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Filipa Silveira Monteiro da Gama Pereira

Dissertação orientada por:

Professor Doutor João Cerqueira, Instituto de Investigação em Ciências da Vida e Saúde

Professora Doutora Ana Crespo, Faculdade de Ciências da Universidade de Lisboa

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Resumo

No dia-a-dia o Homem está sujeito a situações stressantes que põem em causa a sua sobrevivência, afectando-o física e emocionalmente, podendo contribuir para o aparecimento de algumas patologias. Na resposta a estas situações, o cérebro desempenha uma função preponderante. É ele que determina quais as situações que são consideradas ameaçadoras e que desencadeia a resposta fisiológica perante essas ameaças.

A resposta a situações de stress está muito dependente do eixo hipotálamo-pituitária-adrenal. Este eixo é responsável pela activação da síntese de diversas hormonas, como os glucocorticóides. Os glucocorticóides, juntamente com Sistema Nervoso Autónomo, desencadeiam a resposta *fight or flight* que permite ao indivíduo reagir a situações ameaçadoras. Esta resposta é rapidamente activada quando é necessária e é inibida logo depois. Quando isto se verifica, há uma boa adaptação ao stress e a homeostasia é reposta. Em algumas situações, como na exposição ao stress crónico, esta resposta não é a conveniente, promovendo uma má adaptação. Esta resposta não adaptada, deve-se a um aumento crónico dos glucocorticóides na corrente sanguínea. Esta situação é prejudicial ao organismo podendo causar mudanças profundas no comportamento.

O cérebro é um dos alvos desta resposta inadequada ao stress. Tanto o hipotálamo como o córtex pré-frontal, sofrem profundas alterações estruturais, como atrofia neuronal, e limitações funcionais que se traduzem em défices comportamentais.

Outro órgão que parece sofrer com os efeitos nocivos do stress é o coração. Muitos factos sugerem que o stress aumenta o risco de doenças cardiovasculares e até de morte súbita. No entanto, ainda não se sabe ao certo qual o efeito específico do stress no coração. Existe a possibilidade de os danos a nível de estruturas do cérebro, provocados pelo stress, poderem estar na base dos problemas cardiovasculares a ele associados.

A interacção entre o cérebro e o coração regula a nossa actividade cardíaca e tónus vascular. Não é pois de estranhar que doenças neurológicas, como a depressão ou a epilepsia aumentem o risco de doenças cardiovasculares.

Existem evidências de que certas estruturas corticais podem influenciar a regulação cardiovascular.

O córtex insular é uma estrutura muito importante a nível do sistema autónomo e límbico. É responsável por processar estímulos emocionais e respostas fisiológicas. A sua função está intimamente ligada com a regulação neuroendócrina, cardiovascular e gastrointestinal. Para além disso também está associado com outras funções como a tomada de decisões ou a linguagem. Tanto em humanos como em roedores, a insula desempenha funções importante ao nível da regulação cardiovascular. Aparentemente esta estrutura cortical pode desencadear tanto respostas simpáticas, caracterizadas pelo aumento do ritmo cardíaco e da pressão sanguínea, como por respostas parasimpáticas que se caracterizam pelas respostas cardiovasculares opostas. Na prática clinica, danos na ínsula são muitas vezes associados com a morte dos pacientes. Esta possível causa-efeito reforça ainda mais a importância do córtex insular na regulação cardiovascular.

Aparentemente, a insula do hemisfério direito e a do esquerdo desempenham funções diferentes na regulação cardiovascular. O córtex insular direito parece estar associado com a resposta simpática e o esquerdo com a resposta parasimpática. A ínsula direita parece ter um papel dominante na regulação cardiovascular.

Existem também outras estruturas corticais importantes na regulação autónoma, que parecem intervir na modelação cardiovascular, como o córtex infralímbico, no entanto a sua função ainda não está bem esclarecida.

Por tudo o que já foi mencionado, este trabalho pretende aprofundar o conhecimento sobre a regulação neuronal da resposta cardiovascular em roedores. Para tal definiram-se os seguintes objectivos: 1 - estabelecer um protocolo de estimulação do córtex insular com gravação simultânea do electrocardiograma; 2 -

estudar o impacto do stress crónico na ínsula e na regulação cardiovascular, 3 - elucidar a interacção existente entre a ínsula e o córtex infralímbico.

Neste estudo foram utilizados ratos Wistar-Han que foram divididos inicialmente em dois grupos. Um desses grupos funcionou como controlo e o outro foi submetido a um protocolo de stress, o *Chronic Umpredictable Stress protocol*. Todos os animais foram sujeitos ao protocolo de electrofisiologia de estimulação da ínsula, se bem que alguns elementos do grupo de controlo não sofreram a totalidade do protocolo, pois a sua ínsula não foi estimulada. Em seguida foi analisada a activação neuronal do córtex infralímbico devido a essa mesma estimulação. Para isso, analisou-se a expressão do *c-fos*, pois a expressão deste *early gene*, é encarada como sinal de activação celular devido a ser desencadeado por um determinado estímulo.

Os nossos resultados indicam que, quando se estimula o córtex insular, os animais controlo estimulados apresentam bradicardia (diminuição do ritmo cardíaco), contrariamente os animais stressados não apresentam qualquer alteração do ritmo cardíaco quando se dá a estimulação, tal como os animais do grupo de controlo que não foram estimulados.

Analisando a expressão de *c-fos* no córtex infralímbico, verificou-se que os animais controlo, estimulados, apresentam uma expressão, significativamente superior de *c-fos* em comparação com os outros dois grupos de animais. Os outros dois grupos apresentam uma expressão similar de *c-fos*, muito diminuta.

Assim sendo, os nossos resultados comprovam que o córtex insular é um importante centro da regulação cardiovascular. Esta experiência demonstra também que o stress afecta a regulação cardiovascular, uma vez que os animais stressados não apresentam bradicardia, aquando da estimulação da ínsula. Esta evidência sugere que o stress altera ou desregula os mecanismos corticais responsáveis pela regulação cardiovascular. Para além disso, a análise da expressão *c-fos*, parece indicar que o stress provoca danos na ligação entre a ínsula e o córtex infralímbico, podendo assim, esta disrupção, estar na base da falta de regulação do ritmo cardíaco por parte da ínsula nos animais stressados.

Perceber se as alterações funcionais na ligação ínsula - cortex infralímbico se devem a alterações estruturais de alguma destas regiões ou até de outras estruturas é uma das dúvidas lançadas por este estudo.

Palavras-chave: Stress, córtex insular, regulação cardiovascular, electrofisiologia

Abstract

All individuals are constantly subjected to the effects of stress situations. The brain is one of the main organs affected by the deleterious effects of stress, causing functional deficits and behavior impairments. However, stress can affect other organs, like heart. Several studies associated the exposure to stress situations with cardiac pathologies. The cause of these cardiac problems is still unclear, but brain damages, caused by stress, can induce cardiac diseases.

