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Ciclo XXVII

NEGATIVE PSYCHOSOCIAL INFLUENCES ON CARDIAC ACTIVITY IN RATS: THE MEDIATING ROLE OF THE AUTONOMIC NERVOUS SYSTEM

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Preface

In the late 1620s, William Harvey hinted at a link between the brain and the heart when he wrote "..for every affection of the mind that is attended with either pain or pleasure, hope or fear, is the cause of an agitation whose influence extends to the heart.."". The role of psychosocial states in the modulation of cardiac homeostasis is no longer confined to the realms of anecdote. Extensive research has shown that psychosocial factors such as chronic life stress, poor social support, depression, anxiety and hostility/Type A behavior represent important risk factors for the onset and progression of cardiac alterations.

A number of pathophysiological mechanisms have been proposed to explain these relationships. Among these, mounting evidence supports the involvement of autonomic mechanisms. The autonomic nervous system represents the principal neural pathway through which the brain and the heart interact. The same cerebral regions (insular cortex, cingulate cortex, amygdala) involved in the regulation of emotions, mood and social behaviors, are also implicated in the autonomic control of cardiac function via hypothalamic and brain stem centers. While proper functioning of the sympathetic-parasympathetic dynamic balance at rest as well as in response to stress enables adaptive response to environmental demands, conditions of autonomic imbalance, in which typically the sympathetic system is hyperactive and the parasympathetic system is hypoactive, are associated with a lack of dynamic flexibility and increased arrhythmic risk. Thus, the hypothesis of cardiac autonomic imbalance due to alterations in the brain-heart circuitry may provide a theoretical framework for a better understanding of the association between psychosocial risk factors and cardiac disease.

The present series of experiments will address the role of the autonomic nervous system in mediating cardiac disturbances in rat models that reproduce human negative psychosocial conditions.

CHAPTER 1

GENERAL INTRODUCTION

1.1 PSYCHOSOCIAL RISK FACTORS AND CARDIAC DYSFUNCTION – A BRIEF OVERVIEW OF THE HUMAN LITERATURE

Decades of research have shown that emotional experience is dominated by two core and broad dimensions accounting for the variability in individual levels of psychological well being: positive affect and negative affect [1,2]. Positive affect is a dimension that reflects a level of pleasurable engagement with the environment and that involves both emotional and cognitive components, such as joy, enthusiasm, happiness, high energy levels, interest and motivation [3]. These psychological elements are generally associated with healthy patterns of cardiac function, although the role of other potential mediators of this association, such as healthy lifestyles, is of major relevance [4]. In contrast, negative affect is a dimension that reflects aversive emotions, such as sadness, fear, depression, anxiety, frequent anger and hostility [5]. There is increasing recognition that, on an inherited predisposing basis, the expression of negative affective states is ultimately determined by a number of environmental factors such as, for example, acute and chronic life stressors and the absence of social support. Therefore, psychosocial risk factors generally include the presence of negative affective states such as depression and anxiety and personality traits such as anger and hostility, as well as environmental factors that increase the risk of an individual developing them. As reviewed below, each of these psychosocial variables appears to contribute significantly to the pathogenesis of cardiac disease, independently from traditional risk factors such as cholesterol levels, waist fat, body mass index and poor physical activity [6,7,8,9,10].

In humans, major life changes associated with psychological and emotional adjustment are associated with an increased risk of cardiac events. For example, in the months after the death of a spouse, mortality from cardiovascular causes is increased [11]. Similarly, during the months after the terrorist attacks on the World Trade Center in New York in 2001 the rate of defibrillator firings resulted two to three times higher than normal in patients living far from the catastrophe and in those with both ischaemic and non-ischaemic cardiomyopathies [12,13], suggesting that the psychological fallout associated with this terrorism act lasted for weeks. Daily life stress can increase risk of cardiovascular

events. Employees who experience work-related stress and individuals who are socially isolated or lonely have an increased risk of a first coronary heart disease event [14]. For example, in one study of work-related stressors, upcoming deadlines were associated with a six-fold increase in myocardial infarction [15]. Other studies suggest that chronic work-related stress could carry a two to three times higher risk of cardiac events, especially when employees perceive little control over their work environment [16]. Likewise, in women with established coronary disease, marital stress was associated with a risk of recurrent events three times higher than in women with no marital stress [17]. In the Nurses' Health Study, caring for a sick spouse at home nearly doubled the risk of death from coronary events [18].

The presence of chronic, unpredictable, or uncontrollable stressors can also contribute to the development of depressive symptoms. Importantly, strong evidence indicates that depression is associated with an increased risk of cardiovascular morbidity and mortality, both in healthy individuals and in patients with heart disease [19,20]. Population-based studies of individuals without coronary disease have reported that depression predicts subsequent coronary disease independently of other comorbidities [21]. Moreover, the prevalence of major depression is about 20% in cross-sectional studies of patients with known heart disease, and depression is strongly associated with mortality after myocardial infarction [21,22].

Increasing evidence indicate that chronic anxiety is also a predictor of cardiovascular events, both ischaemic heart disease events and sudden death [23,24]. Moreover, a dose-dependent relationship between anxiety levels and the occurrence of cardiac death has been reported [25,26]. Interestingly, anxiety disorders seem to be associated with especially high rates of sudden death, by contrast with depressive disorders which are often characterized by a higher incidence of coronary events [25,26].

Frequent anger and hostility predict incident coronary events [27]. In the Atherosclerosis Risk in Communities Study, normotensive patients with high anger temperament scores (characterized by frequent or long-lasting anger reactions with little or no provocation) had higher risk for fatal or non-fatal cardiac events after adjustment for traditional cardiovascular risk factors [28]. In the last decades,

Type A personality (a personality construct characterized by competition, hostility and exaggerated commitment to work) has received attention as a potential cardiovascular risk factor, but results have been mixed as to whether this personality type is truly associated with cardiovascular events [29,30]. Nevertheless, it appears that specific Type A behaviors such as anger and hostility (as opposed to competitiveness and hypervigilance) are associated with cardiovascular morbidity and mortality [31].

Such cumulative epidemiological evidence supports the conceptual perspective that, although these domains can overlap, chronic stress, depression, anxiety and hostility/Type A behavior have all the potential to adversely affect cardiac function [32]. Pathophysiological mechanisms underlying these relationships fall into three broad categories: (i) behavioral mechanisms, whereby psychosocial conditions contribute to a higher frequency of unhealthy behaviors known to increase cardiac risk (e.g., poor diet and smoking), (ii) genetic mechanisms, and (iii) direct pathophysiological mechanisms, including immune, neuroendocrine and autonomic alterations. This thesis will focus specifically on the role of autonomic nervous system in mediating the link between psychosocial risk factors and cardiac dysfunction.

1.2 THE MEDIATING ROLE OF THE AUTONOMIC NERVOUS SYSTEM

The autonomic nervous system is divided into the opposing sympathetic and parasympathetic divisions that act to control heart rate, cardiac contractility, vasodilatation, and other critical functions. The activity of these branches is normally in dynamic balance, but can be rapidly modulated in response to changing environmental demands. Conditions that cause sympathetic activation include, for example, physical activity, coronary ischemia, heart failure, and mental stress. There is a large body of evidence to suggest that autonomic imbalance, in which typically the sympathetic system is hyperactive and the parasympathetic system is hypoactive, is associated with various pathological conditions [33]. In particular, cardiac autonomic imbalance has long been recognized as one of the most important predictors of ventricular arrhythmias and sudden cardiac death in patients with heart disease, as well as nonsudden fatal and nonfatal cardiac events in the general population

[34,35,36,37]. But what sets cardiac autonomic tone? One possibility is that psychosocial phenomena play an important role.

Considerable evidence from human studies points to autonomic nervous system imbalance in negative emotional states engendered by interactions with environment and social cues. For example, depression has been associated with elevated resting heart rate, decreased heart rate variability (HRV) (a measure of cardiac autonomic innervation by the brain, see next subchapter), impaired vagal control, and elevated levels of plasma norepinephrine [20,38,39], suggesting chronic inappropriate activation of the sympathetic nervous system. Chronic anxiety has been linked to diminished HRV and impaired vagal control [40,41]. Moreover, high levels of hostility have been related to diminished vagal modulation of heart function [42] and high levels of circulating catecholamines [43], suggesting a shift of the autonomic balance toward a sympathetic prevalence.

A starting point for understanding the role of autonomic nervous dysfunction in mediating cardiac disturbances in these psychosocial conditions is to define the central neural circuitry in the brain-heart axis. Psychosocial stress and emotional states are processed in forebrain regions such as the insular cortex, cingulated cortex and amygdala [44]. Such brain regions, which are viewed together as the limbic system, act on frontal, temporal and striatal centers to shape our behaviors and on hypothalamic and brain stem centers to change our bodily arousal state through autonomic efferent nuclei [45,46,47]. The structures of this “central autonomic network” (firstly described by Bennarroch [45]) include paraventricular and related nuclei of the hypothalamus, the periaquaductual gray matter, the parabrachial nucleus, the nucleus ambiguous, the dorsal motor nucleus of the vagus, the rostral ventro-lateral medulla, the raphe pallidus, among others [45,47] (Figure 1). The hypothalamus and higher centers modify the activity of the medullary centers that regulate sympathetic and vagal outflow to the heart in response to emotional states and stress [47,48,49] (Figure 1). The parasympathetic output to the heart comes mainly from neurons in the nucleus ambiguous and to a lesser extent from the dorsal motor nucleus [50], whereas the rostral ventro-lateral medulla is considered as a principal origin of sympathetic cardiac activity [51,52] (Figure 1).

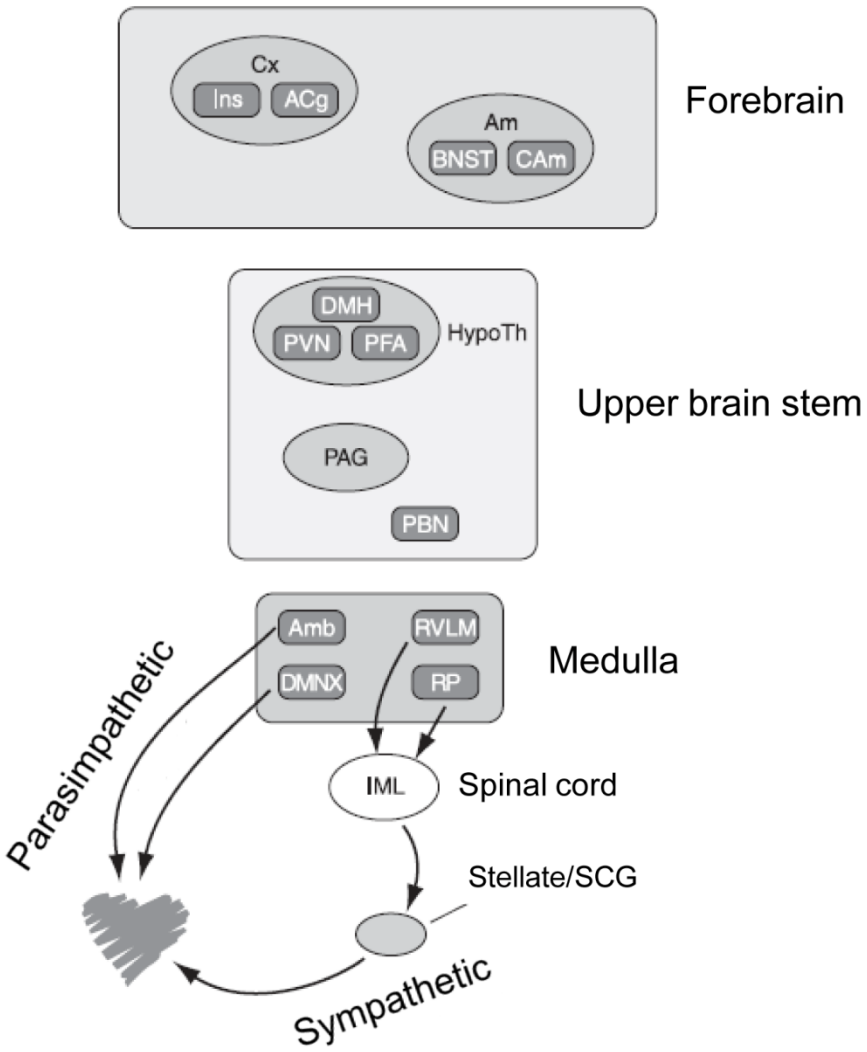

Figure 1. Brain-structures involved in the control of the heart (modified from (48)). Abbreviations: ACg, anterior cingulated; Am, amygdala; BNST, bed nucleus of the stria terminalis; CAm, central amygdala; Cx, cortex; DMH, dorsomedial hypothalamus; DMNX, dorsal motor nucleus of the vagus nerve; IML, intermediolateral column; Ins, insular cortex; PAG, periacqueductal grey; PBN, parabrachial nucleus; PFA, periphornical area; PVN, paraventricular nucleus; RP, raphe pallidus; RVLM, rostral ventro-lateral medulla; SCG, superior cervical ganglia.

Sympathetic and parasympathetic inputs exert modulatory effects on cardiac function. Elevations in sympathetic tone levels increase cardiac chronotropism, inotropism, and coronary constriction. Importantly, sympathetic neural activity predisposes the heart to cardiac arrhythmias and increases the likelihood of ventricular fibrillation [53]. Conditions of excessive sympathetic modulation alter electrophysiology properties in a potentially pro-arrhythmic manner by enhancing the dispersion of repolarization or by generation of delayed after-depolarization [54]. Vagal antagonistic action, by opposing sympathetic action at both pre- and post-junctional levels [55], modulates not only chronotropism but also ventricular performance, intracellular calcium handling and cardiac electrophysiology [56]. However, while sympathetic stimulation has similar effects on both atrial and ventricular myocytes, vagal stimulation does not. In the ventricles, vagal stimulation prolongs action potential duration and effective refractory period [56,57], whereas in the atria vagal activation reduces the atrial effective refractory period [58,59], augments spatial electrophysiological heterogeneity [60], and promotes early after-depolarization toward the end of phase 3 in the action potential [61]. This differential effect may explain why parasympathetic stimulation is pro-arrhythmic in the atria but anti-arrhythmic in the ventricles, whereas sympathetic stimulation seems to be pro-arrhythmic for both chambers [53]. It therefore appears that conditions of sympathetic/parasympathetic imbalance, in which one branch dominates over the other, may increase arrhythmic risk directly by lowering the threshold for arrhythmias or indirectly by causing atherothrombotic disease in the coronary arteries (Figure 2).

Based on these considerations, it is reasonable to hypothesize that psychosocial risk factors may interfere with cardiac health by causing alterations in the autonomic nervous system control over cardiac function.

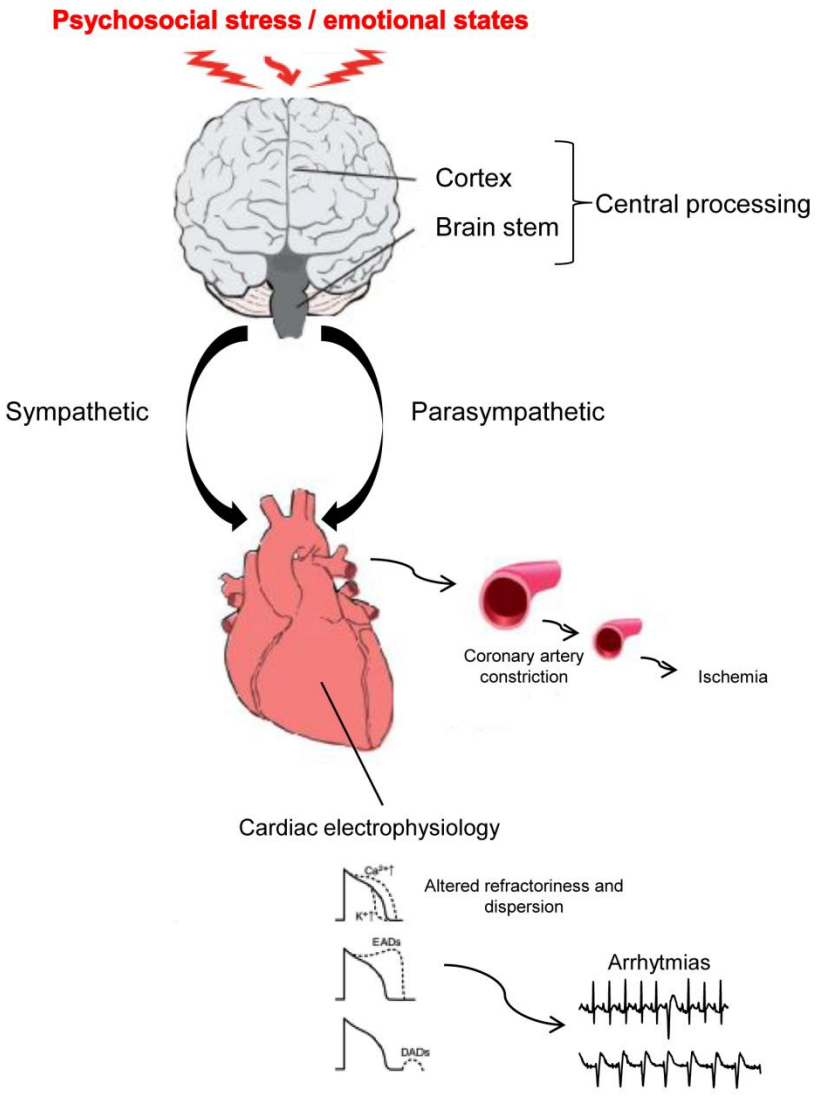

Figure 2. Brain-heart axis and role of autonomic mechanisms in the generation of cardiac arrhythmias. Central neural processing of psychosocial stress and aversive emotions may lead to alterations in the autonomic outflow to the heart, which in turn can cause atherothrombotic disease in the coronary arteries and pro-arrhythmic changes in the electrical properties of the myocardium.

1.3 MEASURING AUTONOMIC INFLUENCES ON THE HEART

Normal heart rate is characterized by an elevated degree of beat-to-beat variability. This physiological phenomenon, which is called HRV, reflects the dynamic balance between the sympathetic and parasympathetic inputs to the intrinsic activity of the sinoatrial node. HRV is largely dependent on respiratory-related mechanisms mediated by the parasympathetic nervous system. Collectively, these mechanisms produce respiratory sinus arrhythmia - rhythmic oscillations of heart rate around its mean value - with increases in heart rate during inspiration as vagal influence is momentarily suppressed, and decreases in heart rate during the early expiration phase as vagal influence resumes. Although heart rate can vary from moment to moment for reasons other than vagal influence (e.g., in response to thermoregulatory changes, physical activity, changes in blood pressure), respiration is reliably periodic. Therefore, the strength of vagal influence can be assessed by measuring the rhythmic oscillation in the intervals between consecutive heart beats that are due to respiratory sinus arrhythmia [62].

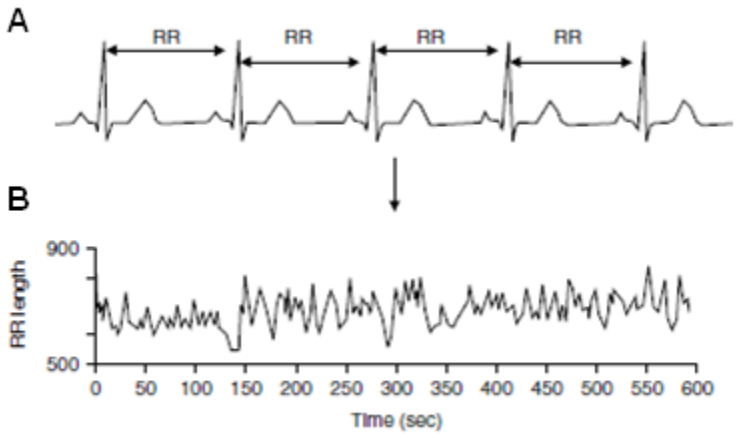

Figure 3. Example of heart rate beat-to-beat changes – heart rate variability (Fig. 3B), determined by RR intervals from QRS complex (Fig. 3A)

HRV can be analyzed by traditional linear methods as well as novel nonlinear methods that describe variability from different aspects. Conventional linear HRV methods fall under the broader description of being either “time-domain” or “frequency-domain” analyses. Among time-domain measures of HRV, which are assessed with calculations based on statistical operations on R-R intervals [63], the standard deviation of R-R intervals (SDNN) and the root mean square of successive R-R interval differences (RMSSD) estimate the total variability and the activity of the parasympathetic nervous system activity, respectively [64]. Frequency-domain measures are based on spectral analysis of a sequence of R-R intervals and provide information on how power (variance) is distributed as a function of frequency. Usually, three oscillatory components are distinguished in the spectral profile: the very low (VLF), the low (LF) and the high (HF) frequency bands. The power of the HF band includes respiration-linked oscillations of HR and therefore reflects the modulation of vagus nerve discharge caused by respiration [65], whereas the LF and VLF bands are related to a more gradual interplay between sympathetic and parasympathetic influences [66]. The power of LF and HF bands is often reported in normalized (relative or fractional) units, which correspond to the relative value of each power in proportion to the total power (usually minus the VLF component). In particular, LF to HF ratio is taken as a putative measure of sympathovagal balance, where higher numbers indicate greater relative sympathetic dominance [62].

In order to evaluate in greater detail the intrinsic complexity of HRV, algorithms based on the chaos theory and nonlinear dynamics have been developed (for an historical overview of the evolution of the concept of HRV and its application see [67]). Nonlinear methods are based on the assumption that the mechanisms involved in heart rate regulation interact with each other in a nonlinear way. The basic concept of nonlinear HRV methods is to try to capture the non-periodic behavior and complexity that exist inside the R-R interval dynamics. Various nonlinear methods have been tested in several sets of R-R interval data [34,68,69,70,71,72,73,74], providing additional prognostic information and complementing traditional time- and frequency-domain analyses.

1.4 THE VALUE OF EXPERIMENTAL INVESTIGATION IN ANIMAL MODELS

Determining autonomic dysfunction in negative psychosocial conditions may yield important insights into the ways in which the central nervous system interacts with the heart to produce arrhythmias and, ultimately, strategies for prevention of sudden cardiac death. In this regard, research in humans is limited by several factors: (i) the difficulty to control and standardize for the individual social history preceding laboratory or clinical assessment; (ii) the potential bias of language based self-reports which are often used to assess the presence of negative psychosocial states; (iii) the ethical concerns and regulations that clearly restrict the application of psychosocial stress stimuli for mere experimental purposes; (iv) the relatively long-span of human cardiovascular pathogenesis. It is here that animal studies become indispensible.

Modeling human negative psychosocial conditions by means of laboratory animals require several criteria, among which face validity (i.e., the phenotype of the model is similar to the physiological and behavioral features described in humans) and construct validity (i.e., the factors which promote the development of such psychosocial state are similar to those in humans) are of major relevance. Because the development of human negative psychosocial conditions is likely to be caused by complex interactions between genetic and environmental factors, the establishment of an appropriate construct is a difficult task. However, the application of psychosocial stressors that have high relevance for the human condition is considered a valid approach for modeling features of stress-induced anxiety and/or depression in experimental animals. Among the variety of stress paradigms, social stress fulfils this demand, because social (and territorial) relationships play, through inter-individual communication and its consequences on the genome and the epigenome, a major ecological role in animals and humans [75,76,77]. On the other hand, there are different approaches to the study of autonomic correlates of a trait behavioral characteristic (for example trait anxiety or stress coping behavior). When we want to obtain genetically selected animals we use certain parameters to select the animals that are directly related to the behavior of interest. The adequacy of the genetic process is evaluated measuring such behavior in each generation and the selection of the

extremes in each generation. We can thus eventually obtain stable lines differences in the measure(s) of interest that are maintained across generations. This is the case of the so-called high anxiety-related behavior (HAB) and low anxiety-related behavior (LAB) rats [78]. Alternatively, we can classify the two extremes of a non-selected outbred population of animals on the basis of the behavioral characteristic of interest (for example aggressiveness) and then study putative differences in cardiac autonomic control presumably related to this trait.

Importantly, experimental investigations aimed at unveiling autonomic mechanisms underlying the link between psychosocial risk factors and cardiac dysfunction could take obvious advantages from: (i) detailed 24 h cardiac monitoring in freely behaving animals by means of radiotelemetry, and (ii) analysis of HRV for obtaining valid cardiac parameters of autonomic control of the heart. Using these methods, animal research has just started investigating the complex interplay between psychosocial risk factors and cardiac autonomic function. For example, in a study by Wood et al. [79], rats exposed to 7 consecutive days of social defeat displayed symptoms of a depressive-like state that were associated with reduced HRV and signs of sympathetic predominance (increased LF to HF ratio). It was not determined whether such autonomic imbalance was due to a decrease in vagal tone and/or an increase in sympathetic tone. Similar changes in cardiac autonomic neural outflow were observed in rats submitted to a chronic mild stress model of depression [81]. Evocation of anxiety states in rats by means of classical conditioning provoked a significant shift of the sympathovagal balance toward a sympathetic prevalence (increased LF to HF ratio) [82]. Cardiac autonomic function in rats displaying symptoms of social stress-evoked anxiety was investigated in a study conducted by Sevoz-Couche and colleagues [83]. In this study, HRV analysis was conducted in rats submitted to intermittent daily episodes of social defeat. Five days after the last defeat, stressed rats showed symptoms of an anxiety-like behavior that were accompanied by signs of reduced vagal modulation of resting HR [83].

Taken together, these changes in rats correspond to changes which are relevant to cardiac diseases in humans under many kinds of negative psychosocial conditions, providing preliminary evidence that

animal research has the potential to provide insights into the mechanisms that influence the association between psychological risk factors and cardiac dysfunction.

1.5 AIMS AND OUTLINE OF THIS THESIS

In view of the above reported considerations, the principal aim of this thesis is to investigate in detail the impact of psychosocial risk factors on cardiac activity in rat models, with special emphasis on the study of autonomic mechanisms underlying these associations.

In **chapter 2** an experiment is described investigating electrical and structural properties of the myocardium in a highly relevant rat model of social stress-induced depression. By means of epicardial mapping, potential enduring cardiac electrophysiological changes relevant to arrhythmogenesis are assessed in socially stressed rats displaying depressive-like symptoms. In the experiment described in **chapter 3,** a similar protocol of social stress is applied to rats with a genetic predisposition to depression. First, it is investigated whether signs of a depressive like-state are associated with (i) changes in the autonomic neural modulation of cardiac function and (ii) increased arrhythmia vulnerability. Second, it is tested whether a pharmacological approach targeting the endocannabinoid system can reverse/prevent the depressive-like symptoms and also exert cardioprotective effects. **Chapters 4** reports about the study of cardiac function in a rat model of trait-anxiety. It is questioned, first, whether rats with high levels of anxiety show changes in the autonomic neural control of heart rate at rest, and, second, what the functional consequences of the observed differences may be in terms of cardiac stress responsiveness and arrhythmia vulnerability. In **chapter 5,** the association between given personality characteristics and cardiac function is investigated in two groups of rats that differ widely in their levels of aggressive behavior. It is tested the hypothesis that high levels of aggressive behavior in rats are related to changes in the autonomic neural modulation of heart rate at rest and during stress that may favor the onset of cardiac arrhythmias. In **chapter 6** the results are summarized, the main findings are discussed, and suggestions for future studies are given.

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CHAPTER 2

STRUCTURAL AND ELECTRICAL MYOCARDIAL REMODELING IN A RODENT MODEL OF DEPRESSION

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ABSTRACT

Objective: Despite a well documented association between stress and depression with cardiac morbidity and mortality, there is no satisfactory explanation of the mechanistic link between affective and cardiac disorders. In this study we determined cardiac electrophysiological properties in an animal model of depression.

Methods: Depression-relevant physiological and behavioral parameters were measured in adult male wild-type rats during and after a period of intermittent social defeat stress (n=12) or empty cage exposure (control condition, n=11). Nine days after the last social defeat/empty cage exposure, animals were anesthetized and high-definition epicardial potential mapping was performed.

Results: Stressed animals versus control counterparts displayed a larger reduction in the circadian amplitude of heart rate (-32±3% *vs.* -13±2%, p=.001) and body temperature (-33±4% *vs.* -5±2%, p=.001) rhythms, had smaller body weight gain (+11±1% *vs.* +17±1%, p<.001) and showed a larger reduction in sucrose solution intake (-19±6% *vs.* -7±4%, p=.006). Epicardial mapping analysis revealed a significant decrease in transversal conduction velocity of the wavefront (0.23±0.00m/s *vs.* 0.27±0.01m/s, p=.02), a shortening of the effective refractory period (86.8±2.1ms *vs.* 95.9±3.0ms, p=.01) and an increase in myocardial excitability (stimulus duration 0.01ms) in stressed animals versus controls. At sacrifice, a moderate fibrosis affecting the left ventricle was observed in the stressed group.

Conclusions: Intermittent social stress procedure is associated with depression-like symptoms and altered myocardial electrical stability in a potentially pro-arrhythmic manner. In particular, reduced myocardial refractoriness and impaired conduction, which are considered major determinants of arrhythmogenesis, represent possible mechanisms underlying cardiac vulnerability.

2.1 INTRODUCTION

Strong evidence indicates that depression is associated with an increased risk of cardiovascular morbidity and mortality, both in healthy individuals and in patients with heart disease (1, 2). Clinical studies of autonomic activity (3, 4) and heart rate variability (HRV) (5, 6) point out that the adverse effects of depression may be due, at least in part, to a dysregulation of the autonomic nervous system. For example, depressive patients have been found to display a predominance of sympathoadrenergic activation and/or reduced parasympathetic (vagal) modulation to the heart (3, 4), or a decreased HRV (5, 6). While these changes in the autonomic neural cardiac outflow may provoke arrhythmogenic effects (7), there is no clear and unequivocal understanding of the patophysiological basis of the association between depression and heart disease. Clearly, elucidation of the underlying mechanisms is crucial for a successful treatment of this comorbidity.

Valuable tools for providing insights into the relationship between depression and cardiovascular disease are methods inducing depressive-like states in experimental animals. Until now, only one study has assessed the heart susceptibility to cardiac arrhythmias in a rodent model of experimental depression (8). In this study, rats exposed to a chronic mild stress paradigm developed depression-like symptoms and showed increased susceptibility to experimentally induced ventricular arrhythmias, suggestive of an altered myocardial electrical stability. However, little is known about the mechanisms by which stress and depression alter myocardial properties in a pro-arrhythmic manner.

Addressing this issue, in the present study we exposed rodents to repeated episodes of social defeat stress. This experimental paradigm has been successfully applied to produce several behavioral and physiological changes that mirror the symptoms of human depression and it is argued to be a valid and reliable animal analog of depression (9). In addition to the relevance of social defeat-based models for investigating depression-relevant symptoms, social defeat in rats has been well characterized as engaging the sympathetic nervous system (10) and is the only experimental paradigm where ventricular arrhythmias have been reported in animals with normal hearts (10). We

thus applied this stress paradigm for the study of cardiac electrical activity in a rodent model of depression.

Our hypothesis was that rats exposed to intermittent homotypic social defeat stress would display pro-depressive symptoms at the behavioral, neuroendocrine and physiological level combined with enduring pro-arrhythmic changes in their myocardial electrical properties. To test this, nine days after the last social defeat episode we assessed potential enduring cardiac electrophysiological changes relevant to arrhythmogenesis by means of epicardial mapping.

