Habituation of the cardiovascular responses to restraint stress is inhibited by exposure to other stressor stimuli and exercise training

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Abbreviations

CVS, chronic variable stress; HPA axis, hypothalamus-pituitary-adrenal axis; HR, heart rate; MAP, mean arterial pressure; RRS, repeated restraint stress.

Key-words: adaptation; blood pressure; heart rate; corticosterone; chronic variable stress; social isolation.

Summary statement

The results reported in the present study provide evidence that diseases evoked by stress might be related to impairment in the habituation process upon repeated exposure to the same stressor.

ABSTRACT

This study evaluated the effect of exposure to either a chronic variable stress (CVS) protocol or social isolation, as well as to treadmill exercise training, in the habituation of the cardiovascular responses upon repeated exposure to restraint stress in rats. The habituation of the corticosterone response to repeated restraint stress was also evaluated. For this, animals were subjected to either acute or 10 daily sessions of 60 min of restraint stress. CVS and social isolation protocols lasted 10 consecutive days, whereas treadmill training was performed 1h/day, 5 days/week for 8 weeks. We observed that serum corticosterone increase was decreased during both the stress and the recovery period of the 10th session of restraint. Habituation of the cardiovascular responses was identified in terms of a faster return of heart rate to baseline values during the recovery period of the 10th session of restraint. The increase in blood pressure and the decrease in tail skin temperature were similar at the first and 10th session of restraint. Exposure to either CVS, social isolation or treadmill exercise training inhibited the habituation of the restraint-evoked tachycardia. Besides, CVS increased the blood pressure response at the 10th session of restraint, whereas social isolation enhanced both the tachycardia during the first session and the drop in skin temperature at the 10th session of restraint. Taken together, these findings provide new evidence that pathologies evoked by stress might be related to impairment in the habituation process to homotypic stressors.

INTRODUCTION

The physiological responses observed during aversive threats (e.g., hypothalamus-pituitary-adrenal (HPA) axis and cardiovascular/autonomic changes) are part of adaptive mechanisms in short-term, thus maintaining homeostasis and survival (Crestani, 2016; Dhabhar, 2019; Sterling, 2012). However, the frequent exposure to these changes during repeated exposure to stress situations might lead to dysfunctions and pathologies (Danese and McEwen, 2012; Dhabhar, 2019; Herman, 2013; McEwen, 1998). The term "habituation" refers to the progressive decrease of the stress responses during repeated exposure to the same aversive stimulus (i.e., homotypic stressor) (McCarty, 2016; Rankin et al., 2009; Thompson and Spencer, 1966). In this sense, the habituation process has been described as a prominent mechanism for adaptation to chronic stressful events, since it dampens the deleterious effects following repeated exposure to aversive stimuli (Grissom and Bhatnagar, 2009; Herman, 2013; McCarty, 2016). Additionally, the progressive decrease of the responses upon repeated exposure to non-life-threatening aversive stimuli conserves body`s energy and resources, thus improving the coping in future threats (Grissom and Bhatnagar, 2009; McCarty, 2016).

Exposure to chronic stressors might also increase the magnitude of the physiological responses during aversive threats, a phenomenon that has been denominated sensitization (Belda et al., 2015; McCarty, 2016). More typically, enhanced physiological responses, including cardiovascular changes (Grippo et al., 2002; Grippo et al., 2006), have been reported in chronically stressed animals when facing a novel stressor (i.e., different from that exposed previously) (Belda et al., 2015; Herman, 2013). In this sense, sensitization has been proposed to underlie several pathologies (Belda et al., 2015). Nevertheless, another possibility in which co-exposure to different stressors might underlie pathologies is impairing the habituation process to homotypic stressors. However, to the best of our knowledge, a possible influence of

co-exposure to other stressors in habituation of the physiological responses to homotypic stressors has never been investigated.

The chronic variable stress (CVS) is a valid and reliable rodent model of stress in which a combination of several mild stressors are applied randomly (Golbidi et al., 2015; Grippo and Johnson, 2009; Willner, 2005; Willner, 2017). Sensitized cardiovascular responses in chronically stressed animals when facing a novel stressor were obtained using the CVS as chronic stressor (Grippo et al., 2002; Grippo et al., 2006). Based on this, the CVS was chosen in the present study to investigate the influence of this stressor in habituation of the cardiovascular responses upon repeated exposure to restraint stress. However, some authors have pointed out the relevance of chronic social stressors in preclinical studies, mainly due to ethological and translational value of these stressors (Carnevali et al., 2017; Sgoifo et al., 2014). Therefore, the social isolation was also used in the present study as a chronic social stressor.

In opposition to chronic emotional stressors, exercise has a positive impact in pathologies etiology and development, so that it has been defined as a "good/protective stressor" (Dhabhar, 2019; Heijnen et al., 2016). Regarding the stress-evoked pathologies, beneficial influence of exercise has been proposed to be mediated by facilitation of adaptative responses (Dhabhar, 2019). Accordingly, studies have documented that physically active subjects and animals exhibit decreased reactivity and accelerated recovery of cardiovascular changes during exposure to acute stressors (Cleroux et al., 1985; Hsu et al., 2016; Masini et al., 2011; Morimoto et al., 2000; Rimmele et al., 2009; Sinyor et al., 1983). Besides, a preclinical study in rats identified that voluntary wheel running enhanced the habituation of heart rate (HR) response to repeated audiogenic stress (Masini et al., 2011). However, a possible influence of other protocols of physical training in habituation of cardiovascular responses to stress has never been evaluated. This is an important issue since, for instance, previous studies reported that treadmill exercise training increased neuroendocrine and brain noradrenaline

responses to acute stressors (Dishman et al., 2000; White-Welkley et al., 1995; White-Welkley et al., 1996). Furthermore, despite reports that the effect of physical training in neuroendocrine responses is stress type–specific (Campeau et al., 2010; Droste et al., 2003; Droste et al., 2006; Droste et al., 2007), the effect in cardiovascular responses to stressors other than audiogenic stress has never been evaluated. Therefore, the treadmill exercise training was chosen as a "good/protective stressor", hypothesizing that it evokes opposite effects in relation to CVS and social isolation in habituation of the cardiovascular responses to restraint stress.

Therefore, our purpose in the present study was to test the hypothesis that exposure to either CVS or social isolation impairs the habituation of the cardiovascular responses to restraint stress in rats, whereas treadmill exercise training evokes opposite effects. We also evaluated the serum corticosterone response upon repeated exposure to restraint stress to reproduce the well-stablished data regarding habituation of the HPA axis activation (Grissom and Bhatnagar, 2009; McCarty, 2016; Rabasa et al., 2015).

MATERIALS AND METHODS

Animals

One hundred seven 60 days old male Wistar rats (weighing approximately 250g) were used in the present study. Animals were obtained from the animal breeding facility of the São Paulo State University (UNESP) (Botucatu, SP, Brazil), and were housed in collective plastic cages (four animals per cage) in a temperature-controlled room (24ºC) in the Animal Facility of the Laboratory of Pharmacology (School of Pharmaceutical Sciences, UNESP). Animals were kept under a 12:12h light-dark cycle (lights on between 07:00–19:00h) and had free access to water and standard laboratory food (Presence, Neovia, São Paulo, Brazil), except during the experimental period. Experimental procedures were carried out following protocols approved by the Ethical Committee for the Use of Animals of the School of Pharmaceutical

Science-UNESP, which complies with Brazilian and international guidelines for animal use and welfare.