There is a link between heart and brain, where insular cortex seems to play an important role. This cortical region can modulate heart rate as well as blood pressure. Other brain regions, like infralimbic cortex, seem to contribute for cardiovascular regulation.

In this work we want to assess the impact of chronic stress in cardiovascular regulation, and we want to clarify the interactions between insula and imfralimbic cortices.

With the above in mind, we designed an experimental setup consisting in deep brain stimulation and simultaneous electrocardiogram recording in anesthetized animals. After this procedure we have analyzed the neuronal activation in the infralimbic cortex due to insula's stimulation.

Our results show that during stimulation protocol, control animals (with stimulation) developed bradycardia, whereas the stressed animals did not. Besides, the *c-fos* analysis reveals that stress disrupt the pathway between insula and infralimbic regions.

Based on data, we conclude that chronic stress can affect cardiovascular regulation, possibly due to the disruption of the pathway between insula and infralimbic cortices.

Keywords: Stress, insular cortex, cardiovascular regulation, electrophysiology

Index

1. INTRODUCTION

1.1 - Stress

1.1.1 - Stress and brain

Common sense tells us that daily routine, experiences involving social interactions or physical challenges have a huge influence in our psychological and physical health. This influence can increase the risk for several diseases [(1)].

We can define a stress situation as a condition which endangers our survival, affecting our emotional and physical stability. Brain plays a key role in these situations [(2)]: it determines what a threat is as well as the start of physiological and behavior responses that aims an adaptation to the new situation and reinstating homeostasis [(3)] [(4)].

In the brain, one of the principal vectors of the stress response is hypothalamic-pituitary-adrenal (HPA) axis who is in charge for activating the synthesis of stress hormones. The hypothalamus is initially responsible for the production of corticotrophin-releasing hormone (CRH), which stimulates the pituitary gland to synthesize and release adrenocorticoid hormone (ATCH) into circulation. The ATCH, in turn, will induce the adrenal cortex to stimulate the production and release of glucocorticoids (GC) [(5)].

In order to face a threat, the GC, in close coordination with the Autonomous Nervous System (ANS), promotes *fight or flight response* - activating gluconeogenesis, mobilizing stored energy to skeletal muscles, improving cardiovascular tone and cognitive functions [(6)]. The shutdown of this HPA axis pathway occurs when glucocorticoids inhibit the synthesis of CRH and ACTH on the brain. Usually this response is rapidly activated when necessary and is efficiently ended afterwards [(4)]. When this happens, a healthy adaptation occurs to the stressor and homeostasis is restored. In some situations (chronic stress exposure for example), the response is inadequate, excessive or prolonged, promoting mal adaptation. This maladaptive response is characterized by a persistent activation of HPA axis and a chronic increase

of GC in the blood stream – mainly cortisol in humans and corticosterone in rats. This situation is harmful for the organism as it inflicts deep behavioral changes in humans and rodents, as depressive-like symptoms, a hyperanxious state and learning/memory deficits [7] [8].

Brain is also a target for stress hormones, particularly to glucocorticoids. These molecules are powerful structure and function modulators of many Central Nervous System regions [9], its overproduction triggers structural and functional deficits in brain areas [7]. Hippocampus is one of the first brain structures affected by overproduction of GC and stress, due to the highest levels of glucocorticoid receptors [10]. Initially it was found that stress and GC could cause neurodegenerative effects on hippocampal neurons, leading to a reduction in the number of cells [11], however it was demonstrated that there was no significant neuronal lost in this area [12] [13]. What stress really promotes is dendritic atrophy and synaptic loss in hippocampus accompanied by behavior deficits like impairments in spacial and learning memory [14]. Stress can also affect other brain regions like prefrontal cortex (PFC).

As in hippocampus, the stress effect on the PFC, can be associated with structural damages and functional impairments. It was proved that stress can disrupt two key processes attributed to PFC - working memory and behavior flexibility. This behavior impairment can be due to volumetric reductions of the upper layers of the PFC [15] [16].

1.1.2 - Stress and heart

When the adaptation to a stress situation fails, several systems of the body can suffer the deleterious effect of maladaptive response.

Extreme emotional and physical situations in daily routine can be associated with a higher susceptibility for cardiovascular diseases [17] or even sudden death [18].

This subject is not new and several studies have been made, mainly in humans. Normal quotidian situations like watching a football game of your favorite team,

having a serious discussion with a family member or professional problems can trigger several cardiac pathologies. Witnessing natural catastrophes, like earthquakes or armed conflicts can be associated with this problematic also [1] [19].

The INTERHEART study analyzed the incidence of several psychosocial stressors as a risk factor for myocardial infarction (MI), in 24767 people from 52 countries. In this study patients who already had a previous MI were compared with patients without cardiac problems. Cardiac patients revealed to be exposed more often to stress situations in their daily routine (at work, at home) than non-cardiac individuals [20]. Other studies indicate also that anger or exciting moments can precede arrhythmias [18] [21] and MI [22] [23]. The pressure of an important decision making or a deadline approach, at work, can also trigger cardiac events like coronary heart disease [24] [25].

The incidence of acute MI and sudden death has been shown to increase following natural disasters. Following an earthquake, the number of patients admitted in hospitals with MI is abnormally high. Patients, who had their heart rate monitored on these events, presented a heart rate instability and blood pressure variability, which persists over a few hours after the earthquakes [26] [27]. During Gulf War (Irak 1990/91), the first missile strike on Israel was associated with the higher incidence of acute MI and sudden cardiac death in areas which were close, but not directed affected by the missiles attacks. In the next 16 days this tendency was not observed, suggesting that the Israeli population had adapted to the stress [28] [29].

Similarity, in laboratory, chronic stressed animals presented increased resting heart rate and a decrease in heart rate variability [30].

The cause for these cardiac problems are still unclear, it might be changes in the heart muscle (for example, metabolic changes), or also because stressed brain can affect heart. Indeed, there is interplay between brain and heart.

1.2 - Brain and heart interaction

Despite anatomically distincts in location and function, heart and brain share an intricate network involving a complex and tight regulation [31].

Common wisdom tells us that, in response to physical activity, threats and emotional state, the interaction between brain and heart regulates our cardiac activity and vasomotor tone [32]. So, it is not surprising that brain dysfunction can interfere with cardiovascular functioning. In fact, a lot of clinical data demonstrate that neurological pathologies, like depression and epilepsy, are able to increase the risk of cardiovascular catastrophic events and sudden death [33] [34]. Is currently observed that depression can contribute to a worse prognosis in patients with heart failure [35] [36] and epileptic seizures can trigger cardiac arrhythmias and electrocardiogram (ECG) abnormalities, increasing the risk of sudden unexpected death [37].

