NMDA receptors in the insular cortex modulate cardiovascular and autonomic but not neuroendocrine responses to restraint stress in rats

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Abstract

Background and Purpose: The insular cortex (IC) is a brain structure involved in the modulation of autonomic, cardiovascular and neuroendocrine adjustments during stress situations. However, the local neurochemical mechanisms involved in the control of these responses by the IC are poorly understood. Glutamate is a prominent excitatory neurotransmitter in the brain. Thus, the current study aimed to investigate the involvement of glutamatergic neurotransmission within the IC in cardiovascular, autonomic and neuroendocrine responses to acute restraint stress. Experimental Approach: The selective NMDA glutamate receptor antagonist LY235959 (1 nmol/100 nL) and the selective non-NMDA glutamate receptor antagonist NBQX (1 nmol/100 nL) were microinjected into the IC 10 min before the onset of restraint stress. Key Results: The antagonism of NMDA receptors within the IC potentiated the restraint-evoked increases in both arterial pressure and heart rate, while non-NMDA blockade had no effect on these parameters. Spontaneous baroreflex analysis demonstrated that microinjection of LY235959 into the IC decreased baroreflex activity during restraint stress. The decrease in tail skin temperature during restraint stress was shifted to an increase in animals treated with the NMDA receptor antagonist. Moreover, the blockade of IC glutamate receptors did not affect the increase in circulating corticosterone levels during restraint stress. Conclusion and Implications: Overall, our findings provide evidence that IC glutamatergic signalling, acting via NMDA receptors, plays a prominent role in the control of autonomic and cardiovascular responses to restraint stress but does not affect neuroendocrine adjustments.

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Running title: Insular cortex modulate cardiovascular responses during stress.

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AUTHOR CONTRIBUTIONS

Goulart : Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft preparation, Visualization.**Busnardo:** Methodology, Formal analysis, Investigation, Writing – review and editing. **Belém-Filho** : Methodology, Formal analysis, Investigation. **Benini** : Methodology, Formal analysis.**Fassini:** Methodology, Formal analysis, Resources, Writing – review and editing. **Crestani** : Methodology, Formal analysis, Resources, Writing – review and editing. **Godoy:** Methodology, Formal analysis, Investigation. **Correa:** Methodology, Formal analysis, Resources, Writing – review and editing. **Alves** : Conceptualization, Methodology, Resources, Data Curation, Writing – review and editing, Visualization, Supervision, Project administration, Funding acquisition.

Ethical Committee - Experimental procedures were carried out following protocols approved by the Ethical Committee for Use of Animals of the Federal University of Lavras (Approval # 025/16), which complies with Brazilian and international guidelines for animal use and welfare

Bullet point summary

What is already known?

The insular cortex modulates the cardiovascular responses to restraint stress.

The glutamatergic neurotransmission into insular cortex modulates the baroreflex system.

What this study adds?

- The antagonism of NMDA receptors within the IC potentiated the restraint-evoked increases in both arterial pressure and heart rate.
- Spontaneous baroreflex analysis demonstrated that NMDA-receptors into the IC decreased baroreflex activity during restraint stress.

- The decrease in tail skin temperature during restraint stress was shifted to an increase in animals treated with the NMDA receptor antagonist.

Clinical significance

The assessment of both, brain areas and neurotransmitters during stress situations may contribute to a more effective planning of therapeutic strategies for reducing stress damages.

ABSTRACT

Background and Purpose: The insular cortex (IC) is a brain structure involved in the modulation of autonomic, cardiovascular and neuroendocrine adjustments during stress situations. However, the local neurochemical mechanisms involved in the control of these responses by the IC are poorly understood. Glutamate is a prominent excitatory neurotransmitter in the brain. Thus, the current study aimed to investigate the involvement of glutamatergic neurotransmission within the IC in cardiovascular, autonomic and neuroendocrine responses to acute restraint stress.

Experimental Approach: The selective NMDA glutamate receptor antagonist LY235959 (1 nmol/100 nL) and the selective non-NMDA glutamate receptor antagonist NBQX (1 nmol/100 nL) were microinjected into the IC 10 min before the onset of restraint stress.

Key Results: The antagonism of NMDA receptors within the IC potentiated the restraint-evoked increases in both arterial pressure and heart rate, while non-NMDA blockade had no effect on these parameters. Spontaneous baroreflex analysis demonstrated that microinjection of LY235959 into the IC decreased baroreflex activity during restraint stress. The decrease in tail skin temperature during restraint stress was shifted to an increase in animals treated with the NMDA receptor antagonist. Moreover, the blockade of IC glutamate receptors did not affect the increase in circulating corticosterone levels during restraint stress.

Conclusion and Implications: Overall, our findings provide evidence that IC glutamatergic signalling, acting via NMDA receptors, plays a prominent role in the control of autonomic and cardiovascular responses to restraint stress but does not affect neuroendocrine adjustments.

Keywords: Insular cortex; Restraint stress; Glutamate; NMDA.

Abbreviations

blood pressure (BP); insular cortex (IC); nitric oxide (NO); neuronal nitric oxide synthase (nNOS); soluble guanylyl cyclase (sGC); cyclic guanosine monophosphate (cGMP); mean arterial pressure (MAP); heart rate (HR); restraint stress (RS); paraventricular nucleus of the hypothalamus (PVN); hypothalamic-pituitary-adrenal (HPA);

INTRODUCTION

Psychological factors such as stressful events induce a coordinated set of behavioural and physiological changes (McEwen, 2000). In the short term, physiological adjustments are important adaptive responses that maintain homeostasis and ensure survival (Sterling, 2012). The physiological responses to stress are mainly characterized by alterations in the autonomic nervous system and cardiovascular system, increases in plasma catecholamine levels and activation of the hypothalamic-pituitary-adrenal (HPA) axis (Herman et al., 2016; Joels & Baram, 2009). Autonomic responses include increases in both blood pressure (BP) and heart rate (HR) (Campeau & Watson, 1997; R. A. Dampney, Horiuchi, & McDowall, 2008), a drop in tail skin temperature as a consequence of sympathetically mediated vasoconstriction in skin beds (Blessing & Seaman, 2003; Nakamura, 2015; Vianna & Carrive, 2005) and baroreflex activity modulation (Crestani, 2016; R. A. L. Dampney, 2017).