Experimental design

All restraint sessions and measurements (i.e., cardiovascular and corticosterone) were performed during the morning period in order to minimize possible circadian rhythm interferences. Figure 1 shows schematic representation of the protocols that evaluated the influence of either CVS, social isolation or treadmill exercise training in habituation of the cardiovascular responses to restraint stress.

Habituation of the corticosterone response upon repeated exposure to restraint stress

This protocol aimed to evaluate the habituation of the HPA axis activation upon repeated exposure to restraint stress. For this, we evaluated the restraint-evoked increase in serum corticosterone concentration in a different set of rats subjected to either (i) an acute session of 60 min of restraint stress (acute group) or (ii) 10 daily trials of 60 min of restraint stress (RRS). Corticosterone responses of the RRS group were evaluated at the 10^{th} session of restraint and compared with values obtained in the acute group. Blood samples (~200 μL) for determination of serum corticosterone concentration were collected from the femoral artery catheter immediately before (pre-stress value) and 15, 45, 60, 80, 100 and 120 min after the onset of restraint session.

Influence of chronic variable stress in habituation of the cardiovascular responses to restraint stress

This protocol aimed to evaluate the impact of the exposure to a CVS protocol in habituation of the cardiovascular responses to restraint stress. Since previous studies that evaluated the impact of CVS in cardiovascular and neuroendocrine reactivity to restraint

performed the chronic stress protocol before the restraint exposure (Flak et al., 2011; Grippo et al., 2002; Grippo et al., 2006; Heck et al., 2020), the RRS started 24h after the last session of the CVS protocol (Fig. 1). Therefore, for evaluation of the influence of CVS, we recorded the restraint-evoked blood pressure and HR increases and the drop in tail skin temperature in different set of rats subjected to either (i) an acute session of 60 min of restraint stress (acute control), (ii) 10 daily trials of 60 min of restraint stress (RRS control), (iii) 10 days of CVS performed before an acute session of 60 min of restraint stress (acute+CVS) and (iv) 10 days of CVS performed before the 10 daily trials of 60 min of restraint stress (CVS+RRS). Animals of the acute group were left undisturbed, except for cleaning the cages, in the animal facility, and were subjected to the acute session of restraint stress on the same day of the chronically stressed animals. Cardiovascular responses of RRS groups were recorded at the 10th session of restraint.

Influence of social isolation in habituation of the cardiovascular responses to restraint stress

This protocol aimed to evaluate the influence of the social isolation stress in habituation of the cardiovascular responses to restraint stress. The influence of social isolation in physiological and behavioral changes evoked by chronic stressors has been evaluated by single-housing the animals during the chronic stress protocol (Heck et al., 2020; Westenbroek et al., 2003a; Westenbroek et al., 2003b). Therefore, in the present study the social isolation was performed concurrently with the daily sessions of restraint (Fig. 1). Thus, for evaluation of the influence of social isolation, we recorded the restraint-evoked blood pressure and HR increases and the drop in tail skin temperature in different set of animals subjected to either (i) an acute session of 60 min of restraint stress (acute control), (ii) 10 daily trials of 60 min of restraint stress (RRS control), (iii) 10 days of social isolation performed before an acute session of 60 min of restraint (acute isolated) and (iv) 10 daily sessions of 60 min of restraint stress performed concurrently with social isolation (RRS isolated). Recording of restraint-evoked

cardiovascular responses in acute and RRS groups was performed as described in the previous protocol.

Influence of treadmill exercise training in the habituation of cardiovascular responses to restraint stress

This protocol aimed to evaluate the influence of treadmill exercise training in habituation of the cardiovascular responses to restraint stress. For this, we investigated the restraint-evoked blood pressure and HR increases and the drop in tail skin temperature in either (i) animals sedentary submitted to an acute session of 60 min of restraint stress (acute sedentary), (ii) rats sedentary subjected to 10 daily sessions of 60 min of restraint stress (RRS sedentary), (iii) animals subjected to 8 weeks of treadmill exercise training and submitted to an acute session of 60 min of restraint stress (acute trained) and (iv) rats subjected to 8 weeks of treadmill training and submitted to 10 daily trails of 60 min of restraint stress in the last 10 days of the treadmill exercise protocol (RRS trained). The sedentary rats were kept in the animal facility for the same period as the rats subjected to the exercise training. Recording of restraint-evoked cardiovascular responses in acute and RRS groups was performed as described in the previous protocol.

For the cardiovascular recording, animals in all experimental protocols were transferred to the recording room in their home cage and were allowed 60 min to adapt to recording room conditions, such as sound and illumination, before starting the data acquisition. The recording room was temperature controlled (24^{o}C) and was acoustically isolated from the other rooms. Blood pressure and HR recordings started at least 30 minutes before the stress session onset and were performed throughout the restraint stress period. Tail skin temperature was measured at 10, 5 and 0 min before the restraint (pre-stress values) and every 10 min during the stress session. Blood pressure, HR and tail skin temperature were also recorded in the home cage after the end of the restraint stress (recovery period). For determination of the pre-stress

levels, the mean of blood pressure, HR and skin temperature measurements throughout the 10 min before the restraint was calculated in all experimental groups.

Restraint stress

Rats were placed individually into a plastic cylindrical restraint tube (diameter 6.5 cm, length 15 cm), ventilated by holes (1 cm diameter) that comprised approximately 20% of the tube surface (Benini et al., 2019; Buynitsky and Mostofsky, 2009). Restraint stress lasted 60 min, and immediately after the end of the restraint session animals were returned to their home cages.

Chronic variable stress

The CVS protocol consisted of exposure to different stressors on a variable schedule for 10 consecutive days (Almeida et al., 2015; Duarte et al., 2015; Vieira et al., 2018). The stressors used in the CVS included: i) open field (10 min); ii) cold (4^{o}C) or room temperature isolation housing; iii) humid sawdust (overnight or all day); iv) food/water deprivation (overnight); v) swim stress (4 min); vi) lights on overnight; and vii) lights off during day (120-180 min); viii) labyrinth open cross (5 min). All stress sessions were performed in an adjacent room to the animal facility. Control rats were kept in the animal facility for the same period as the rats subjected to the CVS protocol.

Social isolation

The rats submitted to social isolation stress were housed individually in plastic cages with free access to water and food for 10 days, while control animals were continually housed four per cage throughout the experiment. Isolated and control rats were housed in the same room, so that isolated rats maintained visual, auditory, and olfactory contact with the other animals (Almeida et al., 2020; Cruz et al., 2016; Fone and Porkess, 2008).

Treadmill exercise training

Initially, rats were familiarized with exercise on the rodent treadmill (AVS Projetos, São Carlos, SP, Brazil) for one week. During this period, all animals ran daily on the treadmill at a speed of 0.3 km/h and 0% grade for 10 min. No electrical stimulation was used to induce them to run (Camargo et al., 2013; Engi et al., 2016). Then, animals were subjected to a progressive maximal exercise test, which consisted of treadmill running with 0.3 km/h of increment each 3 min until exhaustion (Engi et al., 2016). After the first maximal exercise test, animals were randomly allocated in sedentary and trained (both groups possessed the same physical capacity before training onset). Trained groups underwent a low-intensity training (50–60% of maximal exercise capacity, 0% grade) on the treadmill 1 h/day, 5 days/week for 8 weeks (Engi et al., 2016). The sedentary groups were subjected once per week to a short period of mild running (10 min, 0.5 km/h, 0% grade) to keep them familiarized with treadmill environment and experimental procedures. The progressive maximal running test was repeated at weeks 2, 4 and 6 to adjustment of training intensity and evaluation of training efficacy. The maximal running test was also performed at the end of the protocol (week 8) to confirm the training efficacy and evaluate the effect of RRS exposure.