2.2 METHODS

Animals, housing and preliminary surgery

Experiments were conducted on 10-week-old Wild Type Groningen male rats (*Rattus norvegicus*), weighing 300-350g. They were housed individually in Plexiglas cages measuring 39×23×15 cm. Additional older rats, weighing 550-600g, were housed with a sterilized female partner in larger plastic cages (60x35x40 cm) and used as residents in the resident-intruder paradigm (see next subchapter for details). All animals were kept in rooms with controlled temperature (22±2°C) and a reversed light-dark cycle (lights on from 19.00 to 7.00 h), with food and water *ad libitum.* All experimental procedures and protocols were approved by the Veterinarian Animal Care and Use Committee of Parma University, with animals cared for in accordance with the European Community Council Directives of 22 September 2010 (2010/63/UE).

Radiotelemetric transmitters (TA11CTA-F40, Data Sciences International, St. Paul, MN, USA) for recording electrocardiogram (ECG), core body temperature (T, °C) and locomotor activity (LOC, expressed as counts/minute, cpm) were implanted 1 week prior to the commencement of the experiments. Animals were anesthetized with tiletamine hydrochloride + zolazepam hydrochloride (Zoletil 200 mg/kg, s.c.) and the transmitters were implanted according to a surgical procedure that guarantees high quality ECG recordings even during vigorous physical activity (11).

Intermittent homotypic social defeat stress

The social defeat test was based on a classical “resident–intruder” paradigm (12). Before the start of each session, the female partners of the resident rats were removed from the cages. Each rat from the DEF group was transferred from its home cage to the resident’s cage, with a wire mesh partition separating the rats. During this phase (30 min), the DEF rat was protected from direct physical contact but it was in constant sensory contact with the resident. Then, the wire mesh partition was removed allowing physical interaction for 15 min. DEF rats (n=12) were attacked (latency to first attack: 65±9 s; number of attacks: 5.6±1.1) and defeated by the resident, that is, when the intruder rat assumed a supine posture that was held for at least 5 s. To avoid large individual differences in the intensity of received aggression, rats were exposed every time to a different opponent in a rotational design. The DEFs were exposed to 12 social defeat episodes over a period of 25 days, with social defeat sessions occurring daily for 4 days on week 1, twice a week during week 2 and week 3 and then daily for 4 consecutive days (9) (Fig. 1).

In correspondence to each session of defeat, CTR animals (n=11) were introduced to an unfamiliar empty cage (same dimension as the resident’s cage) with clean bedding. They were confined to half of the cage by a wire mesh partition for 30 min, followed by 15 min of free exploration of the cage without the partition. After each session, DEF and CTR rats were returned to their home cages. On two occasions (first and last episode of social defeat/unfamiliar cage exposure), continuous telemetric recordings were performed before (30 min), during (45 min) and after (30 min) the tests.

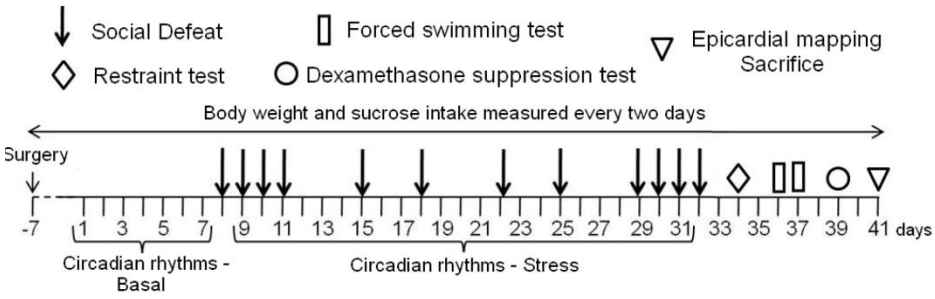

Figure 1. Timeline of the procedures used in the current study.

Body weight and sucrose intake

Body weight (BW) of experimental rats was measured every two days from the beginning to the end of the experiment. Sucrose solution intake was monitored to define anhedonia. Animals were allowed to drink from two preweighed bottles, one filled with 1% sucrose solution and the other one filled with tap water, throughout the experiment. The bottles were removed and weighed every two days, and sucrose solution intake was expressed as the relative percentage of the total liquid intake. In order to avoid place preference, bottle positions were switched every 24h. Anhedonia was defined as a reduction in sucrose solution intake relative to baseline values and relative to the control group.

Circadian rhythms

Heart rate (HR, bpm), T and LOC were sampled continuously for 60 s every 60 min in baseline conditions (days 1-7) and during the intermittent stress period (days 9-31) (Fig.1). The three parameters were quantified as means of 12-h inactive (light) and 12-h activity (dark) phases. For each animal, the daily amplitude of HR, T, and LOC rhythms was calculated as the difference between average active and inactive phase values, respectively.

Restraint test and ECG analysis

Two days after the last social defeat (Fig.1), each animal was introduced into a restrainer (wire-mesh tube; inner diameter: 6 cm, length: 20 cm) for 15 min. After the test, animals were returned to their home cages. Continuous ECG recordings were performed before (30 min), during (15 min) and after (30 min) the test. ECG signals (sampling frequency 1 kHz) were exported from the acquisition system (Data Sciences International, St. Paul, MN) into Chart5 software (ADInstruments, Sydney, Australia). We quantified the average inter-beat-interval duration (RR, ms), the root mean square of successive differences between adjacent RR intervals (r-MSSD, ms) and the percentage of successive interval differences larger than 10 ms (pNN10). R-MSSD and pNN10 quantify short- term, high-frequency variations of RR and therefore estimate the activity of the parasympathetic nervous system (13).

Calculations of these indexes were performed after removal of arrhythmic events and recording artifacts. The occurrence of ventricular and supraventricular premature beats (VPBs and SPBs, respectively) was determined and quantified off-line based on the Lambeth Conventions for the study of experimental arrhythmias (14).

Forced swimming test

An adapted version of the forced swimming test (FST) originally described by Porsolt (15) was used. The FST consisted of a 15-min training session followed 24h later by a 5-min test session (Fig. 1). During both sessions, rats were forced to swim individually in a Plexiglas cylinder (height: 40 cm, diameter: 30 cm) filled with water (temperature: 24±1°C; depth: 30 cm). During the test session, rats' behavior was videotaped, and the overall time spent in immobility was scored by a trained experimenter blind to animals' group. Immobility was defined as the animal floating without struggling and making only those movements necessary to keep its head above the water.

Dexamethasone suppression test

One week after the last defeat (Fig. 1), tail vein blood (0.5 ml) was collected and assayed for basal adrenocorticotropic hormone (ACTH) and corticosterone levels; immediately after, rats were injected subcutaneously with dexamethasone (synthetic glucocorticoid, 30 ug/kg), and four hours later tail vein blood (0.5 ml) was again collected to assess HPA axis reactivity via plasma ACTH and corticosterone level determinations. Blood samples were collected into chilled tubes containing ethylenediaminetetraacetic acid (EDTA). Samples were centrifuged at 4°C for 10 min at 2600 rpm, and 100 µl of the supernatant were stored at -20°C until assayed. ACTH and corticosterone were measured with a commercial RIA kit (MP Biomedicals, Orangeburg, NY, USA).

Epicardial mapping

Nine days after the last defeat (Fig. 1), rats were anesthetized with medetomidine (0.4 mg/kg, i.p.) and ketamine (50 mg/kg, i.p.). Subsequently, the heart was exposed through a longitudinal sternotomy (16). An epicardial electrode array (Fig. 2A) was used to record 64 unipolar epicardial electrograms (EGs) during normal sinus rhythm (NSR) and ventricular pacing (Fig. 2B), as previously described in detail (16).

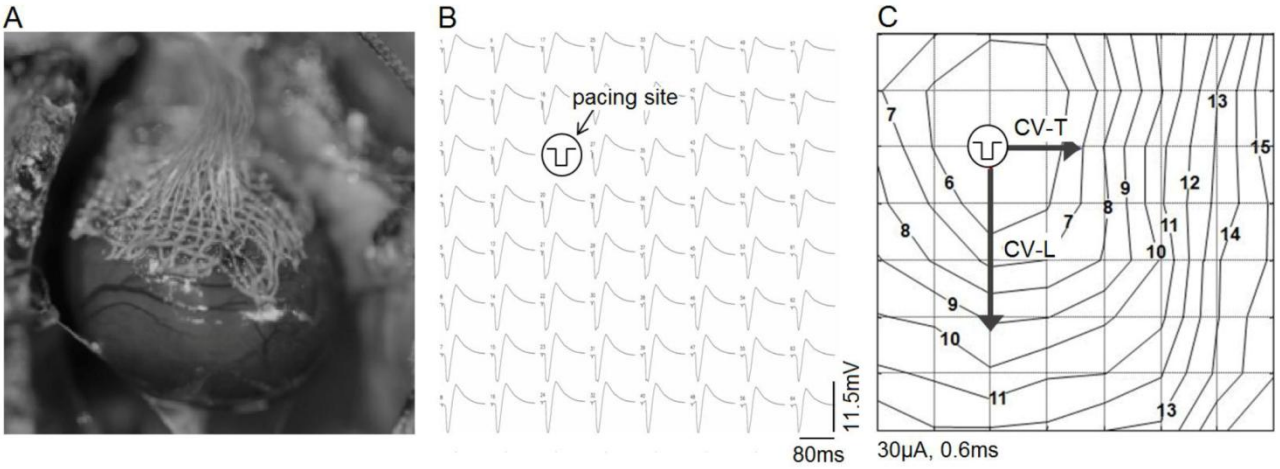

Figure 2. Epicardial mapping data of a representative rat. (A) 8×8 Electrode array on the anterior ventricular surface. (B) Unipolar electrograms collected during ventricular pacing at the electrode indicated by the pulse symbol. (C) Example of paced activation isochrone map used for computing conduction velocity longitudinally (CV-L) and transversally (CV-T) to fiber orientation; numbers on each isochrone line indicate the activation time in ms. Numerals at the bottom of the panels indicate current pulse strength and duration.

Subsequent analysis was conducted as follows:

Excitability. A strength-duration curve was obtained as a measure of cardiac excitability at two electrode positions. The strength-duration curve is represented by the equation I=Rh(1+Chr/T), where I is the threshold current strength, T is the pulse duration, Rh is the rheobase and Chr the chronaxie.

Conduction velocity (CV). Activation sequences (isochrone maps) were computed from the activation times of NSR or paced beats using custom written software and CV longitudinally and transversally to fiber orientation was calculated from them (Fig. 2C), as in (16).

Refractoriness. At 8 regularly selected electrodes of the array, eight baseline stimuli (S1), 1 ms duration and twice diastolic threshold were followed by a premature stimulus (S2), eight times threshold, whose delay from previous S1 was first progressively decremented by 10 ms steps until capture was lost and then progressively incremented by 2 ms steps till capture was resumed. The effective refractory period (ERP) was defined as the shortest S1-S2 time interval at which the excitation from S2 was failed.

Cardiac *intervals*. The duration of R-R interval, P wave, PQ segment, QRS complex, QT segment and corrected QT (normalized to cycle length: QTc) was measured from the root mean square signal computed from all the EGs.

At the end of each experiment, the heart was arrested in diastole by cadmium chloride solution injection (100 mM, i.v.).

Measurements at sacrifice

The heart was removed from the chest and fixed in 10% buffered formalin solution. We determined heart weight (HW), left ventricular weight (LVW), right ventricular weight (RVW) and their values relative to HW. LV free wall thickness and LV transverse diameters were morphometrically computed (Image Pro-plus 4.0 software, Media Cybernetics, USA) and LV chamber volume was calculated as in (17). Wedges of ventricular myocardium were embedded into a paraffin block with the epicardial surface facing upward. Each block was then sectioned with a microtome into 5μm-thick sections. Subsequently, sections were stained with Masson's trichrome in order to evaluate the total amount of fibrosis in the LV, as in (17). Adrenal glands were also removed, trimmed and weighed.

Statistical analysis

Two-way repeated measure ANOVA was applied for comparisons between DEFs and CTRs on data obtained from social defeat/unfamiliar cage tests, restraint test, circadian rhythms, BW gain, sucrose intake and ACTH and corticosterone determinations. Follow-up analyses were conducted using Student's "t" tests, with a Bonferroni correction for multiple comparisons for each outcome variable separately. A priori Student's "t"-tests, after controlling for homogeneity of variance via Levene test, were applied for comparisons between DEFs and CTRs on the occurrence of VPBs and SPBs during restraint and on data obtained from FST, cardiac mapping and measures at sacrifice. Statistical significance was set at $p<0.05$.

2.3 RESULTS

HR and T responses to the first and last social defeat

Before the first test, no differences were observed between the two groups in basal values of HR and T, whereas prior to the last social defeat the DEF rats had lower basal values of HR and T than the CTRs (Table 1). Social defeat provoked tachycardic and hyperthermic responses: indeed, the DEF rats showed higher HR and T values during the sensory contact and the interaction phases and higher T values during the recovery phases than the CTRs in the corresponding control conditions (Table 1). In the DEFs, no differences were observed in HR and T values between the first and the last episode of social defeat before, during and after the tests (Table 1). Mean values and statistical results are presented in Table 1.

Table 1 Heart rate (HR, bpm) and body temperature (T, °C) responses to the first and last social defeat episode in defeated (DEF) and control (CTR) rats.

			Baseline (30 min)	Sensory contact (30 min)	Interaction (15 min)	Recovery (30 min)
DEF (n=12)	First social defeat	HR	380±11	477±7*	514±9*	427±10
		T	37.9±0.1	39.1±0.1*	39.7±0.1*	39.0±0.0*
	Last social defeat	HR	362±6**	472±4*	495±5*	416±4
		T	37.8±0.1*	39.2±0.0*	39.9±0.1*	39.0±0.1*
CTR (n=11)	Unfamiliar empty cage	HR	364±9	446±6	435±8	424±4
		T	37.8±0.1	38.8±0.1	38.9±0.1	38.6±0.1
	Unfamiliar empty cage	HR	393±12	436±13	434±15	422±4
		T	38.1±0.1	38.6±0.1	38.9±0.1	38.5±0.1

*Data are expressed as mean±SEM. DEF= defeated rats; CTR= control rats. For periods corresponding to the sensory contact and the interaction phases, CTRs were exposed to an unfamiliar empty cage. ANOVA: significant effect of recording period (DEF: F_{HR}=97.3, p<.001; F_T=409.0, p<.001; CTR: F_{HR}=33.5, p<.001; F_T=63.8, p<.001). * p<.001 and ** p=.01 versus the respective CTR value (Student't' test).*

BW and sucrose solution intake

On day 7 (last day of pre-stress period) BW was 337±6 g for the DEFs and 355±8 g for the CTRs. During the week after the first social defeat, the DEF rats showed a significantly smaller increase of BW than the CTRs (DEF=3.1±2.0 g *vs.* CTR=15.0±1.6 g, p<.001) (Fig. 3A). This difference persisted until the end of the experiment (body weight gain: DEF=+11±1 % *vs*. CTR=+17±1 %, p=.001) (Fig. 3A).

During the pre-stress period (days 1-7) the DEF and the CTR rats showed a similar preference for the relative consumption of sucrose solution (58±1% and 60±6%, respectively). From day 9 to 40 both groups showed an overall reduction in sucrose solution intake compared to their respective basal levels (DEF=-19±6 % *vs.* CTR=-7±4 %, p=.006). Specifically, the magnitude of this reduction was significantly larger in the DEFs compared to the CTRs during the first (DEF=-25±7 % *vs.* CTR=-6±3 %, p=.04) and third (DEF=-21±5 % *vs.* CTR=-5±4 %, p=.02) weeks (Fig. 3B) of the intermittent stress period.

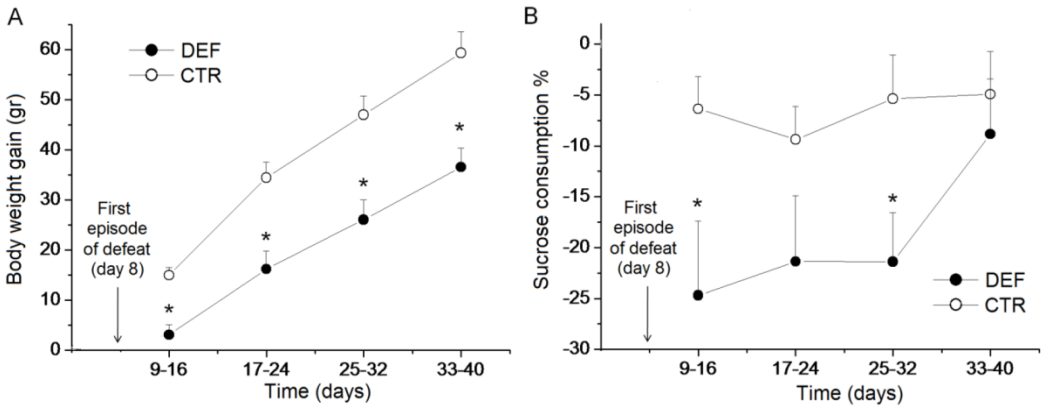

*Figure 3. Time course of body weight changes (panel A) and sucrose solution intake (panel B) in DEF (n=12) and CTR (n=11) rats during and after intermittent social-defeat stress. Body weight and sucrose solution intake were measured every two days, and each point is the mean±SEM of data obtained during the indicated periods. In (A), body weight is expressed as the respective increment to the corresponding pre-stress value (day 7). In (B), sucrose solution intake is expressed as the delta percentage to the pre-stress mean value (days 1-7). * = significantly different from corresponding CTR value (p values are reported in the text).*

Circadian rhythms

No differences between the DEF and the CTR rats were found in baseline circadian values of HR, T (Fig.4) and LOC (data not shown), neither for the absolute light and dark phase values nor for the rhythm amplitude.

From day 9 to 26 of the intermittent stress period, the DEF rats showed significantly higher values of HR than the CTRs during the inactive phases (Fig. 4A), and a consequent larger reduction in the circadian amplitude of HR (DEF=-32±3% *vs.* CTR=-13±2%, p=.001) (Fig. 4C).

Similarly, the DEFs had higher values of T than the CTRs during the inactive phases (Fig. 4B), and a consequent larger reduction in the circadian amplitude of T (DEF=-33±4% *vs.* CTR=-5±2%, p=.001) (Fig. 4D). In the same period, no differences were observed in the circadian rhythm of LOC between the two groups (data not shown).

ECG and T responses to the restraint test

No differences were observed in any basal cardiac parameter beteween the DEFs and the CTRs (RR: DEF=174.7±2.1 ms vs. CTR=167.3±4.6 ms; RMSSD: DEF=2.6±0.2 ms vs. CTR=2.5±0.2 ms; pNN10: DEF=0.6±0.2 % vs. CTR=0.7±0.2 %). Subjecting rats to restraint provoked similar cardiac acceleration (reduced RR index) and vagal withdrawal (reduced RMSSD and pNN10 indices) in the two groups (RR: DEF=128.7±2.3 ms vs. CTR=130.2±1.9ms; RMSSD: DEF=2.1±0.1 ms vs. CTR=2.1±0.1 ms; pNN10: DEF=0.2±0.1 % vs. CTR=0.1±0.0 %). During the restraint, the incidence of both SPBs (DEF=0.8±0.6 vs. CTR=1.0±0.5) and VPBs (DEF=1.1±0.5 vs. CTR=1.1±0.6) was very limited and similar between the DEF and CTR rats. Basal temperature values were identical in the two groups (DEF=37.6±0.0 °C vs. CTR=37.6±0.0 °C). Subjecting rats to restraint provoked hypertermia, with the magnitude of this increase being higher in the DEFs than CTRs both during restraint (DEF=0.9±0.1 °C vs. CTR=0.4±0.1 °C; p=.01) and the recovery phase (DEF= 1.0±0.1 °C vs. CTR=0.7±0.1 °C; p=.03).

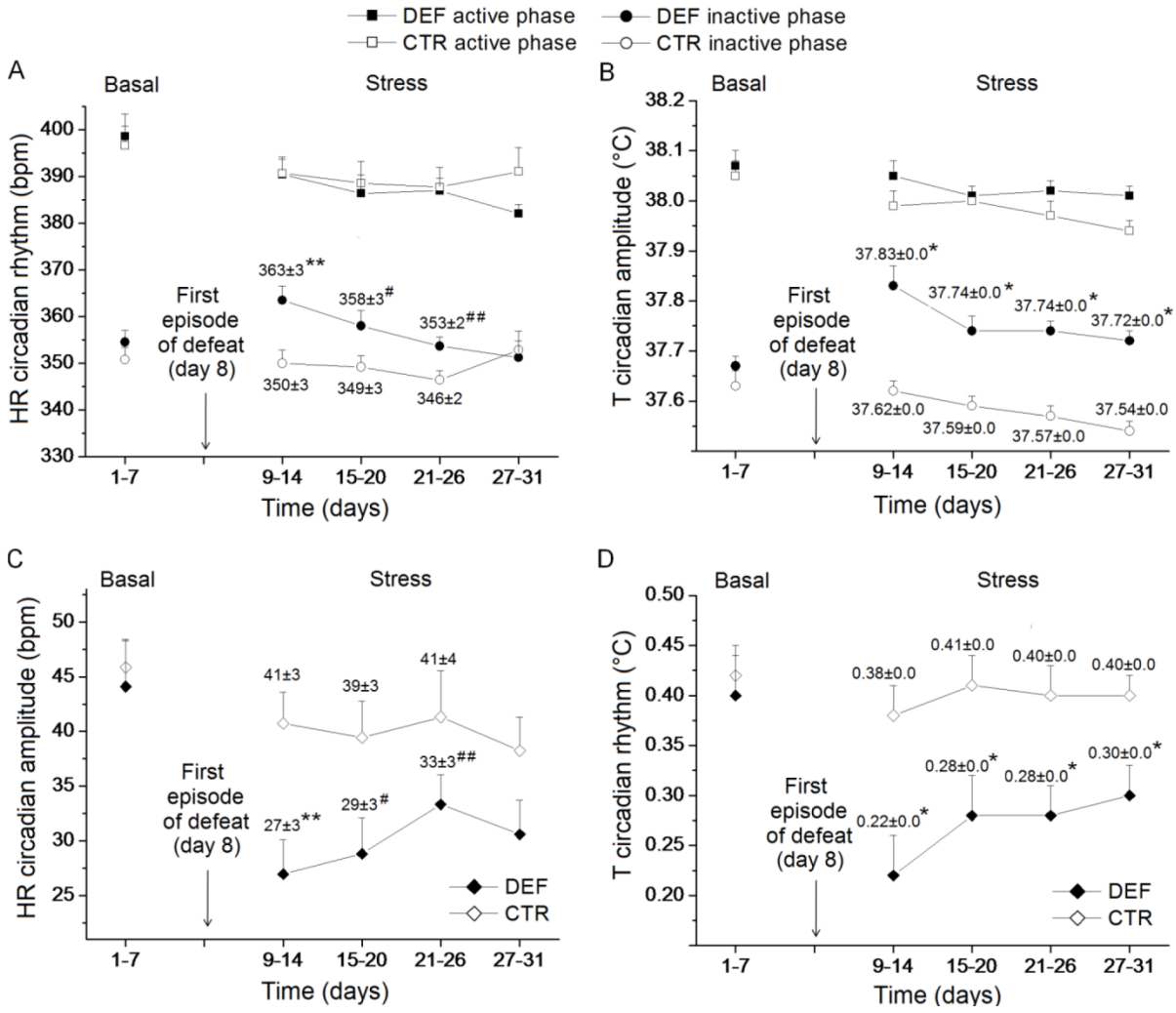

*Figure 4. Changes in circadian rhythms of HR and T in DEF (n=12) and CTR (n=11) rats during the intermittent stress period. (A, B) HR and T during active (squared symbols) and inactive (round symbols) phases of the circadian cycle. (C, D) Circadian rhythm amplitude of HR and T. Each point is the mean±SEM. ANOVA: significant effect of time for active phase (HR: F=12.5, p=.02; T: 17.3, p=.01) inactive phase (T: F=7.4, p=.02) and rhythm amplitude (HR: F=9.7, p=.005) values, significant effect of group for amplitude values (HR: F=6.2, p=.02; T: F=6.7, p=.02) and time x group interaction for inactive phase values (T: F=9.4, p=.007). * p<.001, ** p=.004, # p=.02, ## p=.04 versus the respective CTR group value (Student't' test).*

Forced swimming test

During the test session, the DEF rats spent a significantly longer time immobile then the CTRs (DEF=96±8 s *vs.* CTR=62±5 s, p=.02).

Dexamethasone suppression test

Basal, pre-dexamethasone injection ACTH levels were significantly lower in the DEFs than the CTRs (DEF=124±17 ng/ml *vs.* CTR=231±25 ng/ml, p=.002), whereas no differences were observed in basal corticosterone levels between the two groups (DEF=290±28 ng/ml *vs.* CTR=250±20 ng/ml) (Fig. 5). Dexamethasone injection provoked a significant reduction in ACTH levels compared to basal levels in the CTRs but not in the DEFs (DEF=-34±22 ng/ml; CTR=-147±21 ng/ml, p=.001), although the absolute post-injection values were similar in the two groups (Fig. 5A). Corticosterone levels after dexamethasone administration were similar in the DEF and the CTR rats and significantly lower compared to the respective basal levels (DEF=-236±29 ng/ml, p<.001; CTR=-214±23 ng/ml, p<.001) (Fig. 5B).

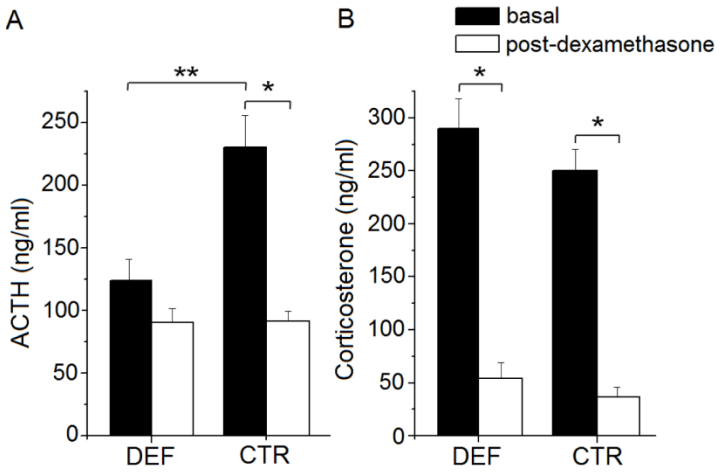

*Figure 5. Plasma ACTH (panel A) and corticosterone (panel B) levels before (basal) and 4h after a dexamethasone injection (post-dexamethasone) in DEF (n=12) and CTR (n=11) rats. HPA axis activity was evaluated one week after the last defeat. Each bar is the mean±SEM. * = significantly different from corresponding basal value and ** = significantly different from corresponding CTR value (p values are reported in the text).*

Epicardial mapping data

The epicardial mapping protocol was prematurely interrupted in six animals because of technical difficulties that precluded accurate recording. Therefore, epicardial mapping data were obtained only from the seventeen rats that completed the protocol.

To measure myocardial excitability, a strength-duration curve was constructed (Fig. 6A) and Rh and Chr values (Fig. 6B) were determined from it. The intensity of pacing stimuli required to excite the ventricle was significantly lower in the DEF than the CTR rats for stimulus duration of 0.01ms (DEF=2026±489 µA *vs.* CTR=3637±677 µA, p=.04). Similar current intensities between the two groups were needed to trigger an action potential for the other stimulus durations.

No differences were observed between the DEF and CTR rats in Rh and Chr values (Fig. 6B).

Analysis of conduction velocities obtained from isochrone maps during epicardial pacing revealed that transversal ventricular CV was significantly slower in the hearts of the DEF rats compared to the CTRs (p=.02, Fig. 6C), whereas no differences were observed in longitudinal ventricular CV between the two groups (Fig. 6C).

ERP was significantly shorter in the hearts of the DEF rats compared to the CTRs (p=.01, Fig. 6D).

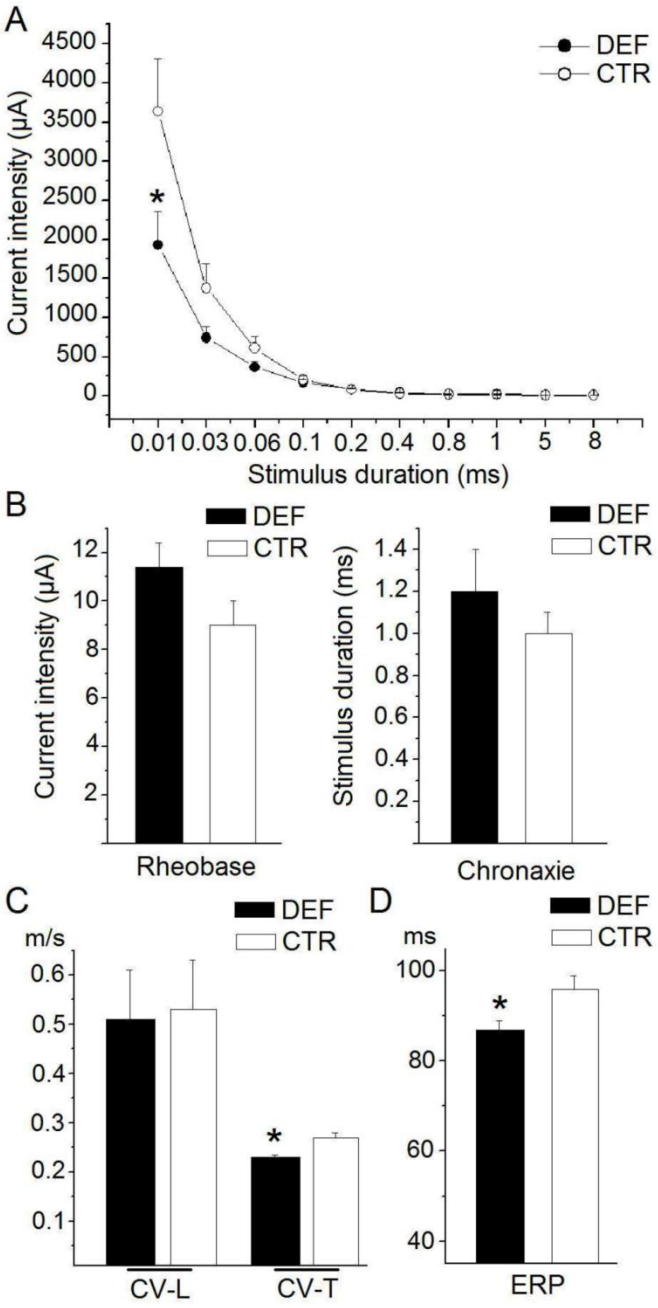

*Figure 6. Epicardial mapping data in DEF (n=9) and CTR (n=8) rats. (A) Strength-duration curve: shown are mean strength-duration values±SEM that were effective for inducing an action potential. (B) Rheobase and chronaxie. (C) Conduction velocity longitudinally (CV-L) and transversally (CV-T) to epicardial fiber direction. (D) Effective refractory period (ERP). The bars illustrate the mean values±SEM. * = significantly different from corresponding CTR value (p values are reported in the text).*

Cardiac interval durations are shown in Table 2. In the hearts of DEF rats we observed significantly larger RR and PQ segment duration values, and significantly smaller QRS wave and QTc segment duration values compared to the control hearts.

Table 2. Epicardial mapping parameters in defeated (DEF) and control (CTR) rats.

	DEF (n=9)	CTR (n=8)
RR interval	637.8±12.1*	572.9±7.8
P wave duration	30.6±0.3	30.0±0.3
PQ segment duration	24.9±0.3*	22.3±0.2
QRS complex duration	18.2±0.2*	18.9±0.1
QT segment duration	32.3±0.2	32.7±0.3
QT normalized to cycle length (QTc)	13.0±0.1*	13.8±0.2

Data are mean±SEM, expressed in ms.

