General Physiology and Biophysics
Revised manuscript #1

Title: Ivabradine reduces baseline and stress-induced increase of heart rate and blood pressure and modulates neuroendocrine stress response in rats depending on stressor intensity
Running title: Ivabradine modulates stress response
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Abstract
Ivabradine, a selective inhibitor of the sinoatrial pacemaker, is used in clinical practice to reduce heart rate. However, its potential effect on the neuroendocrine stress response has not been investigated. Therefore, we determined the effect of administering ivabradine to rats on cardiovascular parameters and plasma levels of epinephrine, norepinephrine, and corticosterone. Ivabradine was administered intraperitoneally 30 min before exposing animals to either handling, restraint, or immobilization stress. Heart rate and blood pressure were monitored telemetrically. Blood samples were collected before, during, and after stressor exposure to determine the extent of the neuroendocrine stress response as reflected by plasma epinephrine, norepinephrine, and corticosterone levels. In animals pretreated with ivabradine, significantly lower values of heart rate and blood pressure were found during both the baseline period and during exposure to stressors, as well as during the rest period following stressor exposure. Ivabradine also significantly reduced handling-induced epinephrine and norepinephrine release into the bloodstream. However, ivabradine significantly potentiated restraint- and immobilization-induced increases of plasma epinephrine levels, whereas stress-induced changes in plasma norepinephrine and corticosterone levels were ambiguous. Our data shows that ivabradine significantly reduces blood pressure in rats during both baseline and stressful conditions, and also affects the neuroendocrine stress response. These findings show that viscerosensory signaling from the cardiovascular system may significantly modulate the neuroendocrine stress response.

Keywords: blood pressure; corticosterone; epinephrine; heart rate; ivabradine; norepinephrine

Changelog
Author's Response to Reviewers

Reviewer #1:

We would like to thank Reviewer #1 for his/her positive comments and suggestions. We followed the recommendations and hope that you find the revised version of the manuscript satisfactory.

1a. Does the ivabradine cross the blood-brain barrier? Please, justify it with scientific reports, since it is a very important issue related to the question raised.
We added into the Introduction of revised version of the manuscript the information that ivabradine does not cross blood-brain barrier (Young et al. 2014). As mentioned in the Discussion, this is an important fact that excludes the direct effect of ivabradine on brain structures involved in regulation of neuroendocrine stress response.

1b. Which is the action mechanism of ivabradine? Specifically, does it act via any mechanism that might affect the hormonal responses evaluated?
Ivabradine represents a highly selective inhibitor of sinoatrial funny current that plays a crucial role in depolarization of cardiomyocytes. There are no data related to ivabradine direct effect on cells of endocrine glands. Moreover, there are no data that ivabradine affects other organs or tissues (exception represents retina).

1c. Cardiovascular system is still responsive (see comments below). Is not possible that these changes might activate viscerosensory nerves?
We are fully agree. We suggest that the transmission of viscerosensory signals from the heart is involved in altered neuroendocrine responses to ivabradine administration. We suggest that ivabradine-related reduction of heart rate attenuate transmission of signals from heart's mechanoreceptors, as mentioned in the Discussion.

2. Introduction: The mechanism (i.e., action mechanism) by which ivabradine evokes sinoatrial pacemaker activity blockade should be included. Additionally, the advantage of this drug to answer the study`s question should be also presented.
Information related to the effect of ivabradine were added into the Introduction. Rational basis related to advantages of this drug to answer the study question are now described in the Introduction in more clear way.

3. Page 5, "fear-inducing stress": The designation of the aversive stimuli as "STRESSORS" is more appropriate than "fear-inducing stress", since no behavioral analysis were performed to evaluate the "fear" component of the aversive stimuli.
Manuscript was significantly revised – now is focused on the role of visceral signalization on neuroendocrine stress response. Only in the Discussion, potential role of visceral signalization in shaping emotion is shortly mentioned. "Fear-inducing stress" and word "fear" was changed to "stress" throughout the manuscript.

4. RESULTS: The pharmacological treatment evokes a massive decrease in cardiovascular parameters (especially the HR) before the stress session. In this sense, evaluation of cardiovascular responses to stress via analysis of absolute values of MAP and HR can evoke misinterpretations. For instance, authors mention throughout the manuscript that MAP and HR responses were decreased. However, it seems that changes (i.e., values immediately before versus during stress) are the same (or even higher in case of MAP response to handling). My suggestion is that changes (i.e., difference in values during stress vs before stress) rather the absolute values is analyzed. It seems that it will change significantly the data interpretation.
We are thankful for this note. Differences between pre-stress value (-5 min interval) and values in stress intervals were calculated and marked in graphs by "+". Related information were added into the Results – last paragraph of the section "The effect of ivabradine on heart rate and mean arterial pressure during handling, restraint stress, or immobilization"

5. RESULTS: How do the authors explain the opposite effect of ivabradine in EPI (increase) and NOR (decrease) responses to restraint and immobilization? It is poorly explored in the discussion.
Text explaining above-mentioned differences were added into the Discussion.