Over the last century accumulated evidences indicate that brain lesions can be correlated with cardiovascular deregulation [38] [39]. Cortical regions seem to be more prone to cause this deregulation [40]. Damages in a particular cortical area, the insular cortex (IC) seems to have a deleterious effect in cardiac functions. A comparison of ECG recordings before and after IC stroke, of the same patients, revealed a higher prevalence of cardiac abnormalities in patients with damage in the IC compared to those in which the IC had been spared. This data indicate that IC may have an important role in cardiac regulation [31].

1.3 - What is the insula and how can it control the heart?

Insular cortex (IC) or insula is a cortical region deeply connected with limbic and autonomic systems, it seems to process emotional and sensory information with physiologic responses [37] [41] [42]. Its neurons respond to different visceral sensations, allowing the adjustment of the internal equilibrium [31] [43]. Its "actions" can affect numerous systems like neuroendocrine, gastrointestinal, respiratory and cardiovascular [43] [44]. Besides that, IC seems to be also an important center in diverse fields like tactile recognition, language, pain and behavior (such as decision-making processes) [5] [43] [45] [46].

In humans, IC is located beneath the frontoparietal and temporal opercula (Figure 1) [43] [47] and is divided in anterior and posterior part, by the middle cerebral artery which lies on its surface. The insula can also be divided histologically, in three parts: a posterior granular insula, an anteroinferior agranular region and a dysgranular

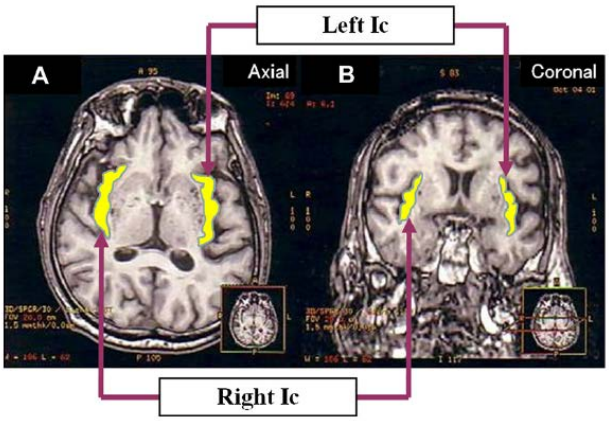

Figure 1 - Axial and coronal views of a brain using magnetic resonance imaging.Yellow color represents the insular cortex [31].

insular cortex [48]. In rats, the IC is located in a different region: it occupies the dorsal bank of the rhinal sulcus and extends to the borders of the primary and secondary somatosensory areas [49] . Like in humans, insula can also be divided in granular,

dysgranular and agranular regions, in which the latter can further be divided in dorsal, ventral and posterior parts (Figure 2) [49].

Several experiments assessed the modulation of different cardiovascular parameters by insula and its regulation of ANS. This brain region affects electrocardiography, cardiac function, blood pressure and baroreflex sensibility [31] [50].

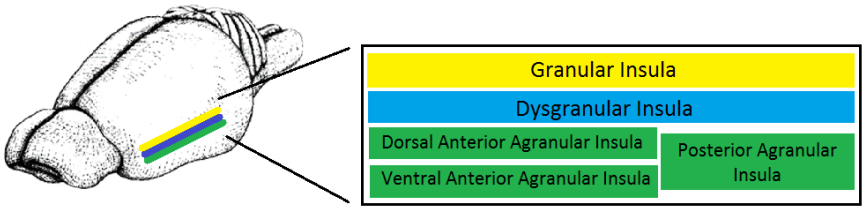

Figure 2 – Schematic layout of location and subdivisions of insula in rats

Insula can be associated with a dual effect on cardiovascular system in animal models. Its stimulation can elicit a sympathetic response – increasing blood pressure and heart rate – and a parasympathetic response associated with the opposite physiological effects [44] [51].

Apparently these opposite responses can be triggered by different insular regions, but this association is not completely understood. A remarkable work developed by Oppenheimer *et al*. (1990) [51] associate a tachycardic effect (heart rate increase) to the stimulation of rostral posterior insula, meanwhile the decrease in heart rate (bradycardia) was related with the caudal part of the posterior insula [51]. Despite the predominance of sympathetic responses upon the stimulation of rostral posterior insula, bradycardic effects were reported too [52] [53].

The evidence that IC mediates cardiac rhythm is also noticed when its function is inhibit, revealing a decrease in heart rate variability and in baroreflex sensibility [53].

In humans, it is difficult to develop studies regarding IC region. Clinical investigation is mainly performed in patients who have insular lesions, or during brain surgeries to treat pathologies like epilepsy. Fortunately, modern bioimagiology tools,

like emission tomography or functional magnetic resonance, allow us to better understand the functional network of IC. During its stimulation in several patients, blood pressure variation and heart rate variability is also observed [31] [37], corroborating the animal studies.

Insular damages can cause in several occasions the patient's death [41]**.** This association between death and IC damages is recurrently done in clinical data, which reinforces the importance of IC in cardiovascular regulation [31] [54] [55]. Moreover, in laboratory, the stimulation of IC can induce profound ECG alterations which culminate also with lethal cardiac arrhythmia [52]. These evidences indicate that this cortical region plays a core role in cardiovascular regulation.

Cardiovascular modulation appears to be, like the majority of cortical functions [56], a lateralized function [37] [57]. Apparently, the right and left insula influence the cardiac regulation in a differential manner. In the majority of studies right insula produces an increase either in heart rate or blood pressure, meanwhile the left IC is associated with the opposite effect, decreasing cardiac function [31] [47]. Besides that, right IC seems to have a dominant role in cardiovascular regulation [41] [58]. In clinics, damages in the right insula are more often related with patients' death, when the lesion occur in left insula the repercussion in patient health is less severe [41] [59]. In addition, the right IC lesions compared with the left IC lesion or no IC lesion at all, increase the risk of all-cause death during the follow-up period [60] [61].

As insula is one important autonomic and limbic center [31], it has been object of detailed neuroanatomic studies to reveal the diverse projections from and to this structure [44] [31] [43]. Insular cortex projects to other brain regions, as infralimbic cortex [44]. This sub region of medial PFC cortex (mPFC) seems to regulate autonomic responses as modulation of arterial blood pressure and cardiac feedbacks [63] [64] [34], however its exact role is still unclear.

All data gathered in human and animal studies seems to evidence the huge importance of the insular cortex in cardiovascular regulation, playing a core role in the link between heart and brain. Due to the above reasons, we decided to analyze the impact of stress in of the heart insula's heart rate control.

2. GOALS

This thesis aims to give a contribution to clarify the neural networks responsible for the regulation of cardiovascular physiology in rats. As stated before, the insular cortex as a key role in cardiovascular modulation, so the following objectives were defined:

- Establish a stimulation protocol of insular cortex with simultaneous electrocardiogram recordings.