** p<.001 versus the respective CTR group value (Student't' test).*

Cardiac anatomy and morphometry

Cardiac anatomy. No differences were observed between the DEF and the CTR rats with respect to the weight of the LV and RV and linear LV and RV parameters. Only LV chamber length was significantly longer in rats exposed to the intermittent social-defeat stress. However, LV chamber volume was unchanged in the DEF rats compared to the CTRs (Table 3).

Tissue morphometry. The volume fraction of myocytes was also unaffected by the social challenge (DEF=91.8±1.4 % *vs.* CTR=93.1±0.7 %). On the other hand, the total amount of myocardial fibrosis in the LV was significantly (3-fold) larger in the DEF rats compared to the CTRs (Table 3). This difference was mostly due to significantly larger perivascular collagen deposition in the heart of the DEFs, whereas no significant differences were observed in the amount of interstitial fibrosis between groups (Table 3).

Table 3. Gross cardiac characteristics and left ventricular myocardial fibrosis in defeated (DEF) and control (CTR) rats.

	DEF (n=12)	CTR (n=11)
HW (mg)	916.4±19.4	976.4±87.5
HW/BW (mg/g)	0.0025±0.0000	0.0024±0.0002
LVW (mg)	730.6±16.9	778.9±19.5
RVW (mg)	185.8±4.2	197.5±8.7
LVW/HW (mg/mg)	0.8±0.0	0.8±0.0
RVW/HW (mg/mg)	0.20±0.00	0.20±0.00
LV chamber length (mm)	11.4±0.3*	10.4±0.2
LV chamber equatorial diameter (mm)	4.8±0.3	4.7±0.1
LV chamber volume (mm^3)	136.6±11.5	126.2±6.8
LV wall thickness (mm)	2.4±0.1	2.6±0.1
RV wall thickness (mm)	1.1±0.1	1.1±0.1
LV total fibrosis (%)	0.55±0.13**	0.18±0.05
LV perivascular fibrosis (%)	0.45±0.12***	0.14±0.04
LV interstitial fibrosis (%)	0.09±0.06	0.04±0.04

*Values are expressed as mean±SEM. * p=.01, ** p=.02, *** p=.03 versus the respective CTR group value (Student't' test). BW= body weight; HW = heart weight; LV = left ventricle; LVW = left ventricular weight; RV = right ventricle; RVW = right ventricular weight.*

Adrenal weight

At sacrifice, adrenals were heavier in the DEFs compared to the CTRs (DEF=70.6±6.1mg *vs.* CTR=52.1±1.3 mg, p=.01). This difference persisted when adrenal weight was corrected for body weight (DEF=0.19±0.02 mg/g *vs.* CTR=0.13±0.01 mg/g, p=.003).

2.4 DISCUSSION

Characterization of stress-induced pro-arrhythmic changes in cardiac function is an important step for understanding the mechanisms underlying depression-related cardiovascular disease. In this study we demonstrated that rats displaying physiological and behavioral depression-like symptoms developed electrical (impaired conduction and reduced refractoriness) and structural (fibrosis) cardiac remodeling, consistent with the view that stress and depression are associated with an increased risk for ventricular arrhythmias and sudden cardiac death (18).

Intermittent social defeat stress provoked depression-like syndrome

Social defeat by an aggressive conspecific animal provoked potent tachycardic and hyperthermic responses, the latter likely due to sympathetically-induced activation of thermogenesis in the brown adipose tissue and to sympathetically-mediated vasoconstriction in the cutaneous vascular bed (19). Tachycardia and hyperthermia did not habituate over the period of intermittent homotypic stress, which is strong evidence of sustained sympathetic activation in rats undergoing social defeat stress.

Stressed animals exhibited enduring changes in several physiological and behavioral parameters that resemble some of the clinical symptoms of depression. First, the DEF animals displayed a long-term reduction in body growth, a common marker of depression in rats (9). Second, the larger decrease of sucrose solution intake observed in the DEFs than CTRs during the intermittent homotypic stress period is interpreted as a sign of anhedonia, a core symptom of depression (8, 20). This difference disappeared at the end of the intermittent stress period. This could be due to the fact that rats had sucrose solution continuously available and therefore they adapted to the taste. Alternatively, it could be argued that our control rats (repeatedly exposed to new unfamiliar cages) did not have “fully unstressed” values of behavioral/physiological parameters at the end of the intermittent stress protocol. Although this could be viewed as an objective limitation of this study, we think that our control group, rather than non-handled controls, was the best for investigating the actual effects of social subordination and defeat and thus did not limit the reliability of our conclusions.

In addition to reduced body weight gain and anhedonia, the DEF rats showed a dampening of the circadian rhythm amplitude of HR and body temperature during the period of intermittent homotypic stress. This was mainly due to a rise in the values of these parameters during the inactive phases. Similar circadian abnormalities are commonly described in depressed subjects (21) and are again strong indicators of sustained sympathetic activity in the DEF rats.

When tested in the forced swimming test, the DEFs displayed prolonged immobility, a relevant behavioral index of decreased motivation and behavioral despair (15).

We also investigated whether the exposure to intermittent homotypic stress also affects the acute response to a heterotypic stressor. During the restraint test, the two groups exhibited a similar decrease in RR values. This stress-induced tachycardia was at least in part mediated by vagal withdrawal in both groups, as indicated by the significant reductions in r-MSSD and pNN10 values (indices of cardiac vagal outflow). However, these data indicate that intermittent social-defeat stress did not affect the dynamics of cardiac autonomic balance in response to a heterotypic stressor. On the other hand, the DEFs showed a larger hyperthermia than the CTRs during and after the restraint. Therefore, it appears that body temperature may be a more sensitive marker than HR for assessing the increased stress responsiveness that often accompanies several psychological disorders (22). This may be due to the fact that body temperature is purely sympathetically regulated, whereas changes in cardiac sympathetic activity may be masked by counteracting changes in cardiac vagal outflow that lead to unaffected HR.

The activity of the HPA axis was evaluated one week after the last defeat. In the DEF rats we did not find the hypercortisolism that we would have expected in repeatedly stressed rats. However, the DEF rats displayed mismatches between ACTH and corticosterone levels compared to the CTRs. The administration of dexamethasone (synthetic glucocorticoid) suppressed corticosterone levels to the same extent in the two groups, whereas ACTH levels were reduced in the CTRs but not in the DEFs, although the absolute post-injection levels were similar between the two groups. This neuroendocrine picture can be explained by hypothesizing that: i) the DEFs developed an increased sensitivity to ACTH at the adrenocortical level; ii) basal ACTH levels in the DEFs were already low and could not be further reduced

by dexamethasone injection. Whatever the actual mechanism, the overall changes observed in the DEFs point to a functional and structural (increased adrenal weight, suggestive of hypertrophy) alteration of the HPA axis, as in several psychiatric conditions including depression (23).

Intermittent social defeat stress caused cardiac remodeling

Given the above presented evidence of the presence of a depressive-like state in rats exposed to repeated social defeat episodes, the central aim of our study was to assess enduring changes in their myocardial electrical properties. Analysis of epicardial electrograms revealed an increase in atrio-ventricular conduction time (PQ segment) in the heart of the DEFs compared to the control hearts. Also, in the DEFs ventricular activation was characterized by significant reduction of the QRS complex and QTc interval durations compared to the CTR animals. It has been demonstrated that β-adrenergic agonists shorten the QRS complex wave by increasing the inward sodium current in ventricular myocytes and consequently shortening the action potential duration (24, 25). It is thus logical to speculate that increased cardiac sympathetic tone may be one of the mechanisms responsible for the shortening of the QRS complex wave in the DEF rats. Other factors, presumably associated with myocardial volume and/or Purkinje-myocardial junction synchronization, may also contribute to QRS interval shortening. Because the sodium current is also a major determinant of conduction, enhanced sympathetic cardiac drive would accelerate CV (conduction velocity) within the ventricles. However, analysis of CV revealed that transverse CV was instead slower in the heart of the DEFs compared to the control heart, while longitudinal CV did not change significantly. Such transverse unidirectional slowdown of CV may favour micro-reentry via the transverse path (26), and thus can be considered as a pro-arrhythmogenic effect of intermittent stress exposure.

By using epicardial multiple-lead recording, we showed a shortening in cardiac refractoriness (ERP) in the DEF rats. Interestingly, some canine studies have demonstrated that sympathetic overactivity shortens ERP in the left ventricular myocardium (27), supporting the idea of increased sympathetic drive in the heart of the DEF rats. Shortening of ERP and reduction in CV are major determinants of

arrhythmogenesis. Their combined effect on impulse propagation is defined by the wavelength L = ERP x CV of the electrical impulse, which represents the distance traveled by the impulse propagation within one refractory period (28). A short wavelength (by reducing ERP and/or CV) increases the likelihood that single or multiple reentrant circuits can be accommodated by the heart. This possibly represents the arrhythmogenic mechanism whereby intermittent stress exposure provokes adverse cardiovascular events.

In addition to the effects on conduction and ERP, alterations in intercellular electrical connection can influence myocardial excitability. In our study, myocardial excitability was documented by computing the strength-duration curve. Rheobase and chronaxie values obtained from the curve were similar in the two groups. However, the curve showed a tendency for increased excitability in the hearts of the DEF rats that reached statistical significance for stimulus duration of 0.01ms. This finding is consistent with structural remodeling of the hearts of the DEF rats. Indeed, it has been reported that myocardial excitability is modulated by the properties of electrical coupling of the interconnected myocytes. In particular, the smallest myocardial region capable of initiating a propagated action potential (liminal length) is inversely related to the extracellular resistance, which largely depends on collagen deposition (29, 30). We hypothesize that the modest increase in fibrosis accumulation observed in the hearts of the DEF animals may have provoked increased extracellular resistance, leading to reduced liminal length and consequent increased myocardial excitability. These changes would reflect arrhythmogenic conditions in the DEF rats (29, 30).

In conclusion, this study provides new insight into the mechanicistic analysis of cardiac dysregulation in a rodent model of psychological depression, as defined by the presence in our stressed rats of some of the physiological and behavioral changes that resemble those observed in clinical patients. In particular, reduced myocardial refractoriness and impaired conduction, considered to be major determinants of arrhythmogenesis, represent long-lasting potentially pro-arrhythmic effects of intermittent stress exposure.

Limitations of the study

It must be recognized that the occurrence of arrhythmias following intermittent homotypic stress was very modest, thus not supporting the hypothesis that the electrophysiological remodeling found at sacrifice is in fact pro-arrhythmic. “Physiological” provocation of arrhythmias was assessed during the restraint test. Evocation of such minor arrhythmic effects during restraint can be explained by either of two mechanisms: (i) the relative mildness of this acute stressor or (ii) the fact that the evaluation of arrhythmia susceptibility was not temporally consistent (7 days before) with the assessment of pro-arrhythmic electrophysiological remodeling in the repeatedly stressed rats.

It also has to be acknowledged that this study does not provide insights into the biophysical mechanisms and the cellular and subcellular basis of the reported remodeling. Larger sample sizes are needed to document whether the magnitude of behavioral changes and their physiological correlates are related to markers of arrhythmic vulnerability and cardiac fibrosis.

One fundamental distinction between animal models of depression and depressed patients is that in humans, negative emotions, traumatic memories and ruminating thoughts/fears of the future are constantly present in the conscious state. In contrast, in our rats, cardiac sympathetic overactivity was limited to periods of aggressive interaction, with relatively rapid return to the baseline. This possibly explains relatively mild functional remodeling in the rat myocardium. Nevertheless, our study provides a foundation for systematic elucidation of detrimental effects of repeated stress exposure on the cardiac electrical stability.

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CHAPTER 3

ANTIDEPRESSANT-LIKE ACTIVITY AND CARDIOPROTECTIVE EFFECTS OF FATTY ACID AMIDE HYDROLASE INHIBITOR URB694 IN SOCIALLY STRESSED WISTAR KYOTO RATS

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ABSTRACT

Background: In humans, depression is mainly triggered by prolonged exposure to psychosocial stressors and is often associated with cardiovascular comorbidity. Mounting evidence suggests a role for endocannabinoid signaling in the regulation of both emotional behavior and cardiovascular function. Here, we examined cardiac activity in a rodent model of social stress-induced depression and investigated whether pharmacological inhibition of the enzyme fatty acid amide hydrolase (FAAH), which terminates signaling of the endocannabinoid anandamide, exerts antidepressant-like and cardioprotective effects.

Methods: Male Wistar Kyoto rats were exposed to five weeks of repeated social stress or control procedure. Starting from the third week, they received daily administration of the selective FAAH inhibitor URB694 (0.1 mg/kg, i.p.) or vehicle. Cardiac activity was recorded by radiotelemetry.

Results: Repeated social stress triggered biological and behavioral changes that mirror symptoms of human depression, such as (i) reductions in body weight gain and sucrose solution preference, (ii) hyperactivity of the hypothalamic-pituitary-adrenocortical axis, and (iii) increased immobility in the forced swim test. Moreover, stressed rats showed (i) alterations in heart rate daily rhythm and cardiac autonomic neural regulation, (ii) a larger incidence of spontaneous arrhythmias, and (iii) signs of cardiac hypertrophy. Daily treatment with URB694 (i) increased central and peripheral anandamide levels, (ii) corrected stress-induced alterations of biological and behavioral parameters, and (iii) protected the heart against the adverse effects of social stress.

Conclusions: Repeated social stress reproduces aspects of depression/cardiovascular comorbidity in Wistar Kyoto rats. Pharmacological enhancement of anandamide signaling might be a promising strategy for the treatment of these comorbid conditions.

3.1 INTRODUCTION

Extensive evidence suggests that depression is a robust and independent predictor of cardiovascular disease incidence and progression [1,2]. While a variety of mechanisms – including genetic and behavioral mechanisms – have been proposed to explain this association, one specific pathophysiological mechanism through which depression is thought to increase cardiac risk is a dysregulation of the autonomic neural control of cardiac function. For example, depressive patients have been found to display a predominance of sympathoadrenergic activation and/or reduced parasympathetic modulation, as evidenced by increases in resting-state heart rate (HR) and decreases in its variability (HRV) [3,4], both of which are considered to be predictive of adverse cardiovascular events [5]. However, other studies have reported contradictory findings, leading to significant debate and discussion as to whether autonomic function is altered in depression [6].

A common precipitating factor for the onset and progression of both depressive and cardiovascular disorders in vulnerable individuals is represented by prolonged or repeated exposure to stressors of psychosocial nature [7,8]. In preclinical settings, the resident-intruder paradigm is considered a relevant rat model of social stress that relies on robust ethological prerequisites to meet construct and etiological validity for the human condition [9]. Rats repeatedly exposed to this social stress paradigm show behavioral and biological changes that mirror human depression [10,11,12]. Importantly, such symptoms have been associated with long-lasting alterations in the autonomic neural modulation of HR [12] and pro-arrhythmic remodeling of electrical and structural properties of the myocardium [11]. Therefore, this stress paradigm appears to be a useful experimental approach for studying the shared pathophysiology and neural substrates that link depression and cardiovascular disease and may be used to identify novel therapeutic strategies for treating this comorbidity.

In this regard, recent years have witnessed an increasing interest in the role of the endocannabinoid (ECB) system in the regulation of emotional behavior [13,14] and cardiovascular function [15,16]. ECBs, which include anandamide (AEA) and 2-arachidonoylglycerol, and their receptors are prevalent throughout neuroanatomical structures and circuits that are implicated in depression, such as the

prefrontal cortex, hippocampus, amygdala and striatum [17,18], as well as throughout the cardiovascular system [19,20]. In the brain, ECBs have been shown to constrain the stress response, acting presynaptically on ECB_1 receptors and, mainly, modulating hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system activity, via down-regulation of excitatory (e.g. of glutamatergic) neurotransmission [21,22,23]. AEA is primarily catabolized *in vivo* by the enzyme fatty acid amide hydrolase (FAAH), which also cleaves the noncannabinoid fatty acid ethanolamides oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) [24]. Preliminary studies have demonstrated that facilitation of AEA signaling via inhibition of FAAH activity exerts antidepressant-like activity in chronically stressed rats [25], and that activation of the ECB pathway with exogenous AEA improves cardiac resistance to arrhythmias in rats [26,27]. Taken together, these findings provide a rationale for the preclinical assessment of the therapeutic potential of FAAH inhibitors for the treatment of depression-cardiovascular comorbidity.

Based on these considerations, in this study we applied a protocol of repeated social stress on animals with a predisposition to stress-related psychopathology (i.e., the Wistar Kyoto (WKY) rats) [28] in order to: (i) verify the presence of behavioral and biological signs of a depressive-like state; (ii) determine whether such symptoms were associated with changes in the autonomic modulation of HR (as indexed by HRV analysis) and increased incidence of spontaneous arrhythmias; (iii) evaluate whether pharmacological treatment with a selective FAAH inhibitor (URB694) exerts antidepressant-like activity and cardioprotective effects.

3.2 METHODS

Animals

Experiments were conducted on 3-month-old Wistar Kyoto male rats (Charles River, Italy). They were singly housed with a 12-h light cycle (lights on at 19.00 h) in climate-controlled rooms (20±2 °C). Food and water were available *ad libitium*, unless otherwise specified. Additional older Wild-type Groningen male rats were housed with an oviduct-legated female partner and used as residents in the resident-intruder paradigm (see below for details). All procedures were approved by the Veterinarian Animal Care and Use Committee of Parma University, with animals cared for in accordance with the European Community Council Directives (2010/63/UE).

Experimental design

Figure 1 displays the timeline of all procedures. We used 32 rats in Experiment 1 and 24 rats in Experiment 2. In each experiment, after baseline determinations, rats were randomly assigned to either a social stress (2 groups) or a control (2 groups) condition. Starting from day 19, stressed (STR) and control (CTR) groups received daily injection of either vehicle (VEH) or the selective FAAH inhibitor URB694. Specific experimental procedures are described in the following sections.

Drug treatment

URB694 was synthesized as previously described [29,30]. The compound was freshly prepared for administration as in [25]. Starting from day 19, CTR and STR rats received daily injections of VEH (vol: 1ml/kg) or URB694 (0.1mg/kg, i.p.) (Figure 1). The dose was chosen based on the available literature data and our experience with FAAH inhibitors [29,30]. All injections were made between 14.00 and 15.00 h.

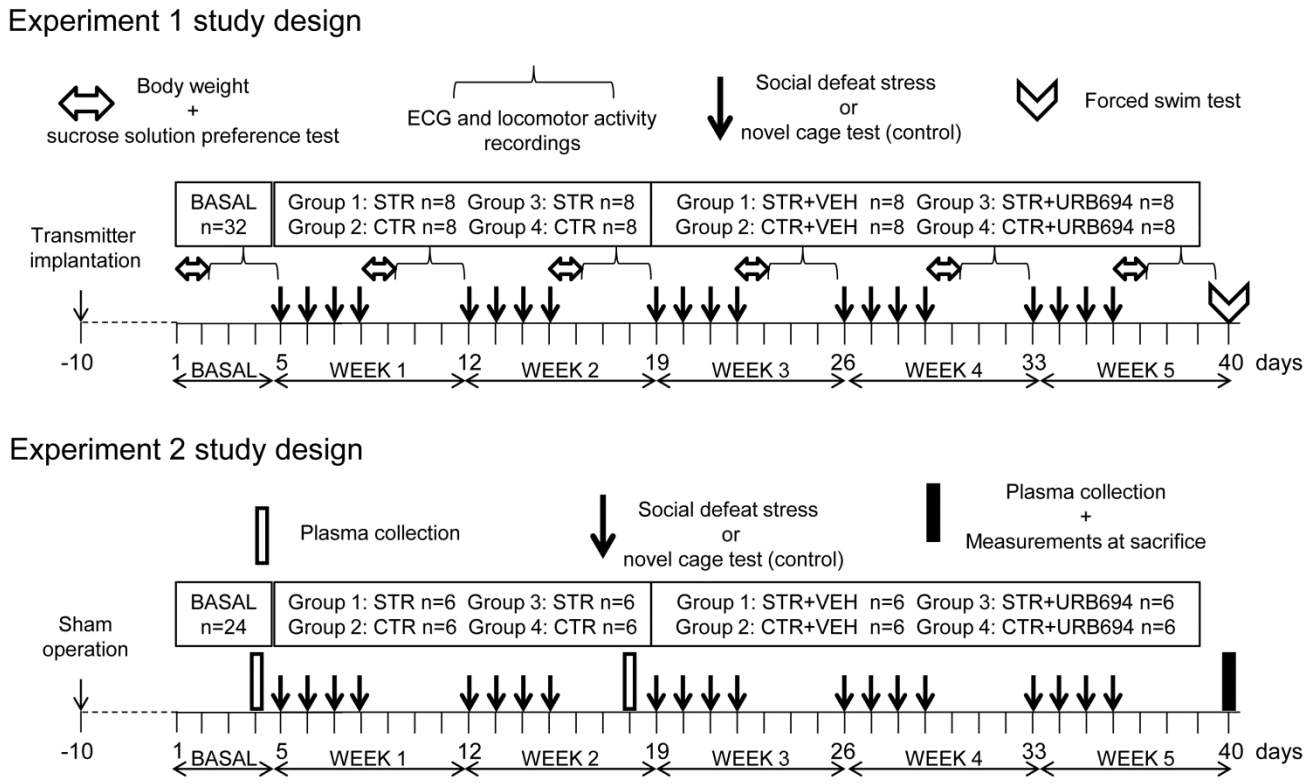

Figure 1. Timeline of experimental procedures in stressed (STR) and control (CTR) rats that were treated with either daily URB694 (0.1 mg/kg) or vehicle (VEH) starting from the beginning of the third week of the experimental protocol.

Surgery

Radiotelemetric transmitters (TA11CTA-F40, Data Sciences International, St. Paul, MN, USA) for ECG and locomotor activity (LOC, expressed as counts/minute, cpm) recordings were implanted in animals intended for Experiment 1 under tiletamine hydrochloride + zolazepam hydrochloride (Zoletil, 20 mg/kg, s.c.) anesthesia, according to [31]. Rats intended for Experiment 2 underwent a similar procedure without transmitter implantation (sham operation). Animals were allowed at least 10 days to recover before the start of recordings (Figure 1).

Social stress

Social stress was based on a classical “resident–intruder” paradigm [32] and was conducted similar to [11]. Briefly, each rat from the stress groups ("intruder") was transferred to the resident’s cage, with a wire mesh partition separating the rats for 30 min. Subsequently, the partition was removed allowing physical interaction for 10 min. Social stress exposure resulted in intruder subordination and defeat (i.e., when the intruder rat assumed a supine posture that was held for at least 5 s). STR rats were exposed to 20 social defeat episodes over a period of 5 weeks, with social defeat sessions occurring daily for 4 consecutive days every week (Figure 1). In the same days, CTRs were placed in a novel cage behind a partition for 30 min followed by 10 min of free exploration. All rats were returned to their home cages after each session, which took place between 9.00 and 12.00 h.

Body weight (BW) and sucrose preference test

BW and sucrose solution consumption preference were measured weekly for the duration of the study (Figure 1). Animals were weighed and subsequently food and water deprived for 15 hours prior to the sucrose solution consumption test [25]. During the test, animals were given access to water and 2% sucrose solution in premeasured bottles. One hour later, the two bottles were removed and weighed again and food and water were placed back in the cage. Sucrose solution intake was expressed as the relative percentage of the total liquid intake, and was taken as an operational index of anhedonia, defined as reduced sucrose preference relative to CTR animals and baseline values.

ECG and LOC data collection and analysis

ECG and LOC were sampled for 2 min every hour in baseline conditions and at the end of each week of the stress protocol (Figure 1). Daily rhythms of HR, HRV indexes and LOC were calculated as previously described [33]. Detailed procedures for HRV analysis are only briefly summarized here. Time- and frequency-domain analysis of HRV was conducted on multiple segments of continuous and stable ECG signals. In the time-domain, we calculated the square root of the mean squared

differences of successive RR intervals (RMSSD, ms), which reflects vagal input to the heart [34]. In the frequency-domain (fast-Fourier transformation), we measured (i) the power of the low (LF; 0.2-0.75 Hz) (ms^2) and the high (HF; 0.75-2.5 Hz) (ms^2) frequency bands, the latter reflecting respiratory-related vagal influences [35], and (ii) the LF to HF ratio, which is taken as a synthetic measure of sympathovagal balance [36].

In addition, the occurrence of arrhythmic events was determined and quantified off-line based on [37,38].

Forced swim test

An adapted version of the forced swim test originally described by Porsolt [39] was used. 24 h after the last URB694/VEH injection (Figure 1), rats were forced to swim individually for 5 min in a Plexiglas cylinder (height: 40cm, diameter: 30cm) filled with water (temperature: 24±1°C; depth: 30cm). During the test, rats' behavior was videotaped, and the overall time spent in immobility was scored by a trained experimenter blind to animals' group. Immobility was defined as the animal floating without struggling and making only those movements necessary to keep its head above the water.

Plasma collection and measurements at sacrifice

Tail vein blood was collected three times during the experimental protocol (Figure 1) and assayed for plasma levels of corticosterone, AEA, PEA and OEA. Blood was centrifuged (2600 g; 4°C; 10 min), and the supernatant was collected and stored at -20°C until analysis. 24 hours after the last URB694/VEH injection, animals were decapitated under anaesthesia (see above). Brains were rapidly removed and selected regions (prefrontal cortex, hippocampus and striatum) were dissected over dry ice using a rat brain atlas [40] as a guide, frozen in liquid nitrogen, and stored at -80°C until analysis. The heart and the adrenal glands were also removed, weighed and stored at -80°C until analysis.

Corticosterone analyses

Plasma was deproteinized by addition of two volumes of organic solvent, containing the internal

standard dexamethasone. After centrifugation (14000 g, 10 min, 4°C), the supernatant was directly injected in the liquid chromatography/tandem mass spectrometry system (HPLC/MS/MS) for quantification of corticosterone levels.

Analysis of fatty acid ethanolamides AEA, OEA, PEA

Fatty acid ethanolamides AEA, OEA and PEA were extracted from (i) the plasma, and (ii) 10% w/v brain tissue and atrial and ventricular homogenates by organic solvent addition and quantified by liquid HPLC/MS/MS spectrometry. The HPLC/MS/MS analytical standards AEA, OEA PEA and the deuterated internal standards, AEA-d_4 and PEA-d_4 were purchased from Cayman Chemicals (Ann Arbor, Michigan) as stock solutions in ethanol.

FAAH assay

For ex vivo determination of FAAH activity, frozen brains were thawed and homogenized in ice-cold Tris buffer (10 volumes, 50 mM, pH 7.5) containing 0.32 M sucrose. The homogenates were centrifuged (1000 g, 10 min, 4°C) and total protein content was quantified in the supernatant by Pierce BCA protein kit. FAAH activity was measured at 37°C for 30 min in 0.5 mL Tris buffer (50 mM, pH 7.5) containing fatty acid-free bovine serum albumin (BSA) (0.05 %, w/v), 50 µg of protein from brain homogenates, 10 µM anandamide and [^{3}H]-anandamide (10000 disintegrations per minute [dpm]). The reactions were stopped with 1 mL $CHCl_3$/MeOH (1:1). After centrifugation (2000 g, 10 min, 4°C), [^{3}H]-ethanolamine was measured in the aqueous phase by liquid scintillation counting. [^{3}H]-arachidonoylethanolamide (specific activity: 60 Ci/mmol), employed as a substrate for ex vivo FAAH assay, was purchased from American Radiolabeled Chemicals (ARC, St. Louis, Missouri).

Statistical analysis

Two-way ANOVA for repeated measures with group as between-subject factor (4 levels) was applied for data obtained from: (i) BW and sucrose solution preference test with time as within-subject factor (6 levels: baseline; stress week 1, 2, 3, 4 and 5); (ii) ECG and LOC recordings separately for dark and light phases, with time as within-subject factor (6 levels: baseline; stress week 1, 2, 3, 4 and 5); (iii)

plasma determinations with time as within-subject factor (3 levels: baseline; stress week 2 and 5). All other data were analyzed with 2 (stress or control exposure) x 2 (URB694 or VEH treatment) factorial design ANOVAs. Follow-up analyses were conducted using Student's "t" tests, with a Bonferroni correction for multiple comparisons for each outcome variable separately. Statistical significance was set at $p<0.05$.

3.3 RESULTS

BW

BW changes are shown in Figure 2A. Two-way ANOVA for repeated measures yielded a significant effect of time ($F=225.0$, $p<0.01$). Baseline BW was similar in the four groups. Starting from the end of the third week of the stress protocol, BW was significantly lower in STR+VEH compared to CTR+VEH rats ($t=2.73$, $p<0.05$). This difference persisted until the end of the experiment (fifth week: $t=3.6$, $p<0.01$). Chronic treatment with URB694 significantly increased BW gain in STR+URB rats compared to STR+VEH rats (fifth week: $t=2.2$, $p<0.05$), whereas it did not significantly modify BW gain in CTR+URB compared to CTR+VEH animals.

Sucrose solution consumption preference

Changes in sucrose solution preference are depicted in Figure 2B. Two-way ANOVA for repeated measures yielded a significant effect of group ($F=10.8$, $p<0.01$). In baseline conditions, the four groups showed a similar preference for the consumption of the sucrose solution. STR+VEH rats showed a reduction in sucrose solution preference compared to (i) CTR+VEH rats starting from the end of the second week ($t=2.3$, $p<0.05$) until the end of the experiment (fifth week: $t=4.8$, $p<0.01$), and (ii) baseline levels at the end of the fourth ($t=2.5$, $p<0.05$) and fifth ($t=2.9$, $p<0.05$) weeks. STR+URB rats showed a significant reduction in sucrose solution consumption preference compared to CTR+URB rats at the end of the second week of the stress protocol ($t=2.4$, $p<0.05$). Daily treatment with URB694 significantly increased sucrose solution preference up to baseline and control levels in STR+URB rats,

whereas it did not significantly modify sucrose solution preference in CTR+URB animals compared to CTR+VEH counterparts.

