of HR variability in animals stressed by an air-jet ⁵⁶. In accordance, we presently observed that administration of an OXT antagonist in the PVN caused a decrease in LF and LF/HF, and an increase of HF during restraint stress. However, it has been reported that oxytocinergic chemogenetic activation in the PVN directly acts on parasympathetic cardiac vagal neurons in the vagal nucleus by co-release of OXT and glutamate, increasing cardiac parasympathetic tone (Dyavanapalli et al., 2020), showing that cardiovascular control mediated by PVN OXT may involve both sympathetic and parasympathetic pathways.

Restraint stress is a model that induces behavioral changes related to psychiatric disorders such as anxiety and depression. Focusing on anxiety, acute restraint stress induces changes evidenced as reductions in the exploration of the open arms in the elevated plus maze ^{8,15,43,76} and the central area in the open field arena ⁹, indicating an anxiogenic phenotype developed by rats. In the present study, we observed RS-induced anxiety-associated behaviors, indicated by a reduction in time spent in the center zone and an increase in the time spent in the peripheral zone of the open field, as well as a reduction in the time spent and number of entries in the open arm of elevated plus maze, in the stressed vehicle group. Additionally, animals treated with the OXT antagonist at the doses 3 and 6 nmol showed reduced entries, time spent and transitions in the light-dark box, whereas the dose of 9 nmol prevented all behavioral changes.

OXT has been extensively reported to have anxiolytic and/or antistress effects ^{51,53,77}, and a positive correlation has been reported between cerebrospinal fluid (CSF) and plasma OXT and higher scores on the

Spence Children's Anxiety Scale that assesses severity of trait anxiety symptoms⁷⁸, suggesting OXT as a possible marker for anxiety disorders. Acute OXT treatment in the PVN caused an increase in time spent in the light side of the box, and in the open arms in elevated plus maze⁷⁹. Nonetheless, there are still many questions about whether PVN OXT positive neurons are activated during acute restraint stress, and even more so during repeated restraint stress²¹. Regarding repeated stress, PVN OXT may be involved in the adaptation process to stress, an idea has been extended to the PVN as a whole⁸⁰. However, PVN OXT action may be more complex and dependent, for example, on the social components of OXT effects. Smith and Wang⁸¹ showed that prairie voles that were restrained for 1 hour plus social isolation (a protocol similar to ours with rats) exhibited an increase in OXT content in the PVN with a decrease in the density of OXT receptors. These animals also displayed behavioral responses such as increased open arm latency, reduced open arms entries and time, and increased plasmatic corticosterone levels. These behavioral and hormonal changes were mitigated when animals recovered in the presence of their social partners.

Smith and Wang (2014) also showed that OXT injected into the PVN could reverse all behavioral effects caused by restraint plus social isolation. Conversely, injection of an OXT antagonist in a restrained-plus-partner group induced anxiogenic effects, and in a high dose, increased plasma corticosterone levels. Similarly, in our protocol, PVN OXT may be required for the mediation of anxiety-associated behaviors, as a maladaptive response to stress. Blockade of OXT receptor in the PVN with 9 nmol L-368,899 (high dose), during the restraint session prevented the late increase in anxiety observed both in the elevated plus maze and the light-dark box.

Interestingly, Yoshida and colleagues⁸² showed that OXT has anxiolytic effects mediated by serotonergic neurons, suggesting that the OXT–serotonin pathway plays an important role in modulating anxiety. Acute chemogenetic activation of PVN OXT reversed the anxiety developed by mice under a high fat diet and previously restrained for 3 days. To evaluate the progression of anxiety-induced by a hypercaloric diet, the same study showed that chemogenetic inhibition of the same OXT neurons was unable to affect the anxiety parameter evaluated⁸³.