Studies using image analysis techniques and Fos protein measurement have demonstrated that stressful stimuli activate neurons in the insular cortex (IC) (Ahn et al., 2015; Imbe, Kimura, Donishi, & Kaneoke, 2014; Uematsu, Kitamura, Iwatsuki, Uneyama, & Tsurugizawa, 2015). In addition, nonselective synaptic blockade in the IC generated by local microinjection of CoCl2 decreased both the increased blood pressure

and tachycardia evoked by restraint stress (Alves, Crestani, & Correa, 2010). Moreover, the inhibition of local IC neurotransmission by CoCl2 attenuated freezing and increased the mean arterial pressure and heart rate in the groups that received CoCl2 either immediately after conditioning or 10 min before re-exposure to the aversive context but not in the group that received CoCl2 before the conditioning session. Regarding the HPA axis response, cortical regions such as the prefrontal cortex are related to the control of the HPA axis (Ekstrand, Hellsten, & Tingstrom, 2008; Gjerstad, Lightman, & Spiga, 2018; Neigh, Owens, Taylor, & Nemeroff, 2010) and participate in negative feedback via intermediate synapses in the PVN (Herman, McKlveen, Solomon, Carvalho-Netto, & Myers, 2012; Ulrich-Lai & Herman, 2009). In limbic areas such as the hippocampus and prefrontal cortex, glutamatergic neurotransmission plays an inhibitory role in the HPA axis (Diorio, Viau, & Meaney, 1993; Figueiredo, Bodie, Tauchi, Dolgas, & Herman, 2003; Ulrich-Lai & Herman, 2009). IC seems to be related to the regulation of cortisol in women with depression (Ottowitz et al., 2004).

The presence of glutamatergic terminals has been demonstrated in the IC (Dori, Dinopoulos, Cavanagh, & Parnavelas, 1992). In addition, microinjection of glutamate into the IC caused cardiovascular responses (Butcher & Cechetto, 1995; Ranjbar, Hatam, & Nasimi, 2015; Ruggiero, Mraovitch, Granata, Anwar, & Reis, 1987), showing that IC glutamatergic neurotransmission participates in the modulation of cardiovascular activity. Glutamatergic neurotransmission via NMDA receptors in the IC also has a facilitatory influence on baroreflex activity (Alves, Crestani, Resstel, & Correa, 2009), suggesting that the control of stress-evoked cardiovascular changes might be mediated by modulation of this cardiovascular reflex mechanism. Thus, in the present study, we tested the hypothesis that cardiovascular, neuroendocrine and autonomic responses to an acute session of restraint stress are mediated by glutamatergic neurotransmission in the IC.

MATERIAL AND METHODS

Animals

Experimental procedures were carried out following protocols approved by the Ethical Committee for Use of Animals of the Federal University of Lavras (Approval # 025/16), which complies with Brazilian and international guidelines for animal use and welfare. Twenty-five male Wistar rats (60 days old, weighing 230–270 g) were used in the present study. The animals were obtained from the animal breeding facility of São Paulo State University (UNESP) (Botucatu, SP, Brazil) and kept in the Animal Care Unit of the Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo. The animals were kept under a 12-h/12-h light/dark cycle (lights on between 7:00 AM and 7:00 PM) and had free access to water and standard rat food. We chose all group size of all experiments shown in the present study based on previously reported data which are comparable with our experiments (Barretto-de-Souza, Benini, Reis-Silva, & Crestani, 2021; Gomes-de-Souza, Costa-Ferreira, Oliveira, Benini, & Crestani, 2020).

The use of wistar rats for these experiments is justified by the fact that this species of animal has some characteristics that allow us to experiment with it, among them: Viability, similarities with human beings (Leong, Ng, & Jaarin, 2015). Moreover, the choice of using male wistar rats for these experiments was to avoid the possible interference of hormonal variation throughout the estrous cycle of female and autonomic responses during restraint stress (Mahmoodzadeh, Fliegner, & Dworatzek, 2013; Miller et al., 2017; Miller et al., 2011).

Surgical procedures

Seven days before the experiments, animals were anaesthetized with 2,2,2-tribromoethanol (250 mg/kg, i.p.) and placed in a stereotaxic apparatus (Stoelting, USA). After local anaesthesia with 2% lidocaine, the skull was exposed through an incision in the skin. The periosteum was removed with a 10% H_2O_2 solution. Stainless-steel guide cannulas (11 mm long, 0.55 mm outside diameter) were implanted bilaterally in the IC according to coordinates obtained from the rat brain atlas of Paxinos and Watson (1997) (anteroposterior, +3.2 mm from bregma; lateral, +3.75 mm from bregma; vertical, -4.5 mm from skull; incisor = -3.2 mm) (Paxinos & Watson, 1997). Self-curing acrylic resin and screws were used for fixation of the cannulas to the skull, and a 0.2 mm diameter mandrel was inserted into the cannulas to avoid obstruction during the animals'

recovery. As a prophylactic measure, the animals received a veterinary pentabiotic (Fontoura Wyeth, Brazil; 80,000 UI, i.m.) and the non-steroidal anti-inflammatory drug flunixin meglumine (Banamine®, Schering-Plough, Brazil; 2.5 mg/kg, s.c.) after the surgery.

Twenty-four hours before the experiments, the animals were anaesthetized with 2,2,2-tribromoethanol (250 mg/kg, i.p.), and a polyethylene catheter was inserted into the inferior abdominal aorta via the femoral artery for cardiovascular recording and blood sampling. The catheter was exteriorized on the animal's dorsum and fixed to the skin by surgical suture. At the end of the surgery, the animals received the non-steroidal anti-inflammatory drug flunixin meglumine (Banamine®, Schering-Plough, Brazil; 2.5 mg/kg, s.c.). The animals were kept in individual cages throughout the postoperative period, and cardiovascular parameters were recorded. On the day of the experiments, microinjections into the IC were randomized in a non-blind manner.

Microinjection of drugs into the insular cortex

Injection needles (12 mm long, 33 G, Small Parts, USA) were connected to a 1 µl syringe (Hamilton, USA) through a polyethylene tube (PE-10) for the microinjection of drugs into the brain. The final volume of microinjection was 100 nL/side (Alves, Crestani, Resstel, & Correa, 2014; Alves et al., 2013).

Cardiovascular recording

The cannula implanted into the femoral artery was connected to a pressure transducer, and pulsatile arterial pressure was recorded using an HP-7754A amplifier (Hewlett-Packard, Palo Alto, CA, USA) and an acquisition board (Biopac M-100, Goleta, CA, USA) connected to a personal computer. Mean arterial pressure (MAP) and HR values were derived from the pulsatile arterial pressure recordings.

Restraint stress

The animals were placed in a PVC tube (15 cm long and 6.5 cm in diameter, with 1 cm holes for ventilation that made up approximately 20% of the tube surface). The restraint session lasted 30 min. After the end of the restraint session, the rats were returned to their home cages. Each animal was subjected to only one restraint session to avoid habituation (Benini, Oliveira, Gomes-de-Souza, & Crestani, 2019; Benini, Oliveira, Gomes-de-Souza, Rodrigues, & Crestani, 2020).

Serum corticosterone

Blood was collected from the catheter implanted into the femoral artery. The first blood sample was collected to measure the baseline value of the hormone corticosterone (basal measurement). Fifteen minutes after the onset of restraint stress, a new sample was taken (stress measurement). The volume obtained for each sample was 0.3 ml. To obtain the serum, the samples remained at room temperature for 1 h to allow clot retraction and were subsequently centrifuged at 2000 xg for 15 min. After centrifugation, the serum was separated and stored in a -80 °C freezer for further analysis. Serum corticosterone was measured by immunoenzymatic assay (ELISA) using a commercially available kit (Cayman Chemical, Michigan, USA).