6. DISCUSSION, page 9, third paragraph: It was demonstrated for several stressors, including the restraint, a sympathetic and parasympathetic coactivation (rather than an opposite change in sympathetic and parasympathetic) (for review, see Crestani, Front Physiol. 7:251, 2016). Please review.
During extensive revision of the manuscript, text related to the role of sympathetic and parasympathetic nerves in regulation of heart rate was excluded.

7. Discussion, page 10: The authors discuss the differences in neuroendocrine responses to the different stressors in terms of the physical activity (struggle) observed in restraint and immobilization, which is absent in handling. However, in the Methods session the authors mentioned that these stressors were chosen because of the different intensities of stress evoked by them.
Description of stressors “severity” was unified throughout the manuscript according to description in the Methods.

8. Discussion, page 11, line 4: This discussion needs to be revised. The idea of "feed-forward" indicates a response that is evoked without any sensorial information, which is exactly the opposite of reflex (responses evoked by a sensory stimulus). The idea of “conditioning” is not appropriately used as well, since it presumes the pairing of two events. It is not clear the two events that are paired during an acute session of stress to evoke the physiological responses.
Text of the Discussion was extensively modified. The idea of "feed-forward" was completely excluded. We hope that specification of conditioned and unconditioned factors make our hypothesis clearer.

Reviewer #2:
We would like to thank Reviewer #2 for his/her positive comments and suggestions. We followed the recommendations and hope that you find the revised version of the manuscript satisfactory.

1. The main problem of the manuscript arises from the author´s effort to focus on fear and emotions. No behavioral tests were used to confirm the presence of fear or anxiety and their level. I suggest to focus on stressor intensity and exclude all parts related to fear.
Parts related to fear were excluded throughout the manuscript. Now we are focusing on the effect of viscerosensitive signaling on neuroendocrine stress response.

2. There is no clear hypothesis. The aim of the study should be formulated as concrete hypotheses, not only “investigation of the effect”.
The last paragraph of the Introduction was modified. We hope that the hypothesis is now more clearly articulated.

3. The description of Results is given in insufficient detail. Results on ANOVA should be written more detailed. Both factors (treatment, time) and interactions between factors should be stated. Degrees of freedom along with a significance level (F=.., p=) for all significant main effects as well as interaction between factors need to be added. The same should be done in case of Student t-test results (both t-values and p-values should be stated).
Above-mentioned statistical description was added in the section Results.

4. The discussion is written too extensively and needs to be re-written in a more concise form. The first three paragraphs should be deleted. I strongly suggest starting the Discussion with the

summary of main findings obtained.
The Discussion was extensively modified and shortened, the first paragraphs were excluded.

5. The authors observed opposite effects of restraint and immobilization on epinephrine and norepinephrine levels. Can you please discuss this finding?
These finding are now discussed in more details in the Discussion.

6. Can you please give more information how the area under the curve was calculated? Was it calculated as an AUC with respect to ground or with respect to increase?
AUC was calculated with respect to ground.

7. I suggest replacing the collocation “fear-inducing stressors” only by “stressors” as no behavioral test was conducted to assess fear or anxiety. The same applies also for “conditions with mild fear”. Handling is a condition of mild stress intensity.
Collocation “fear-inducing stressors” was replaced by word “stressors” throughout the manuscript.

References

Young GT, Emery EC, Mooney ER, Tsantoulas C, McNaughton PA. 2014. Inflammatory and neuropathic pain are rapidly suppressed by peripheral block of hyperpolarisation-activated cyclic nucleotide-gated ion channels. Pain 155: 1708-1719.

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Ivabradine reduces baseline and stress-induced increase of heart rate and blood pressure and modulates neuroendocrine stress response in rats depending on stressor intensity

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Running title: **Ivabradine reduces blood pressure and modulates neuroendocrine stress response**

Abstract

Ivabradine, a selective inhibitor of the sinoatrial pacemaker, is used in clinical practice to reduce heart rate. However, its potential effect on the neuroendocrine stress response has not been investigated. Therefore, we determined the effect of administering ivabradine to rats on cardiovascular parameters and plasma levels of epinephrine, norepinephrine, and corticosterone. Ivabradine was administered intraperitoneally 30 min before exposing animals to either handling, restraint, or immobilization stress. Heart rate and blood pressure were monitored telemetrically. Blood samples were collected before, during, and after stressor exposure to determine the extent of the neuroendocrine stress response as reflected by plasma epinephrine, norepinephrine, and corticosterone levels. In animals pretreated with ivabradine, significantly lower values of heart rate and blood pressure were found during both the baseline period and during exposure to stressors, as well as during the rest period following stressor exposure. Ivabradine also significantly reduced handling-induced epinephrine and norepinephrine release into the bloodstream. However, ivabradine significantly potentiated restraint- and immobilization-induced increases of plasma epinephrine levels, whereas stress-induced changes in plasma norepinephrine and corticosterone levels were ambiguous. Our data shows that ivabradine significantly reduces blood pressure in rats during both baseline and stressful conditions, and also affects the neuroendocrine stress response. These findings show that viscerosensory signaling from the cardiovascular system may significantly modulate the neuroendocrine stress response.