Restraint stressed animals have been reported to show increased ACTH and corticosterone plasma levels indicating activation of the HPA axis. Intracerebroventricular administration of OXT reduced this activation in both parameters²⁶. However, this effect was observed only after a high dose of OXT, and specifically in female SD rats. Our results showed that antagonism of OXT receptor in the PVN did not alter corticosterone release induced by RS.

Using elegant techniques, Zhan and collaborators⁸⁴ showed that PVN OXT neurons are activated during restraint stress session and show a positive correlation with struggling behavior during the restraint session, as evidenced by increased of calcium signaling measured through fiber photometry. Moreover, chemogenetic inhibition of PVN OXT reduced the activation of several other brain regions in response to restraint including the medial prefrontal cortex; piriform cortex; lateral septum; paraventricular nucleus of the thalamus; lateral habenula; basolateral amygdala; CA1 field of the hippocampus, and subiculum ventral part. The authors concluded that strong activation of PVN OXT neurons during restraint impairs social

memory, an effect that can be prevented by inhibiting these neurons during the restraint period. Our findings support their results, as blockade of PVN OXT receptors altered both the late anxiety-like behavior and the cardiovascular responses induced by the restraint stress model.

This study has several limitations that are important to acknowledge and provide context for our findings. First, while our results demonstrate that blocking oxytocin receptors within the PVN is critical for the development of delayed stress-induced anxiety and cardiovascular responses during restraint stress, we did not use an oxytocin agonist to confirm that enhancing oxytocin signaling provides opposite effect. Future studies could explore this possibility to further characterize the therapeutic potential of this system. A second limitation is that our experiments were conducted exclusively on male rats. The oxytocin system is known to exhibit significant sexual dimorphism, and future research is necessary to determine if our findings on PVN oxytocin receptor blockade are comparable in females. Sex differences in neuroendocrine and autonomic regulation are well-documented, and females are also exposed to stressors in which oxytocin plays a modulatory role^{85,86}. In fact, estrogen can influence oxytocin receptor expression and signaling, potentially leading to distinct cardiovascular outcomes in females compared to males^{87,88}.

The last limitation is that our central injection approach enabled precise targeting of the PVN, but does not allow us to infer the effects of systemic oxytocin or its antagonist. We also recognize that stereotaxic surgery may introduce stress. Additional studies using a peripheral route of administration (e.g., intraperitoneal) would be required to compare the effects of central versus systemic oxytocin signaling in these stress-related behaviors.

5. Conclusion

To our knowledge, this is the first study to investigate the role of the oxytocin receptor during restraint stress, with a specific focus on its cardiovascular effects. In conclusion, our results indicate that PVN OXT activation during restraint stress elicits cardiovascular responses, primarily contributing to the tachycardia observed during the stress session. These effects appear to be mediated by autonomic modulation of central control. Antagonism of OXT receptors increases sympathetic vascular tone, reduces sympathetic cardiac activity, and enhances parasympathetic cardiac modulation, ultimately leading to a reduction in tachycardic response to stress.

Regarding behavioral outcomes, our data suggests that PVN OXT receptor activation is involved in the expression anxiety-related behaviors, and administration of a high dose of the OXT receptor antagonist could prevent this behavioral alteration.

Declarations

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Author contributions: CRediT

Belém-Filho IJA: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing – original draft, writing – review and editing. **Tercino AF:** Data curation, formal analysis, investigation and methodology. **Lucena-Silva GV:** Investigation and methodology. **Zangrossi-Júnior H:** methodology, project administration, resources, supervision. **Corrêa FMA:** Conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, resources, supervision, funding sources, writing – review and editing.

Competing Interests

The authors have nothing to disclose.

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Table

Table 1 – Baseline values of mean arterial pressure (MAP) and heart rate (HR) measured before and after the injection of vehicle (100 nL/side) or L-368,899 at doses of 3, 6, or 9 nmol/100 nL per side into the paraventricular nucleus (PVN) of the hypothalamus. Paired t-test was used to compare values obtained 1 minute before injection and 1 minute before the onset of the restraint session. ns = not significant.