Tail skin temperature recording

Tail skin temperature was recorded at a distance of 50 cm using an IRI 4010 multi-purpose thermal imager (InfraRed Systems Ltd., Park Circle, Tithe Barn Way, Swan Valley, Northampton, UK). The temperature was measured at five points on the animal's tail, and the mean value was calculated for each recording.

Spontaneous baroreflex analysis

The sequence method was used to evaluate baroreflex function over the physiological range of fluctuations in arterial pressure without any pharmacological manipulation. For this purpose, the beat-to-beat values of systolic arterial pressure (SAP) and pulse interval (PI) were analysed using CardioSeries v2 software (Lataro, Silva, Silva, Salgado, & Fazan, 2017). The SAP time series were evaluated to find SAP elevations of 4 or more beats that were accompanied by a progressive increase in the R-R interval, which are called UP ramps. DOWN ramps are the opposite and consist of SAP reductions of four or more beats that are accompanied

by a progressive decrease in the R-R interval. Spontaneous baroreflex activity was assessed based on the slope (ms/mmHg) of the linear regression between the SAP and PI for UP, DOWN and average (i.e., mean of UP and DOWN sequences) sequences (Di Rienzo et al., 2001). For this experiment, we used a correlation coefficient (r) greater than 0.80. The baroreflex effectiveness index (BEI) was also calculated as the ratio between the number of SAP ramps followed by the reflex PI response and the total number of SAP ramps (Di Rienzo et al., 2001).

Drugs and solutions

NBQX (Tocris Bioscience, USA; selective non-NMDA glutamate receptor antagonist) and LY235959 (Tocris Bioscience, USA; selective NMDA glutamate receptor antagonist) were dissolved in artificial cerebrospinal fluid (aCSF) (100 mM NaCl; 2 mM Na_3PO_4; 2.5 mM KCl; 1.0 mM $MgCl_2$; 27 mM $NaHCO_3$; 2.5 mM CaC_2; pH 7.4). Urethane (Sigma, USA) and 2,2,2-tribromoethanol (Sigma, USA) were dissolved in saline (0.9% NaCl). The veterinary pentabiotic (Fort Dodge, Campinas, SP, Brazil) and the non-steroidal anti-inflammatory drug flunixin meglumine (Banamine®, Schering-Plough, Brazil) were used as provided.

Experimental design

The rats were brought to the experimental room in their own cages. The animals were allowed at least 60 min to adapt to the experimental room conditions, such as sound and illumination, before starting the experiment. The experimental room was temperature-controlled (25 °C) and acoustically isolated from the other rooms.

After at least 30 min of basal cardiovascular recording, different sets of animals were subjected to bilateral microinjection into the IC of either the selective NMDA glutamate receptor antagonist LY235959 (1 nmol/100 nL), the selective non-NMDA glutamate receptor antagonist NBQX (1 nmol/100 nL) or vehicle (Adami, Barretto-de-Souza, Duarte, Almeida, & Crestani, 2017) (aCSF, 100 nL). Ten minutes after the IC treatment, the animals underwent a 30 min session of restraint stress.

Cardiovascular recordings began at least 30 min before the onset of the restraint and were performed throughout the period of exposure to the restraint stress. Tail skin temperature was recorded immediately after the IC treatment and 7 and 3 min before restraint stress onset (basal measurements). Tail skin temperature was also recorded immediately after restraint stress and every 10 min while the animal remained inside the tube.

The analysis of spontaneous baroreflex activity was performed in 5 min segments of recording at 4 points: before pharmacological treatment (basal), post-treatment and at two points during restraint stress. Analysis during the stress session was performed at 5–10 (point 1) and 20–25 (point 2) min after the onset of restraint. For serum corticosterone measurement, blood was collected immediately before the animal was placed in the restraint tube and 15 min after the onset of restraint stress. The corticosterone measurements were made in the same group of animals that was used for the cardiovascular and temperature measurements.

Histological determination of the microinjection sites

At the end of each experiment, animals were anaesthetized with urethane (1.2 g/kg, i.p.), and Evans blue dye (1%, 100 nL) was microinjected at the site of drug administration in the brain. Then, the animals were perfuse-fixed (intracardiac 0.9% NaCl followed by 10% formalin), and the brains were removed. After fixation in 10% formalin, the brains were sectioned in 40 μm thick frontal cuts for analysis of the injection sites. The actual placement of the microinjection needles was determined upon analysis of serial sections and identified according to the rat brain atlas of Paxinos and Watson (Paxinos & Watson, 1997).

Data and Statistical analysis

All analysis were performed using Prism 6.0 software (GraphPad, USA) and followed the guidelines on experimental design and analysis in pharmacology (Curtis et al., 2018). Data are expressed as the mean ± standard error of the mean (SEM). The paired Student's t test was used to compare basal values of MAP, HR, tail skin temperature and spontaneous baroreflex analysis before and after drug treatment. We used the

unpaired Student's t test to compare baseline corticosterone values (before stress) between the control and drug treatments. Changes in cardiovascular parameters, tail skin temperature, spontaneous baroreflex and corticosterone levels evoked by restraint stress were analysed using two-way ANOVA with treatment as the main factor (vehicle x drugs) and time as repeated measures followed by Bonferroni's*post hoc* test. $P<0.05$ was set as significant.

RESULTS

Fig. 1 shows a photomicrograph of a coronal brain section depicting the bilateral microinjection sites in the IC of a representative animal. Diagrammatic representations of the bilateral microinjection sites of LY235959, NBQX and vehicle into the IC are also presented in Fig. 1.

Effects of microinjection of LY235959 or NBQX in the insular cortex on cardiovascular and tail skin temperature responses to restraint stress.

aCSF - Bilateral microinjection of aCSF (vehicle, 100 nL) into the IC did not change the basal values of MAP, HR or tail skin temperature (Table 1).

LY235959 - Bilateral microinjection of the selective NMDA glutamate receptor antagonist LY235959 (1 nmol/100 nL) into the IC did not change the basal values of MAP, HR or tail skin temperature (Table 1). However, analysis of the time-course curves of cardiovascular responses to restraint stress indicated that LY235959 microinjected into the IC enhanced the restraint-evoked increase in MAP (treatment: $F_{(1,13)} = 9.194$; time: $F_{(40,520)} = 8.073$; interaction:, $F_{(40,520)} = 1.761$) and HR (treatment:, $F_{(1,13)} = 5.171$; time: $F_{(40,520)} = 22.22$; interaction: $F_{(40,520)} = 1.627$) when compared to animals treated with aCSF (Fig. 2 and 3).

The antagonism of the NMDA glutamate receptor within the IC shifted the decrease in tail skin temperature in increase (treatment: $F_{(1,13)} = 8.648$; interaction: $F_{(6,78)} = 5.184$; time: $F_{(6,78)} = 1.931$) (Fig. 4).