Key words: blood pressure; corticosterone; epinephrine; heart rate; ivabradine; norepinephrine

Introduction

The stress response is a highly orchestrated reaction of an organism to external or internal factors (stressors) that disrupt homeostasis [32]. Crucial components of the neuroendocrine stress response include the sympathoadrenal system (SAS) and hypothalamo-pituitary-adrenocortical axis (HPA) [4]. Whereas a balanced neuroendocrine stress response is essential for survival, altered activation of the SAS or HPA axis, in either magnitude or duration, might have detrimental effects on the organism [11, 20, 21]. Therefore, the activity of the SAS and HPA axis are modulated by several mechanisms, including feedback signaling from visceral organs [12].

While the effect of increased SAS activity on cardiovascular system function has been well described [9], the role of signals transmitted from the cardiovascular system to the brain in modulating SAS and HPA axis activity during stressful situations remains unclear. Increased heart rate, accompanied by more frequent activation of heart mechanoreceptors, represents a typical response to psychosocial and physical stressors [19]. While these changes in heart rate detected by cardiac mechanoreceptors are known to be transmitted to the brain [24], it is unclear whether or not these signals affect the neuroendocrine stress response.

Several methodological issues make it difficult to investigate the role of viscerosensory signaling from the heart in modulating the neuroendocrine stress response. Firstly, it is difficult to selectively manipulate heart rate without affecting the regulatory effects of the neuroendocrine stress response on peripheral tissues and organs via the SAS and HPA axis. For example, cardiac denervation in patients after heart transplantation leads to damage of both viscerosensory and autonomic motor nerve fibers [25, 26]. Additionally, pharmacological approaches exert a low degree of selectivity. For example, β-blockers, besides causing a reduction of heart rate, also affect the activity of other visceral organs and tissues, which induces various changes to the internal environment [10] that are subsequently signaled to the brainstem and other structures involved in regulating stress-related neuroendocrine stress responses. Fortunately, ivabradine, a highly selective inhibitor of the sinoatrial funny current, was developed in the 1990´s. Ivabradine selectively blocks f-channels on cardiac pacemaker cells that determine spontaneous electrical pacemaker activity in the sinoatrial node. This reduced current inhibits slow diastolic depolarization, thereby reducing heart rate [1, 7, 30]. It was also found that ivabradine may affect blood flow in some vascular beds [8]. However, there are no data showing that ivabradine has a significant effect on the activity of endocrine glands or other visceral tissues and organs. In

addition, ivabradine does not cross the blood-brain barrier [33] and therefore cannot directly affect brain functions. Based on these data, we propose that this drug provides an opportunity to investigate the effect of selective inhibition of heart rate on various physiological functions, including activity of the SAS and HPA axis.

Base on the above-mentioned facts, using ivabradine, a drug that selectively reduces heart rate without affecting the activity of other visceral organs and tissues in the body [6, 23], we investigated the role of signaling that accompanies a stress-induced rise in heart rate on the extent of the neuroendocrine stress response. In our study, rats pretreated by ivabradine were exposed to handling, restraint stress, or immobilization while plasma epinephrine (EPI), norepinephrine (NE), and corticosterone levels were assayed. Furthermore, to confirm the efficacy of ivabradine-induced attenuation of stress-induced increases in heart rate, cardiovascular parameters were monitored telemetrically.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 250 – 300 g (Charles River Laboratories International Inc., Germany) were housed three per cage under controlled conditions (12/12 h light/dark cycle with lights on at 6:00 AM, temperature 22 ± 1°C), with food and water provided *ad libitum*. Experiments were performed between 08:00 - 12:00 a.m. The experiments were carried out in accordance with the Council Directive 2010/63EU of the European Parliament and the Council of 22nd September 2010 on the protection of animals used for scientific purposes.

Experimental design

We performed two series of experiments (Fig. 1). In the first experiment, the effects of IVA pretreatment on cardiovascular function in rats exposed to fear-inducing stressors were analyzed using telemetric devices. In the second experiment, the effects of ivabradine on neuroendocrine stress responses in rats was determined by measuring plasma EPI, NE, and corticosterone levels using immunoassay methods.

Exposure of animals to stressors

Three stressors; handling, restraint stress, and immobilization, each differing in their extent of activation of the neuroendocrine stress response were used and were performed as previously described [3, 15]. Handling, a mild psychological stressor, was performed by gentle manipulation of the animals. To do this, each animal was removed from the cage and gently manipulated using both hands. Because animals accommodate to this stressor relatively fast, rats in our experiments were only handled for 5 min. Restraint, a stronger stressor with predominant psychological and less intense physical components, was performed by placing the animal into a plastic tube that restricted its movements. Immobilization, a strong stressor with both physical and psychological components, was performed by taping of all four limbs to metal holders attached to an immobilization board. As a result of this the animal was not able to move, breathing was hampered, and acral parts of limbs were ischemic [16, 17].

