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HEART RATE VARIABILITY IN ANIMAL MODELS OF PSYCHOLOGICAL-CARDIOVASCULAR COMORBIDITY

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Preface

Cardiovascular disease and psychopathologies, including depression and anxiety, represent respectively the first and second leading cause of serious illnesses, reduced quality of life, and mortality among the population of the Western countries. Interestingly, epidemiological and clinical studies highlighted a bidirectional association between cardiovascular dysfunction and psychiatric illnesses.

The presence of environmental stressors represents a common factor in the development of both psychopathologies and cardiovascular disease. Psychosocial adverse stimuli seem to represent independent risk factors for cardiovascular pathologies, as important as the traditional risk factors, such as hypertension, hypercholesterolemia, smoking, obesity and physical inactivity. At the same time, it is widely recognized that major life stressful events contribute to the subsequent development of mood and anxiety disorders.

Until now, the mechanistic links between cardiovascular dysfunction and altered mood or anxiety are not completely clarified. One of the most important pathophysiological mechanism proposed is the alteration of autonomic neural regulation of the heart. Cardiac autonomic dysfunction includes increased heart rate, decreased parasympathetic control, sympathetic hyperactivity, reduced heart rate variability, augmented catecholamine release, and reduced sensitivity of the baroreceptor reflex.

Reliable animal models that mimic human psychiatric illnesses may provide further insights on the changes of autonomic neural regulation characterizing comorbid depression/anxiety and cardiovascular dysfunction.

1. Cardiac autonomic regulation

Heart rate is normally determined by the rate of depolarization of the cardiac pacemaker (sinoatrial node, atrioventricular node, Purkinje fibers). In particular, the sinuatrial node is the normal cardiac pacemaker, because its rate of depolarization is faster than that of other pacemaker tissue. However, in order to better adapt cardiac response to the continuous environmental and internal stimuli the central nervous system plays a key role. Actually, the activity of the cardiovascular system is controlled by higher brain centers and control areas in the brainstem through the peripheral activity of the autonomic nervous system (ANS) (Hainsworth, 1998). The ANS comprises sympathetic and parasympathetic nerves and both divisions contain afferent and efferent nerves. The motor neurons forming the vagus nerves originate in the dorsal motor nucleus and in the nucleus ambiguous. Sympathetic nerves originate in the intermediolateral column of the spinal cord in the upper thoracic region.

The two branches of the ANS exert opposite and complementary effects on the cardiovascular system and, usually, their activity is in dynamic balance. Cardiac vagal innervation comprises the sinoatrial node, the atrioventricular conduction pathway and the atrial muscle. The parasympathetic influence on heart rate is mediated by synaptic release of acetylcholine by the vagus nerve. The activation of muscarinic acetylcholine receptors (M2) induces an increase in cell membrane K^+ conductance and also inhibits the hyperpolarization-activated pacemaker current I_f. Acetylcholine possesses a very short latency period and high turnover rate. The rapid response of this biological mechanism enables the parasympathetic nervous system to regulate cardiac function on a beat to beat basis. The effect of parasympathetic stimulation to the sinoatrial node is the decrease of its rate of depolarization resulting in slower heart rate (negative chronotropic effect). In

addition, vagal activity also slows atrio-ventricular conduction (negative dromotropic effect) and decreases the force of contraction of atrial muscle (negative inotropic effect). The right vagus nerve exert a major effect on the sinuatrial node, whereas the left nerve on the atrio-ventricular conduction pathway.

Sympathetic nerves innervate the entire heart (sinoatrial node, atrio-ventricular conduction pathway, atrial and ventricular myocardium) and their effects are due to the stimulation of beta-adrenergic receptors (beta1). This is mediated by synaptic release of noradrenaline which is reabsorbed and metabolized relatively slowly. Changes in cardiovascular function mediated by alterations in sympathetic activity therefore have a slower time course. Activation of beta-adrenergic receptors results in cAMP-mediated phosphorylation of membrane proteins and increases in I_{Ca2++} and I_f currents. The aim effects of sympathetic postganglionic fibers stimulation are the increase of heart rate (positive chronotropic effect), of force of contraction (positive inotropic effect), of atrio-ventricular conduction rate (positive dromotropic effect), of myocardial excitability (positive batmotropic effect) and of diastolic relaxation (positive lusitropic effect). The right sympathetic nerves are more concerned with regulation of heart rate, while the left sympathetic nerves with regulation of cardiac inotropic state.

Normally, there is a tonic activity in both divisions of the ANS and the net effect on heart rate represents the balance between the two antagonist effects (figure 1). However, at rest parasympathetic modulation of cardiac activity is dominant and its influence declines with increasing physical and mental arousal, with a concomitant rise of sympathetic activity. The two branches of the ANS tend to operate at different frequencies, because of the differences in their neurotransmitters' function and, therefore, it is possible to identify and quantify the variation in heart rate related predominantly to changes in sympathetic or parasympathetic activity (Greenwood et al., 1997).

Other physiological mechanisms influence cardiovascular control, including baroreceptors and chemoreceptors activity, local tissue metabolism and circulating hormones (Levy and Martin, 1979). The ANS controls heart rate and force, but also gives rise to reflexes that influence both the heart itself and the state of contraction of the vessels. These neural pathways are also closely linked to baroreceptor reflex activity, with changes in blood pressure playing a key role either increasing or decreasing activity of one or the other pathway (figure 1).

Blood pressure modifications, recognized by baroreceptors, induce opposite variations of heart rate. Baroreceptors are localized in the adventitia of some arteries, particularly the carotid sinuses and the aortic arch and they summate in their reflex effects. Increases in blood pressure stretch the vessels inducing increases in discharge frequency of the receptors. This results in increase in efferent vagal activity and decrease in sympathetic activity inducing slower heart rate. The baroreceptor mediated modifications of heart rate occur at a low frequency (typically six times per minute in humans) and can be significantly modified by sympathetic blockade (Sleight et al., 1995).

Other stretch receptors are localized in the atria near the junctions of the superior and inferior venae cavae and the pulmonary veins. They are stimulated by the stretching due to increase in atrial volume and for this reason their discharge frequency is directly related to atrial pressure. The effect of the reflex mediated by these receptors is to rise heart rate in response to an increase in venous return mediated by an increase in cardiac sympathetic nerves (Bainbridge reflex).

Arterial chemoreceptors are situated in the carotid and aortic bodies and their activity is stimulated by arterial hypoxia, hypercapnia or acidemia. The stimulation of these receptor principally causes increase in the rate and depth of respiration. However, both sets of

receptors also influence heart rate, but in opposite directions. Actually, carotid chemoreceptors slow heart rate, whereas aortic chemoreceptors increase it.

Superimposed on sympathetic and parasympathetic activity are very slow cyclical variations that have not been well characterized, but are thought to be related to changes in autonomic activity associated with thermoregulatory mechanisms (Fleisher et al., 1996) and fluctuations in activity of the renin-angiotensin system (Taylor et al., 1998).

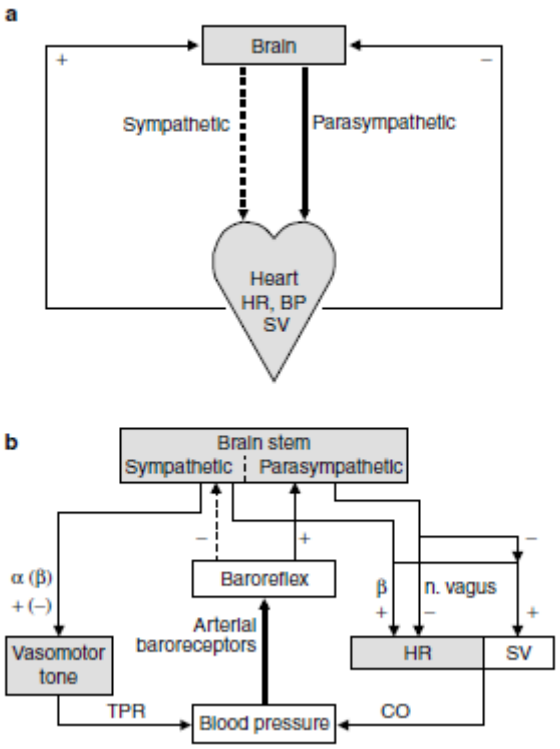


Figure 1. (a) A very simple model illustrating the influence of the sympathetic and parasympathetic nervous activity on heart rate; (b) a more elaborate working model of cardiovascular control mechanisms of heart rate, blood pressure and the feedback mechanism of the baroreflex (from Aubert et al., 2003). BP = blood pressure; CO = cardiac output; HR = heart rate; n.vagus = nervus vagus; SV = stroke volume; TPR = total peripheral resistance; α = α-sympathetic system; β = β-sympathetic system.

Normal heart rate is characterized by an elevated degree of beat-to-beat variability that depends on respiration, physical, environmental and mental factors and circadian variations. The cyclical variation of heart rate in relation to respiration is named respiratory sinus arrhythmia: actually, heart rate increases during inspiration and decreases during expiration. The mechanism linking the variability of heart rate to respiration is complex and involves both central and reflex interactions. This respiratory related variation occurs at a high frequency (typically 15 times per minute at rest) and can be abolished by vagal blockade (Keselbrener and Akselrod, 1998). Sinus arrhythmia is principally due to the activity of the parasympathetic nervous system. Actually, the two branches of the ANS are characterized by different rates of action, with vagal effects being faster than sympathetic ones. Therefore, only parasympathetic modulation is able to adapt heart rate to the cyclic and rapid alternation of inspiration and expiration. The magnitude of the sinus arrhythmia can provide information about the level of cardiac vagal modulation (Eckberg, 1983).

Cardiovascular system is characterized by circadian variation: in particular, in humans heart rate and blood pressure are decreased during the night, whereas in rodents is the opposite (Littler et al., 1975). Circadian variations are due to central control mechanisms (hypothalamus), autonomic regulation and circulating and local hormones.

During physical exercise heart rate rapidly increases and many mechanisms are involved in this immediate response: vagal withdrawal and sympathetic activation, stimulation of metaboreceptors in the muscles, reflexes from lung inflation, inhibition of the baroreceptor reflex. However, a program of strenuous physical training decreases resting heart rate, but unchanging cardiac output (Saltin et al., 1968).

2. Heart Rate Variability (HRV)

Normal heart rate is characterized by beat-to-beat variability over a wide range that is described and quantified by the analysis of heart rate variability (HRV). A high variability in heart rate is considered as a sign of a good adaptability, whereas lower variability is often a marker of abnormal and insufficient adaptability of the autonomic nervous system, implying the probable presence of a physiological malfunction (Pumprla et al., 2002).

As described in details before, the main modulator of heart rate variations is the ANS. Therefore, HRV analysis provides, in a non-invasive way, information about cardiac autonomic modulation (Task Force, 1996). In normal conditions, the two branches of the ANS are in dynamic balance. However, the activity of the sympathetic and parasympathetic components can be rapidly modulated in response to changes in the environmental demands. When these modifications lead to a static imbalance, the organism becomes vulnerable to pathology. Actually, the autonomic imbalance, in which one branch of the ANS predominates over the other, is associated with a lack of dynamic flexibility and health (Thayer et al., 2010). Many evidences suggest that the autonomic imbalance, in which typically the sympathetic system is hyperactive and the parasympathetic system is hypoactive, is associated with different pathological conditions (Thayer and Friedman, 2004). Since the sympathetic system is associated with energy mobilization, the condition in which this branch predominates is characterized by excessive energy demands that ultimately cannot be met. Therefore, autonomic imbalance may increase morbidity and mortality in many conditions and diseases.

Normally, the heart is under tonic inhibitory control by parasympathetic activity. As the parasympathetic system is associated with vegetative and restorative functions, resting cardiac autonomic balance favors energy conservation. In addition, heart rate is

characterized by beat-to-beat variability over a wide range, which is principally due to vagal influence. Therefore, autonomic imbalance in which the activity of the sympathetic branch predominates is associated with a reduction in HRV. Low HRV has been shown to be associated with increased risk of all-cause mortality. Thus, changes in the HRV pattern offers an early and sensitive indicator of compromised health (Decker et al., 1997; Liao et al., 1997; Tsuji et al., 1996).

Analysis of HRV was first used in clinical practice almost 50 years ago. The first application of HRV dates back to 1965 when Hon and Lee (Hon and Lee, 1965) noted that the reduction of HRV preceded fetal distress, in particular hypoxia, before any appreciable change occurred in heart rate itself. Then, in the late 1970s the reduction of HRV was first correlated with increased mortality and arrhythmic events in survivors of myocardial infarction (Wolf et al., 1978). The clinical importance of HRV became appreciated in the late 1980s, when it was confirmed that HRV was a strong and independent predictor of mortality after an acute myocardial infarction (Kleiger et al., 1987; Malik et al., 1989). More recently, HRV analysis has been increasingly used to assess autonomic dysfunction in different pathological conditions.

2.1 Measurement of HRV

The first step for the analysis of HRV is obtaining high quality ECG under stationary conditions. The ECG signals are analogue/digital converted for computer processing and, in order to have a good time resolution, a sampling rate of at least 250 Hz (normally for human ECG signals) and up to 1000 Hz (normally for rat ECG signals) or 2000-5000 Hz (normally for mouse ECG signals) is recommended. Heart rate variability is quantified by analysis of variations of the intervals between consecutive normal heart beats. The usual

definition of a heart beat interval is the time between consecutive R wave peaks. The time-course of the R-R interval is called tachogram and further quantitative analysis of this curve allows to obtain the HRV parameters (figure 2). Advances in computer technology have allowed sequential R-R intervals to be measured accurately and recorded in real time. It is crucial that before processing, these signals are corrected for ectopic and missed beats.

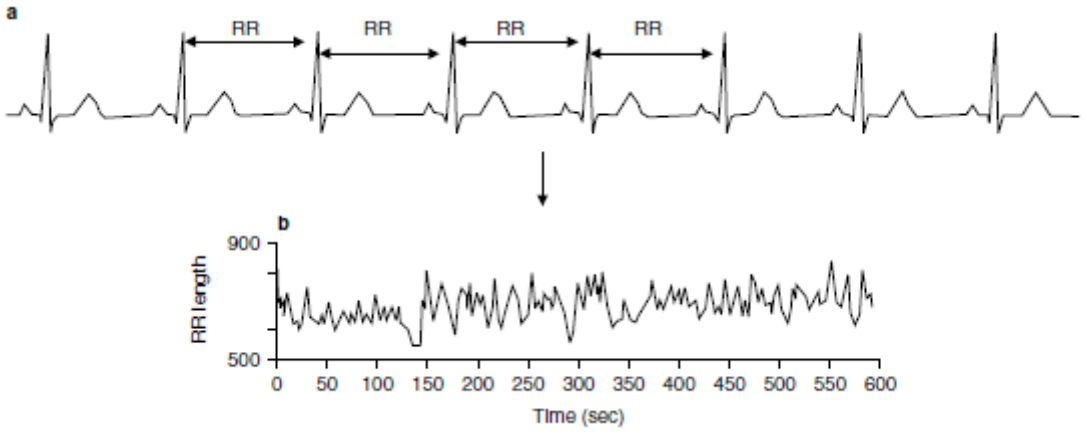


Figure 2. Calculation of consecutive RR intervals (a) on the ECG results in the tachogram (b) (from Aubert et al., 2003).

There are many methods to quantify HRV based on different mathematical approaches, which can be classified in three main categories: time domain, frequency domain and non-linear dynamics methods (Task Force, 1996).

2.1.1 Time domain

The time domain parameters, which represent the simplest methods to perform HRV analysis, are calculated with mathematically simple methods to measure the amount of variability present in a specific time period in a continuous ECG signal.

The most frequently used time domain indices are listed as follows.

Standard deviation (SD, ms) of the RR interval: it is the square root of variance and is mathematically equal to total power of spectral analysis. It reflects all the cyclic components responsible for variability in the period of recording. This index measures the state of the balance between sympathetic and parasympathetic control of heart rate; in other words, it estimates overall heart rate variability and therefore includes the contribution of both branches of the ANS to the heart rate variations. SD depends largely on the duration of the recording and, therefore, values from recordings of different duration should not be compared.

Root mean square of successive differences between adjacent RR intervals (r-MSSD, ms): to obtain the value of this parameter each difference between successive R-R intervals is summed, the result averaged and then the square root obtained. It reflects very short-term HRV measured over a much longer period of time

Percentage of successive RR interval differences larger than 10 or 20 ms for rodents (pNN10-pNN20, %), and 50 ms for humans (pNN50, %): this index is obtained by counting the number of large beat to beat changes that exceed a pre-set threshold in a recording.

Time-domain measures based on beat-to-beat intervals, like SD, are useful clinical tools for detecting abnormalities of autonomic activity, but cannot be used to quantify specific changes in sympathetic or parasympathetic activity (Pumprla et al., 2002). However, r-MSSD and pNN10-20-50 can be considered as vagal indexes because they quantify the short- term, high frequency variations of the R-R interval, which are due to the activity of the parasympathetic nervous system (Stein et al., 1994). Therefore, these indices provide sensitive and specific interchangeable measurements of parasympathetic activity, which are easy to measure both in human ambulatory ECGs (Kleiger et al, 1993) and in rodents' radiotelemetrically recorded ECGs (Sgoifo et al., 1998).

2.1.2 Geometrical methods

Geometrical methods represent another possibility to process RR intervals in the time-domain (Task Force, 1996). These tools estimate the geometrical complexity of the signal and its space filling propensity. The simplest geometrical method is the sample histogram from which it is possible to calculate parameters related to the distribution, such as mode, skewness and kurtosis. Another geometrical tool is the Poincarè or Lorentz map. The Poincarè method plots the duration of each RR interval against the duration of the immediately preceding interval in order to obtain, graphically, an ellipse. From this ellipse it is possible to measure the width and the length, which represent variations that occur at a specific frequency rate. Actually, the index SD1 is calculated as the standard deviation of the distances of the points of the ellipse's width, whereas the index SD2 is calculated as the standard deviation of the distances of the points of the ellipse's length. Experimental evidence indicates that SD1 values reflect the level of short-term variability and, consequently, is able to quantify vagal activity (Brennan et al., 2001).

The major advantage of the geometric methods is their relative insensitivity to the analytical quality of the signal. However, these methods need a reasonable number of RR intervals to construct the geometric pattern. Therefore, in order to correctly perform geometrical indexes calculation, long-lasting recordings should be used. (Task Force, 1996). Up to now, the practical use of the geometrical methods seems to be rather limited.

2.1.3 Frequency domain

Spectral analysis decomposes any time-dependent fluctuating signal into its sinusoidal components and allows to detect and quantify the amount of cyclical variation present at different frequencies (Malliani et al., 1991). Graphically, it is presented by plotting the amount of variation present in a recording on the vertical axis against the frequency at

which it occurs on the horizontal axis. This graph is usually named power spectrum and the measure of the area under the curve at different frequencies expressed as spectral power provides a numerical measure of the amount of high and low frequency variability. Various algorithms can be used to evaluate the oscillatory components and they are generally classified in nonparametric and parametric methods (Task Force, 1996). The most commonly used nonparametric algorithm is the fast Fourier transform (FFT), which is characterized by computational efficiency and simple implementation. FFT is usually employed with a priori selection of the number and frequency range of bands of interest. However, the reliability of this method is affected by the frequency resolution, which is directly related to the duration of the recording period (Aubert et al., 1999). The autoregressive (AR) modeling, the most used parametric algorithm, can decompose the overall spectrum into smoother spectral components, which can be distinguished independent of preselected frequency bands. The AR algorithm allows the automatic calculation of low- and high- frequency power components with an easy identification of the central frequency of each component (Task Force, 1996). The most important advantage of this method is that it can provide a reliable and accurate spectral estimation even with short segments of data.

Spectral analysis of HRV requires stationary recordings of at least 200-500 consecutive heart-beats. The ECG signal should satisfy several requirements in order to obtain a reliable spectral estimation. Moreover, the signal should be stable in order to attribute individual spectral components to well-defined physiological mechanisms, i.e. the mechanisms modulating the heart rate should not change during the recording period. In addition, for a reliable estimation it is important to select the proper sampling rate. Ectopic beats, arrhythmic events, missing data, pauses, non-periodic R-R interval changes and noise may alter the estimation of the power spectral density of HRV. Therefore, artifacts

should be preferentially removed from the signal before performing spectral analysis (Task Force, 1996).

Since from the first studies on power spectral analysis of ECG recordings (Penaz et al., 1968; Sayers, 1973; Akselrod et al., 1981), it appeared clear that the HRV signal contains well-defined rhythms, which correspond to specific physiological mechanisms. In a typical power spectral density curve three main frequency bands can be observed: very low frequency (VLF), low frequency (LF), high frequency (HF) (figure 3).

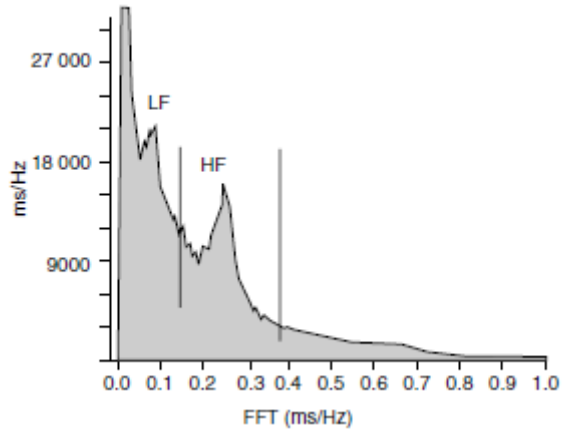


Figure 3. Example of the power spectrum of an ECG signal calculated via fast Fourier transform (from Aubert et al., 2003). FFT = fast Fourier transform; HF = high frequency; LF = low frequency.

These three specific components characterize the spectral profile of many mammals, but the frequency range of each band depends on the heart rate of the species (Aubert et al., 1999). The amplitude of each component is assessed by its power spectral density, measured as the integral of the amplitude-frequency curve and expressed in ms^2. VLF component (normally ranging from 0.00 Hz to 0.03 Hz in humans and from 0.00 Hz to 0.25 Hz in rats) contains long period rhythms and its power is due to long-term regulation mechanisms, such as thermoregulation, renin-angiotensin system, and other humoral factors (Kitney and Rompelman, 1977). The low frequency band is set in the range 0.03 -

0.15 Hz for humans and 0.25 - 0.75 Hz for rats, with a central frequency generally centered respectively at 0.1 Hz and 0.5 Hz. Its physiological interpretation is still controversial. Actually, both sympathetic and parasympathetic contributions are considered to determine LF (Eckberg, 1997). However, an increase in its power has been observed as a consequence of sympathetic activation, such as rest-tilt maneuver, stress, hemorrhage. For this reason, many authors consider the LF power a marker of increased sympathetic activity (Malliani et al., 1991). The high frequency component is set in the range 0.15 – 0.4 Hz for humans and 0.25 – 2.5 Hz, for rats, with a central frequency at the respiratory rate. The parasympathetic activity is considered to be responsible for HF power density. It is also associated with respiration-linked oscillation of heart rate due to the intrathoracic pressure changes and mechanical variations caused by breathing activity. The role of the vagus nerve in determining the HF band of the spectrum was confirmed after experiments with vagotomy (Chess et al., 1975) or after muscarinic receptor blockade (Convertino, 1999).

Power in the LF and HF bands can be expressed in absolute values (ms^2) or normalized units (nu). Normalized units are obtained by dividing the power of a given component by the total power from which VLF has been subtracted and multiplying by 100. The LF/HF ratio estimates the fractional distribution of power, which is taken as an indirect measure of sympathovagal balance.

A clarifying example of the application of spectral analysis of ECG signals is reported in figure 4. The active change of posture from supine (right) to standing (left) represents a simple autonomic provocation. Actually, the ANS responds to changes in posture via blood pressure receptors in the lungs and in the arterial system. The change of position induces an increase in heart rate, cardiac contractility and vascular tone by decreasing parasympathetic input and increasing sympathetic modulation. These changes are

reflected in the power spectrum by an increase of LF power and a decrease of HF power during standing. Hence, the supine position is characterized by a parasympathetic prevalence, which switches to sympathetic predominance while standing (Aubert et al., 2003).

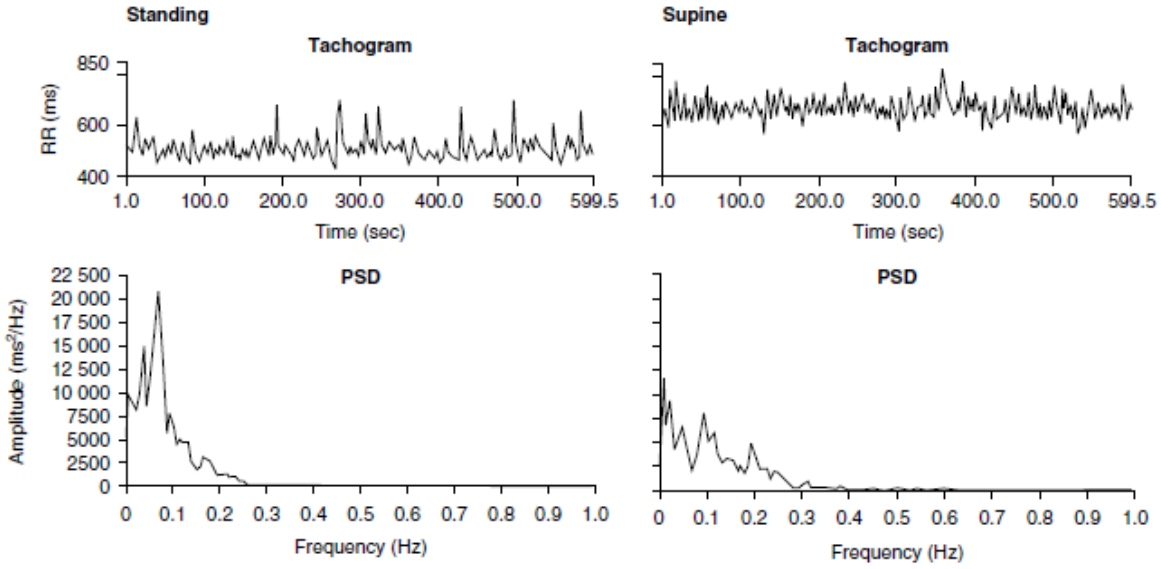


Figure 4. Tachogram and corresponding power spectral density of a standing individual (left) and a supine individual (right). Heart rate rises from supine to standing and high frequency power is depressed compared with supine, whereas low frequency power increases (from Aubert et al., 2003). PSD = power spectral density.

2.1.4 Comparison between time domain and frequency domain parameters

There is a close relation between some time domain and frequency domain HRV parameters (Kleiger et al., 1993). The total spectral power is strongly correlated to the standard deviation of the R-R interval. Actually, both these indices measure the total amount of variability in the signal. In addition, the power of HF component of the spectrum has very strong correlation with the vagal parameters r-MSSD and pNN50. Therefore, these time and frequency domain indices can be used interchangeably. The HRV technique chosen for a particular study may depend on different factors. On the one hand, spectral analysis allows a more precise evaluation of the direction and magnitude of

changes in sympathovagal balance (Montano et al., 2009), but it requires strict mathematical criteria for its application. On the other hand, time domain methods are not characterized by strict requirements of application and, therefore, are easier to apply in a variety of different experimental conditions.

2.1.5 Non-linear methods

In the last years a series of complex techniques has been developed based on non-linear dynamics and chaos theory that could be able to quantify those characteristics that cannot be revealed by linear methods (Merati et al., 2004; Porta et al., 2009). The variability of heart rate is certainly determined also by nonlinear phenomena, which are the result of complex interactions between hemodynamic, electrophysiological, humoral and neural mechanisms. Methods of non-linear dynamics define parameters that quantify complicated interactions of independent and interrelated components. Some evidences suggest that a reduction in complexity of cardiac activity comes along with a decrease in parasympathetic modulation, suggesting that a considerable amount of non-linear behavior is provided by this component of the ANS (Beckers, 2002).

Examples of these new methods based on chaos theory are: fractal dimension, approximate entropy, detrended fluctuation analysis, Lyapunov exponents, symbolic analysis. Non-linear dynamics methods for HRV analysis may provide a more sensitive approach to characterize function or dysfunction of the control mechanisms of the cardiovascular system. Up to now, chaos analysis for evaluation of autonomic regulation has been investigated both in normal subjects and patients with cardiovascular disease (Guzzetti et al., 2000; Kagiyama et al., 1999). However, these techniques are mathematically complicated and require more powerful computing. In addition, they are still under development and evaluation.

2.2 Application of HRV analysis

In the last years, HRV analysis has been extensively applied to assess autonomic balance in both physiological and pathological conditions.

2.2.1 Physiological conditions

The application of HRV measures has helped to clarify the role of the ANS in regulating the cardiovascular response to many physiological modifications. For instance, a shift of the sympathovagal balance from a parasympathetic dominance when supine to a sympathetic dominance when standing is reflected by corresponding changes in HRV variability indexes (figure 4).

During dynamic exercise, the increase in heart rate is due to both a parasympathetic withdrawal and an augmented sympathetic activity and the relative role of the two drives depends on the exercise intensity (Bernardi and Piepoli, 2001; Perini et al., 1990). However, the available data on HRV during exercise are far from consistent (Aubert et al., 2003). Actually, problems arise when time series are not stationary. Interestingly, long-term physical training has been shown to induce sinus bradycardia and a shift of the sympathovagal balance towards a parasympathetic prevalence at rest (Seals and Chase, 1989), suggesting the importance of regular physical training for primary and secondary cardiovascular prevention. Actually, many studies report that patients affected by different cardiovascular dysfunctions (hypertension, ischemic heart disease, chronic heart failure) showed decreased power of the LF component and concomitant increased power on the HF component of the spectrum, when subjected to moderate exercise training (Coats et al., 1992; Lucini et al., 2002; Pagani et al., 1988).

HRV analysis has been also employed to explore the role of the ANS in regulating cardiovascular circadian rhythms, demonstrating sympathetic predominance in the morning and parasympathetic dominance in the night (Ewing et al., 1991). The early morning increase of tonic sympathetic modulation may explain the increase in acute cardiac events which characterizes the first few hours of the morning (Furlan et al., 1990). Many studies have focused on the influence of age and sex on cardiac autonomic control by means of HRV measures. Ageing is associated with a progressive reduction in sympathetic and parasympathetic activity, reflected by a global decrease of HRV indices (Ramaekers et al., 1999; Reardon and Malik, 1996). Therefore, this decline may correspond to reduced responsiveness of autonomic control with age. Furthermore, women have been found to be characterized by lower LF power, suggesting a lower sympathetic tone compared to men (Ramaekers et al., 1998). This evidence might explain why women are somewhat protected against arrhythmias and the development of coronary heart disease. However, it has been demonstrated that the regulation of the autonomic tone varies during the menstrual cycle, with parasympathetic predominance during the follicular phase (Saeki et al., 1997; Sato et al., 1995).

Physical, psychological and mental factors are also able to modify the assessment of cardiac autonomic activity. In general, exposure to stressors induces tachycardic responses (Korte et al., 1992; Kirby and Johnson, 1990, Wan et al., 1990). The increase in heart rate may result from both sympathetic activation and/or vagal withdrawal (Berntson et al., 1991). Therefore, cardiac response to an acute stressor is usually characterized by a shift of sympathovagal balance towards a prevalence of the sympathetic component. Reduction of the vagal indexes of HRV and concomitant increase of LF power and LF/HF ratio occur normally during stress response. Experimental evidence suggests that the

amplitude of cardiovascular reactivity to acute stressors can predict the development of preclinical and clinical cardiovascular morbidity (Treiber et al., 2003).

2.2.2 Pathological conditions

There is growing evidence for the role of the ANS in a number of somatic and mental disorders. HRV analysis allows to assess the impairment of autonomic activity in various pathological conditions either of cardiac or non-cardiac origin.

Cardiovascular diseases

Autonomic dysfunction plays an important role in the pathophysiology of ischaemic heart disease. Acute myocardial infarction is characterized by adverse changes in autonomic regulation, i.e. a decrease in vagal activity which leads to prevalence of sympathetic control and to cardiac electrical instability (Flapan et al., 1993). In particular, marked autonomic dysfunction associated with decreased HRV is a powerful risk stratifier for overall mortality, induced and spontaneous ventricular tachycardia, and sudden death following myocardial infarction (Bigger et al., 1992; Molgaard et al, 1991; Singer et al., 1988). Decreased HRV was reported to be more sensitive and specific as a predictor of mortality than conventional risk factors, such as low ventricular ejection fraction, abnormal exercise test, adverse clinical grading score or the presence of ventricular arrhythmias (Kleiger et al., 1993). Also chronic heart failure has been associated with autonomic dysfunction, reflected by reduced HRV (Nolan et al., 1992). The reduced HRV is associated with the occurrence of significant compensatory neuroendocrine changes, which will predispose to cardiac electrical instability or promote the development of progressive heart failure.

Autonomic dysfunction may play an important role also in the pathogenesis of hypertension (Singh et al., 1998). Actually, essential hypertension is generally characterized by an increase in sympathetic activity reflected by a reduction of HRV. Moreover, HRV has a prognostic value and its increase is associated with the reduction in cardiac morbidity and mortality due to hypertension (Ylitalo et al., 1999).

Patients with a recent heart transplant show an extremely reduced HRV and it is apparent that HRV can increase with time in long-term transplant survivors, indicating that reinnervation has occurred (Lord et al., 1997). Some evidences also indicate that episodes of rejection are associated with changes in HRV, which may be useful to identify patients developing allograft rejection (Zbilut et al., 1988).

The ANS plays an important role in regulating myocardial electrical stability. In fact, sympathetic activity favors the onset of life-threatening ventricular tachyarrhythmias, whereas parasympathetic modulation exerts a protective and antifibrillatory effect. Abnormalities in HRV have been demonstrated to occur immediately prior to the onset of ventricular tachycardia, indicating adverse changes in autonomic activity (Lombardi et al., 2000). In addition, variations in the autonomic balance could play a role also in the onset of atrial fibrillation (Klingenheben et al., 1999; Lombardi et al., 2001). Actually, parasympathetic stimulation decreases the atrial refractory period, facilitating the development of re-entrant wavelets, while sympathetic activation predisposes to electrical instability by shortening action potential duration and increasing automaticity and triggered activity. Recently, HRV analysis has been proposed to assess autonomic modulation before the onset of arrhythmias in patients with cardiac diseases and this information can also help in guiding antiarrhythmic therapy.

Many studies suggest that autonomic impairment and decreased HRV are associated with some of the risk factors for cardiovascular diseases. For example, there are evidences that

low HRV is linked to high cholesterol levels (Christensen et al., 1999). Moreover, poor lifestyle choices, including lack of physical activity, smoking and being overweight, have been shown to be associated with autonomic imbalance and decreased parasympathetic modulation (Hayano et al., 1990; Karason et al., 1999; Sloan et al., 2009). On the other hand, smoking cessation, physical exercise, and weight loss are all associated with increased HRV (Carter et al., 2003; Karason et al., 1999; Minami et al., 1999). In addition, some other healthy habits, such as dietary changes, including the consumption of fruit and vegetables and intake of omega-3-fatty acids and vitamin D, are associated with a reduced risk for cardiovascular diseases and some suggestive evidence links them to increased HRV (Mozzafarian et al., 2008; Park et al., 2009).

Diseases of non-cardiac origin

A common complication of diabetes mellitus is autonomic neuropathy, which is characterized by early and widespread neuronal degeneration of small nerve fibers of both sympathetic and parasympathetic tracts. Diabetic patients with autonomic neuropathy have a substantially increased risk of premature death (O'Brien et al., 1991). Since early subclinical detection of autonomic dysfunction is important for risk stratification and subsequent management, HRV analysis represents an efficacy tool for detecting autonomic neuropathy (Ewing et al., 1991). Furthermore, it has been demonstrated that HRV is inversely correlated to fasting glucose and hemoglobin A1c levels, independently of many traditional cardiovascular risk factors (Thayer and Fischer, 2005).

Many other chronic pathological conditions are associated with autonomic dysfunction leading to reduced HRV, such as renal disease (Vita et al., 1988), chronic liver disease (Dillon et al., 1994), chronic respiratory disease (Watson et al., 1999), HIV infection (Neild et al., 2000). In addition, it has been shown a progressive reduction of HRV in patients in

intensive therapy units who develop brain deaths and this phenomenon may help to identify candidates for organ donation (Rapenne et al., 2000).

Alterations in autonomic regulation and HRV have been associated with immune dysfunction and inflammation, which have been implicated in a number of conditions and diseases. The common mechanism may involve excess proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and -6 (IL-6), and C-reactive protein. In particular, sympathetic hyperactivity and decreased HRV have been shown to be associated with the increased release of these proinflammatory cytokines, whereas increased parasympathetic tone attenuates their production (Maier and Watkins, 1998; Tracey, 2002).

Many studies have provided strong evidence linking negative affective states and impaired autonomic regulation: decreased parasympathetic activity has been implicated as possible mediator in this link (Kiecolt-Glaser et al., 2002; Rozansky et al., 1999; Verrier and Mittleman, 2000). Reductions in HRV have been found in panic disorders (Friedman and Thayer, 1998), depression (Thayer et al., 1998), generalized anxiety disorders (Thayer et al., 1996), and posttraumatic stress disorders (Cohen et al., 1999).

3. Stress and cardiovascular disease

Cardiovascular disease (CVD), including coronary artery disease (CAD), ischemic heart disease, hypertension, and infarction, is the leading cause of serious illness, death and reduced quality of life in both men and women in most Western countries and a major concern for developing economies (American Heart Association, 2006; Domanski et al.,

2002; Mathers and Loncar, 2002; Yusuf et al., 2001). In particular, CAD represents the largest single cause of death in Europe, regarding the 21% of male and 22% of female deaths (Allender et al., 2008). In addition, it is the leading cause of death in people aged less than 75 years, i.e. 20% and 19% of male and female deaths respectively. However, in many countries mortality due to CVD has declined in the past three decades, due to prevention and increased survival following acute coronary events (American Heart Association, 2006).

Development of CVD is multifactorial and determined by the interaction of genetic, physiological, environmental and behavioral factors, many of which depend on individual's lifestyle which can be controlled and modified. Interestingly, genetic and non-genetic factors act in an interactive manner and therefore, the effect of many genes will depend on the environment in which they are expressed. In other words, the interindividual variability is fundamental in determining the response to environmental factors, with the impact of a given factor often dependent on the individual's genetic predisposition (Schwartz et al., 2003).

A wide range of risk factors for CVD have been identified. In particular, up to now eight are the recognized risk factors, which can be distinguished in six modifiable and two non-modifiable variables. Three of the modifiable factors are defined biological factors and include high blood pressure, diabetes and high cholesterol levels. The three other modifiable variables can be considered lifestyle factors and include smoking, physical inactivity and obesity. The two non-modifiable risk factors are age and family history of early heart disease.

Recently, other psychosocial factors have been considered as emerging risk factors for CVD, including stressful life events, general stress, depression, and anxiety (Kubzansky and Kawachi, 2000; MacLeod et al., 2002; Yan et al., 2003; Yusuf et al., 2004).

Many case studies have long reported a relationship between acute stress and the development of CVD (Green et al., 1972; Myers and Dewar, 1975). An acute stressor associated with increased rates of cardiac events is bereavement. For example, one study reported that in 95,647 individuals followed up for 4 to 5 years the highest relative mortality occurred immediately after bereavement, with a 2-fold higher risk for men and 3-fold higher risk for women (Kaprio et al., 1987). Cardiac event rates are also increased in the immediate after of other acute life stressors, such as earthquakes and terrorist activities (Brown, 1999; Leor et al., 1996; Meisel et al., 1991).