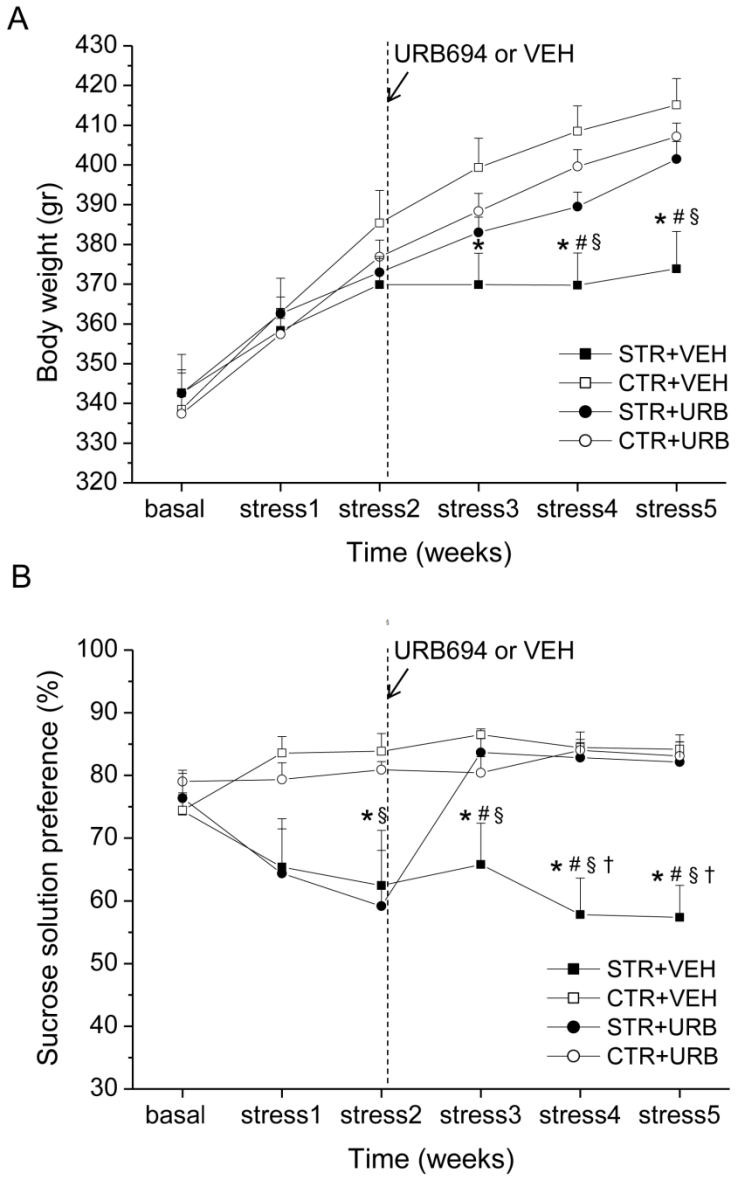

*Figure 2. Time course of changes in body weight (panel A) and sucrose solution preference (panel B) in baseline conditions and during the five weeks of repeated social stress. Data are reported as mean ± SEM for stressed (STR) and control (CTR) rats treated with either daily URB694 (URB) or vehicle (VEH) (n=8 per group). Significant differences (Bonferroni test, p values are reported in the text): * = versus CTR+VEH value; # = versus STR+URB value; § = versus CTR+URB value; † = versus baseline value.*

HPA axis activity

Changes in plasma corticosterone levels are reported in Figure 3. Two-way ANOVA for repeated measures yielded a significant effect of (i) time ($F=24.2$, $p<0.01$), and (ii) group ($F=4.6$, $p<0.05$). The four groups had similar baseline plasma corticosterone levels. Post-hoc analyses revealed that at the end of the stress protocol, STR+VEH rats showed significantly higher plasma corticosterone levels compared to (i) baseline levels ($t=4.0$, $p<0.01$), and (ii) CTR+VEH ($t=2.6$, $p<0.05$), CTR+URB ($t=3.2$, $p<0.05$) and STR+URB ($t=3.2$, $p<0.05$) rats.

In addition, adrenal weight corrected for BW was significantly heavier in STR+VEH rats compared to the other groups ($t=6.1$, $p<0.01$ vs. CTR+VEH; $t=3.5$, $p<0.01$ vs. STR+URB; $t=4.5$, $p<0.01$ vs. CTR+URB) (Table 1).

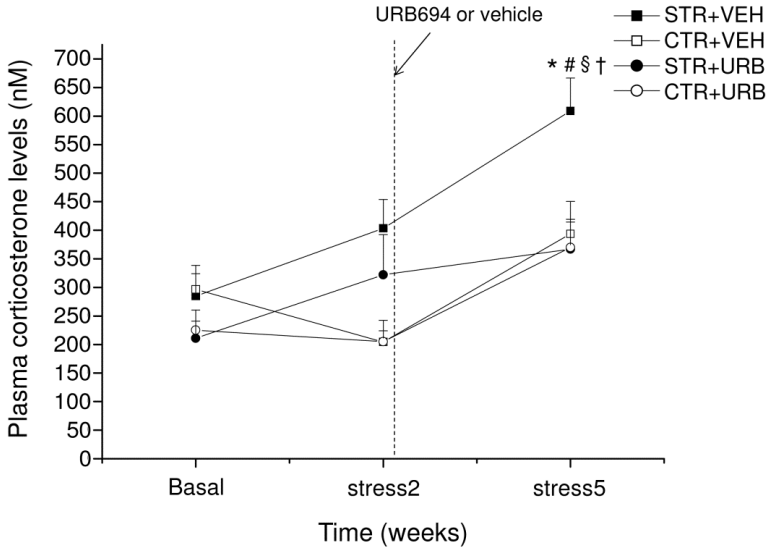

*Figure 3. Time course of changes in plasma corticosterone levels in baseline conditions and at the end of the second and fifth week of the stress protocol. Data are reported as mean ± SEM for stressed (STR) and control (CTR) rats treated with either daily URB694 (URB) or vehicle (VEH) (n=6 per group). Significant differences (Bonferroni test, p values are reported in the text): * = versus CTR+VEH value; # = versus STR+URB value; § = versus CTR+URB value; † = versus baseline value.*

Forced swim test

Behavior during the forced swimming test is illustrated in Figure 4. ANOVA yielded a significant effect of stress exposure ($F=28.7$, $p<0.01$) and drug treatment ($F=13.3$, $p<0.01$), and a stress x treatment interaction ($F=28.7$, $p<0.01$). Follow-up analyses revealed that STR+VEH rats spent more time in immobility compared to the other groups ($t= 5.2$, $p<0.01$ vs. CTR+VEH; $t= 3.9$, $p<0.01$ vs. STR+URB; $t= 5.1$, $p<0.01$ vs. CTR+URB).

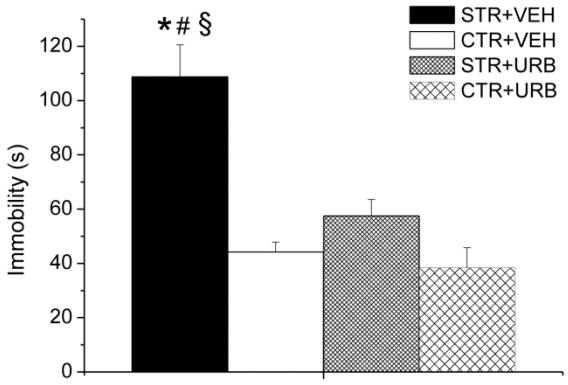

Figure 4. *Immobility during the forced swim test in stressed (STR) and control (CTR) rats treated with either daily URB694 (URB) or vehicle (VEH) (n=8 per group). Data are reported as mean ± SEM. Significant differences (Bonferroni test, p values are reported in the text): * = versus CTR+VEH value;* $^{\#}$ *= versus STR+URB value;* § *= versus CTR+URB value.*

HR daily rhythm

Changes in HR daily rhythm and its amplitude are shown in Figure 5A. Two-way ANOVA for repeated measures yielded: (i) a significant effect of time for dark phase HR ($F=20.0$, $p<0.01$) values; (ii) a significant effect of group for dark phase HR values ($F=3.4$, $p<0.05$) and HR rhythm amplitude ($F=3.6$, $p<0.05$); (iii) a time x group interaction for dark phase HR values ($F=3.4$, $p<0.05$). Follow-up analyses revealed that during the dark phase of the fifth week, STR+VEH rats had significantly lower HR values compared to baseline ($t=3.2$, $p<0.05$) and CTR+VEH ($t=2.9$, $p<0.05$) values, whereas no changes were observed between STR+URB and CTR+URB animals. In the same period, HR rhythm amplitude resulted significantly reduced in STR+VEH rats compared to the respective baseline value ($t=3.5$, $p<0.01$) and the other groups ($t=3.9$, $p<0.01$ vs. CTR+VEH; $t=3.8$, $p<0.01$ vs. STR+URB; $t=3.5$, $p<0.01$ vs. CTR+URB).

HRV parameters

HRV parameters during the dark phases of the baseline period and the fifth week of the stress protocol are illustrated in Figure 5B. Two-way ANOVA for repeated measures yielded a significant effect of time ($F=5.0$, $p<0.05$) and (ii) a time x group interaction ($F=4.5$ $p<0.05$) for dark phase LF/HF values. Follow-up analyses revealed that STR+VEH rats showed significantly lower LF to HF ratio during the fifth week compared to (i) the respective baseline value ($t=3.6$, $p<0.01$), and (ii) CTR+VEH rats ($t=3.0$, $p<0.05$), whereas no changes were observed between STR+URB and CTR+URB animals (Fig. 5B). There were no significant differences in RMSSD and HF values among the groups; therefore, no follow-up tests were performed.

LOC activity

Two-way ANOVAs for repeated measures yielded a significant effect of time ($F=4.8$, $p<0.05$) and (ii) a time x group interaction ($F=4.7$ $p<0.05$) for dark phase LOC values. Follow up analyses revealed that during the dark phase of the fifth week, STR+VEH rats showed significantly lower LOC values

compared to (i) CTR+VEH (t=2.2, p<0.05) and CTR+URB (t=2.2, p<0.05) values, and (ii) the respective baseline value (t=2.5, p<0.05) (Figure 5B).

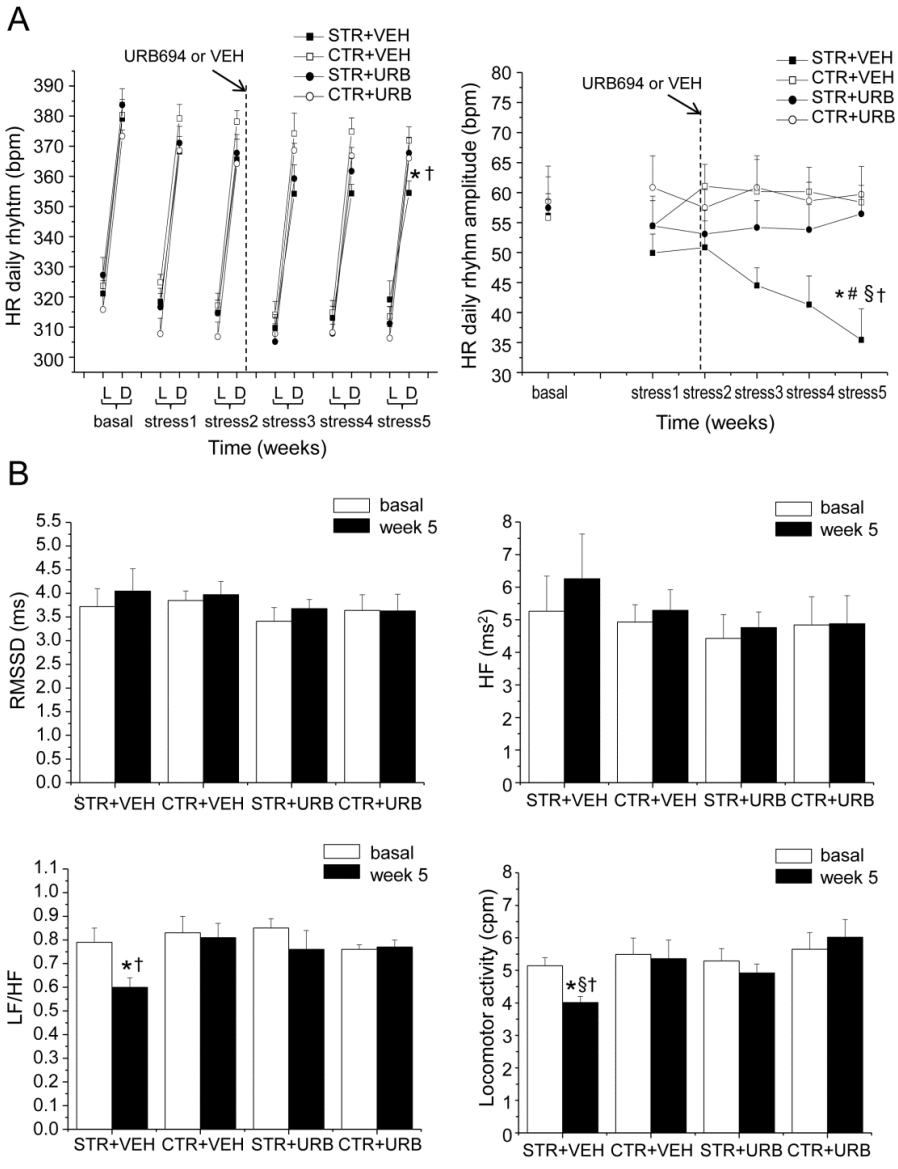

Figure 5. Panel A: time course of changes in (i) heart rate (HR) rhythm during the 12-h light (L) and 12-h dark (D) phases of the daily cycle (left), and (ii) HR daily rhythm amplitude (right). Panel B: heart rate variability and locomotor activity data collected during the dark phases of the basal period and at the end of the fifth week of the stress protocol. Data are reported as mean ± SEM for stressed (STR) and control (CTR) rats treated with either daily URB694 (URB) or vehicle (VEH) (n=8 per group).

*Significant differences (Bonferroni test, p values are reported in the text): * = versus CTR+VEH value; # = versus STR+URB value; § = versus CTR+URB value; † = versus baseline value.*

Vulnerability to cardiac arrhythmias

Examples of the most common forms of arrhythmias found in this study are reported in Figure 6A. Arrhythmia vulnerability was almost completely absent in baseline conditions (total incidence of arrhythmias: CTR+VEH= 0.38±0.25, STR+VEH= 0.56±0.37, CTR+URB= 1.03±0.58, CTR+VEH= 0.66±0.56). At the end of the fifth week of the stress protocol, the total incidence of arrhythmias was significantly higher in STR+VEH rats compared to the other groups (t=3.8, p<0.01 vs. CTR+VEH; t=3.6, p<0.01 vs. STR+URB; t=3.9, p<0.01 vs. CTR+URB) (Figure 6B). This was mainly due to a significantly higher incidence of sinus pauses in STR+VEH rats compared to the other groups (t=3.2, p<0.01 vs. CTR+VEH; t=2.5, p<0.05 vs. STR+URB; t=2.9, p<0.05 vs. CTR+URB) (Figure 6B).

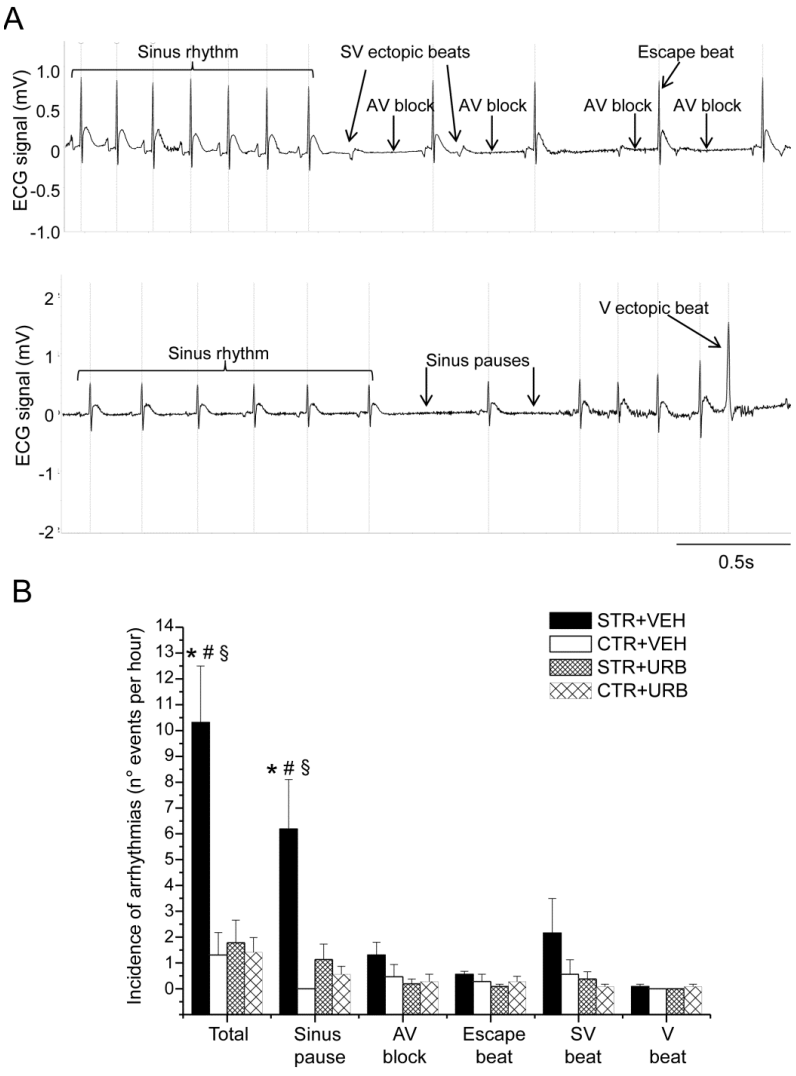

*Figure 6. Panel A: Examples of the most common arrhythmias found in this study from a representative STR+VEH rat (abbreviations: AV= atrioventricular; SV= supraventricular; V= ventricular). Panel B: Incidence of arrhythmias during the fifth week of the stress protocol in stressed (STR) and control (CTR) rats treated with either daily URB694 (URB) or vehicle (VEH) (n=8 per group). Data are reported as mean ± SEM. Significant differences (Bonferroni test, p values are reported in the text): * = versus CTR+VEH value; # = versus STR+URB value; § = versus CTR+URB value.*

Heart weight

Heart weight corrected for BW was significantly heavier in STR+VEH rats compared to the other groups (t=2.4, p<0.05 vs. CTR+VEH; t=2.8, p<0.05 vs. STR+URB; t=2.2, p=0.05 vs. CTR+URB) (Table 1).

Table 1. Body weight, adrenal weight and heart weight in stressed (STR) and control (CTR) rats treated with either daily URB694 (URB) or vehicle (VEH) (n=6 per group).

	BW (g)	AW (mg)	AW/BW (mg/g)	HW (g)	HW/BW (g/g)
STR+VEH	327±18*	34±2$^{\#}$	0.104±0.002*$^{\#\S}$	1.13±0.04	0.00347±0.00011*$^{\#\S}$
CTR+VEH	390±12	25±2	0.065±0.004	1.24±0.04	0.00317±0.00005
STR+URB	356±10	24±3	0.069±0.009	1.12±0.03	0.00315±0.00003
CTR+URB	357±4	25±2	0.071±0.006	1.13±0.04	0.00317±0.00008

*Data are reported as mean ± SEM. BW = body weight; AW= adrenal weight; HW= heart weight. Significant differences (Bonferroni test, p values are reported in the text): * = versus CTR+VEH value;* $^{\#}$ *= versus STR+URB value;* § *= versus CTR+URB value.*

FAAH activity

FAAH activity levels in the brain are depicted in Figure 7. There were no significant effects of stress exposure on FAAH activity levels in the prefrontal cortex, hippocampus and striatum. Daily treatment with URB694 significantly decreased FAAH activity in the (i) prefrontal cortex, (ii) hippocampus, and (iii) striatum. Results of post-hoc analyses are reported in Fig.7 legend.

Fatty acid ethanolamides levels

Brain levels

Brain AEA, OEA and PEA levels at the end of the experimental protocol are illustrated in Figure 7. There were no significant effects of stress exposure on AEA, PEA and OEA levels in the prefrontal cortex, hippocampus and striatum. Daily treatment with URB694 significantly increased (i) AEA levels

in the prefrontal cortex (F=30.2, p<0.01), hippocampus (F=28.9, p<0.01) and striatum (F=61.7, p<0.01), (ii) OEA levels in the prefrontal cortex (F=61.7, p<0.01), hippocampus (F=25.38, p<0.01) and striatum (F=8.2, p<0.01), and (iii) PEA levels in the prefrontal cortex (F=10.9, p<0.01) and hippocampus (F=25.4, p<0.01). Results of post-hoc analyses are reported in Fig.7 legend.

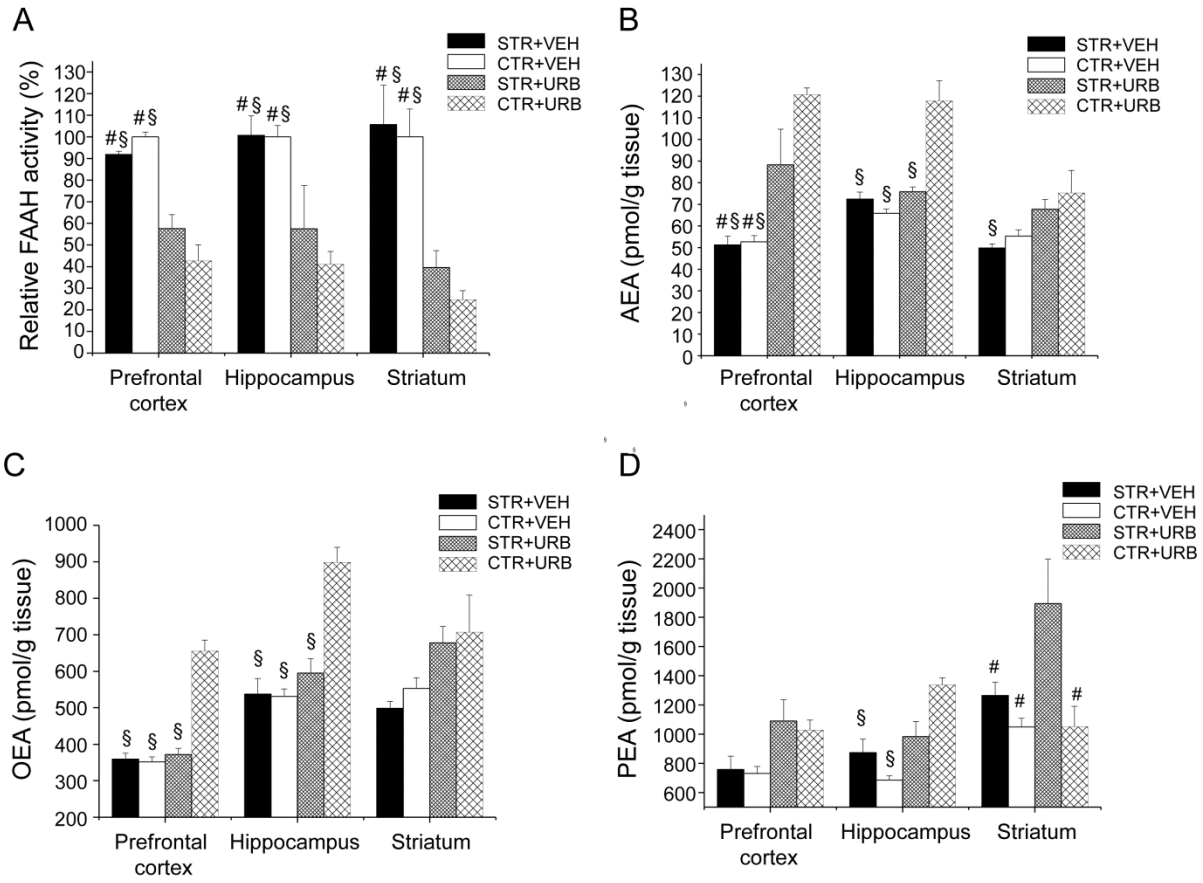

*Figure 7. Fatty acid amide hydrolase (FAAH) activity (panel A) and anandamide (AEA, panel B), oleoythanolamide (OEA, panel C) and palmitoylethanolamide (PEA, panel D) levels in the prefrontal cortex, hippocampus and striatum, 24 h after the last URB694/vehicle injection. Data are reported as mean ± SEM for stressed (STR) and control (CTR) rats treated with either daily URB694 (URB) or vehicle (VEH) (n=6 per group). FAAH activity was calculated as the relative % to CTR+VEH value. Significant differences (Bonferroni test, p values are reported in the text): * = versus CTR+VEH value; # = versus STR+URB value; § = versus CTR+URB value.*

Plasma levels

Changes in plasma AEA, OEA and PEA levels are reported in Table 2. Two-way ANOVAs for repeated measured yielded: (i) a significant effect of time for AEA ($F=6.9$, $p<0.05$), OEA ($F=8.9$, $p<0.01$) and PEA ($F=59.1$, $p<0.01$) levels; (ii) a significant effect of group for AEA ($F=5.9$, $p<0.01$), OEA ($F=6.2$, $p<0.01$) and PEA ($F=5.8$, $p<0.01$) levels; (iii) a time x group interaction for PEA levels ($F=7.7$, $p<0.01$). Follow-up analysis revealed that at the end of the experimental protocol (week 5), STR+VEH and CTR+VEH had significantly lower plasma levels of AEA, PEA and OEA compared to (i) the respective baseline levels, and (ii) STR+URB and CTR+URB levels (p values are reported in the legend of the figure). In addition, daily administration of URB694 increased plasma levels of AEA in STR+URB and CTR+URB rats compared to pre-treatment (second week) levels (p values are reported in Table 2 legend).

*Table 2. Plasma levels of AEA, OEA and PEA in stressed (STR) and control (CTR) rats treated with either daily URB694 (URB) or vehicle (VEH) (n=6 per group), in basal condition and at the end of the 2nd and 5th week of the experimental protoco*l.

		Basal	week 2	week 5
AEA (nM)	STR+VEH	0.94±0.10	0.53±0.05†	0.61±0.07$^{\#\S\dagger}$
	CTR+VEH	1.18±0.18	0.72±0.05†	0.69±0.05$^{\#\S\dagger}$
	STR+URB	0.97±0.09	0.76±0.12†	1.02±0.09‡
	CTR+URB	1.09±0.15	0.69±0.06†	1.12±0.08‡
OEA (nM)	STR+VEH	8.46±0.33	7.78±0.49	7.20±0.43$^{\#\S}$
	CTR+VEH	8.08±0.77	8.92±0.73	7.19±0.35$^{\#\S}$
	STR+URB	9.03±0.78	8.28±0.90	9.41±0.42
	CTR+URB	12.19±1.01	10.20±0.48	9.71±0.06
PEA (nM)	STR+VEH	10.86±0.43	11.47±0.35	4.95±1.06$^{\#\S\dagger}$
	CTR+VEH	10.98±0.73	10.50±0.94	5.38±0.73$^{\#\S\dagger}$
	STR+URB	11.83±0.49	11.18±0.92	8.58±0.88†
	CTR+URB	12.43±0.82	10.65±0.64	12.32±0.68

Data are reported as mean ± SEM. AEA = anandamide; OEA= oleoythanolamide; PEA= palmitoylethanolamide. Significant differences (Bonferroni test): $^{\#}$ = p<0.05 versus STR+URB value; § = p<0.05 versus CTR+URB value; † = p<0.05 versus baseline value; ‡ = p<0.05 versus week 2 value.

Heart levels

In atrial homogenates, there were no significant effects of stress on AEA, OEA and PEA levels (Table 3). Daily treatment with URB694 significantly increased AEA ($F=14.8$, $p<0.01$), OEA ($F= 29.9$, $p<0.01$) and PEA ($F=42.8$, $p<0.01$) levels (Table 3). In ventricular homogenates, AEA levels were significantly increased by daily treatment with URB694 ($F=5.1$, $p<0.05$) and were not affected by stress exposure. Moreover, PEA levels were significantly reduced by stress exposure ($F=10.2$, $p<0.01$) and increased by daily treatment with URB694 ($F=7.8$, $p<0.05$) (Table 3). Results of post-hoc analyses are reported in Table 3 legend.

Table 3. AEA, OEA and PEA levels in atrial and ventricular homogenates of stressed (STR) and control (CTR) rats treated with either daily URB694 (URB) or vehicle (VEH) (n=6 per group).

		Atria	Ventricles
AEA (pmol/g)	STR+VEH	28.2±2.2$^{\#}$	22.7±1.3§
	CTR+VEH	23.1±2.5$^{\#}$	33.6±4.4
	STR+URB	40.3±2.8	34.7±4.0
	CTR+URB	31.0±2.6	38.4±4.2
OEA (pmol/g)	STR+VEH	64.7±5.5$^{\#\S}$	66.4±1.4
	CTR+VEH	57.4±8.1$^{\#\S}$	79.2±5.2
	STR+URB	102.5±4.1	86.6±2.8
	CTR+URB	100.0±10.1	74.3±6.6
PEA (pmol/g)	STR+VEH	66.3±4.0$^{\#\S}$	71.7±3.6§
	CTR+VEH	56.2±8.7$^{\#\S}$	87.5±7.8§
	STR+URB	106.2±8.2	100.9±7.6§
	CTR+URB	178.5±21.4	172.0±22.5

Data are reported as mean ± SEM. AEA = anandamide; OEA= oleoythanolamide; PEA= palmitoylethanolamide. Significant differences (Bonferroni test): $^{\#}$ = $p<0.05$ versus STR+URB value; § = $p<0.05$ versus CTR+URB value.

3.4 DISCUSSION

The major and novel finding of the present study is that the potent and selective FAAH inhibitor URB694 normalizes (i) biological and behavioral depressive-like symptoms, and (ii) cardiac alterations elicited by repeated social stress in WKY rats.

Depressive-like syndrome in socially stressed WKY rats

There is incresing recognition that, on an inherited predisposing basis, the development of mood disorders may be precipitated by a number of environmental factors, such as, for example, chronic life stressors [41]. Therefore, the application of a social stress paradigm to animals with a predisposition to a depressive-like phenotype (WKY rats) [28] is, in our view, a valid experimental approach to model biological and behavioral changes reminiscent of those observed in human depression.

Stressed WKY rats treated with vehicle displayed a long-term reduction in body weight growth, a common marker of depression in rats [10]. Moreover, they exhibited an increase in plasma corticosterone levels and adrenal gland weight. Of note, such changes were observed four days after the last defeat, suggesting chronic HPA axis hyperactivity, a well documented finding in depression [42]. In addition, the decrease in sucrose solution preference in stressed animals may reflect the onset of an anhedonic state, a core symptom of depression [43]. Finally, their prolonged immobility in the forced swim test may be regarded as a relevant index of the depressive-like symptom of helplessness [44]. Taken together, these findings indicate that repeated social stress induced persistent changes in a variety of parameters relevant to depression in WKY rats.

Antidepressant-like activity of URB694

Previous studies have reported that chronic stress impairs ECB signaling, at either the ligands or the receptor levels [45,46]. Here, we did not find significant effects of repeated social stress exposure on central levels of AEA, similarly to another study [25]. A possible explanation is that control animals also presumably experienced certain amount of stress (e.g. novel cage test, daily injections). Indeed,

at the end of the experimental protocol plasma AEA levels resulted significantly lower in both stressed and control animals injected with vehicle compared to the respective baseline levels. One possible limitation of this study is that we did not determine the expression of ECB receptors, which would have provided a more complete picture on the effects of this social stress protocol on ECB neurotransmission. As expected, daily administration of the potent and selective FAAH inhibitor URB694 provoked a long-lasting reduction in FAAH activity in the prefrontal cortex, hippocampus and striatum. This, in turn, resulted in an increase in AEA levels both in the selected brain areas (especially in the prefrontal cortex) and in the periphery. Importantly, chronic treatment with URB694 normalized the effects of social stress on body weight growth, sucrose solution preference and behavior in the forced swim test. Of note, this drug regimen had no effects on controls, suggesting that the increase of AEA levels via inhibition of its degradative enzyme (FAAH) did not affect normal biological processes and behavioral responses. Preclinical research regarding the role of the ECB system in the regulation of stress and emotional behavior creates a compelling argument that ECB signaling in the prefrontal cortex is crucial for constraining activation of the neuroendocrine and behavioral response to stress [14,23,47]. For example, it has been shown that ECB_1 activation in the prefrontal cortex suppresses glucocorticoid secretion after cessation of a stressor [48]. Accordingly, in this study daily treatment with URB694 also prevented the hypercorticosterolemia and adrenal hypertrophy observed in stressed rats treated with vehicle. Therefore, our hypothesis is that the beneficial effects of URB694 on the neuroendocrine and behavioral adverse consequences of repeated stress exposure may be due to the ability of FAAH inhibitors to magnify endogenous AEA signaling at ECB_1 receptors, particularly in the prefrontal cortex [14]. This is also supported by (i) experimental evidence showing that the ECB_1 antagonist rimonabant prevents the mood-enhancing actions of a similar FAAH inhibitor (URB597) [49,50], and (ii) the fact that other targets of FAAH, such as OEA and PEA, do not activate ECB receptors, but are thought to influence ECB signaling by competing with AEA for catabolic activity of FAAH: the so called “entourage effect” [51].