- Assess the impact of chronic stress on insular cortex and its modulation of cardiovascular function.

- Elucidate the interaction between the insular and infralimbic cortices and evaluate the relevance of this interaction in the integration of emotional and stressful stimulus in cardiovascular response.

3. Methods

3.1 - Animals and treatments

Experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and National Institutes of Health guidelines on animal care and experimentation.

Thirty-seven male Wistar-Han rats (Charles River laboratories, Barcelona, Spain), weighting 300-400g and aged 3 months were used in this study. Animals were housed in groups of two under standard laboratory conditions (room temperature 22^{o}C, relative humidity of 55%, 12 hours light cycle beginning at 8 A.M., food and water *ad libitum*).

3.2 – Stress Protocol

The animals were divided in two different groups. One group (n=15) was submitted to a protocol of Chronic Unpredictable Stress (CUS) as previously described by Cerqueira *et al* (2007) [16]. Briefly, the animals were randomly exposed to different kinds of stressors. The stressors applied consisted of:

- Restraint in a small place – two rats were placed in a small plastic box with practically no space to move, for one hour.
- Shaking – five animals were inserted in a plastic bag and shake repeatedly for 15 minutes.
- Overcrowding – five animals were placed in one cage that usually holds only two animals, for one hour.
- Cold water – animals were placed in a container with approximately 5 cm of water (18^{o}C) for one hour.
- Exposure to a hot drier – the upper part of the cages was removed and for a period of 30 minutes, the animals were exposed to a hot air stream.

In order to avoid adaptation from the animals, the stressors were applied in a random way changing not only the stressor applied but also the duration of the exposure and the time of the day it was performed. The unpredictability of the protocol has the additional advantage of mimicking the unexpectable daily stressful situations experienced in real life by humans [65].

The other group was formed by control animals handled twice a week. Since the manipulation itself is a stressful procedure, handling enables the animals to familiarize with the operator, diminishing the stress effect associated during the experimental procedures. In addition this guaranties that possible differences observed between animal groups are not due to manipulation [66].

3.3 – Stress monitoring

To monitor the efficacy of the stress protocol we evaluated two parameters.

Chronic stress is associated with a slower weight gain, independently of food intake [66]. Therefore, during the stress protocol, body weight was measured three times per week.

Furthermore, it is well known that chronic stress activates the HPA axis [67] which will lead to the production of corticosterone. This hormone secretion rise sharply after experimental negative stimuli [30] and therefore its measurement in basal conditions is a useful tool to evaluate the stress effects in rats. In this context, the plasma levels of corticosterone were measured using a radioimmunoassay (ELISA kit, R & D Systems), in serum samples. The blood was collected the day before any experimental proceeding, between 9 and 10 A.M. by tail venepuncture, bearing in mind the normal circadian values of corticosterone plasma levels.

3.4 – Electrophysiology

As previously described and in accordance with the literature, stimulation of insular cortex induces variations on heart rate and blood pressure. The most common technical approaches to stimulate a specific brain region are local electrical stimulation or pharmacological activation of excitatory pathways using several neurotransmitters, like glutamate. In this study, an electrical stimulation protocol was designed, since it has a better temporal resolution than the pharmacological approach. The response observed during electrical stimulation is precise and instantaneous, unlike the pharmacological effect which is progressive over time [(50)] that imposes difficulties in establishing a causal relation between stimulus and effect.

3.4.1 - Electrophysiology protocol implementation

The design of the insular cortex stimulation protocol with simultaneous electrocardiogram recording was based on the experimental procedure described by Yasui *et al*. (1991) [(44)]. During the process of protocol implementation 30 animals were used to optimize the surgical technique and the quality of acquired data. In order to optimize and determine the best location to deliver the appropriate stimulus several coordinates were initially tested in the dorsal-ventral axis of posterior agranular IC. Stimulation began 1.2 mm above the IC and repeated every 0.3 mm until the electrode reached 0.9 mm below the ventral coordinate of IC. The major variations of heart rate were observed between coordinates -0.6 and -0.9 and therefore the stimulus was performed only in these areas during the rest of the experimental procedure.

The stimulation of the insular cortex was performed with two types of bipolar concentric electrodes: tungsten electrodes and platinum-iridium electrodes. Although both materials are well suited for acute stimulation protocols (both are non-toxic for tissues) the latter was chosen given the fact that platinum-iridium alloy is more resistant to electrolytic degradation and can therefore last much longer in extensive

and repeated stimulation protocols. The platinum-iridium electrodes also had a slightly bigger tip radius which should result in a decreased magnitude of current density at the tip (for equivalent stimulus) with consequent reduced tissue damage [68]. The only disadvantage in using the platinum-iridium electrodes over the tungsten is that the former are more expensive.

The electrocardiographic recordings for measuring heart rate can be obtained from several locations. Once more in order to obtain the most accurate signal several attempts were performed using several approaches. The need to remove animal hair to increase the contact between the electrodes and the skin of the thorax precluded the use of these locations for this kind of recordings. In this sense, using the limbs for measuring electrical activity from the heart came as a good approach to overcome this problem. Hereupon we decided to place the electrodes on the paws, as they have no fur and it is easy to hold the electrodes with tape. To improve electric conductivity we applied an electrolytic gel to the electrode-skin interface.

The electrocardiographic recording presented are the result of the electrical differential between the two opposite paws (hind and front paw). A ground electrode was placed in the in the other hind paw. To instantly convert the raw ECG recording signals to heart rate (heart beats per minute), we created a script software tool which allowed us to observe, along the stimulation protocol, if any heart rate alteration upon stimulation had occurred. For posterior graphical analysis of the data we also designed a spreadsheet for easy heart rate variation analysis.

The next problem to overcome was the anesthesia. The first approach consisted of the use of Sodium Pentobarbital (0.025mL per 100g of body weight). However, Pentobarbital is a barbiturate, acting as a depressant of central nervous system, inducing respiratory and cardiovascular depression. This may influence the neuronal response to deep brain stimulation. As an alternative Ketamine was used. This drug is a non-barbiturate anesthetic that in conjunction with other drugs, such as medetomidine, provides good analgesia for surgical procedure. Dose-dependent respiratory depression and hypotension may occur also with ketamine but only in larger doses [69]. The later mix was therefore chosen as the experimental anesthetic.

During the recording sessions, the filter and amplifier settings had to be programmed to filter-out undesired electrical noise and to obtain a cleaner ECG signal. A stainless steel faraday cage was built and placed over all electronic equipment and the stereotaxic frame holding the rat to minimize the interference of electromagnetic noise in the ECG recordings. Despite the use of the faraday cage background noise due to electrical contamination from the power source and ground persisted during all the procedures. In the majority of the experimental trials these interferences were negligible, but on rare occasions they would force halt or prevent the trial to be carried out.