	MAP (mmHg)						HR (bpm)				
	n	Before	After	t	P	*	Before	After	t	P	*
Vehicle	7	106.6 ± 3.4	108.8 ± 2.4	0.5665	0.5918	ns	340.7 ± 14.6	365.6 ± 12.8	2.281	0.0627	ns
L-368,899 3 nmol	8	101.8 ± 2.3	101.2 ± 3.0	0.2969	0.7752	ns	355.4 ± 9.6	345.2 ± 8.7	0.7717	0.4656	ns
L-368,899 6 nmol	7	108.4 ± 5.3	114.3 ± 2.9	1.661	0.1478	ns	325.6 ± 12.1	363.7 ± 11.1	2.896	0.0275	*
L-368,899 9 nmol	8	107.0 ± 3.0	109.3 ± 2.6	0.9335	0.3816	ns	361.4 ± 21.0	375.4 ± 14.3	0.8699	0.4132	ns

Figures

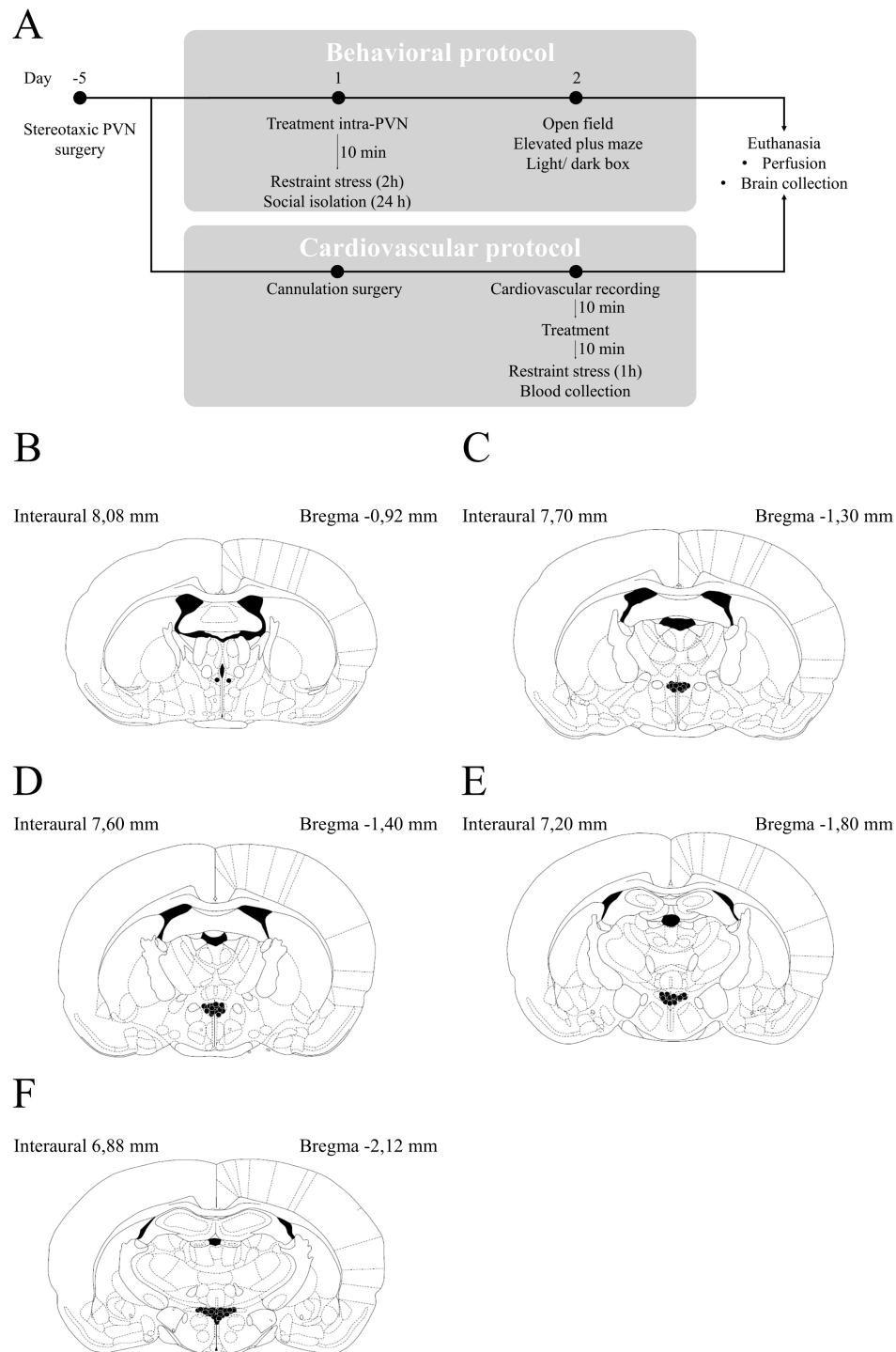


Figure 1

Schematic representation of the experimental protocol and diagrammatic representation of site of microinjection. A: Schematic representation of behavioral and cardiovascular protocol to study oxytocin receptors into paraventricular nucleus (PVN) of rats during restrain stress. B-F: Diagrammatic representation of site injection represented by filled circles of L-368,899 (3, 6 or 9 nmol/100nL.side⁻¹) or vehicle (100nL/side) into PVN.