Social stress-induced cardiac abnormalities

Stressed rats treated with vehicle showed a progressive dampening of the daily amplitude of HR rhythm, which was mainly due to a reduction of HR values during the dark phase of the daily cycle. Such an effect may be ascribed to lower levels of somatomotor activity in these animals. However, we did not find a significant correlation between the magnitudes of HR and LOC decreases. This suggests that other factors likely concurred to determine stress-induced bradycardia. Indeed, HRV analysis revealed that repeated social stress induced a shift of the sympathovagal balance towards parasympathetic prevalence (decreased LF to HF ratio) in vehicle-treated rats, without an increase in the absolute levels of vagal tone (RMSSD and HF indexes). These HRV data suggest that sympathetic influences on cardiac pacemaker activity were likely reduced in these animals, causing bradycardia. This was an unexpected phenomenon, as human depression is often associated with sympathetic hyperactivity [3,4]. However, in a recent study depressive patients have been shown to exhibit signs of parasympathetic prevalence (decreased LF to HF ratio) during mental stress compared to controls [52]. The authors suggested that this finding may signal the accumulative effects of depression on the cardiovascular system in real life stress situations. Like psychosocial stress in humans, social stress in rats has been well characterized as engaging the sympathetic nervous system [53]. Therefore, our hypothesis is that repeated episodes of social defeat might have determined a desensitization/downregulation of cardiac β-adrenoreceptors in vehicle-treated animals. This would explain why cardiac sympathetic influences were reduced in these animals. These specific patterns of cardiac autonomic modulation were coupled with an increased incidence of spontaneous arrhythmias, particularly sinus pauses. Sinus pauses, alternatively known as sinus arrest or atrial standstill, are due to cessation of the sinus node impulse and result in absent PQRST complexes unless an escape site becomes the pacemaker [38]. These arrhythmic events are generally attributed to conditions of excessive vagal modulation [38,54], indicating that persistent vagal predominance may have predisposed the heart of stressed rats to sinus node dysfunction. However, we cannot exclude that arrhythmogenesis was due to stress-induced alterations in the structural characteristics

(such as the reported cardiac hypertrophy) and/or in the electrophysiological properties of the myocardium [11]. It may be argued that the incidence and severity of the arrhythmic events reported in this study were rather moderate. However, it should be kept in mind that arrhythmia susceptibility was assessed in young, otherwise healthy rats during undisturbed resting conditions. In addition, an important distinction between animal models of depression and depressive patients is that, in humans, negative emotions, traumatic memories and ruminating thoughts are constantly present in the conscious state. In contrast, in our rats, cardiac sympathetic hyperactivity was likely limited to periods of aggressive interaction [11,53]. This may explain the relatively mild effects of this social stress protocol on cardiac electrical stability.

Cardioprotective effects of URB694

Chronic treatment with URB694 normalized resting heart rate, restored the autonomic balance and prevented arrhythmia occurrence in stressed animals. The drug regimen had no effects on controls, in line with the idea that ECBs do not mediate tonic control over the cardiovascular system in normal physiology [15]. ECB_1 receptors are present on pre-synaptic sympathetic nerve terminals and *in vitro* studies report that their activation decrease sympathetic outflow by inhibition of noradrenaline release [55]. It is therefore tempting to speculate that the cardioprotective effects of URB694 might be due to its ability to dampen stress-induced sympathetic hyperactivity via enhancement of AEA signaling at these receptors. However, we cannot exclude that the effects of the FAAH inhibitor are mediated directly on the myocardium. Indeed, our data indicate that administration of URB694 determined a general increase in AEA, OEA and PEA levels in heart homogenates. Moreover, preliminary studies have shown that exogenous application of either AEA or PEA confers cardiac protection [56,57] and increases cardiac resistance to the arrhythmogenic effects of ischemia and reperfusion [26], with the underlying molecular and electrophysiological mechanisms that have yet to be elucidated. Further investigation is required in order to clarify whether URB694 exerts its anti-arrhythmic effects directly on the myocardium by increasing the threshold for arrhythmias and/or indirectly by restoring the

autonomic balance either at the central or peripheral level. Whatever the mechanism prevails, our results suggest that inhibition of FAAH activity improves cardiac status in WKY rats exposed to repeated social defeat stress.

Conclusion and perspectives

This study provides solid evidence that facilitation of AEA signaling with the potent and selective FAAH inhibitor URB694 corrects a variety of biological and behavioral depressive-like changes induced by repeated social stress in WKY rats. Intriguingly, this pharmacological approach appears to confer also cardioprotection against the adverse effects of social stress in this animal model. Clearly, more needs to be known about the physiological roles of AEA as well as of other fatty acid ethanolamides such as OEA and PEA in order to predict possible additional benefits or unwanted side effects of FAAH inhibitors. In interpreting our results, it must also be acknowledged that WKY rats have been shown to have higher levels of brain FAAH compared to Wistar rats [58] and therefore might be hyper-responsive to the treatment with the FAAH inhibitor used in our study. Nevertheless, our findings provide a strong basis for further investigation aimed at determining whether FAAH might represent an effective therapeutic target for the treatment of depression-cardiovascular comorbidity under chronic stress conditions.

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CHAPTER 4

LOW VAGALLY-MEDIATED HEART RATE VARIABILITY AND INCREASED SUSCEPTIBILITY TO VENTRICULAR ARRHYTHMIAS IN RATS BRED FOR HIGH ANXIETY

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ABSTRACT

In humans, there is a documented association between anxiety disorders and cardiovascular disease. Putative underlying mechanisms may include an impairment of the autonomic nervous system control of cardiac function. The primary objective of the present study was to characterize cardiac autonomic modulation and susceptibility to arrhythmias in genetic lines of rats that differ largely in their anxiety level. To reach this goal, electrocardiographic recordings were performed in high-anxiety behavior (HAB, n=10) and low-anxiety behavior (LAB, n=10) rats at rest, during stressful stimuli and under autonomic pharmacological manipulations, and analyzed by means of time- and frequency-domain indexes of heart rate variability. During resting conditions, HAB rats displayed a reduced heart rate variability, mostly in terms of lower parasympathetic (vagal) modulation compared to LAB rats. In HAB rats, this relatively low cardiac vagal control was associated with smaller heart rate responsiveness to acute stressors compared to LAB counterparts. In addition, beta-adrenergic pharmacological stimulation induced a larger incidence of ventricular tachyarrhythmias in HABs compared to LABs. At sacrifice, a moderate increase in heart-body weight ratio was observed in HAB rats. We conclude that high levels of anxiety-related behavior in rats are associated with signs of i) impaired autonomic modulation of heart rate (low vagally-mediated heart rate variability), ii) poor adaptive heart rate responsiveness to stressful stimuli, iii) increased arrhythmia susceptibility, and iv) cardiac hypertrophy. These results highlight the utility of the HAB/LAB model for investigating the mechanistic basis of the comorbidity between anxiety disorders and cardiovascular disease.

4.1 INTRODUCTION

Converging evidence from both epidemiological and experimental studies indicates that there is a bidirectional association between anxiety disorders and cardiovascular disease [1, 2]. Patients suffering from anxiety disorders are at higher risk of morbidity and mortality related to heart disease [3-5], and cardiovascular disease may induce anxiety [6, 7]. Despite this, the mechanisms that underlie these associations are far from being completely understood.

Alterations of the autonomic neural control of cardiac function may play a mediating role in the link between anxiety disorders and cardiac pathophysiology. Basal and stress-induced changes in the autonomic modulation of heart rate (HR) have been described in humans with anxiety disorders [8], and they are also common in heart disease [9]. In particular, the notion that anxious individuals are often characterized by a relatively low vagal component of heart rate variability (HRV) has long-standing support in the literature [9-11].

Research into the study of cardiac autonomic modulation that characterizes anxiety in its state, trait and psychopathological forms may provide insights into the mechanisms underlying its comorbidity with cardiovascular disorders. Traditionally, preclinical research has focused on the study of autonomic correlates of stress-evoked anxiety. Specifically, animals displaying anxiety-like states in response to psychological stressors have been shown to be characterized by a cardiac autonomic imbalance in the sympathetic direction, as indexed by HRV indexes [12, 13]. On the other hand, the investigation of cardiac autonomic function in animal models of trait anxiety has been conducted only sporadically and provided preliminary evidence of an altered autonomic regulation of HR in subjects with high levels of trait anxiety [14, 15].

Given these considerations, the principal objective of this study was to characterize in detail the autonomic neural modulation of HR in two Wistar rat lines selectively bred for either high (HAB) or low (LAB) anxiety-related behavior. The HAB/LAB rats have been proved to display robust, consistent and reliable differences in their level of baseline anxiety (for a review see [16, 17]). In addition, this rat model has been particularly useful for unveiling the neurobiological, neuroendocrine and physiological

correlates of high trait anxiety [18-20]. Therefore, the use of these psychogenetically selected rats offers, in our view, a valid and reliable methodological approach for investigating the autonomic correlates of extremes in anxiety-related behavior.

In the current study we tested the hypothesis that high levels of trait anxiety in rats would be associated to specific features of autonomic neural modulation of HR that would support the use of this rat model for the study of the mechanisms mediating anxiety and cardiovascular disorder comorbidity. Sympathetic and parasympathetic (vagal) influences on the heart were assessed during resting and stress conditions via time- and frequency-domain analysis of HRV. Pharmacological autonomic manipulations were conducted i) to assess the relative contribution of sympathetic and vagal components, using beta-adrenoceptor and muscarinic receptor antagonists, respectively, and ii) to investigate susceptibility to cardiac arrhythmias following pharmacological stimulation of β-adrenoreceptors. Finally, cardiac structural analysis was performed in order to verify whether given autonomic features were related to specific gross characteristics of the heart.

4.2 METHODS

Ethics statement and animals

The experimental protocol described here was approved by the Veterinarian Animal Care and Use Committee of Parma University, and carried out in accordance with the European Community Council Directives of 22 September 2010 (2010/63/UE).

Experiments were carried out on 5-month-old male Wistar rats (380-420 g body weight) selectively bred for either high (HAB) or low (LAB) anxiety-related behavior in the elevated plus-maze test [16]. The animals were obtained from the animal facilities of the University of Regensburg (Germany). The HAB (n=10) and LAB (n=10) rats used in this study were housed in groups of 3-4 per cage and kept in rooms with controlled temperature (22±2°C) and a reversed light-dark cycle (light on from 19:00 to 7:00 h), with free access to food and water.

Anxiety-related behavior

Initially, HAB and LAB rats were tested on the elevated plus-maze to confirm their anxiety-related phenotype. The elevated plus-maze, validated for measuring anxiety [21], is based on creating a conflict between the rat's exploratory drive and its innate fear of open and exposed areas. The plus maze consisted of 4 elevated arms (100 cm above the floor, 50 cm long and 10 cm wide) arranged in a cross-like position, with two opposite arms being enclosed (by means of 40 cm high walls), and two being open, including at their intersection a central square platform (10×10 cm) which gave access to the four arms. Each rat was initially placed on the central platform facing one closed arm and behaved freely for 5 min. The behavior during the test was recorded using a video camera positioned above the maze. The following behavioral parameters were calculated using the Ethovision 6.0 software (Noldus, The Netherlands): i) number of entries in the open arms (% of total entries), ii) latency to enter an open arm (s), and iii) time spent in the open arms (% of total time).

Surgery: radiotransmitter implantation

One week after the behavioral testing, HAB and LAB rats were anesthetized with tiletamine hydrochloride + zolazepam hydrochloride (Zoletil 200 mg/kg, s.c.). Radiotelemetric transmitters (TA11CTA-F40, Data Sciences International, St. Paul, MN, USA) for recording electrocardiogram (ECG), core body temperature (T, °C) and locomotor activity (LOC, expressed as counts/minute, cpm) were implanted according to a procedure described by Sgoifo and colleagues [22]. The transmitter body was placed in the abdominal cavity; one electrode was fixed to the dorsal surface of the xyphoid process and another electrode was placed in the anterior mediastinum close to the right atrium. Such electrode location guarantees high-quality ECG recordings (Figure 1), even during vigorous physical activity. Immediately after surgery, rats were individually housed, injected for 2 days with gentamicin sulfate (Aagent, Fatro, 0.2ml/kg, s.c.) and allowed 10 days of recovery before the start of experimental recordings.

Experimental protocol and radiotelemetric recordings

Following recovery from surgery, animals were left undisturbed in their home cages for 7 days for collection of baseline daily rhythms of HR, HRV, T and LOC (see section '2.5. Baseline daily rhythms' for details). Subsequently, rats were submitted on different days to: i) pharmacological manipulations (days 1, 3, 5 and 7), and ii) restraint test (day 9). These tests (described below) were carried out between 10:00 and 14:00 (i.e., the dark phase of the light/dark cycle).

ECG waves (sampling frequency 1000 Hz), T and LOC signals (sampling frequency 256 Hz) were picked up by a radiotelemetry receiver (RPC-1) and recorded via ART-Gold 1.10 data acquisition system (Data Sciences Int., St. Paul, MN, USA) [23].

Baseline daily rhythms

ECG, T and LOC were sampled around-the-clock for 2 minutes every hour over a period of 7 days for collection of baseline daily rhythms. Data analysis was performed as follows. Initially, separate

estimates of HR, HRV indexes (see section '2.8. Quantification of HRV' for details), T and LOC were generated for each 2-min recording period. Subsequently, radiotelemetric and HRV parameters were averaged as mean values of 12h-light and 12h-dark daily phases. These parameters were then further averaged as means of the 7 days of the light and dark phases. Finally, the rhythm amplitude of each parameter was calculated as the difference between mean values of the dark and the previous light phase, respectively.

Selective pharmacological autonomic challenges

On different days, HAB and LAB rats received subcutaneous injections of: 1) vehicle (0.9% NaCl, vol: 1ml/kg; day 1); 2) methylscopolamine (muscarinic receptor antagonist, at a dose of 0.05 mg/kg; day 3); 3) atenolol (β1-adrenergic receptor antagonist, at a dose of 2 mg/kg, day 5); 4) isoproterenol (β-adrenoceptor agonist, at a dose of 0.02 mg/kg, day 7). Drug doses were selected on the basis of a previous study [24]. Each drug injection was separated by a 2-day washout period. ECG recordings were performed prior to (30 min, baseline conditions) and following (15 min) the injections. Data analysis was conducted as follows. Initially, we split each recording period in 5-min epochs (0-5 min, 5-10, etc.). For each epoch, separate estimates of HR and HRV indexes (see section '2.8. Quantification of HRV' for details) were generated. In addition, the overall effects of drug administration on HR were evaluated by computing the area under the response time curve above baseline (AUC).

Restraint test

Each animal was introduced for 15 min into a restrainer fitted closely to the body size (wire-mesh tube; inner diameter: 6 cm, length: 20 cm) [25]. After the test, animals were returned to their home cages. Continuous ECG, T and LOC recordings were performed in baseline conditions (30 min, prior to the test), during the restraint test (15 min) and throughout the recovery period (45 min). Data analysis was conducted as follows. Initially, we split each recording period in 5-min epochs (0-5 min, 5-10, etc.). For

each epoch, separate estimates of HR, HRV indexes (see section '2.8. Quantification of HRV' for details), T and LOC were generated. Subsequently, cardiac autonomic response to the restraint test was evaluated by computing the AUC.

Quantification of HRV

Initially, each raw ECG signal was visually inspected to ensure that all R-waves were correctly detected. HR (reported in beats per minute; bpm) and time- and frequency-domain parameters of HRV were then quantified using ChartPro 5.0 software (ADInstruments, Sydney, Australia). In the time-domain, we obtained the square root of the mean squared differences of successive RR intervals (RMSSD, ms), which quantifies short-term, high-frequency variations of RR and therefore estimates the activity of the parasympathetic nervous system [26]. For spectral (frequency-domain) analysis of HRV, the power spectrum was obtained with a fast Fourier transform-based method (Welch's periodogram: 256 points, 50% overlap, and Hamming window). We considered the total power of the spectrum (ms^2), which reflects all the cyclic components responsible for variability, and the power of the low frequency (LF; 0.2-0.75 Hz) and high frequency (HF; 0.75-2.5 Hz) bands in absolute values (ms^2). The power of LF band is a non-specific index as it contains contributions of both the sympathetic and parasympathetic influences [27]; the power of HF band is due to the activity of the parasympathetic nervous system and includes respiration-linked oscillations of HR [28]. The low frequency/high frequency ratio (LF/HF) estimates the fractional distribution of power and is taken as a synthetic measure of sympathovagal balance [29]. Those parts of ECG recordings which were non-stationary and/or exhibited recording artifacts were excluded from the analysis in accordance to an automatic test checking stationarity of the mean and variance of HR [29, 30].

Quantification of arrhythmic events

The occurrence of arrhythmic events was determined and quantified off-line based on the Lambeth Conventions for the study of experimental arrhythmias [31]. We determined and quantified the

separate occurrence of supraventricular and ventricular ectopic beats (Figure 1B, C) and the total number of tachyarrhythmic events.

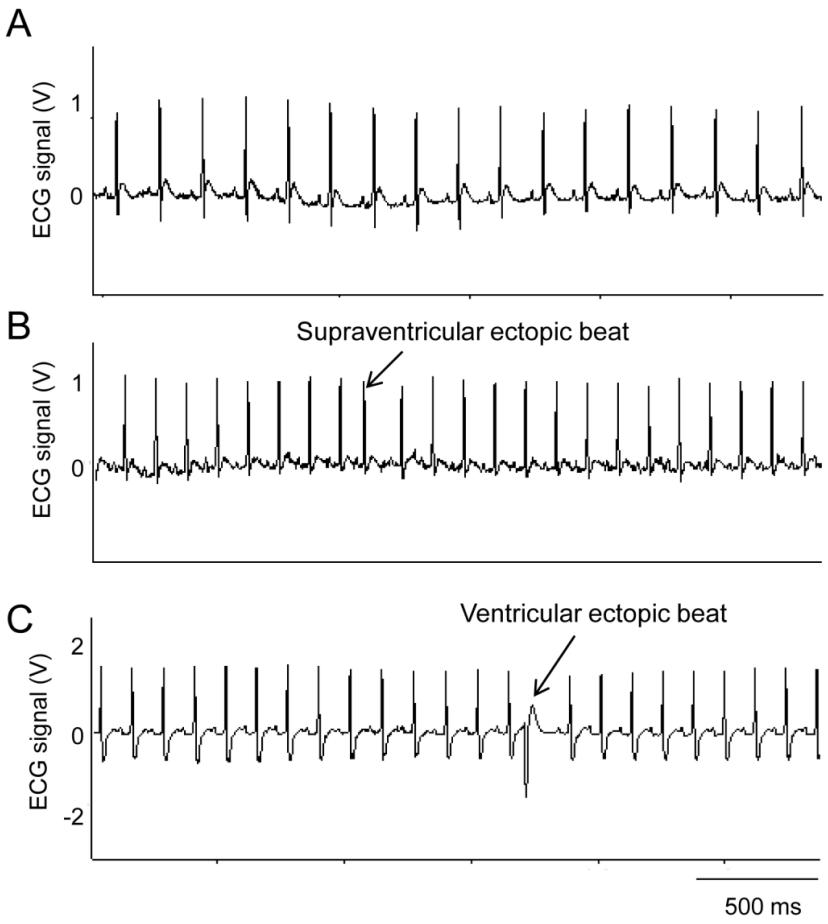

Figure 1. Examples of ECG traces belonging to a representative HAB rat. Panel A: normal ECG. Panel B and C: ECG with isolated premature supraventricular and ventricular ectopic beats, respectively.

Post mortem measurements

Two days after the restraint test, rats were euthanized. Under anesthesia (tiletamine hydrochloride + zolazepam hydrochloride, Zoletil 200 mg/kg, s.c.), the heart was arrested in diastole with cadmium chloride solution (100mM, i.v.) and excised for subsequent morphological/morphometric analysis, as described previously in detail [32, 33]. Briefly, the heart was removed from the chest and fixed in 10%

buffered formalin solution. We determined: i) heart weight (HW) and its value relative to body weight (BW); ii) left ventricular (LV) and right ventricular (RV) weight, and their values relative to HW; iii) LV chamber length, volume, and transverse diameter; iv) LV free wall thickness. In addition, wedges of the ventricular myocardium were embedded into a paraffin block with the epicardial surface facing upward. Each block was then sectioned with a microtome into 5-µm-thick sections. Subsequently, sections were stained with Masson's trichrome in order to evaluate the total amount of fibrosis in the left ventricle [32, 33].

Statistics

All statistical analyses were performed using the software package SPSS (version 20). Two-way ANOVA for repeated measures with group as between-subject factor (2 levels: HAB and LAB) was applied for data obtained from: i) baseline daily rhythms, with time as within-subject factor (2 levels: light and dark phases); ii) pharmacological manipulations, with time as within-subject factor (4 levels: baseline; post-injection 1, 2, and 3); iii) restraint, with time as within-subject factor (5 levels: baseline; test; recovery 1, 2, and 3). Follow-up analyses were conducted using Student's "t" tests, with a Bonferroni correction for multiple comparisons for each outcome variable separately. A priori Student's "t"-tests, after controlling for homogeneity of variance via Levene test, were applied for comparisons between HAB and LAB rats on: i) data obtained from the elevated plus maze test; ii) the occurrence of arrhythmic events; iii) measurements at sacrifice. Data are presented as means ± standard error of the mean (SEM). Statistical significance was set at $p<0.05$.

4.3 RESULTS

Anxiety-related behavior

The elevated plus-maze test was conducted as a validation criterion for the relative anxiety phenotype of HAB and LAB rats. HAB rats were more anxious than LABs as reflected by a lower percentage of time spent on open arms (HAB=2.1±1.1 % vs. LAB=64.8±1.1 % of total time, t=-15.4, p<0.01) and a lower percentage of open arm entries than LABs (HAB=18.3±7.8 % vs. LAB=55.1±2.1 % of total entries, t=-4.6, p<0.01). In addition, the average latency time to enter an open arm was longer in HABs compared to LABs (HAB=201±44 s vs. LAB=15±5 s, t=4.2, p<0.01).

Daily rhythms of radiotelemetric parameters

The daily rhythms of HR, HRV parameters, T and LOC under resting conditions are depicted in Figure 2 and presented in Table 1.

Two-way ANOVA yielded main effects of: i) time for HR values (F=690.9, p<0.01), total spectral power (F=25.0, p<0.01), RMSSD values (F=16.6, p<0.01), spectral power in HF (F=16.7, p<0.05) and LF (F=4.3, p<0.05) bands, LF to HF ratio (F=28.3, p<0.01), T values (F=1266.0, p<0.01) and LOC values (F=455.9, p<0.01); ii) group for total spectral power (F=19.4, p<0.01), RMSSD values (F=16.4, p<0.01), spectral power in HF (F=17.48, p<0.01) and LF (F=10.0, p<0.05) bands, and T values (F=38.03, p<0.01).

HAB and LAB rats had similar mean HR values in both phases of the light-dark cycle (Figure 2A and Table 1). However, HAB rats exhibited significantly lower values of total spectral power than LAB rats during both the light (t=-4.37, p<0.01) and the dark (t=-2.22, p<0.05) phases (Figure 2B and Table 1), and showed a significantly smaller rhythm amplitude of this parameter compared to LAB rats (t=-2.69, p<0.05) (Table 1). Time-domain analysis of HRV revealed that HAB rats had significantly lower values of RMSSD than LAB rats in both phases of the light-dark cycle (light: t=-3.26, p<0.05; dark: t=-4.63, p<0.01) (Figure 2C and Table 1), and a tendentially smaller rhythm amplitude of this parameter compared to LAB rats (t=-2.0, p=0.07) (Table 1). Likewise, frequency-domain analysis of HRV

indicated that spectral power in HF band was significantly lower in HAB rats compared to LAB rats in both the light ($t=-12.32$, $p<0.05$) and the dark ($t=-13.53$, $p<0.01$) phases (Figure 2D and Table 1). In addition, HAB rats exhibited a significantly lower rhythm amplitude of HF ($t=-2.56$, $p<0.05$) spectral band compared to LABs (Table 1). No differences between groups were observed for LF/HF values (Figure 2E and Table 1), in accordance with the absence of significant differences in HR.

In addition, HAB rats had higher T values than LABs in both phases of the light-dark cycle (light: $t=6.48$, $p<0.01$; dark: $t=4.68$, $p<0.01$) (Figure 2F and Table 1), whereas LOC values were similar between the two groups (Table 1).

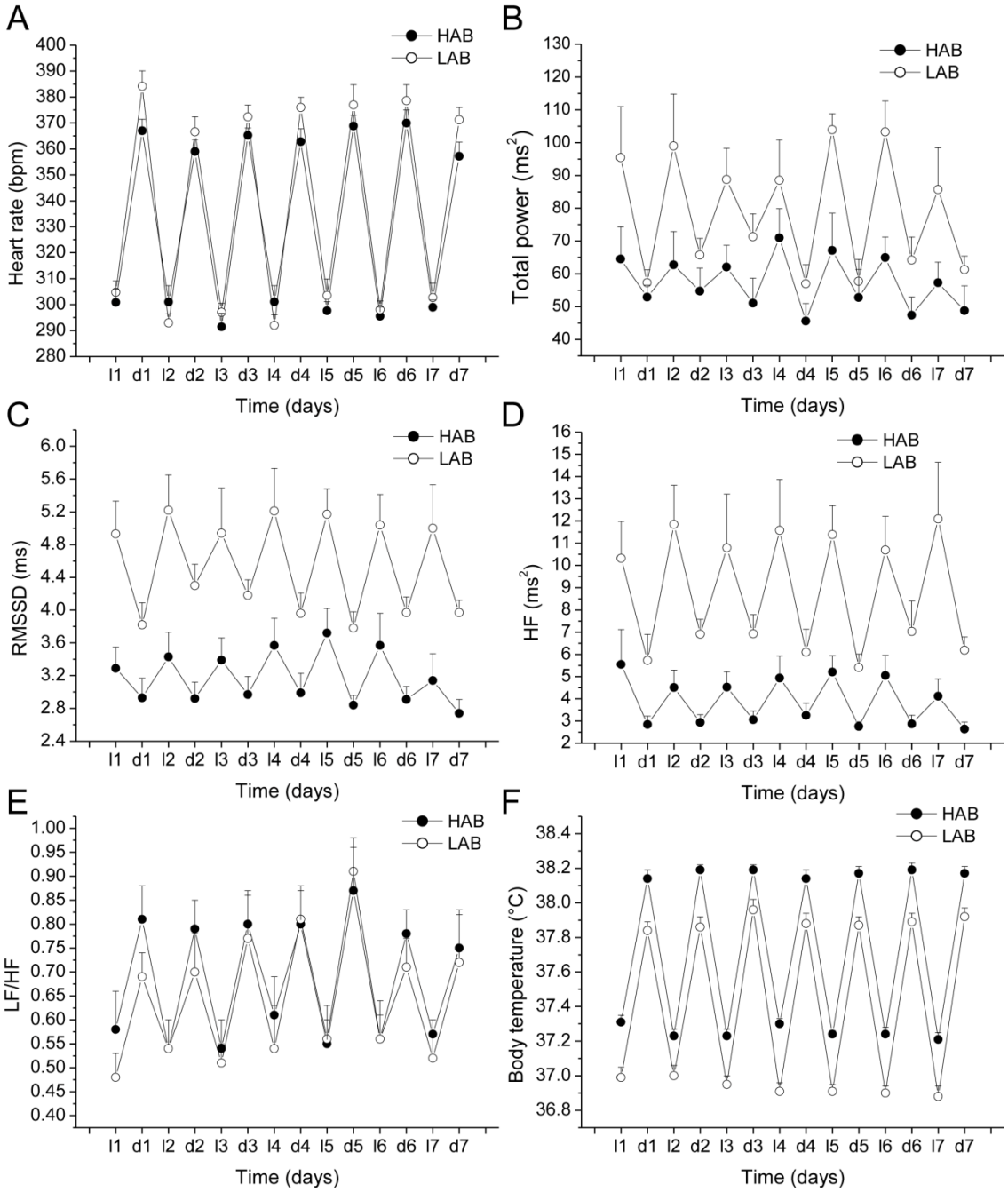

Figure 2. Time course of changes in heart rate, heart rate variability parameters and body temperature values during the light and dark phases of baseline daily rhythm recordings (7 days), in HAB (n=10) and LAB (n=10) rats. For the 12h-light and 12h-dark phases, values are reported as means ± SEM of data obtained by averaging multiple 2-min segments acquired every hour. Abbreviations: RMSSD = root mean square of successive R-R interval differences; HF = high-frequency; LF= low-frequency. Statistical results are reported in the text.

Table 1. Daily rhythms of radiotelemetric and HRV parameters.

		Light	Dark	Amplitude
HR (bpm)	HAB	298±4	365±3	67±3
	LAB	299±3	375±4	76±4
Total power (ms^2)	HAB	62.7±3.7#	53.2±4.2*	9.5±4.4*
	LAB	96.4±6.8	64.8±3.2	31.6±7
RMSSD (ms)	HAB	3.44±0.29#	2.90±0.16*	0.54±0.15
	LAB	5.07±0.40	4.00±0.17	1.07±0.37
HF power (ms^2)	HAB	4.84±0.77#	2.91±0.31*	1.93±0.52*
	LAB	11.24±1.77	6.33±0.59	4.92±1.59
LF power (ms^2)	HAB	2.23±0.27#	2.15±0.25#	0.08±0.18*
	LAB	5.52±1.04	4.10±0.64	1.42±0.70
LF/HF	HAB	0.51±0.05	0.76±0.06	0.25±0.04
	LAB	0.50±0.06	0.63±0.06	0.13±0.06
T (°C)	HAB	37.24±0.03*	38.17±0.04*	0.93±0.03
	LAB	36.90±0.04	37.88±0.05	0.98±0.05
LOC (cpm)	HAB	2.1±0.2	5.3±0.2	3.2±0.2
	LAB	2.3±0.2	5.7±0.3	3.4±0.2

*For the 12h-light and 12h-dark phases, values are reported as means ± SEM of data obtained by averaging multiple 2-min segments acquired every hour over a period of 7 days, in HAB (n=10) and LAB (n=10) rats. Abbreviations: HRV = heart rate variability; HR = heart rate; RMSSD = root mean square of successive R-R interval differences; LF = low-frequency; HF = high-frequency; T = body temperature; LOC = locomotor activity. * and # indicate a significant difference between HAB and LAB rats ($p<0.05$ and $p<0.01$, respectively).*

Selective pharmacological autonomic challenges

Vehicle injection

During baseline, pre-injection ECG recordings HAB and LAB rats had similar HR values (Figure 3A). However, HAB rats had significantly lower values of RMSSD (HAB= 3.1±0.2 ms vs. LAB= 4.1±0.3 ms, t=-2.84, p<0.05) and spectral power in HF band (HAB= 3.7±0.6 ms^2 vs. LAB= 6.8±0.9 ms^2, t=-2.13, p<0.05) than LABs. As indicated by AUC values (Figure 3A, inner graph), injection of vehicle provoked a smaller increment of HR in HAB rats compared to LABs (t=-2.81, p<0.05). Specifically, after vehicle administration HR was significantly lower in HABs than LABs during the first (t=-5.45, p<0.01), second (t=-3.55, p<0.01) and third (t=-2.72, p<0.05) 5-min period (Figure 3A). In addition, injection of vehicle provoked a reduction of RMSSD values and HF spectral power, with the magnitude of this decrement being smaller in HAB than LAB rats (RMSSD: HAB= -0.5±0.2 ms vs. LAB= -2.1±0.5 ms, t=-3.1, p<0.01; HF: HAB= -0.6±0.5 ms^2 vs. LAB= -3.3±0.6 ms^2, t=-2.11, p<0.05). Further analysis revealed a very modest incidence of supraventricular (HAB= 0.1±0.1 vs. LAB= 0.2±0.1) and ventricular (HAB= 1.1±0.5 vs. LAB= 0.6±0.4) arrhythmias following injection of vehicle, with no group differences.