Surgical preparation

Twenty-four hours before the cardiovascular recording, animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and a polyethylene cannula (a 4 cm segment of PE-10 bound to a 13 cm segment of PE-50) (Clay Adams, Parsippany, New Jersey, USA) was implanted into the abdominal aorta via the femoral artery for cardiovascular recording. The catheter was exteriorized on the animal's dorsum. After surgery, rats were treated with the non-steroidal anti-inflammatory drug flunixin meglumine for post-surgical analgesia (0.5 mg/mL/kg, s.c.) and with a poly-antibiotic formulation containing streptomycins and

penicillins to prevent infection (560 mg/mL/kg, i.m.). The animals were kept in individual cages after the surgery.

Arterial pressure and heart rate recording

The cannula implanted into the femoral artery was connected to a pressure transducer (DPT100, Utah Medical Products Inc., Midvale, Utah, USA). Pulsatile arterial pressure was recorded using an amplifier (Quad Bridge Amp, ML224, ADInstruments, New South Wales, Australia) and an acquisition board (PowerLab 4/30, ML866/P, ADInstruments, New South Wales, Australia) connected to a personal computer. Mean arterial pressure (MAP) and HR values were obtained from the pulsatile arterial pressure recordings.

Tail cutaneous temperature measurement

Increase in vasomotor sympathetic activity during stressful events drops cutaneous blood flow (Blessing, 2003), which in turn decreases the skin temperature (Benini et al., 2019; Vianna and Carrive, 2005). Therefore, restraint-evoked decrease in tail cutaneous temperature was evaluated as an indirect measurement of vasomotor sympathetic response in cutaneous beds (Benini et al., 2019; Vianna and Carrive, 2005). For this, tail cutaneous temperature was recorded using a Multi-Purpose Thermal Imager (IRI4010, InfraRed Integrated Systems Ltd, Northampton, UK). For analyzing the images, the temperature was measured on five points along the animal's tail, and a mean of the values was calculated for each recording (Benini et al., 2019; Oliveira et al., 2015).

Serum corticosterone measurement

Samples were collected in plastic tubes and were left undisturbed for 60 min to clot. Then, samples were centrifugated at 2,000 x g for 15 min, and serum was stored at -80^{o}C until quantification. Serum corticosterone concentration was measured using a commercial corticosterone enzyme-linked immunosorbent assay (ELISA), according to manufacturer

instructions (corticosterone ELISA kit, item # 511320, Cayman Chemical, Ann Arbor, MI, USA).

Statistical analysis

Data are presented as mean±standard error of the mean (SEM). The pre-stress values of MAP, HR, and tail skin temperature were compared using two-way ANOVA, with restraint (acute vs repeated) and stress (control vs CVS/isolation/exercise) as independent factors, followed by Bonferroni's *post hoc* test. Time-course curves of corticosterone response were compared using the two-way ANOVA, with restraint (acute vs repeated) as the main factor and time as repeated measurement. The time-course curves of cardiovascular changes were analyzed using three-way ANOVA, with restraint (acute vs repeated) and stress (control vs CVS/isolation/exercise) as main factors and time as repeated measurement, followed by Bonferroni's *post hoc* test. The mean of all points across either the restraint or the recovery periods in the time-course curves were also calculated (i.e., mean response throughout "stress" and "recovery" period), and these values were compared using three-way ANOVA, with restraint and stress as main factors and period (restraint vs recovery) as a repeated measurement; followed by Bonferroni's *post hoc* test. The significance was set at $P<0.05$.

RESULTS

Habituation of the serum corticosterone response upon repeated exposure to restraint stress

Analysis of the pre-stress values of serum corticosterone did not indicate differences in animals subjected to acute and repeated restraint stress (RRS) (15±0.5 vs 16±0.4 ng/ml, $t=0.99$, $P=0.335$). However, analysis of the time-course curves of serum corticosterone concentration indicated that values of the RRS group were lower in relation to those obtained in the acute group ($P=0.005$) (Fig. 2A). Analysis of the mean serum corticosterone responses

throughout the restraint and recovery period indicated that the RRS group presented decreased values during both restraint (P<0.0001) and recovery (P=0.049) periods (Fig. 2B).

Influence of chronic variable stress in habituation of the cardiovascular responses to restraint stress

Analysis of the pre-stress values of mean arterial pressure (MAP) and HR did not indicate effect of either RRS (MAP: P=0.420; HR: P=0.688) or CVS (MAP: P>0.656; HR: P>0.062), when compared with the acute control group (Table 1). Pre-stress values of tail skin temperature of both acute+CVS (P=0.048) and RRS+CVS (P=0.002) groups were higher in relation to respective control groups (Table 1).

Analysis of the time-course curves of the MAP response indicated effect of restraint (P<0.001), and CVS increased the response in both acute+CVS (P<0.05) and RRS+CVS (P<0.05) groups, when compared with the acute control group (Fig. 3A). Analysis of the time-course curves of HR and tail skin temperature responses did not indicate effect of either RRS (HR: P=0.389, temperature: P=0.348) or CVS (HR: P=0.930, temperature: P=0.241) (Fig. 3B and 3C).

Analysis of the mean MAP responses throughout the restraint and recovery period indicated that CVS increased MAP response during restraint in the RRS+CVS group (P=0.045), when compared with the acute control group (Fig. 3D). Analysis of the mean HR responses throughout the restraint and recovery period indicated that values of the RRS control group during recovery period was lower in relation to those of the acute control group (P=0.007) (Fig. 3E). Analysis of the tail skin temperature response did not indicate effect of either RRS (P=0.348) or CVS (P=0.241) (Fig. 3F).

Influence of social isolation in habituation of the cardiovascular responses to restraint stress

Analysis of the pre-stress values of MAP and HR did not indicate effect of either RRS (MAP: P=0.064; HR: P=0.099) or isolation (MAP: P=0.167; HR: P=0.246) in relation to values of the acute control group (Table 1). Pre-stress levels of tail skin temperature in both acute isolated (P=0.002) and RRS isolated (P=0.003) groups were higher in relation to respective control groups (Table 1).

Analysis of the time-course curves of MAP did not indicate effect of either RRS (P=0.932) or isolation (P=0.089) (Fig. 4A). However, evaluation of the time-course curves of HR indicated enhanced response in the acute isolated group (P<0.01) and decreased values in the RRS control group (P<0.01), when compared with the acute control group, in specific moments of the stress and recovery periods, respectively (Fig. 4B). Analysis of the time-course curves of tail skin temperature indicated increased tail skin temperature response in RRS isolated group during specific moments of the restraint and recovery periods (P<0.01), when compared with the acute control group (Fig. 4C).