Chronic stress includes stress in the work place and aspects of the social environment such as social isolation and low social support, mental stress, marital stress, and caregiver strain. The INTERHEART study, which investigated the association between chronic stress and the incidence of myocardial infarction in a sample of 25,000 people from 52 countries, revealed that those who reported permanent stress at work or at home had a 2.1-fold increased risk for developing CVD, after adjusting for age, gender, geographic region and smoking (Rosengreen et al., 2004).

In the last years work stress has been consider as a major problem of public health associated with cardiovascular morbidity and mortality (Duijts et al., 2007; Harter et. al., 2002). An increasing epidemiologic literature indicates that job-strain and effort-reward imbalance are associated with an increased number of adverse clinical outcomes (Johnson et al., 1989; Kivimaki et al., 2002). Some studies reported that job-stressed patients are primed to hyper-respond to stressors even outside of the work environment (Steptoe at al., 2003; Thomas et al., 2004).

The presence of high levels of social support is known to promote psychological and physical health, whereas low level of social support has been shown to be associated with illness (Rozanski et al., 2005). For example, living alone (Case et al., 1992), lacking a

confidant (Williams et al., 1992), suffering from social isolation (Ruberman et al., 1984), and low emotional support (Berkman et al., 1992) have been associated with increased mortality. Aside from social factors, also low socioeconomic status (low education, low income level) is a significant contributor to increased risk in healthy persons and a contributor toward poor prognosis in patients with established CAD (Kaplan and Keil, 1993).

Marital stress, a specific form of social conflict, represents a chronic stressor with pathophysiological effects. For example, in a study of 292 female coronary patients aged 30-62 years, women who reported severe marital stress had a 3-fold greater likelihood of recurrent coronary events over a 4-year follow-up, compared to women who reported low or no marital stress (Orth-Gomer et al., 2000).

Finally, caregiving strain is an increasingly prevalent stressor that has been shown to be associated with potential cardiovascular risk and sequelae. For example, the Caregiver Health Effects Study reported that caregivers had a 63% higher mortality rate that non-caregiving control subjects (Schultz and Beach, 1999).

Some studies with animal models, particularly monkeys, show the importance of the interaction of psychosocial factors and other risk factors in developing CVD. Kaplan and colleagues (Kaplan et al., 1982) reported that cynomolgus macaques fed with an atherogenic diet similar to that consumed by typical North Americans, developed coronary artery atherosclerosis only when exposed to an unstable social environment that provided recurrent behavioral challenges, i.e. the presence of unfamiliar monkeys.

Acute and chronic stress contribute to the pathogenesis of CVD in a direct way, but also promoting unhealthy lifestyle behaviors, such as higher rates of smoking, poor diet, more sedentary life, excess consumption of alcohol, pure compliance with medical regimens. In addition, when these unhealthy lifestyles are established, the presence of psychosocial

stress worsen the situation leading to recurrent cardiac events (Rozanski et al., 1999). However, the excess of CVD risk associated with psychosocial factors is not adequately explained by the only presence of unhealthy lifestyles and largely persists following statistical adjustment for known cardiovascular risk factors. Therefore, there are biologically plausible mechanisms by which stress can influence CVD (Everson-Rose and Lewis, 2005)

The rapid growth of research on the association between psychosocial factors and CVD morbidity and mortality over the past few decades has brought about a greater focus on the pathophysiological mechanisms or pathways by which psychosocial factors can influence the development and progression of cardiac disease. The proposed principal mechanisms involved in this relationship are the activation of the autonomic nervous system and of the hypotalamic-pituitary- adrenal (HPA) axis, serotonergic dysfunction, secretion of proinflammatory cytokines and platelet activation (figure 5).

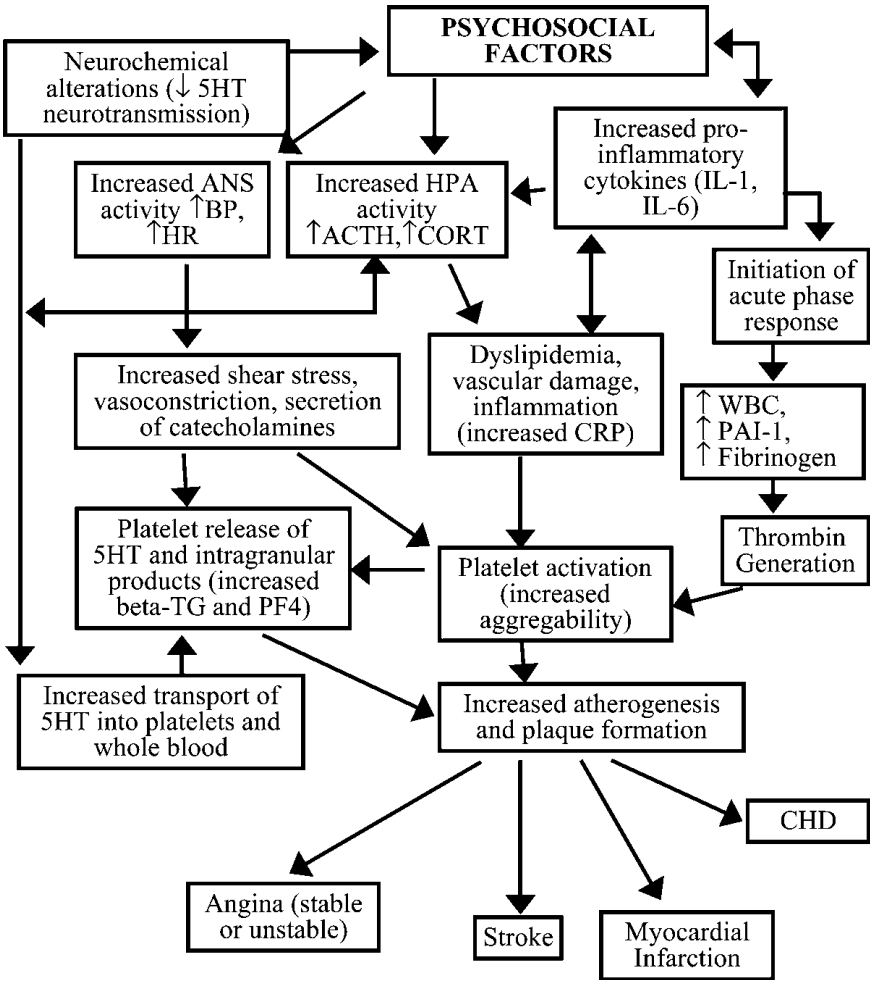


Figure 5. Proposed model of the causal pathways between psychosocial factors and cardiovascular disease (from Everson-Rose and Lewis, 2005). 5HT = serotonin; ANS = autonomic nervous system; BP = blood pressure; HR = heart rate; ACTH = adrenocorticotropin hormone; CORT = cortisol; IL-1 = interleukin-1; IL-6 = interleukin-6; CRP = C-reactive protein; WBC = white blood cell count; PAI-1 = plasminogen activator inhibitor; ß-TG = beta-thromboglobulin; PF4 = platelet-factor 4; CHD = coronary heart disease.

Stressors may influence cardiovascular function and promote atherogenesis through the HPA axis and ANS activation. In fact, HPA dysregulation, including hypercortisolemia or excess glucorticoid secretion, can contribute to hypertension, insulin resistance, visceral obesity, coagulating changes, and increased lipid levels, which are precursors to CVD (Chrousos and Gold, 1998). In addition, low vagal tone and exaggerated stress-induced sympathetic responses, associated with reduced HRV, combine as potential physiological

mediators linking psychosocial factors to cardiac risk (Carroll et al., 2001; Thayer and Lane, 2007). Actually, acute and chronic stress are associated with higher circulating catecholamines, exaggerated blood pressure and heart rate responses. To better understand the effects of stress-mediated autonomic regulation of cardiac response, the interrelation between brain, autonomic nerves and heart is represented in figure 6. In the brain many areas are engaged in autonomic processes, including cortical regions, thalamus, hypothalamus, and brainstem regions. Stress or emotion are processed in a network involving these areas, resulting in sympathetic and vagal neural input to the heart. Autonomic outflows modulate coronary artery and microvascular tone and thus myocardial perfusion, leading to possible ischemia. Elevated blood pressure and vascular resistance increase shear stress on blood vessels, which can cause vulnerable atherosclerotic plaques to rupture. The sympathetic and parasympathetic input to the heart influences a number of electrophysiological parameters including action potential duration, refractoriness and delayed after-depolarisations. These electrophysiological alterations, mostly due to sympathetic activation, may lead to enhanced arrhythmogenesis which characterizes mental and psychological stress (Hemingway et al., 2001). Also studies on animal models have reported that sympathetic activation and vagal withdrawal, which characterize acute stress response, are associated with an increased risk of cardiac arrhythmias that depends on the type of stressor. In particular, in rodents, cardiac arrhythmias have been shown to be more frequent in social compared with nonsocial stress conditions (Sgoifo et al., 1999).

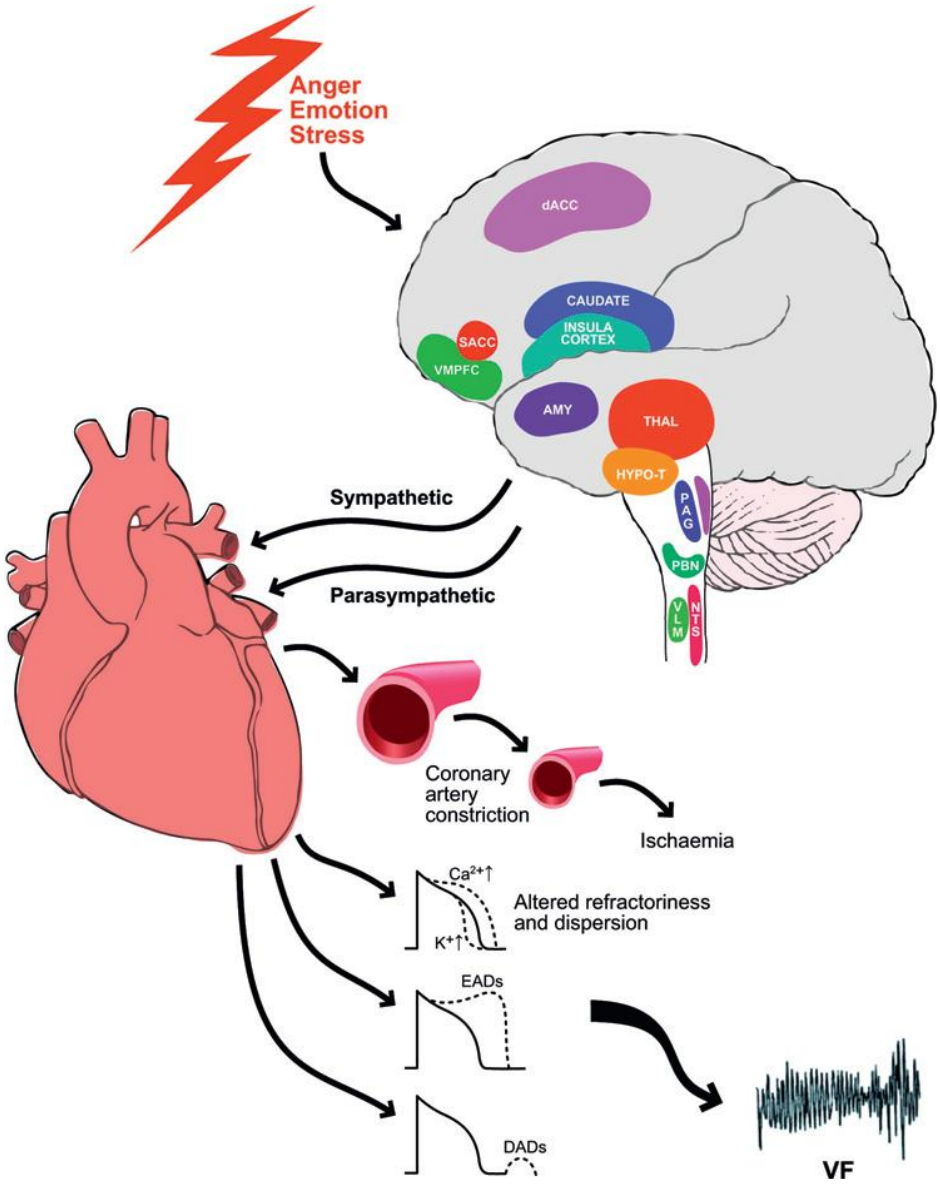


Figure 6. Overview of brain – autonomic nerves and the heart as an interactive system (from Taggart et al., 2011). AMY = amygdala; dACC = dorsal anterior cingulate cortex; DAD = delayed afterpolarisation; EAD = early afterdepolarisation; HYPO-T = hypothalamus; NTS = nucleus tractus solitarius; PAG = periaqueduct grey; PBN = parabrachial nucleus; SACC = subgenual anterior cingulate cortex; THAL = thalamus; VF = ventricular fibrillation; VLM = ventrolateral medulla; VMPFC = ventromedial prefrontal cortex.

Abnormalities in serotonergic function may be another mechanism by which stress may lead to cardiovascular dysfunction. Serotonin is critical in the regulation of mood, emotions and behavior, but, at the same time, is involved in the regulation of thrombovascular processes. Actually, serotonin is characterized by vasoactive properties and is involved in thrombogenesis, platelet activation and hypertension (De Clerck, 1990; Fetkowska et al., 1988). Stress exposure has been shown to produce alterations of serotonin levels and function (McEwen and Mendelson, 1993). In particular, results obtained in animal studies suggest that chronic stress causes alterations in the density and activity of brain serotonin receptors (Flugge, 1995; Lanfumey et al., 1999).

Enhanced platelet response represents a key mechanism whereby psychosocial stress may trigger acute ischemic events and contribute to the development and progression of CVD (Markovitz and Matthews, 1991). Platelets play a central role in hemostasis, thrombosis and the development of atherosclerosis and acute coronary syndromes (Lefkovits et al., 1995). As mentioned above, the alteration of serotonergic function may mediate the association between stress and platelet activation and reactivity.

In addition to impaired inhibition of platelet aggregation, also endothelial dysfunction, impairment of vasodilation or even paradoxical vasoconstriction, seem to be common pathways whereby stress affects the cardiovascular system (Sherwood et al., 1999; Yeung et al., 1991).

Finally, another mechanism involved in the relationship between stress and CVD is the secretion of pro-inflammatory cytokines. The induction of cytokines, vasoactive molecules and growth factors provokes endothelial injury which may result in a cascade of atherogenic changes. The association between stress and activation of pro-inflammatory cytokines is bidirectional. On the one hand, cytokines can induce behavioral and psychological expressions of stress (Sapolsky et al., 1987). On the other hand, conditions

of psychological and social stress have been shown to increase inflammatory cytokines, such as IL-6 (Kiecolt-Glaser et al., 2003; Zhou et al., 1993).

4. Depression, anxiety and cardiovascular dysfunction

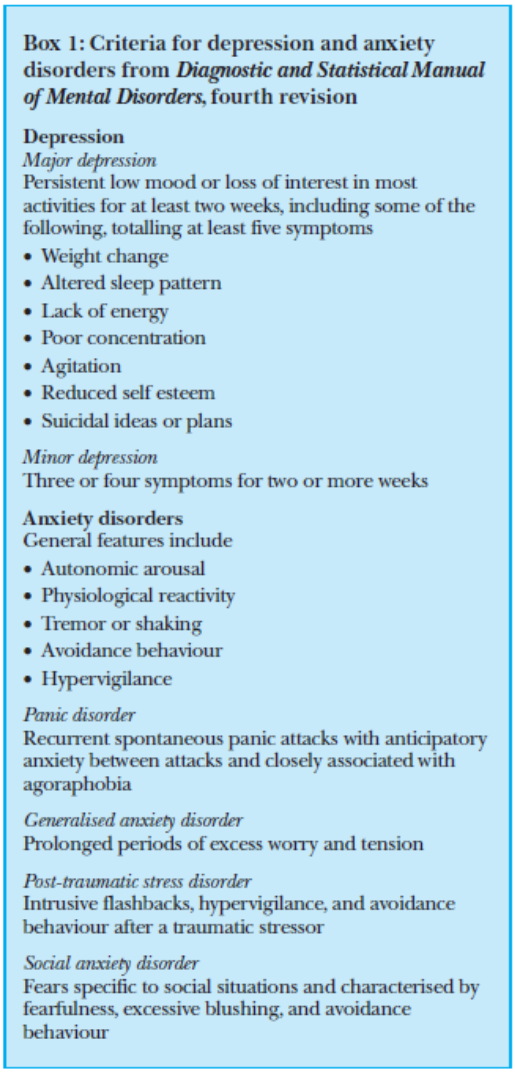
Psychopathologies, such as depression and anxiety disorders, are important leading cause of disability worldwide and represent a major public health problem (World Health Organization report, 2005).

Depressive disorders affect about 120 million people worldwide and are the second leading cause of disability, after ischemic heart disease (Murray and Lopez, 1996). The lifetime incidence of depression is estimated at nearly 12% in men and 20% in women respectively (Kessler et al., 2003). In agreement with the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2000), depression is a multifaceted psychological disorder characterized by behavioral, neuroendocrine, and physiological alterations. Episodes of major depression are characterized by the presence of depressed mood, markedly decreased interest in all activities, inability to experience pleasure (anhedonia), changes in appetite, sleep disturbances, fatigue, psychomotor retardation or agitation, slowing of speech and actions, and suicidal thoughts (figure 7). These behavioral attitudes are accompanied by perturbations of most of the major physiological and neuroendocrine systems. In particular, depressive disorders have been shown to be associated with monoamine dysregulation, HPA axis dysfunction, altered immune system function, biological rhythm disturbances, and body weight alterations (American

Psychiatric Association 2000; Asnis, 1987; Cunningham and De Souza, 1993; Meltzer, 1990).

Anxiety may be considered as an emotional anticipation of an aversive situation and is characterized by species-specific behavioral fear responses to stressful and threatening stimuli (Neumann et al., 2010). In other words, anxiety arises out of a sense of threat and is characterized by a perceived inability to predict, control, or gain the preferred results (Barlow, 1988). Trait anxiety could be defined as innate anxiety and is mostly determined by genetic background. Conversely, the contextual activation due to a specific situation is termed state anxiety. From an evolutionary perspective, adequate levels of trait and state anxiety can be adaptive when they trigger coping responses that protect an individual from threats. However, anxiety may become maladaptive when it increases or persists to such a degree that the individual can no longer function effectively in everyday life (Moser, 2007). According to the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2000), a variety of anxiety disorders have been classified as formal clinical diagnoses: generalized anxiety disorders, phobic anxiety, panic disorder, post-traumatic stress disorder, social anxiety disorders. The lifetime prevalence of anxiety disorders, such as panic disorder or general anxiety, is about 17% (Somers et al., 2006), with social anxiety disorder, like social phobia, being the most common one (Kessler et al., 1994). The most common features characterizing episodes of anxiety are physiological arousal and reactivity, avoidance behavior, excess worry and tension, tremor or shaking, hypervigilance (figure 7). Despite the variety of manifestations of anxiety, evidence suggests that anxiety reactions at all stages have similar cognitive, neurobiological, and behavioral characteristics; therefore, clinically diagnosed anxiety and subclinical anxiety are not fundamentally different phenomena (Barlow, 1988; Lewis 1993). Anxiety disorders represent some of the major health problems in the Western world in term of costs of

healthcare, sick-leave from work, disabilities and premature mortality (World Health Organization, 2004). Epidemiological and clinical evidence indicates that patients with anxiety disorders often have multiple medical comorbidities, including chronic pain conditions, gastrointestinal, cardiovascular, endocrine and respiratory disorders (Culpepper, 2009).



Box 1: Criteria for depression and anxiety disorders from *Diagnostic and Statistical Manual of Mental Disorders*, fourth revision

Depression

Major depression

Persistent low mood or loss of interest in most activities for at least two weeks, including some of the following, totalling at least five symptoms

- Weight change
- Altered sleep pattern
- Lack of energy
- Poor concentration
- Agitation
- Reduced self esteem
- Suicidal ideas or plans

Minor depression

Three or four symptoms for two or more weeks

Anxiety disorders

General features include

- Autonomic arousal
- Physiological reactivity
- Tremor or shaking
- Avoidance behaviour
- Hypervigilance

Panic disorder

Recurrent spontaneous panic attacks with anticipatory anxiety between attacks and closely associated with agoraphobia

Generalised anxiety disorder

Prolonged periods of excess worry and tension

Post-traumatic stress disorder

Intrusive flashbacks, hypervigilance, and avoidance behaviour after a traumatic stressor

Social anxiety disorder

Fears specific to social situations and characterised by fearfulness, excessive blushing, and avoidance behaviour

Figure 7. Summary of criteria for depression and anxiety disorders from the Diagnostic and Statistical Manual of Mental Disorders (from Davies et al., 2004).

Converging evidence from both experimental and epidemiological studies indicates that there is a bidirectional association between psychopathologies and cardiovascular

diseases. Patients affected by a variety of mental disorders, including depression, anxiety, and schizophrenia, are at significantly higher risk for cardiac morbidity and mortality than general population (Rosengreen et al., 2004; Rozanski et al., 1999; Sowden and Huffman, 2009).

Many studies have shown depression to be an independent risk factor for the development and progression of ischemic heart disease (Jiang et al., 2002; Lippi et al., 2009). In addition, it has been reported that approximately 20% to 50% of patients who die from myocardial infarction may have experienced an episode of depression prior to the heart attack (Glassman and Shapiro, 1998; Green et al., 1972). Depressed patients are at higher risk of death due to cardiac events for up to 10 years following the diagnosis of established CVD than non-depressed subjects (Barefoot et al., 1996). The adverse outcomes after an acute cardiac event, such as myocardial infarction, have been shown to be associated with the presence of mild depressive symptoms (Bush et al., 2002; Lesperance et al., 2004). For example, in the study of Lesperance and colleagues (Lesperance et al., 2004) 896 postmyocardial infarction patients were divided into four categories based on the Beck Depression Inventory (BDI), ranging from no depressive symptoms (BDI<5) to severe depressive symptoms (BDI≥19). During the 5-year follow-up period, a gradient relationship was observed between the magnitude of depressive symptoms and the frequency of deaths, with increased events occurring even in patients with mild depressive symptoms (figure 8). Moreover, the presence of depressive symptoms in healthy individuals increases the risk for developing coronary artery disease (Glassman and Shapiro, 1998).

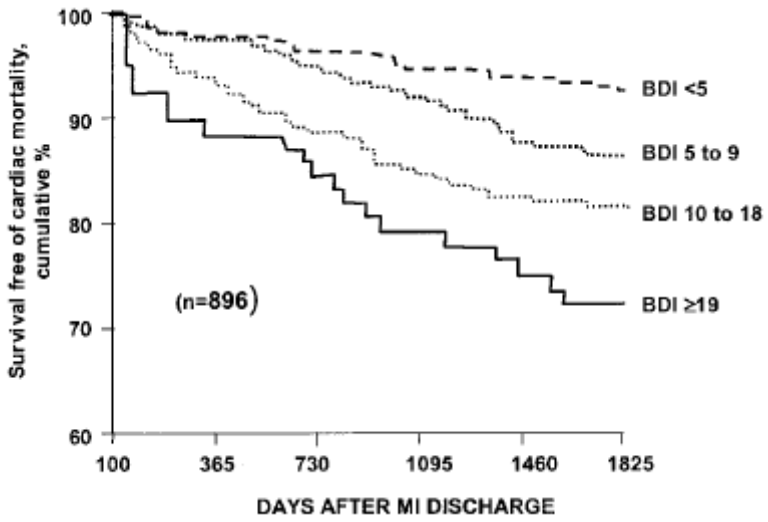


Figure 8. Survival rate in postmyocardial infarction patients divided in four categories (1-4) based on the Beck Depression Inventory (from Lesperance et al., 2004). BDI = Beck Depression Inventory; MI = myocardial infarction.

On the other hand, the prevalence of depressive disorders in patients following a myocardial infarction may rise to approximately 45% and might be even higher in patients with chronic CVD, such as congestive heart failure (Freedland et al., 2003; Schleifer et al., 1989). It has been estimated that between 17% and 27% of patients with coronary heart disease suffer from major depression, and a significantly higher percentage has subsyndromal symptoms of depression (Rudish and Nemeroff, 2003).

Increasing evidence has linked anxiety disorders to the development of CVD in the general population. In particular, several studies have noted a relationship between phobic anxiety and sudden cardiac death (Kawachi et al., 1994; Kubzansky and Kawachi 2000; Watkins et al., 2006). Also panic disorders have been reported to be associated with increased incidence of cardiovascular morbidity (Coryell et al., 1982; Kahn et al., 1987; Weissmann et al., 1990). Panic attacks are a common cause of non-cardiac chest pain, (Fleet et al., 2000), but panic disorder may also confer as much as a 3-4 fold increased risk of myocardial infarction relative to the general population (Smoller et al., 2007). The relation

between posttraumatic stress disorders and CVD has been demonstrated in veterans exposed to war-related traumatic events (Falger et al., 1992). Moreover, high levels of worry, an important cognitive component of anxiety, have been shown to be associated with a 2.5 fold increased risk for myocardial infarction (Kubzansky et al., 1997). The presence of general anxiety has been shown to predict major cardiac events in a 2-year follow-up of patients with coronary artery disease (Frasure-Smith and Lesperance, 2008). Finally, generalized anxiety disorder is associated with elevated rates of CVD risk factors, including smoking, hypercholesterolemia, and diabetes (Barger and Sydeman, 2005). Anxiety disorder is common among patients with chronic CVD and among those coping with recovery from acute cardiac events. Actually, the prevalence of anxiety is approximately 70% to 80% in patients who have experienced an acute cardiac event and persists over the long term in about 20% to 25% of patients (Crowe et al., 1996; Moser and Dracup, 1996). Anxiety may be considered as an expected reaction to an acute cardiac event, but, when persisting, it can have negative consequences for patients' health (Kubzansky et al., 1997; Kubzansky et al., 1998; Moser and Dracup, 1995). In particular, anxiety disorder is predictive of worse quality of life among patients with CVD, including difficulties adhering to prescription for medication, activity, and diet (Lane et al., 2000; Rose et al., 1994). Evidence indicates sex-differences in the level of anxiety perceived after an acute cardiac event. Women reported mean anxiety levels 25% higher than those reported by men and this finding is consistent across the Western and Eastern cultures (Moser at al., 2003). Finally, posttraumatic stress disorders can occur after a severe cardiac event (Pedersen, 2001) and panic disorder presents a prevalence of 10% to 50% in patients with CVD (Fleet et al., 2000).

4.1 Pathophysiological mechanisms

The bidirectional association between mental illness and CVD is multifaceted, involving an integration of several central and peripheral processes, and may be explained by a complex combination of behavioral and pathophysiological mechanisms. Unfortunately, the precise mechanisms by which psychopathologies and cardiovascular dysfunctions are linked remain largely unknown. In particular, the direction of causation is not clearly understood. However, the most reliable hypothesis is that CVD and depression or anxiety share common underlying factors (Johnson and Grippo, 2006; Mosovich et al., 2008). The comprehension of these common biological mechanisms is important to lead to effective prevention and treatment of both these pathological conditions.

Up to now, many studies have highlighted some of the underlying mechanistic links between CVD and psychopathologies, including the presence of exogenous stressors, alterations of autonomic regulation, neurohumoral dysregulation, platelet and endothelial dysfunction, impaired immune regulation and dysfunction of neurotransmitter systems (Grippo, 2009; Rozansky et al., 1999).

4.1.1 Stress reactivity

The presence of environmental stressors represents a common factor in the development of both psychopathologies and CVD. Major life stressful events may sensitize individual to subsequent development of depression and anxiety disorder (Kendler et al., 1999; McEwen, 2003). The exposure of animals to uncontrollable and unpredictable stressors represents one of the most used strategies to induce physiological and behavioral correlates of human psychiatric illness in animal models (Anisman and Matheson, 2005). The possible mechanisms by which psychosocial factors, including stress and altered

emotional states, lead to CVD have already been described in section 3. (“Stress and cardiovascular disease”). Actually, depression and anxiety seem to act in a similar way inducing central and peripheral modifications which can bring about cardiovascular dysfunction. At the same time, stress-induced cardiovascular dysfunction involves the same physiological and behavioral alterations that are observed at the onset of altered affective states. Exposure to exogenous stressors induces alterations in central processes involving serotonin, norepinephrine and corticotropin-releasing factor (CRF), and activation of neuroendocrine, immune and autonomic nervous systems (Adell et al., 1988; Kiecolt-Glaser et al., 2003; Sgoifo et al., 2001). Furthermore, environmental stressors contribute to cardiovascular dysfunction inducing hypertension, changes in vascular resistance, arrhythmias, endothelial dysfunction, and platelet reactivity (see section 3. for details).

4.1.2 Autonomic dysfunction

Alterations in autonomic nervous system function characterize the presence of both psychiatric illnesses, including depression and anxiety disorders, and CVD. In particular, the autonomic imbalance is determined by increased sympathetic activity, decreased parasympathetic tone, or both. Such condition is normally characterized by a reduction of HRV. As above explained in details (see paragraph 2.2.2 “Application of HRV – *Pathological conditions* –“), autonomic imbalance and reduced HRV are prognostic indicators for morbidity and mortality related to CVD. In addition, evidence of ANS dysfunction has been found also in patients affected by depression or anxiety (Rozansky et al., 1999).

Over the past decades, many studies have investigated the link between impaired autonomic function and anxiety and taken together these results suggest that anxiety disorders are commonly associated with ANS dysregulation, in particular with increased

sympathetic activity (Cohen and Benjamin, 2006). The presence of anxiety symptoms in healthy subjects has been associated with decreased parasympathetic outflow and increased sympathetic prevalence, resulting in reduced HRV (Kawachi et al., 1995; Piccirillo et al., 1997; Virtanen et al., 2003). A similar autonomic pattern has been found also in patients suffering panic attacks, i.e. sympathetic predominance associated with increased power of the LF component and concomitant decreased power of the HF component of the power spectrum (Yeragani et al., 1993; Yeragani et al., 1998). Also posttraumatic stress disorder patients have been shown to present increased sympathetic activity in resting conditions, whereas they failed to respond to stressful stimuli (Cohen et al., 1998; Cohen et al., 2000; Sahar et al., 2001). Reduced vagal control and increased sympathetic activation observed in anxiety have been linked to reduced baroreflex activity (Watkins et al., 1998), an important risk factor for sudden cardiac death (Billman et al., 1982). Finally, several studies have indicated that pharmacological treatment of anxiety disorder partly corrects autonomic dysfunction, resulting in increased HRV parameters (Pohl et al., 2003; Slaap et al., 2002; Yeragani et al., 1998).

Depression has been observed to be associated with alterations of cardiac autonomic regulation, including activation of the sympathetic nervous system and withdrawal of the vagal tone (Barton et al., 2007; Hausberg et al., 2007). Therefore, depressed patients both with and without CVD are characterized by reduced HRV (Carney et al., 1995; Rechlin et al., 1994; Stein et al., 2000). The reduction of HRV appears to be related with the severity of depressive symptoms, with a lower degree of HRV in patients with higher levels of depression (Krittayaphong et al., 1997).

The higher sympathetic tone and reduced vagal activity lead to elevations in resting heart rate and systemic arterial pressure (Carney et al., 2005; Rozansky et al., 1988). Evidence also indicates that elevated heart rate characterizes both hypertensive and normotensive

depressed patients (Goldstein, 1983; Lechin et al., 1995). In addition, the increase of heart rate in depression has been observed to be independent of the presence of other cardiovascular dysfunctions (Carney et al., 1988; Forbes and Chaney, 1980). The presence of high levels of depressive symptoms has been also associated with increased systemic vascular resistance, leading to elevated blood pressure at rest (Matthews et al., 2005). The effects of increased sympathetic tone seen in depression may be due to the increase of catecholamine levels, which has been found in depressed patients both in the plasma and in the cerebrospinal fluid (Gold et al., 2005). In addition, stress and anxiety have been associated with increased plasma and urinary norepinephrine and epinephrine levels in healthy adults (Bedi and Arora, 2007; Lader, 1974). Finally, the increased sympathetic tone may lead, as just reported for anxiety disorder, to reduced sensitivity of the baroreceptor reflex, which has been observed in depressed patients (Watkins and Grossmann, 1999). Impaired baroreceptor reflex has been shown to have a prognostic value in patients with CVD (La Rovere et al., 1998).

4.1.3 HPA axis dysregulation

The activity of the HPA axis plays a fundamental role in linking stress response, emotional states and cardiovascular function. In particular, HPA axis dysfunction has been extensively studied in depression. Depressive disorders are characterized by several alterations of adrenocortical activity (Plotsky et al., 1998). In particular, depressed patients exhibit alterations of corticotropin-releasing factor (CRF) levels in the cerebrospinal fluid, hypothalamus, and locus coeruleus (Banki et al., 1992; Bissette et al., 2003; Raadsheer et al., 1995). Moreover, depressive disorders have been shown to be associated with dysregulated adrenocorticotropic hormone (ACTH) response to CRF, increased adrenal response to ACTH, hypercortisolemia and lack of cortisol suppression in response to

dexamethasone (Asnis et al., 1987; Maes et al., 1998). Evidence of HPA axis activity dysregulation associated with depression in humans is supported by studies in animal models, in which similar modifications have been observed (Froger et al., 2004: Grippo et al., 2005). The presence of hypercortisolemia in depression has been shown to be linked with blunted growth and sex hormones and may promote central obesity, leading to an increase of peripheral fatty acids. These metabolic modifications may contribute to insulin resistance and diabetic complications, associated with cardiovascular dysfunction (De Groot et al., 2001; Weber-Hamann et al., 2002).

Compared to the findings in depression, relatively little is known about HPA axis dysregulation in anxiety disorders. However, most evidence have suggested that also anxiety disorders, like generalized anxiety disorders, panic disorders and posttraumatic stress disorders, are associated with HPA axis abnormalities (Abelson and Curtis, 1996; Kellner et al., 2004; Sauttler et al., 2003). Studies in animal models of anxiety may help understanding the role of neuroendocrine dysfunction in anxiety disorders. Actually, impaired HPA axis activity have been found in rodents with high levels of anxiety-related behavior (Landgraf and Wigger, 2002).

4.1.4 Immune dysfunction

Immune dysregulation may play a fundamental role in linking psychosocial factors to CVD. Actually, pro-inflammatory cytokines levels are elevated both in mood and cardiovascular disorders. Several studies have reported that depression and anxiety are associated with increased release of IL-1 and IL-6, C-reactive protein, and TNF (Anisman and Merali, 2002; Maes et al., 1998; Maes et al 1999; Sluzeweska et al., 1996). Similar findings of immune alterations have been observed in animal models of both depression (Grippo et al., 2005; Willner, 2005) and CVD (Felder et al., 2003). The interaction between

neuroendocrine processes, such as the activation of the HPA axis, and cytokines plays a fundamental role in regulating both cardiovascular function and affective states. In fact, the high levels of hormones secreted in depression and anxiety disorders, including cortisol and catecholamines, have been shown to increase inflammation processes (Gotthardt et al., 1995; Kop et al., 2002; Maes et al., 1999). In particular, the prolonged elevation of the glucorticoid levels, which characterizes depressive disorder, may lead to a desensitization of the central and macrophagic glucorticoid receptors (Carney et al., 2002). Finally, depressive and anxiety disorders have been associated with other immune changes, including reduced lymphocyte number and proliferation (Kemeny et al., 1989; Koh et al., 1998)

4.1.5 Platelet activation

Platelets abnormalities have been associated with depressive symptoms, in particular increased platelet activation and reactivity (Musselman et al., 1996). Patients with ischemic heart disease and comorbid depression are characterized by increased plasma concentrations of platelet-specific proteins, like beta-thromboglobulin and platelet factor 4 (Laghrissi-Thode et al., 1997). Depression is also associated with enhanced incidence of endothelial dysfunction (Rajagopalan et al., 2001). Moreover, anxiety disorders have been associated with increased platelet activity and hypercoagulable state (von Kanel et al., 2001; Yapislar et al., 2011). As previously mentioned in paragraph 3. (“Stress and cardiovascular disease”), platelet activation and endothelial dysfunction are key mechanisms in the development and progression of CVD.

4.1.6 Central neurotransmitter systems

Both psychological and cardiovascular disorders are characterized by impairment of several central nervous system processes. In particular, alteration in monoamine function, in particular norepinephrine, has been shown to be implicated in the pathophysiology of depression and anxiety disorder (Esler et al., 2006; Lambert et al., 2000; Ressler and Nemeroff, 2001). The overactivation of the norepinephrine system, which characterizes anxiety and depression, is associated with increased sympathetic activity, which may lead to CVD. Also the serotonergic system plays a fundamental role in psychiatric disorders. Dysregulation of the norepinephrine and serotonin systems have been observed to mediate many behavioral and physiological symptoms of depression and anxiety, including autonomic alterations, hypersensitive stress and fear responses, anhedonia, appetite and sleep disturbances (Lucki, 1998; Ressler and Nemeroff, 2000).

Depression and anxiety are associated with reduced brain concentration of serotonin and to several changes of serotonin receptors and transporters (Cryan et al., 2005; Maes and Meltzer, 1995; Ressler and Nemeroff, 2000; Schins et al., 2003). As discussed above (see paragraph “Stress and cardiovascular disease”), serotonin is also fundamental for the regulation of cardiovascular function, suggesting that dysregulation of serotonin processes plays a key role in the link between depression, anxiety and CVD. For example, it has been reported that alteration in the serotonin receptors 1A density in specific regions of the central nervous system may represent one of the possible mechanisms linking mental and cardiac disorders (Nalivaiko, 2006).

5. Rodent models of psychopathology, for studying cardiovascular comorbidity

In combination with clinical and epidemiological studies in humans, experimental approaches with animal disease models provide novel and useful methods for investigating the causal and common pathophysiological substrates linking psychological factors and cardiovascular dysfunction. Experimental paradigms that focus on reliable and valid animal models could provide valuable translational results and offer insight into neural, physiological and behavioral mechanisms underlying this link.

Animal models should be characterized by three types of validity: face validity implies that the phenotype of the model is similar to the physiological and behavioral features described in humans; predictive validity requires that the model should be sensitive to manipulations known to influence human pathology, for example pharmacological treatments; construct validity relates to the similarity between the theoretical rationale underlying animal model and human behavior and implies that the human disease considered and the animal model share common pathological substrates.

5.1 Rodent models of depression

Several animal models of depression have been developed and reasonably well validated, both in non-human primates and in rodents. In particular, the most used preclinical models in rodents for the study of mood disorders are learned helplessness, behavioral despair, chronic mild stress and paradigms based on social stress.

The paradigm of learned helplessness was developed by Seligman (Seligman et al., 1980) and it consists in the exposure of the animal to an aversive stimulus that cannot be escaped, controlled or predicted. Indeed, the term “learned helplessness” refers to a constellation of behavioral changes that follows exposure to stressors that are non-controllable. This model is based on the cognitive theory of depression which suggests that negative and uncontrollable events may lead to reactions and behaviors of vulnerability. Actually, this kind of behavior is thought to occur in depression. The behavioral patterns which characterize this experimental procedure are long-lasting inability to escape in subsequent tests where escape is possible, immobility, passivity and increased vocalizations. The biggest limitation of this paradigm is the lack of evidence that learned helplessness is a cognitive behavioral process that characterizes also human depression (Geyer and Markou, 1995).