3.5 - Surgery

For this part of the experiment, the control group was divided in two. The first subgroup (n=15) named controls with stimulation (Ctrl w/ stimulation) performed the exact protocol as described below. The other control subgroup (Ctrl w/o stimulation) performed the same protocol with just one difference, their insula was not stimulated.

Animals were anesthetized with a mixture of Ketamine (75mg/kg) / Medetomidine (0,5mg/Kg) interperitoneal injected in the animal facility, to minimize the stress of this invasive method. As they became unconscious, the animals were taken to the electrophysiology room. Animals were then placed in a David Kopf® stereotaxic frame (Germany), and their body temperature was maintained at 37^{o}C by means of a homeothermic blanket (Stoelting, Ireland) and a rectal probe.

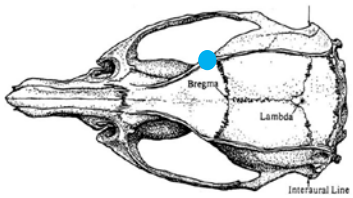

Figure 3 – Scheme of the rat's skull, showing the bregma and lambda points and the approximate localization of the IC (adapted from Watson and Paxinos 2005).

A scalp portion was removed, exposing the cranium. A small hole was drilled in the insular cortex region, using the topographic references Lambda and Bregma, as showed in Figure 3 (adapted from Paxinos and Watson, 2005) [70].

A concentric bipolar platinum-iridium electrode (WPI, U.S.A.) was placed in the right insular cortex, namely in the Posterior Agranular Insula (PAI) (coordenates: bregma's level rostrocaudally; 5.8 mm lateral to the midline and 7.4 mm below cortical surface) [70].

3.6 - ECG recording and stimulation protocol

The electrocardiographic signal (to monitor heart rate) was acquired using three Ag/AgCl electrodes (Easycap, Germany) placed in the two hind paws and the right front paw. To improve the electrical contact between paw and electrode, an electrolytic gel (Easycap, Germany) was used and an adhesive tape helped to maintain physical contact.

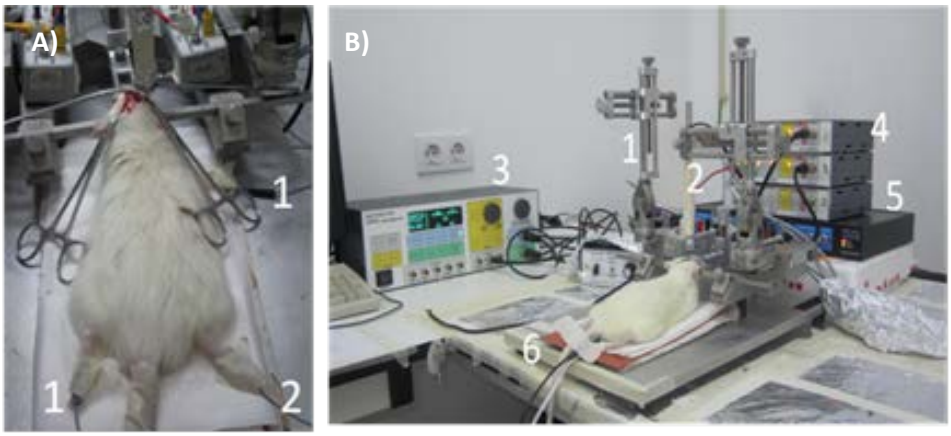

Figure 4 – **A)** Rat with ECG electrodes: 1- Differential recording electrods; 2- Ground electrode. **B)** Rat in the stereotaxic frame and acquisition system apparatus: 1 – Stereotaxic frame; 2 – insular cortex electrode; 3 –Stimulator; 4 – Amplifiers; 5 – Analogue to digital converter; 6 – Homeothermic blanket.

The ECG signal results from the differential between the “left hind paw” electrode and the “right front paw” electrode. The third electrode (right hind paw) functioned as the ground reference, to electrically match the acquisition system and the animal’s body diminishing the artifact from the acquisition (Figure 4a).

The signal acquired from ECG electrodes was amplified (50k), filtered (0.1-100Hz), and recorded on a personal computer running Signal 4.08 (CED, UK), Figure 4b.

At the beginning of each experiment, the heart rate (HR) of the rat was recorded for 10 minutes, in order to obtain a baseline measurement. To acquire heart rate a Signal script was created to measure the distance (time) between two consecutive “QRS” complexes (Figure 5). Since this distance is equivalent to one heart beat, the heart rate was automatically calculated according to the following formula *“HR in beats per minute = 60 / (average QRS distance in seconds)”.*

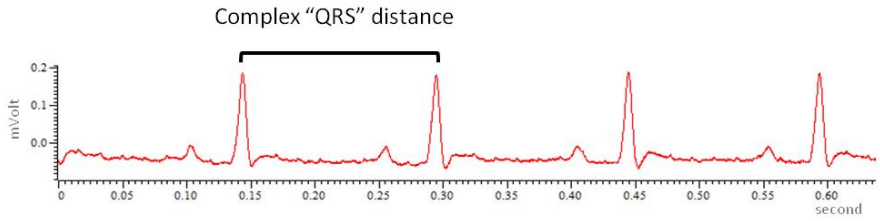

Figure 5 – Distance between “QRS complexes”

After this period, a bipolar electrode was inserted in the PAI region, to deliver 1 train of 1 ms-long square pulses at 50 Hz, during 30 seconds, at the intensity of 200 µA. This stimulation was repeated two additional times, with a 2 minutes interval.

The animals were left unconscious and at rest for 90 minutes after the end of the stimulation protocol (see section below 3.7.1). After this period, a different electrical stimulus (a single biphasic 1 mA 10s long-pulse) was applied, in order to mark the brain tissue in contact with the electrode and facilitate the histological determination of its position.

Finally, the animals were perfused with 4% paraformaldeyde solution to fix the tissue in order to maintain its original architecture. Brains were then carefully removed

and placed in the same fixative solution, for 48 hours. Until sectioning, brains were preserved in a 8% sucrose in phosphate buffer solution (PBS), at 4^{o}C.

The brains were then sectioned in 50µm coronal slices in a vibratome (Leica). Brain slices containing the PAI were stained with a light Giemsa solution to better identify this region and confirm the exact position of the electrode. The slices containing mPFC were submitted to an immunohistochemestry procedure to detect nuclear expression of c-Fos protein (see next section).

Whenever the electrode failed the target position (PAI), recordings were discarded.