NBQX - Bilateral microinjection of the selective non-NMDA glutamate receptor antagonist NBQX (1 nmol/100 nL) into the IC did not change the basal values of MAP, HR or tail skin temperature (Table 1). Analysis of the time-course curves of cardiovascular responses to restraint stress also did not indicate a significant effect of IC treatment with NBQX on the restraint-evoked increase in MAP (treatment: $F_{(1,13)} = 2.402$; time: $F_{(40,520)} = 15.87$; interaction: $F_{(40,520)} = 1.727$) and HR (treatment: $F_{(1,13)} = 0.9897$; time: $F_{(40,520)} = 25.02$; interaction: $F_{(40,520)} = 1.125$) (Fig. 2 and 3). The blockade of non-NMDA glutamate receptors within the IC did not change the tail skin temperature values compared to animals treated with aCSF (treatment: $F_{(1,13)} = 0.002649$; time: $F_{(6,78)} = 13.14$; interaction: $F_{(6,78)} = 0.8501$) (Fig. 4).

Effects of microinjection of LY235959 or NBQX into the insular cortex on baroreflex activity during restraint stress

aCSF - Bilateral microinjection of artificial cerebrospinal fluid (aCSF, 100 nL) into the IC did not change the basal values (before stress) of the baroreflex effectiveness index (Table 2) or the baroreflex gain (Table 3).

LY235959 – Bilateral microinjection of LY235959 did not change the basal values (before stress) of the baroreflex effectiveness index (Table 2) or the baroreflex gain (Table 3). Spontaneous baroreflex analysis demonstrated that microinjection of the selective NMDA glutamate receptor antagonist LY235959 (1 nmol/100 nL) into the IC decreased the BEI for the UP ($F_{(1,10)} = 10.09$), ALL ($F_{(1.10)} = 11.46$) and DOWN sequences ($F_{(1,10)} = 7.85$) (Fig. 5). The baroreflex gains of the UP, DOWN and ALL sequences were not altered by IC treatment with LY235959 (UP: $F_{(1,10)} = 1.984$; DOWN: $F_{(1,10)} = 1.245$; and ALL: $F_{(1,10)} = 4.689$) (Fig. 5).

NBQX – Bilateral microinjection of NBQX did not change the basal values (before stress) of the baroreflex effectiveness index (Table 2) or the baroreflex gain (Table 3)**.** Bilateral microinjection of the selective non-NMDA antagonist NBQX (1 nmol/100 nL) into the IC did not change the BEI in any of the sequences (UP: ($F_{(1,8)} = 0.8254$; DOWN: $F_{(1,8)} = 0.07898$; and ALL: $F_{(1,8)} = 0.3413$). The baroreflex gains for the

UP, DOWN and ALL sequences were also not changed by IC treatment with NBQX (UP: $F_{(1,8)} = 0.9174$; DOWN: $F_{(1,8)} = 1.305$; and ALL: $F_{(1,8)} = 0.4202$) (Fig. 5).

Effects of microinjection of LY235959 or NBQX into the insular cortex on the increase in circulating corticosterone evoked by restraint stress

LY235959 – Bilateral microinjection of the selective NMDA antagonist LY235959 (1 nmol/100 nL) did not alter the increase in circulating corticosterone caused by restraint stress (treatment: $F_{(1,11)} = 0.1106$; time: $F_{(1,\ 11)} = 22.91$; interaction: $F_{(1,11)} = 1.168$) (Fig. 6). The baseline corticosterone release (before stress) of animals treated with LY235959 was not different from that of animals treated with aCSF ($t = 1.219$).

NBQX – Bilateral microinjection of the selective non-NMDA antagonist NBQX (1 nmol/100 nL) did not change the increase in corticosterone triggered by restraint stress (treatment: $F_{(1,11)} = 1.256$; time: $F_{(1,11)} = 34.61$; interaction: $F_{(1,11)} = 1.698$) (Fig. 6). NBQX treatment did not change the baseline corticosterone release (before stress) of animals treated with NBQX compared to animals treated with aCSF ($t = 0.1818$).

DISCUSSION

The results obtained in the present study supported the hypothesis that IC glutamatergic neurotransmission controls cardiovascular and autonomic responses during restraint stress. Indeed, we observed that blockade of NMDA glutamate receptors within the IC potentiated the pressor and tachycardiac responses to acute restraint stress and inhibited the decrease in tail skin temperature. In addition, we found that IC NMDA glutamate receptors played a facilitatory role in spontaneous baroreflex activity. On the other hand, IC glutamatergic neurotransmission mediated by local non-NMDA glutamate receptors did not seem to control these adjustments. Additionally, contrary to our initial hypothesis, the present data do not indicate an involvement of IC glutamatergic neurotransmission present in restraint-evoked increases in circulating corticosterone during restraint stress.

Restraint stress in animal models causes autonomic changes, such as increased blood pressure and HR, sympathetically mediated cutaneous vasoconstriction resulting in a drop in tail skin temperature, and modulation of baroreflex function (Crestani, 2016; Dos Reis, Fortaleza, Tavares, & Correa, 2014). This stressor also activates the HPA axis, which results in increased circulating corticosterone levels in rodents (Bali & Jaggi, 2015; Buynitsky & Mostofsky, 2009). These physiological adjustments are highly reproductible between different laboratories around the world; thus, restraint is one of most commonly used models of stress in rodents (Bali & Jaggi, 2015; Buynitsky & Mostofsky, 2009). Therefore, the restraint stress model is an excellent model for studying neurobiological mechanisms involved in physiological adjustments to stress.

Based on the combination of sensory inputs and limbic connectivity, the IC has been described as an important cortical centre for the integration of autonomic and behavioural responses during aversive threats (Gogolla, 2017; Oppenheimer & Cechetto, 2016; Verberne & Owens, 1998). Indeed, the IC has been implicated in the modulation of stress responses, such as restraint (Alves, Crestani, & Correa, 2010; Myers, 2017; Nagai, Hoshide, & Kario, 2010; Oppenheimer & Cechetto, 2016), contextual fear conditioning (Alves et al., 2013), and fear-induced underestimation (Kamada & Hata, 2018). In the contextual fear conditioning test, microinjection of cobalt chloride (a nonselective synapse blocker) into the IC before re-exposure to an aversive context attenuated the blood pressure and heart rate increases evoked by the conditioned stimulus (Alves et al., 2013). Accordingly, IC treatment with $CoCl_2$ also greatly attenuated both pressor and tachycardiac responses evoked by acute restraint stress (Alves et al., 2010). We further demonstrated similar effects following IC treatment with either α_1- or α_2-adrenoceptor antagonists (Alves et al., 2014), thus demonstrating a role of local noradrenergic neurotransmission in the IC-mediated control of restraint-evoked cardiovascular changes.