The behavioral despair paradigm, also named forced swimming test, was first proposed by Porsolt (Porsolt et al., 1978). In this procedure, the experimental animal is forced to swim in a confined space from which it cannot escape on one or several successive times. Behavioral despair is estimating by quantifying immobility, defined as the animal floating in the water without struggling and making other movements, except for those necessary to keep its head above the water. Indeed, the rodent, after unsuccessful attempts to escape, becomes immobile. This paradigm is the most frequently used model for investigating antidepressant potential. Actually, a number of antidepressant drugs has been shown to decrease the duration of immobility (Petit-Demouliere et al., 2005). However, the major problem of this paradigm is the interpretation of immobility as a failure to cope. In fact, immobility may be also interpreted as a successful adaptive strategy that conserves energy and allows the animal to survive longer (Geyer and Markou, 1995).

The chronic mild stress paradigm is a rodent model of depression developed by Katz (Katz et al., 1982) and further elaborated by Willner (Willner et al., 1991). It is based on the exposure of rodents (mostly mice and rats) to a period of mild and unpredictable stressors. In the model of Katz, the stressors are electrical shock, immersion in cold water, reversal of light/dark cycle, fasting, isolation, tail pinch, being shaken, being moved from cage to cage, usually repeated over a period of three weeks. Willner and colleagues modified the paradigm using milder stressors, such as stroboscopic illumination, paired housing, white noise, acute water deprivation, damp bedding, for a period of at least two weeks. Chronic mild stress has been shown to induced anhedonia in rodents (Willner et al., 1991). Anhedonia, defined as decreased responsiveness to pleasurable stimuli, is a core component of human depressive disorder (Loas, 1996). The chronic mild stress model characterizes anhedonia principally by a reduced consumption of palatable solutions, such as sucrose or saccharin. The paradigm of chronic mild stress has a high degree of predictive validity and has been successfully used for identification of antidepressant drugs (Moureau et al., 1992; Muscat et al., 1992; Willner et al., 1987). In recent years, the chronic mild stress model has been employed to study the effects of depression on physiological and cardiovascular variables (Grippo et al., 2002; Grippo et al., 2003; Grippo et al., 2004; Grippo et al., 2008; Grippo, 2009). Grippo and colleagues found that four weeks of chronic mild stress are associated with behavioral symptoms of depression, such as anhedonia and reduced locomotor activity and highlighted the association with cardiovascular modifications, including exaggerated pressure and heart rate reactivity (Grippo et al., 2002; Grippo et al., 2003), reduced heart rate variability (Grippo et al., 2002; Grippo et al., 2003) and increased risk of arrhythmic events (Grippo et al., 2004) .

Although the animal models described until now are reliable experimental paradigms for the study of the role of stress in determining depression, they have limited resemblance to

the challenges that animals would usually face in a natural setting (Willner, 1984). In addition, the main sources of stressful stimuli for humans, which can lead to the development of psychological disorders, are of social nature (Brown, 1993). For this reason, animal models that involve some sort of social challenge seem to be more appropriate as they represent situations that characterize individuals' everyday lives. In the last years newer models in rodents have focused on the role of social environment in mediating affective states (Bartolomucci et al., 2003; Grippo et al., 2007; Sgoifo et al., 2001). The most widely used social stressors in rodent models are defeat and isolation. Social defeat, obtained via the resident-intruder paradigm (Miczeck, 1979), consists in introducing the experimental animal into the territory of an aggressive male conspecific by which it is investigated, attacked and finally submitted. Social defeat has been shown to produce intense acute and long-lasting behavioral and physiological responses (Koolhaas et al., 1997). Short-term effects include increase in body temperature and heart rate, robust activation of the symaptho-adrenomedullary system and the pituitary-adrenocortical axis, shift of the sympathovagal balance towards a sympathetic prevalence and increased occurrence of cardiac arrhythmias. Furthermore, long-lasting physiological and behavioral effects can persist up to weeks and include changes in body weight, food intake and preference, circadian rhythmicity of heart rate, body temperature and physical activity, social and exploratory behavior. Acute or chronic social defeat may also lead to psychopathological changes mimicking certain aspects of human depression (Buwalda et al., 2005; Fuchs and Flugge, 2002). The utilization of anti-depressant pharmacological treatment has been shown to reduce the behavioral, physiological, neuroendocrine and neurobiological changes following defeat. In particular, the chronic exposure to social defeat has been employed as a model of depression in rodents. The studies of Rygula et al. (2005) and of Becker et al. (2008) validated chronic social defeat as a paradigm

capable to induce physiological and behavioral depression–like symptoms, including anhedonia, increased immobility in the forced swim test, reduced locomotor and exploratory activity, hyperactivity of the HPA axis, decreased body weight and reduced hippocampal volume.

In Chapter 2 I employed an experimental paradigm based on social defeat and isolation in rats, in order to investigate some of the behavioral, cardiovascular and hormonal alterations occurring in a depression-like state. In Chapter 3 I applied a protocol consisting of 12 episodes of social defeat in rats, to study the impact of depression on cardiac function and structure.

5.2 Rodent models of anxiety

When considering animal models of anxiety, it is important to distinguish between the tests used to reveal the level of anxiety and the paradigms which are thought to evoke the pathology (Beuzen and Belzung, 1995; Kalueff et al., 2007).

Numerous behavioral tests have been developed to assess the level of anxiety-related behavior in rodents (figure 9). In general, behavioral and physiological responses to stressful events can be used to assess anxiety responses of laboratory animals. Naturally aversive situations for rats and mice include unfamiliar open spaces, heights or bright lights. The traditional animal models employed to test anxiety are based on the exploration of novel environments and include the open field and the elevated plus maze test. The open field test allows the evaluation of general locomotor and exploratory behavior, which is thought to be inversely related to the level of anxiety (Kennet et al., 1985). The elevated plus maze test (EPM) was validated by Pellow (Pellow et al., 1985) and is based on the conflict between rodent's exploratory drive and its innate fear of bright and open spaces. A

high level of anxiety is expressed by avoidance of the unprotected arms of the test apparatus. In another type of test for anxiety based on social behavior (social interaction), the level of anxiety is measured by the decrease in social interaction between pairs of animals placed in an open area (File, 1985).

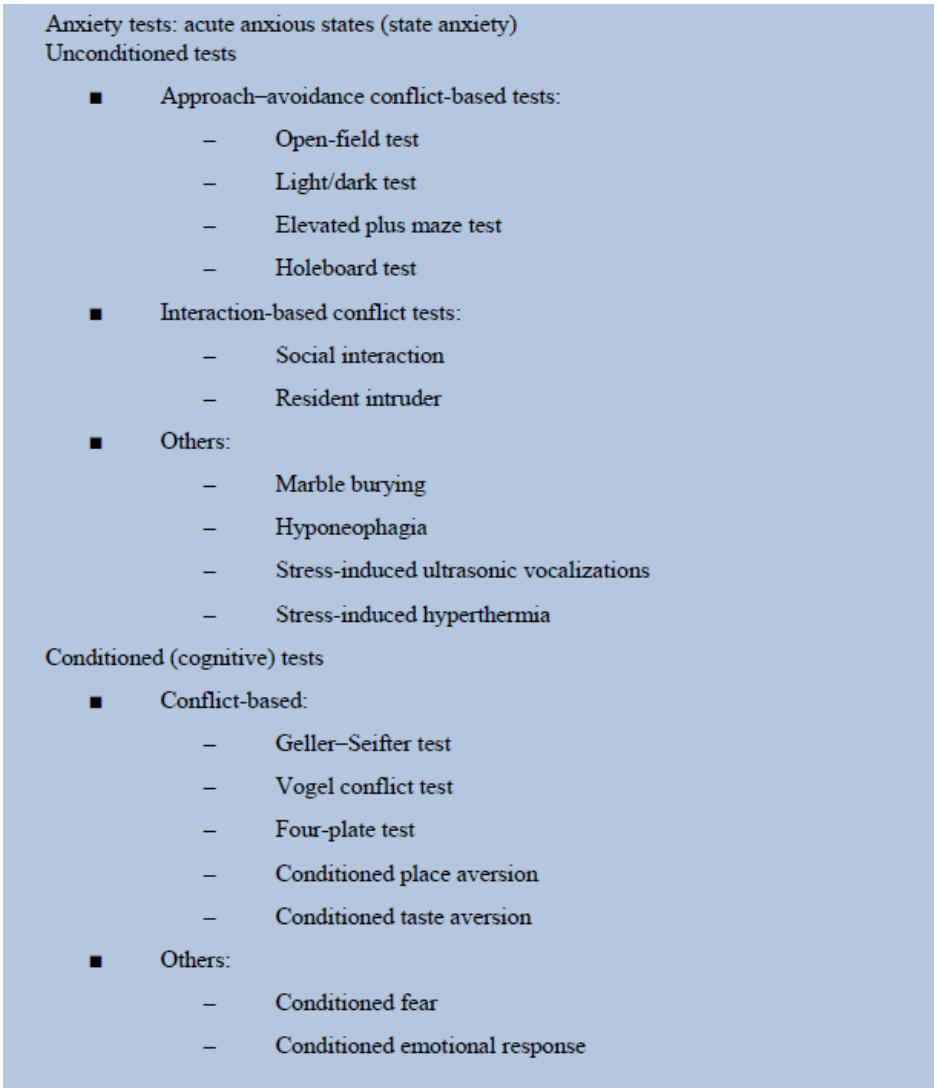


Figure 9. List of the experimental tests most commonly used in rodents to evaluate the levels of anxiety-related behaviors (from Sartori et al., 2011).

The rodent models aimed at mimicking the pathophysiology of human anxiety are obtained by experimentally manipulating the environment and/or the animal neurophysiology, neurochemistry and genome. Rodent models for anxiety-related behavior include exposure to early life stressors (see the paragraph 5.4 *"Early life stress"* for details), chronic stress in adulthood, transgenic modifications and selective breeding for extremes in trait anxiety. In particular, selective breeding for a specific phenotype within one strain have proved to represent a powerful tool for investigating the genetic variability of complex, polygenetic traits such as anxiety (Swallow and Garland, 2005). The breeding protocol usually starts with a heterogeneous population of rats or mice that is tested for the particular trait of interest. Then, individuals displaying extreme levels of the selected trait are mated. Selective breeding has allowed to obtain rats and mice with extreme levels of anxiety behavior: HAB (high anxiety-related behavior) and LAB (low anxiety-related behavior). In particular, rats with opposite levels of trait anxiety represent useful model as they (i) allow the identification of neural pathways and circuits underlying anxiety-related behavior and of candidate genes responsible of trait anxiety and (ii) are sensitive to pharmacological treatments (Landgraf and Wigger, 2002). The selection criterion for the HAB and LAB lines is the behavior in the elevated plus maze test. The animals are normally tested at 9 weeks of age. Male and female HABs and LABs are selected for further breeding only if the percentage of time spent in the open arms of the EPM test is below 5% and above 40-45%, respectively. For experimental purposes, the selection criterion is less than 10% of time spent in the open arms for HAB and more than 35% for LAB rats (Neumann et al., 2010). Differences between HAB and LAB in anxiety-related behavior are independent of gender (Bosch et al., 2005) and age (Landgraf and Wigger, 2002), and have been shown to be consistent over different years (Neumann et al., 2010) (figure 10). In addition, the opposite level of anxiety in HAB and LAB has been confirmed

in other validated tests of anxiety (Henniger et al., 2000; Muigg et al., 2008; Ohl et al., 2001). Noteworthy, HAB rats are characterized also by depression-related behaviors (Keck et al., 2003; Slattery and Neumann, 2010).

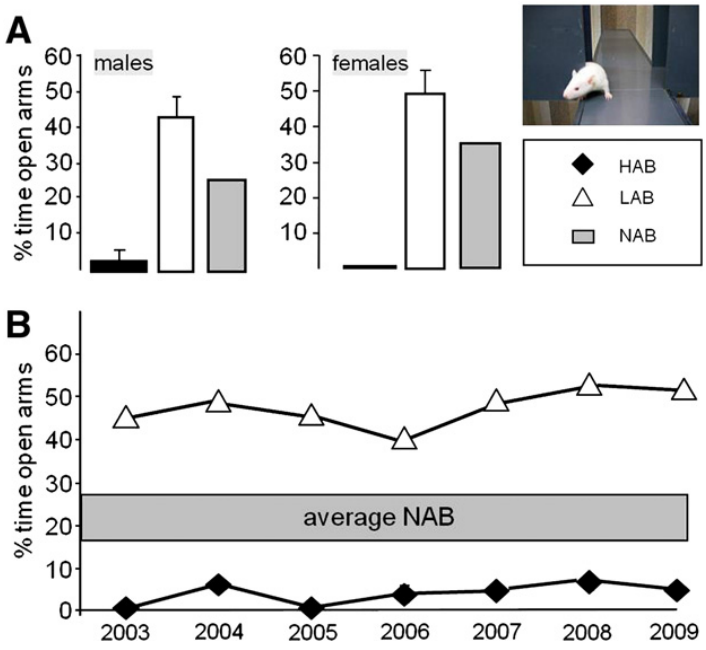


Figure 10. Anxiety-related behavior of male and female HAB, LAB and non-selected NAB rats at the age of 9 weeks in the elevated plus-maze (A) which is consistent over the years between 2003 and 2009 (B). The percentage of time spent in the open arms of the maze has been taken as the main anxiety parameter (from Neumann et al., 2011). EPM = elevated plus maze test; HAB = high anxiety-related behavior; LAB = low anxiety-related behavior; NAB = normal anxiety-related behavior.

Behavioral differences between HAB and LAB rats are accompanied by distinct neuroendocrine and neuronal features. HAB rats display a hyper-responsiveness of the HPA axis to a mild emotional stressor (Landgraf et al., 1999), whereas the neuroendocrine response to social stimuli is enhanced in LAB rats (Veenema et al., 2007). HAB rats are also characterized by increased release of vasopressin in the hypothalamus (Wigger et al., 2004) and reduced serotonergic transmission (Keck et al., 2005). Moreover, HAB and LAB

are different in stress coping strategy, with HAB being more passive, susceptible and vulnerable to stress conditions (Landgraf et al., 1999).

In Chapter 4 I studied the characteristics of cardiac autonomic regulation and stress responsivity in the HAB/LAB model of anxiety.

5.3 Early life stress

Early life adverse experiences can enhance stress responsiveness and lead to greater susceptibility to psychopathologies throughout life (Heim and Nemeroff, 2001). A variety of evidence in humans suggests that early life stress constitutes a major risk factor for the development and persistence of mental disorders, including major depression, posttraumatic stress disorder and general anxiety disorder (Agid et al., 1999; Famularo et al., 1992). The animal models of early life stress are principally based on the disruption of the mother-infant relationship. In particular, in rodents the most used paradigm is maternal separation, in which the pups are removed from maternal nest during weaning for a variable period of time. The long-term effects of this early life manipulation strongly depend on the duration of the separation. Actually, brief maternal separation (15 min) has been shown to attenuate adrenal and behavioral response to stress in adulthood and to reduce anxiety-related behaviors (Levine, 1957). On the other hand, long maternal separation (3-4 h) and maternal deprivation (24 h) have been associated with enhanced anxiety- and depression-like behaviors and with increased activity of the HPA axis (Aisa et al., 2007; Plotsky and Meaney, 1993; van Oers et al., 1998). In particular, rats exposed to long maternal separation showed anhedonia, decreased exploration of a novel environment, increased freezing, higher ACTH and corticosterone response to a variety of stressors, increased CRF levels in specific brain areas (Caldji et al., 1998; Ladd et al., 2000; Plotsky

and Meaney, 1993). Finally, postnatal stress has been shown to affect the expression of neurotrophines, which may be important factors involved in mediating the effects of early life stressful or traumatic experiences on behavioral dysfunction and psychopathologies (Cirulli et al., 2009).

In Chapter 5 I used a protocol of long maternal separation in rats, to study the long term effects of early life stress on cardiac autonomic balance and stress reactivity.

6. Outline of this thesis

The studies included in this thesis focused on the investigation of the pathophysiological mechanisms linking psychopathology and cardiovascular disease. In the experiments described different rat models of human psychopathologies were used. The common aim was to investigate the alterations of cardiac autonomic regulation, which are considered a key mechanistic link between psychological disorders and cardiovascular disease.

The studies described in Chapter 2 and Chapter 3 were designed to explore the association between depression and cardiac functional and structural alterations. In particular, two different experimental paradigms based on social stress were implemented to induce depressive-like symptoms in rats. In the experiment described in Chapter 2, depression was provoked by exposing the male rat to an adverse social episode, i.e. social defeat, followed by a prolonged period of social isolation. Physiological and behavioral signs of depression and their association with changes of autonomic neural input to the heart (established by means of HRV indexes) were described. In particular, the effects of this stress protocol were explored on (i) adrenocortical reactivity, (ii) anhedonic

behavior, (iii) daily rhythms of heart rate, body temperature and physical activity, (iv) cardiac autonomic stress reactivity, and (v) myocardial structure and morphology. In Chapter 3 a model of depression based on chronic social stress, i.e. 12 episodes of social defeat, was applied. The onset of a depression-like state was again evaluated considering a number of physiological and behavioral features: (i) body weight gain, (ii) hypothalamic-pituitary-adrenocortical (HPA) axis activity, (iii) circadian rhythmicity of heart rate, body temperature and locomotor activity, (iv) anhedonic behavior, (v) immobility in the forced swimming test. Then, it was assessed whether these parameters were related to (i) cardiac autonomic stress reactivity, (ii) cardiac electrophysiological properties and (iii) myocardial structure.

The study described in Chapter 4 was aimed at investigating possible signs of cardiovascular dysfunction in rats with opposite levels of anxiety-related behavior. In particular, these two lines of rats were compared for: (i) cardiac autonomic regulation in baseline conditions, (ii) cardiac autonomic responsivity to different stressors, (iii) effects of autonomic pharmacological manipulation, (iv) daily rhythms of heart rate, body temperature and physical activity, (v) cardiac structure and morphology.

Since early life adverse experiences are thought to play a major role in the susceptibility to psychopathologies, in Chapter 5 a protocol of maternal separation in rats was applied. The aim of this study was to clarify whether maternal separation may represent a useful rodent model to study the relationship between early life stress and adult cardiovascular dysfunction. To reach this purpose, the long-term effects of maternal separation were evaluated on (i) cardiac autonomic regulation in resting conditions, (ii) cardiac autonomic reactivity to a repeated stressor, (iii) response to pharmacological autonomic blockade, (iv) circadian rhythmicity of heart rate and vagal activity, (v) cardiac structure and morphology.

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CHAPTER 2

SOCIAL DEFEAT AND ISOLATION INDUCE CLEAR SIGNS OF A DEPRESSION-LIKE STATE, BUT MODEST CARDIAC ALTERATIONS IN WILD-TYPE RATS

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Chapter 2

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Abstract

Adverse social environments play a relevant role in the onset and progression of mood disorders. On the other hand, depression is an independent risk factor for cardiovascular morbidity. This study was aimed at (i) corroborating the validity of a rat model of depression based on a negative social episode followed by social isolation and (ii) verifying its impact on cardiac function and structure. Pair housed, wild-type (Rattus norvegicus) rats were implanted with radiotransmitters for ECG, temperature and activity recordings. They were either exposed to a social defeat episode followed by 4-week isolation or left undisturbed with their female partners. The social challenge induced a series of biological changes that are commonly taken as markers of depression in rats, including decreased body weight gain and reduced preference for sucrose consumption, functional and structural changes of the hypothalamic-pituitary-adrenocortical axis, increased anxiety in the elevated plus maze test. The cardiovascular alterations consisted in (i) transitory heart rate circadian rhythm alterations, (ii) lack of habituation of cardiac autonomic responsivity (tachycardia and vagal withdrawal) to an acute stressor, and (iii) moderate hypertrophy affecting the right ventricle of the heart. These results indicate that a depression-like state induced via this model of social challenge was associated with modest cardiovascular changes. Further studies are required to confirm the validity of this rat model of depression as a valid preclinical approach to the comprehension of the biological substrates underlying depression-cardiovascular comorbidity.

1. Introduction

Depression is a common psychiatric disorder affecting about 120 million people worldwide, and statistics clearly identify it as a major public health problem (World Health Organization, global report, 2005). Depression is characterized by disruptions in the functioning of a number of behavioral, neuroendocrine and physiological processes. Among these, low mood or inability to experience pleasure (anhedonia), slowing of speech and action, hypothalamic-pituitary-adrenocortical (HPA) axis dysfunction, sleep and biological rhythm disturbances, and body weight alterations constitute prevalent signs of depressive illness (American Psychiatry Association, 2000).

Clinical and experimental evidence points to a robust association between depressive disorders and cardiovascular dysfunction. In particular, patients with depression have a significantly higher risk for cardiac morbidity and mortality (Khawaja et al., 2009; Lippi et al., 2009), independently from traditional risk factors such as hypertension, smoking, elevated cholesterol, physical inactivity and elevated body mass index (Sowden et al., 2009; Whooley, 2006). Although many studies have highlighted the association between depression and heart disease, the underlying mechanistic links remain unclear. One possible pathophysiological mechanism appears to be an alteration of cardiac sympathovagal balance, due to elevated cardiac sympathetic and/or reduced cardiac vagal tone (Barton et al., 2007; Carnet et al., 1988; Kemp et al., 2010; Pitzalis et al., 2001; Rechlin et al., 1994). Perturbations of the autonomic nervous system and its imbalance may result in ventricular tachyarrhythmias and sudden cardiac death, the latter being one of the leading causes of cardiovascular mortality (Zipes and Wellens, 1998).

Stress is a critical environmental factor for the development of both clinical depression (Kendler et al., 1999) and cardiovascular disease (Steptoe and Brydon, 2009). Major life

events, for instance loss of a family member, social isolation or job strain, may sensitize individuals to subsequent stress and thereby increase the risk of developing such disorders (Post, 1992).

To better understand the associations among stress, depression, and cardiovascular dysfunction, experimental approaches that focus on reliable animal models could provide valuable translational results, and offer insight into causal and common mechanisms (neural, physiological and behavioral) underlying these links (Nestler and Hyman, 2010).

Among a number of different preclinical models of depression, we believe that a social challenge based on a single episode of social defeat followed by a period of isolation is the experimental paradigm that best relies on robust theoretical prerequisites to meet construct and etiological validity for the target syndrome. Indeed, in real life situations human subjects frequently cope with stimuli generated from the interaction with conspecifics (social stressors) (Bjorkvist, 2001), and social challenge is probably the most pervasive stressor in humans and social animals (Bartolomucci et al., 2005; Sachser et al., 1998). One of the most frequently used model in rodent studies is the acute or chronic social defeat paradigm (Koolhaas et al., 1997; Rygula et al., 2005). Social defeat induces a classical acute stress response, with robust cardiovascular and neuroendocrine activations, hyperthermia, and a prominent behavioral reaction (Buwalda et al., 2005; Sgoifo et al., 1999; Tornatzky and Miczeck, 1994), which lasts up to a few hours. In addition, several studies have reported long-lasting adverse effects of social defeat on a number of physiological and behavioral features, including body weight gain, circadian rhythmicity of heart rate, body temperature and locomotion, cardiac electrical activity, neuroendocrine functioning and behavioral response to novel stressors (Koolhaas et al, 1997; Meerlo et al., 1996; Sgoifo et al., 2002). These functional changes have been interpreted as reliable signs mimicking certain aspects of mood disorders in humans.

Like social subordination, social isolation is a stressful condition that plays an important role in triggering psychological disturbances such as depression (Thoits, 1995; Underwood, 2000). Several studies in rodents suggest that social deprivation has a long-term behavioral and physiological burden. For example, rats deprived of social contact have a characteristic hyperactive response to novelty stress (de Jong et al., 2005; Serra et al., 2005; Sharp et al., 2002), whereas individuals housed in small groups are better protected against excessive neuroendocrine activation elicited by acute stressors (Seeman and McEwen, 1996).

Given these considerations, in this study we anticipated that rats exposed to a model of social challenge based on a single episode of social defeat followed by four weeks of isolation would display pro-depressive physiological and behavioral symptoms. Our principal aim was to verify whether the induction of a depression-like state was accompanied by functional and structural changes at the level of the heart, that would justify the use of this stress paradigm in preclinical studies exploring the biological substrates linking depression and cardiovascular morbidity.

2. Methods

2.1 Animals and housing

Fourty-four adult male rats (Rattus norvegicus, Wild type Groningen strain) were used in this study; these animals were originally derived from the University of Groningen (The Netherlands) and bred in our department under conventionally clean conditions. This strain

has a complex social structure and is characterized by remarkable autonomic and neuroendocrine stress reactivity (Sgoifo et al., 1996a; Sgoifo et al., 1997). Experiments were conducted in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC), and were approved by the University of Parma Animal Welfare Committee. Rats were housed in unisex groups of four individuals from weaning until the onset of experiments (15 weeks of age). Before and during the experimental procedure, they were kept in rooms with controlled temperature (22±2°C) and lighting (lights on from 17:00 h to 05:00 h). Food and water were freely available throughout the experiment and the bedding consisted of standard saw dust.

2.2 Surgery: transmitter implantation

The radiotelemetry system employed in this study enabled the recording of the electrocardiogram (ECG), body temperature (T) and locomotor activity (Act) from freely behaving animals. It consisted of flat transmitters measuring 25x15x8 mm (TA11CTA-F40, Data Sciences Int., St Paul, MN, USA) and platform receivers (RPC-1, Data Sciences Int., 32x22x3 cm). Two weeks prior to the start of recording sessions, rats 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 sustained physical activity (Sgoifo et al., 1996b). Briefly, the body of the transmitter was placed into the abdominal cavity, and the two electrodes were fixed respectively to the dorsal surface of the xyphoid process and in the anterior mediastinum close to the right atrium. Subsequently, rats were prophylactically injected for two days with gentamicine sulfate (Aagent, Fatro, 0.2 ml/kg, s.c.).

2.3 Experimental groups

Animals were randomly assigned to two experimental treatments, namely social defeat and isolation (SDI, n=22) and control conditions (CTR, n=22). Since two weeks prior to surgery until the social defeat (or control) test (day 0), both SDI and CTR rats were paired with an ovariectomized female partner in plexiglas cages measuring 60x35x40 cm. Immediately after surgery, cages were divided transversely in two equal subzones (35x30x40 cm) by a wire mesh partition allowing olfactory, visual, and acoustic contact with the female partner, but no actual socio-sexual interaction. SDI rats were exposed to a 30-min social defeat test immediately followed by a 30-min psychosocial challenge. The defeat test was based on a classical “resident–intruder” test (Miczeck, 1979): the experimental animal (intruder) was introduced to the home cage of a dominant male (resident), after temporary removal of the dominant’s female partner; once there, it was vigorously attacked and finally subordinated by the resident animal (social defeat) (Buwalda et al., 2005). The residents were made aggressive by housing them for 2 weeks with sterilized female partners and their level of aggression was tested 4 times before the defeat test. The psychosocial challenge was obtained by inserting a wire mesh partition dividing the interaction cage in two equal halves, thus allowing sensory contact between the opponents but hampering further conflict. CTR animals were introduced to an unfamiliar cage with clean bedding (30 min of free exploration, 30 min confined to half of the cage by a wire mesh partition). Both the social challenge and the unfamiliar-cage test took place in a cage measuring 60x35x40 cm. After the test, SDI and CTR rats were returned to their home cages and confined to half of their housing cage as previously described. However, SDI rats also had their female partner removed from the cage and remained for 4 weeks in individual housing; during this period, olfactory isolation was

guaranteed by filtered covers and visual isolation by white paper walls surrounding the cage. On the contrary, CTR rats went back to the previous housing condition (with the female partner).

2.4 Outline of the experimental protocol

Figure 1 reports schematically the main tests and measurements performed across the experimental protocol. Day 0 corresponds to either the social defeat test (SDI group) or the unfamiliar-cage test (CTR group). Body weight was measured seven times across the experimental protocol: twice before, on the same day, and four times after the social defeat (or control) test. A dexamethasone suppression test was performed 10 days after defeat, whereas the sucrose solution preference test was performed twice before defeat and three times afterwards. The open field test was performed twice: 9 and 23 days after defeat, respectively. Similarly, the elevated plus-maze test was conducted twice following the defeat episode, namely on day 7 and 21. All these test/measurements were performed during the dark phase.

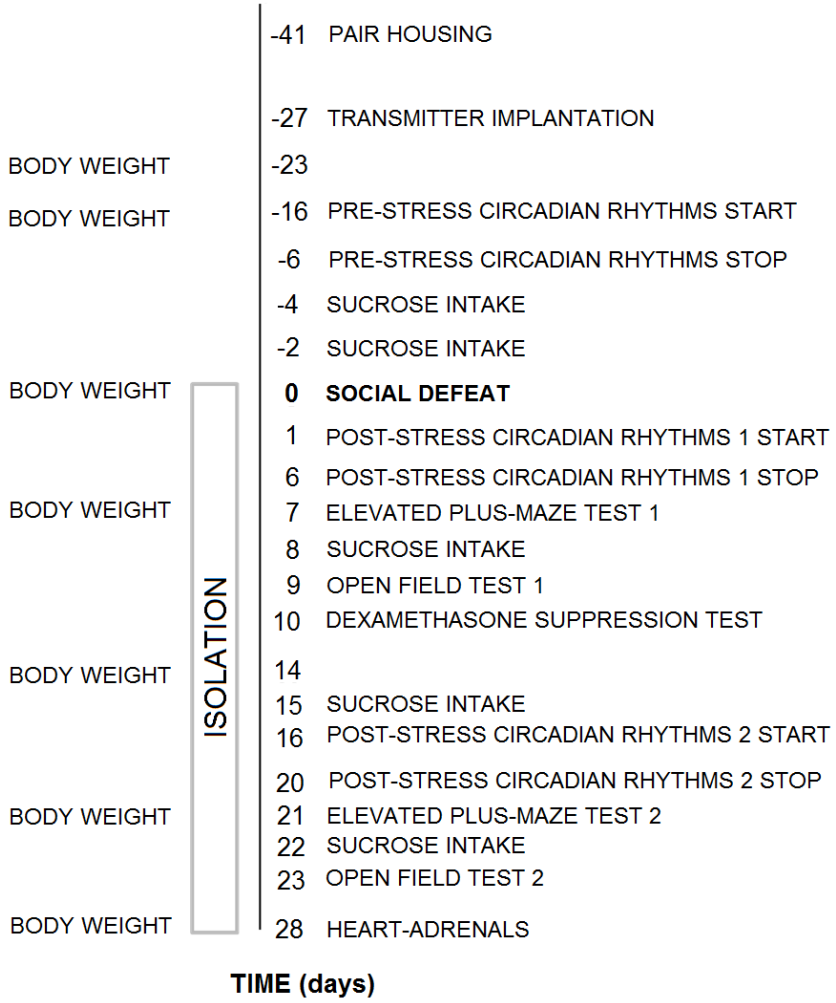

Figure 1. Schematic diagram of the procedures used in this study.

2.5 Sucrose preference test

The intake of water and sucrose solution (1%) was measured as an operational index of anhedonia (reduced responsiveness to a pleasurable stimulus). Ad libitum 1% sucrose solution was available to the animals for four days following surgery, to allow for adaptation to its taste. All fluid intake tests were performed during the dark phase. Two baseline tests were conducted on days -4 and -2 and the results were averaged; the fluid intake test was then repeated on days 8, 15, and 22 following the stress episode (figure 1).

Tap water was removed 20 h prior to each preference test; water and 1% sucrose solution were then placed on each cage in premeasured bottles, and fluid intake was monitored for 1 h (Grippo et al., 2007). Sucrose solution consumption (% of the total amount of fluid intake) was then quantified and used as an index of hedonic behavior.

2.6 Behavioral measurements

Open field test. The open field test allows for the evaluation of general locomotor and exploratory behavior of rats (Kennett et al., 1985). The open field apparatus was square-shaped (100x100x50 cm), with walls made of white plastic and the floor painted white and divided into 16 equal sectors by black lines. Each rat was placed in the center of the open field and behaved freely for 10 min; its behavior was recorded using a video camera placed above the maze. The following behavioral variables were collected using the Ethovision 6.0 software (Noldus, The Netherlands): number of entries (n) and cumulative time (s) spent in (i) the outer squares (the 12 peripheral sectors: periphery), (ii) the 4 corners of the arena (4 sectors: corner) and (iii) the inner squares (the 4 central sectors: center). In addition, the latency (s) to access the corner zone and the remaining part of the periphery (corner excluded) was quantified, as well as the overall distance traveled (cm). The test was performed in the same room where the experimental animals were housed.

Elevated plus-maze test. The elevated plus-maze, validated for measuring anxiety (Pellow et al., 1985), consisted of 4 elevated arms (100 cm above the floor, 50 cm long and 10 cm wide). The arms were arranged in a cross-like position, with two opposite arms being enclosed (by means of 40-cm high walls; closed zone), and two being open (open zone), including at their intersection a central square platform (10x10 cm) which gave access to

the four arms. All floor surfaces were black and made of polyvinyl carbonate. 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 variables were collected using the Observer XT 6.0 software (Noldus, The Netherlands): number of entries (n) and time spent (s) inside each zone or the central platform, and latency (s) to access the open and closed zones. As for the open-field, the elevated plus-maze test was performed in the same room where the experimental animals were housed.

2.7 Dexamethasone suppression test, blood sampling and corticosterone determination

HPA axis reactivity was assessed using a dexamethasone (30 µg/kg, SC) suppression test via plasma corticosterone level determinations 240 min after the injection. Blood samples (0.5 ml) were taken from the tail vein and collected into chilled tubes containing 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. Corticosterone was measured with a RIA kit (RIA Immuchem^{TM} Double antibody^{125} I RIA kit, MP Biomedicals, Orangeburg, NY, USA).

2.8 24-hour sampling of heart rate, body temperature and physical activity

Heart rate (HR, bpm), body temperature (T, °C) and locomotor activity behavior (Act, counts per min, cpm) were recorded in the three following phases: (i) pre-stress, from day -16 to -6; (ii) post-stress1, from day 1 to day 6; (iii) post-stress2, from day 16 to 20 (figure 1). HR, T, and Act were sampled continuously for 60 s every 60 min, in order to assess

their circadian rhythmicity. The three parameters were quantified as means of the 12-h resting phase (light), and 12-h active phase (dark). For each animal, the daily amplitude of the rhythms of HR, T, and Act was calculated as the difference between average activity and resting phase values, respectively (Meerlo et al., 1999).

2.9 ECG data acquisition and analysis

Continuous ECG recordings were performed before (10 min), during (10 min) and after (10 min) the two open field tests (day 9 and 23 after defeat; figure 1). ECG analysis was performed using a software package developed in our lab for quantification of time-domain indices of heart rate variability (Sgoifo et al., 2001). The following parameters were quantified: (i) the mean R–R interval duration (RR, ms) and (ii) the root-mean square of successive R–R interval differences (r-MSSD, ms). R-MSSD reflects the magnitude of heart rate changes between consecutive beats and is a widely accepted index of cardiac vagal activity (Stein et al., 1994). RR and r-MSSD calculations were performed after removal of arrhythmic events and recording artifacts, and expressed as means of 1-min recording periods.

2.10 Post-mortem measurements

Heart remodelling and morphometry. The hearts of 8 anesthetized SDI and 8 CTR animals were arrested in diastole by an injection of cadmium chloride solution (100 mM, IV) and excised. The two atria, the right ventricle (RV) and the left ventricle (LV) inclusive of the septum were separately weighed, fixed in 10% buffered formalin solution, and used for morphometric studies. The following parameters were determined: heart weight (HW) to

body weight (BW) ratio, LV to BW, and RV to BW ratios. LV free wall thickness and LV transversal diameters were morphometrically computed (Image Pro-plus). The LV chamber volume was calculated according to the Dodge equation (Dodge and Baxley, 1969). Subsequently, from the equatorial slice embedded in paraffin, 5 μm-thick sections were cut and used for the following analyses. One section, stained with Masson's trichrome, was analyzed by optical microscopy (magnification 250X) in order to evaluate the total amount of interstitial and reparative fibrosis (Beltrami et al., 1994) in the LV myocardium. According to a procedure previously described (Costoli et al., 2004), for each section a quantitative evaluation of the fibrotic tissue was performed in 60 randomly selected fields from the subendocardium, midmyocardium and subepicardium, with the aid of a grid defining a tissue area of 0.160 mm^2 and containing 42 sampling points, each covering an area of 0.0038 mm^2. To define the volume fraction of fibrosis in the three layers of the ventricular wall, the number of points overlaying myocardial scarrings were counted and expressed as a percentage of the total number of points explored. For reparative fibrosis, the numerical density of fibrotic foci per unit area of myocardium was also determined.

Adrenal weight. Adrenal glands were also removed, carefully trimmed, and weighed to evaluate possible hypertrophic and/or hyperplastic effects due to social defeat and isolation.

2.11 Data analysis and statistics

All parameters were expressed as mean ± SEM. Statistical significance for all tests was set at $p<0.05$. All statistics were performed using SPSS 11.5 software package (SPSS

Inc., Chicago, IL, USA). For technical reasons, sample sizes are not the same for all types of measurements collected.

Two-way ANOVA for repeated measures, with “group” as the between-subject factor (two levels: CTR and SDI) was applied for: (i) % sucrose solution consumption (within-subject factor “time”: four time points); (ii) RR and r-MSSD values in the open-field test (within-subject factor “time”: two time points, i.e. test 1 and 2); (iii) behavioral parameters in the open-field and elevated plus-maze test (within-subject factor “time”: two time points, i.e. test 1 and 2); (iv) circadian rhythm amplitude of HR, T, and Act (within-subject factor “time”: 12 time points). Follow-up analyses were conducted using Student’s “t”-tests, with a Bonferroni correction for multiple comparisons. *A priori* Student’s “t”-tests, after controlling for homogeneity of the variance via a Levene test, were performed for comparisons between SDI and CTR rats on: (i) AUC values for RR and r-MSSD in the open-field test; (ii) corticosterone levels in the dexamethasone suppression test, (iii) adrenal weight to body weight ratio, (iv) AUC values for body weight, and (v) cardiac anatomy and morphometry parameters.

3. Results

3.1 Aggression received during social defeat test

During the 30-min defeat test, SDI intruders received on average 17.4 ± 2.3 attacks, with a mean latency time to first attack of 21.1 ± 4.9 s. At the end of the test, careful external inspection of defeated animals did not reveal major signs of wounding.

3.2 Body weight

Body weight just before defeat (or unfamiliar cage test) was 447.7±8.9 g for SDI and 441.9±9.6 g for CTR rats; at sacrifice, 456.1±8.6 g for SDIs and 462.5±8.9 g for CTRs. Overall body weight gain for each SDI and CTR rat was assessed by means of AUC compared to pre-defeat value. The average AUC value of SDI animals (n=21) was significantly lower when compared to the corresponding CTR (n=22) value (SDI: -14.7±139.9 g*x*day^{-1} vs. CTR: 340.4±81.4 g*x*day^{-1}; $t(41)=2.19$, $p<0.05$). This evidence suggests an overall attenuation of body weight gain due to the social challenge.

3.3 Sucrose preference

Figure 2 reports the temporal dynamics of sucrose solution intake before and after social defeat. Average sucrose intake gradually (not significantly) increased in both groups up to 15 days after defeat, but decreased in SDI rats on day 22, where it was significantly lower than corresponding CTR value ($F_{timexgroup}(3,123)=4.10$, $p<0.05$; $t_{day22}(41)=2.17$, $p<0.05$). These data point to the establishment of an anhedonic state in rats exposed to the social challenge, which becomes evident only three weeks after social defeat.