3.7 - *c-fos* detection

3.7.1 - Immunohistochemistry protocol

The "cause-effect" relationship between activation of a particular region and the expression of the immediate early gene (IEG) *c-fos* has been well documented not only in the spinal cord but also in several brain regions [71] [72] [73] [74] . *c-fos* is the most frequently activated IEG in the central nervous system, following various internal or external stimuli, such as electrical or pharmacological stimulation, stress or learning experience [75] [76] [77]. This proto-oncogene encodes a transcription factor, Fos, which regulates the expression of many late-response genes. Thus, visualization of *c-fos* could serve as a sensitive indicator of gene activation in individual target neurons activated following a given stimulus.

After the stimulation protocol the animals were left unconscious and at rest for 90 minutes to guarantee that the *c-fos* activation detected by imunohistochemistry is a specific consequence of the electrical stimulation [78]. To detect the *c-fos* expression, slices containing the mPFC were submitted to a free floating immunohistochemical standard procedure as based on Dragunow *et al*.(1987) [79]. The primary antibody used was a rabbit polyclonal antibody against *c-fos* at 1:2000 dilution (Calbiochem, USA),

whereas a biotinylated swine anti-rabbit IgG (DakoCytomation) at 1:200 was used as the secondary antibody.

Briefly, in this protocol, a series of free floating sections were washed several times in PBS, 0.1M. Afterwards the endogenous peroxidases were inhibit in a hydrogen peroxide 3.3% in PBS solution, and cellular membranes were permeabilized in a series of PBS-T (0.3% triton X-100 with PBS) rinses. To increase the specificity of antigen-antibody reaction, the brain sections were immersed in a fetal bovine serum with PBS-T. After incubation with primary antibody, the slices were washed in PBS-T and incubated with the secondary antibody solution. The bound chromogen was revealed using 50% 3.3-diaminobenzidine (DAB; Sigma Alrich) and 0.02% hydrogen peroxide in Trizma base solution.

3.7.2 - Quantification of *c-fos* staining

In the present work the neuronal activation of the infralimbic region was analyzed, using the Visiopharm Integrator System (VIS) software and a camera (Pixelink PL-A622, Canada) coupled to a motorized optical microscope (Olympus BX51TF, Japan).

The infralimbic area was outlined according to the rat brain atlas of Paxinos and Watson (2005) [(70)]. All cells that marked positive for *c-fos* were counted. A positive stained nucleus is considered, when it presented an oval/circular form and evident clear brown staining, that was due to *c-fos* expression (Figure 6).

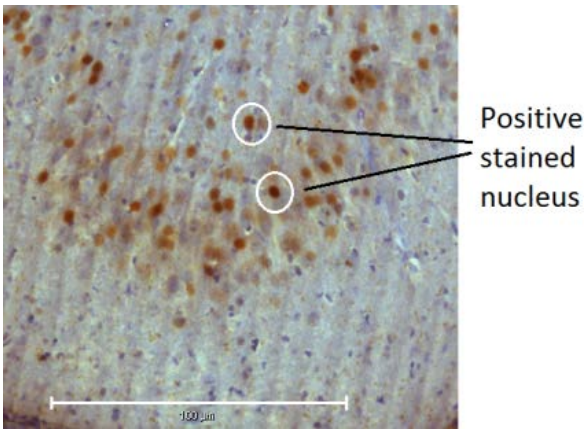

Figure 6 – Example of positive stained nucleus for *c-fos* in brain tissue. Scale bar = 100 µm.

Total cell numbers were then normalized for area and expressed as staining density (*c-fos*-labeled neurons/mm^2).

3.8 - Statistics

Data was analyzed using ANOVA or repeated measures ANOVA when appropriate, using IBM SPSS 19.0 software (Statistical Package for Social Sciences, SPSS Inc., USA), followed by Tukey's honestly significant differences post-hoc tests to identify paired differences between groups. T-test for paired samples was used for analyzing the heart rate before and during the stimulation for each group. Results were considered significant whenever $p<0.05$. All results are presented as mean +/- standard error of the mean, unless otherwise stated.

C-fos data where normalized for the contralateral (non-stimulated) hemisphere (as a ratio), to control for differences introduced by the staining procedure.

4. Results

4.1 - Biometric parameters and hormonal determinations

Physiological alterations caused by chronic stress can be evaluated by several parameters, such as body weight variations or elevation on the corticosterone serum levels and as previously described these physiological parameters may be used as a control for the chronic unpredictable stress protocol efficacy.

In this sense, animals' weight was monitored during the experimental period (56 days). Figure 7 shows weight evolution of stressed (CUS) and control (Ctrl) animals.

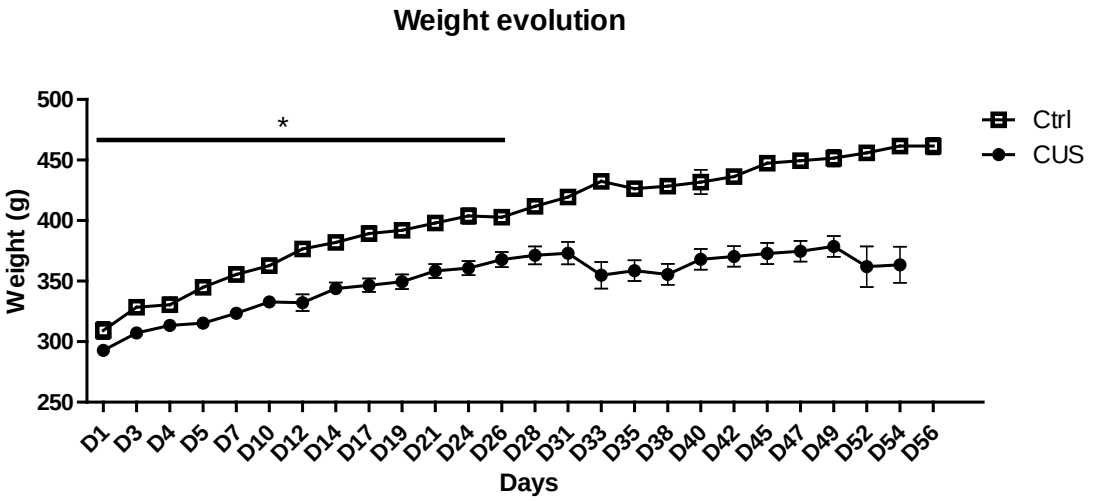

Figure 7 –Stressed animals gain less weight over time. Results are expressed as mean ± SE *p<0.05.

Stressed animals tend to gain less weight than controls over time. The difference observed in the body weight gain between both groups was statistically significant for the first 26 days (p=0.0245). Data from day 26 to day 56 was not included in the statistical analysis because individual tracking of each animal body weight was not possible as some animals were progressively being sacrificed due to other experimental procedures (electrophysiology studies).

The levels of corticosterone in the serum of stressed and control animals were also measured and the results are displayed in Figure 8. Although the small number of animals precludes an adequate statistical analyses, results clearly show that stressed animals present higher levels of corticosterone in serum.