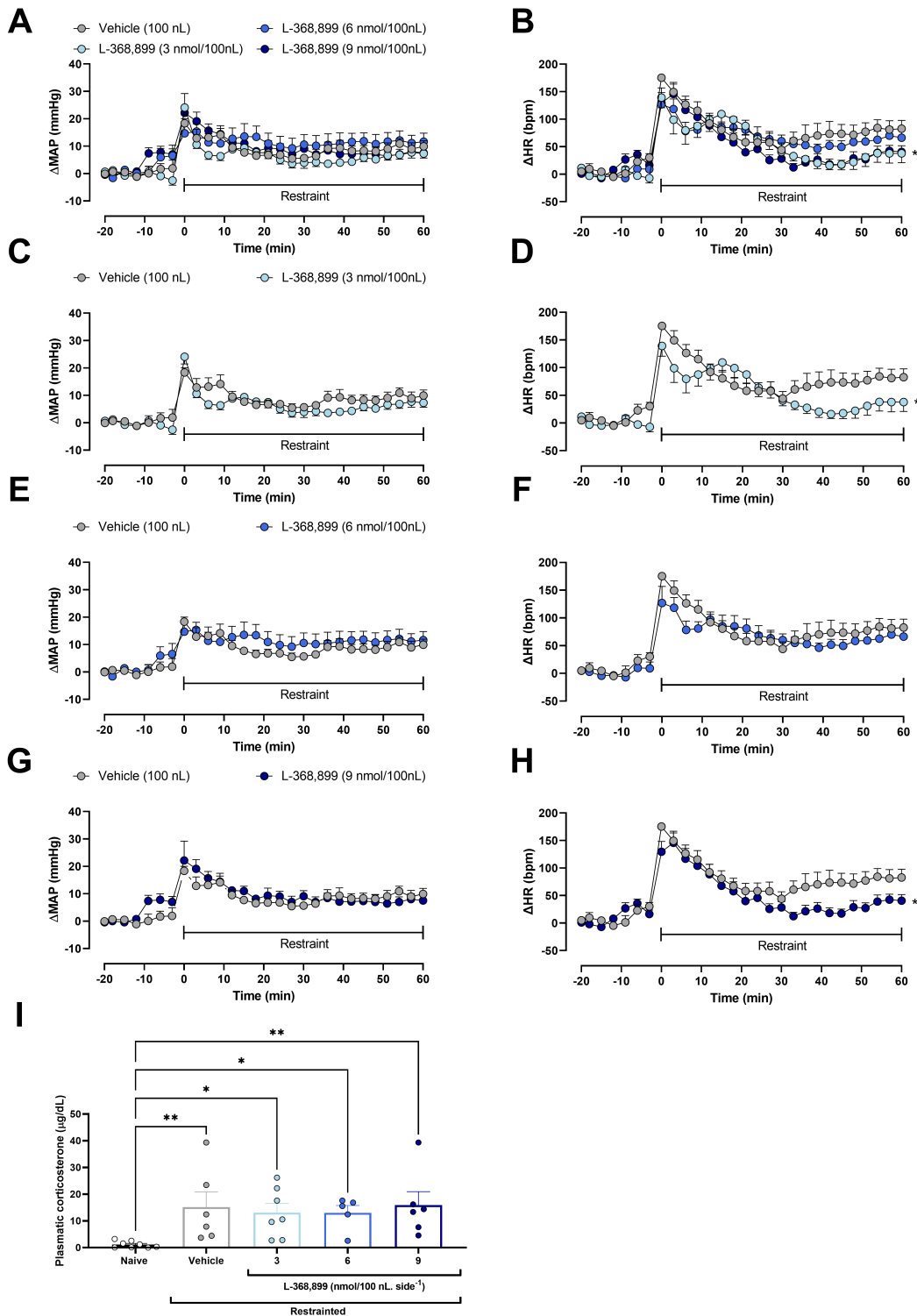


Figure 2

Cardiovascular and neuroendocrine responses evoked by restraint stress in rats pretreated with vehicle (100 nL/side; n=7; gray bar and filled circles) or L-368,899 at the doses of 3 (n=8, light blue filled circles); 6 (n=7, medium blue filled circles) and 9 nmol/100 nL each side (n=8, dark blue filled circles) into paraventricular nucleus (PVN) of hypothalamus. (A,C,E and G) Changes in mean arterial pressure (Δ MAP) (B,D,F and H) and heart rate (Δ HR) over time during restraint in rats. The time of injection was in -10 minute.

On time zero, animals were subjected to restraint stress. Average of Δ MAP (B) and Δ HR (D) during 60 min of restraint stress expressed as % of control response. (I) Dosage of plasmatic corticosterone hormone in groups: naïve (basal values, n=8, white bar and empty circles), rats pretreated with vehicle (n=6, gray bar and filled circles) or L-368,899 3 (n=7, light blue and filled circles), 6 (n=5, medium blue and filled circles) or 9 nmol (n=6, dark blue and filled circles) subjected to restraint over 60 min. Mean $\pm$ S.E.M. Two-way RM ANOVA followed by Fischer's post-test. One-way ANOVA followed by Fischer's post-test. * Significantly different from ASCF, P < 0.05. # significantly different from naïve group, P < 0.05.