Attenuation of stress-induced increase of heart rate by ivabradine pretreatment

Ivabradine (Procoralan 5 mg, Les Laboratoires Servier, Neuillysur-Seine, France) was dissolved in saline and injected intraperitoneally (5 mg/kg bw) in rats (IVA group). The vehicle treated rats were given an intraperitoneal injection of saline (SAL group). Ivabradine or saline in a volume of 2 mL/kg were administered 30 min before exposure of animals to stressor. The dose of ivabradine was chosen based on our pilot experiment in which we have found that this dose is sufficient to prevent stress-induced increase of heart rate.

Monitoring of cardiovascular system activity

In the first experiment, rats were randomly divided into IVA and SAL groups and exposed to 5 min handling (n = 4), 60 min restraint (n = 4), or 60 min immobilization (n = 4). Heart rate and mean arterial pressure were recorded by telemetric devices with Millar catheters (model TRM54P, Telemetry Research, Auckland, New Zealand) implanted into the abdominal aorta seven days prior to the exposure of rats to the stressors (for details of implantation of telemetry devices see [22]). Briefly, animals were anesthetized with an intramuscular administration of a ketamine (Narkamon 5%; 1.2 mL/kg) and xylazine (Rometar 2%; 0.4 mL/kg) mixture. The tip of the Millar catheter was placed into the aorta above the bifurcation of its abdominal part. The transmitter was then placed into the abdominal cavity and attached to the peritoneum using

sutures. The abdomen was then closed in two layers with interrupted sutures. To avoid post-operational infection, intramuscular injection of antibiotics (penicillin) was given immediately after implantation. After surgery, rats were housed separately with free access to tap water and pelleted food.

The recording of cardiovascular parameters started 60 min prior to the exposure to stressors. This interval represented baseline values analogical to the control interval used during blood sampling in the second experiment (see below). These cardiovascular parameters were continuously recorded during the entire exposure to stressors and also for the next 120 min (handling) or 60 min (restraint, immobilization) as an analogy to the rest interval during blood sampling (see below).

Determination of neuroendocrine stress response

In the second experiment, rats were randomly divided into IVA and SAL groups and exposed to handling (n = 17), restraint (n = 18), or immobilization (n = 13) stress, respectively. Blood samples (0.4 mL) were collected at defined time intervals via a cannula implanted into the jugular vein one day prior to the exposure of animals to stressors. The cannulation of the jugular vein allows for repeated blood sampling without inducing any stress effects and was performed as previously described [31]. Briefly, animals were anesthetized with a mixture of ketamine-xylazine as described above. The rats were then fixed in a supine position to an acrylic surgery platform. The area of incision (15 mm long) was on the right shoulder close to the base of the neck. To reach the jugular vein it was necessary to separate the surrounding muscle and membranous tissue. The exposed jugular vein was cut with spring-scissors on the upper surface and a polyethylene tube (silicon tubing, PE 50; Becton-Dickinson, Parsippany, NJ) filled with heparinized saline (300 IU/mL) was carefully placed into the right jugular vein. About 0.1 mL of the heparinized saline was then infused through the catheter and a slow withdrawal of blood was attempted to confirm correct cannulation procedure. The cannula was tied to the vein with rostral and caudal ligatures to avoid occluding the cannula. Following this, the rat was removed from the platform and we made a small incision in the center of the rat's nape with a trochar. The end of the cannula was then advanced into the trochar and exteriorized at the other end of the ventral incision, and was then attached with medical thread. After cannulation, the rats were housed individually. Exposure to stressors and blood sampling started on the next day after the overnight

recovery. The duration of the 1 day recovery was selected according to our previous experiments and findings showing that jugular vein cannulation does not increase baseline corticosterone levels 1 day after cannulation and does not alter subsequent stress responses in animals exposed to stressors 1 or more days after the surgical procedure [18].

Approximately 30 min before of the start of blood sampling, the free end of the jugular catheter was connected with a longer cannula to allow unstressed blood collection. Before ivabradine injection, the first blood samples (control samples) were collected from unstressed rats. Afterwards, the blood samples were collected via cannula at the time points of 0 and 5 min during handling and at 15, 30, 60, and 120 min after handling (rest phase). During restraint and immobilization, blood samples were collected at the 5, 15, 30, and 60 min time points during exposure, as well as 60 min after the end of exposure to stressor (rest phase). To replace the lost volume of blood (0.4 mL) each rat was administered an equal volume of heparinized saline (50 IU/mL) by cannula.