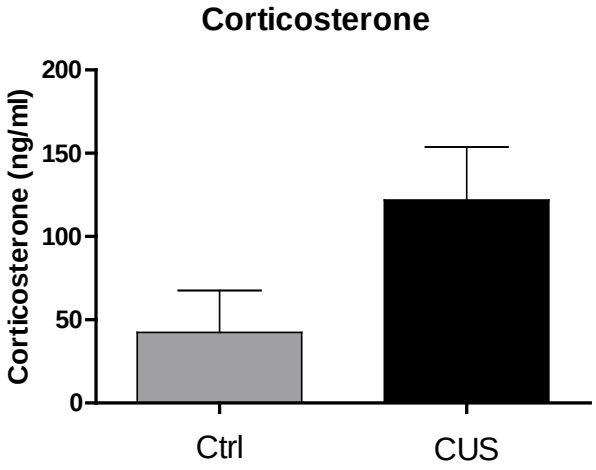

Figure 8 - Stressed animals present higher levels of serum corticosterone. Results are expressed as mean ± SE

4.2 - ECG recording and stimulation protocol

In order to assess how stress exposure would affect the control of the heart rate by the insular cortex in both groups, we continuously monitored the ECG of anesthetized control and chronically stressed animals while stimulating the insula. In addition, to control any non-specific effects of electrode placement upon heart rate, a group of animals that had an electrode implanted in the insula but which were not stimulated was formed (Ctrl w/o stimulation). Figure 9 plots the average heart rate of each group during the experimental procedures, starting 30 seconds before the beginning of stimulation (first vertical line) until 30 seconds after the end of stimulation (second vertical line), to obtain both baseline and recovery profiles. Importantly, whenever the data was not successfully recorded or whenever the electrode failed to be placed in the correct coordinates for the insula, data was discarded

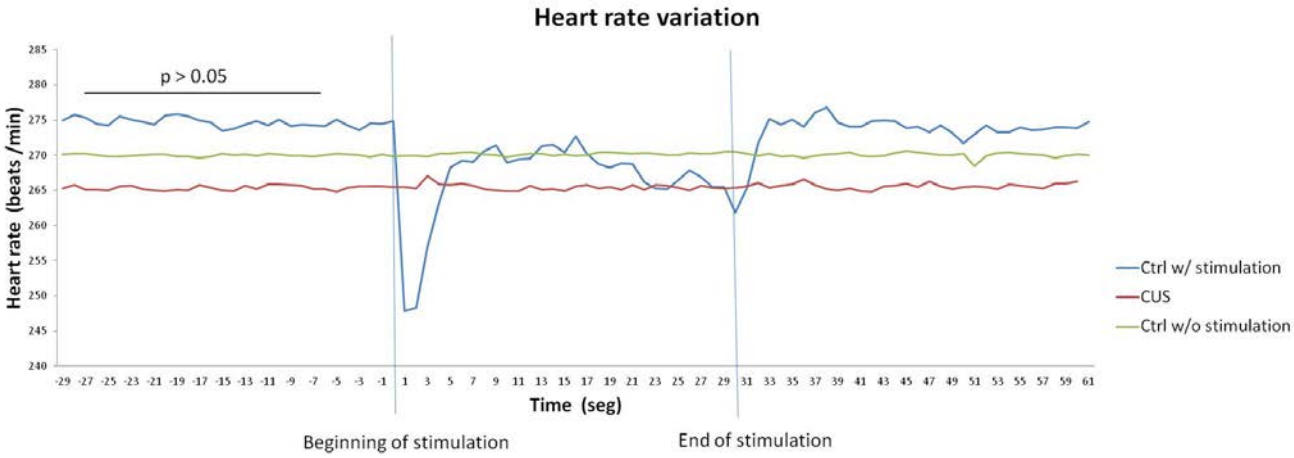

Figure 9 - In Ctrl without stimulation and in stressed animals, stimulation of the insula failed to induce bradycardia. Only Crtl with stimulation shows a decrease in heart rate.

Before stimulation, the heart rate was not significantly different between the three groups (F = 0.6408; p>0.05). A within group comparison, showed that only stimulated control group had a significantly different heart rate during stimulation, when compared with his baseline (6% lower). Neither stressed animals nor non-stimulated control presents this heart rate difference (Figure 10) (Ctrl w/o stimulation t=0.7283 p=0.5422; Ctrl w/ stimulation t=7.632 p=0.0167; CUS t=0.8587 p=0.5210).

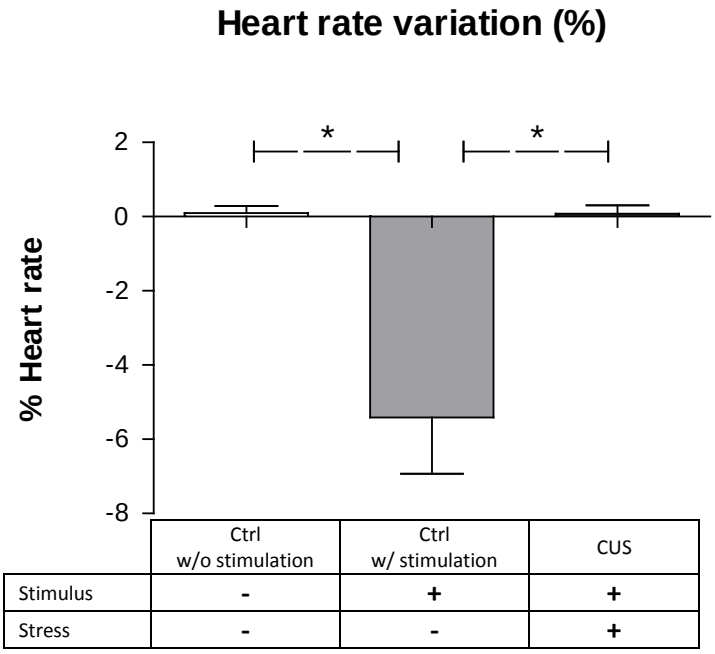

Figure 10 - Stimulation of the insular cortex only has produced an effect in the heart rate of control animals with stimulation. Results are expressed as mean ± SE *p<0.05

4.3 - *C-fos* analysis

In response to specific stimulus, some neurons start the expression of early immediate genes, such as *c-fos*, and therefore this can be regarded as a marker of cell activity. An increase of *c-fos* expression in specific brain regions can indicate neuronal activation in response to a particular stimulus.

The number of *c-fos* positive cells in the infralimbic cortex was counted after electrophysiological procedures, in order to analyze if the neuronal activation was

dependent or not of insula stimulation. Since stimulation was unilateral and the immunohistochemical procedures were done at different times, results of *c-fos* positive cells are not presented as absolute densities (number of positive cells per section area) but rather as interhemispheric ratios, which controls for technical differences in staining (Figure 11).