Analysis of the mean MAP throughout the restraint and recovery period did not indicate effect of either RRS (P=0.967) or isolation (P=0.078) (Fig. 4D). Analysis of the mean HR response throughout the restraint and recovery period indicated that values during restraint in the acute isolated group was enhanced in relation to acute control (P<0.01) and RRS control (P<0.01) groups (Fig. 4E). Besides, HR values during recovery period were lower in the RRS control group in relation to acute control (P=0.024) and RRS isolated (P=0.015) groups (Fig. 4E). Analysis of the tail skin temperature indicated that the response during restraint was enhanced in the RRS isolated group in relation to the acute control group (P=0.028) (Fig. 4F).

Influence of treadmill exercise training in habituation of the cardiovascular responses to restraint stress

Comparison of the values obtained in sedentary and trained before the onset of RRS protocol (i.e., at weeks 0, 2, 4 and 6) indicated that training increased the running speed on the treadmill at weeks 2 ($P<0.01$), 4 ($P<0.0001$) and 6 ($P<0.0001$) (Fig. 5A). Analysis of the maximal running speed after the RRS (i.e., at the week 8) indicated that training increased running speed on the treadmill in both control ($P<0.0001$) and RRS ($P<0.0001$) groups (Fig. 5B).

Analysis of the pre-stress values of MAP and HR did not indicate effect of either RRS (MAP: $P=0.781$; HR: $P=0.198$, temperature: $P=0.080$) or exercise (MAP: $P=0.131$; HR: $P=0.307$, temperature: $P=0.106$) in relation to values of the acute sedentary group (Table 1). Analysis of the time-course curves of MAP, HR and tail skin temperature did not indicate effect of either exercise (MAP: $P=0.052$, HR: $P=0.363$, temperature: $P=0.053$) or RRS (MAP: $P=0.312$, HR:$P=0.194$, temperature: $P=0.083$) (Fig. 6A, 6B and 6C). Analysis of the mean HR response throughout the restraint and recovery period indicated that values during the recovery period of the RRS sedentary group were lower when compared with the acute sedentary group ($P=0.048$) (Fig. 6E). Analysis of the mean MAP (Fig. 6D) and skin temperature (Fig. 6F) responses throughout the restraint and recovery period did not indicate effect of either exercise (MAP: $P=0.055$, temperature: $P=0.062$) or RRS (MAP: $P=0.302$, temperature: $P=0.083$).

DISCUSSION

The results reported here provide the first evidence that exposure to other chronic aversive stimuli, as well as treadmill exercise training, impairs the development of habituation of the physiological responses to homotypic stressors. Indeed, we identified that habituation of the cardiovascular responses identified at the 10th session of 60 min of restraint was mainly

characterized as a faster return of HR to baseline values during the post-stress period. The habituation of HR response was completely inhibited in animals exposure to either CVS, social isolation stress or treadmill training. Besides, CVS increased the MAP response in animals subjected to RRS, whereas social isolation enhanced both the tachycardia during an acute session of restraint and the drop in tail skin temperature in rats submitted to RRS. Evaluation of the corticosterone response indicated that the increase at the 10th session of 60 min of restraint was smaller during both the restraint and the recovery periods in relation to the response obtained in acutely stressed animals.

The vast majority of information regarding the habituation process upon repeated exposure to the same stressor has been obtained from studies that evaluated the sympathetic-adrenal medullary response and HPA axis activation (Grissom and Bhatnagar, 2009; McCarty, 2016; Rabasa et al., 2015). Regarding the latter, habituation was documented in rodents to various stressors, including restraint (Grissom and Bhatnagar, 2009). Such as observed in the present study (Fig. 2), the habituation of the corticosterone response to restraint stress is perceived as a reduced increase during the restraint session, which in turn evokes decreased values during the recovery period (Grissom and Bhatnagar, 2009). This pattern of habituation is such different from those identified for the cardiovascular responses. Indeed, such as reported in a recent report (Benini et al., 2019), we identified here that habituation of the cardiovascular responses to restraint was mainly observed in terms of a faster return of HR to baseline values during the recovery period of the restraint stress. However, the HR values during the 10th session were similar to that observed during the acute restraint session. Besides, the MAP increase and the drop in tail skin temperature did not demonstrate any sign of habituation. Taken together, the corticosterone and cardiovascular responses to stress indicate that habituation is system-specific rather than a generalized body response. Accordingly, some authors have proposed that physiological systems other than the HPA axis, including the

cardiovascular system, are less sensitive to the habituation process (Rabasa et al., 2015). An explanation for the lesser habituation of cardiovascular response might be the dramatic impact that an insufficient response can evoke in homeostasis of organs and systems.

The landmark paper of Thompson and Spencer (1966), later revised by Rankin et al (2009), defined a list of characteristics to the habituation process. Although some of these criteria have not yet been experimentally tested (McCarty, 2016; Rabasa et al., 2015), these definitions have provided a theoretical framework for evaluation and discussion of the habituation process to aversive stimuli (Grissom and Bhatnagar, 2009; McCarty, 2016). One of the criteria (characteristic # 8) proposes that "*presentation of another (usually strong) stimulus results in recovery of the habituated response (dishabituation)*" (Rankin et al., 2009; Thompson and Spencer, 1966). Although this criterion has not been directly addressed, even in terms of neuroendocrine responses (McCarty, 2016), this dishabituation, as defined by Thompson and Spencer, proposed that a determined habituated response to a specific stimulus should be presented normal during exposure to a novel stressor. In this sense, the hypothesis tested in the present study is different from this criterion, since we investigated the impact of exposure to other chronic stressors in the acquisition of the habituation rather than in the "dishabituation" of a habituated response. Thus, to the best of our knowledge, the present study provides the first evidence that exposure to other aversive stimuli might impact the habituation process.

The CVS is a non-habituating stressor that has been demonstrated to evoke changes in HPA axis, anxiety- and depressive-like behaviors and cardiovascular function (Almeida et al., 2015; Costa-Ferreira et al., 2016; Duarte et al., 2015; Grippo and Johnson, 2009; Vieira et al., 2018; Willner, 2005; Willner, 2017). Besides, this chronic stressor has been reported to increase physiological responses to novel stressors (i.e., different from those presented during the protocol) (Crestani, 2016; Herman, 2013). In this sense, previous studies identified

increased blood pressure and HR responses to air-jet stress in rats previously exposure to a CVS protocol (Grippo et al., 2002; Grippo et al., 2006). Our results of increased restraint-evoked MAP response in acute+CVS group (Fig. 3) are in line with these previous findings. Additionally, the increased MAP response observed in the RRS+CVS group (Fig. 3) indicates that CVS impact is still observed after repeated exposure to the novel stressor. More importantly, present results provide new evidence that CVS impairs the habituation process of the cardiovascular responses. Our data constitute the first evidence that CVS affects the development of habituation to homotypic stressors.