Biochemical analysis of plasma

Immediately after collecting blood samples, Eppendorf vials containing the collected blood were placed on ice and centrifuged at 3000*g* for 15 min at 4°C to separate plasma. Plasma samples were stored at -70°C until analyzed. Plasma catecholamine concentrations were determined by a commercially available enzyme immunoassay kit (2-CAT (A-N) Research ELISA, LDN, Nordhorn, Germany). The minimum detection limit for catecholamines in this ELISA kit is 8.1 pg/mL for EPI and 5.4 pg/mL for NE (depending on sample volume). Plasma corticosterone concentration was determined by a commercially available radioimmunoassay kit (Corticosterone rat/mouse RIA kit, DRG Diagnostic, Germany). The minimum detection limit for corticosterone in these RIA kits is 7.7 ng/mL.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 program (GraphPad Software, San Diego CA, USA). Statistical differences between the groups were determined by two-way analyses of variance (ANOVA) with repeated measures followed by post hoc, pair wise comparisons using Bonferroni's correction and an unpaired Student´s t-test. Data are presented as mean ± SEM and represent the mean for 4 rats in experiments monitoring cardiovascular activity

and 8 – 10 rats in experiments determining the neuroendocrine stress response. The *p* value of < 0.05 was taken as indicative of statistical significance. The area under the curve (AUC) was calculated based on the concentration × time as a measure of the magnitude of the response. The AUC represents an integrated value of the total amount of hormone released during the 5 min of handling, 60 min of restraint stress, or 60 min of immobilization.

Results

The effect of ivabradine on heart rate and mean arterial pressure during handling, restraint stress, or immobilization

Continual telemetric recording demonstrated that heart rate and mean arterial pressure increased only slightly after intraperitoneal injection of saline (-30 min interval) compared to pre-injection period (-35 min interval). Furthermore, before exposure to stressors (handling, restrain or immobilization; -5 min interval) values of these cardiovascular parameters slightly lower or even reached control values in animals injected by saline (Fig. 2A, 3A, 4A). In contrast, injection of ivabradine 30 min prior to exposure to the stressor significantly reduced values of heart rate and mean arterial pressure during the time intervals prior to the exposure of animals to stressors (-15 min and -5 min; Fig. 2A, 3A, 4A).

Whereas exposure of saline-treated rats to stressors induced increase of heart rate and blood pressure, animals injected with ivabradine showed significantly reduced values of heart rate and mean arterial pressure during exposure to stressors, as well as during following rest period (Handling HR: $F_{1,60}$=6496.45, $p < 0.001$, MAP: $F_{1,60}$=721.53, $p < 0.001$, Fig. 2A; Restrain HR: $F_{1,54}$=1477.63, $p < 0.001$, Restrain MAP: $F_{1,54}$=257.58, $p < 0.001$; Fig. 3A; Immobilization HR: $F_{1,60}$=434.72, $p < 0.001$, Immobilization MAP: $F_{1,54}$=298.72, $p < 0.001$; Fig. 4A).

When comparing the -5 min, pre-stress interval with the subsequent stress and post-stress rest intervals, exposure to stressors induces a significant increase of HR and MAP in both saline and ivabradine pretreated rats. This increase was more prominent in saline treated animals than those injected with ivabradine. In handled rats this increase was found at the beginning of the handling procedure, whereas by the end of handling, HR values did not differ from the pre-stress -5 min interval (Fig. 2A). In rats exposed to restraint, increased values of the observed cardiovascular parameters were found in several stress intervals (Fig. 3A), whereas in immobilized animals HR and MAP values were increased during the entire period of immobilization (Fig. 4A).

The effect of ivabradine on plasma catecholamines and corticosterone levels during handling, restraint stress, or immobilization

In rats exposed to handling, ivabradine pretreatment significantly decreased the total amount of EPI and NE released during the 5 min handling period compared to the SAL group (EPI AUC: t_{16}=2.668, $p < 0.05$, NE AUC: t_{16}=5.217, $p < 0.001$; Fig. 2B, C). On the other hand, ivabradine pretreatment significantly exaggerated restraint- and immobilization-induced increases of plasma EPI (Restrain EPI: $F_{1,96}$=70.62, $p < 0.001$, Fig. 3B; Immobilization EPI: $F_{1,66}$=13.94, $p < 0.001$, Fig. 4B). Also, the total amount of EPI released during the 60 min restraint stress was significantly higher in the IVA group compared to saline treated animals (t_{16}=9.010, $p < 0.001$; Fig. 3B), as well as during the 60 min immobilization (t_{11}=2.452, $p < 0.05$; Fig. 4B). Similar to handling, the total amount of released NE was significantly decreased in ivabradine-pretreated rats compared to the SAL group during restraint stress (t_{16}=2.172, $p < 0.05$; Fig. 3C). In immobilized rats, the total amount of released NE increased only slightly in rats pretreated by ivabradine (t_{16} = 1.488, p = 0.1488, SAL: 119 ± 7 ng/mL/min vs. IVA: 106 ± 5 ng/mL/min; Fig. 4C). However, stress-induced increases in plasma corticosterone levels did not differ between the IVA and SAL groups during exposure to the stressors (Fig. 2D, 3D, 4D).