A previous study on urethane-anaesthetized rats identified that glutamate microinjection into the IC produced different types of cardiovascular responses, including long oscillatory, pressor, depressor, bradycardiac and tachycardiac responses (Ranjbar, Hatam, & Nasimi, 2015). These results are intriguing and demonstrate that the IC generates ambiguous responses when stimulated with glutamate. Aiming to understand the role

of glutamatergic neurotransmission within the IC in autonomic responses during stress situations, we evaluated the participation of the glutamatergic ionotropic NMDA and non-NMDA receptors within the IC using the selective antagonists LY235959 and NBQX, respectively. IC treatment with LY235959 potentiated the pressor and tachycardiac responses evoked by restraint stress, confirming the involvement of NMDA receptors in the IC in the modulation of cardiovascular responses during restraint. It is important to note that microinjection of the NMDA receptor antagonist in the IC did not alter the basal values of blood pressure, heart rate or baroreflex parameters, suggesting that IC glutamatergic neurotransmission does not tonically modulate these parameters. In contrast to the effect identified following non-selective neurotransmission blockade in the IC, which indicated a facilitatory influence of this cortical structure on cardiovascular responses induced by restraint stress, the results reported here indicate that glutamatergic neurotransmission in the IC has an inhibitory influence on the increases in BP and HR observed during restraint stress. One explanation for these discrepancies might be that there are both facilitatory and inhibitory

neurochemical mechanisms within the IC controlling cardiovascular responses during restraint stress; thus, the nonselective blockade caused by $CoCl_2$ is not able to reveal the specific role of the different local mechanisms. IC treatment with NBQX did not affect the increases in arterial pressure or HR induced by restraint, suggesting that non-NMDA receptors within the IC do not participate in the cardiovascular changes observed during acute stress.

The increase in blood pressure observed during restraint is concomitant with increases in heart rate and sympathetic activity. A previous study published by our laboratory showed that rats with sinoaortic baroreceptor denervation presented exacerbated increases in blood pressure and HR when subjected to restraint stress (Dos Reis et al., 2014), showing the active role of the baroreflex in controlling cardiovascular function during this aversive situation. Likewise, (Crestani, Tavares, Alves, Resstel, & Correa, 2010) demonstrated that the heart rate reflex curve was shifted upward and to the right during restraint stress. Based on previous evidence that glutamatergic neurotransmission within the IC plays an excitatory role in the modulation of baroreflex function (Alves, Crestani, Resstel, & Correa, 2009), in the present study, we evaluated spontaneous baroreflex activity during the restraint stress session. We observed a decrease in BEI during restraint stress in the group treated with the NMDA receptor antagonist in the IC. These data provide additional evidence that NMDA receptors in the IC are also involved in the control of reflex responses during more discrete changes in arterial pressure. Corroborating the present findings, we demonstrated previously that local IC treatment with a selective NMDA receptor antagonist (but not with a non-NMDA glutamate receptor antagonist) decreased the reflex bradycardia response evoked by an increase in blood pressure caused by intravenous infusion of phenylephrine (Alves et al., 2009). Therefore, taken together, the cardiovascular and spontaneous baroreflex findings in the present study indicate that the inhibitory influence of IC NMDA receptors in the pressor and tachycardiac responses evoked by restraint stress might be mediated, at least partly, via facilitation of baroreflex function.

In addition to modulation of baroreflex function, restraint stress also triggers other autonomic responses, including sympathetically mediated cutaneous vasoconstriction, which in turn causes a drop in tail skin temperature (Brasil, Fassini, & Correa, 2018; Busnardo et al., 2019; Vianna & Carrive, 2005). Previous studies demonstrated that IC modulates sympathetic nerve activity (Cechetto & Chen, 1990), thus contributing to cutaneous vasodilation or vasoconstriction and changes in tail skin temperature. The participation of glutamatergic neurotransmission in modulating autonomic responses involving a drop in skin temperature has been described in other areas in animals subjected to stress (Moraes-Neto, Scopinho, Biojone, Correa, & Resstel, 2014). Moreover, IC inactivation using bupivacaine potentiated hypothermia and bradycardic and hypertensive responses to hypoxia (Casanova, Contreras, Moya, Torrealba, & Iturriaga, 2013). In the present study, we observed that blockade of NMDA receptors in the IC shifted the drop in tail skin temperature to an increase (see Fig. 4). The increase in tail temperature was unexpected, since NMDA receptors in the IC appear to have an inhibitory influence on cardiovascular responses to restraint. Thus, we expected that this glutamatergic receptor would have a similar role in the control of the tail skin temperature response. The present results provide evidence of the existence of different sympathetic mechanisms controlling cutaneous and other vascular beds, such as the muscular, renal, and splanchnic circulations. Another possible

explanation is that glutamatergic neurotransmission through NMDA receptors within the IC modulates thermoregulatory centres and causes increased temperature by increasing metabolism, which is reflected as an increase in the tail skin temperature due to heat dissipation. The IC has reciprocal connections with several brain areas responsible for controlling sympathetic activities and thermogenesis, including the lateral hypothalamus, periaqueductal grey and parabrachial nucleus (Gogolla, 2017). Additionally, the parabrachial nucleus has been associated with temperature control related to behaviour via projections to the dorsal raphe nucleus (Yahiro, Kataoka, Nakamura, & Nakamura, 2017). On the other hand, the lateral hypothalamus participates in body temperature regulation (de Vrind, Rozeboom, Wolterink-Donselaar, Luijendijk-Berg, & Adan, 2019) and brown adipose tissue-mediated thermogenesis (You, Chu, Guo, & Lu, 2020), while the periaqueductal grey is an important relay in the descending pathways regulating thermogenesis (de Git et al., 2018). However, more studies are necessary to clarify the involvement of the IC in the control of temperature adjustments during acute restraint stress. In the present study, we only evaluated the autonomic responses evoked by restraint stress and did not measure thermoregulatory responses.

One of the main responses triggered by exposure to a stressor stimulus is the activation of the HPA axis and the consequent release of glucocorticoids into the circulation (Selye, 1951). Glucocorticoids are essential to preparation for physiological, environmental and psychological challenges. One of the main functions of glucocorticoids is energy redistribution to optimize survival when facing a challenge (Herman et al., 2016). Treatment of the IC with either NMDA or non-NMDA receptor antagonist did not change the increase in corticosterone levels during stress. Taken together with the results of the other parameters analysed in the present study, these findings provide further evidence indicating that the central pathways and neurochemical mechanisms controlling circulating corticosterone are distinct from those regulating autonomic and cardiovascular responses during restraint stress (Busnardo et al., 2019; Gouveia et al., 2016).

In summary, the results reported in the present study suggest that glutamatergic neurotransmission present in the IC differentially modulates cardiovascular and autonomic responses during aversive threats. Our data indicate that IC NMDA glutamate receptors have an inhibitory effect on tachycardic and pressor responses to restraint stress that seems to be mediated, at least partially, by the facilitation of baroreflex function. Furthermore, the data reported here suggest that NMDA glutamatergic receptors in the IC are involved in the drop in tail skin temperature during acute restraint. Finally, the present findings provide evidence that HPA axis control during stress is mediated by mechanisms other than glutamatergic neurotransmission in the IC.

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FIGURE LEGENDS

Figure 1 - Representation of injection sites.(A) Photomicrograph of a coronal brain section depicting bilateral microinjection sites into the IC of a representative animal.**(B)** Diagrammatic representations of the bilateral microinjection sites into the IC of LY235959 (1nmol/100nL (n = 7) - blue circles), NBQX (1nmol/100nL (n = 7) - pink circles) and vehicle aCSF (100nL (n = 8) - black circles).