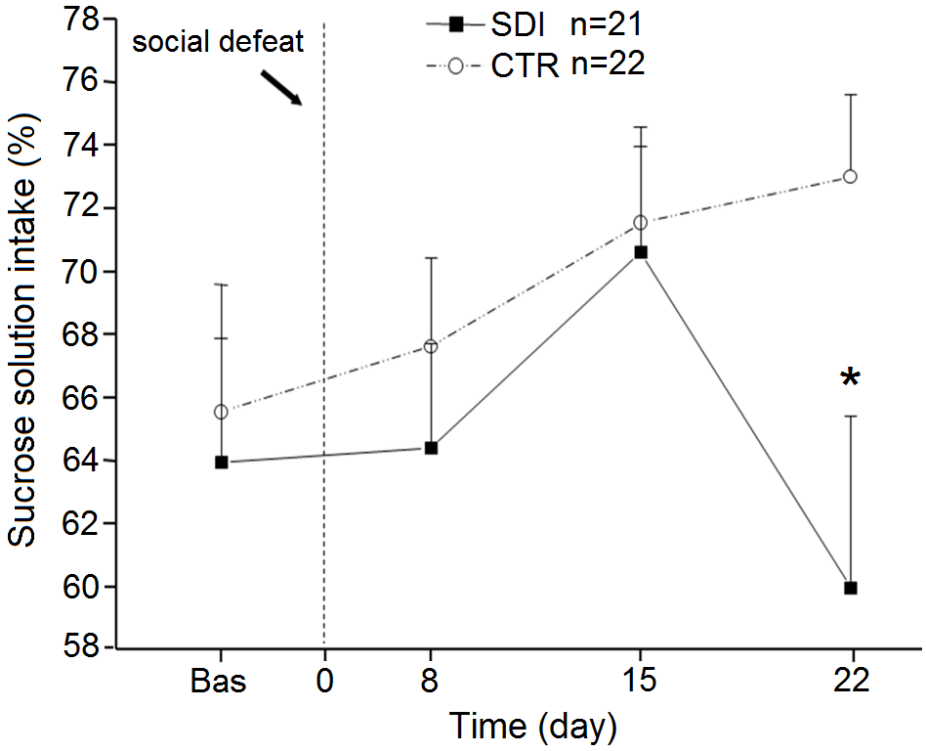

Figure 2. Sucrose consumption (%) at baseline and after a single social defeat episode (days 8, 15 and 22) in defeated+isolated (SDI) and control (CTR) rats. Baseline values represent the means of the values collected on days -4 and -2. *: significantly different ($p<0.05$, Student "*t*" test) from corresponding CTR value.

3.4 Behavior in the open-field test

Table 1 summarizes CTR and SDI rat behavioral response to the open-field test performed 9 and 23 days after social defeat. Two-way ANOVA for repeated measures on the cumulative time spent in and the latency to reach the corners of the arena indicated a significant effect of time (time spent: $F_{time}(1,41)=6.23$, $p<0.05$; latency: $F_{time}(1,41)=3.86$, $p<0.05$), thus suggesting an overall increased tendency to prefer this zone upon re-exposure to this exploration test, with no differences between groups. Similarly, the cumulative time spent in the entire peripheral zone was increased in the second open-field test ($F_{time}(1,41)=4.45$, $p<0.05$), but again SDIs did not differ from CTRs. Concerning the

centre section, a significant effect of time was found for the cumulative time spent and the number of entries exhibited (time spent: $F_{time}(1,41)=4.88$, $p<0.05$; entries: $F_{time}(1,41)=5.62$, $p<0.05$), indicating an overall reduced tendency to occupy this part of the arena upon re-exposure to the open-field test, but with no group differences.

Table 1. Behavioral responses during the open field test 1 (9 days after the social defeat) and open field test 2 (23 days after the social defeat) in defeated+isolated (SDI, n=22) and control (CTR, n=21) rats. Values are reported as mean ± SEM.

	1ST TEST		2ND TEST	
	SDI	**CTR**	**SDI**	**CTR**
ENTRIES CORNER (n)	30.5±3.2	25.1±1.6	18.2±2.5	21.4±5.3
TIME SPENT CORNER (s)	431.8±19.3	466.9±13.4	519.9±12.1	537.8±7.2
LATENCY CORNER (s)	3.4±0.5	3.7±0.9	2.8±0.6	2.9±1.0
ENTRIES CENTER (n)	4.8±0.8	4.0±0.8	2.4±0.7	1.9±0.3
TIME SPENT CENTER (s)	18.5±3.2	20.9±5.2	7.4±2.7	5.3±0.9
ENTRIES PERIPHERY (n)	11.3±3.7	7.2±1.1	4.2±0.8	9.6±5.3
TIME SPENT PERIPHERY (s)	581.5±3.3	579.1±5.2	592.6±2.7	594.7±1.1
LATENCY PERIPHERY (s)	1.4±0.3	1.6±0.5	1.3±0.4	0.9±0.5
TOTAL DISTANCE MOVED (cm)	3449±235	2957±135	2724±194	2732±182

3.5 Behavior in the elevated plus-maze test

Table 2 summarizes CTR and SDI rat behavioral response to the elevated plus-maze test performed 7 and 21 days after defeat. Two-way ANOVA for repeated measures on the time spent in the open arms revealed a significant effect of group ($F(1,41)=3.64$, $p<0.05$) and a time x group interaction ($F(1,41)=5.32$, $p<0.05$). SDI rats spent significantly less time in the open arms during both the 1st and 2nd test ($t(41)=2.54$ and $t(41)=3.34$, respectively;

p<0.05). A significant effect of time was found also for the amount of time spent in the centre of the maze (F(1,41)=4.78, p<0.05), with no differences between groups. A significant effect of group (F(1,41)=6.21, p<0.05) and a time x group interaction (F(1,41)=6.67, p<0.05) was observed for the number of entries in the open arms, with SDI rats exhibiting significantly lower values during both the 1st and 2nd test (t(41)=3.12 and t(41)=4.54, respectively; p<0.05). A significant effect of group was found for the latency to access the open arms (F(1,41)=4.56, p<0.05), with SDI rats showing significantly larger values during both tests (t(41)=2.24 and t(41)=2.21, respectively; p<0.05). A significant effect of time was observed for the latency to enter the closed arms (F(1,41)=3.86, p<0.05), with no group differences.

Table 2. Behavioral responses during the elevated plus maze Test 1 (7 days after the social defeat) and the elevated plus maze Test 2 (21 days after the social defeat) in defeated+isolated (SDI, n=22) and control (CTR, n=21) rats.

	1ST TEST		2ND TEST	
	SDI	**CTR**	**SDI**	**CTR**
ENTRIES CLOSED ARMS (n)	4.9±0.5	5.2±0.5	4.9±0.6	5.1±0.5
TIME SPENT CLOSED ARMS (s)	254.0±9.2	232.8±11.3	271.2±7.1	262.7±10.5
LATENCY CLOSED ARMS (s)	10.2±2.6	16.2±4.0	6.5±1.6	4.1±0.9
ENTRIES OPEN ARMS (n)	0.9±0.2 *	1.6±0.4	0.4±0.2 *	1.3±0.4
TIME SPENT OPEN ARMS (s)	11.9±3.3 *	23.9±6.5	5.6±2.6 *	15.5±6.0
LATENCY OPEN ARMS (n)	216.3±24.5 *	158.2±27.2	243.8±20.2 *	191.2±27.2
ENTRIES CENTER (s)	5.9±0.7	6.9±0.7	5.4±0.7	6.4±0.8
TIME SPENT CENTER (s)	34.1±6.4	43.3±6.7	23.2±6.1	21.8±4.8

Values are reported as mean ± SEM. * : significantly different (p<0.05) from corresponding CTR value.

3.6 Adrenocortical response to dexamethasone suppression test

Ten days after social defeat, plasma corticosterone levels in response to the dexamethasone suppression test were significantly higher in SDI rats (n=20) when compared to the CTR counterparts (n=18) (SDI=14.3±3.5 ng/ml vs. CTR=6.9±0.5 ng/ml; $t(36)=2.09$, $p<0.05$). This evidence suggests that the animals undergoing defeat and isolation developed an alteration of the negative feedback mechanisms modulating HPA axis activity.

3.7 Biological rhythms

Figure 3 summarizes the effect of a social defeat episode followed by long-term isolation on circadian rhythmicity of HR, T and Act. Compared to CTR counterparts, rats exposed to the adverse social condition exhibited a significantly larger reduction of the daily rhythm amplitude for several days: namely, day 1-6 and 18 for HR (figure 3), day 1-6 and 16-20 for Act (figure 3), and day 1 for T (figure 3) (statistical details in figure 3 legend). In other words, the social challenge negatively influenced the circadian rhythmicity of these parameters, though the intensity and duration of the effect varied depending on the parameter considered (HR, Act or T).

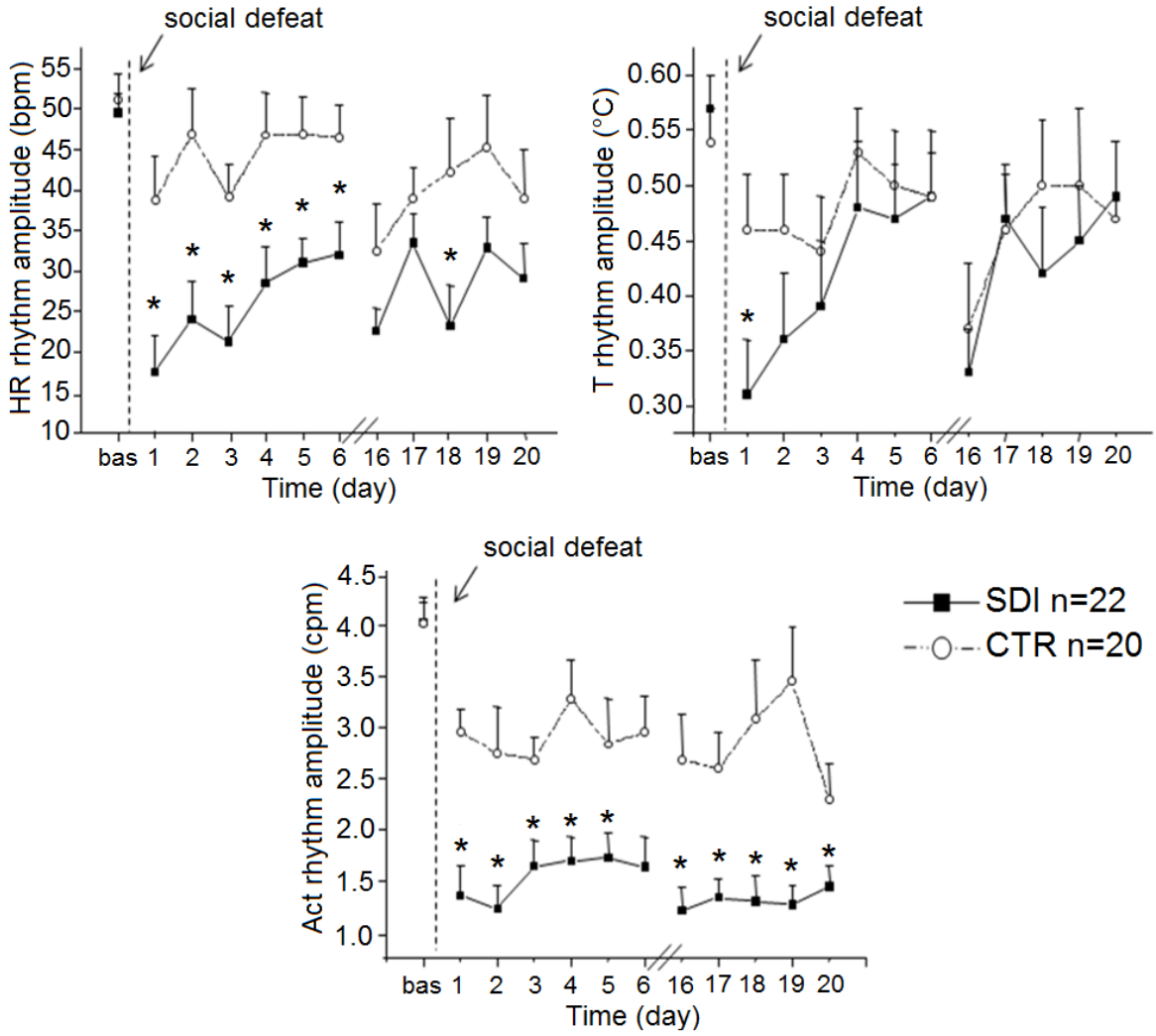

Figure 3. Effects of social defeat and subsequent isolation on circadian rhythms of heart rate (HR), body temperature (T) and physical activity (Act) (first and third week after the defeat episode). Data are expressed as daily amplitude (difference between corresponding night and day value).

ANOVA. Significant effect of "recording day" (Act: $F(11,440)=15.72$, $p<0.01$), significant effect of "group" (HR: $F(1,40)=15.3$, $p<0.01$; Act: $F(1,40)=30.36$, $p<0.01$) and "recording day x group" interaction (Act: $F(11,440)=4.87$, $p<0.05$).

Follow-up analysis (Student 't' test') between SDIs and CTRs revealed the following significant differences: day 1 (HR: $t(40)=3.01$, $p<0.01$; T: $t(40)=2.13$, $p<0.05$; Act: $t(40)=4.33$, $p<0.01$); day 2 (HR: $t(40)=3.13$, $p<0.01$; Act: $t(40)=3.04$, $p<0.01$); day 3 (HR: $t(40)=2.72$, $p<0.01$; Act: $t(40)=2.57$, $p<0.05$); day 4 (HR: $t(40)=2.68$, $p<0.05$; Act: $t(40)=3.55$, $p<0.01$); day 5 (HR: $t(40)=2.87$, $p<0.01$; Act: $t(40)=2.25$, $p<0.05$); day 6 (HR: $t(40)=2.54$, $p<0.05$; Act: $t(40)=2.83$, $p<0.01$); day 16 (Act: $t(40)=3.07$, $p<0.01$); day 17 (Act: $t(40)=3.19$, $p<0.01$); day 18 (HR: $t(40)=2.33$, $p<0.05$; Act: $t(40)=2.85$, $p<0.01$); day 19 (Act: $t(40)=3.89$, $p<0.01$); day 20 (Act: $t(40)=2.08$, $p<0.05$).

*: significantly different from corresponding control value.

3.8 Cardiac autonomic response in the open-field test

Cardiac autonomic responses (RR and r-MSSD values) to the open-field tests are detailed in figure 4. For both parameters, the individual overall responses to the 1st and 2nd test (9 and 23 days after defeat, respectively) were expressed as AUC compared to pre-test baseline value. SDI rats did not differ from the CTR counterparts during either the 1st or 2nd open-field exposures. However, delta AUC values ($AUC_{open\text{-}field2}$ – $AUC_{open\text{-}field1}$) for RR and r-MSSD of SDI rats were significantly lower compared to CTR counterparts (delta AUC for RR: CTR=131.26±66.69 vs. SDI=-79.91±66.28 ms×min, $t(40)=2.24$, $p<0.05$; delta AUC for r-MSSD: CTR=15.09±5.19 vs. SDI=-7.92±7.39, $t(40)=2.49$, $p<0.05$). In other words, CTR rats showed a clear habituation of heart rate acceleration (RR interval) and vagal withdrawal response (r-MSSD) that was not observed in the SDI counterparts.

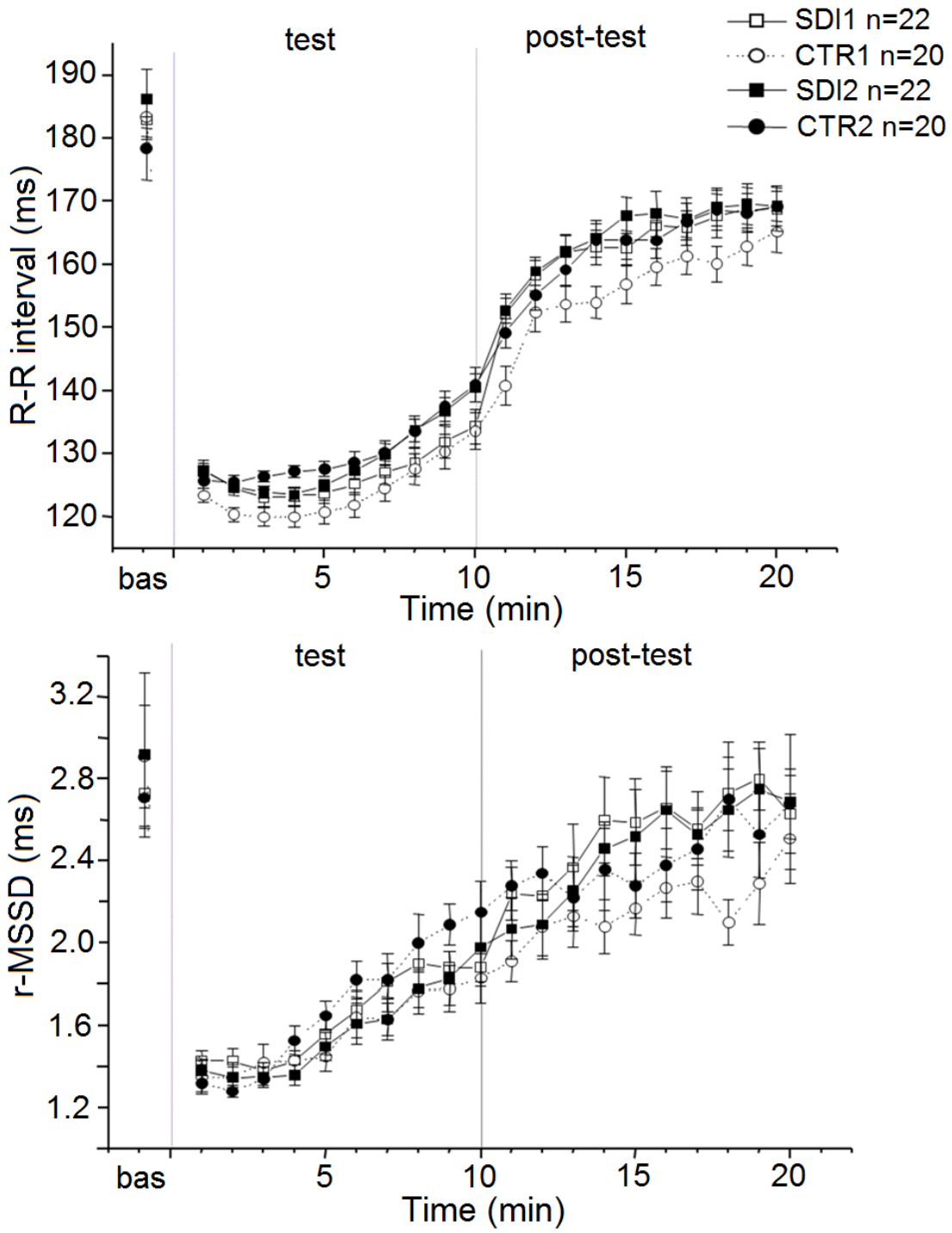

Figure 4. Time course of heart rate parameters in defeated+isolated (SDI) and control (CTR) rats before (baseline, bas: mean of 30-min recording) and during the first (day 9) and second (day 23) open-field test. RR = mean R-R interval duration (indicating heart rate); r-MSSD = root mean square of successive R-R interval differences (indicating cardiac vagal activity).

3.9 Adrenal weight

At the time of sacrifice, SDI rats had significantly heavier adrenals when compared to the CTR counterparts ($t(41)=3.16$, $p < 0.01$). SDI adrenal weight corrected for body weight was 0.119±0.004 mg/g (n=22); the corresponding CTR value was 0.102±0.004 mg/g (n=21). Thus, rats experiencing acute social defeat and a prolonged period of isolation likely developed adrenal hypertrophy.

3.10 Cardiac anatomy and morphometry

Cardiac anatomy. As shown in Table 3, no differences were observed between the SDI and CTR groups with respect to the weight of the LV and linear LV parameters. Only LV wall thickness was slightly (although not significantly) reduced in rats exposed to social defeat ($t(14)=1.86$, $p=0.06$). LV chamber volume was unchanged and LV mass-to-chamber volume ratio was only moderately increased in SDI rats. In contrast, both RV weight and its ratio to heart weight were significantly higher in SDI than in CTR rats (+10 % and +8 % respectively; Table 3). Thus, social defeat and isolation did not significantly affect LV anatomy, but produced a moderate RV hypertrophy.

Tissue morphometry. No significant differences were observed between the two groups of animals in terms of total amount of myocardial fibrosis in the left ventricle (SDI=1.21±0.08 % vs. CTR=1.32±0.27 % of the LV myocardium). The volume fraction of myocytes was also unaffected by social challenge (SDI=85±1.49 % vs. CTR=86.49±2.06 %). Negligible values of perivascular and interstitial fibrosis, including small foci of collagen accumulation distributed in the myocardium, were observed in the LV of both SDI and CTR hearts.

However, the two forms of collagen accumulation were differently expressed in experimental animals; interstitial fibrosis was higher while perivascular fibrosis was lower in SDI rats when compared to CTRs (Table 3). Interestingly, interstitial collagen accumulation in the LV of SDI hearts was predominantly distributed in the endo-myocardium and mid-myocardium and was significantly larger when compared to CTR group (endo-myocardium SDI=0.42±0.16 % vs. CTR=0.22±0.1 %, $t(14)=2.33$, $p<0.05$; mid-myocardium SDI=0.99±0.27 % vs. CTR=0.5±0.24 %, $t(14)=3.07$, $p<0.05$). Perivascular fibrosis was modest in SDI hearts and more abundant in the mid-myocardium of CTR hearts (CTR=1.34±0.41 % vs. SDI=0.54±0.18 %, $t(14)=4.01$, $p<0.01$). Such forms of myocardial damage were absent in the RV.

Table 3. Gross cardiac characteristics and left ventricular myocardial fibrosis in defeated+isolated (SDI, n=8) and control (CTR, n=8) rats.

	CTR (n=8)	SDI (n=8)
LVW (mg)	962.1±35.1	950.2±48.9
RVW (mg)	201.5±9.8	223.7±14.8 *
LVW/HW (mg/mg)	0.76±0.01	0.74±0.01
LVW/BW (mg/g)	2.01±0.96	2.06±0.93
RVW/HW (mg/mg)	0.16±0.01	0.19±0.01 *
RVW/BW (mg/g)	0.42±0.06	0.48±0.08
LV chamber length (mm)	13.20±0.25	12.92±0.33
LV chamber equatorial diameter (mm)	5.96±0.20	5.83±0.14
LV chamber volume (mm^3)	247.0±18.1	229.0±7.4
LV wall thickness (mm)	2.55±0.12	2.34±0.04
LV mass/chamber volume	3.67±1.82	3.91±1.64
RV wall thickness (mm)	0.80±0.06	1.09±0.08 *
LV perivascular fibrosis (%)	1.01±0.28	0.70±0.08 *
LV interstitial fibrosis (%)	0.31±0.06	0.51±0.14 *

Values are reported as mean ± SEM. BW = body weight; HW = heart weight; LVW = left ventricular weight; RVW = right ventricular weight.

*: significantly different ($p<0.05$) from corresponding CTR value.

4. Discussion

The preliminary aim of the present study was to verify the induction of depression-like symptoms via a stress paradigm based on a negative social episode followed by prolonged social isolation. Although defeat and isolation have been separately used in many rodent studies aiming at demonstrating their pro-depressive effects, there is only limited information concerning the effects of the combination of a negative social episode with prolonged isolation (de Jong et al., 2005; Von Frijtag et al., 2000). We believe that the depression-like signs obtained through such a combination of factors might have important translational value, as human conditions promoting mood disorders are often based on a traumatic social event (loss of job, of social status, of close relative or partner) followed by social isolation.

The main objective was to verify whether social stress-induced “depression” was associated with alterations of cardiac function and structure that would justify the use of this model of social challenge in preclinical studies exploring the biological substrates linking depression and cardiovascular morbidity.

To the best of our knowledge, this is the first study in rodents that combines a number of behavioral/physiological measures aimed at documenting a depression-like state induced via social challenge with markers of functional and structural remodeling at the level of the heart.

Indeed, we found a series of biological changes that are commonly taken as markers of depression in rats: decreased body weight gain and reduced preference for sucrose consumption, biological rhythm disturbances, functional and structural changes of the

hypothalamic-pituitary-adrenocortical axis, increased anxiety in the elevated plus maze test.

Nonetheless, we found only modest functional and structural changes affecting cardiovascular function. Specifically, stressed rats showed transient disturbance of heart rate circadian rhythmicity and failed to develop habituation of cardiac autonomic responsivity (tachycardia and vagal withdrawal) upon re-exposure to a homotypic acute stressor (open field). Their left ventricular anatomy was not significantly affected, but a moderate hypertrophy was observed in the right ventricle of the heart.

In the present study, overall analysis of body weight changes during post-defeat isolation period revealed an attenuation of body weight gain of SDI rats when compared to CTR counterparts. The retardation of growth may be at least partly due to the fact that food intake may be reduced after a social defeat episode (Meerlo et al., 1996; Merlo et al., 1997). Also, an increase in metabolic processes may be involved (Martì et al., 1994), as explained by stress-induced release of catabolic hormones such as corticosterone and adrenaline from the adrenal cortex and medulla respectively (Armario et al., 1986; Frankenhaeuser, 1971). This study does not provide information regarding whether reduced weight gain is due to decreased food consumption or catabolic hormones, or both; however both of these processes are associated with stress. Whatever the explanation, this long-term reduction in body growth is one of the most consistently found effects following social defeat and is considered an index of a depression-like state (Meerlo et al., 1996; Ruis et al., 1999).

SDI rats also showed disturbed physiological and behavioral rhythms. Changes in rhythmicity were evidenced by a larger reduction of the daily amplitude of heart rate and locomotor activity both during the first and the third week following defeat, whereas the difference in the day-night oscillation of body temperature was limited to the very first day

after defeat. This discrepancy may be explained by differences in the nature of these parameters. Indeed, body temperature is a homeostatic parameter, maintained at a relatively stable level within an optimal narrow window in a broad range of environmental conditions, as a result of its critical role for proper physiological functioning in homeothermic species (McEwen, 2000). In contrast, allostatic parameters such as heart rate, blood pressure, physical activity and tissue mediators like neurotransmitters and hormones largely fluctuate in a changing environment, to optimize stability of homeostatic parameters such as body temperature, pH or pO_2 (McEwen, 2000). Our results are in line with the idea that most stress responses represent challenges to the body's allostasis, not challenges to its homeostasis (McEwen, 2000). Biological rhythm abnormalities, in particular blunted circadian amplitude, have been linked to depressive disorders in both clinical and experimental studies (Souêtre et al., 1989). We hypothesize that heart rate circadian rhythm disturbances might also reflect pathological changes in the autonomic control of the heart, thus representing a sensitive index of the increased cardiac vulnerability that often accompanies depression.

In terms of neuroendocrine activation, compelling evidence has accumulated that the HPA axis is hyperactive in major depressive disorder: high levels of plasma cortisol were found in depressed patients, suggesting that glucocorticoids hypersecretion is a core symptom of the depressive state (Holsboer, 2000). In animal models, the dexamethasone suppression test has been used extensively to quantify dysregulation of the HPA axis (Carroll, 1980). Dexamethasone is a synthetic glucocorticoid that mimics corticosterone by inducing negative feedback to the pituitary, hypothalamus and hippocampus. In our study, after a dexamethasone injection, SDI rats showed significantly higher plasma corticosterone levels than CTRs, suggesting the development of an impaired negative feedback ability to regulate the HPA axis at either the pituitary or the adrenocortical level. Besides functional

abnormalities, we also found structural changes in this essential stress responsive axis, as shown by increased adrenal gland weight of SDI rats. Nevertheless, our data do not clarify the differential role of hypertrophy and hyperplasia in determining this increase in adrenal volume. Hyperplasia and hypertrophy are two distinct processes that frequently occur together, and may well be triggered by chronic stress (Ulrich-Lai et al., 2006).

The negative social episode followed by prolonged isolation induced behavioral changes relevant to affective disorders. Firstly, SDI rats developed a reduced preference for sucrose solution. In the present study, baseline, starting levels of percent sucrose solution intake (65%) point to a lower preference as compared to other recent studies using the same sucrose solution concentration (see for example Dagyte et al., 2010: close to 100%). The different strain used (Wild-type-Groningen vs. Wistar) might partly explain this difference. Indeed, other articles report widely changing baseline preference for sucrose solution: Rygula and colleagues (2005) reported a 70% preference with a 0.8% solution in Wistar rats; Grippo and colleagues (2003) described an 80% preference of 1% solution in Sprague-Dawley rats. Apart from these considerations, our data point to a clear decrease in sucrose preference in SDI rats three weeks after defeat. A similar time course in the establishment of reduced hedonic behavior was found in previous reports (Grippo et al., 2003; Rygula et al., 2005; Willner et al., 1996). The decrease of sucrose solution preference is generally interpreted as a valid and operational index of anhedonia, which is considered a core symptom for the diagnosis of major depression (Loas, 1996).

When rats were exposed 7 and 21 days after defeat to the elevated plus-maze test, SDIs spent significantly less time in the open, unprotected arms of the maze than CTRs. Additionally, the latency to enter the open arms was increased in SDI rats, while the number of entries decreased compared to CTRs. This indicates that defeated/isolated rats were more anxious than their control counterparts, as open/unprotected arms are

interpreted as more threatening than the closed/protected ones. It should be noted that, since the comorbidity between anxiety and depression is a remarkable issue in human behavioral disorders (American Psychiatric Association, 2000), a possible relationship between the behavior seen in the plus maze test and the development of a depressive-like state in SDI animals is of great relevance.

We also examined explorative behavior in the open field tests performed 9 and 23 days after defeat. During the second open field exposure, both groups showed a reduction in the number of entries and time spent in the centre zone and seemed to be more prone to occupy the corners of the arena (increased time spent and reduced latency to reach them) compared to the first test, with no differences between the two groups. The total distance traveled in the two open field tests was similar between the two groups, suggesting unchanged locomotor activity. This is in disagreement with the home cage locomotor behavior of the two groups, with SDI rats showing larger reduction of locomotor activity than CTRs during the first and third week after the defeat episode. This discrepancy can be attributed to the fact that, while locomotor activity in the home cage reflects basal motor function, locomotor activity in the open field test rather determines exploratory behavior in novel environment under acute challenge. Taken together these data indicate that there were no significant differences between groups in the expression of behavioral coping strategy during the two tests.

Although social defeat and isolation did not affect behavioral response to the open field test, they did produce some cardiovascular changes. Comparison of cardiac autonomic responses between the first and second open field test revealed a habituation-like effect for R-R interval values in CTR rats. In other words, stress-induced heart rate acceleration was gradually reduced from the first to the second acute challenge. Interestingly, this gradual reduction was not found in SDI rats. Similarly, a habituation-like effect for vagal

activity (i.e. reduced vagal withdrawal, r-MSSD index) to repeated open field testing was observed in CTR but not in SDI rats. Results from our control group and several other studies (Costoli et al., 2004; De Boer et al., 1990; McCarty and Pacak, 2000) indicate the occurrence of a gradual decline in some physiological responses of the organism when a homotypic stressor is repeatedly applied (habituation). Conversely, in defeated and isolated rats we found a lack of habituation of cardiac autonomic responsiveness, both in terms of tachycardia and vagal withdrawal, upon re-exposure to an open field test. This suggests that defeated/isolated animals did not adapt in terms of cardiac stress responsivity, although the stressor was unchanged over time. Persisting, high cardiovascular reactivity to environmental stressors has been described in patients suffering from depression and may represent a marker of increased proneness to cardiovascular disease (Carney et al., 1995).

Finally, morphometric analysis at sacrifice revealed that social defeat and isolation did not induce severe myocardial damage, with collagen accumulation that slightly affected only the left ventricle. Similarly, social defeat and isolation did not significantly alter ventricular anatomy, although a moderate hypertrophy was observed in the right ventricle. Global cardiac hypertrophy has been described in human and animal studies after chronic systemic exposure to adrenocortical hormones (Lumbers et al., 2005; Molketin and Dorn, 2001). The apparent lateral effect on the heart observed here may instead be related to a minimal loss of myocardial mass replaced by fibrosis present only in the LV, which has to support a higher workload. Overall, it appears that this type of social challenge does not affect dramatically the anatomical and structural integrity of the rat myocardium.

In conclusion, this model of social challenge induced a series of biological changes that are commonly taken as markers of depression in rats, and therefore represents a preclinical rat model with interesting translational implications for the human syndrome.

The design of this study does not allow differentiating between the effects of social defeat, isolation, and exposure to acute stressors on outcomes in subsequent test situations. This distinction is important in order to establish the duration of isolation required for depression-associated outcomes to become evident in the absence of intervening/confounding stressors, and represents the major limitation of the model. The cardiovascular alterations associated to clear signs of a depression-like state consisted in (i) transitory heart rate circadian rhythm alterations, (ii) lack of habituation of cardiac autonomic responsivity to an acute stressor, (iii) moderate hypertrophy affecting the right ventricle of the heart. Whether these relatively mild changes are physiologically meaningful remains to be determined. Further studies are required to confirm the validity of this rat model of depression as a preclinical tool for the study of the mechanisms of cardiac comorbidity in depression. A valid strategic approach could be the use of more sensitive indices (e.g. myocardial electrical properties) for the analysis of cardiac function in defeated and isolated rats. Alternatively, more substantial alterations of cardiac function and structure could be brought about by the implementation of repeated defeat episodes and/or longer isolation periods.

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CHAPTER 3

STRUCTURAL AND ELECTRICAL MYOCARDIAL REMODELLING IN RODENT MODEL OF DEPRESSION

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Chapter 3

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Abstract

While there is well documented association between chronic stress and depression with cardiac morbidity and mortality, there is no satisfactory explanation of the mechanistic link between affective and cardiac disorders. Addressing this question, in the present study we determined cardiac electrophysiological properties in an animal model of depression. Adult male wild-type rats were subjected to chronic social stress, consisting of 12 sessions of social defeat by an aggressive conspecific over a period of 4 weeks; control rats were exposed to empty unfamiliar cages. During chronic stress period, defeated animals had substantial reduction in the circadian amplitude of heart rate (-28%) and body temperature (-23%) rhythms, had smaller body weight gain (-41%) and showed a reduced preference for sucrose solution (-11%) compared to controls. After termination of chronic stress, defeated rats showed significantly longer periods of immobility in the forced swimming test (+55%) and developed functional and structural abnormalities of the hypothalamic-pituitary-adrenal axis. Ten days after termination of the stress protocol, animals were anesthetized and high-definition epicardial potential mapping was performed. Stressed animals showed a significant decrease in transversal conduction velocity (-15%), shortening of the effective refractory period (-10%), increase in myocardial excitability (105%, stimulus duration 0.01ms) and, at sacrifice, moderate fibrosis affecting the left ventricle. These data demonstrate that a depression-like state induced via chronic social stress is associated with 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.

1. Introduction

Chronic psychological stress and depression have long been recognized as independent risk factors for cardiovascular mortality, both in healthy individuals and in patients with heart disease (Barth et al., 2004; Lett et al., 2004). Despite the extensive evidence linking chronic stress and depression with impaired cardiovascular function, the underlying pathophysiological mechanisms are difficult to identify. They may involve arrhythmogenic effects provoked by changes in the autonomic cardiac outflow (Frasure-Smith et al., 1995). Direct evidence for exaggerated cardiac sympathetic hyperactivity has been found in a subset of patients with major depression, who had extraordinarily high levels of cardiac noradrenaline spillover (Barton et al., 2007). Along with increased cardiac sympathetic activity, parasympathetic (vagal) tone is reduced in depression, resulting in an alteration of the cardiac vago-sympathetic balance that is revealed by reduced heart rate variability (HRV) (Koschke et al., 2009; Veith et al., 1994). While it is recognized that such autonomic changes increase the risk of ventricular arrhythmias (Carney et al., 2002; Verrier and Lown, 1982), the major cause of sudden cardiac death (Milner et al., 1985), there is no current understanding of the mechanisms by which chronic stress and depression alter myocardial properties in a pro-arrhythmic manner. Two main reasons can explain this knowledge gap. Firstly, preclinical mechanistic studies relevant to affective disorders are usually focused on the enduring behavioral but not cardiac changes following chronic stress. Secondly, cardiac vulnerability has been extensively examined only in the context of the acute stress response (Corbalan et al., 1974; Liang et al., 1979), which offers poor help when it comes to explore lasting consequences of chronic stress and depression. The progress in the field is hampered by the lack of adequate animal models. Until now, there is only one study that has assessed the heart susceptibility to ventricular arrhythmia in a

rodent model of experimental depression (Grippo et al., 2004). In this study, rats exposed to a chronic mild stress paradigm developed depression-like symptoms and showed increased susceptibility to experimentally induced ventricular arrhythmia, suggestive of an altered electrical stability of the myocardium. While this study did not look into enduring cardiac changes, it demonstrates that animal models of chronic stress have potential for providing valuable insights into the relationship between depression and cardiovascular disease.

In the present study, we have used a paradigm of chronic social stress (CSS), a well documented animal model of depression (Becker et al., 2008), for the study of the mechanistic basis of the pro-arrhythmic effects of chronic stress. The CSS model involves exposing rodents to repeated episodes of social defeat. 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 (Becker et al., 2008). In addition to the relevance of social defeat-based models for investigating depression-relevant symptoms, social defeat is the only experimental paradigm where ventricular arrhythmias have been reported in animals with normal hearts (Sgoifo et al., 1999). We thus applied this ethologically-relevant paradigm for the study of cardiac electrical activity in a rodent model of depression.

Our hypothesis was that rats exposed to CSS would display pro-depressive symptoms at the behavioral (anhedonia, learned helplessness), neuroendocrine (hypothalamic-pituitary-adrenocortical (HPA) axis dysfunction) and physiological level (biological rhythm disturbances and body weight alterations) combined with enduring pro-arrhythmic changes in myocardial electrical properties. To test this, we assessed cardiac electrophysiological changes relevant to arrhythmogenesis in chronically stressed animals by means of epicardial mapping. This technique allows in vivo recording of unipolar electrograms from

the anterior ventricular surface of exposed rat hearts and assessment of cardiac electrophysiological parameters and spread of ventricular activation during normal sinus rhythm and ventricular pacing, using high-density epicardial electrode arrays (Rossi et al., 2008). Importantly, epicardial mapping studies were performed ten days after termination of the CSS period, in order to assess potential enduring effects of chronic stress on cardiac function.

2. Methods

2.1 Ethical approval and animal housing

The experiments and procedures used were submitted to and 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 24 November 1996 (86/609/EEC). Wild Type Groningen male rats (*Rattus norvegicus*) descending from an original colony derived from the University of Groningen (The Nederlands) were bred in our department under conventionally clean conditions. This strain has a complex social hierarchy and is characterized by remarkable autonomic and neuroendocrine stress reactivity (Sgoifo et al., 1996a; Sgoifo et al., 1997). Animals were housed in unisex groups of 4 individuals from weaning until the onset of experiments (10 weeks of age), when they were housed individually in Plexiglas cages measuring 39×23×15 cm; at this time their body weight was 300-350 g. Additional older rats, weighing 550-600 g, were used as residents in the resident-intruder paradigm (see section “Social defeat” for details). They were housed with a sterilized female partner for 2 months in a larger plastic cage

(60x35x40 cm). Then, they were trained to be aggressive by repeated introduction (five sessions) of an intruder (belonging to a separate group of rats specifically used for this task) into their home cages; the 12 most aggressive rats were eventually chosen as resident rats in confrontation encounters and they were used for all the successive series of experiments. All animals were 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 food and water *ad libitum.*

2.2 Radiotelemetry system

Radiotelemetry was used for recording of electrocardiograms, core body temperature (T, °C) and locomotor activity (LOC, expressed as counts/minute, cpm). The telemetry system consisted of implantable transmitters (TA11CTA-F40, Data Sciences Int., St. Paul, MN, USA), two types of receivers (one for small cages: RPC-1, Data Sciences Int., 32 x 22 x 3 cm; the other manufactured by the Electronic Department in the Biological Centre of the University of Groningen, The Netherlands, for large cage recordings, 74 x 47 x 3) and an acquisition system (ART-Silver 1.10, Data Sciences Int., St. Paul, MN, USA). One week before the start of the experiments, animals were anesthetized with tiletamine hydrochloride + zolazepam hydrochloride (Zoletil 200 mg/kg, s.c.) and the transmitters were chronically implanted according to a surgical procedure described by Sgoifo and colleagues (Sgoifo et al., 1996b). 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. After surgery, rats were injected for 2 days with gentamicide sulfate (Aagent, Fatro, 0.2 ml/kg, s.c.).