Interaction with members of the same species is a prominent social factor. Accordingly, clinical and preclinical studies have provided evidence that disruption of social bonds and perceived isolation (loneliness) might increase cardiovascular morbidity and mortality (Cruz et al., 2016; Rozanski et al., 1999; Steptoe and Kivimäki, 2012). In this sense, similarly to CVS, social isolation enhanced restraint-evoked cardiovascular responses (Fig. 4), as evidenced by increase in tachycardia and drop in tail skin temperature in the acute isolated and RRS isolated groups, respectively. This finding is line with previous evidence of increased cardiovascular changes in single-housed rats during environmental challenges (Azar et al., 2011; Sharp et al., 2002). Nevertheless, a previous study identified that 16 daily sessions of 1h of social isolation followed by partner change enhanced restraint-evoked corticosterone response in female but not male rats (McCormick et al., 2005). Also, corticosterone increase caused by restraint was enhanced in adult female rats subjected to social isolation during adolescence, while the response was decreased in adult male animals (Weintraub et al., 2010). Despite differences in protocols of social isolation, as well as some evidence that social isolation during adolescence increased HPA axis response to other stressors (e.g., swim stress and startle) (Mathews et al., 2008; Weiss et al., 2004), the results described above together with

data reported here indicate that restraint-evoked HPA axis and cardiovascular response are differently affected by this social stressor in adult male rats.

We also identified that social isolation impaired the habituation of the HR response upon repeated exposure to restraint stress (Fig. 4B and 4E). Our findings contrast with previous evidence that post-weaning social isolation did not affect the habituation of the acoustic startle reactivity in different rat strains (Varty and Geyer, 1998). A possible influence of social isolation in habituation of the physiological responses during exposure to homotypic stressors has never been evaluated previously. Therefore, despite the difference in the social isolation protocol and stressor evaluated, the evidence mentioned above together with data reported here indicate that social isolation differently affects the habituation of behavioral and cardiovascular responses to stress. Additionally, CVS (Fig. 3) and social isolation (Fig. 4) similarly inhibited the decrease in HR response identified in the RRS control groups in relation to the acute control groups, thus providing evidence that the impact of co-exposure to other aversive stimuli in the habituation of cardiovascular responses is independent of the paradigm of stress.

Although exercise activates stress responses, such as HPA axis and cardiovascular changes, a distinction with emotional stress has been proposed in terms of “good stress” (e.g., exercise) and “bad stress” (e.g., emotional stress) based on physiological changes and impact in pathologies etiology and development (Dhabhar, 2019; Heijnen et al., 2016). To the best of our knowledge, the present study is the first to investigate the effect of treadmill training in cardiovascular responses to stress in rodents. However, the absence of effect of treadmill training in restraint-evoked cardiovascular changes (Fig. 6) contrasts with previous evidence that voluntary wheel running decreased cardiovascular responses to an acute session of open field, cage switch and restraint stress (Masini et al., 2011; Morimoto et al., 2000). The inhibition of the habituation of restraint-evoked tachycardia in treadmill-trained rats (Fig. 6) also contrasts with evidence that voluntary wheel running enhanced the habituation of the

cardiovascular responses observed upon repeated exposure to audiogenic stress (Masini et al., 2011). The discrepancies with previous studies might be due to differences in the type of physical training and/or aversive stimulus. Regarding the stressor type, previous evidence have indicated that impact of exercise is stress type-specific (Campeau et al., 2010; Droste et al., 2003; Droste et al., 2006; Droste et al., 2007). In fact, studies evaluating the neuroendocrine responses identified that spontaneous wheel running reduced the HPA axis responses to lower-intensity stressors (Campeau et al., 2010; Droste et al., 2003; Droste et al., 2006; Droste et al., 2007), whereas this response was enhanced in more intense stressors, such as restraint (Droste et al., 2003; Droste et al., 2006). Taken together with evidence reported here, these results suggest that cardiovascular (no effect) and HPA axis (enhanced) responses to restraint are differently affected by exercise. Regarding the type of physical training, although treadmill exercise might be potentially more stressful than voluntary wheel running, this possible stressful component seems not to explain the discrepancies. As stated above, previous studies identified increased corticosterone response to restraint stress in animals subjected to voluntary wheel running (Droste et al., 2003; Droste et al., 2006). Furthermore, previous studies reported habituation of rats to the treadmill training procedures (White-Welkley et al., 1995). Therefore, further studies directly comparing different training protocols are necessary for evaluation of the influence of the type of physical training in cardiovascular responses to stress.

Sensitization of physiological responses to stress have been mainly reported in terms of enhanced responses in animals chronically stressed when facing a novel stressor (Rabasa et al., 2015), so that enhanced cardiovascular responses to restraint stress reported here in animals subjected to either CVS (Fig. 3) or social isolation (Fig. 4) are in line with previous evidence. Sensitization has been reported to underlie several stress-related disorders, including cardiovascular pathologies (Rabasa et al., 2015; Ursin, 2014). In fact, studies have documented that exaggerated stress-evoked blood pressure increase is related to enhanced cardiovascular

disease risk (Jennings et al., 2004; Steptoe et al., 1996). Therefore, the present findings obtained in animals subjected to CVS and social isolation provides further evidence that enhanced cardiovascular reactivity to novel stressors might be a prominent mechanism related to cardiovascular dysfunctions related to chronic stress. Additionally, the different impact of treadmill training (absence of effect) versus CVS and social isolation (sensitization) supports the idea of the exercise as a "good/protective stressor" (Dhabhar, 2019; Heijnen et al., 2016).

Regarding the habituation process, as stated in the introduction, the habituation process has been described as a prominent mechanism for adaptation to chronic stressful events (Grissom and Bhatnagar, 2009; Herman, 2013; McCarty, 2016). In this sense, the results reported in the present study provide new evidence that stress-related pathologies might be consequence of an impairment of the habituation process to homotypic stressors. Nevertheless, contrary to our hypothesis, treadmill exercise training also inhibited the habituation of the cardiovascular responses to restraint. The meaning of this finding is not clear, since exercise has been described as a protective factor for stress-related diseases (Dhabhar, 2019), so that the similar impact of exercise and the chronic stressors in habituation of the cardiovascular responses to restraint stress contrasts with this idea. Therefore, further studies are necessary to better explore the relevance of the exercise data reported here in terms of etiology of the stress-evoked pathologies.

In summary, our data indicate that exposure to other chronic stressors, such as CVS and social isolation, as well as to treadmill exercise training, inhibits habituation of the cardiovascular responses upon repeated exposure to restraint stress. These findings provide new evidence that in addition to sensitization of the responses to novel stressors, pathologies evoked by stress might also be related to impairment in the habituation process to homotypic stressors.

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COMPETING INTERESTS

No competing interests declared.