Discussion

In our experiments, we have confirmed that ivabradine pretreatment significantly reduces heart rate during unstressed conditions. However, contrary to the generally accepted assumption that ivabradine does not affect blood pressure, we have shown that ivabradine administration even in dose of 5 mg/kg bw also significantly reduced baseline blood pressure in rats. Moreover, we found that ivabradine also attenuated stress-induced increases in heart rate and blood pressure. In rats exposed to handling (a mild stressor), ivabradine administration significantly reduced stressor-induced activation of SAS as shown by the reduced levels of EPI and NE in ivabradine treated rats compared to saline injected ones. However, handling-induced activation of the HPA axis, as determined by plasma corticosterone levels, was not affected by ivabradine. On the contrary, ivabradine significantly potentiated restraint- and immobilization-induced increases of plasma EPI levels whereas plasma NE and corticosterone levels exhibited ambiguous changes.

To our knowledge, there is only one study in humans investigating the effect of IVA on plasma EPI and NE levels in healthy volunteers at basal conditions, as well as during tilt and physical activity. They found out that ivabradine administration was associated with decreased levels of heart rate and mean arterial pressure together with increased levels of EPI and NE during exercises with sympathetic stimulation [13].

We hypothesize that the mechanisms responsible for reduced activation of SAS in rats pretreated with ivabradine and exposed to a mild stressor (handling) are related to mechanisms of learning by conditioning (for review see [27]). Exposure of an organism to stressors usually leads to an increase in heart rate. Because activation of the neuroendocrine stress response and increases in heart rate are frequently coupled, it is possible that this response develops via conditioning. This conditioned response has the neuroendocrine stress response as the unconditioned "factor" and increases in heart rate as conditioned "factor". In this way, an increase in heart rate is able to potentiate the neuroendocrine stress response by itself. The reduction of heart rate by ivabradine also reduces the effectiveness of this conditioned response and therefore may be responsible for the attenuated activity of SAS detected in handled rats pretreated by ivabradine.

In contrast to handling, plasma EPI was increased in rats pretreated by ivabradine and exposed to an intermediate stressor such as restraint stress, or a strong stressor such as immobilization. We hypothesize that this is because restraint stress and immobilization represent stressors that are accompanied by physical activity when animals try to escape from the restraint cylinder or immobilization board, as compared to handling, which does not. This physical activity may be accompanied by mild hypoxia in muscles that represents a potent stimulus for the adrenal medulla, whereas sympathetic nerves releasing NE are not significantly stimulated [34].

In addition, we hypothesize that the ivabradine-induced reduction of heart rate in animals exposed to intermediate (restraint) or intensive (immobilization) stressors is associated with insufficient peripheral responses to released catecholamines into the bloodstream. For example, the ivabradine-induced decrease in heart rate and blood pressure may consequently lead to reduced perfusion of organs and muscles. This insufficient peripheral response to excessive catecholamine release provokes additional stimulation of SAS and subsequent EPI release as documented by the increased plasma EPI in ivabradine-injected rats exposed to restraint or

297 immobilization. Published data indicate that activation of baroreflex may play a pivotal role in
298 this compensatory response [5].
299 Ivabradine administration did not significantly affect stress-induced increases in plasma
300 corticosterone levels. Therefore, we suggest that afferent signaling from the cardiovascular
301 system plays a more important role in the regulation of SAS activity than the HPA axis.
302 Furthermore, these data suggest that HPA axis activity is mainly under the influence of the
303 forebrain structures activated predominantly by psychological stressors [14, 35].
304 Our findings of an attenuated neuroendocrine stress response as a consequence of reduced
305 heart rate during exposure to a mild stressor supports the assumption that while stress related
306 processes in the brain, including emotions, drive changes in the activity of visceral organs,
307 activation of visceral organs may in turn shape neuroendocrine stress response [2]. Because
308 ivabradine does not cross blood-brain barrier [33], its central effect on neuroendocrine stress
309 response may be excluded. Based on our data, we suggest that the heart is activated during stress
310 response by central commands originating in the brain, but the heart itself also modulates
311 neuroendocrine and emotional responses in the brain, as proposed the James-Lange theory of
312 emotions.
313 Transmission of signals from the heart to the brain is altered in patients who have received a
314 heart transplant. However, published data indicate that the stress response is not affected in these
315 patients [28]. This discrepancy between our findings and the above-mentioned data might be
316 related to several factors. In our experiments, we selectively prevented the stress-induced
317 increase in heart rate, while in patients with a transplanted heart, a mild stressor (Stroop test)
318 induces a slight increase in heart rate and blood pressure that may lead to activation of several
319 receptors in the cardiovascular system (e.g. mechanoreceptors in the heart, baroreceptors). In
320 addition, re-innervation occurs in a transplanted heart. Moreover, these patients are treated by
321 several drugs also affecting the heart itself. All of these factors may affect transmission of signals
322 from the transplanted heart to the brain, consequently affecting the stress response and may
323 explain the discrepancies between published data and our findings.
324 To our knowledge, this is the first study investigating the effect of attenuating stress-induced
325 heart activity by ivabradine during the neuroendocrine stress response in laboratory animals
326 exposed to different stressors. We have shown that attenuation of the stress-induced increase in
327 heart rate during conditions of mild stress reduces the response of the SAS. These data indicate

that afferent signals from the heart play a significant role in modulating the neuroendocrine stress response, which may highlight the importance of visceral signals in modulating emotions, as originally proposed by the James-Lange theory. Therefore, psychological approaches to reducing the stress-induced rise in heart rate may be of importance in neurotic individuals with an exaggerated response to stressors [29]. In addition, pharmacological attenuation of heart rate during stressful conditions may represent a protective strategy useful in preventing the adverse effects of exaggerated SAS activation, particularly in patients with cardiovascular diseases.