2.3 General experimental outline

The sequence of interventions and measurements employed in the current study is shown in figure 1; specific experimental procedures and data analysis are described in the following sections. Animals were randomly assigned to two experimental groups: defeated (DEF, n=12) and control (CTR, n=11) rats. The DEF rats were exposed to 12 episodes of social defeat over a period of 25 days (chronic social stress, CSS), 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 (Becker et al., 2008). In correspondence to each session of social defeat, the CTR rats were exposed to an unfamiliar empty cage. Circadian rhythms of heart rate (HR, bpm), T and LOC were recorded before and during CSS. During the week after CSS, rats were exposed to several challenges: i) restraint stress test (RST), ii) forced swimming test (FST), and iii) dexamethasone suppression test (DST) (figure 1). Body weight and sucrose solution intake (sucrose preference test, an index of anhedonia) of experimental rats were measured every two days from the beginning to the end of the experiment. Ten days after the end of CSS, animals were anesthetized, and high-definition epicardial mapping was performed. Finally, at sacrifice the adrenal glands and the heart were removed for morphometrical analysis.

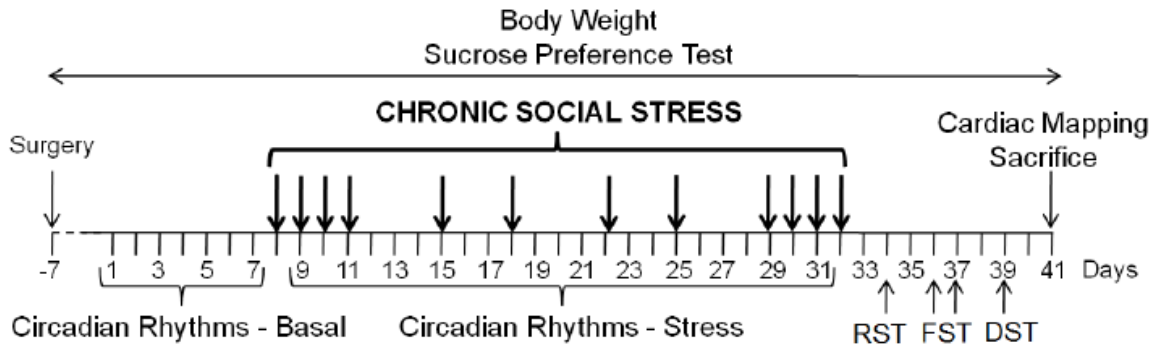

Figure 1. Timeline of procedures used in the current study: black arrows indicate social defeat sessions. DST = dexamethasone suppression test, FST = forced swimming test, RST = restraint stress test.

2.4. Social defeat

The social defeat was based on a classical “resident–intruder” paradigm (Miczeck, 1979). 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 (figure 2A). During this phase of ‘sensory contact’ (30 min), the DEF rat was protected from direct physical contact but it was in olfactory, auditory and visual contact with the resident. Then, the wire mesh partition was removed allowing physical interaction for 15 min (figure 2B). DEF rats were usually attacked by the resident within the 1st min of interaction (overall number of attacks: 5.6±1.1), and social defeat was assessed based on the exhibition of specific submissive postures (Blanchard et al., 2001). To avoid large individual differences in the intensity of received aggression, rats were exposed every time to a different opponent in a rotational design.

A) B)

Figure 2. Example of an episode of social defeat. A) Sensory contact phase; B) Physical interaction.

In correspondence to each session of defeat, the CTR animals 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, the DEF and the 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, with the animal in its home cage), during (30 min sensory contact + 15 min interaction) and after (30 min, with the animal back to its home cage) the tests.

2.5 Restraint stress test (RST)

Each animal was introduced into a restrainer (wire-mesh tube; inner diameter: 6 cm, length: 20cm) for 15 min. After the test, animals were returned to their home cages. ECG recordings were performed before (30 min, with the animal in its home cage), during (15

min) and after (30 min, with the animal back to its home cage) the test. ECG signals (sampling frequency 1 kHz) were exported from the acquisition system into Chart5 software (ADInstruments, Sydney, Australia) for HRV analysis. In the time-domain, the average R-R interval duration (average inter-beat-interval, 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) were calculated. Frequency-domain (fast Fourier transform) indexes were also collected. We measured total power of low frequency (LF; 0.2-0.75 Hz) and high frequency (HF; 0.75-2.5 Hz) bands of the spectrum. Calculations of time- and frequency-domain indexes were performed after removal of arrhythmic events and recording artifacts. The occurrence of ventricular and supraventricular premature beats was determined off-line, and quantified as number of events. The identification of these rhythm disturbances was based on the classical definition of arrhythmias in man and on the Lambeth Conventions for the study of experimental arrhythmias (Walker et al., 1982).

2.6 Forced swimming test (FST)

A modified version of the FST originally described by Porsolt and colleagues (Porsolt et al., 1977) was used. The FST consisted of a 15-min training session followed 24h later by a 5-min test session. During both sessions, rats were forced to swim individually in a Plexiglas cylinder (height: 40cm, diameter: 30cm) filled with water (temperature: 24±1°C; depth: 30cm). At the end of each swimming session, animals were removed from the cylinder, gently dried with paper towels and returned to their home cage. Water was changed after each single test. During the test session, rats' behavior was videotaped, and the overall time spent in immobility during the 5-min test session 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.

2.7 Dexamethasone suppression test (DST)

Initially, blood samples (0.5 ml) were collected from the tail vein of each rat in order to assess basal levels of ACTH and corticosterone; immediately after, rats were injected subcutaneously with dexamethasone (synthetic glucocorticoid, 30ug/kg), and four hours later blood samples (0.5 ml) were again collected from the tail to assess HPA axis reactivity via plasma ACTH and corticosterone level determinations. Blood samples were collected into chilled tubes containing 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 RIA kit (RIA Immuchem^{TM} Double antibody^{125} I RIA kit, MP Biomedicals, Orangeburg, NY, USA).

2.8 Sucrose preference test

Sucrose solution intake was monitored to operationally define anhedonia. Animals of both groups were allowed to drink from two preweighed bottles, one filled with 1% sucrose solution and the other one filled with 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.

2.9 Circadian rhythms

HR, T and LOC were sampled continuously for 60s every 60 min with the animal in its own home cage in baseline conditions (days 1-7) and during CSS (days 9-31) (figure 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 the rhythms of HR, T, and LOC was calculated as the difference between average active and inactive phase values, respectively (Meerlo et al., 1999).

2.10 Epicardial mapping

Rats were anesthetized with intraperitoneal injection of medetomidine (0.4 mg/kg) and ketamine (50 mg/kg). A tracheal tube was inserted via tracheotomy and animals were artificially ventilated with room air using a rodent ventilator (rodent ventilator 7025, Ugo Basile, Comerio, Italy). Subsequently, the heart was exposed through a longitudinal sternotomy and suspended in a pericardial cradle. Body temperature was maintained constant at 37°C with infrared lamp. Supplemental doses of anesthetic were given when necessary. The epicardial electrode array and the mapping system used in this study have been described in detail previously (Macchi et al., 1998; Rossi et al., 2008). Briefly, a 64-electrode (8x8, 1 mm resolution) array was firmly positioned to the epicardium, with all electrodes establishing a stable contact. The electrode array covered part of the anterior surface of the right and left ventricles (figure 3A). In each experiment, 64 unipolar epicardial electrograms (EGs) were recorded between each array electrode and a common reference electrode placed on the left hind leg, during normal sinus rhythm (NSR) and ventricular pacing (figure 3B). Hearts were paced with cathodal current pulses from

one of the electrodes and EGs were measured from all other electrodes at a frequency slightly higher than spontaneous sinus rhythm. For each pulse duration, threshold current was measured by decreasing pulse strength till capture was lost. The indifferent electrode for cathodal current pulses was placed subcutaneously in the chest wall.

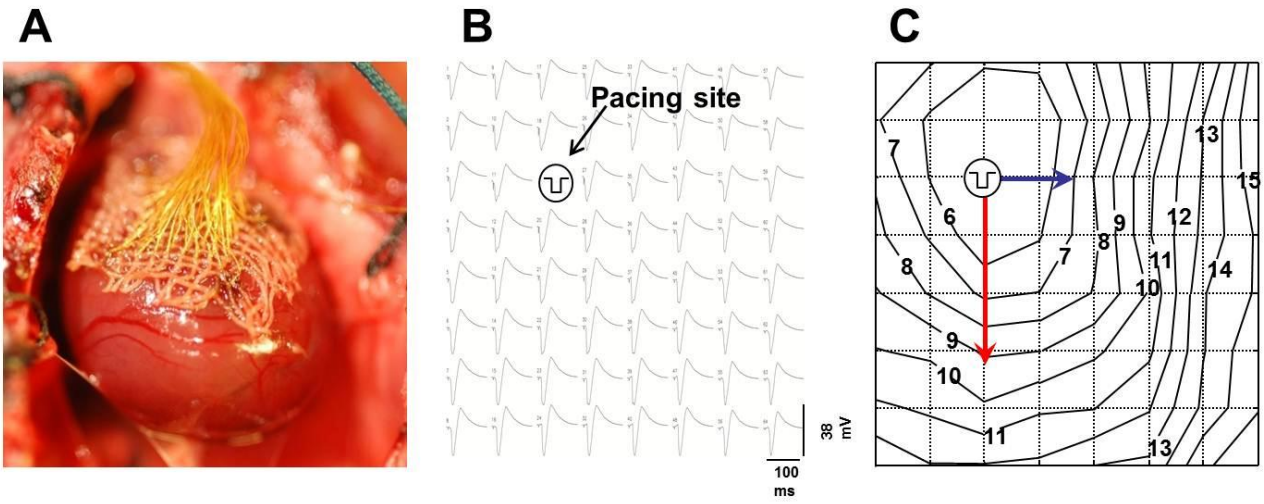

Figure 3. Epicardial mapping data of a representative rat. (A) 8×8 Electrode array on the anterior ventricular surface. (B) Unipolar electrograms collected during ventricular pacing at the electrode indicated by the pulse symbol. (C) Example of paced activation isochrone map used for computing conduction 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.

The following measurements were carried out:

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

Conduction velocity (CV). Activation times were estimated during NSR using the instant of the minimum time derivative of unipolar EGs during QRS, and were referenced to QRS

onset during NSR or stimulus onset during pacing. An activation sequence (isochrone map) was computed from the activation times of NSR or paced beats using custom written software (figure 3). CV longitudinally and transversally to fiber orientation was computed from the isochrones maps (figure 3C). Specifically, longitudinal CV was evaluated from electrodes distant from the pacing site on the long axis of the elliptical wavefront. Transversal CV was evaluated from electrodes on a line perpendicular to the long axis of the elliptical wavefront. It is known that the long axis of the elliptical wavefront is parallel to the local fiber direction at the pacing site (Taccardi et al., 2008).

Excitability. A strength-duration curve was obtained as a measure of cardiac excitability at two electrode positions. The strength-duration curve represents the threshold current which was measured for the following pulse durations, orderly: 8 – 5 – 1 – 0.8 – 0.4 – 0.2 – 0.1 – 0.06 – 0.03 – 0.01 ms. The strength-duration curve is represented by the equation I=Rh(1+Chr/T) (25), where I is the threshold current strength, T is the pulse duration, Rh is the rheobase (i.e. the lowest current strenght with infinite pulse duration which succeeds in eliciting a propagated response in excitable tissues) and Chr the chronaxie (i.e. the pulse duration having a threshold twice that of Rh).

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.

At the end of each experiment, the heart was arrested in diastole by an injection of cadmium chloride solution (100mM, i.v.). The epicardial position of the electrode array was labeled by marks burned onto the tissue by constant current through four corner

electrodes of the array. Thereafter, the heart was rapidly removed from the chest, weighed, and fixed in 10% buffered formalin solution for 24–48 h.

2.11 Measurements at sacrifice

Cardiac anatomy and morphometry. The two atria, the right (RV) and left (LV) ventricles inclusive of the septum were separately weighed and subsequently fixed in 10% buffered formalin solution for morphometric studies. The following parameters were determined: heart weight (HW), LV weight (LVW), RV 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). The LV chamber volume was calculated according to the Dodge equation (Dodge and Baxley, 1969). 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 myocardium by means of optical microscopy (magnification 250X). According to a procedure previously described (Costoli et al., 2004), quantitative evaluation of fibrotic tissue was performed in 10 randomly selected fields from the subendocardium, midmyocardium and subepicardium, with the aid of a grid defining a tissue area of 0.56 mm^2 and containing 42 sampling points, each covering an area of 0.013 mm^2. To define the volume fraction of fibrosis in the three layers of the ventricular wall, the number of points overlaying myocardial scarring were counted and expressed as a percentage of the total number of points explored.

Adrenal weight. Adrenal glands were also removed, carefully trimmed, and weighed.

2.12 Statistical analysis

Statistical analyses were performed using SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA) and statistical significance for all tests was set at $p \leq 0.05$. Values of all parameters were expressed as mean ± SEM. Two-way ANOVA for repeated measures with “time” as between-subject factor (2 levels: first and last episode) and “recording periods” (4 levels: baseline, sensory contact, interaction and recovery) as within-subject factor was separately applied to both rat groups for data obtained from the first and last episode of social defeat/unfamiliar cage tests. Two-way ANOVA for repeated measures with “group” as between-subject factor (2 levels: DEF and CTR) was applied on data obtained from: (i) social defeat/unfamiliar cage tests, with within-subject factor “recording periods” (4 levels: baseline, sensory contact, interaction and recovery); (ii) RST, with within-subject factor “recording periods” (3 levels: baseline, restraint and recovery); (iii) circadian rhythms during CSS, with within-subject factor ‘time’ (5 levels: days 1-7, 9-14, 15-20, 21-26, 27-31); (iv) body weight gain and sucrose preference test, with within-subject factor “time” (4 levels: days 9-16, 17-24, 25-32, 33-40). Follow-up analyses were conducted using Student “*t”* tests (hypothesis-driven, statistically justified comparisons only) after checking for variance homogeneity by means of Levene test. Student’s “*t*”-tests, again after Levene test, were applied for comparisons between DEFs and CTRs on the occurrence of ventricular and supraventricular premature beats (VPBs and SPBs, respectively) during RST and for data obtained from FST, DST, cardiac mapping and measures at sacrifice.

3. Results

3.1 Body weight and sucrose solution intake

Changes in body weight and sucrose solution intake during and after CSS are shown in figure 4. On day 7 (last day of pre-stress period) body weight was 337±6 g for the DEFs and 355±8 g for the CTRs. During the first week (days 9-16) of the CSS (1st defeat episode on day 8), the DEF rats showed a significantly smaller increase of body weight than the CTRs (DEF=3.1±2.0 g *vs.* CTR=15.0±1.6 g, $p<0.01$). This difference persisted until the end of the experiment ($F=17.26$, $p<0.01$) (figure 4A).

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). After the onset of CSS, both groups showed a reduction in sucrose solution intake compared to respective basal levels, with the magnitude of this reduction being larger in the DEFs compared to the CTRs during the first (DEF=-24.7±7.2 % *vs.* CTR=-6.4±3.2 %, $p<0.05$) and third (DEF=-21.4±4.8% *vs.* CTR=-5.4±4.3 %, $p<0.05$) weeks of chronic stress (figure 4B).

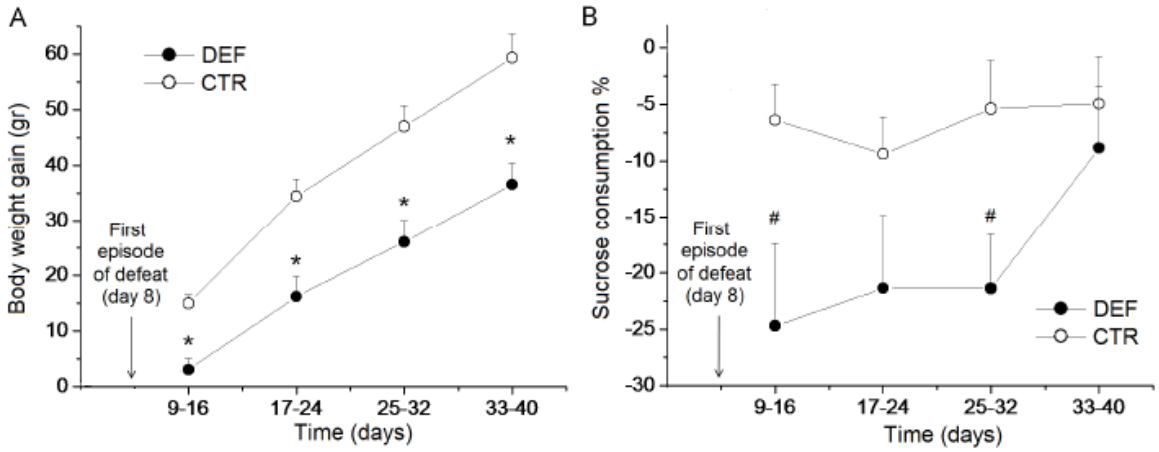

Figure 4. Time course of body weight changes (panel A) and sucrose solution intake (panel B) in defeated (DEF, n=12) and control (CTR, n=11) rats during and after chronic social stress. Body weight and sucrose solution intake were measured every two days, and each point is the mean±SE of data obtained 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). * and # : significantly different from corresponding CTR value ($p<0.01$ and $p<0.05$, respectively).

3.2 Circadian rhythms

No differences between the DEF and the CTR rats were found in baseline circadian values of HR, T (figure 5) and LOC (active phase: DEF=3.8±0.5 cpm *vs.* CTR=3.8±0.3 cpm; inactive phase: DEF=2.0±0.2 cpm *vs*. CTR=2.2±0.2 cpm; amplitude: DEF=1.8±0.4 cpm *vs.* CTR=1.6±0.2 cpm; $p>0.05$ for all).

During the first 17 days of CSS, the DEF rats showed significantly higher values of HR than the CTRs during the inactive phases of the circadian cycle (figure 5A), and a consequent reduction in the circadian amplitude of HR compared to the CTRs (figure 5C).

Similarly, the DEFs had higher values of T then the CTRs during the inactive phase of the CSS period (figure 5B), and a consequent reduction in the circadian amplitude of T compared to the CTRs (figure 5D). Mean values and statistical analysis for these parameters are presented in figure 5.

During CSS, no differences were observed in the circadian rhythm of LOC between the two groups (data not shown).

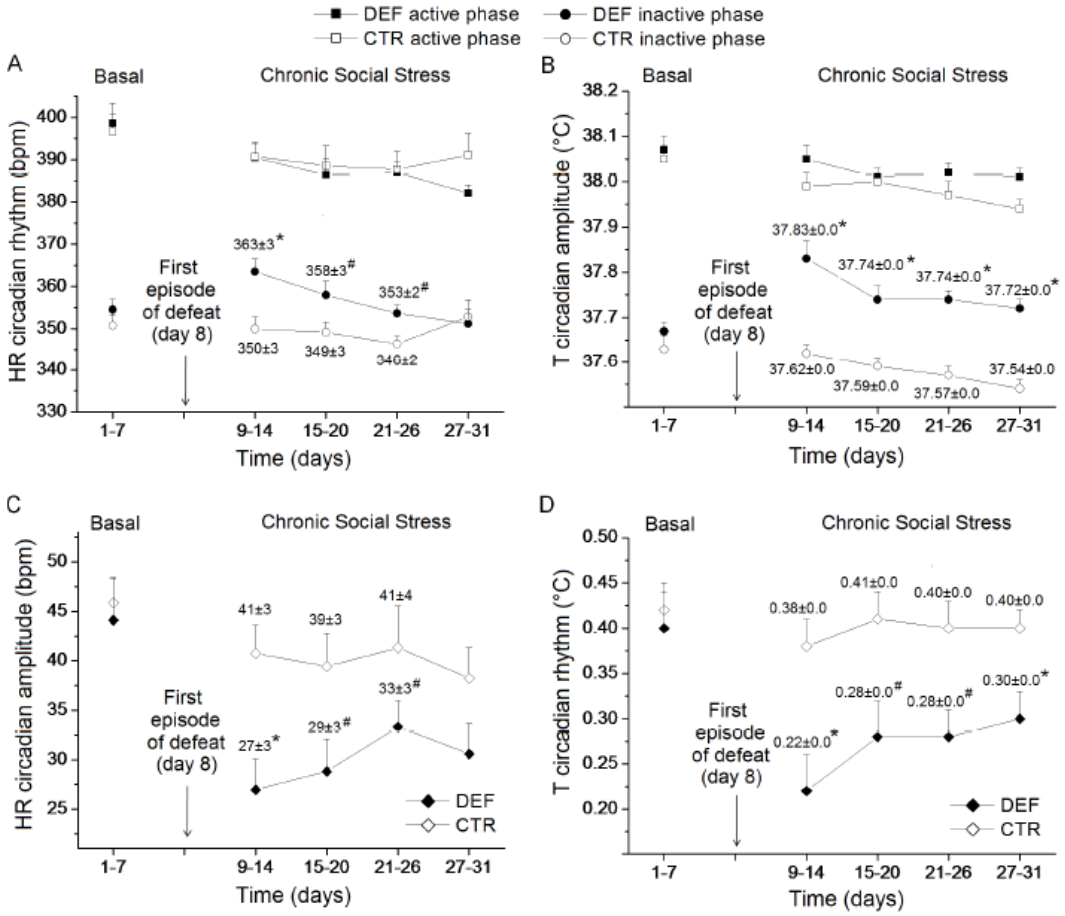

Figure 5. Changes in circadian rhythms of HR and T in defeated (DEF, n=12) and control (CTR, n=11) rats during the CSS. (A, B) HR and T during active (squared symbols) and inactive (round symbols) phases of the circadian cycle. (C, D) Circadian amplitude of HR and T. Each point is the mean±SE of data obtained during the indicated periods. * and #: significantly different from corresponding CTR value ($p<0.01$ and $p<0.05$, respectively). Results of ANOVA applied on HR and T values revealed a significant effect of time for active phase (HR: $F=12.5$, $p<0.01$; T: 17.3, $p<0.01$) inactive phase (T: $F=7.4$, $p<0.05$) and amplitude (HR: $F=9.7$, $p<0.01$) values, a significant effect of group for amplitude values (HR: $F=6.2$, $p<0.05$; T: $F=6.7$, $p<0.05$) and a time x group interaction for inactive phase values (T: $F=9.4$, $p<0.01$).

3.3 Heart rate and body temperature 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). As expected, social defeat provoked profound tachycardic and hyperthermic responses: indeed, the DEF rats showed higher values of HR and T during the sensory contact and the agonistic phases and higher values of T 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). The CTR rats had higher T values in baseline conditions of day 32 than day 8, and did not show any difference in HR and T values between the first and the last unfamiliar cage exposure (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 episode of social defeat.

			Baseline (30 min)	Sensory contact (30 min)	Interaction (15 min)	Recovery (30 min)
DEF (n=12)	**First social defeat** (day 8)	**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 (day 32)	**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** (day 8)	**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 (day 32)	**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±SE of the indicated recording periods. For periods corresponding to the sensory contact and the interaction of the DEFs with an aggressive rat, the CTRs were exposed to a new empty cage. * and †: significant difference between the DEFs and the CTRs ($p<0.01$ and $p<0.05$, respectively); ‡: significant difference between day 8 and day 32 in the CTRs ($p<0.05$). Results of ANOVA: (i) for the DEFs — significant effects of recording period (HR: $F=97.3$, $p<0.01$; T: $F=409.0$, $p<0.01$); (ii) for the CTRs — significant effects of recording period (HR: $F=33.5$, $p<0.01$; T: $F=63.8$, $p<0.01$).

3.4 Restraint stress test (RST) and ECG recordings

No differences were observed in any basal parameter beteween the DEFs and the CTRs (Table 2). The DEFs and the CTRs showed lower values of RR compared to the basal levels during the restraint and the recovery phases, with no differences between groups. R-MSSD and pNN10 were lower during the restraint compared to the baseline in the DEF and CTR rats. In both groups, during the recovery phase LF power was higher and HF power was lower compared to the basal levels, resulting in a significant increase in LF/HF

ratio compared to the pre-stress value. However, no differences were observed between groups in any of the HRV indexes (Table 2). During the restraint phase, 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. Temperature values during the restraint and the recovery phases were higher in both groups compared to the basal level, with the absolute values being higher in the DEFs than in the CTRs in both phases (Table 2). Mean values and results of ANOVA and post-hoc tests for these variables are presented in Table 2.

Table 2. R-R interval (RR), time domain HRV parameters (r-MSSD; pNN10), frequency domain HRV parameters (LF; HF; LF/HF) and body temperature (T) values in defeated (DEF; n=12) and control (CTR; n=11) rats during the RST.

		Baseline (30 min)	Restraint (15 min)	Recovery (30 min)
RR (ms)	**DEF**	**174.7±2.1**	**128.7±2.3**†	**153.8±2.2**†
	CTR	**167.3±4.6**	**130.2±1.9**†	**149.8±2.3**†
r-MSSD (ms)	**DEF**	**2.6±0.2**	**2.1±0.1**‡	**2.2±0.1**
	CTR	**2.5±0.2**	**2.1±0.1**	**2.2±0.1**
pNN10 (%)	**DEF**	**0.6±0.2**	**0.2±0.1**	**0.3±0.1**
	CTR	**0.7±0.2**	**0.1±0.0**†	**0.3±0.1**
LF (n.u.)	**DEF**	**40.8±2.6**	**44.7±3.1**	**52.3±2.4**†
	CTR	**43.7±1.9**	**44.3±2.8**	**54.3±1.7**†
HF (n.u.)	**DEF**	**59.2±2.6**	**55.3±3.1**	**47.7±2.4**†
	CTR	**56.3±1.9**	**55.7±2.8**	**45.8±1.5**†
LF/HF	**DEF**	**0.8±0.1**	**0.9±0.1**	**1.2±0.1**†
	CTR	**0.8±0.0**	**0.9±0.1**	**1.2±0.1**†
T (°C)	**DEF**	**37.6±0.0**	**38.5±0.1***,†	**38.6 ± 0.1***,†
	CTR	**37.6±0.0**	**38.1 ± 0.1**†	**38.4 ± 0.1**†

Data are expressed as mean±SE of the indicated recording periods. * significantly different from the corresponding CTR group value ($p<0.05$); † and ‡ significantly different from the respective basal values ($p<0.01$ and $p<0.05$, respectively). Results of ANOVA: (i) for RR interval — significant effect of time ($F=97.3$, $p<0.01$); (ii) for T — significant effects of time ($F=28.9$, $p<0.01$), and group ($F=5.6$, $p<0.05$); for LF — significant effect of time ($F=7.5$, $p<0.01$); for HF — significant effect of time ($F=7.5$, $p<0.01$).

3.5 Forced swimming test (FST)

During the FST, performed five days after the last episode of defeat (day 37), the DEF rats spent a significantly longer time immobile then the CTRs (DEF=96±8 s *vs.* CTR=62±5 s, $p<0.01$).

3.6 Dexamethasone suppression test (DST)

Figure 6 shows the hypophyseal and adrenocortical responses to the DST that was performed seven days after the last episode of defeat (day 39). Basal plasma ACTH levels were significantly lower in the DEFs then the CTRs (DEF=124±17 ng/ml *vs.* CTR=231±25 ng/ml, $p<0.01$), whereas no differences were observed in basal plasma corticosterone levels between the two groups (DEF=290±28 ng/ml *vs.* CTR=250±20 ng/ml). The injection of dexamethasone provoked a significant reduction in plasma ACTH levels compared to basal levels in the CTRs but not in the DEFs (DEF=-34±22 ng/ml, $p>0.05$; CTR=-147±21 ng/ml, $p<0.01$), although the absolute post-injection values were similar in the two groups. Plasma 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 and CTR=-214±23 ng/ml).

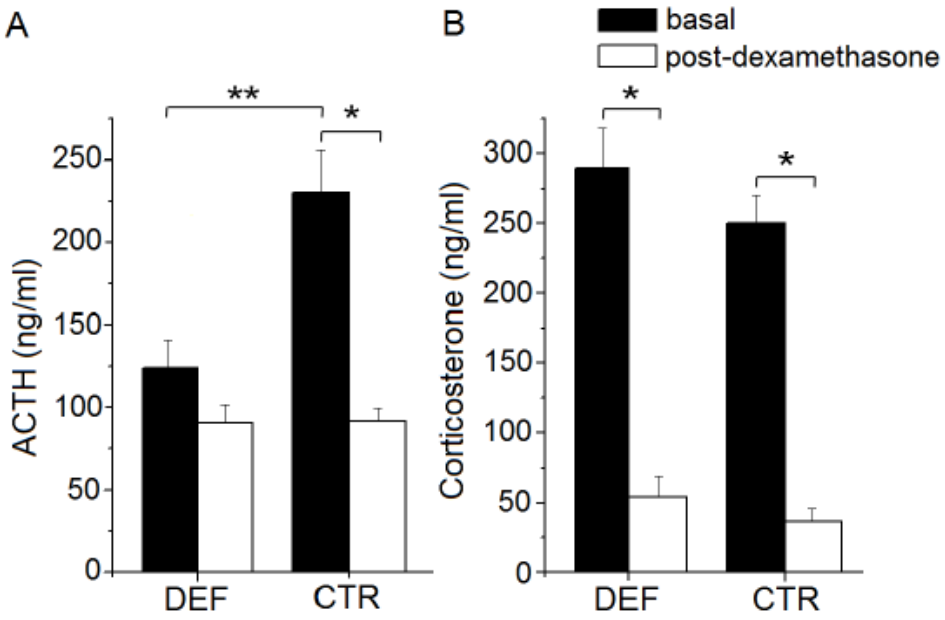

Figure 6. Plasma ACTH (panel A) and corticosterone (panel B) levels before and after (4h) the injection of dexamethasone in defeated (DEF, n=12) and control (CTR, n=11) rats. Each bar is the mean±SE. * Significantly different from corresponding basal value ($p<0.01$). **: significantly different from corresponding CTR value ($p<0.01$).

3.7 Adrenal weight

At the time of sacrifice, adrenal weight, corrected for body weight, was heavier in the DEFs compared to the CTRs (DEF=0.19±0.02 mg/g *vs.* CTR=0.13±0.01 mg/g, $p<0.01$).

3.8 Epicardial mapping recording

To measure myocardial excitability, a strength-duration curve was constructed (figure 7A) and Rh and Chr values (figure 7B) were determined from it. As shown by the curve, 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<0.05). Similar current intensities between the two groups were needed to trigger an action potential at stimulus durations of 8, 5, 1, 0.8, 0.4, 0.2, 0.1, 0.06 and 0.03 ms. No differences were observed between the DEF and CTR rats in Rh (DEF=11.4±1.0 μA *vs.* CTR=9.0±0.7 μA, P>0.05) and Chr (DEF=1.2±0.2ms *vs.* CTR=1.0±0.1ms, p>0.05) values.

Analysis of conduction velocities obtained from isochrones maps during epicardial pacing revealed that transversal ventricular CV was slower in the hearts of the DEF rats compared to the control counterparts (DEF=0.23±0.0 m/s *vs.* CTR=0.27±0.1 m/s, P<0.01) (figure 7C), whereas no differences were observed in longitudinal ventricular CV between the two groups (DEF=0.51±0.1 m/s *vs.* CTR=0.53±0.1 m/s, p>0.05) (figure 7C).

ERP was significantly shorter in the hearts of the DEF rats compared to the CTRs (DEF=86.8±2.1 ms *vs.* CTR=95.9±3.0 ms, p=0.01) (figure 7D).

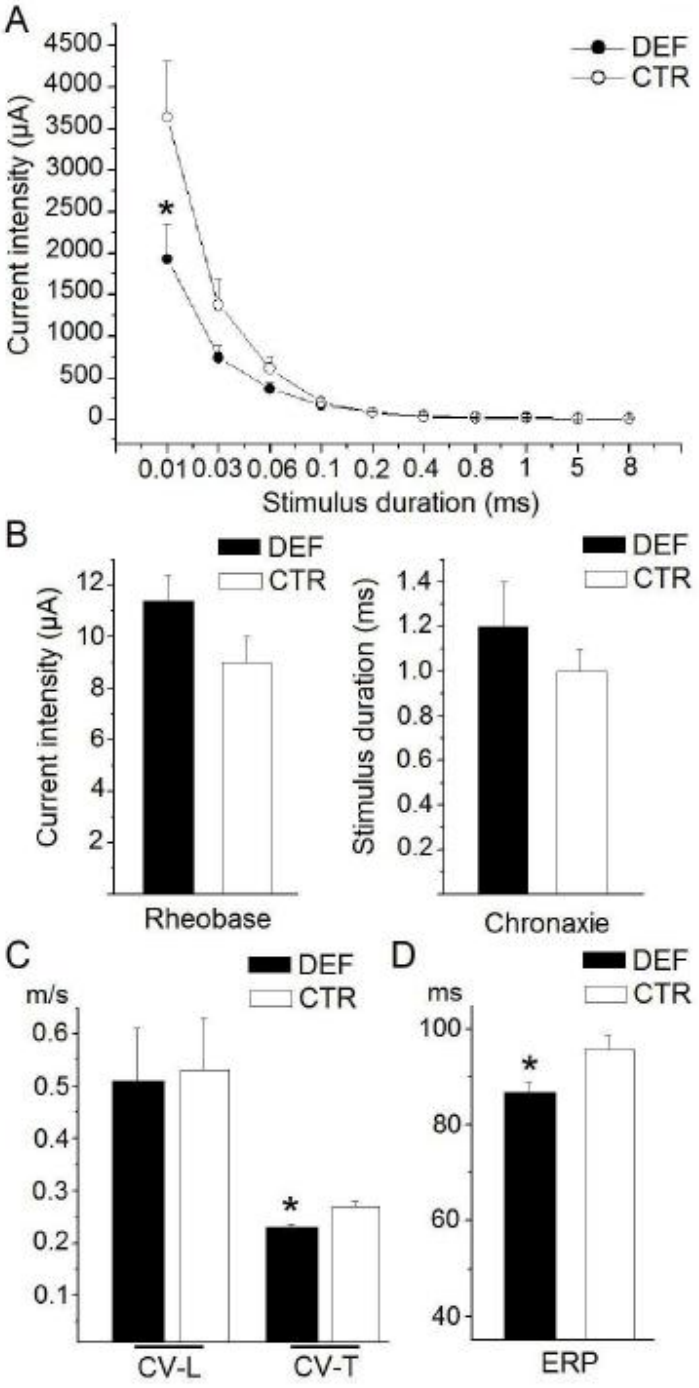

Figure 7. Epicardial mapping data in defeated (DEF, n=9) and control (CTR, n=8) rats. (A) Strength-duration curve: shown are mean strength-duration values±SE that were effective for inducing an action potential. (B) Rheobase and chronaxie. (C) Conduction velocity longitudinally (CV-L) and transversally (CV-T) epicardial fiber direction. (D) Effective refractory period (ERP). The bars illustrate the mean values±SE. *: significantly different from corresponding CTR value ($p<0.05$).

Cardiac intervals obtained from epicardial electrogram recordings are shown in Table 3. In the hearts of DEF rats we observed a significant increase of RR and PQ segment duration values, and a significant decrease in QRS wave and QTc segment duration values compared to the control hearts.

Table 3. Epicardial mapping parameters in the DEF and CTR rats

	DEF (n=9)	CTR (n=8)
RR	**637.8±12.1***	**572.9±7.8**
P	**30.6±0.3**	**30.0±0.3**
PQ	**24.9±0.3***	**22.3±0.2**
QRS	**18.2±0.2***	**18.9±0.1**
QT	**32.3±0.2**	**32.7±0.3**
QTc	**13.0±0.1***	**13.8±0.2**

Data are mean±SE, expressed as ms. *: significantly different from corresponding CTR value ($p<0.01$).

3.9 Cardiac anatomy and morphometry

Cardiac anatomy. As shown in Table 4, 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 CSS. LV chamber volume was unchanged in the DEF rats compared to the CTRs.

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 left ventricle was significantly larger in the DEF rats compared to

the CTRs. This difference was due to larger perivascular collagen deposition in the heart of the DEFs, whereas no differences were observed between groups in the amount of interstitial fibrosis (Table 4).

Table 4. Gross cardiac characteristics and left ventricular myocardial fibrosis in defeated (DEF) and control (CTR) rats.

	DEF (n=12)	CTR (n=11)
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 reported as mean±SE. *: significantly different ($p<0.05$) from CTR corresponding value.

4. Discussion

Characterization of pro-arrhythmic changes in cardiac function induced by chronic stress is an important step for understanding the mechanisms underlying depression-related cardiovascular disease. In this study we attempted to shed new light on this issue by determining cardiac electrophysiological properties in a previously validated rodent model of chronic stress and depression. Indeed, our results demonstrate that rats exposed to CSS show a set of core behavioral and physiological symptoms that characterize depressed individuals. Our major novel finding is that CSS provokes electrical (impaired conduction and reduced refractoriness) and structural (fibrosis) cardiac remodelling, consistent with the view that chronic stress and depression are associated with an increased risk for ventricular arrhythmia and sudden cardiac death (Khawaja et al., 2009).

4.1 CSS provoked depression-like syndrome

Social defeat by an aggressive conspecific provoked potent tachycardic and hyperthermic responses that did not habituate over the CSS period, confirming the high adversity of this stress paradigm (Koscke et al., 2009). The validity of intermittent social-defeat procedure as a rodent model of depression was confirmed by the presence of profound enduring changes in several physiological and behavioral parameters that resemble those observed in chronically stressed and depressed individuals. First, repeatedly defeated animals had reduced weight gain compared to control ones. A reduction in food intake has been reported for several days in rats exposed to repeated episodes of defeat (Becker et al., 2008), and is likely responsible for this long-term reduction in body growth, a commonly taken marker of depression in rats (Ruis et al., 1999). Second, the substantial decrease of

sucrose solution intake observed in the DEF animals during the CSS period is interpreted as a sign of anhedonia, a core symptom of depression (Willner, 1997). Third, the prolonged immobility of the DEF rats in the forced swimming test is regarded as a relevant behavioral index of the depression-like symptoms of decreased motivation and behavioral despair (Porsolt et al., 1977). Fourth, during CSS the DEF rats showed a dampening of the circadian rhythm amplitude of HR and body temperature that 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 (Soutre et al., 1989) and are interpreted as the physiological manifestation of emotional stress and anxiety (Friedman and Thayer, 1998).