Methylscopolamine injection

In baseline conditions, HAB and LAB rats had similar baseline HR values (Figure 3B). However, HAB rats had significantly lower values of RMSSD (HAB= 3.2±0.2 ms vs. LAB= 4.3±0.3 ms, t=-2.77, p<0.05) and spectral power in HF band (HAB= 3.7±0.6 ms^2 vs. LAB= 6.8±0.9 ms^2, t=-2.77, p<0.05) than LAB rats. As indicated by AUC values (Figure 3B, inner graph), injection of muscarinic receptor antagonist methylscopolamine provoked a smaller increment of HR in HAB rats compared to LABs (t=-3.88, p<0.01). Specifically, after methylscopolamine administration HR was significantly lower in HABs than LABs during the first (t=-3.79, p<0.01), second (t=-4.50, p<0.01) and third (t=-4.52, p<0.01) 5-min period (Figure 3B). As expected, muscarinic receptor blockade provoked a marked reduction of RMSSD values and HF spectral power, with the magnitude of this decrement being smaller in HAB than LAB rats (RMSSD: HAB= -2.4±0.3 ms vs. LAB= -3.9±0.3 ms, t=-3.01, p<0.01; HF: HAB= -3.5±0.6 ms^2 vs. LAB= -6.7±0.9 ms^2, t=-2.86, p<0.05). Further analysis revealed a modest incidence of

supraventricular (HAB=0.4±0.2 vs. LAB=0.3±0.1) and ventricular (HAB=2.4±0.8 vs. LAB=1.3±0.5) arrhythmias following methylscopolamine administration, with no group differences.

Atenolol injection

In baseline conditions, HAB and LAB rats had similar baseline HR values (Figure 3C). However, HAB rats had significantly lower values of RMSSD (HAB= 3.1±0.2 ms vs. LAB= 4.3±0.4 ms, t=-2.62, p<0.05) and spectral power in HF band (HAB= 3.5±0.6 ms^2 vs. LAB= 6.9±1.5 ms^2, t=-2.13, p<0.05) than LAB rats. No differences between groups were observed for LF/HF values (HAB= 0.80±0.08 vs. LAB= 0.81±0.12, p=n.s.), in accordance with the absence of significant differences in baseline HR.

Injection of β1-adrenergic receptor antagonist atenolol provoked transient increment in HR in LAB rats, but not in HABs (Figure 3C). Consequently, AUC values (Figure 3C, inner graph), resulted significantly lower in HAB rats than LABs (t=-4.40, p<0.01). Specifically, after atenolol administration HR was significantly lower in HABs than LABs during the first (t=-7.98, p<0.01), second (t=-3.92, p<0.01) and third (t=-3.68, p<0.01) 5-min period (Figure 3C). In addition, atenolol injection provoked i) a reduction of RMSSD values and HF spectral power in both groups, with the magnitude of this decrement being much smaller in HAB than LAB rats (RMSSD: HAB= -0.5±0.1 ms vs. LAB= -1.9±0.3 ms, t=-3.7, p<0.01; HF: HAB= -1.1±0.2 ms^2 vs. LAB= -3.7±0.6 ms^2, t=-3.6, p<0.01), and ii) a moderate increase in LF to HF ratio in LAB rats, but not in HAB rats (HAB= +0.01±0.07 vs. LAB= +0.27±0.11, t=1.9, p=0.07). Further analysis revealed a modest incidence of supraventricular (HAB=0.1±0.1 vs. LAB=0.1±0.1) and ventricular (HAB=1.0±0.6 vs. LAB=0.2±0.1) arrhythmias following atenolol administration, with no group differences.

Isoproterenol injection

In baseline conditions, HAB and LAB rats had similar baseline HR values (Figure 3D). As indicated by AUC values (Figure 3D, inner graph), injection of β-adrenoceptor agonist isoproterenol provoked a similar increment of HR between HAB and LAB rats. However, after isoproterenol administration HR was significantly lower in HABs than LABs during the first (t=-2.64, p<0.05) and third (t=-2.18, p<0.05) 5-min period (Figure 3D). In addition, after β-adrenoceptor stimulation with isoproterenol, HAB rats

showed a significantly larger incidence of ventricular arrhythmias compared to LAB rats (t=2.76, p<0.05) (Figure 4), whereas the occurrence of supraventricular arrhythmias was modest and similar between the two groups (HAB=0.9±0.3 vs. LAB=0.9±0.3).

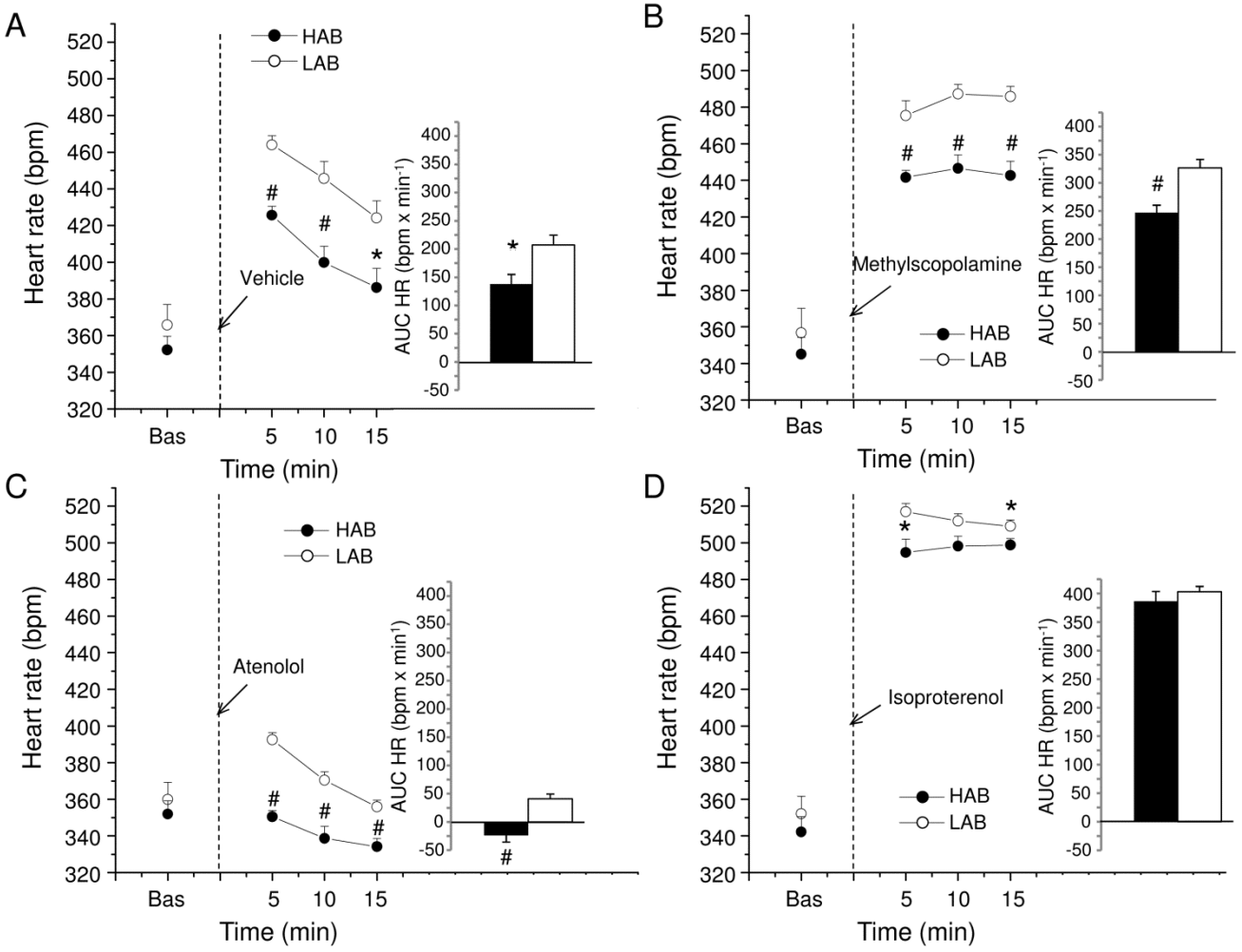

*Figure 3. Time course of changes in heart rate after injection of vehicle (panel A), methylscopolamine (panel B), atenolol (panel C) and isoproterenol (panel D) in HAB (n=10) and LAB (n=10) rats. Baseline reference value (bas) is the mean value of the six 5-min time points in resting conditions. Inner graphs represent the area under the response time curve above baseline (AUC) of heart rate during the respective post-injection phases. * and # indicate a significant difference between HAB and LAB rats (p<0.05 and p<0.01, respectively).*

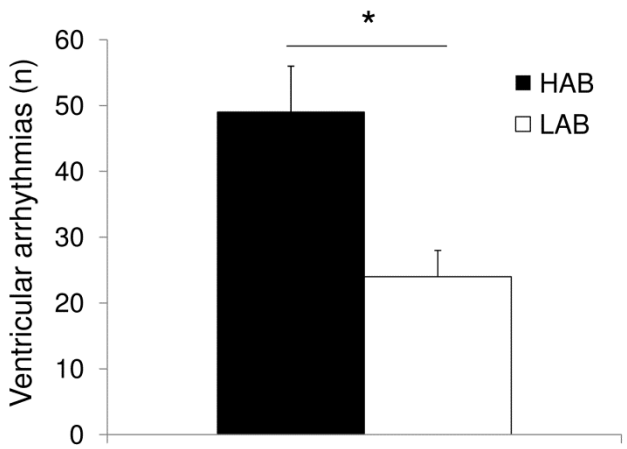

*Figure 4. Incidence of ventricular arrhythmias following (15 min) pharmacological stimulation of β-adrenoceptors with isoproterenol, in HAB (n=10) and LAB (n=10) rats. * indicates a significant difference between HAB and LAB rats (p<0.05).*

Restraint test

Cardiac autonomic responses to the restraint test are depicted in Figure 5 and detailed in Table 2.

Two-way ANOVA yielded main effects of: i) group for HR values ($F=8.18$, $p<0.05$), and spectral power in HF band ($F=6.0$, $p<0.05$), and ii) time for T ($F=24.98$, $p<0.01$) and LOC ($F=16.03$, $p<0.05$) values.

Before the test, baseline HR was similar between the two groups (Figure 5A and Table 2). However, HAB rats showed lower values of RMSSD ($t=-2.91$, $p<0.01$) and spectral power in HF band ($t=-2.85$, $p<0.05$) than LAB rats ($t=-2.91$, $p<0.01$) (Figure 5C, E and Table 2).

Submitting rats to restraint provoked a smaller increment of HR in HABs than LABs, as indicated by AUC values ($t=-4.28$, $p<0.01$) (Figure 5B). Specifically, during restraint HR was significantly lower in HABs than LABs during the first ($t=-3.01$, $p<0.01$), second ($t=-6.52$, $p<0.01$) and third ($t=-6.85$, $p<0.01$) 5-min period (Figure 5A). In the same period, RMSSD and HF values were similar between the two groups (Figure 5C, E and Table 2). However, AUC analysis revealed that HAB rats exhibited a smaller reduction of RMSSD and HF indexes compared to LABs (AUC RMSSD: $t=-4.29$, $p<0.01$; AUC HF: $t=-3.18$, $p<0.01$) (Figure 5D, F).

During the first 15 min of the recovery phase, HR values were significantly lower in HAB than LAB rats ($t=-4.39$, $p<0.01$) (Figure 5A and Table 2). In addition, RMSSD values and spectral power in HF band were significantly lower in HAB than LAB rats during the last 15 min of the recovery phase (RMSSD: $t=-2.89$, $p<0.05$; HF: $t=-2.23$, $p<0.05$) (Figure 5C, E and Table 2).

In addition, during the restraint test the incidence of ventricular arrhythmias was larger, although not significantly, in HAB than LAB rats (HAB=6.6±3.9 vs. LAB=0.9±0.4). On the other hand, the occurrence of supraventricular arrhythmias was very modest and similar between the two groups (HAB=0.2±0.2 vs. LAB=0.3±0.2).

No differences between the two groups were observed for T and LOC values, neither during baseline recordings nor during and after the test (Table 2).

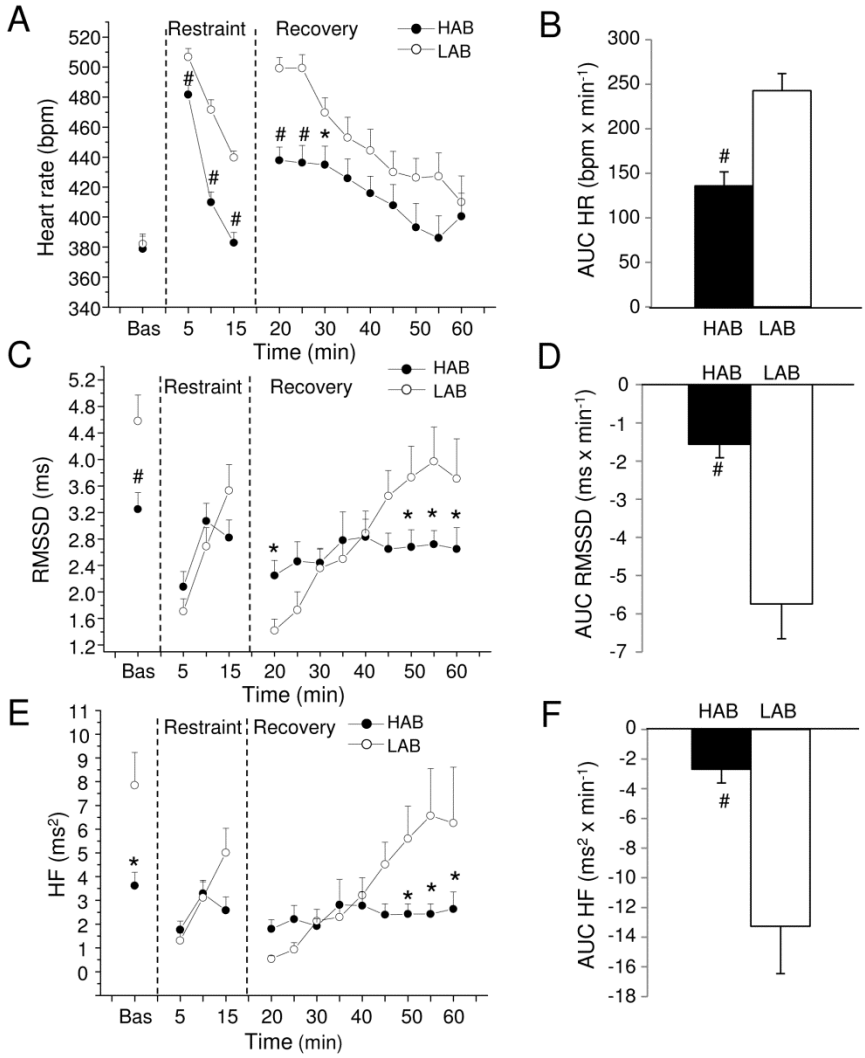

*Figure 5. Cardiac autonomic response to the restraint test in HAB (n=10) and LAB (n=10) rats. Panels on the left show the time course of changes in heart rate (A), RMSSD values (C) and high-frequency (HF) spectral power (E), in baseline conditions (bas), during the restraint and the recovery phase. Baseline reference value (bas) is the mean value of the six 5-min time points in resting conditions. Panels on the right represent the area under the response time curve above baseline (AUC) of heart rate (B), RMSSD (D) and high-frequency (HF) spectral power during the restraint phase. Values are expressed as mean ± SEM. Abbreviations: RMSSD = root mean square of successive R-R interval differences; HF = high-frequency. * and # indicate a significant difference between HAB and LAB rats ($p<0.05$ and $p<0.01$, respectively).*

Table 2. Radiotelemetric and HRV parameters during the restraint test.

		Baseline	Restraint	Recovery (min 0-15)	Recovery (min 15-30)	Recovery (min 30-45)
HR (bpm)	HAB	379±9	425±6$^{\#}$	436±10$^{\#}$	417±12	393±15
	LAB	382±7	473±4	489±6	442±13	421±15
Total Power (ms^2)	HAB	67.7±8.2	37.6±4.3	46.0±5.7	51.5±6.1	54.5±5.6
	LAB	84.2±12.0	32.9±5.5	26.9±5.9	40.1±5.4	52.1±8.0
RMSSD (ms)	HAB	3.25±0.25$^{\#}$	2.66±0.20	2.38±0.24	2.75±0.29	2.68±0.25*
	LAB	4.58±0.39	2.75±0.25	1.84±0.19	2.94±0.29	3.80±0.50
HF Power (ms^2)	HAB	3.62±0.56*	2.57±0.39	1.97±0.42	2.66±0.63	2.50±0.48*
	LAB	7.85±1.38	3.15±0.51	1.20±0.22	3.34±0.57	6.15±1.82
LF Power (ms^2)	HAB	2.55±0.41	2.56±0.45	2.33±0.50	2.61±0.64	2.17±0.38
	LAB	4.65±0.75	2.80±0.69	1.26±0.17	2.45±0.39	3.45±0.59
LF/HF	HAB	0.74±0.07	1.02±0.13	1.20±0.07	1.02±0.09	0.95±0.10
	LAB	0.64±0.07	0.91±0.19	1.03±0.11	0.78±0.08	0.71±0.10
T (°C)	HAB	38.2±0.1	38.3±0.1	38.8±0.1	38.8±0.1	38.6±0.1
	LAB	38.0±0.1	38.2±0.2	38.7±0.1	38.7±0.1	38.5±0.1
LOC (cpm)	HAB	5.6±0.5	1.5±0.3	22.3±1.6	11.3±1.9	8.3±2.1
	LAB	7.4±1.4	2.2±0.4	27.5±2.6	13.8±1.5	10.0±1.5

*Values are reported as means ± SEM of data obtained by averaging multiple 5-min segments acquired in baseline conditions (30 min), during the restraint (15 min) and the recovery phase (45 min), in HAB (n=10) and LAB (n=10) rats. Abbreviations: HRV = heart rate variability; HR = heart rate; RMSSD = root mean square of successive R-R interval differences; HF = high-frequency; LF = low-frequency; T = body temperature; LOC = locomotor activity. * and $^{\#}$ indicate a significant difference between HAB and LAB rats ($p<0.05$ and $p<0.01$, respectively).*

Measurements at sacrifice

Before euthanasia, BW was tendentially lower in HAB than LAB rats (HAB= 400±9g vs. LAB= 436±11g, t=-1.9, p=0.07).

3.5.1. Cardiac Anatomy

The HW per se was not significantly different in HAB rats compared to LAB counterparts (Table 3). However, HW corrected for BW (HW/BW ratio) resulted significantly heavier in HABs than LABs (t=4.45, p<0.01). No significant differences in LV weight, RV weight, and linear LV parameters were observed between HAB and LAB rats (Table 3).

3.5.2. Tissue morphometry

Morphometric analysis revealed a similar volume fraction of myocytes (HAB=88.7±1.5% vs. LAB=90.6±1.0%) and a similar amount of myocardial fibrosis (HAB=1.5±0.3% vs. LAB=2.3±0.4%) in the LV myocardium of HAB and LAB rats.

Table 3. Gross cardiac characteristics.

	HAB (n=10)	LAB(n=10)	% of variation
HW (mg)	908.8±36.9	864.1±24.6	+5
HW/BW (mg/g)	2.27±0.05$^{\#}$	1.98±0.05	+15
LVW (mg)	710.6±26.59	676.5±16.93	+5
LVW/HW (mg/mg)	0.783±0.012	0.784±0.007	0
RVW (mg)	197.9±16.37	187.7±10.11	+5
RVW/HW (mg/mg)	0.217±0.012	0.216±0.007	0
LV chamber length (mm)	12.79±0.50	12.28±0.35	+4
LV chamber volume (mm^3)	99.99±19.25	95.67±9.53	+5
LV chamber diameter (mm)	3.76±0.24	3.83±0.19	-6
LV wall thickness (mm)	2.64±0.10	2.46±0.08	+7

Values are reported as means ± SEM. Abbreviations: LV = left ventricle; HW= heart weight; BW=body weight; LVW = LV weight; RV = right ventricle; RVW = RV weight. Values are expressed as means ± SEM. $^{\#}$ indicates a significant difference between HAB and LAB rats (p<0.01).

4.4 DISCUSSION

The purpose of this study was to characterize cardiac autonomic regulation in a unique model of trait-anxiety, the HAB/LAB rats. Our major novel finding is that HAB rats show signs of i) impaired cardiac autonomic modulation (low vagally-mediated HRV), ii) poor adaptive HR responsiveness to stressful stimuli, and iii) increased susceptibility to arrhythmias compared to LAB counterparts. These findings are consistent with the view that high levels of anxiety-related behavior in rats are associated with specific features of cardiac autonomic regulation that may predict increased vulnerability to cardiac morbidity and mortality.

The behavior of HAB/LAB rats on the elevated plus maze is consistent with extensive literature documenting divergent and opposite levels of anxiety between these two rat lines [16, 17]. In order to investigate the autonomic correlates of extremes in anxiety-related behavior, HRV analysis was applied as a window into the cardiac autonomic control that characterizes HAB/LAB rats. In particular, measures of HRV in both the time and frequency domains have been used successfully to index vagal modulation [34], which is considered a useful indicator of the behavioral and physiological flexibility of an organism and its ability to adaptively respond to stress [35].

Initially, HRV parameters were evaluated during baseline rhythm recordings (7 days) in order to obtain a valid characterization of cardiac autonomic regulation under normal undisturbed resting conditions. We found that HRV was significantly reduced in HAB rats compared to LABs in both phases of the light-dark cycle. This phenomenon was clearly related to a much lower vagal modulation of HR in HAB rats compared to LAB counterparts, as indexed by RMSSD and HF power values. The smaller increase in HR observed in HABs compared to LABs following vagal blockade with methylscopolamine is a reliable pharmacological confirmation of a lower relative contribution of the vagal control over resting HR in HAB rats. The fact that vagal blockade also provoked a milder reduction of RMSSD and HF power values in HAB than LAB rats was due to significant differences in baseline values of these vagal indexes, which were lower in HAB rats. Despite the difference in cardiac vagal tone, HAB and LAB rats had similar mean HR values at rest. This finding led us to

hypothesize that the reduced cardiac vagal tone observed in HAB rats was coupled with a decreased cardiac sympathetic influence on the sino-atrial node compared to LAB rats. This is supported by the fact that the two groups also did not differ for LF to HF ratio (index of sympathovagal balance), suggesting that the regulatory influences of the vagal and sympathetic components on cardiac pacemaker activity were similarly balanced in the two groups, leading to similar HR values. This is in apparent contradiction with the notion that anxiety in humans is often accompanied by somatic manifestations of increased sympathetic activation, such as rapid HR, shortness of breathing and sweating [36]. However, we did find that body temperature was higher in HABs than LABs during both phases of the light/dark cycle. Of note, this difference could not be attributed to a different level of somatomotor activity between HAB and LAB rats. These findings indicate that body temperature in this rat model may be a more reliable marker than HR for assessing the increased sympathetic activation that often accompanies anxiety. This may be due to the fact that body temperature is purely sympathetically regulated, whereas simple estimates of HR may oversimplify and misrepresent the complexity of the dynamic balance between the sympathetic and parasympathetic influences on the sino-atrial node. Interestingly, HAB rats had tendentially lower body weight compared to LAB rats. We therefore hypothesize a role of increased sympathetic activity in HAB rats in promoting lipolysis and thermogenesis in adipose tissue and muscle to explain the differences in body weight and body temperature between HAB and LAB rats.

Similarly to what was observed in HAB rats, a negative relationship between cardiac vagal control, as indexed by HRV parameters, and trait anxiety has been found in humans [7, 37, 38]. The general consensus is that low vagal tone is a marker for poor behavioral and physiological flexibility and for inability to adequately respond to stress [35]. In particular, a low degree of vagal control would not allow proper responsiveness and sensitivity of HR to changing environmental demands [39] and, therefore, could represent an important determinant for increased vulnerability to cardiovascular disorders [10]. Accordingly, we found that HAB rats showed a reduced HR responsiveness to acute stressors (i.e., vehicle injection and restraint) compared to LAB counterparts. Importantly, this

difference in HR stress responsiveness was also observed following the injection of the β-blocker atenolol (of note, the tachycardic effect of stress injection was absent in HAB rats under this condition). Interestingly, after β-adrenoreceptor blockade with atenolol LAB rats exhibited a moderate shift of the sympathovagal balance towards a sympathetic prevalence (increase in LF to HF ratio).This phenomenon was due to a marked vagal withdrawal response to injection stress and was absent in HAB rats. Taken together, these results may be interpreted as a sign of reduced vagal flexibility in HAB rats in response to stress. Consistent with this, we found that HAB rats showed a smaller vagal withdrawal (reduction of RMSSD and HF power values) than LABs in response to the restraint stress. This was due to significant differences in baseline values of these vagal indexes, which were indeed much lower in HAB rats. These findings support the view that a low tonic vagal modulation of HR in HAB rats may have determined an inability to flexibly generate adequate HR responses to environmental demands. In addition, we found that the peak HR reached in response to potent β-adrenergic stimulation with isoproterenol was once again lower in HAB rats compared to LAB rats. Therefore, we cannot exclude that the reduced HR responsiveness observed in HAB rats was also due to a desensitization/downregulation of adrenergic receptors in the sino-atrial node.

Importantly, following β-adrenergic pharmacological stimulation with isoproterenol, we found an increased susceptibility to arrhythmias in HAB rats. Arrhythmias were almost exclusively of ventricular origin, whereas supraventricular arrhythmias were just occasionally noted. These findings suggest that HAB rats might have a higher sensitivity/density of ventricular β-adrenergic receptors compared to LAB rats. Signs of increased, although modest, vulnerability to ventricular arrhythmias were also found in HAB rats during the restraint test. Similarly to what has been observed in our previous study on high-aggressive wild-type rats [24], we speculate that HAB rats might be characterized by changes in the electrical properties of the ventricular myocardium (e.g., altered refractoriness and/or conduction [40]) that could potentially be pro-arrhythmic under conditions of strong β-adrenergic stimulation.

Finally, we found that HAB rats were characterized by a higher heart-body weight ratio compared to LABs. Increased heart-body weight ratios are described in rodent models of heart failure and are associated with pathological conditions, such us LV hypertrophy [41, 42]. Here, we found a modest increment of LV wall thickness in HAB rats that was proportional to the increase in LV chamber volume. Moreover, morphological analysis revealed that the moderate increase of LV thickness in HAB rats was not due to a larger accumulation of fibrotic tissue within the LV myocardium. Therefore, the structural remodeling observed in HAB rats appears to rather reflect a nonpathological condition (i.e., increased muscle mass).

Conclusions and perspectives

This study provides a detailed investigation of cardiac autonomic regulation in a rat model of anxiety. We demonstrate that, similarly to what has been observed in anxious individuals, high anxiety-related behavior rats are characterized by a low-vagally mediated HRV. Such deficiency in tonic vagal modulation was coupled with a dampened HR responsiveness to acute stressors and with an increased vulnerability to pharmacologically-induced arrhythmias. These results are consistent with the view that high levels of anxiety-related behavior are associated with specific features of cardiac autonomic modulation and increased susceptibility to arrhythmias that may predict vulnerability to cardiac morbidity and mortality. Furthermore, these findings provide a strong basis for future mechanistic investigations aimed at i) defining, using this rat model, the central neural determinants of the reported vagal control impairment, and ii) increasing our understanding of the mechanistic basis of the comorbidity between anxiety disorders and cardiovascular disease.

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CHAPTER 5

VAGAL WITHDRAWAL AND SUSCEPTIBILITY TO CARDIAC ARRHYTHMIAS IN RATS WITH HIGH TRAIT AGGRESSIVENESS

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ABSTRACT

Personality characteristics, e.g. aggressiveness, have long been associated with an increased risk of cardiac disease. However, the underlying mechanisms remain unclear. In this study we used a rodent model for characterizing cardiac autonomic modulation in rats that differ widely in their level of aggressive behavior. To reach this goal, high-aggressive (HA, n=10) and non-aggressive (NA, n=10) rats were selected from a population (n=121) of adult male wild-type Groningen rats on the basis of their latency time to attack (ALT, s) a male intruder in a resident-intruder test lasting 600 s. In order to obtain information on their cardiac autonomic modulation, ECG recordings were subsequently obtained via radiotelemetry at rest, during stressful stimuli and under autonomic pharmacological manipulations, and analyzed by means of time- and frequency-domain indexes of heart rate variability. During resting conditions, HA rats (ALT<90s) displayed reduced heart rate variability, mostly in terms of lower vagal modulation compared to NA rats (ALT>600s). Exposure to stressful stimuli (i.e. restraint and psychosocial stress) provoked similar tachycardic responses between the two groups. However, under stress conditions HA rats displayed a reduced vagal antagonism and an increased incidence of tachyarrhythmias compared to NA rats. In addition, beta-adrenergic pharmacological stimulation induced a much larger incidence of ventricular tachyarrhythmias in HA rats compared to NA counterparts. These findings are consistent with the view that high levels of aggressive behavior in rats are associated to signs of cardiac autonomic impairment and increased arrhythmogenic susceptibility that may predict vulnerability to cardiac morbidity and mortality.

5.1 INTRODUCTION

In humans, there are large individual differences in the susceptibility for cardiac disorders. The hypothesis that this inter-individual variability may be, in part, influenced by aspects of personality has been subject of debate and research for a long time. The groundbreaking work of Friedman and Rosenman in the late 1950s still represents a hallmark on this regard [1]. They identified two types of behavior patterns (types A and B) that relate to individual stress responsivity and found that type A individuals (aggressive, hostile, impatient, competitive, achievement striving) were more vulnerable to heart disease than type B counterparts (relative absence of type A characteristics). Several failures to replicate this finding later questioned the role of the type A behavior pattern as a cardiac risk factor [2] [3]. This prompted researchers to examine individual components of the multifaceted type A behavior, as inconsistent association between the pattern and cardiac disease might indicate that only certain traits of type A personality influenced cardiac health. In particular, over the last twenty years a considerable number of studies has supported a relationship between the individual tendency to exhibit antagonistic interpersonal type A behaviors such us aggressiveness and hostility and increased risk for the onset and progression of cardiac disease [4-8].

Despite this, a mechanistic hypothesis has not yet been addressed conclusively in human studies. Putative pathophysiological mechanisms may include an impairment of autonomic nervous system control over cardiac function. Abundant evidence demonstrates that reduced autonomic modulation of the heart, as shown by heart rate variability (HRV) measurements, predicts the development of heart disease in initially healthy subjects [9] [10], as well as poorer survival rate in patients with myocardial infarction [11] [12] or heart failure [13].

A deeper insight into the underlying mechanisms might be facilitated by the use of an objective and unbiased animal model since most described behavioral traits in humans are identifiable in animal models as well. Like humans, the way most animal species cope with stressful situations shows a high variability in behavioral responses. In feral rodent populations, a major feature of behavioral coping is the individual tendency to exhibit aggressive intraspecific behaviors [14]. In line with the

characterization of personality in many other animal species [15-18], high levels of aggression in rodents are considered an important indicator and component of a more general proactive coping style, whereas low levels of aggression are believed to be a reflection of a reactive coping style [19] [20]. These divergent behavioral coping styles have frequently been associated with different patterns of both autonomic nervous and endocrine (re)activity [14] [19]. However, the investigation of the cardiac autonomic control of these distinct behavioral and physiological coping styles has been conducted only sporadically and provided inconclusive evidence [21] [22].