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Conflict of interests

The authors declare that they have no conflicts of interest.

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Legends to figures

Figure 1. Schematic illustration of the experimental design.

Figure 2. The effect of saline (SAL; ○) or ivabradine (IVA; ●) pretreatment on heart rate and mean arterial pressure (A), plasma epinephrine (B), norepinephrine (C), and corticosterone (D) levels in rats exposed to handling (grey areas). Areas under the curve (AUC) were measured from 0 to 5 min of handling for the plasma catecholamines or corticosterone concentrations. Each value is the mean ± SEM (n = 4 for cardiovascular parameters; n = 8 – 9 for hormones). Statistical significance compared to saline pretreated group: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; differences between corresponding pre-stress interval (-5 min) and following intervals: $^{++}p < 0.01$; $^{+++}p < 0.001$.

Figure 3. The effect of saline (SAL; ○) or ivabradine (IVA; ●) pretreatment on heart rate and mean arterial pressure (A), plasma epinephrine (B), norepinephrine (C), and corticosterone (D) levels in rats exposed to restraint stress (grey areas). Areas under the curve (AUC) were measured from 0 to 60 min of restraint stress for plasma catecholamines and corticosterone concentrations. Each value is the mean ± SEM (n = 4 for cardiovascular parameters; n = 8 – 10 for hormones). Statistical significance compared to saline pretreated group: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; differences between corresponding pre-stress interval (-5 min) and following intervals: $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$.

Figure 4. The effect of saline (SAL; ○) or ivabradine (IVA; ●) pretreatment on heart rate and mean arterial pressure (A), plasma epinephrine (B), norepinephrine (C), and corticosterone (D) levels in rats exposed to immobilization (grey areas). Areas under the curve (AUC) were measured from 0 to 60 min of immobilization for plasma catecholamines and corticosterone concentrations. Each value is the mean ± SEM (n = 4 for cardiovascular parameters; n = 6 – 7 for hormones). Statistical significance compared to the saline pretreated group: $*p < 0.05$; $**p < 0.01$; ***p < 0.001; differences between corresponding pre-stress interval (-5 min) and following intervals $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$.

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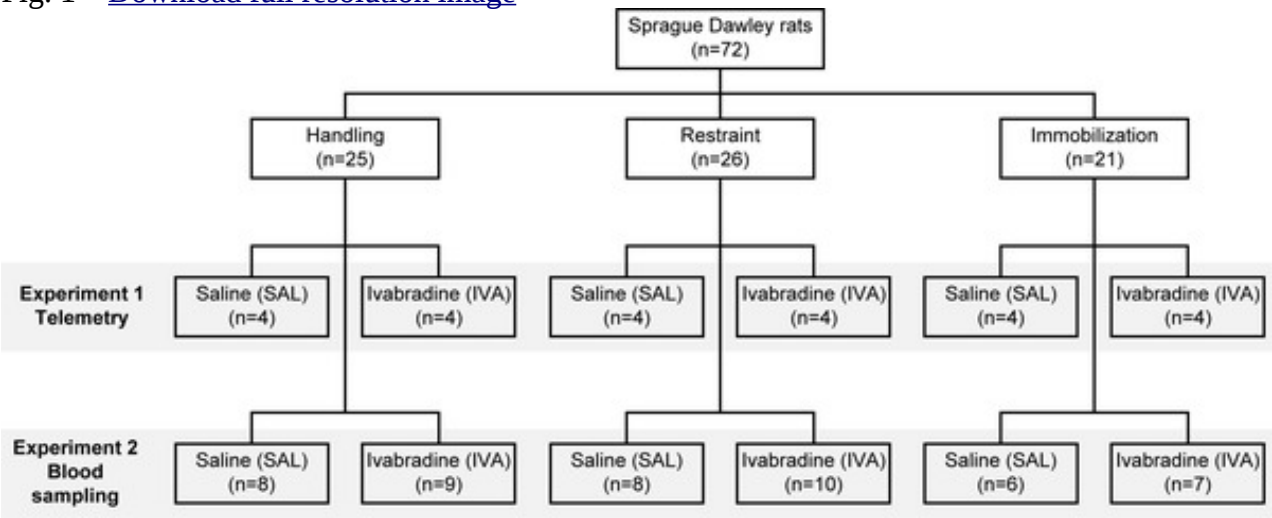