To investigate whether the exposure to CSS also affects the acute response to a heterotypic stressor, animals were submitted to a restraint test two days after the last defeat episode. During the 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 the DEF and the CTR rats, as indicated by the significant reductions in r-MSSD and pNN10 values (indices of cardiac vagal outflow). However, overall HRV data during the restraint and the recovery periods indicate that CSS did not affect the dynamics of cardiac autonomic balance in response to a heterotypic stressor. On the other hand, the DEF rats showed larger hyperthermia than the CTRs during and after the restraint. Stress-induced hyperthermia is primarily due to sympathetically-induced activation of thermogenesis in the brown adipose tissue and to sympathetically-mediated vasoconstriction in the cutaneous vascular bed (Ootsuka et al., 2007), the latter preventing heat dissipation. While our method did not allow determining the relative contribution of these two factors, our data demonstrate that hyperthermia was more pronounced in the DEF than the CTR rats. Therefore, it appears that body temperature is a more sensitive marker than HR for

assessing the increased stress responsiveness that often accompanies many psychological disorders (Post, 1992). 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 episode of social defeat. In basal conditions, our DEF rats had lower ACTH levels than the CTRs. Despite the discrepancy in ACTH levels, the two groups had similar corticosterone levels. Mismatches between ACTH and corticosterone levels are frequently observed in rats after chronic stress (Gomez et al., 1996; Pecoraro et al., 2006). The general interpretation of this phenomenon is that exposure to chronic stress results in both adrenal hypertrophy and hyperplasia (Ulrich-Lai et al., 2006), as indirectly confirmed in our study by the heavier adrenal weight of the DEF rats. This leads to an enhanced maximal response to ACTH without affecting adrenal sensitivity, and, consequently, chronically stressed animals would release significantly more corticosterone in response to a given level of ACTH stimulation (Bornstein et al., 2008). The dexamethasone test is commonly used in clinical practice to characterize neuroendocrine aberrations in depressed patients, who typically show signs of non-suppression of ACTH and/or corticosterone upon dexamethasone (synthetic glucocorticoid) injection. In our study, administration of dexamethasone 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 can be explained by either of two mechanisms: by the fact that basal ACTH levels in the DEFs were already low and could not be further reduced, or by the development of a resistance to negative feedback mechanisms at the adrenocortical level. Whatever the causes, the overall changes observed in the DEF rats point to a dysregulation of the HPA axis. Noteworthy, a dissociation of ACTH and

glucocorticoid levels has been found in several psychiatric conditions, including depression (Bornstein et al., 2006, Carroll et al., 2007).

4.2 CSS caused cardiac remodelling

Given the above presented extensive evidence of a depressive-like state in rats exposed to CSS, the central question of our study was to assess enduring changes in their myocardial electrical properties. Towards this end, ten days after termination of the CSS period animals were submitted to epicardial mapping. 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 DEF rats ventricular activation was characterized by significant reduction of the QRS complex duration, and consequently of the QTc interval, 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 (Arnar et al., 1997; Wang et al., 1982). 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 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. We hypothesize that the expected CV acceleration could have been counteracted by a reduction in connexin (Cx) 43 expression (i.e. the most important gap junctional protein in

the ventricle). The gap junction conductance is indeed an important determinant of intercellular resistance, and alterations could have an effect on CV (Kleber, 1999). Cx43 expression and distribution was not evaluated in this study. However, it has been reported that even a 30-50% reduction in Cx43 is itself not sufficient to reduce CV and that very large changes of electrical coupling are required to impair conduction (van Rijen et al., 2006). It is presumably the combination with other factors, such us the moderate collagen deposition observed in the heart of the DEF rats that caused impaired intercellular electrical coupling especially in transverse direction, resulting in a reduction of transversal CV and increasing the likelihood of conduction blocks. Such transverse unidirectional slowdown of CV may favor micro-reentry via the transverse path (Kleber, 1999), and thus can be considered as a pro-arrhythmogenic effect of CSS.

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 (Inoue and Zipes, 1987), 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 (Wiener and Rosenblueth, 1982). 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 chronic stress 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 did not differ between 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 remodelling 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 (Fozzard, 2001; Fozzard and Schoenberg, 1972). We hypothesized 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 (Fozzard, 2001; Fozzard and Schoenberg, 1972).

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 of a set of physiological and behavioral changes in rats that resemble those observed in depressed and chronically stressed individuals. Whereas previous work had focused on cardiac vulnerability immediately after chronic stress (Grippo et al., 2004), this study examined for the first time changes in myocardial electrical properties that lasted well beyond (10 days) the end of CSS. In particular, reduced myocardial refractoriness and impaired conduction, considered to be major determinants of arrhythmogenesis, represent long-lasting potentially pro-arrhythmic effects of chronic stress.

4.3 Limitations of the study

It must be recognized that the occurrence of arrhythmias following CSS was very modest, thus not supporting the hypothesis that the electrophysiological remodelling 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 remodelling in the chronically 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 remodelling. These are the major limitations of the present study.

4.4 Relevance to human studies

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 remodelling in the rat myocardium. Nevertheless, our study provides a foundation for systematic elucidation of detrimental effects of chronic stress on the heart in the rodent model.

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CHAPTER 4

CARDIAC AUTONOMIC REGULATION IN HIGH AND LOW ANXIETY-RELATED BEHAVIOR WISTAR RATS

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To be submitted

Chapter 4

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Abstract

Many epidemiological and experimental evidences highlight a bidirectional association between anxiety disorders and cardiovascular dysfunction. One of the main pathophysiological mechanism linking anxiety and cardiovascular disorders is the impairment of autonomic regulation. The aim of this study was to investigate the autonomic control of heart rate and cardiac morphological/morphometrical characteristics in a rodent model of trait anxiety, i.e. rats selectively bred for high (HAB) and low (LAB) anxiety-related behavior. To reach this purpose, heart rate and both time and frequency domain indexes of heart rate variability (HRV) were measured at rest and under challenging conditions, namely non social (restraint test) and social stressful stimuli and autonomic pharmacological manipulation.

HAB and LAB showed different levels of cardiac autonomic regulation in baseline conditions, with HAB characterized by lower hear rate and HRV. The pharmacological blockade of autonomic neural regulation revealed a significantly lower intrinsic heart rate in HAB rats, suggesting a shift of cardiac sympathovagal balance towards a sympathetic predominance at rest. Cardiac stress reactivity, i.e. tachycardia and vagal withdrawal, was more pronounced in LAB rats, in particular when exposed to a psychosocial stressor, reflecting differences between the two rat lines not only in autonomic responsivity, but also in the behavioral strategy of coping with stressful situations. Pharmacological beta-adrenergic stimulation induced an enhanced arrhythmogenesis in HAB rats. Finally, no significant differences between the two groups were found in cardiac morphological characteristics and myocardial damage.

1. Introduction

Anxiety is defined as the response to an undetermined, potentially threatening situation and is characterized by a perceived inability to predict, control or obtain the preferred results (Barlow, 1988). From an evolutionary perspective, adequate levels of anxiety are useful in order to protect an individual from threats and allow to successfully cope with challenges. However, when anxiety reactions become persistent, uncontrollable and inappropriate, they can negatively influence the quality of everyday life and lead to a pathological feature. Today, anxiety disorders are the most common psychopathologies in the Western countries and they represent one of the major health problems (World Health Organization, 2004). According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, American Psychiatric Association, 2000), a variety of anxiety disorders have been classified as formal clinical diagnoses, including panic disorder, phobic anxiety, generalized anxiety disorder, post-traumatic stress disorder.

Converging evidence from both epidemiological and experimental studies indicates that there is a bidirectional association between anxiety disorders and cardiovascular diseases (Moser, 2007; Rozanski et al., 1999). On the one hand, patients suffering from anxiety disorders are at higher risk of cardiovascular morbidity and mortality than the general population (Albert et al., 2005; Dunner, 1985; Eaker et al., 2005; Frasure-Smith and Lesperance, 2008; Kawachi et al., 1994; Mykletun et al., 2007; Szekely et al., 2007).On the other hand, the presence of anxiety is very common among patients with chronic cardiovascular disease (Crowe et al., 1996; Jannuzzi et al., 2000; Moser and Dracup, 1996; Sirois and Burg, 2003). In addition, epidemiological evidence has suggested that anxiety disorders are associated with elevated rates of cardiovascular disease risk factors,

including, diabetes, hypercholesterolemia, smoking, obesity, and sedentary life (Barger and Sydeman, 2005; Birkenaes et al., 2006).

Although, the bidirectional relationship between anxiety and cardiovascular dysfunction is well established, the knowledge about the underlying mechanistic links is far to be consistent. One of the most important hypothesized pathophysiological mechanisms is the impairment of autonomic cardiac regulation (Rozansky et al., 1999). Actually, the dysfunction of autonomic nervous system (ANS) characterizes both cardiovascular diseases (Flapan et al., 1993; Palatini and Julius, 2004; Singh et al., 1998) and anxiety disorders (Kiecolt-Glaser et al., 2002; Verrier and Mittleman, 2000). The alterations of cardiac autonomic control can be evaluated by the application of the analysis of heart rate variability (HRV), which provides in a non-invasive way information about the level of activity of the two branches of the ANS to the heart (Task Force, 1996). Reduced HRV, normally associated with a shift of cardiac autonomic balance towards a sympathetic prevalence, is a powerful risk stratifier for overall cardiac morbidity and mortality (Bigger et al., 1992; Kleiger et al., 1987; Kleiger et al., 1993). In addition, many clinical studies have reported that anxiety-related pathologies are associated with decreased HRV (Cohen and Benjamin, 2006; Friedman, 2007; Kawachi et al., 1995; Licht et al., 2009).

Animal models which behaviorally and physiologically phenotype clinical anxiety represent a useful tool for better investigating the causal and common mechanisms underlying the link between anxiety disorders and cardiovascular dysfunction. One of the most reliable rodent model for anxiety is based on the selective breeding of rats characterized by opposite levels of trait anxiety (Landgraf and Wigger, 2002; Liebsch et al., 1998a; Liebsch et al., 1998b). This selection has allowed to obtain two different rat lines, termed high anxiety-related behavior (HAB) and low anxiety-related behavior (LAB). The selection criterion is the behavior in the elevated plus maze test, a well validated experimental

paradigm to test anxiety in rodents (Pellow et al., 1985). The behavioral profile of HAB and LAB rats is independent of sex (Bosch and Neumann, 2008) and age (Landgraf and Wigger, 2002), is present in over all seasons (Neumann et al., 2010) and is confirmed by other behavioral tests for both unconditioned and conditioned anxiety (Muigg et al., 2008; Ohl et al., 2001; Slattery and Neumann, 2010). HAB and LAB rats differ in their stress vulnerability and coping strategies, with HAB animals being more susceptible to stressors and adopting more passive coping strategies (Landgraf and Wigger, 2002). In addition, the two rat strains are characterized by distinct neuroendocrine and neuronal patterns (Neumann et al., 2011).

Although many physiological and behavioral characteristics of HAB/LAB rats have been investigated, up to now there is little information about cardiac autonomic modulation in this rodent model of anxiety. A recent study of Gaburro and colleagues (Gaburro et al., 2011) reported in mice selectively bred for high anxiety reduced heart rate variability, which has been proposed as a highly sensitive and specific marker to distinguish between normal and high anxiety trait.

The aim of the present study was to investigate possible differences in cardiac autonomic modulation between rats with opposite levels of trait anxiety. The assessment of autonomic regulation of heart rate was performed through the quantification of HRV in baseline conditions, during and after the exposure to non-social and psychsocial stressful stimuli, and in response to autonomic pharmacological challenges. In addition, we evaluated also the association between opposite levels of anxiety and cardiac morphological/morphometrical characteristics.

2. Methods

2.1 Animals and housing

Experiments were carried out on 5-month-old male Wistar rats obtained from the animal facilities of the University of Regensburg (Germany). The animals belonged to two lines selectively bred since 1993 for high (HAB) or low (LAB) anxiety-related behavior, as described previously in detail (Landgraf and Wigger, 2002; Liebsch et al., 1998a; Liebsch et al., 1998b). At the age of 10 weeks, the HAB (n=10) and LAB (n=10) rats used in this study were tested at the University of Regensburg (Germany) on the elevated plus-maze (EPM) to confirm their anxiety-related phenotype (Liebsch et al., 1998a; Liebsch et al., 1998b). At their arrival in our laboratory, they were housed in groups of 3-4 per cage and kept in rooms with controlled temperature (22±2°C) at a reversed light-dark cycle (light on from 19:00 to 07:00 h), with free access to food and water. 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 24 November 1996 (86/609/EEC).

2.2 Radiotelemetry system

Radiotelemetry was used for recording electrocardiograms, core body temperature (T, °C) and locomotor activity (ACT, expressed as counts/minute, cpm). The telemetry system employed in this study consisted of flat transmitters measuring 25 x 15 x 8 mm (TA11CTA–F40, Data Science International, St. Paul, MN, USA) and platform receivers. At 5 months of age, the animals were anesthetized with tiletamine hydrochloride + zolazepam

hydrochloride (Zoletil, 200 mg/Kg, s.c.) and the transmitters chronically implanted according to a surgical procedure that guarantees high-quality ECG recordings also during sustained physical activity (Sgoifo et al., 1996). Briefly, the body of the transmitter was placed into 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. Immediately after surgery, rats were individually housed and injected for 2 days with gentamicine sulfate (Aagent, Fatro, 0.2 ml/Kg, s.c.). The animals were allowed 10 days of recovery before the start of experimental recordings.

2.3 Outline of the experimental protocol

An overview and time line of the measurements performed is provided in figure 1. HAB and LAB rats were submitted to two different stressors on two consecutive days, i.e. restraint stress, and psychosocial stress. Before and after the two stress episodes, the circadian rhythms of heart rate, body temperature and physical activity were recorded for seven days. After post-stress rhythm recording, two pharmacological challenges were performed, i.e. (i) the combined double blockade of the two branches of the autonomic nervous system, and (ii) the stimulation followed by blockade of the sympathetic system. Since the day of surgery, the animals were weighed on a weekly basis until the day of sacrifice between 09:00 and 10:00 h. The two stress episodes, pharmacological challenges and euthanasia took place between 09:00 and 14:00 h.

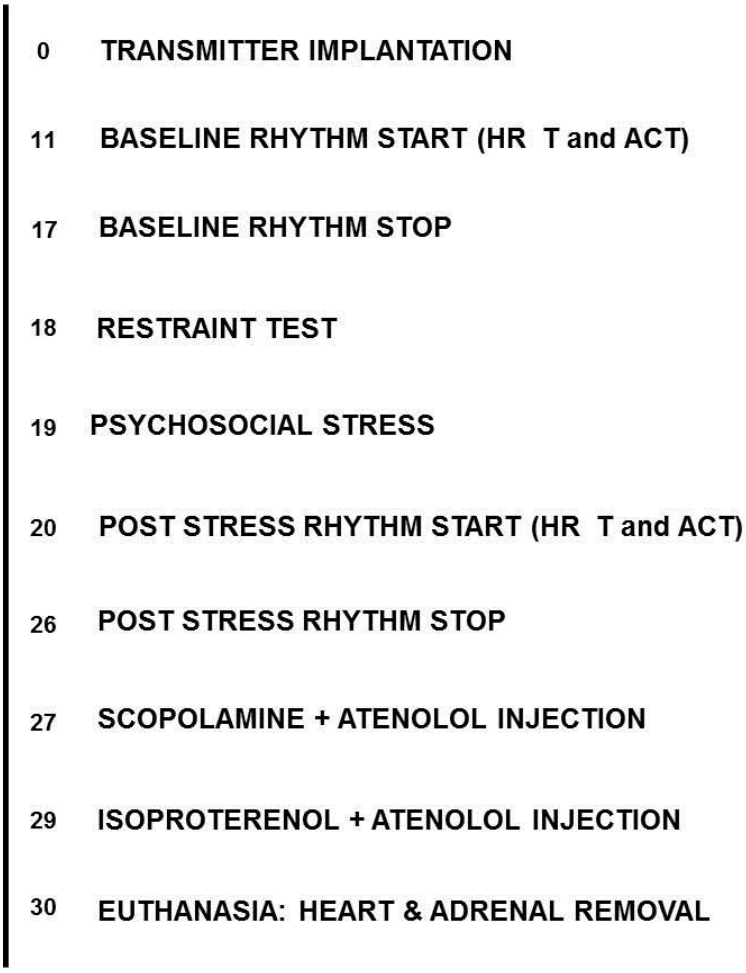

Figure 1. Outline of the experimental protocol. ACT=physical activity; HR=heart rate; T=core body temperature.

2.4 Stress procedures

The restraint session consisted in the confinement of each rat in a wire-mesh tube for 15 min (inner diameter 6 cm, length: 20 cm). After the test, animals were returned to their home cages. Psychosocial stress was obtained by introducing an unfamiliar conspecific male previously confined in a restrainer into the cage of the experimental animal (figure 2). This procedure allowed olfactory, visual and acoustic contact with the intruder, but protected the experimental animal from direct physical contact (Sgoifo et al., 1998).

Figure 2. Detail of the psychosocial stress procedure employed in this study. The male conspecific intruder is confined in a wire mesh tube.

2.5 Pharmacological challenges

In a first pharmacological test, competitive muscarinic receptor antagonist methylscopolamine (50 µg/kg) and sympathetic blocker atenolol (2 mg/kg) (Sigma, St.Louis, MO, USA) (Ngampramuan et al., 2008) were injected s.c. to block vagal and sympathetic influences to the heart in HAB and LAB rats. After baseline ECG recording, methyscoplamine was injected and the ECG recorded to evaluate the effect of parasympathetic blockade; 15 min afterwards, atenolol was administered to the same animals to determine intrinsic heart rate. Intrinsic heart rate is established when the cardiac autonomic nervous system is completely blocked, which is supposed to take place approximately 15-20 min after the sympathetic blocker injection (Safa-Tisseront et al., 1998; Sant'Ana et al., 2011; Souza et al., 2009). In the second pharmacological challenge, beta-adrenergic receptor agonist isoproterenol (20 µg/kg) (Sigma, St.Louis, MO, USA) was

administered s.c. (Kung and Blau, 1978) and the ECG recorded to determine the effect of sympathetic stimulation; 15 min afterwards, atenolol was injected in the same rat to block sympathetic influences to the heart (Whalen and Lewis, 1999).

2.6 Electrocardiographic data collection and analysis

ECG waves were acquired on a PC via ART-Silver 1.10 data acquisition system (Data Sciences Int., St. Paul, MN, USA) with 1000 Hz sampling frequency. Continuous ECG recordings were performed during the acute stress episodes (restraint stress and psychosocial stress) and the pharmacological tests (figure 1) according to the following schedule: (i) restraint stress and psychosocial stress: 30 min baseline, 15 min test, 45 min recovery; (ii) pharmacological double blockade: 30 min baseline, 15 min following scopolamine injection, 45 min following atenolol injection; (iii) sympathetic stimulation and blockade: 30 min baseline, 15 min following isoproterenol injection, 45 min following atenolol injection.

Chart5 software (ADInstruments, Sidney, Australia) was employed to calculate the average R-R interval duration (RR, ms), which corresponds to the average inter-beat-interval in a given time period. In addition, time-domain and frequency-domain parameters of HRV were quantified. The time domain indexes used in this study were 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 (Stein et al.,1994). Frequency domain (fast Fourier transform) indexes were collected in accordance to the guidelines for frequency-domain computations of HRV (Task Force, 1996). 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. LF and HF power was quantified as absolute (ms^2) and normalized units (n.u.). The power of LF represents the activity of both branches of the autonomic nervous system (Eckberg, 1991); the power of HF is due to the activity of the parasympathetic nervous system and includes respiration-linked oscillations of heart rate (Chess et al., 1975). The low frequency/high frequency ratio (LF/HF) estimates the fractional distribution of power. A stationary ECG signal is recommended to reliably perform short-term frequency-domain HRV analysis and the presence of artifacts could influence significantly the results (Task Force, 1996). For these reasons, those parts of ECG recordings which were non stationary and/or exhibited recording artifacts were excluded from the analysis.

During the double pharmacological autonomic blockade intrinsic heart rate, expressed as the intrinsic RR interval (RR_0), was determined in the time interval 15-20 min after the administration of atenolol. Cardiac sympathovagal balance can also be assessed by means of the index named vagal-sympathetic effect (VSE), which is defined as the ratio of the RR interval to the intrinsic RR interval (RR_0) ($VSE=RR/RR_0$) (Goldberger, 1999). The value of VSE reflects the net effects of the sympathetic and parasympathetic influences on the heart rate. In particular, a VSE > 1 corresponds to parasympathetic predominance, whereas VSE < 1 to sympathetic predominance. In this study, we assessed cardiac symapthovagal balance at rest by the ratio between RR interval in baseline conditions (RR_{bas}) and RR_0 ($VSE_{bas}= RR_{bas}/RR_0$).

The occurrence of ventricular premature beats was determined off-line, and quantified as number of events. The identification of these rhythm disturbances was based on the classical definition of arrhythmias in man and on the Lambeth Conventions for the study of experimental arrhythmias (Walker et al., 1982).

2.7 Daily rhythm data collection and analysis

Heart rate (HR), body temperature (T) and physical activity (ACT) were sampled continuously for 120s every 60 min with the animal in its own home cage on two different periods (figure 1): (i) baseline – 7 days – starting after full recovery from surgery (days 11-17), and (ii) post stress – 7 days – starting on the day after the psychosocial stress (days 20-26). The three parameters were quantified as means of 12-h inactive (light) and 12-h active (dark) phases. For each animal, the daily amplitude of the rhythms of HR, T, and ACT was calculated as the difference between average active and inactive phase values, respectively (Meerlo et al., 1999).

2.8 Post-mortem measurements

The day after the last pharmacological challenge (figure 1), rats were euthanised. Under anesthesia (tiletamine hydrochloride + zolazepam hydrochloride s.c.; Zoletil, 200 mg/kg) the heart was arrested in diastole with cadmium chloride solution (100 mM IV) and excised for subsequent morphological/morphometric analysis. Following heart removal, adrenals were also excised and weighed.

2.8.1 Cardiac remodelling and morphometry

The two atria, the right ventricle (RV) and the left ventricle (LV) inclusive of the septum were separately weighed, fixed in 10% buffered formalin solution, and used for morphometric studies. The following parameters were determined: heart weight (HW) and its value relative to body weight (BW), LV weight (LVW), RV weight (RVW) and their values relative to heart weight. LV and RV free wall thickness and LV transverse diameters

were morphometrically computed (Image Pro-plus). The LV chamber volume was calculated according to the Dodge equation (Dodge, 1969). Subsequently, from each heart embedded in paraffin, 5 μm-thick left ventricular sections were cut from the equatorial slice. Sections stained with Masson's trichrome were analysed by optical microscopy (magnification 250X) in order to evaluate the total amount of interstitial and reparative fibrosis (Beltrami et al., 1994) in the LV myocardium. According to a procedure previously described (Costoli et al., 2004), quantitative evaluation of fibrotic tissue was performed in 60 randomly selected fields from the subendocardium, midmyocardium and subepicardium, with the aid of a grid defining a tissue area of 0.160 mm^2 and containing 42 sampling points, each covering an area of 0.0038 mm^2. To define the volume fraction of fibrosis in the three layers of the ventricular wall, the number of points overlaying myocardial scarring were counted and expressed as a percentage of the total number of points explored. For reparative fibrosis, the numerical density of fibrotic foci per unit area of myocardium was also determined.

2.9 Data analysis and statistics

Values of all parameters were expressed as mean ± SEM. Statistical analyses were performed using SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA) and statistical significance for all tests was set at $p \leq 0.05$.

In order to detect possible differences at rest between the two experimental groups, the mean values of RR interval and HRV indexes of 30 min baseline recordings preceding acute stressors and pharmacological challenges were statistically analyzed by means of two-way analysis of variance (ANOVA), with "group" as between-subject factor (2 levels:

HAB and LAB rats) and “time” as within-subject factor (4 levels: restraint stress, social stress, double pharmacological blockade, sympathetic stimulation and blockade).

RR interval and HRV parameters were quantified as the average value of the 30-min baseline recording and of every 5-min time point of the test (t5-t15) and recovery period (r5-r45) for restraint and psychosocial stress and of the period after drug administration (scopolamine: s5-s15; isoproterenol: i5-i15; atenolol: a5-a45) for pharmacological challenges. Two-way ANOVA for repeated measures with “group” as between-subject factor (2 levels: HAB and LAB rats) and “time” as within-subject factor (13 levels: 13 time points of the recording period) was applied on data obtained from: (i) restraint stress; (ii) social stress; (iii) double pharmacological blockade; (iv) sympathetic stimulation and blockade.

Body temperature and physical activity were recorded during restraint and psychosocial stress and measured as the average value of baseline, test and recovery period. The data obtained were statistically analyzed via two way ANOVA with “group” as between-subject factor (2 levels: HAB and LAB rats) and “time” as within-subject factor (3 levels: baseline, test, recovery).

Average light and dark phase values of heart rate, body temperature and physical activity were calculated and expressed as mean of the 7-day baseline and 7-day post-stress recording periods. Statistical analysis on day and night circadian data was performed separately via two-way ANOVA with “group” as between-subject factor (2 levels: HAB and LAB rats) and “time” as within-subject factor (2 levels: baseline, post-stress).

Values of body weight were analyzed via two-way ANOVA for repeated measures, with “group” as between-subject factor (2 levels: HAB and LAB rats) and “time” as within-subject factor (5 levels: 5 weekly measurements).

Follow-up analyses were conducted using Student “t” tests after checking for variance homogeneity by means of Levene test.

The weight of adrenal glands at sacrifice was expressed as the ratio to the animal body weight (mg adrenal gland weight / 100 g body weight).

Comparisons between HAB and LAB rats were performed via Student’s “t” tests, again following a Levene test, for: (i) elevated plus maze data, (ii) vagal-sympathetic effect index (VSE), (iii) number of ventricular ectopic beats occurring during pharmacological sympathetic stimulation, (iv) adrenal weight, and (v) cardiac structural measures.

3. Results

3.1 Behavior in the elevated plus maze

The elevated plus maze test was conducted as the validation criterion for relative anxiety phenotype of the rats employed in this study. HAB rats spent less percent time in the open arms (HAB=2±1% vs. LAB=65±4% of total time, $t(18)=15.4$, $p<0.01$) and entered them less frequently (HAB=18±8% vs. LAB=55±2% of total entries, $t(18)=4.6$, $p<0.01$) than LABs. In addition, the latency to enter an open arm was increased in HAB compared to LAB rats (HAB=201±44s vs. LAB=15±5s, $t(18)=4.2$, $p<0.01$). These results confirmed that HAB rats were more anxious than LABs, since decreased open/unprotected-arms exploration indicates enhanced anxiety-related behavior (Crawley and Goodwin, 1980).

3.2 Cardiac autonomic regulation in baseline conditions

Table 1 reports the resting values of RR interval and all HRV indexes in HAB and LAB rats expressed as the average value of the 30 min of baseline recording performed before each of the four tests, i.e. restraint, psychosocial, pharmacological double blockade, pharmacological sympathetic stimulation and blockade. Two-way ANOVA revealed a significant effect of time only for RR ($F(3,54)=17.08$, $p<0.05$). A significant effect of group was found for the vagal index r-MSSD and for the power in absolute units of the LF and HF bands of the power spectrum (r-MSSD: $F(1,18)=5.26$, $p<0.05$; LF: $F(1,18)=4.97$, $p<0.05$; HF: $F(1,18)=4.59$, $p<0.05$). Rats characterized by high levels of anxiety-related behavior showed in resting conditions a trend towards lower heart rate than LAB rats, but reaching statistical significance only in the recording preceding the psychosocial stress ($t(18)=2.51$, $p<0.05$). In contrast to the heart rate values, the vagal indexes r-MSSD and pNN10 were higher in LABs and significantly different from HABs' values in the baseline recordings preceding restraint test ($t_{r\text{-}MSSD}$ $(18)=2.91$, $p<0.05$; $t_{pNN10}(18)=3.22$, $p<0.05$) and double pharmacological blockade ($t_{r\text{-}MSSD}$ $(18)=2.77$, $p<0.05$; $t_{pNN10}(18)=2.63$, $p<0.05$). In addition, LF and HF power expressed in absolute units were larger in LAB rats compared to the more anxious counterparts, reaching statistical significance in the days of the restraint stress ($t_{LF}(18)=2.48$, $p<0.05$; $t_{HF}(18)=2.85$, $p<0.05$) and the first pharmacological challenge ($t_{LF}(18)=2.01$, $p<0.05$; $t_{HF}(18)=2.77$, $p<0.05$). Taking together, the results obtained from ECG recordings in baseline conditions suggest a reduced heart rate variability in the rats with high anxiety.

Table 1. Mean values of RR interval (ms), time-domain indexes r-MSSD (ms) and pNN10 (%), frequency-domain parameters total power (ms^2) and power in absolute (ms^2) and normalized units (n.u.) of LF and HF bands of 30 min-recording period preceding each acute test performed in the experimental procedure: restraint stress (RESTRAINT), psychosocial stress (PSYCHOSOCIAL STRESS), autonomic double pharmacological blockade (DOUBLE BLOCKADE), pharmacological sympathetic stimulation and blockade (ISOPROTERENOL).

TEST	GROUP	HRV parameters								
		RR (ms)	r-MSSD (ms)	pNN10 (%)	Tot. Pow. (ms^2)	LF (ms^2)	LF n.u.	HF (ms^2)	HF n.u.	LF/HF
RESTRAINT	HAB	163.3±4.42	3.25±0.25*	1.18±0.32*	64.68±8.22	2.55±0,41*	41.26±2,12	3.62±0,56*	58.74±2.12	0.75±0.06
	LAB	155.19±3.34	4.58±0.39	5.42±1.27	84.21±12.02	4.65±0.75	39.26±2.92	7.85±1.38	60.74±2.92	0.70±0.08
PSYCHOSOCIAL STRESS	HAB	168.27±2.56*	3.04±0.34	1.67±0.79	63.24±9.55	2.41±0.57#	41.82±2.18	3.60±1.07	58.18±2.18	0.77±0.06
	LAB	156.89±3.74	4.04±0.48	4.03±1.53	75.40±9.99	4.24±0.79	42.18±2.44	6.69±1.84	57.82±2.44	0.79±0.07
DOUBLE BLOCKADE	HAB	175.04±4.84	3.25±0.28*	1.59±0.51*	70.75±10.10	2.58±0.42*	41.41±2.07	3.74±0.62*	58.59±2.07	0.74±0.07
	LAB	170.66±7.27	4.34±0.28	3.97±0.75	79.00±6.82	4.27±0.73	39.39±2.92	6,83±0.93	60.61±2.92	0,71±0.08
ISOPROTERENOL	HAB	176.21±4.26	3.51±0.34	4.23±2.32	71.08±11.90	2.57±0.41	37.37±2.92	4.51±0.95	62.63±2.92	0.65±0.07
	LAB	171.59±4.88	4.40±0.64	5.37±2.52	89.20±16.75	3.75±0.65	37.21±3.55	9.62±3.57	62,79±3.55	0.67±0.09

All values are expressed as mean ± SEM. *: significant differences between HAB and LAB rats ($p<0.05$); #: trend towards significant difference between HAB and LAB rats ($0.05 \leq p \leq 0.07$).

3.3 Electrocardiographic response to restraint stress

As reported in figure 3 (panel A), rats with low levels of anxiety-related behavior (LAB) showed a larger tachicardic response compared to HAB rats, during the test and the initial phase (r5-r15) of the recovery period. Statistical analysis of RR values via two-way ANOVA revealed a significant effect of time ($F(12,216)=5.05$, $p<0.05$) and group ($F(1, 18)=8.38$, $p<0.05$) (details of Student's "t" test results in figure 3). LAB animals were characterized by high levels of parasympathetic modulation in baseline conditions and displayed a typical stress-induced vagal withdrawal (figure 3, panel B). Statistical analysis via two-way ANOVA did not detect any effect of time or group for r-MSSD. However, anxious rats showed a larger shift of cardiac sympathovagal balance towards sympathetic prevalence during the test and the recovery phase, as revealed by the higher values of the spectral index LF/HF (figure 3, panel C) (group effect: $F(1,18)=5.56$, $p<0.05$; details of Student's "t" test results reported in figure 3).

Physical activity (ACT) and body temperature (T) were not different between the two experimental groups before (ACT: HAB, 5.55±0.51 cpm; LAB, 7.42±1.35 cpm; T: HAB, 38.24±0.09 °C; LAB, 38.09±0.14 °C), during (ACT: HAB, 1.47±0.31 cpm; LAB, 2.19±0.43 cpm; T: HAB, 38.32±0.09 °C; LAB, 38.25±0.15 °C) and after (ACT: HAB, 13.95±1.58 cpm; LAB, 17.06±1.19 cpm; T: HAB, 38.72±0.06 °C; LAB, 38.68±0.06 °C) the restraint test.

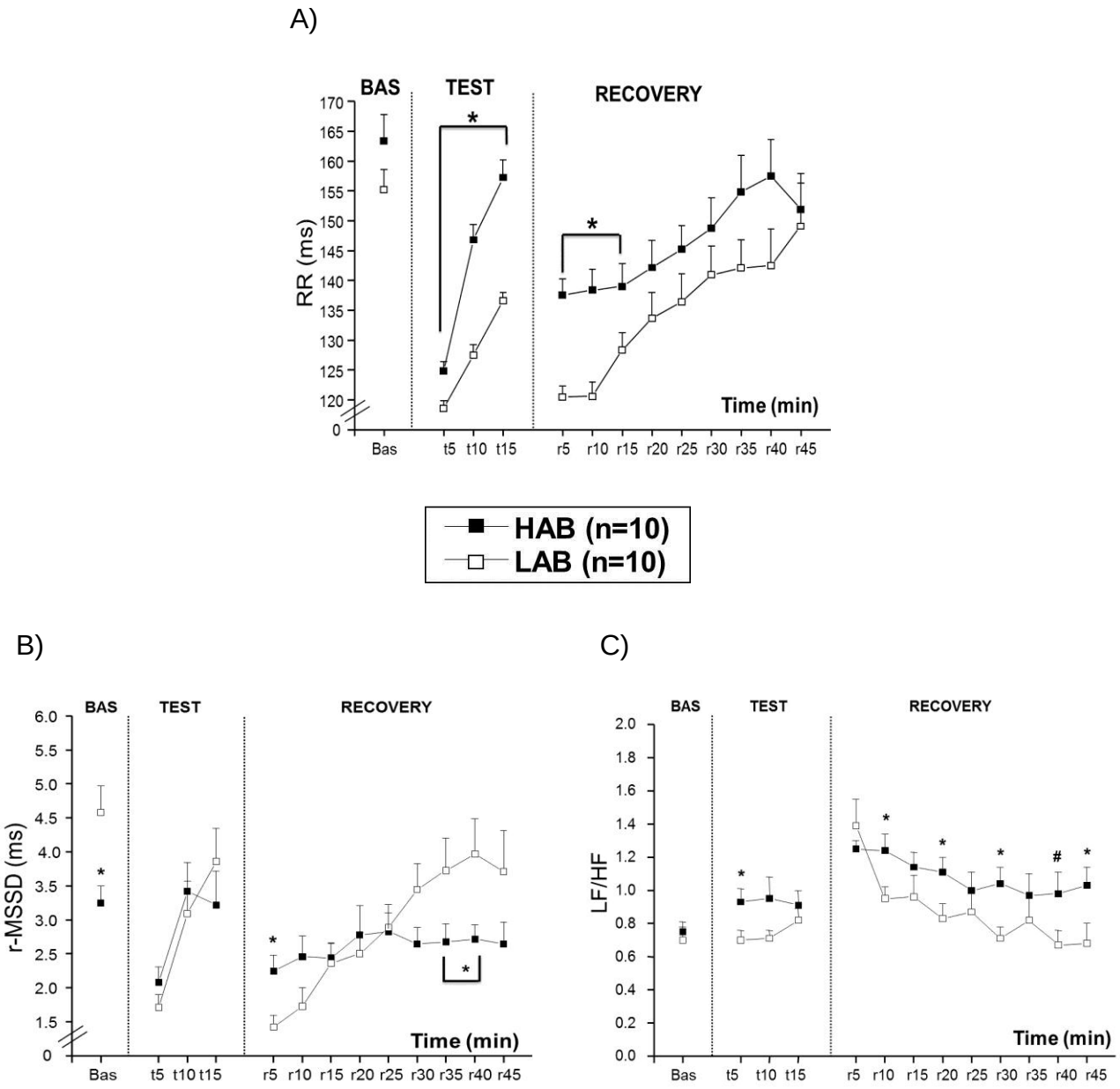

Figure 3. Time course of RR interval (panel A, ms), time-domain index r-MSSD (panel B, ms) and frequency-domain index LF/HF (panel C) before (BAS), during (TEST, t5-t15) and after (RECOVERY, r5-r45) restraint stress in HAB (n=10) and LAB (n=10) rats. *: significant differences between HAB and LAB rats ($p<0.05$); $^{\#}$: trend towards significant differences between HAB and LAB rats ($0.05\leq p\leq 0.07$). Student's "t" test: RR: $3.07\leq t_{t5\text{-}t15}(18)\leq 6.39$, $p<0.05$; $2.21\leq t_{r5\text{-}r15}(18)\leq 5.28$, $p<0.05$; r-MSSD: $t_{bas}(18)=2.91$, $p<0.05$; $t_{r5}(18)=2.89$, $p<0.05$; $2.03\leq t_{r35\text{-}r40}(18)\leq 2.22$; LF/HF: $t_{bas}(18)=2.33$, $p<0.05$; $t_{r10}(18)=2.30$, $p<0.05$; $t_{r20}(18)=2.17$, $p<0.05$; $t_{r30}(18)=2.66$, $p<0.05$; $t_{r40}(18)=2.01$, $p=0.06$; $t_{r45}(18)=2.15$, $p<0.05$.

3.4 Electrocardiographic response to psychosocial stress

The difference in the chronotropic response to an acute stressor between rats with different levels of anxious behavior, already observed for restraint stress, was further increased when the animals were exposed to an acute social challenge (figure 4, panel A). For RR interval values, two way ANOVA found a significant effect of time ($F(12, 216)=25.77$, $p<0.05$), group ($F(1,18)=50.74$, $p<0.05$) and group x time interaction ($F(12, 216)=6.74$, $p<0.05$). Heart rate was significantly higher in LABs in baseline conditions, during the test and the recovery phase (details of Student's "t" test results are reported in figure 4). As expected, in LAB rats the time course of r-MSSD values reflected the vagal withdrawal occurring typically during stress response, that was not detectable in HABs' response (figure 4, panel B). Statistical analysis via two-way ANOVA did not detect any time or group effect for r-MSSD values. Cardiac autonomic balance appeared to be similar in the two experimental groups (figure 4, panel C). Two-way ANOVA applied to frequency-domain parameter LF/HF values revealed a significant effect of time ($F(12,216)=38.13$, $p<0.05$), but not of group.

Although the basal levels of physical activity were similar in the two rat strains (HAB: 4.97±0.98 cpm; LAB: 6.54±0.80; p=n.s.), the locomotor response was different in HABs and LABs during the phase of sensory contact (HAB: 12.34±1.53 cpm; LAB: 25.52±2.83 cpm; $t(18)=4.10$, $p<0.05$) and during the recovery period (HAB: 10.62±0.89 cpm; LAB: 17.17±1.83 cpm; $t(18)=3.22$, $p<0.05$) (ANOVA: effect of time, $F(2,36)=45.40$; effect of group, $F(1,18)=21.58$; group x time interaction, $F(2,36)=4.26$, $p<0.05$ for all). These results reflected a different behavioral response of HAB and LAB rats to a stress condition that involves social contact, with less anxious animals being more physically active.

Two-way ANOVA revealed a significant effect of time ($F(2,36)=136.46$, $p<0.05$) and group x time interaction ($F(2,36)=13.08$, $p<0.05$) for body temperature values. HAB rats displayed higher body temperature in baseline conditions (HAB, 38.23±0.09 °C; LAB, 37.92±0.09 °C; $t(18)=2.44$, $p<0.05$), but stress exposure induced a larger rise in body temperature in LAB animals (test phase: HAB, 38.40±0.07 °C; LAB, 38.68±0.11 °C; $t(18)=2.18$, $p<0.05$; recovery phase: HAB, 38.70±0.05 °C; LAB, 39.10±0.10 °C; $t(18)=3.42$, $p<0.05$).