Given these considerations, in the current study we sought to characterize in detail the autonomic neural modulation of heart rate in two groups of male wild-type Groningen rats (Rattus norvegicus) that differed largely in their level of aggressive behavior. This rat strain was chosen because, in contrast to other laboratory rat strains, it shows a wide and consistent individual variation in aggressive behavior [14]. The behavioral categorization was based on a trait-like characteristic, i.e. high-aggressive or non-aggressive towards a male unfamiliar conspecific intruder. This study included several key goals: (a) to assess sympathetic and parasympathetic influences on heart rate via time- and frequency-domain analysis of HRV at rest and during stress conditions, (b) to assess the relative contribution of vagal control over heart rate by means of cholinergic muscarinic blockade with methylscopolamine; (c) to quantify intrinsic heart rate under double pharmacological autonomic blockade, via beta-adrenoceptor and muscarinic receptor antagonists and (d) to investigate susceptibility to cardiac arrhythmias under stress conditions and following β-receptor pharmacological stimulation. We tested the hypothesis that high levels of aggressive behavior in rats would be directly related to specific features of autonomic neural modulation of heart rate, which would justify the use of this rodent model in preclinical studies investigating the mechanisms underlying the link between aggressiveness and cardiac disease vulnerability.

5.2 METHODS

Ethics statement and animals

Experimental procedures and protocols were approved by the Veterinarian Animal Care and Use Committee of Parma University, with animals cared for in accordance with the European Community Council Directives of 22 September 2010 (2010/63/UE).

In this study we used 4-month-old male Wild type Groningen rats (*Rattus norvegicus*) weighing approximately 380 g. This rat population, originally derived from the University of Groningen (the Netherlands), is currently bred in our laboratory under conventional conditions, at ambient temperature of 22±2°C and on a reversed 12:12 light-dark cycle (light on at 19:00 h), with food and water available *ad libitium*.

Preliminary behavioral testing for aggressiveness

121 Wild type rats were assessed for the display of aggressive behavior towards male unfamiliar conspecific intruders using a standard resident-intruder aggression test [23]. Individual experiments were carried out in 6 different cohorts of animals over a period of 16 months using 20-21 animals in each experiment.

Ten days before the test, each rat was housed with a conspecific oviduct-ligated female partner to stimulate territorial behavior [24] [23]. Before the start of the test, the female partner was removed (approximately 15 min in advance) and an unfamiliar male Wistar rat was introduced into the home cage of the experimental rat. The intruder Wistar rats weighed on average 250 g (3 months old) and were socially housed. The test was repeated on three consecutive days, using every time a different intruder, in order to avoid familiarity between the opponents and obtain a reliable characterization of aggressive traits [14].

All tests lasted 10 min and the latency to the first attack towards the intruder (in s) was measured. The attack latency (average of 3 tests) was used as an index of individual aggressive behavior. As commonly seen in this rat strain [14], individual male resident rats differed widely in their level of

aggression towards unfamiliar intruder males (Figure 1). The ten most aggressive rats (average attack latency= 75±4 s; average number of attacks= 7.4±0.6) were selected and classified as high-aggressive (HA) rats (Figure 1). Ten rats showed no overt aggression at all towards the intruder during the 600-s confrontations and were selected and classified as non-aggressive (NA) rats (Figure 1). HA and NA rats were then used for the following experimental procedures.

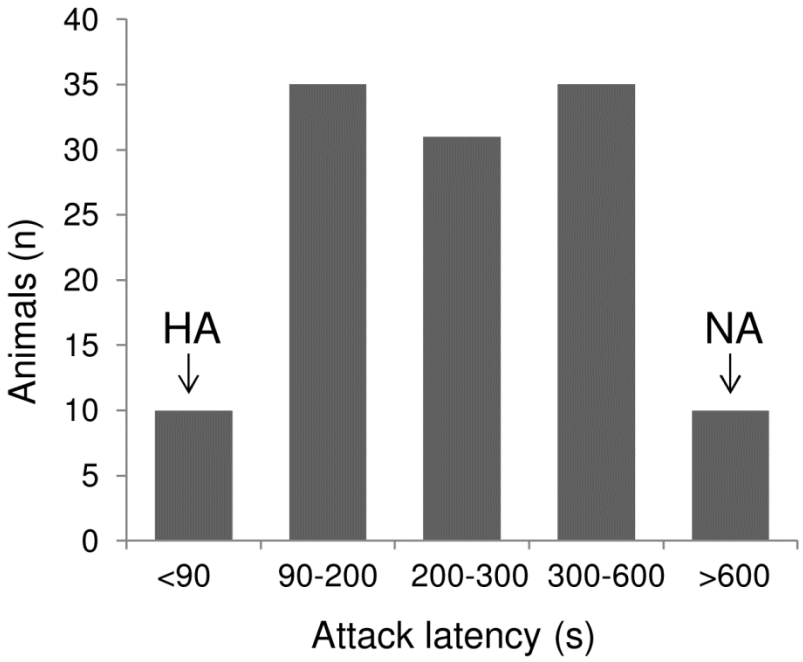

Figure 1. Individual variability in aggressiveness. Distribution of the individual variation in attack latency time towards an unfamiliar intruder within the population (n=121) of wild-type rats. On the x-axis the average (3 tests) attack latency to first attack the intruder rat is categorized. On the y-axis the number of rats in each category is presented. Only rats classified as high-aggressive (HA, attack latency <90s) and non-aggressive (NA, no overt aggression at all during the 10 min-duration of the tests) were subsequently used for the experimental procedures.

Surgery: radiotransmitter implantation

Following the preliminary behavioral testing, HA and NA rats were anesthetized with tiletamine hydrochloride + zolazepam hydrochloride (Zoletil 200 mg/kg, s.c.) Radiotelemetric transmitters (TA11CTA-F40, Data Sciences International, St. Paul, MN) for recording electrocardiogram (ECG), core body temperature (T, °C) and locomotor activity (LOC, expressed as counts/minute, cpm) were implanted according to a procedure described by Sgoifo and colleagues [25]. The transmitter body was placed in the abdominal cavity; one electrode was fixed to the dorsal surface of the xyphoid process and another electrode was placed in the anterior mediastinum close to the right atrium. Such electrode location guarantees high-quality ECG recordings, even during vigorous physical activity. Immediately after surgery, rats were individually housed, injected for 2 days with gentamicin sulfate (Aagent, Fatro, 0.2ml/kg, s.c.) and allowed 10 days of recovery before the start of experimental recordings.

Experimental protocol and radiotelemetric recordings

Following recovery from surgery, animals were left undisturbed in their home cages for 6 days for collection of daily rhythms of heart rate (HR, bpm), T and LOC. Subsequently, rats were submitted on different days to: i) pharmacological autonomic blockade (day 1), ii) restraint test (day 2), iii) psychosocial stress test (day 3), and iv) β-adrenoceptor pharmacological stimulation (day 4). These tests (described below) were carried between 10:00 and 14:00 (i.e. the dark phase of the light/dark cycle). ECG waves (sampling frequency 1000 Hz), T and LOC signals (sampling frequency 256 Hz) were picked up by a radiotelemetry receiver (RPC-1) and recorded via ART-Silver 1.10 data acquisition system (Data Sciences Int., St. Paul, MN, USA).

Baseline daily rhythms

ECG, T and LOC were sampled around-the-clock for 2 minutes every hour over a period of 6 days for collection of baseline daily rhythms. Each day, separate estimates of HR, HRV indexes (see section ‘Quantification of HRV’ for details), T and LOC were generated as average values of 12h-light and

12h-dark phases. These parameters were then further averaged as means of the 6 days of the light and dark phases.

Pharmacological autonomic blockade

The muscarinic receptor antagonist methylscopolamine (0.05 mg/kg, Sigma, St Louis, MO) and the β1-adrenergic receptor antagonist atenolol (2 mg/kg; Sigma, St Louis, MO, USA) were injected s.c. to block vagal and sympathetic influences to the heart in HA and NA rats. After baseline ECG recording (30 min), methylscopolamine was injected to evaluate the relative contribution of the vagal control over HR (vagal modulation), which was calculated as the difference between HR under vagal blockade with methylscopolamine (15-min mean) and resting HR (30-min mean). Fifteen min after methylscopolamine injection, atenolol was administered to the same rats to determine intrinsic heart rate (IHR). The doses and time courses of methylscopolamine and atenolol injections were selected on the basis of the available literature data [26]. IHR is established when the cardiac autonomic nervous system is completely blocked, which, in this instance, is supposed to take place approximately 15 min after the sympathetic blocker injection [27] [28].

Restraint test

Each animal was introduced for 15 min into a restrainer fitted closely to the body size (wire-mesh tube; inner diameter: 6 cm, length: 20 cm). After the test, animals were returned to their home cages. Continuous ECG, T and LOC recordings were performed in baseline conditions (30 min, prior to the test), during the restraint test (15 min) and throughout the recovery period (45 min).

Psychosocial stress test

A male Wistar rat was transferred to the cage of each experimental rat, with a wire mesh partition separating the two opponents in order to avoid direct physical contact. During this phase (15 min), HA and NA resident rats were in constant sensory contact with the intruder. As demonstrated in a

previous study, the mere presence of a potentially antagonist individual in the home cage elicits a strong psychosocial stress response (elevation of plasma corticosterone levels) in both aggressive and nonaggressive resident rats [29]. Moreover, cardiac autonomic reactivity during psychosocial stress test (adverse social contact without overt fighting) in intruder rats was shown to be as large as during an actual fight experience [30]. Continuous ECG, T and LOC recordings were performed in baseline conditions (30 min, prior to the test), during the test (15 min) and throughout the recovery period (45 min).

β-adrenoceptor pharmacological stimulation

After baseline ECG recording (30 min), the β-adrenoceptor agonist isoproterenol (0.02 mg/kg, Sigma, St Louis, MO) was injected s.c. to HA and NA rats. The dose of isoproterenol injection was selected on the basis of the available literature data [31]. ECG recordings were performed for 15 min after isoproterenol administration, to evaluate the chronotropic and proarrhythmic effects of β-adrenoceptor stimulation.

Quantification of HRV

HRV analysis was conducted on multiple segments of stable, continuous ECG signals recorded during: i) baseline daily rhythms (segment duration: 2 min) and ii) restraint test, psychosocial stress test and pharmacological manipulations (segment duration: 5 min). Initially, each raw ECG signal was manually inspected to ensure that all R-waves were correctly detected. We then calculated HR by plotting the number of R waves per unit time (reported in beats per minute; bpm). Subsequently, we quantified time- and frequency-domain parameters of HRV. In the time-domain, we obtained the square root of the mean squared differences of successive RR intervals (RMSSD, ms), which quantifies short-term, high-frequency variations of RR and therefore estimates the activity of the parasympathetic nervous system [32]. For spectral (frequency-domain) analysis of HRV, the power spectrum was obtained with a fast Fourier transform-based method (Welch's periodogram: 256 points,

50% overlap, and Hamming window). We considered the total power of the spectrum (ms^2) and the power of the low frequency (LF; 0.2-0.75 Hz) and high frequency (HF; 0.75-2.5 Hz) bands in absolute values (ms^2). The power of LF band is a non-specific index as it contains contributions of both the sympathetic and parasympathetic influences [33]; the power of HF band is due to the activity of the parasympathetic nervous system and includes respiration-linked oscillations of HR [34]. The low frequency/high frequency ratio (LF/HF) estimates the fractional distribution of power and is taken as a synthetic measure of sympathovagal balance [35]. Those parts of ECG recordings which were non-stationary and/or exhibited recording artifacts were excluded from the analysis in accordance to an automatic test checking stationarity of the mean and variance of HR [36] [35].

Quantification of arrhythmic events

The occurrence of arrhythmic events in baseline conditions, under stressful stimuli and following β-adrenoceptor pharmacological stimulation was determined and quantified off-line based on the Lambeth Conventions for the study of experimental arrhythmias [36]. We determined and quantified the total number of arrhythmic events and the separate occurrence of supraventricular and ventricular ectopic beats, either as isolated or grouped events.

Data analyses

Data are presented as means ± standard error of the mean (SEM) for all analyses, tables and figures. Two-way ANOVA for repeated measures was applied for data obtained from: i) baseline daily rhythms, with group as between-subject factor (2 levels: HA and NA) and time as within-subject factor (2 levels: light and dark phases); ii) restraint and psychosocial stress, with group as between-subject factor (2 levels: HA and NA) and time as within-subject factor (5 levels: baseline; test; recovery 1, 2, and 3). Follow-up analyses were conducted using Student's "t" tests, with a Bonferroni correction for multiple comparisons for each outcome variable separately. A priori Student's "t"-tests, after controlling for homogeneity of variance via Levene test, were applied for comparisons between HA and NA rats on

the occurrence of arrhythmic events and pharmacological manipulations. Statistical significance was set at $p<0.05$.

5.3 RESULTS

Daily rhythms of radiotelemetric parameters

The daily rhythms of HR, HRV, T and LOC under resting conditions are presented in Table 1.

Two-way ANOVA yielded main effects of: i) time for HR values ($F=166.7$, $p<0.01$), RMSSD values ($F=35.4$, $p<0.01$), total spectral power ($F=28.7$, $p<0.01$), spectral power in LF and HF band (LF=, 35.4, $p<0.01$; HF=39.4, $p<0.01$), T ($F=161.9$, $p<0.01$) and LOC values ($F=41.8$, $p<0.01$); and ii) group for RMSSD values ($F=6.4$, $p<0.05$), total spectral power ($F=7.2$, $p<0.05$) and spectral power in LF band ($F=6.4$, $p<0.05$).

HA and NA rats had similar HR values in both phases of the light-dark cycle (Table 1). However, time-domain analysis of HRV indicated that HA rats had significantly lower values of RMMSD than NA rats during both the light ($t=-2.14$, $p<0.05$) and the dark ($t=-2.86$, $p<0.01$) phases (Table 1). Frequency-domain analysis of HRV revealed that: i) total spectral power was significantly lower in HA rats than NA rats during the light ($t=-2.56$, $p<0.05$) and the dark ($t=-2.51$, $p<0.05$) phases (Table 1); ii) spectral power in LF band resulted significantly lower in HA rats compared to NA rats in both phases (light: $t=-2.2$, $p<0.05$; dark: $t=-2.59$, $p<0.05$) and iii) spectral power in HF band was also lower in HA rats, although statistical significance was reached only during the dark phase ($t=-2.25$, $p<0.05$) (Table 1). No differences between groups were observed for LF/HF values (Table 1), in accordance with the absence of significant differences in HR.

HA and NA rats had similar T and LOC values during both the light and the dark phases (Table 1).

Table 1. Daily rhythms of radiotelemetric and HRV parameters.

		Light	Dark
HR (bpm)	HA	334±4	371±6
	NA	329±10	365±9
RMSSD (ms)	HA	2.34±0.19*	1.91±0.14$^{\#}$
	NA	2.94±0.20	2.45±0.12
Total Power (ms^2)	HA	53.0±5.7*	36.8±3.5*
	NA	66.7±4.9	56.3±7.9
LF Power (ms^2)	HA	1.91±0.33*	1.31±0.25*
	NA	3.27±0.49	2.38±0.31
HF Power (ms^2)	HA	2.27±0.29	1.49±0.19*
	NA	3.22±0.42	2.24±0.27
LF/HF	HA	0.84±0.09	0.88±0.08
	NA	1.01±0.08	1.06±0.09
T (°C)	HA	37.7±0.1	38.0±0.1
	NA	37.6±0.1	37.9±0.1
LOC (cpm)	HA	2.7±0.2	3.8±0.2
	NA	2.9±0.3	4.2±0.2

For the 12h-light and 12h-dark phases, values are reported as means ± SEM of data obtained by averaging multiple 2-min segments acquired every hour over a period of 6 days, in high-aggressive (HA, n=10) and non-aggressive (NA, n=10) rats. Abbreviations: HRV = heart rate variability; HR = heart rate; RMSSD = square root of the mean squared differences of successive RR intervals; LF = low-frequency; HF = high-frequency; T = body temperature; LOC = locomotor activity.

** and $^{\#}$ indicate a significant difference between HA and NA rats ($p<0.05$ and $p<0.01$, respectively).*

Pharmacological autonomic blockade

During baseline, pre-injection ECG recordings HA and NA rats had similar HR (Figure 2A). However, HA rats had significantly lower values of RMSSD ($t=-3.42$, $p<0.01$) and spectral power in HF band ($t=-2.72$, $p<0.05$) than NA rats (Figure 2B, C). No differences between groups were observed for LF/HF values (HA=1.05±0.14 vs. NA=1.13±0.12), in accordance with the absence of significant differences in baseline HR.

Injection of the muscarinic antagonist methylscopolamine provoked a rapid rise in HR, with the magnitude of this HR increment (vagal modulation) being smaller in HA than NA rats ($t=-2.1$, $p<0.05$) (Figure 2A). As expected, muscarinic receptor blockade provoked a marked reduction of RMSSD values and HF spectral power, with the magnitude of this decrement being smaller in HA than NA rats (RMSSD: $t=-3.4$, $p<0.05$; HF: $t=-2.8$, $p<0.05$) (Figure 2B, C).

IHR, evaluated after blockade of autonomic neural modulation with methylscopolamine and atenolol, was similar between the two groups (HA=362±6 bpm vs. NA=374±7 bpm).

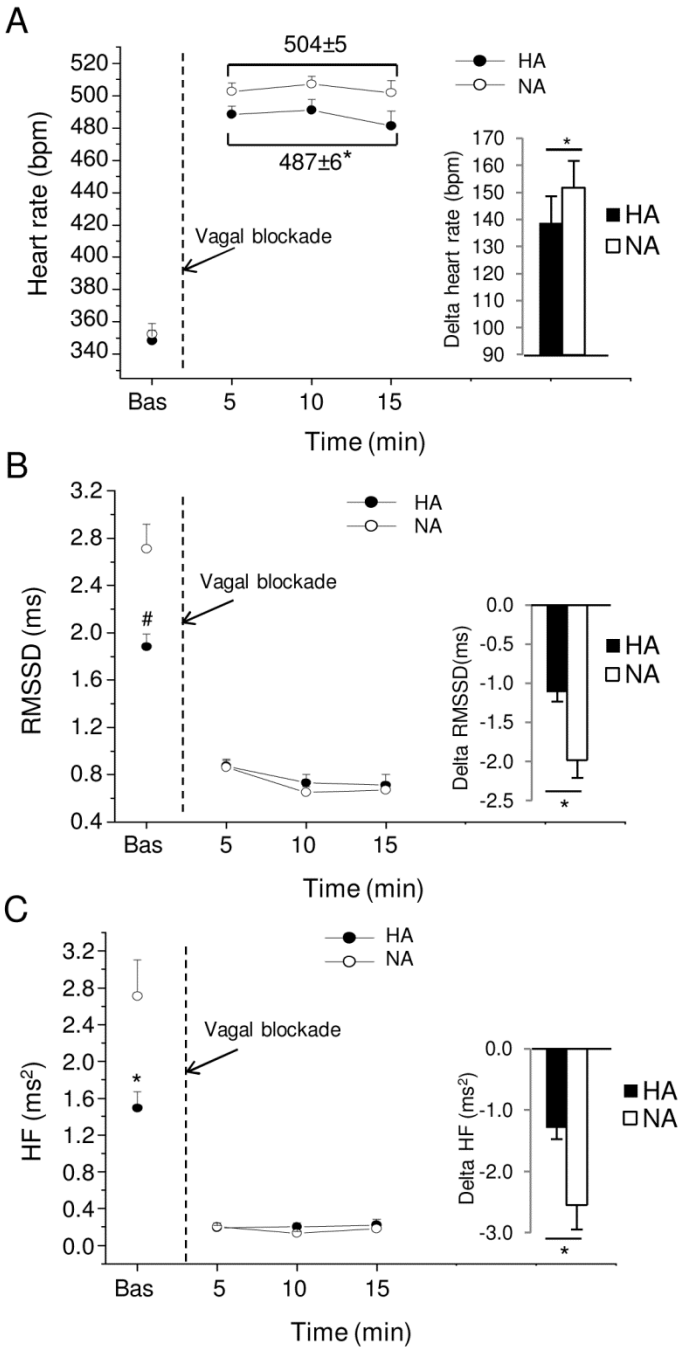

Figure 2. Effects of vagal blockade. Time course of changes in heart rate (panel A), RMSSD values (panel B) and high-frequency (HF) spectral power (panel C), in baseline conditions (bas) and after vagal blockade with metylscopolamine, in high-aggressive (HA, n=10) and non-aggressive (NA, n=10) rats. Baseline reference value (bas) is the mean value of the six 5-min time points in resting conditions. Values are expressed as mean ± SEM. Inner graphs in (A), (B) and (C) represent delta values, which were calculated as the difference between 15-min mean values under vagal blockade and basal values obtained during pre-injection recordings (30 min).

** and # indicate a significant difference between HA and NA rats (p<0.05 and p<0.01, respectively).*

Restraint test

Cardiac autonomic responses to the restraint test are depicted in Figure 3 and detailed in Table 2.

Two-way ANOVA yielded main effects of: i) time for HR values ($F=14.4$, $p<0.01$), total spectral power ($F=5.3$, $p<0.05$) and spectral power in LF band ($F=7.5$, $p<0.01$), and ii) group for RMSSD values ($F=6.1$, $p<0.05$) and spectral power in HF band ($F=6.4$, $p<0.05$).

Before the test, baseline HR was similar between the two groups (Figure 3A and Table 2). However, RMSSD values were significantly lower in HA than NA rats ($t=-3.7$, $p<0.01$) (Figure 3B and Table 2). Similarly, spectral power in LF and HF bands was significantly lower in HA than NA rats (LF: $t=-2.1$, $p<0.05$; HF: $t=-3.0$, $p<0.05$) (Figure 3C and Table 2). During restraint HR was similar between the two groups (Figure 3A and Table 2). However, in the same period RMSSD values and spectral power in HF band were significantly lower in HA than NA rats (RMSSD: $t=-2.1$ $p<0.05$; HF: $t=-2.1$ $p<0.05$) (Figure 3B, C and Table 2). No differences between groups were observed for LF/HF values (Table 2), in accordance with the absence of significant differences in HR. During the recovery phase, HR values were similar between HA and NA rats (Figure 3A and Table 2). RMSSD values and spectral power in HF band were significantly lower in HA than NA rats during the second (RMSSD: $t=-2.1$, $p<0,05$; HF: $t=-2.3$, $p<0.05$) and third (RMSSD: $t=-2.8$, $p<0,05$; HF: $t=-2.3$, $p<0.05$) 15-min recovery phase (Figure 3B, C and Table 2).

In addition, during the restraint test HA rats displayed a significantly higher incidence of arrhythmic events compared to NA rats ($t=2.1$, $p<0.05$) (Figure 4A). No differences between groups were found when ventricular and supraventricular arrhythmic events were considered separately (Figure 4A).

No differences between the two groups were observed for T and LOC values, neither during baseline recordings nor during and after the test (Table 2).

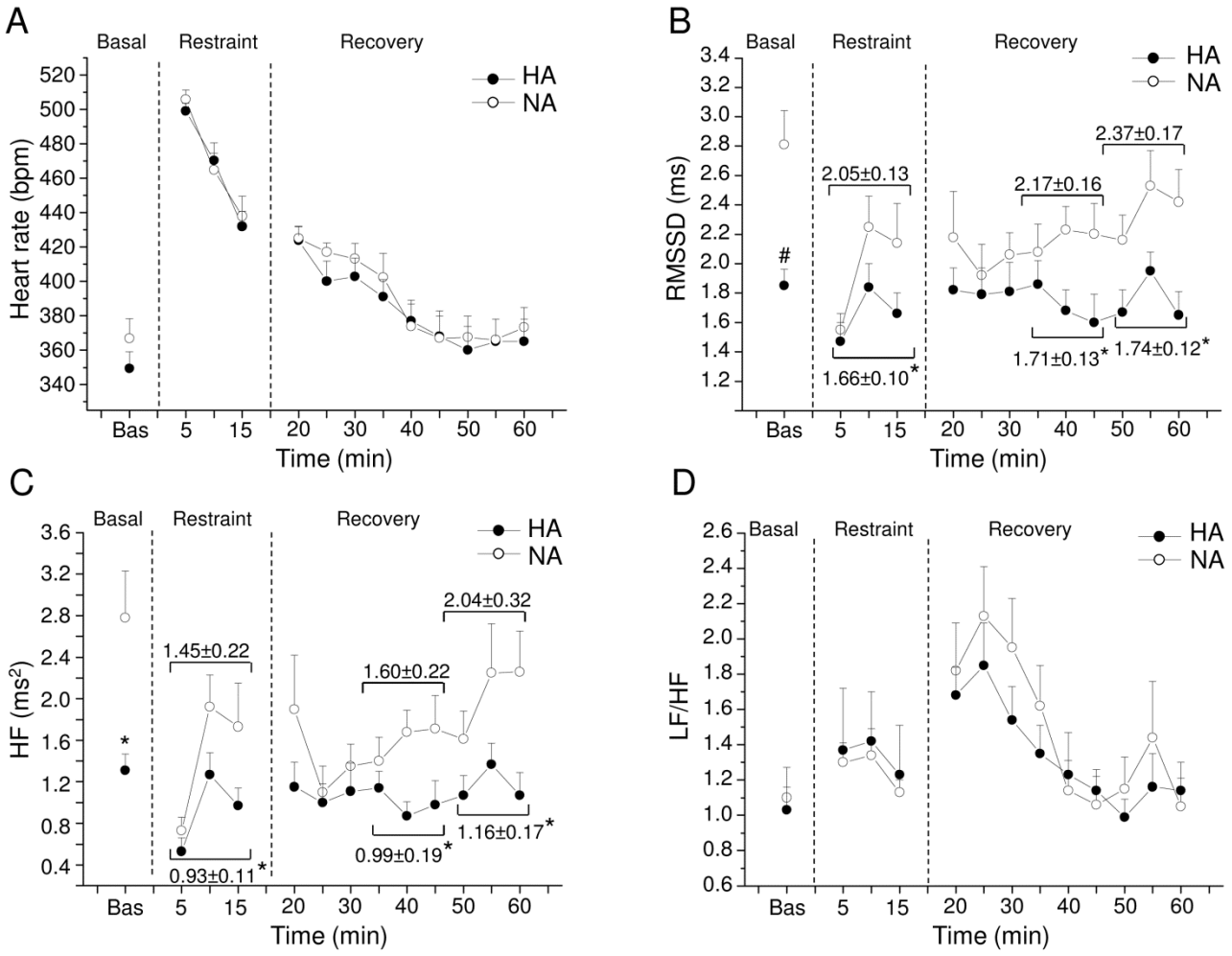

*Figure 3. Cardiac autonomic response to the restrain test. Time course of changes in heart rate (panel A), RMSSD values (panel B), high-frequency (HF) spectral power (panel C), and LF to HF ratio (panel D) in baseline conditions (bas), during the restraint and the recovery phase, in high-aggressive (HA, n=10) and non-aggressive (NA, n=10) rats. Baseline reference value (bas) is the mean value of the six 5-min time points in resting conditions. Values are expressed as mean ± SEM. * and # indicate a significant difference between HA and NA rats (p<0.05 and p<0.01, respectively).*

Table 2. Radiotelemetric and HRV parameters during the restraint test.

		Baseline	Restraint	Recovery (min 0-15)	Recovery (min 15-30)	Recovery (min 30-45)
HR (bpm)	HA	349±9	467±6	409±9	379±8	365±12
	NA	367±11	470±7	418±6	381±13	368±9
RMSSD (ms)	HA	1.85±0.10$^{\#}$	1.66±0.10*	1.81±0.16	1.71±0.13*	1.74±0.12*
	NA	2.81±0.22	2.05±0.13	2.05±0.19	2.17±0.16	2.37±0.17
Total Power (ms^2)	HA	68.9±9.8	23.6±3.2	31.6±3.9	35.9±4.9	43.8±6.1
	NA	73.9±11.5	27.3±3.3	38.7±5.7	33.1±3.2	63.1±13
LF Power (ms^2)	HA	1.38±0.29*	1.36±0.15	1.71±0.26	1.24±0.20	1.14±0.15$^{\#}$
	NA	2.95±0.61	1.83±0.29	2.53±0.36	1.80±0.27	2.12±0.25
HF Power (ms^2)	HA	1.31±0.15*	0.93±0.11*	1.09±0.20	0.99±0.13*	1.16±0.17*
	NA	2.78±0.43	1.45±0.22	1.45±0.28	1.60±0.22	2.04±0.32
LF/HF	HA	1.03±0.13	1.35±0.32	1.68±0.15	1.24±0.12	0.98±0.13
	NA	1.10±0.17	1.26±0.08	1.92±0.23	1.22±0.16	1.22±0.20
T (°C)	HA	37.8±0.1	38.1±0.1	38.7±0.1	38.4±0.1	38.1±0.1
	NA	37.9±0.1	38.3±0.1	38.8±0.1	38.5±0.1	38.2±0.2
LOC (cpm)	HA	2.4±0.7	4.0±0.6	10.0±1.4	2.4±0.6	2.7±0.7
	NA	3.4±0.9	4.6±0.6	12.6±1.1	4.1±0.9	3.5±1.1

Values are reported as means ± SEM of data obtained by averaging multiple 5-min segments acquired in baseline conditions (30 min), during the restraint (15 min) and the recovery phase (45 min), in high-aggressive (HA, n=10) and non-aggressive (NA, n=10) rats.

Abbreviations: HRV = heart rate variability; HR = heart rate; RMSSD = square root of the mean squared differences of successive RR intervals; LF = low-frequency; HF = high-frequency; T = body temperature; LOC = locomotor activity.

* and $^{\#}$ indicate a significant difference between HA and NA rats ($p<0.05$ and $p<0.01$, respectively).

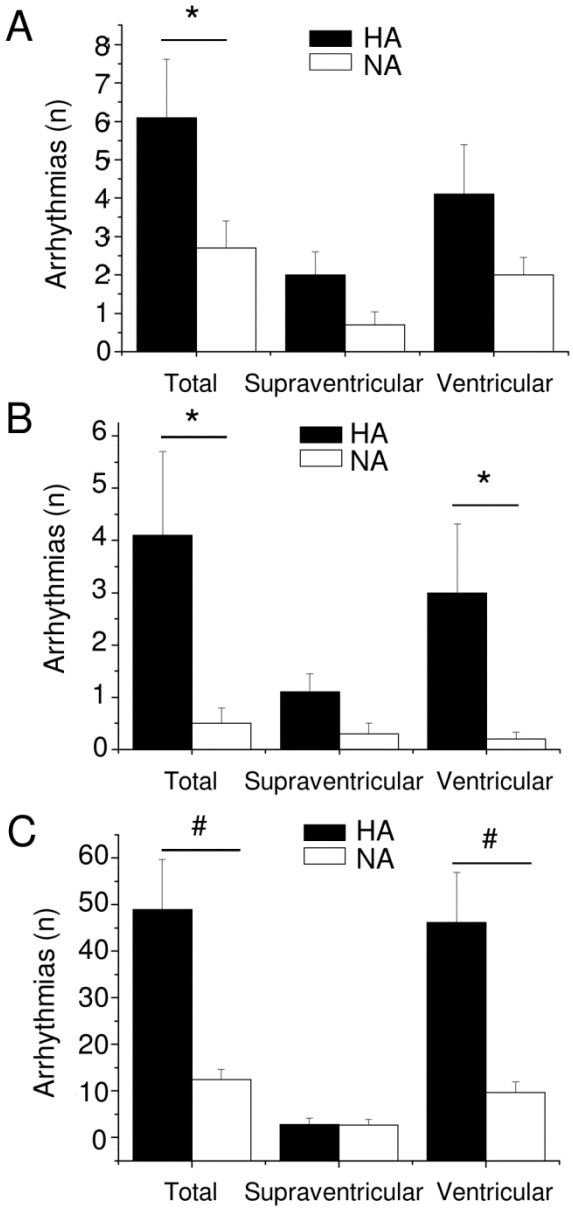

*Figure 4. Susceptibility to cardiac arrhythmias. Incidence of arrhythmias during the restraint (panel A), the psychosocial stress (panel B) and following β-adrenoceptor pharmacological stimulation (panel C), in high-aggressive (HA, n=10) and non-aggressive (NA, n=10) rats. Values are reported as mean ± SEM of number of events (n) per 15-min recording period. * and # indicate a significant difference between HA and NA rats (p<0.05 and p<0.01, respectively).*

Psychosocial stress test

Cardiac autonomic responses to the psychosocial stress test are depicted in Figure 5 and detailed in Table 3.