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Figures

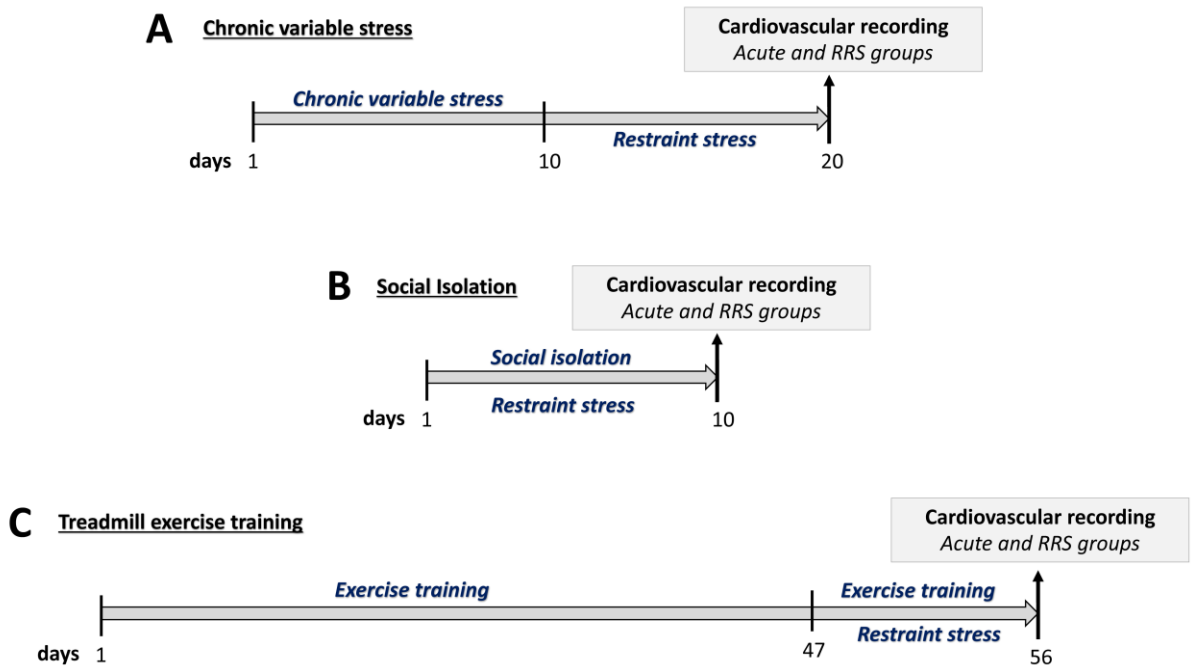

Figure 1 – Schematic representations of the protocols for evaluation of the influence of chronic variable stress (CVS), social isolation and treadmill exercise training on habituation of the cardiovascular responses to restraint stress. (A) For evaluation of the influence of CVS animals were subjected to 10 days of CVS before the 10 daily trials of 60 min of restraint stress. Restraint stress sessions started 24h after the last CVS session. **(B)** For evaluation of the influence of social isolation, animals of the "isolated groups" were single-housed concurrently with the 10 daily sessions of 60 min of restraint stress. Control animals were continually housed four per cage throughout the experiment **(C)** For evaluation of the influence of treadmill exercise training rats were subjected to 8 weeks of treadmill training and submitted to 10 daily trails of 60 min of restraint stress in the last 10 days of the treadmill exercise protocol. In all experimental protocols acute animals were kept in the animal facility for the same period as the rats subjected to the daily sessions of restraint (i.e., RRS groups), so that restraint-evoked cardiovascular responses in acute and RRS groups were performed on the same day.

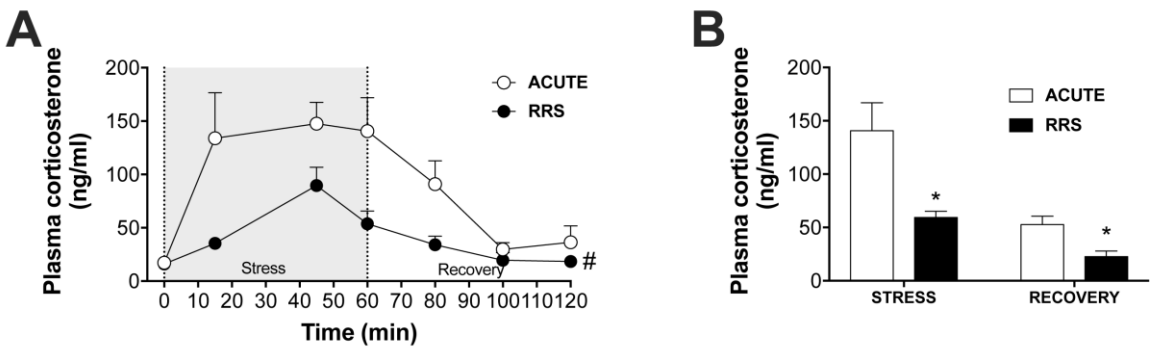

Figure 2 - Habituation of the corticosterone response to restraint stress. (A) Time-course curves of changes in serum corticosterone concentration at first (acute, white circles, n=9) and 10th session (RRS, black circles, n=9) of 60 min of restraint stress. Shaded area indicates the period of restraint. Circles represent the mean and the bars the SEM. # $P<0.05$ over the whole period compared to acute group, two-way ANOVA followed by Bonferroni *post-hoc* test. **(B)** Mean serum corticosterone values throughout the restraint and recovery period at first (acute, white bars, n=9) and 10th session (RRS, black bars, n=9) of 60 min of restraint stress. The bars represent the mean±SEM. * $P<0.05$ versus the acute control group within the same period, two-way ANOVA followed by Bonferroni *post-hoc* test.