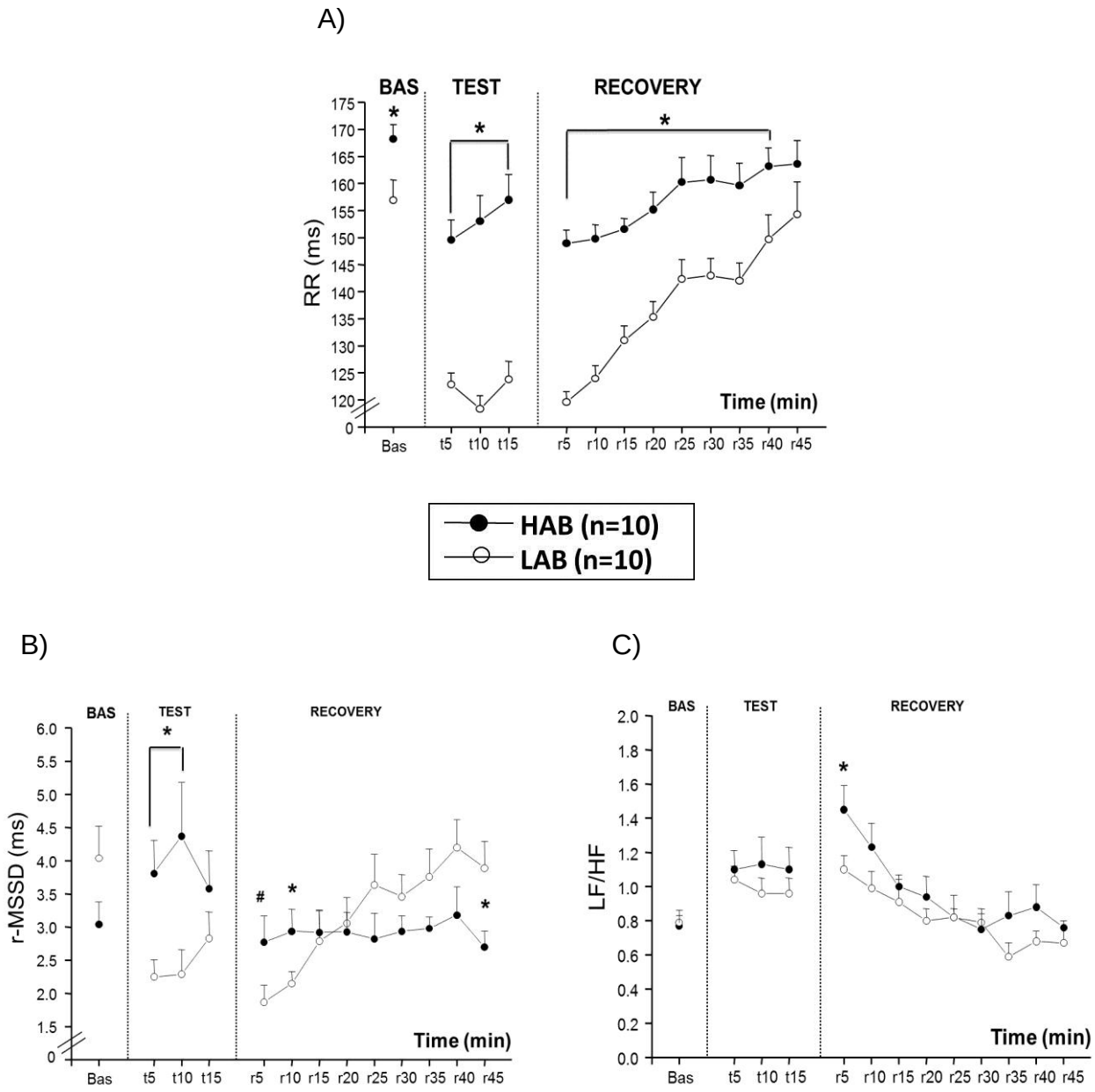

Figure 4. Time course of RR interval (panel A, ms), time-domain index r-MSSD (panel B, ms) and frequency-domain index LF/HF (panel C) before (BAS), during (TEST, t5-t15) and after (RECOVERY, r5-r45) psychosocial stress in HAB (n=10) and LAB (n=10) rats. *: significant differences between HAB and LAB rats ($p<0.05$); #: trend towards significant differences between HAB and LAB rats ($0.05\leq p\leq 0.07$). Student's "t" test: RR: $t_{bas}(18)=2.51$, $p<0.05$; $2.43\leq t_{t5\text{-}r40}(18)\leq 9.55$, $p<0.05$; r-MSSD: $2,34\leq t_{t5\text{-}t10}(18)\leq 2.77$, $p<0.05$; $t_{r5}(18)=1.90$, $p=0.07$; $t_{r10}(18)=2.10$, $p<0.05$; $t_{r45}(18)=2.58$, $p<0.05$; LF/HF: $t_{r5}(18)=2.19$, $p<0.05$.

3.5 Circadian rhythms

Table 2 depicts the physiological parameters heart rate, body temperature and physical activity, expressed as mean value of the active (dark) and inactive (light) phase of the light-dark cycle, recorded before (Baseline) and after (Post-stress) the two days of stress exposure. Two-way ANOVA on heart rate values revealed a significant effect of group only for the dark phase ($F(1,18)=11.31$, $p<0.05$) and no effect of time. Heart rate in the active phase was significantly larger in LAB rats compared to HABs, both before and after the stress period ($t_{Baseline}(18)=2.89$, $t_{Post\text{-}stress}(18)=3.55$, $p<0.05$ for both). Statistical analysis on body temperature values showed a significant effect of time for the active phase ($F(1,18)=5.92$, $p<0.05$) and a significant effect of group for both the active ($F(1,18)=27.19$, $p<0.05$) and inactive phases ($F(1,18)=50.35$, $p<0.05$). Anxious rats were characterized by higher body temperature values both in the dark and the light phase of the circadian rhythm (light phase: $t_{Baseline}(18)=6.48$, $t_{Post\text{-}stress}(18)=6.88$, $p<0.05$); dark phase: $t_{Baseline}(18)=4.68$, $t_{Post\text{-}stress}(18)=5.55$, $p<0.05$). No significant differences between HAB and LAB rats were found for physical activity, neither in the active nor in the passive phase, neither before nor after stress exposure. Statistical analysis did not show significant differences between pre- and post-stress heart rate, temperature and physical activity values neither in LAB nor in HAB animals. In other words, the exposure of the experimental animals to two consecutive stressors did not modify the circadian rhythmicity of these physiological parameters.

Table 2. Mean values of heart rate, body temperature and physical activity in the inactive (light) and active (dark) phases of the circadian rhythm before (Baseline) and after (Post-stress) the stressors, in HAB (n=10) and LAB (n=10) rats.

	Group	Heart Rate (bpm)		Temperature (°C)		Activity (cpm)	
		light phase	dark phase	light phase	dark phase	light phase	dark phase
Baseline	**HAB**	299.74±2.97	355.77±3.26*	37.24±0.03*	38.17±0.04*	2.12±0.20	5.26±0.23
	LAB	306.65±2.58	370.31±3.84	36.90±0.04	37.88±0.05	2.30±0.18	5.65±0.28
Post-stress	**HAB**	301.52±3.39	355.31±3.04*	37.24±0.03*	38.13±0.04*	2.02±0.17	4,89±0.25
	LAB	309.48±4.12	371.40±3.36	36.97±0.03	37.85±0.04	2.16±0.17	5.47±0.33

All values are expressed as mean ± SEM. *: significant differences between HAB and LAB rats ($p<0.05$).

Amplitudes of the daily rhythm of heart rate, body temperature and physical activity, expressed as mean values of pre-stress and post-stress periods are reported in table 3. Statistical analysis did not reveal any significant differences between the two experimental groups in the daily amplitude of heart rate, temperature and activity, neither before nor after the two stressors.

Table 3. Mean values of the daily amplitude of heart rate, body temperature and physical activity before (Baseline) and after (Post-stress) the stressors, in HAB (n=10) and LAB (n=10) rats.

	Group	Amplitude Heart Rate (bpm)	Amplitude Temperature (°C)	Amplitude Activity (cpm)
Baseline	**HAB**	56.03±2.10	0.93±0.03	3.15±0.20
	LAB	63.09±4.32	0.96±0.04	3,28±0.26
Post-stress	**HAB**	53.79±2.89	0.90±0.03	2.87±0.21
	LAB	61.92±4.58	0.88±0.02	3,30±0.29

All values are expressed as mean ± SEM.

3.6 Double pharmacological blockade

As reported in figure 5 (panel A), the injection of the muscarinic receptor antagonist induced a larger tachicardic response in LAB rats. In addition, the administration of the beta-adrenergic receptor blocker induced higher RR interval values in HABs. Two-way ANOVA detected a significant effect of time ($F(12,216)=227.66$, $p<0.05$) and group ($F(1,18)=33.22$, $p<0.05$) (Student's "t" test results are reported in details in figure 5). The two experimental groups showed different intrinsic RR interval (RR_0), that was measured approximately 15-20 min after the administration of atenolol (figure 5, panel A). Anxious rats were characterized by higher RR_0 compared to LABs (HAB: 180.27±1.93 ms; LAB: 164.44±2.18 ms, $t(18)=6.14$, $p<0.05$). The parameter VSE calculated in baseline conditions was significantly lower in LAB rats (HAB: 0.95±0.01; LAB: 1.01±0.02; $t(18)=2.39$, $p<0.05$). In particular, in animals with high levels of anxiety-related behavior VSE was < 1, suggesting a predominance of sympathetic modulation of heart rate at rest. On the other hand, in LABs VSE was > 1, indicating a shift of cardiac autonomic balance towards a parasympathetic prevalence in baseline conditions.

The parasympathetic blockade induced a larger reduction of the vagal index r-MSSD in LAB rats, whereas r-MSSD values were higher in HABs after the sympathetic blockade (figure 5, panel B). The baseline level of vagal activity was not recovered in the two experimental groups at the end of the recording period. A significant effect of time ($F(12,216)=25.97$, $p<0.05$) and group x time interaction ($F(12,216)=12.09$, $p<0.05$) were found for r-MSSD values via two-way ANOVA (details of Student's "t" test results are reported in figure 5). The time course of LF/HF values revealed a more rapid return of cardiac autonomic balance to basal level after sympathetic blockade in HAB rats (figure 5,

panel C) (ANOVA: time effect, F(12,216)=4.85; group x time interaction, F(12,216)=6.66, p<0.05 for both; Student's "t" test results are reported in figure 5).

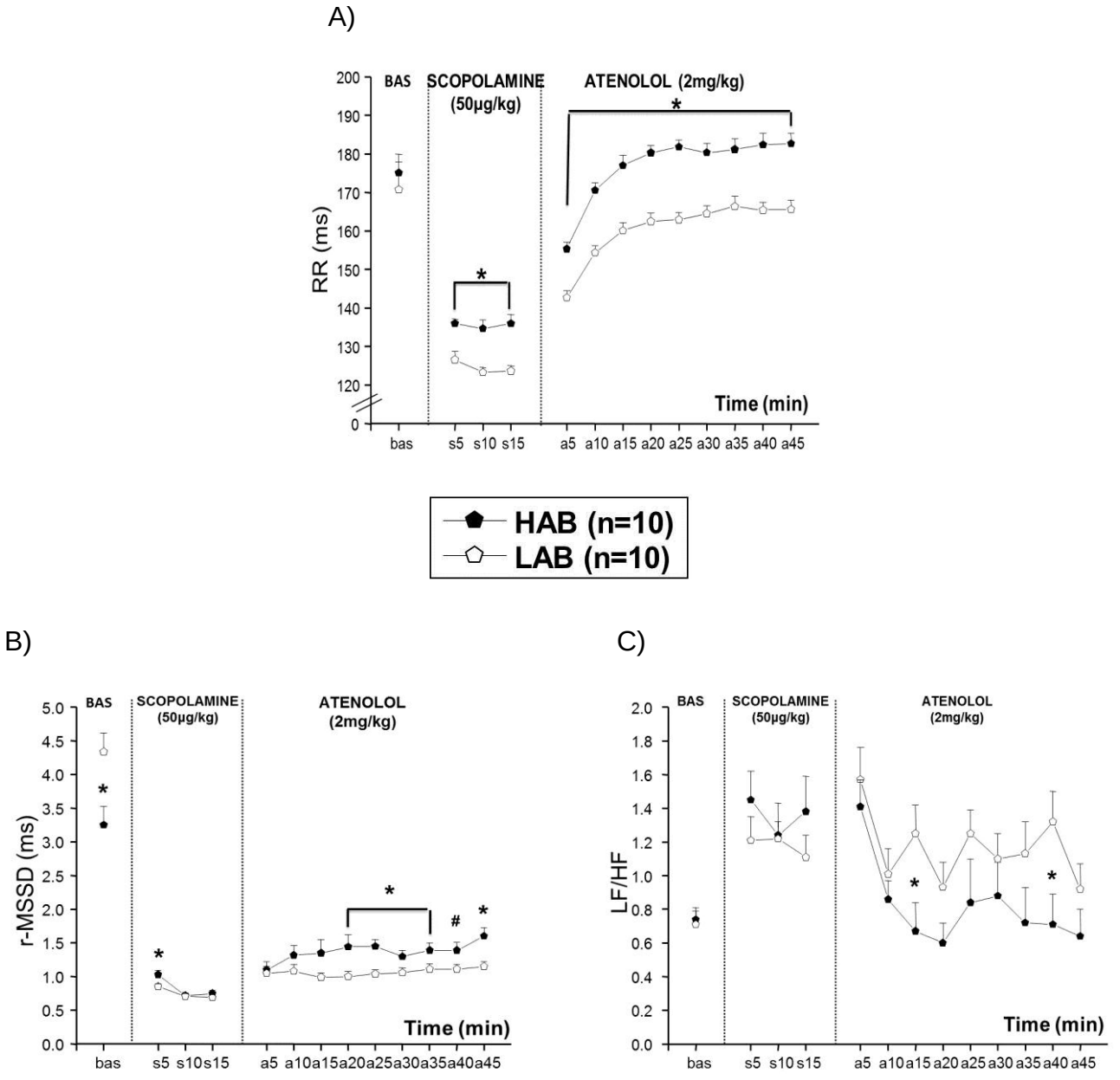

Figure 5. Time course of RR interval (panel A, ms), time-domain index r-MSSD (panel B, ms) and frequency-domain index LF/HF (panel C) before (BAS), after scopolamine administration (SCOPOLAMINE, s5-s15) and after subsequent atenolol injection (ATENOLOL, a5-a45) in HAB (n=10) and LAB (n=10) rats. *: significant differences between HAB and LAB rats (p<0.05); $^{\#}$: trend towards significant differences between HAB and LAB rats (0.05≤p≤0.07). Student's "t" test: RR: $3.77 \leq t_{t5\text{-}r45}(18) \leq 7.10$, p<0.05; r-MSSD: $t_{bas}(18)=2.77$, p<0.05; $t_{s5}(18)=2.53$, p<0.05; $2.19 \leq t_{a20\text{-}a35}(18) \leq 3.46$; $t_{a40}(18)=1.98$, p=0.06; $t_{a45}(18)=3.12$, p<0.05; LF/HF: $t_{a15}(18)=2.42$, p<0.05; $t_{a40}(18)=2.43$, p<0.05.

3.7 Sympathetic stimulation and blockade

The injection of the beta-adrenergic receptor agonist induced a similar increase of heart rate in the two experimental groups (figure 6, panel A). However, heart rate returned more rapidly to basal value in HAB rats after sympathetic blockade. For RR, significant effects of time ($F(12,216)=415.11$, $p<0.05$) and group ($F(1,18)=11.66$, $p<0.05$) were revealed by two-way ANOVA (Student's "t" test results are reported in details in figure 6). The sympathetic stimulation caused the reduction of the vagal index r-MSSD and its value increased after the subsequent injection of the beta-blocker, not reaching baseline level, in both experimental groups (figure 6, panel B) (ANOVA: time effect, $F(12,216)=31.44$, $p<0.05$; no significant effect of group). The time course of the frequency-domain index LF/HF, increased after sympathetic stimulation and decreased to baseline value after sympathetic blockade (figure 6, panel C), reflected a similar modification of cardiac sympathovagal regulation during the pharmacological challenge in the two rat lines (ANOVA: time effect, $F(12,216)=21.88$, $p<0.05$; no significant effect of group).

The incidence of ventricular ectopic beats during pharmacological sympathetic activation was higher in HAB rats, although not reaching statistical significance (HAB: 36.20±8.00; LAB: 20.40±3.15; $t(18)=1.84$, $p=0.08$). This result suggests that massive cardiac beta-adrenergic stimulation can bring about larger electrical instability in the animals characterized by high levels of anxiety-related behavior.

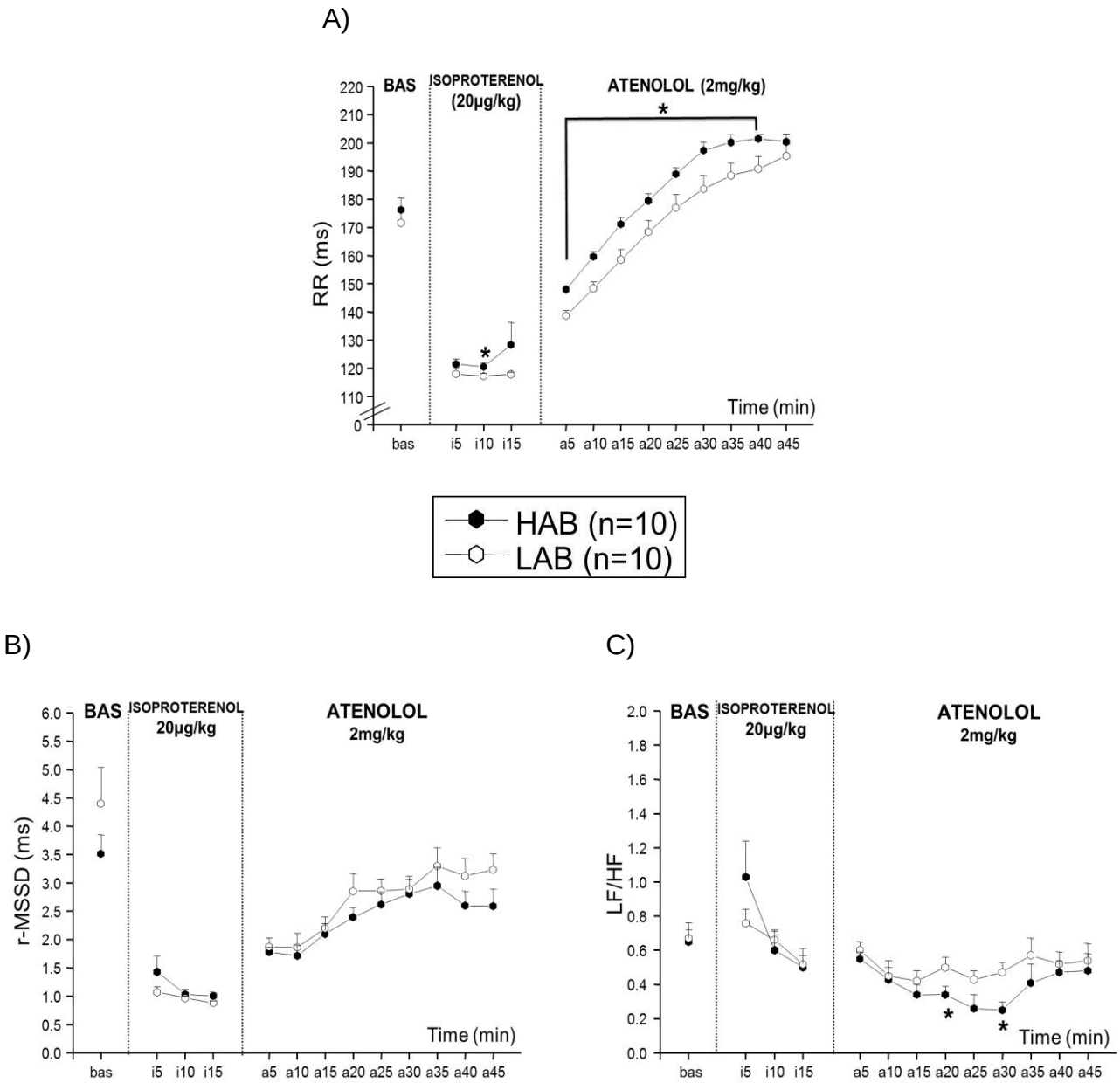

Figure 6. Time course of RR interval (panel A, ms), time-domain index r-MSSD (panel B, ms) and frequency-domain index LF/HF (panel C) before (BAS), after isoproterenol administration (ISOPROTERENOL, i5-i15) and after subsequent atenolol injection (ATENOLOL, a5-a45) in HAB (n=10) and LAB (n=10) rats. *: significant differences between HAB and LAB rats ($p<0.05$); Student's "t" test: RR: $t_{i10}(18)=2.03$, $p<0.05$; $2.28 \leq t_{a5\text{-}a40}(18) \leq 4.35$, $p<0.05$; LF/HF: $t_{a20}(18)=2.03$, $p<0.05$; $t_{a30}(18)=3.02$, $p<0.05$.

3.8 Body weight and adrenal gland weight

HAB and LAB rats were weighed on a weekly basis from the day of surgery (BW_0) until the end of the experimental protocol (BW_4). Two way ANOVA revealed a significant effect of time ($F(4,72)=22.67$, $p<0.05$) due to post-surgery weight decline. In addition, statistical analysis showed a significant effect of group ($F(1,18)=8.41$, $p<0.05$). Although body weight was similar in HAB and LAB rats at the time of transmitter implantation (BW_0: HAB, 383.90±7.76 g; LAB, 411.90±15.21 g; $t(18)=1.64$, p=n.s.), in the successive measurements LABs weighed more than HABs (BW_1: HAB, 378.40±7.37 g; LAB, 420.40±9.93 g; BW_2: HAB, 391.50±7.25 g, LAB, 430.40±10.46 g; BW_3: HAB, 395.70±6.88 g, LAB, 436.00±10.86 g; BW_4: HAB, 400.10±7.84 g, LAB, 437.20±10.52 g, $2.83 \leq t(18) \leq 3.40$, $p<0.05$).

At sacrifice, adrenal weight (corrected per body weight) was similar between HAB and LAB rats (HAB: 10.48±0.23 mg/100g; LAB: 10.48±0.23; $t(18)=1.18$, p=n.s.).

3.9 Cardiac anatomy and myocardial structure

As shown in Table 4, no clear differences were observed between HAB and LAB rats with respect to the weight of the left ventricle (LV) and right ventricle (RV). Only HW/BW was significantly increased in HAB rats ($t(16)=4.45$, $p<0.05$). In addition, the auricle weight was higher in LAB animals ($t(16)=2.07$, $p<0.05$). No significant differences were found in LV linear parameters, including length, thickness and chamber diameter and volume. Thus, opposite levels of trait anxiety did not affect in an evident manner heart anatomy.

Statistical analysis did not detect significant differences between HAB and LAB rats in the total amount of myocardial fibrosis and volume fraction of myocytes (Table 4). Negligible

values of perivascular and interstitial fibrosis, including small foci of collagen accumulation distributed in the myocardium, were observed in the LV of both HAB and LAB hearts. However, interstitial and perivascular fibrosis appeared to be slightly increased in low anxiety rats.

Table 4. Gross cardiac characteristics and left ventricular myocardial fibrosis in HAB (n=9) and LAB (n=9) rats.

	HAB	**LAB**
HW, mg	908.8±36.93	864.11±24.63
HW/BW	0.00227±0.00005*	0.00198±0.00005
LVW, mg	710.6±26.59	676.5±16.93
LVW/HW	0.783±0.012	0.784±0.007
RVW, mg	197.9±16.37	187.7±10.11
RVW/HW	0.217±0.012	0.216±0.007
Auricle weight, mg	89.96±7.32*	109.44±7.14
LV chamber Lenght, mm	12.79±0.50	12.28±0.35
LV chamber Volume, mm^3	99.99±19.25	95.67±9.53
LV chamber diameter, mm	3.76±0.24	3.83±0.19
LV wall thickness, mm	2.64±0.12	2.46±0.08
RV wall thickness, mm	1.00±0.11	1.12±0.05
Myocytes, %	88.72±1.50	90.57±0.99
LV total fibrosis, %	1.51±0.27	2.30±0.41
LV interstilial fibrosis, %	0.81±0.18	0.96±0.26
LV perivascular fibrosis, %	0.70±0.18	1.34±0.28

All values are expressed as mean ± SEM. BW= body weight; HW= heart weight; LV=left ventricle; LVW= left ventricular weight; RV=right ventricle; RVW=right ventricular weight.
*: significant differences between HAB and LAB rats ($p<0.05$).

4. Discussion

The main objective of this study was to investigate the relationship between opposite levels of trait anxiety and (i) autonomic neural regulation of heart rate and (ii) cardiac morphologica/morphometrical characteristics. To reach this purpose, we used a rodent model of trait anxiety based on the selective breeding of rats with high (HAB) or low (LAB) anxiety-related behavior. The basal level of anxiety of the HAB and LAB rats employed in this study was tested by means of the elevated plus maze test.

The results obtained in this study suggest that rats with opposite levels of anxiety-related behavior were characterized by differences in cardiac autonomic regulation at rest and under challenging conditions, namely stress exposure and pharmacological manipulation of ANS. On the other hand, no significant differences in cardiac structural and morphological characteristics were detected.

So far, many behavioral and physiological characteristics of HAB and LAB rats have been investigated (Landgraf and Wigger, 2002), but little is known about their cardiac autonomic regulation and stress responsivity.

The analysis of baseline ECG recordings showed that HAB rats were characterized by lower heart rate compared to LABs, but also by reduced cardiac autonomic regulation, as revealed by HRV parameters. In particular, high levels of anxiety seemed to be associated with decreased parasympathetic input to the heart, as revealed by low values of the vagal indexes r-MSSD and pNN10 (Kleiger et al., 1993). Although LAB rats had similar or even higher heart rate values compared to more anxious animals, they were characterized by elevated parasympathetic control of heart rate in baseline conditions. To explain this discrepancy, one may hypothesize that the elevated parasympathetic drive to the heart was accompanied by a concomitant enhancement of the sympathetic tone. Actually,

spectral analysis of baseline ECG recordings showed in LAB rats elevated values of the power of both LF and HF spectral components. The presence of higher heart rate values in LAB rats was also confirmed by baseline circadian recording. Actually, heart rate was significantly lower in HABs, in particular during the active phase of the light-dark cycle.

HAB and LAB rats differed also in cardiac autonomic stress reactivity. The experimental animals were exposed to two different types of stressors, of both non-social and social nature. Cardiac chronotropic response to both restraint test and psychosocial stress was larger in LAB rats. In particular, the difference in heart rate response between rats with high and low anxiety was even markedly evident when the animals were exposed to a stressor that involved social interaction. The higher tachicardic stress response of LAB rats was accompanied in both stressful conditions by a clear reduction of the parasympathetic input to the heart, as revealed by lower values of r-MSSD during the test and recovery phases. On the other hand, the low vagal activity present at rest in anxious rats did not change in an evident manner following stress exposure. In contrast to heart rate and r-MSSD values, LF/HF values during and after stress exposure were higher in HAB rats. Since the ratio LF to HF power can be considered an indirect measurement of cardiac sympathovagal balance (Montano et al., 1994), its time course indicated a shift of the autonomic balance towards a sympathetic prevalence during stress more pronounced in rats with highly anxious behavior.

In a previous study (Landgraf and Wigger, 2002), no differences in basal blood pressure and heart rate between HAB and LAB rats were reported, whereas we found a trend towards higher heart rate in LABs in resting conditions. In the same study, stress exposure, i.e. a novel environment, induced a rise of heart rate that was less pronounced in HAB rats, despite similar blood pressure increase. Although the stress procedures were different, the blunted tachycardia that characterizes HABs' stress response is in

agreement with our findings. The authors linked the lower rise in heart rate to a more passive coping strategy in rats with high anxiety. In particular, they observed that HAB animals displayed decreased locomotor activity and enhanced freezing behavior during stress exposure than LABs. Actually, experimental evidence has associated freezing to reduced heart rate, mainly due to increased cardiac parasympathetic control (Carrive, 2000; Nijsen et al., 1998). This hypothesis may in part explain also our results. We did not monitor the behavior of the animals during and after the stressors, but we measured the level of physical activity. The locomotor response was significantly different between the two lines during and after the psychosocial stressor. In addition, the time course of the vagal index r-MSSD in HABs, that was substantially unmodified during the sensory contact phase, did not indicate withdrawal of parasympathetic control during stress response and may be associated with the low level of physical activity that characterizes HABs' stress coping strategy (Nijsen et al., 1998). However, the blunted tachicardic response of HAB rats during restraint test is not justify by differences in freezing behavior (the animals were immobilized into the restraint cylinder). Furthermore, physical activity was not significantly different between the two experimental groups even during the recovery phase, when HABs returned more rapidly to basal heart rate. Around –the-clock recording of heart rate and physical activity further supports the idea that decreased heart rate is not a consequence of reduced somatomotor activity: HABs showed, particularly during the night phase, lower heart rate values compared to LAB rats, but no differences in locomotor activity. Therefore, the more passive coping strategy exhibited by animals with high anxiety-related behavior may only partially explain the differences in cardiac autonomic stress responsivity.

So far, stress reactivity of HAB and LAB rats has been investigated in particular considering HPA axis activity (Landgraf et al., 1999; Liebsh et al., 1998a). Experimental

evidence suggests that HAB and LAB lines are characterized by stressor-dependent differences in neuroendocrine response. On the one hand, ACTH and corticosterone levels, which are similar between HAB and LAB rats in baseline conditions, were reported to increase significantly stronger in HABs when exposed to a weak emotional non-social stressor, i.e. an open arm of the elevated plus maze or a novel environment (Landgraf et al., 1999; Liebsh et al., 1998a; Neumann et al., 2005). On the other hand, exposure to stressors involving social environment seems to be more threatening for LAB rats (Veenema and Neumann, 2007). For example, social defeat induced higher ACTH and corticosterone responses in LAB animals compared to both HAB and NAB (normally anxiety-related behavior) rats (Veenema et al., 2007). HAB and LAB male rats have been shown to differ in the level of aggression, with LAB characterized by high and abnormal forms of aggressive behaviors (Neumann et al., 2010). The higher responsiveness to social stimuli of LAB males may explain the large tachycardia displayed by these animals during sensory contact with a conspecific in our experimental protocol. However, it is important to consider that in the present study LAB rats showed higher cardiac stress reactivity also when exposed to a non-social stressor, i.e. restraint test.

Two consecutive stressors did not affect circadian rhythmicity of heart rate, neither in HAB nor in LAB rats. The same evidence was found also for body temperature and physical activity. Animals with high anxiety-related behavior were characterized by higher temperature during both the active and inactive phase of the light-dark cycle before and after the stressors, although no differences in physical activity were detected. In a previous study, Liebsch and colleagues (1998a) reported that HAB and LAB lines did not differ in body temperature and locomotor activity neither at rest nor on the days following a stress test (elevated plus maze). In the same experiment, the rise of body temperature immediately following stress exposure (elevated plus maze test and social defeat) was

larger in LAB rats, but rapidly decreased. Conversely, body temperature remained elevated for almost 3 h after stress in HAB rats. In our study, body temperature stress response was different between the two lines only during social confrontation, where LAB rats exhibited a larger activity response. Conversely, the higher body temperature values measured during around-the-clock recording in HAB rats were not accompanied by significant between group differences in the level of physical activity. The elevated temperature values of HAB rats at rest may be explained by higher levels of sympathetic drive, a neural mechanism crucially involved in the control of core body temperature (Cannon and Nedergaard, 2004; Nakamura, 2011, Silva, 2006).

The expected increase in heart rate after the injection of a cholinergic muscarinic blocker (methylscopolamine) (Aubert et al., 1999; Japundzic et al., 1990) was larger in rats with low anxiety. In addition, the robust rise in heart rate was accompanied by a clear reduction of r-MSSD and a robust increase of LF/HF in both groups. Subsequent injection of a beta-blocker (atenolol) caused the return of heart rate and spectral index of HRV to their basal values in the two experimental lines, though more rapidly in HAB rats. Conversely, the time domain parameter remained suppressed. These results seem to support the presence of a larger parasympathetic regulation in rats with low levels of anxiety. Furthermore, the more efficient return of the electrocardiographic parameters to baseline values following sympathetic blockade in HABs may suggest an enhanced sympathetic control of cardiac activity in this rat strain. The double pharmacological blockade allowed to measure the intrinsic heart rate, which was found to be lower in HAB than LAB rats. In rats cardiac pacemaker is normally under tonic inhibitory vagal control and, therefore, intrinsic heart rate should be higher than its basal (modulated) value. This was the case in LABs, but not in HAB rats, where intrinsic heart rate was similar, or even lower, to the value recorded at rest. This may suggest a low level of autonomic control of heart rate in

anxious rats, in agreement with the lower values of HRV indexes in baseline conditions. In addition, the vagal-sympathetic effect index at rest was < 1in HAB rats, indicating that this rat strain is characterized by a sympathetic prevalence in baseline conditions (Goldberger, 1999). In contrast, the VSE > 1 in LAB animals further support the idea that these rats have a larger parasympathetic drive.

As expected (Whalen and Lewis, 1999), the injection of a beta-adrenergic agonist (isoproterenol) followed by the administration of a beta blocker (atenolol) induced, firstly, a robust increase of heart rate and, successively, the return to its basal value. The tachycardia due to isoproterenol was similar in the two rat lines, but the subsequent return to basal heart rate was more rapid in HABs, as just reported for the double pharmacological blockade. The pharmacological challenge induced the reduction of r-MSSD values, which remained substantially suppressed until the end of the recording period. In addition, also the time course of LF/HF, which increased after isoproterenol injection and returned to basal values following atenolol administration, was similar in HAB and LAB rats. The pharmacological stimulation of beta-adrenergic receptors enhanced arrhythmogenesis in both groups. Interestingly, the number of ventricular ectopic beats was higher in HAB rats, although not significantly. This result suggests a larger electrical instability in the animals with high anxiety-related behavior, likely associated to enhanced sympathetic regulation. Interestingly, ventricular arrhythmias has been proposed as one of the most reliable mechanisms for cardiac death among individuals with anxiety disorders (Rozanski et al., 1999). This hypothesis is supported by the fact that patients suffering from anxiety disorders are characterized by reduced HRV and, therefore, alterations in cardiac autonomic tone, i.e. increase sympathetic drive and/or impaired vagal control (Cohen and Benjamin, 2006). Actually, both alterations have been linked to the occurrence

of arrhythmias and increased cardiac mortality (Farrell et al., 1987; Lown et al., 1973; Rich et al., 1988).

Finally, morphometric analysis at sacrifice revealed that the two opposite trait anxiety levels did not affect significantly cardiac anatomy, except for a larger heart weight to body weight ratio in HAB rats, suggesting a slight degree of hypertrophy. HAB and LAB hearts did not show severe myocardial damage, as revealed by the slight amount of fibrosis.

Altogether, the data reported suggest that rats with opposite levels of anxiety-related behavior differ in cardiac autonomic regulation both in baseline and stressful conditions. Low-anxiety animals seem to be characterized by higher level of cardiac autonomic regulation at rest, although with a trend towards higher heart rate. The vagal indexes of HRV and the response to parasympathetic pharmacological blockade point to an elevated parasympathetic input to the heart in this rat line. In contrast, rats with high anxiety-related behavior show reduced HRV and lower heart rate values, which may be partly explained by the low values of intrinsic heart rate. The evaluation of cardiac sympathovagal balance in resting conditions seems to indicate a sympathetic prevalence in HAB animals, also supported by the results obtained in the pharmacological challenges. Cardiac stress reactivity is more pronounced in LAB rats when exposed to both social and non social stimuli, according to their active coping style. On the other hand, anxious rats do respond less to a stressful condition and this may be interpreted as a lack of adaptability to stressors.

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CHAPTER 5

EARLY MATERNAL SEPARATION HAS MILD EFFECTS ON CARDIAC AUTONOMIC BALANCE AND HEART STRUCTURE IN ADULT MALE RATS

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Stress; in press

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Abstract

Early life adverse experiences have long-term physiological and behavioral effects and enhance stress sensitivity. This study examined the effects of maternal separation (MS) on cardiac stress responsivity and structure in adulthood. Male Wistar rats were separated from the dams for 3 h per day from postnatal day 2 through 15. When exposed to 5-day intermittent restraint stress (IRS) as adults, MS and control rats showed similar acute modifications of cardiac sympathovagal balance, quantified via heart rate variability (HRV) analysis. In addition, MS had no effect on cardiac pacemaker intrinsic activity (as revealed by autonomic blockade with scopolamine and atenolol) and did not affect the circadian rhythmicity of heart rate, neither before nor after IRS. However, MS differed from control rats in cardiac parasympathetic drive following IRS, that was heightened in the latter but remained unchanged in the former, both during the light and dark phases of the daily rhythm. The evaluation of adult cardiac structure indicated that stress experienced during a crucial developmental period induced only modest changes, involving cardiomyocite hypertrophy, increased density of vascular structures, and myocardial fibrosis. The mildness of these functional/structural effects questions the validity of MS as a model for early stress-induced cardiac disease in humans.

1. Introduction

It is widely accepted that stressful events occurring early during postnatal life may alter neuroendocrine and behavioral stress responsiveness and lead to greater susceptibility for psychopathology throughout life (Heim and Nemeroff, 2001; Cirulli et al., 2009).

Animal models based on the disruption of mother-infant relationship have been used for a long time to better understand the short- and long-term effects of early adverse experiences (Faturi et al., 2010; Lehmann and Feldon, 2000; Levine, 2001). In rodents, one of the most commonly used experimental paradigms of early life stress is maternal separation, in which pups are removed from maternal nest repeatedly for a variable time period during the lactation phase. Brief maternal separation (3-15 min per day for several days), also called early handling (EH), has rather robust developmental effects and leads to attenuated adrenal and behavioral response to stress in adulthood (Levine, 1957; Levine et al., 1967; Meerlo et al., 1999). However, prolonged maternal separation (MS) (3h or more per day, for several days) has long term effects that appear to be highly variable, depending on details of the procedures and rat strains used (for review, see Lehmann and Feldon, 2000). A number of studies suggests that animals submitted to MS develop a phenotype that is opposite to that of individuals exposed to EH (Meaney et al., 1996; Plotsky and Meaney, 1993). In particular, maternally separated rats were reported to display larger hypothalamic-pituitary-adrenal (HPA) axis reactivity to acute challenges and higher levels of anxiety in adulthood (Aisa et al., 2007; Huot et al., 2002; Plotsky et al., 2005), potentially resulting in a larger vulnerability to stress-related disease. However, other studies report no major effects of maternal separation on adult adrenocortical activity and anxiety-like behavior (Hulshof et al., 2011; Slotten et al., 2006).

In recent years there is a growing interest in the effects of early adverse experiences on cardiovascular system function and structure. Individual features of cardiovascular regulation result from a dynamic interaction between genetically programmed developmental processes and environmental conditions (Tucker et al., 1984). During ontogeny, cardiac development and maturation partly depend on and interact with input from the autonomic nervous system (ANS) (Claycomb, 1976; Larson and Porges, 1982; Tucker and Johnson, 1984a; Tucker, 1985). For these reasons, stress experienced in this crucial phase may interfere with proper autonomic fiber distribution to the myocardial tissue, which in turn might lead to persistent changes in the functional and morphological characteristics of the heart (Tucker et al., 1984; Tucker and Johnson, 1984b).