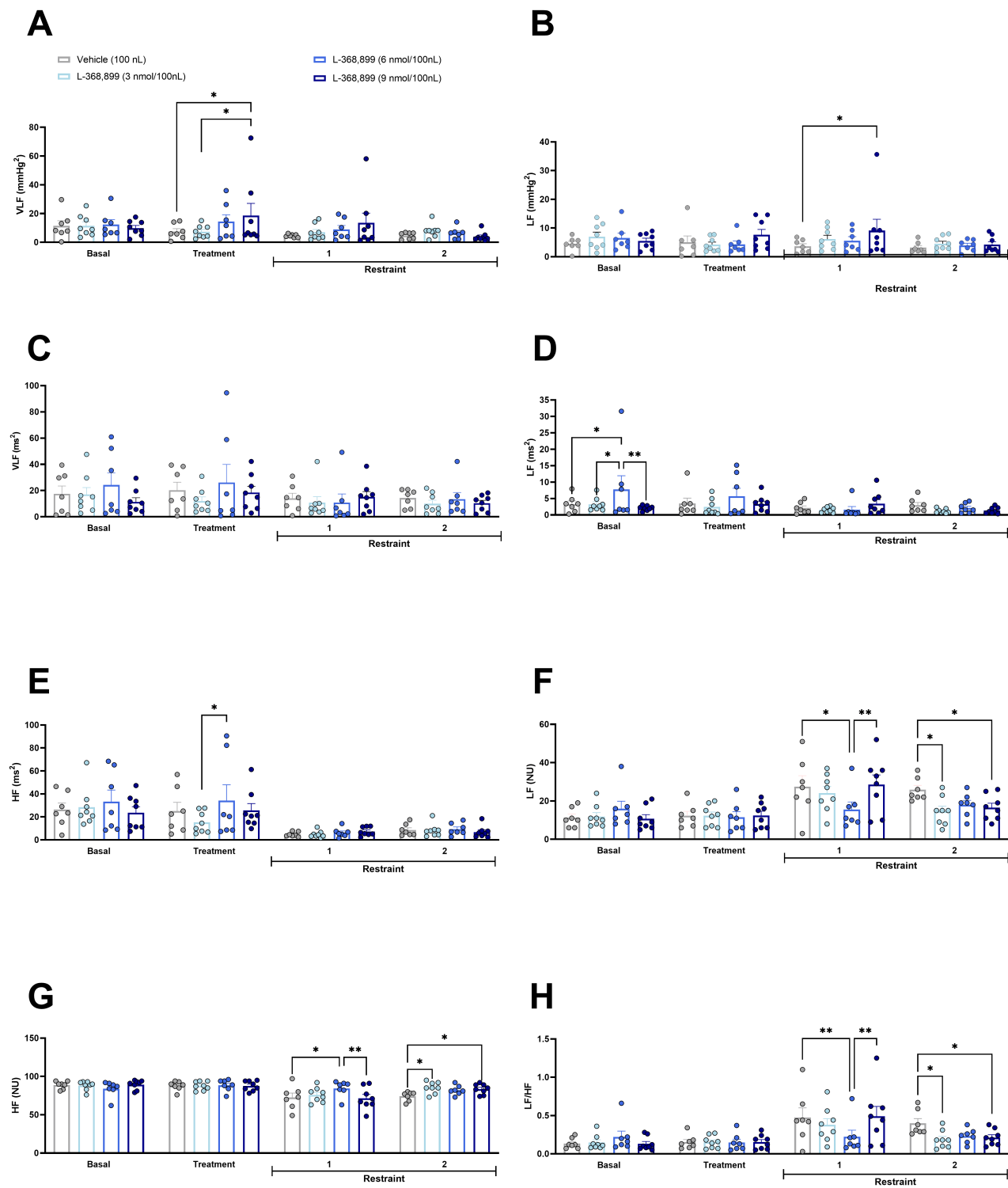


Figure 3

Blood pressure variability (A and B) and heart rate variability (C, D, E, F, G and H) of animals treated with either vehicle (100 nL/side; n=7; gray bar and filled circles) or L-368,899 at the doses of 3 (n=8, light blue bar and filled circles); 6 (n=7, medium blue bar and filled circles) and 9 nmol/100 nL each side (n=8, dark blue bar and filled circles). Mean $\pm$ S.E.M. Two-way RM ANOVA followed by Fischer's post-test. Borderline results were expressed on top of the group. * Significantly different, $P < 0.05$. ** $P < 0.01$. Very low-frequency (VLF), low frequency (LF) and high frequency (HF) oscillations; sympathovagal balance (LF/HF); normalized units (NU).