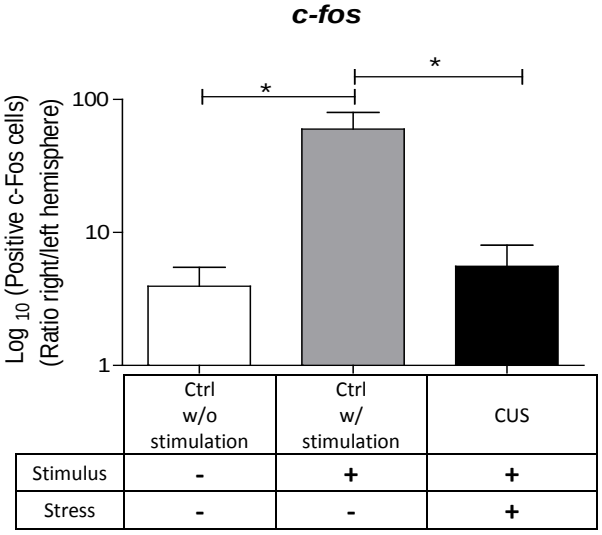

Figure 11 – Stimulation of the insular cortex has only increased the number of *c-fos* positive-cells in the infralimbic cortex in the control animals with stimulation. Results are expressed as mean ± SE. *p<0.05.

Of notice controls without stimulation and stressed animals had a very similar *c-fos* expression in both hemispheres because the ratio is nearly one. This is in contrast with the results from control animals with stimulation, which present a significantly higher activation in the right (ipsilateral) hemisphere (Ctrl w/o stimulation p=0.0347; CUS p=0.0332).

5. Discussion and conclusions

As previously described insular cortex seems to be an undeniable intervenient in cardiac tone. Moreover, this particular structure has been associated with dual control of sympathetic and parasympathetic responses of heart rate, inducing tachycardia or bradycardia according to individual needs [41] [37].

As expected, our experimental data are in accordance with the evidence showing the importance of IC in cardiovascular regulation, as proved by the fact that heart rate decreases upon stimulation of right posterior agranular insula in control animals. Interestingly some differences were found when compared with other similar studies. According to Oppenheimer and colleagues [51] stimulation of this area should elicit a tachycardic response. By the contrary, our results show that stimulation of this area induced bradycardia rather than a tachycardia. This difference has already been reported in literature [47] [52] and may be dependent on experimental differences, namely the kind of anesthetic agent used [47] or the specific region of the IC stimulated. Regarding this last fact, it is important to notice that IC function is lateralized [80] with left side regulating parasympathetic system and right side sympathetic division [31]. This can be the reason for our different results, because we stimulated the right insula instead of the left one as other studies did [51] [50]. This thematic is surrounded by questions and doubts and many controversial data are coming out. The current study focused on the right insula, more precisely in its rostral half of the agranular posterior part, because evidence shows that right IC has a dominant role in autonomic regulation [37] [41] [42] [43] [44]. This is supported by clinical data collected in stroke patients. Data shows that cardiac arrhythmia, produced by unbalanced cardiac autonomic activity, favoring the sympathetic system, was more common after cerebral infarction on the right hemisphere [61].More studies have to be developed to better understand the relationship between specific insula's regions and particular heart rate alterations.

The stress is one important modulator of central nervous system and, as described, can affect several brain regions leading to neuronal atrophy and consequently causing functional neuronal deficits and behavior impairment [7]. To study the impact of stress in cardiovascular regulation, we submitted a group of animals to a chronic stress protocol. Our results confirm the efficacy of the CUS

protocol, given the fact that stressed animals presented a higher corticosterone level than the controls and in addition tend to gain less weight over time than controls.

The heart rate analysis of the stressed and control animals, during stimulation, shows that chronic stress inhibits the insula's effect in cardiac function. In controls (with stimulation), a bradicardic effect is triggered by IC stimulation by opposition, during insula's stimulation, the stressed animals did not present any cardiac alterations. Many theories can be hypothesized which mechanism, pathway or structure is affected by stress. In literature this subject is not very often discussed, but the harmful effect of stress on cardiac function had been associated to an overactivation of sympathetic function in basal conditions [30] [81].

Grippo *et al*. (2002) [30] observed in stressed animals an increase in heart rate compared to controls, which justify the theory that alterations in heart rate due to stress are associated with overactivation of sympathetic system. Interestingly the basal heart rate in the current study was quite similar between the groups, but this analysis was done with the animals already under the effect of the anesthetic agents. However, when the IC was stimulated, stressed animal did not display the expected bradicardic response obtained during the stimulation of IC. This difference suggests that after stress, the cortical mechanisms regulating cardiovascular function are altered in a way that resembles sympathetic system stimulation or on the other side parasympathetic inhibition. As previously stated, it is believed [47] [80] [82] that right IC has an inhibitory effect over sympathetic system and these results clearly support this vision. Independently of which vision prevails it seems that stress promotes an adverse autonomic system imbalance toward sympathetic division, that may contribute to an adverse cardiovascular outcome. Furthermore, it seems that this imbalance is dependent on central nervous system circuits what raises the next question and objective of the current work.

Like previously discussed, there are other brain regions contributing to cardiac regulation. From all the regions involved, IL is clearly gaining weight in this debate and several data has shown its importance in autonomic regulation.

Whether IC action is dependent on IL or not, is still unknown. In that sense, the current study analyzed the neuronal activation of this last region (IL) upon IC stimulation. The *c-fos* analyze of control animals (with stimulation) corroborate the existence of a pathway from IC to IL. The significance of which is given by the fact that stressed animals who do not display a bradycardic response showed a significant inferior activation of this region after IC stimulation. Whether these functional changes are dependent on structural alterations of IC itself, IL or even other regions is still to be clarified and should be a target for further studies. To guarantee that *c-fos* expression in the infralimbic was due to the insular stimulation, we selected animals to perform the same electrophysiological protocol without the stimulation period. As expected, this group did not present neuronal activation. Thus, we can conclude that the *c-fos* activation observed in the controls with stimulation is due to the stimulation itself.

In conclusion, our data corroborates the preponderant role of insular cortex in the cardiovascular regulation. This work also shows that stress can affect cardiovascular regulation, promoting an adverse autonomic system imbalance toward sympathetic division. It seems that stress disrupts the pathway between insular cortex and infralimbic cortex, and this can lead to an absence of cardiac regulation by insula.

In future, it would be interesting to understand how and where stress disrupts insula and infralimbic cortex connection, if it affects only one or both structures or even other regions that might be involved in this pathway. The development of more detail study about how infralimbic region can influence cardiac modulation is other challenging possibility. Another line of research would the study of others insula's connections.

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