Φιγυρε 2 - ἄριατιον οφ μεαν αρτεριαλ πρεσσυρε (ΔΜΑΠ) ανδ ηεαρτ ρατε (ΔΗΡ) ιν ανιμαλς συβθεςτεδ το ρεστραιντ στρεσς. Time-course curves of ΔMAP and ΔHR in animals treated with artificial cerebrospinal fluid aCSF (100nL (n = 8); white circles), the selective NMDA receptor antagonist LY235959 (1nmol/100nL (n = 7); black circles), or the selective non-NMDA receptor antagonist NBQX (1nmol/100nL (n = 7); gray circles). At time 0 the animals were submitted to restraint stress. The circles represent the mean values ± SEM. # $p < 0.05$ indicates that treatment differs from control (aCSF) after two-way ANOVA. * $p < 0.05$ indicates the time when the group showed a difference compared to the aCSF group, by the Bonferroni's post-test of multiple comparisons. Abbreviations: bpm - beats per minute; mmHg - millimeters of mercury; ΔMAP - mean blood pressure delta; ΔHR – heart rate delta.

Figure 3 – Representative recordings of pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR). Records throughout the experiment, including the restraint stress, in animals treated with aCSF (100nL), LY235959 (1nmol/100nL) or NBQX (1nmol/100nL). Abbreviations: bpm - beats per minute; mmHg - millimeters of mercury; MAP - mean blood pressure; HR – heart rate; PAP - pulsatile blood pressure

Φιγυρε 4 - ἄριατιον οφ ταιλ σκιν τεμπερατυρε (Δ ταιλ σκιν τεμπερατυρε) οφ ανιμαλς συβθεςτεδ το ρεστραιντ στρεσς. (A ανδ B) Time-course curve of Δ tail skin temperature in animals treated with in animals treated with artificial cerebrospinal fluid (aCSF - 100nL (n = 8); white circles), the selective NMDA receptor antagonist LY235959 (1nmol/100nL (n = 7); black circles), or the selective non-NMDA receptor antagonist NBQX (1nmol/100nL (n = 7); gray circles). At time 0 the animals were submitted to restraint stress. The circles represent the mean values ± SEM. . # $p < 0.05$ indicates that treatment differs from control (aCSF) after two-way ANOVA. * * $p < 0.05$ indicates the time when the group showed a difference compared to the aCSF group, by the Bonferroni's post-test of multiple comparisons.**(C)**

Tail skin infrared digital images of representative rats showing the tail skin temperature before, at first, 10, 20 and 30 minutes of restraint stress in animals treated intra-IC with either saline, the selective NMDA antagonist or the NON-NMDA antagonist. All images use the same color coding to indicate the temperature.

Figure 5 – Baroreflex activity assessed by the sequence analysis technique. Analysis was performed during before IC treatment (basal); after IC treatment (treatment) and during restraint stress at periods 5-10 min (point 1) and 20-25 min (point 2) in animals treated with artificial cerebrospinal fluid (aCSF - 100nL (n = 5); white bars), the selective NMDA receptor antagonist LY235959 (1nmol/100nL (n = 7); black bars) or the selective non-NMDA receptor antagonist NBQX (1nmol/100nL (n = 5); grey bars). Spontaneous baroreflex sensitivity was assessed during increases (up sequence) and decreases (down sequence) of SAP, as well as the average of all sequences (mean up and down sequences). **(TOP)** Baroreflex effectivenness index (BEI)**(BOTTOM)** Baroreflex gain. The bars represent the mean values ± SEM. * $p < 0.05$ indicates difference compared to the aCSF group after two-way ANOVA.

Figure 6 - Serum corticosterone concentration in animals subjected to restraint stress. Circulating corticosterone concentration before during restraint (stress, 15min after restraint onset) in animals treated with artificial cerebrospinal fluid (aCSF - 100nL (n = 5); white bars), the selective NMDA receptor antagonist LY235959 (1nmol/100nL (n = 8); black bars) or the selective non-NMDA receptor antagonist NBQX (1nmol/100nL (n = 8); grey bars). The bars represent the mean values ± SEM. The groups were compared using two-way ANOVA.

Table Legend -

Table 1 – Control animals (aCSF) and treated with an NMDA receptor antagonist (LY235959 - 1nmol/100nL) and a non-NMDA receptor antagonist (NBQX - 1nmol/100nL). Result of the test of difference of means (student t test) of baseline values of MAP, HR and TST, before and after drug injection. Values expressed as mean ±SEM.

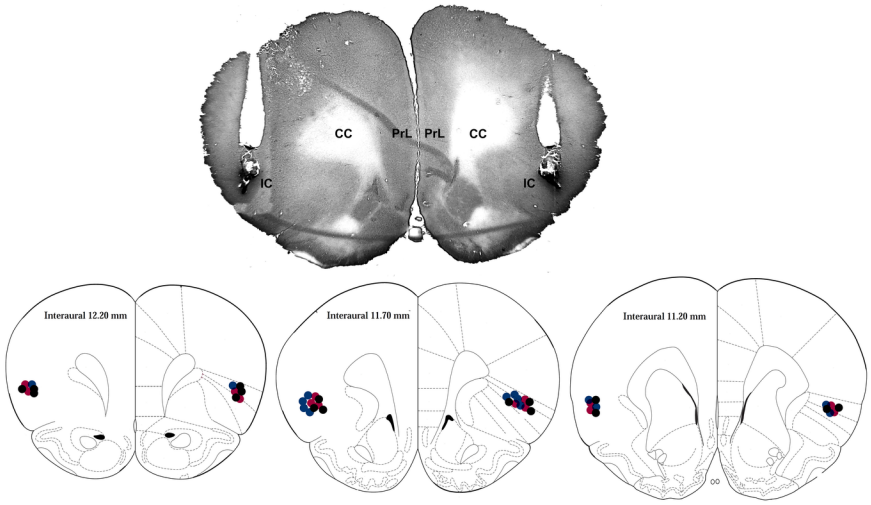
Footnotes – aCSF - artificial cerebrospinal fluid; NBQX - 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide; LY235959 - [3S-(3α,4aα,6β,8aα)]-Decahydro-6-(phosphonomethyl)-3-isoquinolinecarboxylic acid); MAP - mean blood pressure; HR - heart rate; TST – tail skin temperature; bpm - beats per minute; mmHg - millimeters of mercury; ^{o}C - degree Celsius.