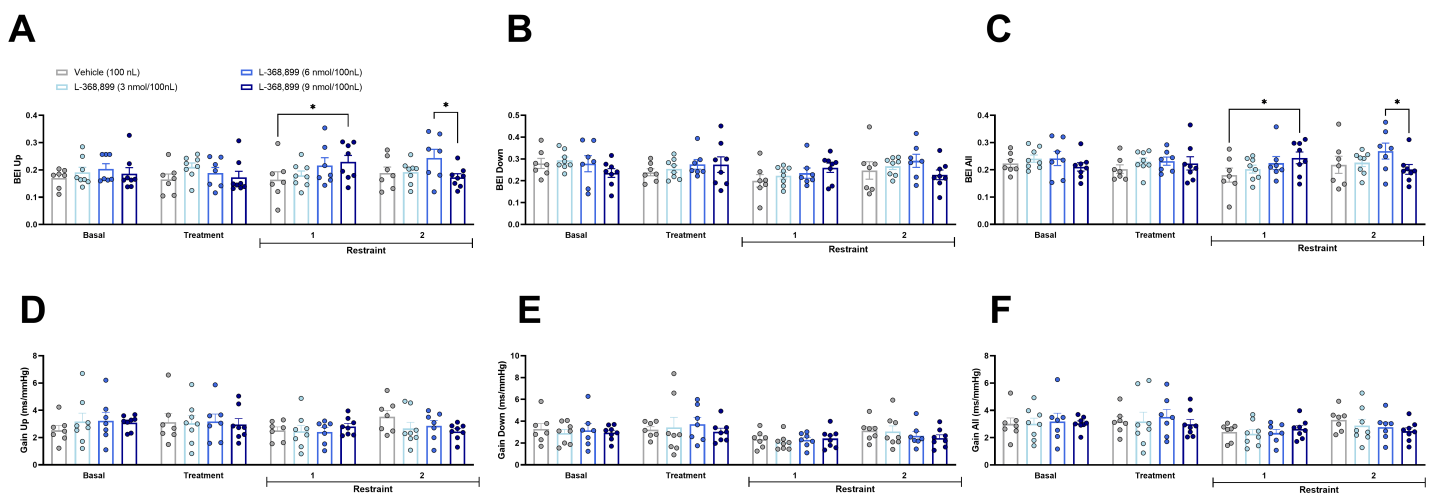


Figure 4

Spontaneously baroreflex activity (A, B, C, D, E, F) of animals treated with either vehicle (100 nL/side; n=7; gray bar and filled circles) or L-368,899 at the doses of 3 (n=8, light blue bar and filled circles); 6 (n=7, medium blue bar and filled circles) and 9 nmol/100 nL each side (n=8, dark blue bar and filled circles). Mean $\pm$ S.E.M. Two-way RM ANOVA followed by Fischer's post-test. Borderline results were expressed on top of the group. * Significantly different, $P < 0.05$. ** $P < 0.01$. Baroreflex effectiveness index (BEI) Up (I), Down (J) and All (K). Baroreflex gain Up (L), Down (M) and All (N).