Two-way ANOVA yielded main effects of: i) time for HR values ($F=5.4$, $p<0.05$) and total spectral power ($F=5.9$, $p<0.05$)), and ii) group for RMSSD values ($F=6.1$, $p<0.05$).

During psychosocial stress HR was similar between the two groups (Figure 5A and Table 3). However, in the same period RMSSD values and spectral power in LF and HF bands were significantly lower in HA than NA rats (RMSSD: $t=-2.1$ $p<0.05$; LF: $t=-2.5$ $p<0.05$; HF: $t=-2.1$ $p<0.05$) (Figure 4B, C and Table 3). No differences between groups were observed for LF/HF values (Figure 4D and Table 3), in accordance with the absence of significant differences in HR. During the recovery phase HR values were similar between HA and NA rats (Figure 5A and Table 3), while RMSSD values and spectral power in HF band were significantly lower in HA than NA rats only during the second 15-min recovery phase (RMSSD: $t=-2.4$, $p<0,05$; HF: $t=-2.2$, $p<0.05$) (Figure 5B, C and Table 3).

In addition, during the psychosocial stress test HA rats displayed a significantly higher incidence of arrhythmias ($t=2.2$, $p<0.05$) (Figure 4B), which was mainly due to a significantly larger incidence of ventricular ectopic events ($t=2.1$, $p<0.05$) compared to NA rats (Figure 4B).

No differences between the two groups were observed for T and LOC values, neither during baseline recordings nor during and after the test (Table 3).

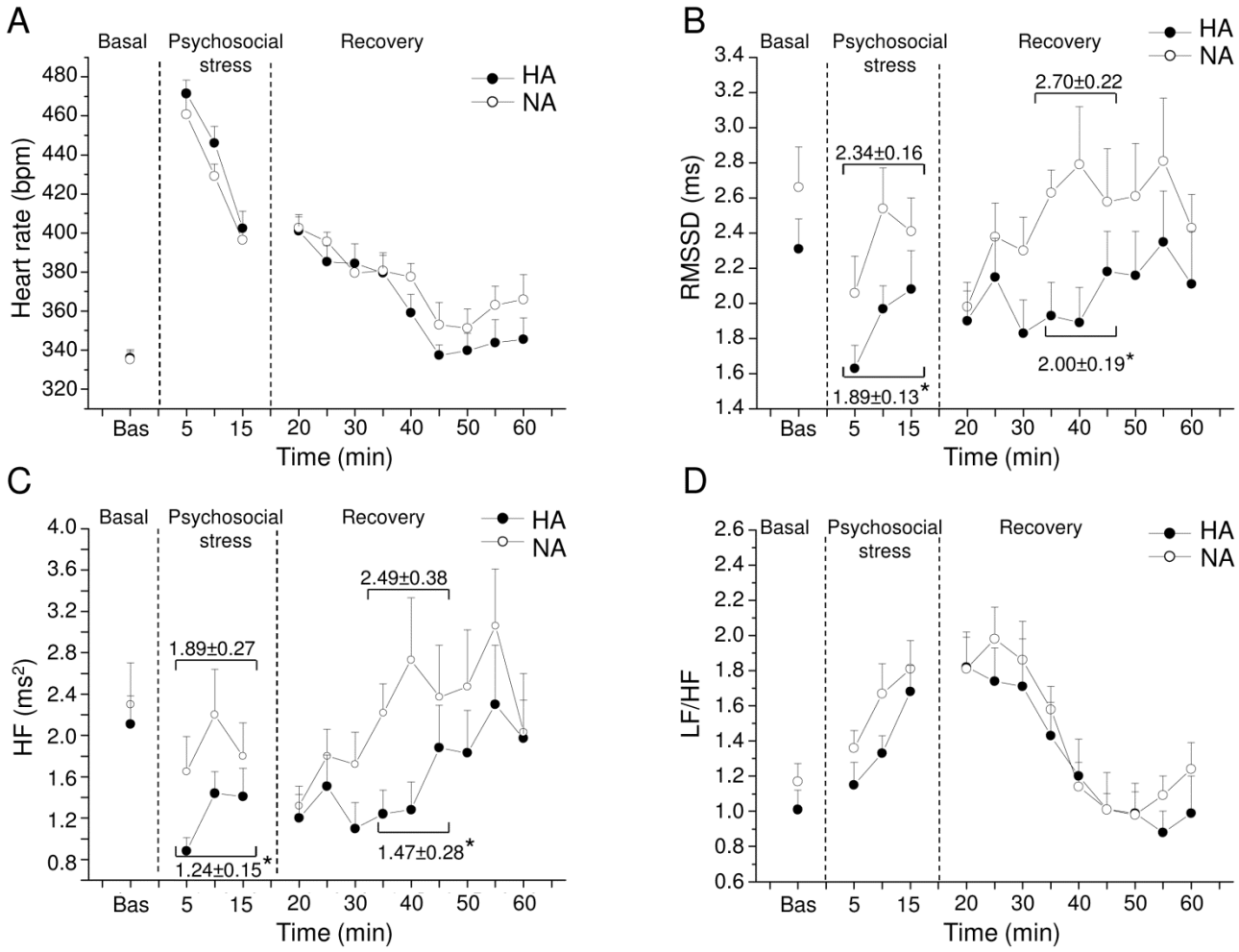

*Figure 5. Cardiac autonomic response to the psychosocial stress test. Time course of changes in heart rate (panel A), RMSSD values (panel B), high-frequency (HF) spectral power (panel C), and LF to HF ratio (panel D) in baseline conditions (bas), during the psychosocial stress and the recovery phase, in high-aggressive (HA, n=10) and non-aggressive (NA, n=10) rats. Baseline reference value (bas) is the mean value of the six 5-min time points in resting conditions. Values are expressed as mean ± SEM. * indicates a significant difference between HA and NA rats (p<0.05).*

Table 3. Radiotelemetric and HRV parameters during the psychosocial stress test.

		Baseline	Psychosocial stress	Recovery (min 0-15)	Recovery (min 15-30)	Recovery (min 30-45)
HR (bpm)	HA	336±4	440±7	390±8	361±8	343±9
	NA	335±4	430±7	392±2	367±8	360±9
RMSSD (ms)	HA	2.31±0.17	1.89±0.13*	1.96±0.17	2.00±0.19*	2.21±0.19
	NA	2.66±0.23	2.34±0.16	2.22±0.13	2.70±0.22	2.62±0.25
Total Power (ms^2)	HA	82.8±8.7	30.6±3.6	35.5±4.4	48.2±7.1	59.7±10.0
	NA	91.0±13.4	42.2±6.0	49.9±7.6	49.7±7.2	60.5±9.0
LF (ms^2)	HA	2.14±0.30	1.73±0.25*	2.14±0.41	1.57±0.31*	1.59±0.31
	NA	2.68±0.41	2.99±0.44	3.04±0.57	3.20±0.59	2.72±0.62
HF (ms^2)	HA	2.11±0.27	1.24±0.15*	1.27±0.21	1.47±0.28*	2.03±0.52
	NA	2.30±0.40	1.89±0.27	1.61±0.19	2.49±0.38	2.52±0.51
LF/HF	HA	1.01±0.11	1.40±0.10	1.69±0.17	1.07±0.17	0.78±0.17
	NA	1.17±0.10	1.58±0.11	1.88±0.15	1.29±0.10	1.08±0.15
T (°C)	HA	37.8±0.1	38.2±0.1	39.0±0.1	38.7±0.1	38.3±0.1
	NA	37.7±0.1	38.3±0.1	39.0±0.1	38.7±0.1	38.2±0.1
LOC (cpm)	HA	1.8±0.4	15.5±3.4	14.4±1.3	5.2±1.2	2.4±0.8
	NA	2.1±0.5	19.0±2.3	16.0±1.8	5.3±1.7	4.6±1.2

Values are reported as means ± SEM of data obtained by averaging multiple 5-min segments acquired in baseline conditions (30 min), during the psychosocial stress (15 min), and the recovery phase (45 min), in high-aggressive (HA, n=10) and non-aggressive (NA, n=10) rats.

Abbreviations: HRV = heart rate variability; HR = heart rate; RMSSD = square root of the mean squared differences of successive RR intervals; LF = low-frequency; HF = high-frequency; T = body temperature; LOC = locomotor activity.

** and # indicate a significant difference between HA and NA rats ($p<0.05$ and $p<0.01$, respectively).*

β-adrenoceptor pharmacological stimulation

HA and NA rats had similar HR during baseline, pre-injection ECG recordings (HA= 339±10 bpm vs. NA=323±7 bpm, p=ns).

After injection of the β-adrenoceptor agonist isoproterenol, HR was similar between the two groups (HA=488±4 bpm vs. NA=475±10 bpm, p=ns). However, following β-adrenoceptor stimulation with isoproterenol, HA rats displayed a dramatically higher incidence of arrhythmias compared to NA rats (t=3.5, p<0.01) (Figure 5C). The increased arrhythmogenesis in HA rats was exclusively due to a significantly larger incidence of ventricular arrhythmias compared to NA rats (t=3.5, p<0.01), whereas the occurrence of supraventricular arrhythmias was modest and similar between the two groups (Figure 5C). In addition, after β-adrenoceptor stimulation with isoproterenol, one HA rat displayed sustained ventricular tachycardia (Figure 6) that led to death.

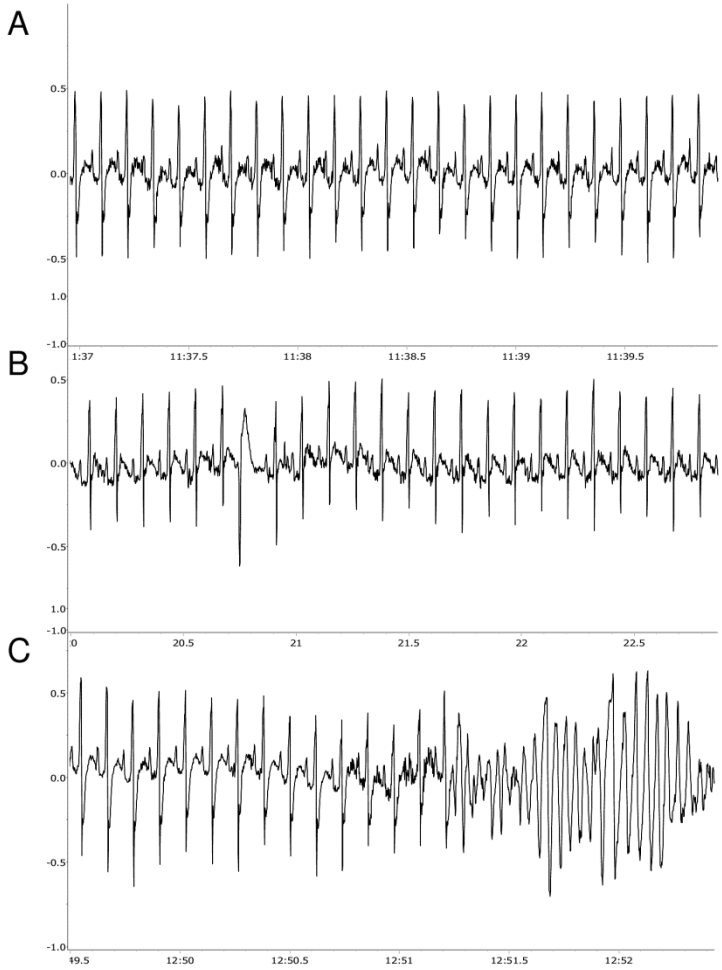

Figure 6. Lethal ventricular tachycardia in a high-aggressive rat. ECG traces belonging to a high-aggressive rat that died following β-adrenoceptor stimulation with isoproterenol. A: normal ECG. B: ECG with an isolated premature ventricular complex. C: ECG before rat's death with sustained ventricular tachycardia.

5.4 DISCUSSION

The purpose of this study was to characterize cardiac autonomic activity in wild-type rats that differed widely in their level of aggressive behavior. To reach this goal, autonomic neural modulation was studied at rest and under different stress conditions by means of HRV analysis, which relies on the principle that the pattern of beat-to-beat control of the sinoatrial (SA) node provides a reflection of cardiac autonomic activity. We found that high-aggressive rats had: i) lower HRV in resting conditions, ii) lower vagal modulation of heart rate at rest and under stress conditions, and iii) larger incidence of arrhythmias induced by stress or by pharmacological β-adrenergic stimulation compared to non-aggressive rats. These findings are consistent with the view that high levels of aggressive behavior in rats are associated with specific features of cardiac autonomic modulation and increased arrhythmogenic susceptibility that may predict vulnerability to cardiac morbidity and mortality.

The wild-type Groningen rat is a rodent species that shows a high individual variability in the tendency to behave aggressively or cope (pro)actively in response to stressful stimuli [19]. In our view, the selection of high-aggressive and non-aggressive subjects within this rat strain is a reliable approach for investigating the autonomic correlates of extremes in aggressive behavior [14]. The analysis of HRV is a widely used tool for obtaining detailed information on the relative contribution of sympathetic and vagal regulation of cardiac (re)activity [38]. Although there is an ongoing debate regarding the suitability of using HRV parameters to estimate sympathetic modulation [39], this approach produces reliable measures of vagal tone during both undisturbed resting and stress conditions [34] [39]. Particularly, parasympathetic/vagal modulation is a useful indicator for determining the behavioral and physiological flexibility of an organism and for measuring its ability to adequately respond to stress [40].

Initially, HRV parameters were evaluated during baseline rhythm recordings (6 days) in order to obtain a valid characterization of cardiac autonomic regulation under normal undisturbed resting conditions. Overall, we found that high-aggressive rats exhibited a reduced HRV compared to non-aggressive counterparts during both the light and dark phases of the circadian rhythm. As suggested by RMSSD

and HF power indexes, high-aggressive rats were characterized by a lower vagal modulation of heart rate. An important pharmacological confirmation of a reduced vagal modulation of heart rate in high-aggressive rats resulted from pharmacological challenge with methylscopolamine. The injection of this vagal blocker led to a lower heart rate increase in high-aggressive rats, confirming a lower relative contribution of the vagal control over resting heart rate compared to non-aggressive counterparts. The fact that vagal blockade provoked a milder reduction of RMSSD and HF power values in HA than NA rats was due to significant differences in baseline values, which were much lower in HA rats. Despite the difference in cardiac vagal tone, high-aggressive and non-aggressive rats had similar heart rate values at rest. One possible explanation for this apparent discrepancy may be that in high-aggressive rats the reduced cardiac vagal tone was coupled with decreased cardiac sympathetic influence on the SA node compared to non-aggressive rats. This is supported by the fact that the two groups did not differ for LF to HF ratio (index of sympathovagal balance), suggesting that the regulatory influences of the vagal and sympathetic components on cardiac pacemaker activity were similarly balanced in the two groups, leading to similar heart rate values. In addition, heart rate, measured after double autonomic blockade and therefore free from autonomic modulation, was similar between the two groups, suggesting that high-aggressive and non-aggressive rats had similar intrinsic automaticity of the SA node.

Subsequently, we tested the two groups under stress conditions. We found that HA and NA rats showed similar heart rate and body temperature responses to the restraint and psychosocial stress tests. This is an interesting and unexpected phenomenon, as previous studies have clearly demonstrated that high-aggressive wild-type rats have a larger sympatho-adrenomedullary reactivity (plasma noradrenaline and adrenaline levels) to acute stress challenges compared to less aggressive rats [21] [22]. Therefore, one would expect larger tachycardic and hyperthermic stress responses in high-aggressive rats. Given that the autonomic physiological target-organ responses (i.e., heart rate, body temperature) were instead not different between HA and NA animals, our first hypothesis was that vagal (re)activity was also higher in HA animals. This, however, was not the case, as the vagal

indices of RMSSD and HF power indicated that HA rats had lower cardiac vagal modulation compared to non-aggressive rats. Based on these observations, we hypothesize that adrenergic receptors in the SA node and skin vessels are desensitized/downregulated in HA animals. This adrenergic receptor remodeling would also explain why the influences of sympathetic and vagal modulations on the pacemaker region were similarly balanced in the two groups (LF to HF ratio index).

Interestingly, during the restraint and the psychosocial stress tests, high-aggressive rats showed a larger susceptibility to tachyarrhythmias compared to non-aggressive counterparts. Arrhythmogenesis was clearly stress-induced, as no arrhythmic events were noted during pre-stress recordings. Alterations in cardiac autonomic modulation are thought to exert a potent influence on arrhythmogenesis (for a review see [41]). Increases in sympathetic nerve activity, through the influence of noradrenaline on beta-adrenergic receptors, participate in the genesis of ventricular tachyarrhythmias. On the other hand, decreases in vagal tone leave the heart exposed to unopposed stimulation by the sympathetic nervous system, and consequently vulnerable to ventricular arrhythmia and sudden death [42]. In our study, high-aggressive rats exhibited a lower vagal antagonism during stress compared to non-aggressive rats, supporting the hypothesis that vagal tone impairment may represent a potential triggering mechanism for the increased arrhythmogenesis observed during stress in high-aggressive rats. However, we cannot exclude that the larger incidence of arrhythmias induced by stress in high-aggressive rats was due to a combination of a vagal dysfunction and the effects of elevated levels of circulating catecholamines [21] [22] on the electrical stability of the ventricular myocardium.

The arrhythmogenic susceptibility in high-aggressive rats was even more dramatically evident after β-adrenergic pharmacological stimulation with isoproterenol. In one high-aggressive rat the injection of the β-receptor agonist provoked sustained ventricular tachycardia that led to death from cardiac arrest. It is interesting to note that arrhythmias were almost exclusively of ventricular origin, whereas supraventricular arrhythmias were just occasionally noted. In addition, heart rate response to β-adrenergic pharmacological stimulation was similar between the two groups. These findings suggest

that high-aggressive rats might have a higher sensitivity/density of ventricular β-adrenergic receptors compared to non-aggressive rats. On the other hand, we speculate that high-aggressive rats might be characterized by changes in the electrical properties of the ventricular myocardium (e.g., altered refractoriness and/or conduction [43]) that could potentially be pro-arrhythmic under conditions of strong β-adrenergic stimulation.

Conclusion and perspectives

In humans, there is strong prospective evidence that reduced heart rate variability and suppressed vagal control over cardiac function contribute to arrhythmogenesis and predict premature cardiac morbidity and mortality [11] [44] [10]. In this study on wild-type rats we document an association among high levels of aggressive behavior, specific patterns of cardiac autonomic neural modulation, and increased arrhythmogenic susceptibility that may predict vulnerability to cardiac morbidity and mortality. We demonstrate that high-aggressive wild-type rats are characterized by lower vagal activity at rest and poorer vagal antagonism during stress, and by a much larger susceptibility to stress- and pharmacologically-induced arrhythmias. These results highlight the relevance of this rodent model for the study of the link between aggressive behavior and cardiac disease vulnerability. Further, these findings provide a strong foundation for future mechanistic experiments that will determine: i) the central neural determinants of the described vagal control impairment; and ii) the cellular and subcellular bases of the reported arrhythmogenesis.

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CHAPTER 6

SUMMARY AND DISCUSSION

6.1 SUMMARY OF THE RESULTS

The studies reported in this thesis aimed at investigating the impact of psychosocial risk factors on cardiac activity in rat models, with special emphasis on the study of autonomic mechanisms underlying these associations.

In **chapter 2**, electrophysiological and structural properties of the myocardium were examined in rats in relation to depression. We provided evidence that rats exposed to a protocol of repeated social defeat stress exhibited signs of a depressive-like state on a variety of biological and behavioral readouts. Using multi-electrode epicardial mapping, we demonstrated that such depressive-like symptoms were associated with a reduction in myocardial refractoriness as well as impaired excitatory wave conduction, both of which are considered major determinants of arrhythmogenesis. Therefore, this stress paradigm seemed to represent a useful approach for investigating whether autonomic mechanisms exert their effects on the predisposed myocardium to trigger cardiac arrhythmias in animals showing depressive-like symptoms. This issue was addressed in the study described in **chapter 3.** Cardiac autonomic activity and arrhythmia vulnerability were investigated in Wistar Kyoto rats exposed to a similar protocol of repeated social defeat stress. We found that stressed rats showed biological and behavioral alterations that mirror human depression. From an autonomic point of view, stressed rats were characterized by a reduction in sympathetic influences to the heart, which consequently determined lower hear rate values. Notably, such autonomic changes were associated with an increased incidence of spontaneous arrhythmias. Possible explanations for the alterations in cardiac autonomic outflow reported in this study and their role in triggering cardiac arrhythmias were discussed. Importantly, we found that enhancement of endocannabinoid signaling via pharmacological inhibition of the enzyme responsible for the degradation of the endocannabinoid anandamide exerted antidepressant-like activity and cardioprotective effects in this rat model.

The study of cardiac autonomic activity in rats that differ widely in their levels of trait-anxiety was described in **chapter 4**. We demonstrated that high levels of anxiety in rats were associated with lower vagal modulation of resting heart rate and increased incidence of pharmacologically-induced

ventricular arrhythmias. Interestingly, in this rat model of trait-anxiety low tonic vagal modulation of resting heart rate appeared to determine an inability to flexibly generate adequate heart rate responses to stress.

In **chapter 5,** the hypothesis that specific personality traits may confer higher cardiac risk was tested. We selected two groups of rats that represented the extremes of the population in terms of aggressive behavior exhibited toward unfamiliar conspecific intruders. We demonstrated that high levels of aggressive behavior and dominance in rats were associated with signs of cardiac autonomic impairment (reduced heart rate variability, mostly in terms of lower vagal modulation) and increased susceptibility to stress- and pharmacologically-induced arrhythmias, which may predict vulnerability to cardiac morbidity and mortality.

6.2 RAT MODELS OF PSYCHOSOCIAL / CARDIAC DISTURBANCES. WHAT CAN WE LEARN FROM THEM?

Research in humans has clearly revealed a link between psychosocial risk factors and cardiovascular dysfunctional states. Chronic life stressors, psychological alterations such as anxiety and depression, personality traits such as anger and hostility, have all been shown to interfere with and modulate the onset and progression of cardiovascular alterations [1]. Preclinical research has just started to investigate the underlying biological bases. The mechanisms involved embrace neuroscience, physiology of the autonomic nervous system and cardiac and molecular electrophysiology, which are usually investigated and reported by investigators from different disciplines. Although the electrophysiological and molecular mechanisms involved in the susceptibility to cardiac arrhythmias are relatively well understood, great strides still need to be made to unravel the contribution of central autonomic pathways in this abnormal heart activity. Animal models that reproduce human negative psychosocial states can offer a valid interdisciplinary approach to this field.

Existing literature in humans is inconsistent as to whether autonomic nervous system function is altered in depressive patients. Some researchers found that depressive patients exhibit a shift of the

simpathovagal balance toward sympathetic dominance (e.g. [2,3,4,5]), while others have been unable to replicate this finding (e.g. [6,7,8]). Such controversy may reflect the difficulty to study the accumulative effects of depression on cardiac autonomic function in real life stress conditions, which can be chronic, intermittent, and psychosocial in nature. In addition, there is active debate concerning the extent to which antidepressant medications influence autonomic function [9,10]. From the studies reported in this thesis, it is quite evident that rat models of social stress-induced depression that bear high translational relevance for the human condition have the potential to shed new light on the autonomic changes that characterize depressive-like states and their impact on the electrical stability of the myocardium.

A great deal of research has highlighted the importance of individual differences in resting heart rate variability that are associated with emotional and personality characteristics to explain the large inter-individual variability in the vulnerability to cardiac disorders. The general consensus is that high levels of resting vagal tone are a sign of autonomic flexibility, the capability of the parasympathetic nervous system to generate adequate responses to environmental challenges by modifying heart rate, respiration and arousal [11,12,13,14]. On the contrary, low levels of vagal modulation may predict mismatches between environmental demands and cardiac (re)-activity, thus increasing vulnerability to cardiac arrhythmias and sudden cardiac death [15,16,17]. The rat models described in this thesis that reproduce trait behavioral characteristics such as anxiety and aggressiveness seem to successfully replicate aspects of cardiac autonomic dysregulation that are often described in anxious and hostile/type A individuals, providing valuable insights into the mechanisms of interaction between autonomic patterns at rest and cardiac reactivity and arrhythmia susceptibility in response to stress.

In our view, these rat models could be further exploited in order to (i) define the central neural determinants of the reported autonomic alterations, and (ii) increase our understanding of the mechanistic bases of the link between psychosocial risk factors and cardiac dysfunctional states.

6.3 DIRECTIONS FOR FUTURE RESEARCH

Given the increased likelihood of cardiovascular problems in patients with depression and anxiety, it is not only important to understand the mechanistic bases of these associations, but also to develop therapeutic treatments for affective disorders that do not have adverse cardiovascular side effects and, more desirably, improve cardiovascular function.

For over half a century investigation of the neurobiological bases of emotional behavior and affective disorders has predominantly focused on the role of monoaminergic neurotransmission, and the vast majority of current pharmacological approaches target monoaminergic systems, such as tricyclic antidepressants or serotonin-specific reuptake inhibitors (SSRIs). However, tricyclic antidepressants might be safe for depressed patients without history of cardiovascular problems, but the cardiotoxic effects of these drugs sometimes preclude their use in depressed patients with cardiovascular disease [18]. In addition, some negative cardiovascular effects of SSRIs, such as vasoconstriction and QT prolongation, have been reported [19,20]. If autonomic modulation is altered in otherwise healthy patients with affective disorders, then another important question is whether treatments for these disorders are able to ameliorate autonomic function. In a meta-analysis conducted by Kemp and colleagues [3] no evidence was found for the SSRIs or other antidepressant medications to improve (or worsen) autonomic function (as indexed by heart rate variability analysis), even when patients responded to treatment. A recent 2-year longitudinal study [21] reported that all classes of antidepressants including the tricyclic antidepressants, the SSRIs and the selective serotonin and noradrenaline reuptake inhibitors were associated with reductions in heart rate variability. These findings suggest that autonomic function might be altered by conventional antidepressant medications, therefore increasing the likelihood of adverse cardiovascular outcomes. This warrants the search for alternative pharmacological approaches for restoring the balance in cardiac autonomic control in psychiatric populations.

Recent investigations have started to draw attention to alternative neurochemical systems in the regulation of mood and anxiety disorders including neuropeptides, cytokines and bioactive lipids.

Endogenous cannabinoids, which include anandamide and 2-arachidonoylglycerol, are one class of bioactive lipids produced in the brain and periphery that exert biological actions via activation of cannabinoid type 1 and 2 receptors. The study of the role of endocannabinoid neurotransmission in the regulation of emotion and stress is exponentially growing. One common theme of endocannabinoid action is downregulation of excitatory (e.g. of glutamatergic) transmission, which prevents overexcitation in neural circuits responsible for hormonal and behavioral stress responses. Importantly, deficiency in the endocannabinoid signaling is thought to play a significant role in the etiology of depression [22]. Therapeutic hopes are derived from the discovery that pharmacological blockade of endocannabinoid-degrading enzymes exerts anxiolytic and antidepressant-like actions in a variety of animal models [23,24,25,26]. On the other hand, there is little evidence supporting significant endocannabinoid-mediated tonic control over the cardiovascular system in normal physiology, although its involvement in disease conditions is highly probable (for reviews see [27,28,29]).

In chapter 3, for the first time we provided evidence that pharmacological blockade of anandamide degradation in rats exerts not only antidepressant-like effects but also cardioprotection against the adverse consequences of repeated social stress exposure on autonomic function and arrhythmia vulnerability. Given the availability of relevant animal models of psychopathologies, the widespread use of heart rate variability analysis as a window into cardiac autonomic control as well as of electrophysiological and molecular techniques for evaluating the intrinsic properties of the myocardium, I believe that it is now timely for preclinical research to evaluate extensively the potential therapeutic value of pharmacological approaches that target the endocannabinoid system for the treatment of the comorbidity between affective and cardiac disorders, particularly under chronic stress conditions (Figure 1). This will hopefully be the work of my future research activity.

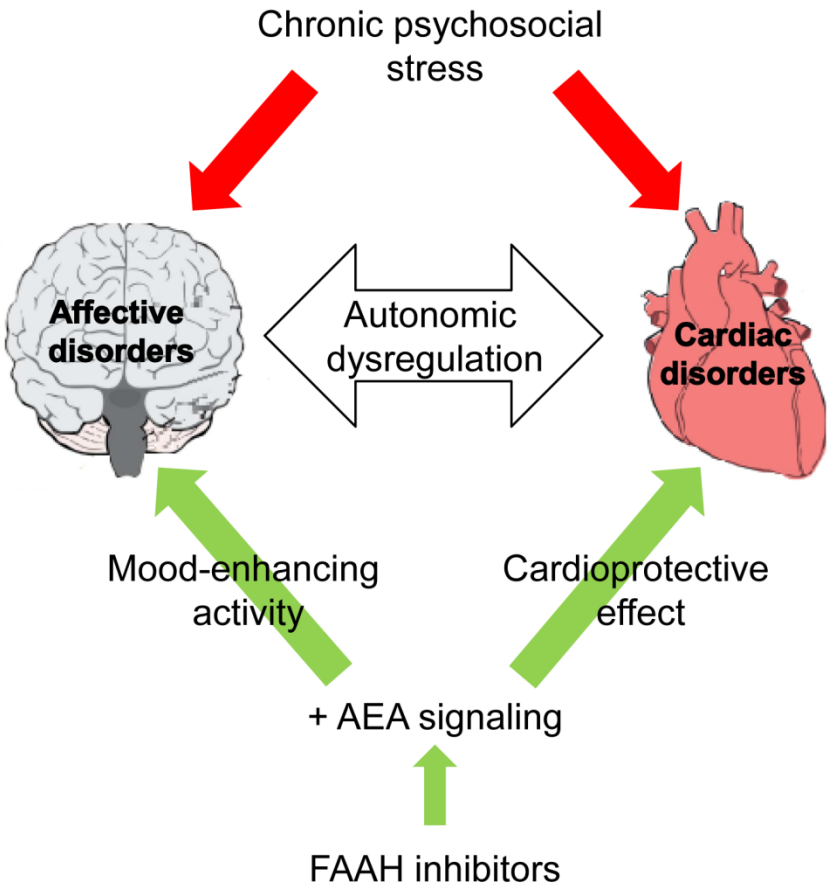

Figure 1. Working hypotheses. Chronic exposure to stressors of psychosocial nature represents a risk factor for the onset and progression of both affective and cardiac disorders, which are linked by a bidirectional association. Pathophysiological mechanisms underlying this comorbidity include a dysregulation of the autonomic neural control over cardiac function. Pharmacological approaches aimed at enhancing anandamide (AEA) signaling via inhibition of the enzyme responsible for AEA degradation (fatty acid amide hydrolase, FAAH) may improve psychological and cardiovascular function. FAAH inhibitors may exert cardioprotective effects (i) directly, by modifying cardiac electrical properties relevant to arrhythmogenesis, and/or (ii) indirectly, by restoring the autonomic balance.

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