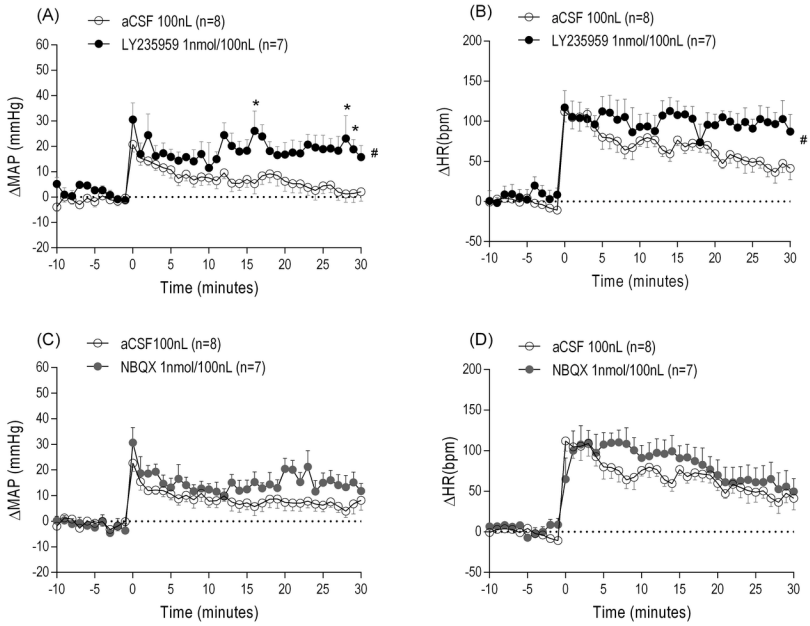
Table 2 – Control animals (aCSF) and treated with an NMDA receptor antagonist (LY235959 - 1nmol/100nL) and a non-NMDA receptor antagonist (NBQX - 1nmol/100nL). Result of the test of difference of means (student t test) of baseline values in the sequences of BEI (Up), BEI (Down) and BEI (All), before and after drug injection. Values expressed as mean ±SEM.

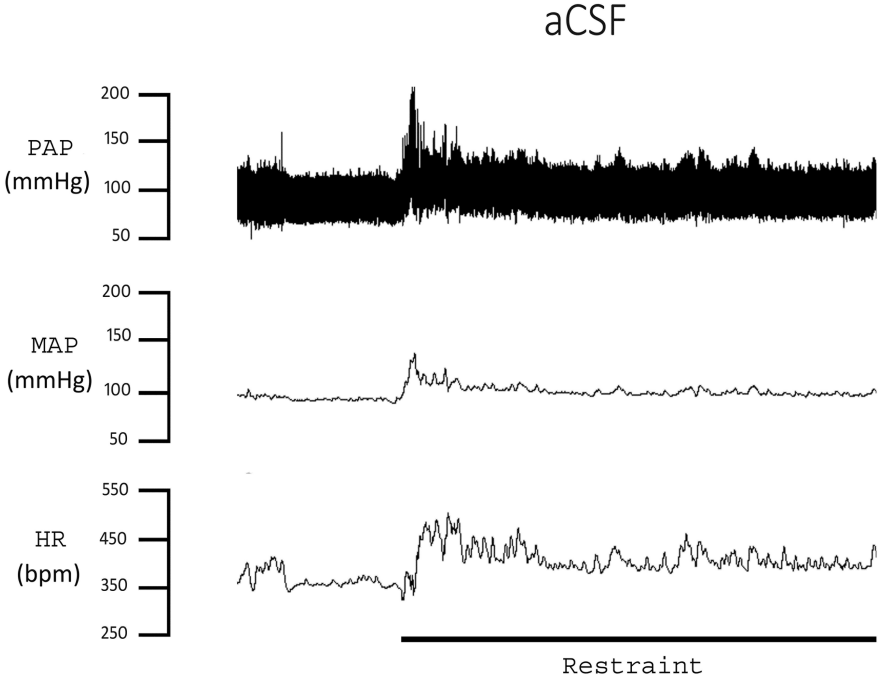
Footnotes – aCSF - artificial cerebrospinal fluid; NBQX - 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide; LY235959 - [3S-(3α,4aα,6β,8aα)]-Decahydro-6-(phosphonomethyl)-3-isoquinolinecarboxylic acid); BEI - baroreflex effectiveness index

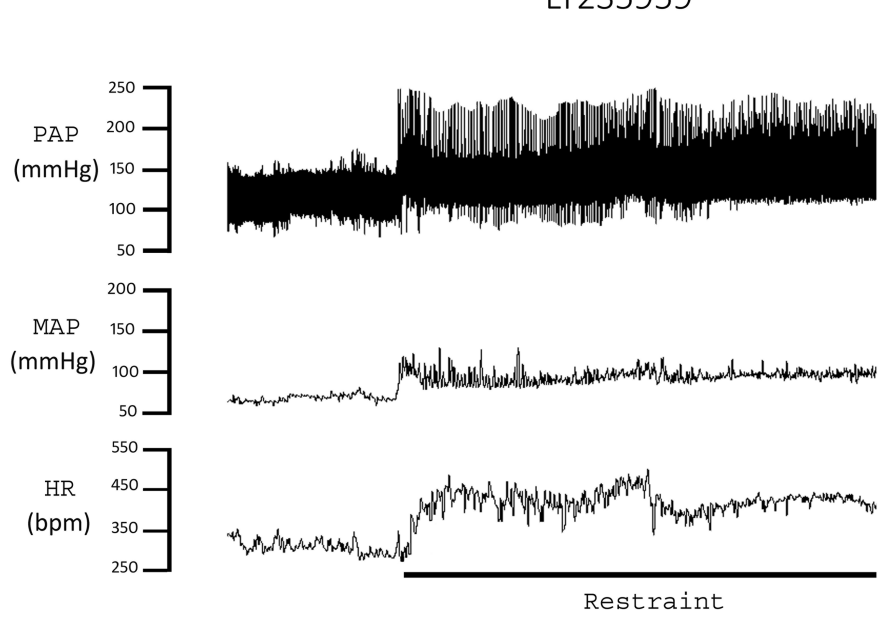
Table 3 - Control animals (aCSF) and treated with an NMDA receptor antagonist (LY235959 - 1nmol/100nL) and a non-NMDA receptor antagonist (NBQX - 1nmol/100nL). Result of the test of difference of means (student t test) of baseline values in the sequences of Gain (Up), Gain (Down) and Gain (All), before and after drug injection. Values expressed as mean ± SEM

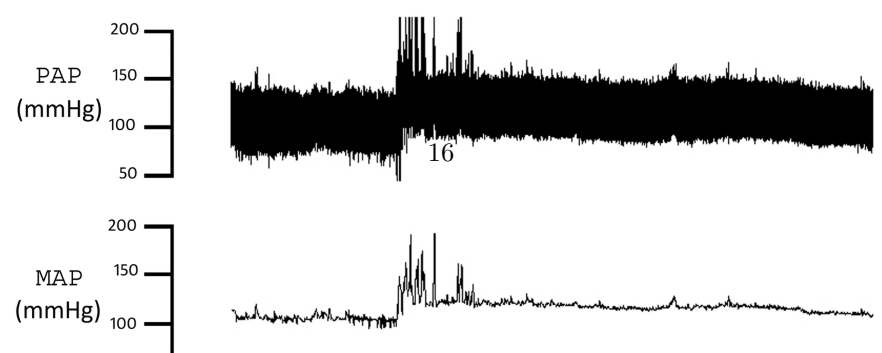
Footnotes – aCSF - artificial cerebrospinal fluid; NBQX - 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide; LY235959 - [3S-(3α,4aα,6β,8aα)]-Decahydro-6-(phosphonomethyl)-3-isoquinolinecarboxylic acid);