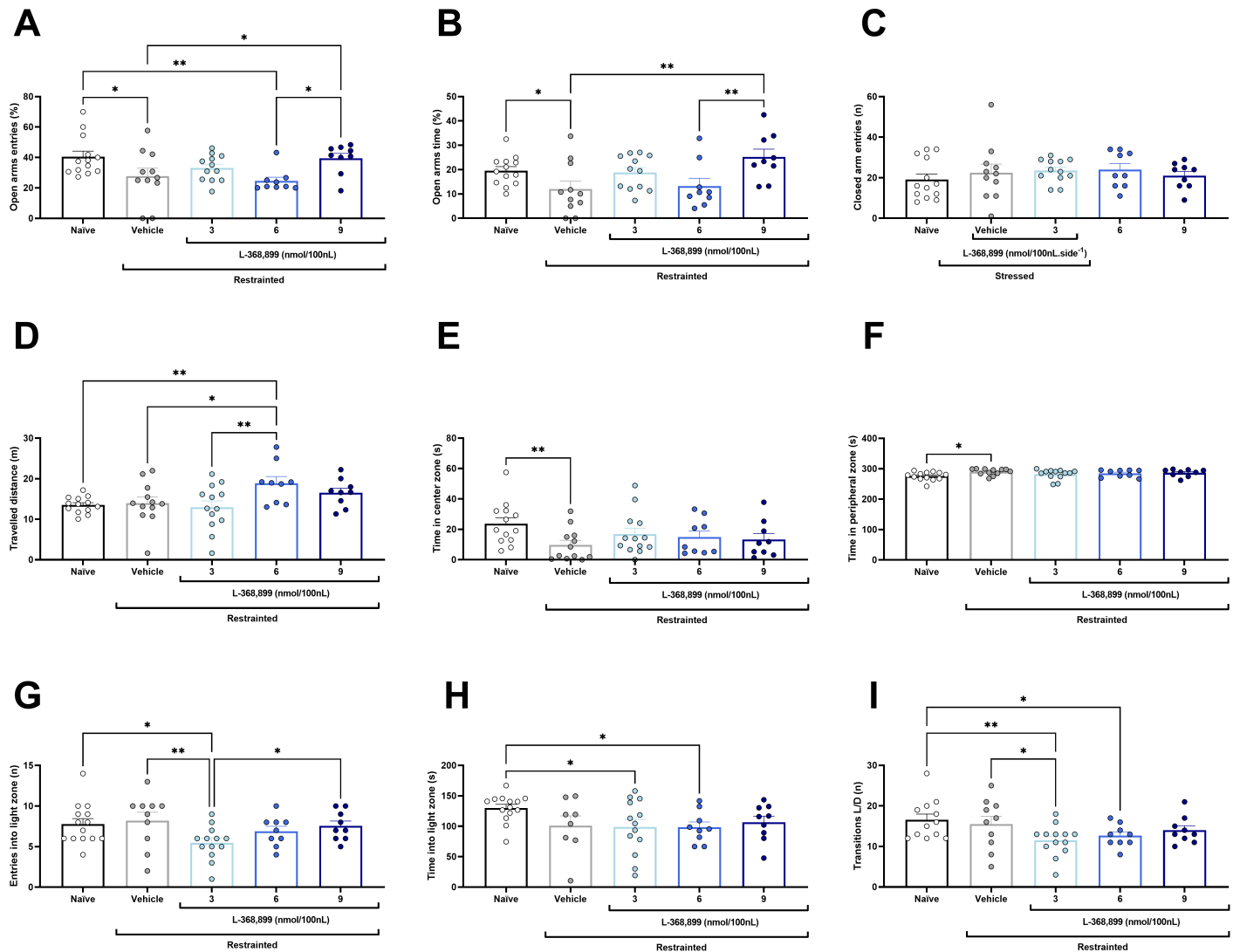


Figure 5

Late behavioral responses on elevated plus maze, open field arena and light dark box of Naïve group (n=14, white bar and empty circles) or evoked by restraint stress during 120 minutes in rats pretreated with vehicle (100 nL/side; n=12; gray bar and filled circles) or L-368,899 at the doses of 3 (n=12-13, light blue bar and filled circles); 6 (n=9, medium blue bar and filled circles bar) and 9 nmol/100 nL each side (n=9, dark blue bar and filled circles) into paraventricular nucleus (PVN) of hypothalamus. (A) Open arms entries in percentage. (B) Open arms time in percentage. (C) Number of entries into closed arms. (D) Locomotor activity measured by travelled distance. (E) Total time spent in the center zone of open field. (F) Total time spent in the peripheral zone of open field. (G) Number of entries into the light side of light dark box. (H) Time spent into the light side of light dark box. (I) Number of transitions of light and dark (L/D) sides of light dark box. Mean $\pm$ S.E.M. One-way ANOVA followed by Fischer's post-test. * Significantly different, $P < 0.05$. ** $P < 0.01$.