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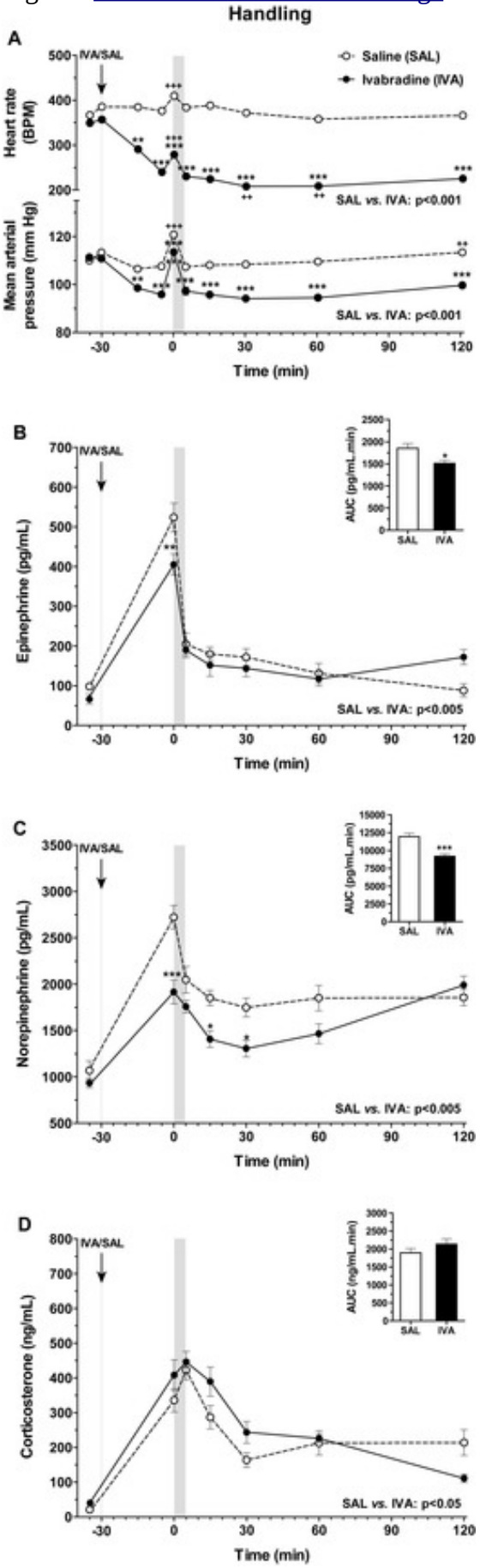

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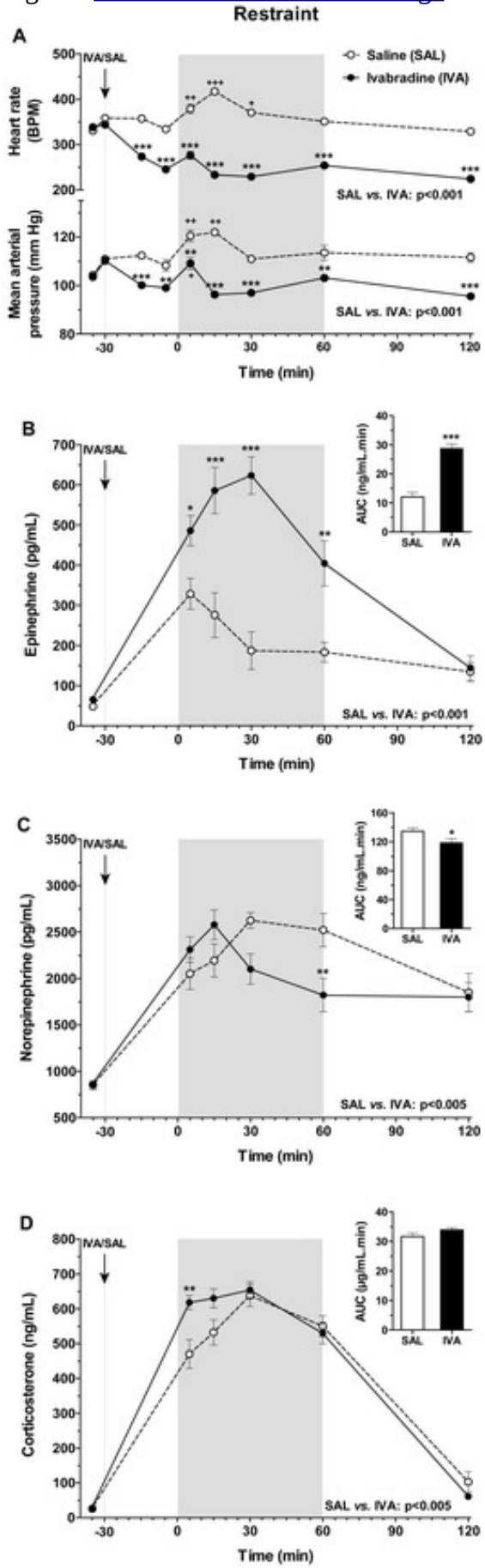

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