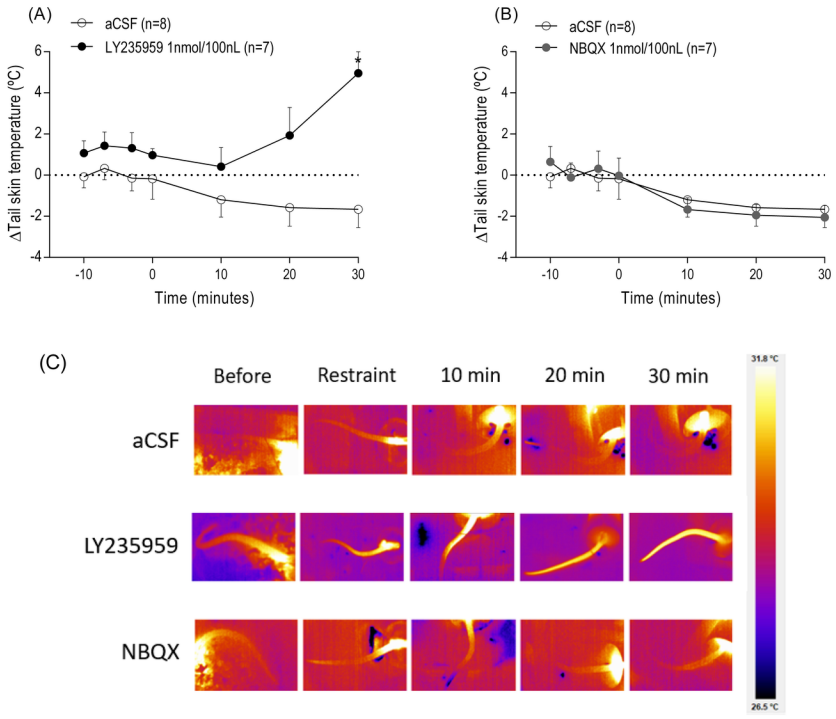


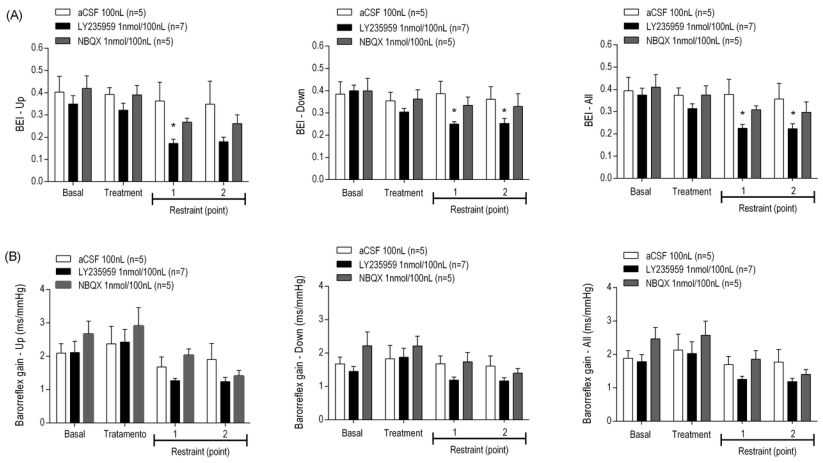












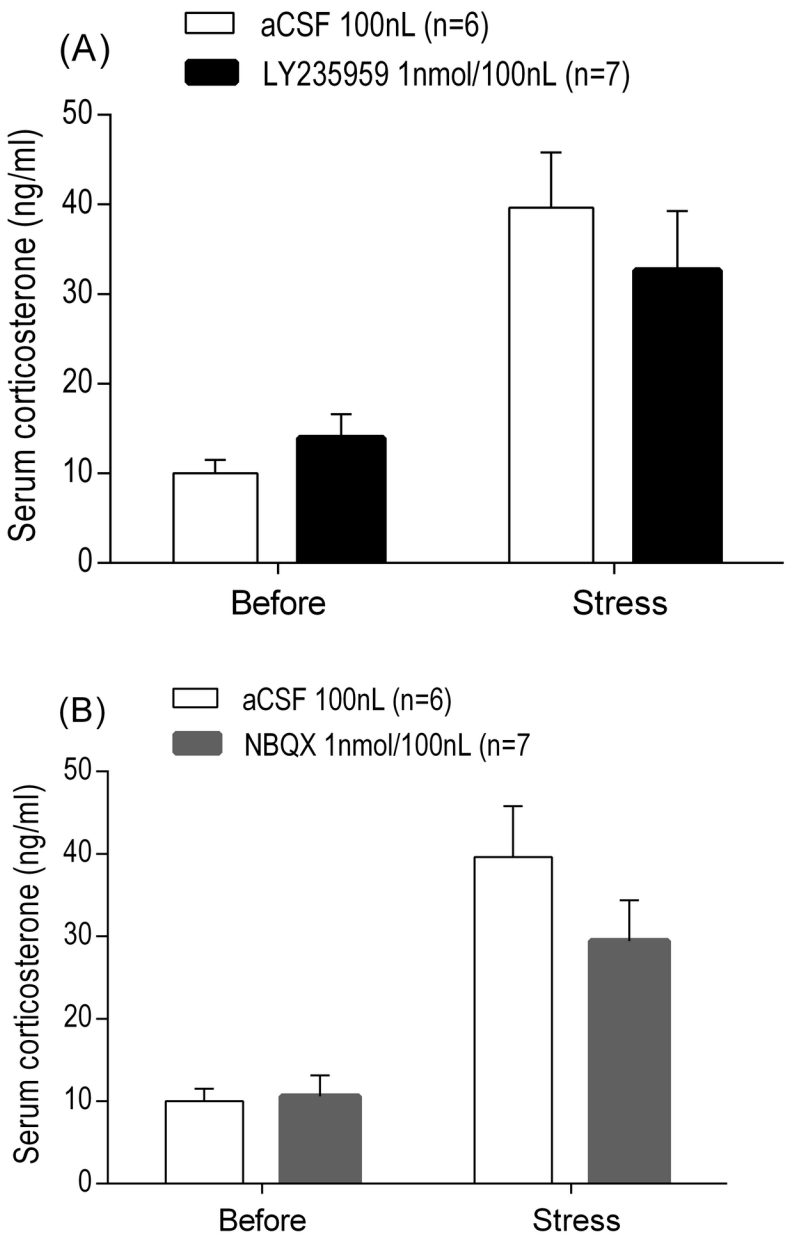


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Table 1.pdf available at https://authorea.com/users/403779/articles/515139-nmda-receptors-in-the-insular-cortex-modulate-cardiovascular-and-autonomic-but-not-neuroendocrine-responses-to-restraint-stress-in-rats

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Table 2.pdf available at https://authorea.com/users/403779/articles/515139-nmda-receptors-in-the-insular-cortex-modulate-cardiovascular-and-autonomic-but-not-neuroendocrine-

responses-to-restraint-stress-in-rats

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Table 3.pdf available at https://authorea.com/users/403779/articles/515139-nmda-receptors-in-the-insular-cortex-modulate-cardiovascular-and-autonomic-but-not-neuroendocrine-responses-to-restraint-stress-in-rats