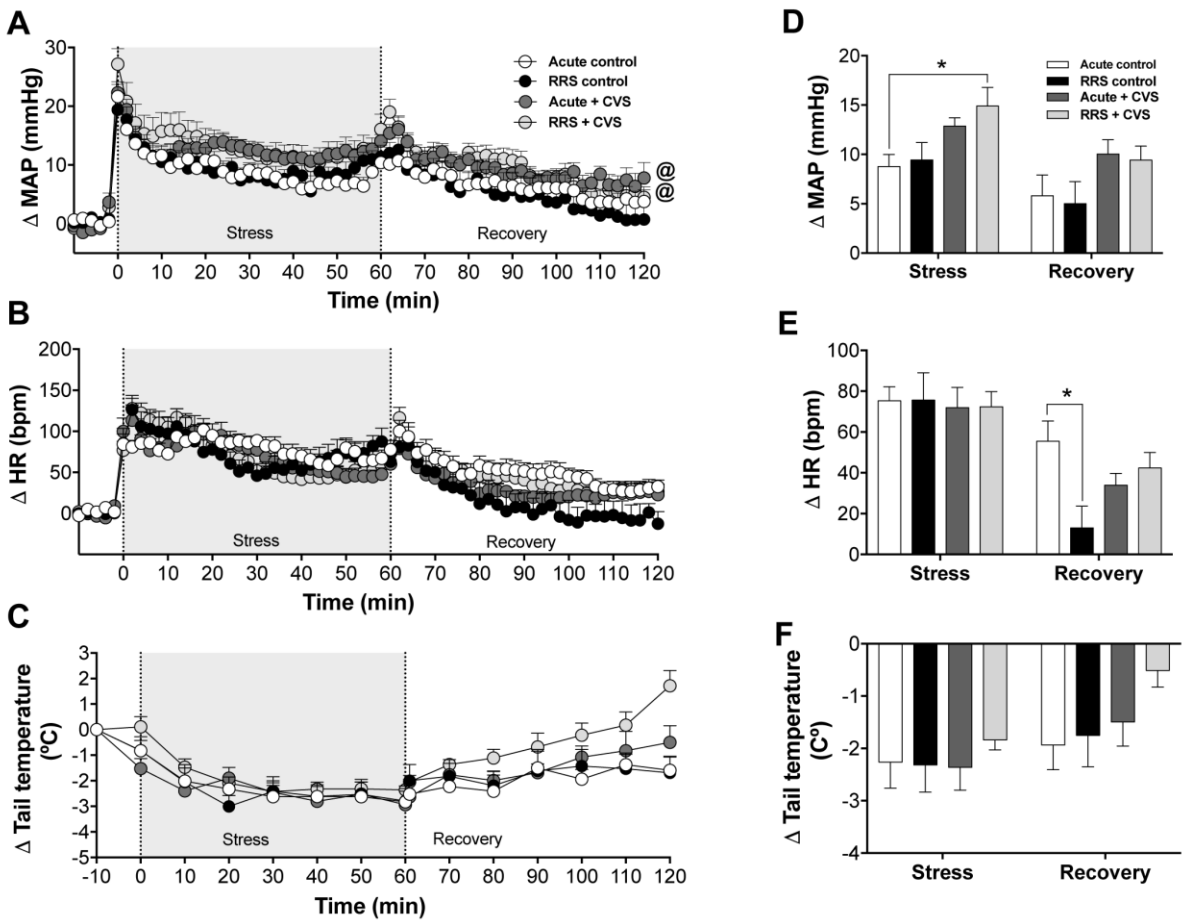

Figure 3 - Influence of chronic variable stress (CVS) in habituation of the cardiovascular responses to restraint stress. (A, B, C) Time-course curves of changes on mean arterial pressure (ΔMAP), heart rate (ΔHR) and tail skin temperature (Δ tail temperature) in animals control at first (acute control, white circles, n=10) and 10th session (RRS control, black squares, n=9) of restraint, as well as in rats submitted to CVS at first (acute+CVS, dark grey squares, n=10) and 10th session (RRS+CVS, light grey squares, n=10) of restraint. Shaded area indicates the period of restraint. Circles represent the mean and the bars the SEM. # P<0.05 over the whole recording period compared to acute control group, three-way ANOVA followed by Bonferroni *post-hoc* test. **(D, E, F)** Mean ΔMAP, ΔHR and Δ tail temperature throughout the restraint and recovery period in groups acute control (white bars, n=10), RRS control (black bars, n=9), acute+CVS (dark grey bars, n=10) and RRS+CVS (light grey bars, n=10). The bars

represent the mean±SEM. * P<0.05 versus the acute control group within the same period, three-way ANOVA followed by Bonferroni *post-hoc* test. Acute session and all trials of restraint in RRS protocol lasted 60 min.

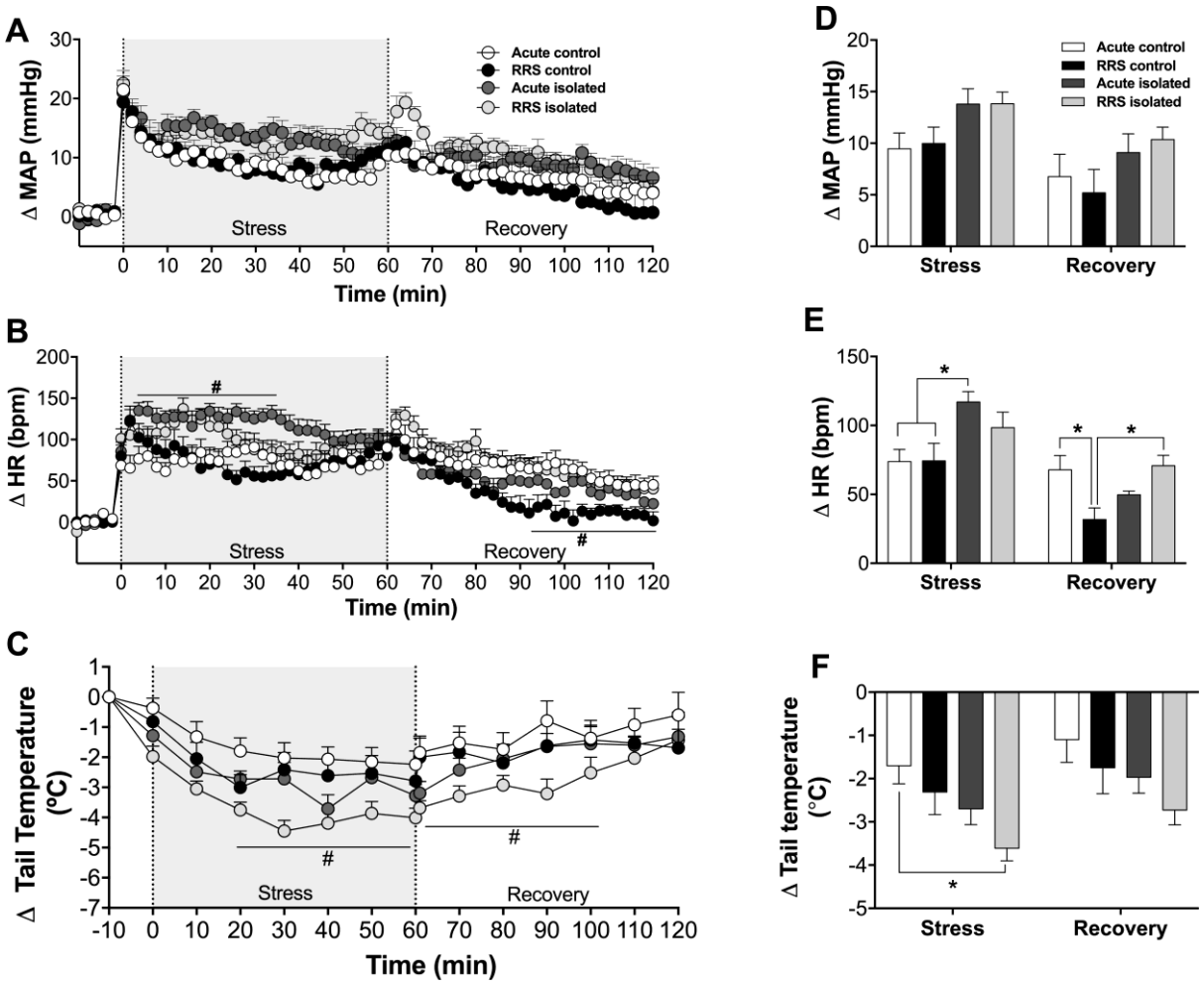

Figure 4 - Influence of social isolation in habituation of the cardiovascular responses to restraint stress. (A, B, C) Time-course curves of change on mean arterial pressure (ΔMAP), heart rate (ΔHR) and tail skin temperature (Δ tail temperature) in animals control at first (acute control, white circles, n=9) ant 10th session (RRS control, black squares, n=9) of restraint, as well as in animals submitted to social isolation at first (acute isolated, dark grey squares, n=9) and 10th session (RRS isolated, light grey squares, n=9) of restraint stress. Shaded area indicates the period of restraint. Circles represent the mean and the bars the SEM. # P<0.05 versus the acute control group, three-way ANOVA followed by Bonferroni *post-hoc* test. **(D, E, F)** Mean ΔMAP, ΔHR and Δ tail temperature throughout the stress and recovery period in groups acute control (white circles, n=9), RRS control (black squares, n=9), acute isolated (dark grey

squares, n=9) and RRS isolated (light grey squares, n=9). The bars represent the mean±SEM. * P<0.05 versus the acute control group within the same period, three-way ANOVA followed by Bonferroni *post-hoc* test. Acute session and all trials of restraint in RRS protocol lasted 60 min.

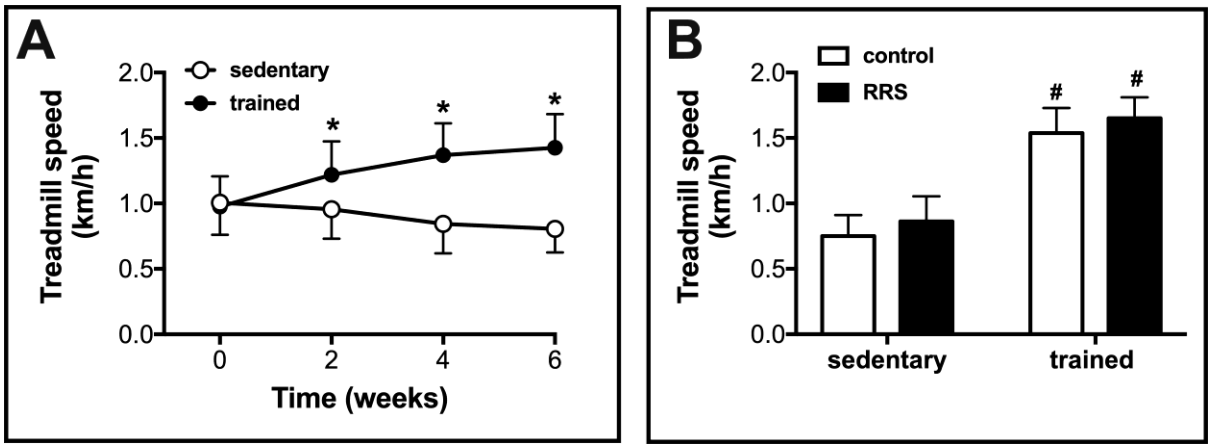

Figure 5 - Maximal running speed (km/h) in maximal exercise tests. (A) Treadmill performance in sedentary and trained rats at weeks 0, 2, 4 and 6 of the treadmill exercise training protocol (i.e., before the onset of the RRS protocol). Circles represent the mean and the bars the SEM. * $P<0.05$ versus the sedentary group, two-way ANOVA followed by Bonferroni *post-hoc* test. **(B)** Treadmill performance after completion of RRS in animals sedentary and trained. The bars represent the mean±SEM. # $P<0.05$ versus the respective sedentary group, two-way ANOVA followed by Bonferroni *post-hoc* test.

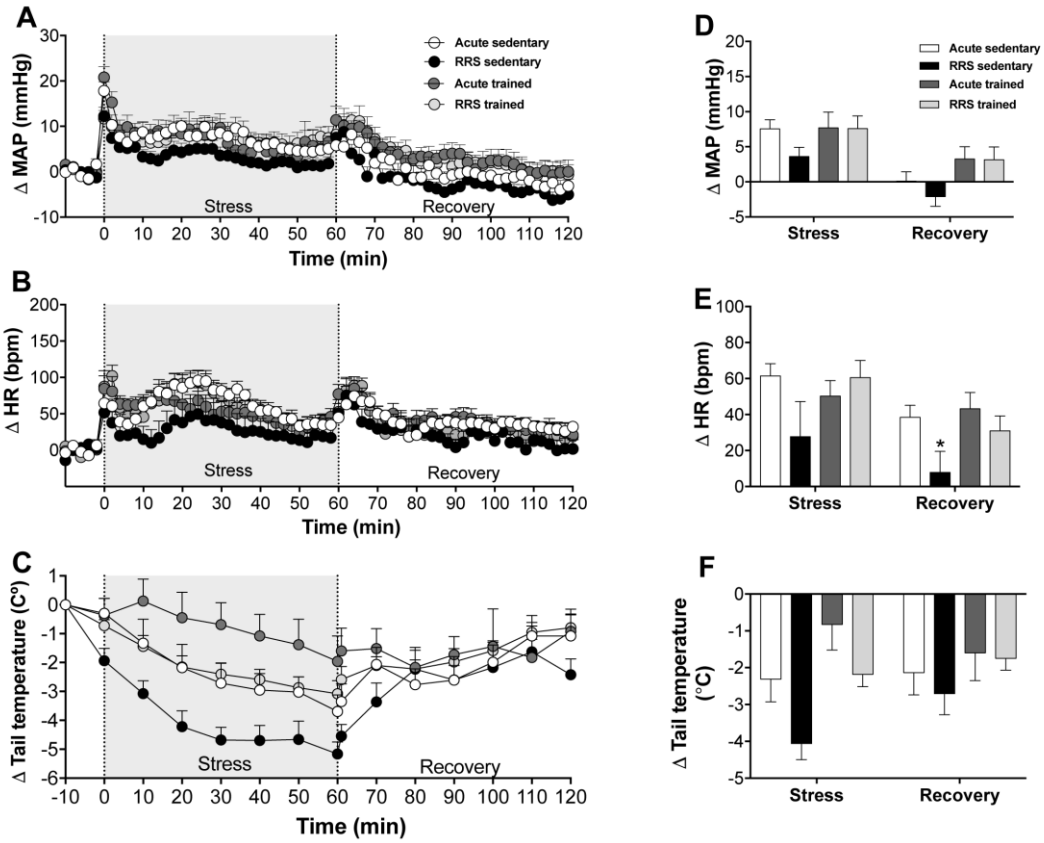

Figure 6 - Influence of treadmill exercise training in habituation of the cardiovascular responses to restraint stress. (A, B, C) Time-course curves of change on mean arterial pressure (ΔMAP), heart rate (ΔHR) and tail skin temperature (Δ tail temperature) in animals sedentary at first (acute sedentary, white circles, n=8) and 10th session (RRS sedentary, black squares, n=8) of restraint, as well as in treadmill trained rats at first (acute trained, dark grey squares, n=8) and 10th session (RRS trained, light grey squares, n=8) of restraint stress. Shaded area indicates the period of restraint. Circles represent the mean and the bars the SEM. Three-way ANOVA. **(D, E, F)** Mean ΔMAP, ΔHR and Δ tail temperature throughout the restraint and recovery period in groups acute sedentary (white circles, n=8), RRS sedentary (black squares, n=8), acute trained (dark grey squares, n=8) and RRS trained (light grey squares, n=8). The bars represent the mean±SEM. * P<0.05 versus the acute sedentary group within the same period, three-way ANOVA. Acute session and all trials of restraint in RRS protocol lasted 60 min.

Table 1 – Pre-stress values of mean arterial pressure (MAP), heart rate (HR) and tail skin temperature (T).

	MAP (mmHg)	*HR (bpm)*	*T (ºC)*	*N*
Chronic variable stress				
Acute control	105±1.1	357±10	28.8±0.5	10
RRS control	109±1.3	362±9	28.0±0.5	9
Acute + CVS	107±1.6	342±8	30.7±0.6 #	10
RRS + CVS	105±2.1	343±8	30.8±0.2 #	10
Exercise				
Acute sedentary	107±1.4	344±5	31.7±0.6	8
RRS sedentary	111±2.4	361±6	33.0±0.2	8
Acute trained	114±2.8	344±8	31.8±0.6	8
RRS trained	111±1.8	346±8	30.7±0.5	8
Social Isolation				
Acute control	104±1.3	353±7	28.0±0.6	9
RRS control	108±1.3	363±10	28.5±0.5	9
Acute isolated	103±1	343±5	30.0±0.4 #	9
RRS isolated	105±2	356±4	30.7±0.4 #	9

Values are the mean of all measurements performed throughout the 10 min before the restraint onset. # $P<0.05$ vs respective control group. Two-way ANOVA followed by Bonferroni *post-hoc* test. CVS – chronic variable stress, RRS – repeated restraint stress.