In humans, epidemiological evidence suggests that unfavorable events experienced early in life are associated with an increased susceptibility to develop heart disease in adulthood. For instance, the Adverse Childhood Experiences Study reported that childhood abuse and neglect are closely associated with the most important risk factors for ischemic heart disease, such as smoking, obesity, physical inactivity and depression (Dong et al., 2004). However, such epidemiological studies provide no evidence for causal relationships and so far only few experimental studies with animal models have investigated the direct link between early adverse experiences and cardiovascular (dys)function (Loria et al., 2010a; Loria et al., 2010b; Sanders and Anticevic, 2007). On the one hand, results of these studies suggest that maternal separation does not influence baseline values of heart rate and blood pressure. On the other hand, in one study maternal separation increased heart rate responsivity to an acute stressor in adult borderline hypertensive rats, with no significant changes in blood pressure response (Sanders and Anticevic, 2007). Furthermore, early life stress rendered adult rats more susceptible to Angiotensin II-induced hypertension, tachycardia and vascular inflammation, which may

contribute to the pathogenesis of cardiovascular disease (Loria et al., 2010a,b). Altogether, the data collected so far suggest that maternal separation may contribute to adult cardiovascular morbidity; nonetheless, it is still unknown whether the autonomic control of cardiac function is affected, at rest and during exposure to acute environmental stimuli and whether the early life adverse experience of maternal separation may alter the development of adult heart morphology and tissue anatomy.

Studies in both humans and animals report that impaired cardiac autonomic regulation characterizes many pathological conditions of cardiac (Brook and Julius, 2000; Thayer et al., 2010) and non cardiac origin (Ewing et al., 1985; Thayer et al., 1996). In humans, the analysis of heart rate variability (HRV), which describes the small beat-to-beat differences in heart rate is a non-invasive approach to gather information about the modulation of the two branches of the ANS to the heart (Task Force, 1996). The same approach has also been successfully applied to rat ECG recordings (Aubert et al., 1999; Sgoifo et al., 1998).

In this study, we tested the hypothesis that adverse events experienced early during postnatal life may interfere with the development of the heart and its autonomic neural control, possibly leading to an increased stress susceptibility in adulthood. In particular, we assessed whether maternal separation in rats induces long-lasting modifications of: (i) cardiac autonomic regulation, both at rest and under challenging conditions, namely restraint stress and pharmacological autonomic blockade, and (ii) cardiac architecture, in terms of gross morphological changes, vascular density and myocardial structural damage.

2. Methods

2.1 Animals and housing

Twenty-four male Wistar rats were used in this study. All efforts were made to reduce animal pain or discomfort. Experiments were conducted in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the University of Parma Animal Welfare Committee. Female and male Wistar rats (Charles River, Calco, Italy) were paired for a period of 10 days, after which the males were removed. Since then, pregnant females were left undisturbed, except for a daily visual check for the presence of pups. The day of birth was defined as pups being present by 10:00h and was designated as day 0. On postnatal day 1, the litters were culled to eight pups, four males and four females. At weaning (3 weeks of age), they were housed with same-sex siblings. No more than two male pups from each litter were used for any given measure. During the entire experiment, all animals were kept under controlled temperature (22 ± 2 °C) and lighting (lights on from 07:00h to 19:00h) conditions. Ad libitum access to food and water was provided throughout the study.

2.2 Maternal separation

Maternal separation (MS), which consisted of daily separation of the litter from the dam for 3 h (9:00h-12:00h), was performed from postnatal day 2 to postnatal day 15, according to previous studies (Plotsky and Meaney, 1993, Hulshof et al., 2011). Each litter was removed from the nest and transferred to another room, to prevent vocal communication between mother and pups. During this 3-hour period, the pups were placed with their

siblings in glass beakers in a water bath set at 32-33°C, consistent with normal nest temperature (Schmidt et al., 1986), in order to prevent body temperature drop (figure 1). In fact, the reduction of litter's body temperature was shown to increase maternal care upon reunion (Leon et al., 1978; Stern and Johnson, 1990), which was found to reduce behavioral and neuroendocrine stress reactivity (Francis et al., 1999; Liu et al., 1997).

During the separation period, the dams remained in the home-cage. Following 3-hour MS period, pups were returned to the home cage. Control pups belonged to other (independent) litters and were left undisturbed all throughout the preweaning period in their mother's nest.

Figure 1. Pups in the glass beaker in the water bath during the MS period.

2.3 Radiotelemetry system

The radiotelemetry system employed in this study consisted of flat transmitters measuring 25 x 15 x 8 mm (TA11CTA–F40, Data Science International, St. Paul, MN, USA) and

platform receivers. At 4 months of age, the animals were anesthetized with tiletamine hydrochloride + zolazepam hydrochloride (Zoletil, 200 mg/Kg, s.c.) and the transmitters chronically implanted according to a surgical procedure that guarantees high-quality ECG recordings also during sustained physical activity (Sgoifo et al., 1996). Immediately after surgery, rats were individually housed and injected for 2 days with gentamicine sulfate (Aagent, Fatro, 0.2 ml/Kg, s.c.). The animals were allowed 10 days of recovery before the start of experimental recordings.

2.4 Outline of adult manipulations

An overview and time line of the measurements that were performed in adulthood is provided in figure 2. Four-month-old MS (n=12) and control (n=12) rats were exposed to an intermittent restraint stress (IRS) protocol. Animals were subjected to restraint stress on 5 consecutive days. Each restraint session consisted of confinement in a wire-mesh tube for 15 min (inner diameter 6 cm, length: 20 cm). Eight days before and after IRS protocol, pharmacological blockade of the two branches of the ANS was performed (see below for details). The weeks in between the pharmacological tests and the IRS protocol were used to assess the daily rhythmicity of heart rate. From the day of surgery, the animals were weighed on a weekly basis until the day of sacrifice.

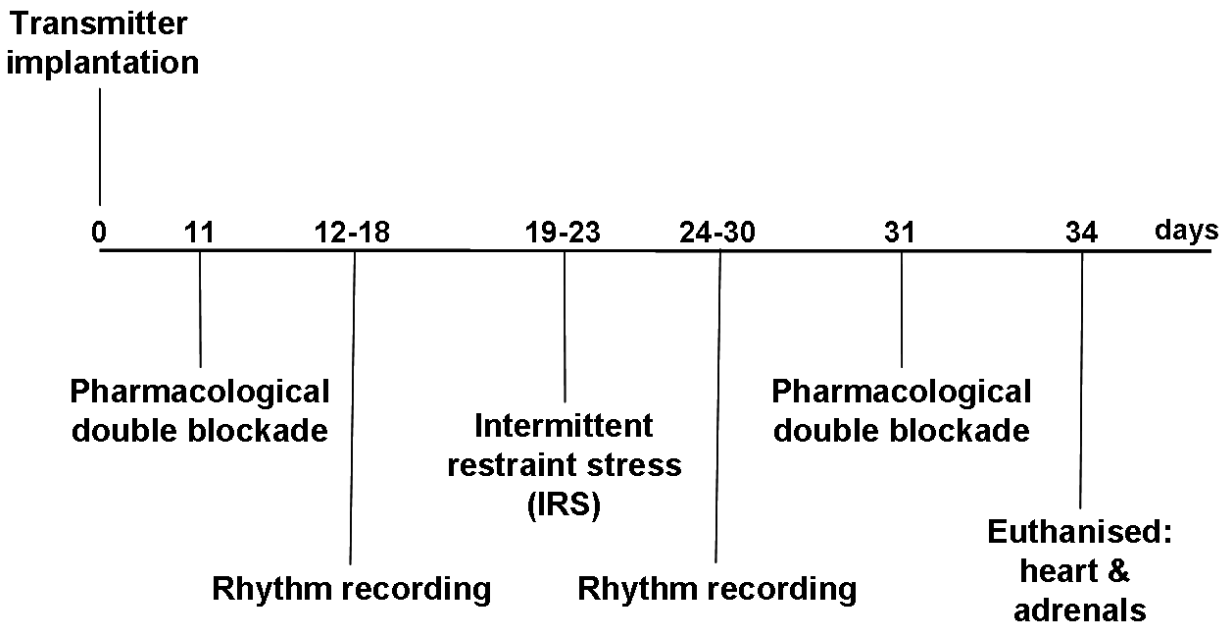

Figure 2. Schematic diagram of the experimental protocol applied to control and maternally separated rats in adulthood.

Transmitter implantation = surgical chronic implantation of transmitters for radiotelemetric ECG recordings.

Pharmacological double blockade = pharmacological blockade of the two branches of the autonomic nervous system with methylscopolamine (muscarinic receptor antagonist) and atenolol (beta-adrenergic receptor antagonist).

Intermittent restraint stress (IRS) = daily exposure to restraint stress for 5 consecutive days.

Rhythm recording = around-the-clock, radiotelemetric ECG recording for HR rhythmicity evaluation.

Euthanised, heart & adrenals = euthanasia of the rats and subsequent removal of hearts and adrenals.

2.5 Pharmacological autonomic blockade

Competitive muscarinic receptor antagonist methylscopolamine (0.05 mg/kg) and sympathetic blocker atenolol (2 mg/kg) (Sigma, St.Louis, MO, USA) (Ngampramuan et al., 2008) were injected s.c. to block vagal and sympathetic influences to the heart in MS and control animals. After baseline ECG recording, methylscopolamine was injected and the ECG recorded to evaluate the effect of parasympathetic blockade; 15 min afterwards,

atenolol was administered into the same animals to determine intrinsic heart rate. Intrinsic heart rate is established when the cardiac autonomic nervous system is completely blocked, which is supposed to take place approximately 10-15 min after the sympathetic blocker injection (Safa-Tisseront et al., 1998; Sant'Ana et al., 2011; Souza et al., 2009). The pharmacological test was performed twice, 8 days before and 8 days after IRS (figure 2).

2.6 Electrocardiographic data collection and analysis

ECG waves were acquired on a personal computer via ART-Silver 1.10 data acquisition system (Data Sciences Int., St. Paul, MN, USA) with 1000 Hz sampling frequency. Continuous ECG recordings were performed during the 1st and 5th restraint stress episodes and the two pharmacological tests (figure 2) according to the following schedule: (i) restraint stress: 30 min baseline, 15 min test, 30 min recovery; (ii) pharmacological double blockade: 30 min baseline, 15 min following scopolamine injection, 45 min following atenolol injection. Chart5 software (ADInstruments, Sidney, Australia) was employed to calculate the average R-R interval duration (RR, ms), which corresponds to the average inter-beat-interval in a given time period. In addition, time-domain and frequency-domain parameters of HRV were quantified. The time domain indexes used in this study were the root mean square of successive differences between adjacent RR intervals (r-MSSD, ms) and the percentage of successive interval differences larger than 20 ms (pNN20, %). R-MSSD and pNN20 quantify short-term, high-frequency variations of RR and therefore estimate the activity of the parasympathetic nervous system (Stein et al.,1994). Frequency domain (fast Fourier transform) indexes were collected in accordance to the guidelines for frequency-domain computations of HRV (Task Force, 1996). We considered only low

frequency (LF; 0.2-0.75 Hz) and high frequency (HF; 0.75-2.5 Hz) bands of the spectrum, and their power was quantified as normalized units (n.u.). The power of LF represents the activity of both branches of the autonomic nervous system (Eckberg, 1991); the power of HF is due to the activity of the parasympathetic nervous system and includes respiration-linked oscillations of heart rate (Chess et al., 1975). The low frequency/high frequency ratio (LF/HF) estimates the fractional distribution of power, which is taken as an indirect measure of sympathovagal balance (Task Force, 1996). A stationary ECG signal is recommended to reliably perform short-term frequency-domain HRV analysis and the presence of artifacts could influence significantly the results (Task Force, 1996). For these reasons, those parts of ECG recordings which were non stationary and/or exhibited recording artifacts were excluded from the analysis.

2.7 Daily rhythm data collection and analysis

ECG waves were sampled around-the-clock for 60 s every 60 min in two different periods: (i) pre-IRS – 7 days – starting on the day after the first pharmacological test, and (ii) post-IRS – 7 days – starting on the day after the last restraint test (figure 2). ECG recordings were analyzed by means of a software package developed in our laboratory (Sgoifo, 2001) and RR and r-MSSD values were quantified as means of the 12-h light (resting) phase and 12-h dark (activity) phase.

2.8 Post-mortem measurements

Three days after post-IRS pharmacological blockade (figure 2), animals were euthanised. Under anesthesia (tiletamine hydrochloride + zolazepam hydrochloride, Zoletil, 200 mg/Kg,

s.c.) the heart was arrested in diastole with cadmium chloride solution (100 mM IV) and excised for subsequent morphological/morphometric analysis. Following heart removal, adrenals were also excised and weighed. Adrenal glands were also obtained from 20 additional five-months old rats (MS, n=10; control, n=10), which were not exposed to adult experimental manipulation.

2.8.1 Cardiac remodeling

The two atria, the right ventricle (RV) and the left ventricle (LV) inclusive of the septum were separately weighed, fixed in 10% buffered formalin solution, and used for morphometric studies. The following parameters were determined: heart weight (HW), LV weight (LVW), RV weight (RVW), LVW/HW and RVW/HW. LV free wall thickness and LV transverse diameters were morphometrically computed (Image Pro-plus). The LV chamber volume was calculated according to the Dodge equation (Dodge and Baxley, 1969). From each heart embedded in paraffin, 5 μm-thick left ventricular sections were cut from the equatorial slice and used for subsequent analyses.

2.8.2 Morphometric analysis

Sections stained with Masson's trichrome were analyzed by optical microscopy (magnification 250X) in order to evaluate the total amount of interstitial and reparative fibrosis (Beltrami et al., 1994) in the LV myocardium. According to a procedure previously described (Costoli et al., 2004), quantitative evaluation of fibrotic tissue was performed in 60 randomly selected fields from the subendocardium, midmyocardium and subepicardium, with the aid of a grid defining a tissue area of 0.160 mm^2 and containing 42 sampling points, each covering an area of 0.0038 mm^2. To define the volume fraction of fibrosis in the three layers of the ventricular wall, the number of points overlaying

myocardial scarring were counted and expressed as a percentage of the total number of points explored. For reparative fibrosis, the numerical density of fibrotic foci per unit area of myocardium was also determined.

2.8.3 Myocyte cell size

The cross-sectional area (CSA) of myocytes was determined by measuring the cell diameter in transversally oriented myocytes, but only in those cells where the entire nuclear profile was clearly defined. To obtain CSA, two diameters were measured and their mean value was used to compute the area. For each LV, 120 to 250 cardiomyocytes were analysed at a magnification of X1000.

2.8.4 Vessel density

The quantification of capillaries and venules was performed in sections stained with polyclonal rabbit anti-vW factor (dilution 1:100, Dako), that recognizes endothelial cells, followed by FITC-conjugated anti-rabbit secondary antibodies (Jackson Laboratory, Baltimore, PA, USA). Nuclei were recognized by bisbenzimide staining (Hoechst No. 33258, Sigma, St Louis, MO, USA). Morphometric sampling at X1000 magnification consisted of counting the number of capillary and venule profiles in a measured area of tissue sections of both the epimyocardium and endomyocardium in which myocytes are transversally oriented. Capillaries were distinguished according to their luminal diameter (range 4-6 µm) from venules (range 6-10 µm) in which vW factor positive profiles lack multiple layers of smooth muscle cells. The number of capillaries and venules per unit area of myocytes was computed. This approach was followed to eliminate the effects of variations caused by changes in the interstitial compartment. Sampling of vessel

measurements involved a minimum of 20 and a maximum of 30 microscopic fields for the LV of each animal (Maestri et al., 2003).

2.9 Data analysis and statistics

Values of all parameters were expressed as mean ± SEM. Statistical analyses were performed using SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA) and statistical significance for all tests was set at $p<0.05$.

In order to detect possible differences at rest between the two experimental groups, the values of RR interval and HRV indexes of 30 min baseline recordings preceding restraint and pharmacological challenges were statistically analyzed by means of two-way analysis of variance (ANOVA), with “postnatal treatment” as between-subject factor (2 levels: MS and control animals) and “time” as within-subject factor (2 levels: first and fifth restraint test; pre-IRS and post-IRS autonomic blockade). In order to analyze the effects of postnatal treatment on cardiac response to restraint and autonomic blockade we calculated delta values for each 5-min time point relative to the baseline and named them delta1, delta2, …delta9 for restraint tests, and delta1, delta2, …delta12 for pharmacological challenges. Statistical analysis was then performed on delta instead of absolute values to abolish possible group differences in stressor responsivity due to differences in baseline. Two-way ANOVA was applied to delta values, with “postnatal treatment” as between-subject factor (2 levels: MS and control animals) and “time” as within-subject factor (9 levels for restraint, 12 levels for pharmacological challenge).

Average light and dark phase values of RR and r-MSSD on each day before and after IRS were calculated. Control and MS values of RR and r-MSSD in baseline conditions (pre-IRS) during the light and dark phases were compared via Student’s “t”-test. Delta values

between each post-IRS day and average pre-IRS value of light and dark phases were computed. Statistical analysis on delta values was performed by means of two way ANOVA, with “postnatal treatment” as between-subject factor (2 levels: MS and control animals) and “time” as within-subject factor (7 days).

Following ANOVAs, posthoc analysis on ECG data was applied where appropriate using Student’s “t”-test, after checking for variance homogeneity by means of Levene test.

Values of body weight were analyzed via two-way ANOVA for repeated measures, with “postnatal treatment” as between-subject factor (2 levels: MS and control animals) and “time” as within-subject factor (6 weeks). The weight of adrenal glands at sacrifice was expressed as a ratio to the animal body weight (mg adrenal gland weight / 100 g body weight) and statistically compared by Student’s “t”-test. Statistical analysis of cardiac structural measures was also performed via Student’s “t” test when 2 groups were analyzed, or via ANOVA followed by “t” test for multiple comparisons.

3. Results

3.1 Electrocardiographic response to restraint

Two-way ANOVA applied to baseline values of RR and HRV parameters measured just before the 1st and 5th restraint episode did not reveal significant effects of group, time, or group x time interaction. This suggests that maternal separation did not induce clear changes in resting HRV indexes, neither at the beginning (1st episode) nor at the end (5th episode) of adult intermittent restraint period (Table 1).

Table 1 also reports the absolute values of RR and HRV parameters for each 5-min time point during and after the 1st and 5th restraint episode. Two-way ANOVA on delta values (values of each 5-min time point during restraint test and recovery relative to baseline) for the 1st restraint test revealed only a significant effect of time for RR ($F(8,176)=109.5$, $p<0.01$) and r-MSSD ($F(8,176)=9.3$, $p<0.01$). No significant effect of group was observed for any of the parameters considered. In other words, maternal separation did not affect the magnitude and temporal dynamics of the changes in sympathovagal balance recorded during and immediately after an acute stressor in adulthood. At the fifth (last) restraint test, the time course of RR and HRV parameters was again similar in MS and control rats (Table 1). Two way ANOVA on delta values revealed a significant effect of time for RR ($F(8,176)=91.9$, $p<0.01$), r-MSSD ($F(8,176)=16.6$, $p<0.01$), LF ($F(8,176)=5.2$, $p<0.05$), HF ($F(8,176)=5.2$, $p<0.05$) and LF/HF ($F(8,176)=7.3$, $p<0.05$). However, a significant effect of postnatal treatment was found only for r-MSSD ($F(1,22)=5.1$, $p<0.05$). Post hoc analysis on r-MSSD delta values revealed only sporadic differences between the two experimental groups, which were limited to a few time points in the recovery phase ($t_{delta5}(22)=2.06$, $p<0.05$; $t_{delta6}(22)=2.32$, $p<0.05$; $t_{delta8}(22)=2.61$, $p<0.05$; $t_{delta9}(22)=2.60$, $p<0.05$).

Table 1. Values (mean ± SEM) of average R-R interval (RR), time domain HRV parameters (r-MSSD; pNN20), frequency domain HRV parameters (LF; HF; LF/HF) during baseline conditions (30-min average value), restraint test (15 min) and recovery phase (45 min), in maternally separated (MS; n=12) and control (n=12) rats, at restraint tests 1 and 5.

	Recording Period	Group	RR (ms)		r-MSSD (ms)		pNN20 (%)		LF (n.u.)		HF (n.u.)		LF/HF	
			Restraint1	Restraint5	Restraint1	Restraint5	Restraint1	Restraint5	Restraint1	Restraint5	Restraint1	Restraint5	Restraint1	Restraint5
	Baseline	MS	210.8±4.43	211.8±5.14	6.87±0.45	7.58±0.83	1.10±0.22	1.87±0.57	30.63±2.53	29.88±3.06	69.37±2.53	70.12±3.06	0.48±0.07	0.42±0.05
		Control	209.9±3.45	207.7±5.90	5.84±0.68	5.50±0.77	1.19±0.32	1.06±0.54	32.88±3.38	32.04±3.35	67.12±3.38	67.96±3.35	0.57±0.10	0.56±0.09
Test	1 (0-5 min)	MS	122.8±0.97	125.5±1.60	3.47±0.77	2.24±0.34	0.29±0.16	0.22±0.09	47.85±2.85	47.89±2.23	52.15±2.85	52.11±2.23	0.98±0.11	0.96±0.09
		Control	121.2±1.08	122.1±1.23	1.69±0.17	2.07±0.22	0.12±0.08	0.10±0.05	43.01±1.84	48.06±1.61	56.99±1.84	51.94±1.61	0.77±0.06	0.90±0.07
	2 (5-10) min	MS	122.3±1.24	129.2±1.92	1.88±0.16	3.19±0.30	0.06±0.03	0.14±0.04	54.01±1.79	49.31±2.36	45.99±1.79	50.69±2.36	1.15±0.11	1.02±0.10
		Control	123.2±2.15	131.3±2.97	2.12±0.24	2.63±0.29	0.20±0.18	0.12±0.06	51.02±1.42	49.91±2.01	48.98±1.42	50.09±2.01	1.01±0.08	0.98±0.10
	3 (10-15 min)	MS	132.1±1.98	139.1±2.80	3.47±0.31	4.09±0.27	0.20±0.07	0.29±0.09	47.52±2.46	46.76±2.83	52.48±2.46	53.24±2.83	0.95±0.09	0.93±0.10
		Control	138.0±2.24	145.4±3.95	3.15±0.37	3.45±0.52	0.22±0.07	0.36±0.20	50.17±2.33	47.29±2.01	49.83±2.33	52.71±2.01	1.06±0.11	0.80±0.09
Recovery	4 (15-20 min)	MS	143.6±2.80	147.0±2.14	5.09±0.47	4.64±0.26	1.12±0.32	1.16±0.30	48.73±3.69	47.18±2.12	51.27±3.69	52.82±2.12	1.09±0.19	0.93±0.09
		Control	142.6±2.60	145.4±2.16	3.66±0.42	3.75±0.42	0.55±0.20	0.50±0.16	52.67±1.67	54.88±1.79	47.33±1.67	45.12±1.79	1.14±0.07	1.15±0.13
	5 (20-25 min)	MS	152.1±3.33	153.6±2.70	4.79±0.45	4.03±0.28	0.84±0.22	0.45±0.08	52.62±3.04	51.95±2.50	47.38±3.04	48.05±2.50	1.08±0.11	1.14±0.10
		Control	146.8±2.64	152.4±1.98	3.68±0.38	3.93±0.54	0.53±0.17	0.60±0.21	53.35±2.31	54.67±2.31	46.65±2.31	45.33±.2.31	1.19±0.11	1.16±0.15
	6 (25-30 min)	MS	152.6±3.41	154.5±2.51	5.00±0.43	4.32±0.39	0.99±0.26	0.60±0.17	46.96±3.62	45.34±2.45	53.04±3.62	54.66±2.45	0.99±0.16	0.87±0.09
		Control	147.1±2.92	154.9±1.52	3.51±0.24	4.03±0.62	0.37±0.1	0.57±0.25	53.44±2.20	50.73±1.14	46.56±2.20	49.27±1.14	1.09±0.15	1.00±0.06
	7 (30-35 min)	MS	154.5±3.79	151.8±2.20	4.46±0.46	4.39±0.36	0.98±0.26	0.88±0.22	44.34±3.32	43.75±3.24	55.66±3.32	56.25±3.24	0.77±0.08	0.85±0.13
		Control	151.7±1.96	154.7±3.03	4.07±0.40	3.49±0.35	1.10±0.32	0.50±0.18	50.15±1.98	46.60±2.35	49.85±1.98	53.40±2.35	1.04±0.08	0.91±0.08
	8 (35-40 min)	MS	157.5±3.85	160.5±3.77	4.05±0.39	4.53±0.24	0.48±0.15	0.42±0.11	47.45±2.85	46.72±1.77	52.55±2.85	53.28±1.77	0.97±0.12	0.91±0.06
		Control	149.7±2.30	159.8±3.66	3.64±0.43	4.19±0.51	0.54±0.25	0.55±0.22	50.57±2.71	44.33±2.02	49.43±2.71	57.67±2.02	1.09±0.13	0.82±0.06
	9 (40-45 min)	MS	158.8±4.75	168.4±5.72	4.06±0.36	4.09±0.28	0.34±0.14	0.33±0.11	42.18±3.83	41.92±3.64	57.82±3.83	58.08±3.64	0.82±0.14	0.71±0.09
		Control	158.4±3.63	174.1±5.65	4.49±0.55	4.14±0.51	0.72±0.38	0.54±0.15	43.14±3.19	41.13±3.35	56.86±3.19	58.87±3.35	0.73±0.09	0.76±0.09

3.2 Electrocardiographic response to pharmacological autonomic blockade

Two-way ANOVA applied to baseline values of RR and HRV parameters did not reveal any significant effect of group, time, or group x time interaction. Maternal separation did not induce changes in resting HRV indices, neither at pre-IRS nor at post-IRS pharmacological autonomic challenge (Table 2).

Table 2 also reports the absolute values of RR and HRV parameters for each 5-min time point during the 1st and 2nd pharmacological challenge. Two-way ANOVA on delta values applied to the 1st pharmacological challenge revealed a significant effect of time for RR ($F(11,242)=401.6$, $p<0.01$), r-MSSD ($F(11,242)=7.9$, $p<0.05$), LF ($F(11,242)=21.7$, $p<0.01$), HF ($F(11,242)=21.7$, $p<0.01$), LF/HF ($F(11,242)=16.8$, $p<0.01$). When applied to the 2nd pharmacological challenge, two-way ANOVA on delta values revealed a significant effect of time for RR ($F(11,242)=915.2$, $p<0.01$), LF ($F(11,242)=14.1$, $p<0.01$), HF ($F(11,242)=14.2$ $p<0.01$) and LF/HF ($F(11,242)=14.9$, $p<0.01$). However, significant effects of group were not observed; in other words, maternal separation did not affect cardiac autonomic responsiveness to vagal and sympathetic blockade, neither before nor after the intermittent restraint stress.

Table 2. Values (mean ± SEM) of average R-R interval (RR), time domain (r-MSSD; pNN20) and frequency domain HRV parameters (LF; HF; LF/HF), in baseline conditions (30-min average value) and after scopolamine (15 min) and atenolol (45 min) injection, in maternally separated (MS; n=12) and control (n=12) rats, at pharmacological challenges 1 and 2 (Test1 and Test2).

Recording period	Group	RR (ms)		r-MSSD (ms)		pNN20 (%)		LF (n.u.)		HF (n.u.)		LF/HF	
		Test1	Test2	Test1	Test2	Test1	Test2	Test1	Test2	Test1	Test2	Test1	Test2
Baseline	MS	203.6±3.89	205.9±5.41	6.47±0.70	6.01±0.61	1.39±0.38	1.72±0.57	36.29±3.50	31.55±2.35	63.71±3.50	68.45±2.35	0.57±0.07	0.50±0.06
	Control	205.3±4.23	200.7±5.76	5.38±0.56	5.60±0.57	0.93±0.26	1.57±0.44	36.86±1.96	38.78±1.83	63.14±1.96	61.22±1.83	0.61±0.05	0.64±0.07
Scopolamine ► 1 (0-5 min)	MS	136.8±2.51	138.5±1.09	1.47±0.17	1.69±0.16	0.09±0.04	0.05±0.02	47.28±2.39	48.34±2.62	52.72±2.39	51.66±2.62	0.94±0.09	0.99±0.11
	Control	136.7±3.65	137.1±2.39	1.66±0.20	1.73±0.19	0.06±0.02	0.08±0.04	50.28±1.95	48.36±3.26	49.72±1.95	51.64±3.26	1.05±0.08	1.01±0.11
2 (5-10 min)	MS	133.5±2.14	134.6±2.05	0.78±0.06	0.87±0.07	0.00±0.00	0.00±0.00	48.48±2.02	46.36±2.21	51.52±2.02	53.64±2.21	0.98±0.08	0.89±0.08
	Control	135.8±3.26	131.6±2.22	0.90±0.08	1.10±0.13	0.01±0.08	0.04±0.02	49.05±1.86	54.45±2.94	50.95±1.86	45.55±2.94	0.94±0.08	1.29±0.13
3 (10-15 min)	MS	131.9±1.70	135.8±2.52	0.99±0.14	0.76±0.06	0.01±0.01	0.00±0.00	48.69±3.29	48.49±3.73	51.31±3.29	51.51±3.73	1.03±0.12	0.94±0.12
Atenolol ►	Control	137.4±3.15	133.5±2.75	0.96±0.16	0.95±0.12	0.05±0.04	0.00±0.00	53.95±2.32	58.59±1.67	46.05±2.32	41.41±1.67	1.16±0.13	1.37±0.11
4 (15-20 min)	MS	155.2±1.42	154.7±1.28	1.79±0.24	2.76±0.45	0.28±0.09	0.50±0.19	54.32±2.45	55.67±2.34	45.68±2.45	44.33±2.34	1.18±0.12	1.32±0.12
	Control	155.2±2.22	155.6±1.77	1.93±0.34	1.72±0.19	0.26±0.12	0.12±0.03	48.24±2.90	50.23±3.27	51.76±2.90	49.77±3.27	1.00±0.11	1.01±0.12
5 (20-25 min)	MS	174.8±2.69	173.6±1.12	1.22±0.14	1.57±0.15	0.03±0.02	0.02±0.01	46.61±1.56	42.17±3.42	53.39±1.56	57.83±3.42	0.84±0.07	0.72±0.08
	Control	177.2±2.26	174.0±2.65	1.10±0.07	1.15±0.09	0.01±0.01	0.00±0.00	41.01±2.30	42.91±4.25	58.99±2.30	57.09±4.25	0.72±0.06	0.84±0.12
6 (25-30 min)	MS	185.5±2.09	181.4±1.87	1.25±0.14	1.48±0.15	0.04±0.02	0.04±0.04	46.43±2.77	38.13±3.16	53.57±2.77	61.87±3.16	0.91±0.09	0.66±0.08
	Control	185.2±2.55	180.1±2.86	1.38±0.09	1.50±0.15	0.02±0.01	0.01±0.01	38.28±1.94	39.88±4.84	61.72±1.94	60.12±4.84	0.64±0.05	0.68±0.11
7 (30-35 min)	MS	189.6±2.62	185.5±2.31	1.82±0.30	1.60±0.16	0.10±0.05	0.08±0.04	36.43±3.86	37.53±3.76	63.57±3.86	62.47±3.76	0.56±0.07	0.66±0.10
	Control	185.8±1.68	182.1±3.24	2.19±0.21	1.66±0.20	0.14±0.05	0.07±0.06	44.25±3.22	42.95±3.54	55.75±3.22	57.05±3.54	0.86±0.11	0.76±0.12
8 (35-40 min)	MS	191.0±2.73	186.9±2.42	1.65±0.28	1.52±0.13	0.07±0.04	0.00±0.00	41.67±4.27	38.89±4.01	58.33±4.27	61.11±4.01	0.83±0.15	0.63±0.08
	Control	187.5±1.49	185.9±3.22	1.50±0.17	1.54±0.16	0.04±0.02	0.02±0.01	33.21±3.80	36.98±2.52	66.79±3.80	63.02±2.52	0.49±0.07	0.61±0.05
9 (40-45 min)	MS	193.3±2.63	190.1±2.75	1.88±0.24	1.42±0.20	0.05±0.02	0.01±0.01	38.30±4.36	33.74±3.42	61.70±4.36	66.26±3.42	0.69±0.10	0.55±0.08
	Control	189.6±2.20	188.2±3.05	1.37±0.11	1.34±0.16	0.01±0.01	0.00±0.00	38.46±2.59	35.26±2.74	61.54±2.59	64.74±2.74	0.61±0.07	0.58±0.07
10 (45-50 min)	MS	196.5±3.20	191.8±2.39	1.68±0.17	1.32±0.14	0.09±0.06	0.03±0.03	45.18±1.56	32.19±5.57	54.82±1.56	67.81±5.57	0.78±0.08	0.48±0.11
	Control	191.7±3.13	189.9±3.22	1.44±0.12	1.31±0.11	0.08±0.04	0.03±0.02	41.89±2.75	37.24±5.12	58.11±2.75	62.76±5.12	0.64±0.08	0.73±0.17
11 (50-55 min)	MS	194.9±2.93	193.6±2.27	2.38±0.23	1.29±0.11	0.12±0.04	0.01±0.01	42.70±5.19	31.07±4.93	57.30±5.19	68.93±4.93	0.86±0.13	0.55±0.13
	Control	193.5±3.53	189.5±3.58	1.46±0.14	1.37±0.17	0.04±0.02	0.00±0.00	38.57±3.61	42.77±5.54	61.43±3.61	57.23±5.54	0.60±0.08	0.79±0.15
12 (55-60 min)	MS	196.7±2.65	195.4±2.76	2.10±0.28	1.37±0.10	0.11±0.05	0.00±0.00	34.98±4.35	34.00±5.00	65.02±4.35	66.00±5.00	0.70±0.10	0.60±0.12
	Control	196.9±3.04	189.2±3.77	1.27±0.16	1.09±0.14	0.00±0.00	0.00±0.00	29.37±3.42	39.00±5.22	70.63±3.42	61.00±5.22	0.46±0.08	0.82±0.19

3.3 Daily rhythms in cardiac activity

MS and control rats exhibited similar light and dark phase values of heart rate (RR) and vagal activity (r-MSSD) under baseline conditions, i.e. before the intermittent restraint stress period (RR_{light}: $t(22)=0.72$, p=n.s.; RR_{dark}: $t(22)=1.20$, p=n.s.; r-$MSSD_{light}$: $t(22)=0.51$, p=n.s.; r-$MSSD_{dark}$: $t(22)=1.45$, p=n.s.) (figure 3A,C).

Two way ANOVA on RR delta values (value at each post-IRS day relative to the pre-IRS reference value) (figure 3B) did not reveal any significant effect of group, time or group x time interaction, neither for the light nor for the dark phase. In other words, maternal separation did not affect daily heart rate rhythmicity, neither before nor after the intermittent restraint stress.

However, two way ANOVA on delta values of r-MSSD (figure 3D) revealed a significant effect of postnatal treatment for both the light and dark phase of the daily rhythm (r-$MSSD_{light}$: group, $F(1,22)=10.8$, $p<0.01$; r-$MSSD_{dark}$: group, $F(1,22)=36.5$, $p<0.01$). Control rats exhibited higher delta values of r-MSSD in the active (dark) and passive (light) phase of the circadian rhythm, with significant differences compared to MS counterparts on all days (1 to 7) following IRS for the dark phase ($2.6 \leq t(22) \leq 4.6$; $p<0.05$) and on days 1,2,3,4, and 6 following IRS for the light phase ($2.7 \leq t(22) \leq 4.4$; $p<0.05$) (figure 3D).

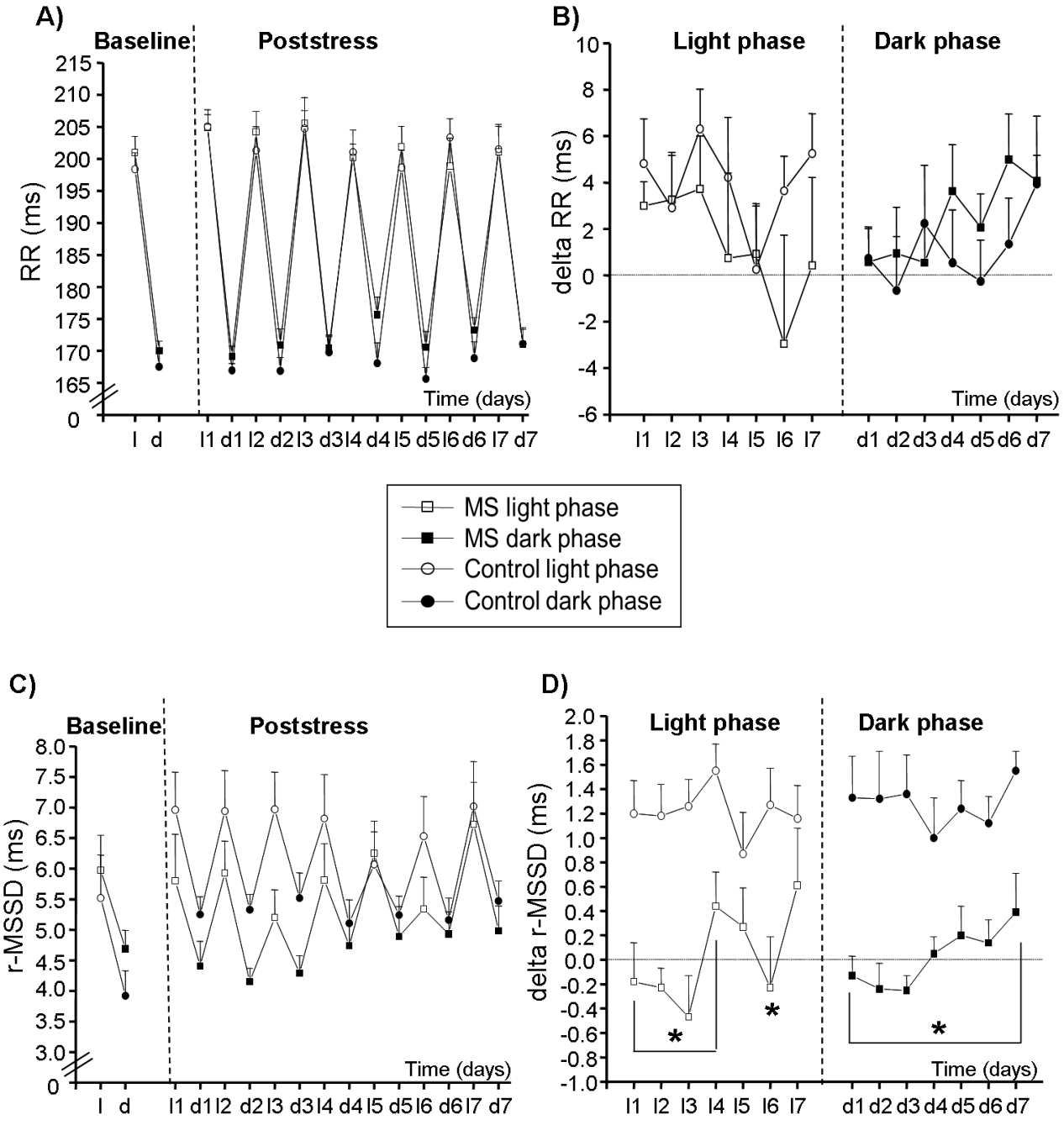

Figure 3. Daily rhythmicity of heart rate before and after the intermittent restraint protocol, in maternally separated and control rats. Average RR values (panel A) and r-MSSD values (panel C) for the 12h light phases (open symbols) and dark phases (solid symbols) before (baseline: l and d) and after (poststress: l1-l7 and d1-d7) the intermittent restraint stress (IRS). Delta values between each post-IRS day and the average pre-IRS value of light and dark phases are also reported (panel B and D), for both control (n=12) and maternally separated (MS, n=12) rats. *: significant differences between MS and CTR animals, $p<0.05$ (Student's "t" test).

3.4 Body weight and adrenal gland weight

Body weight values were similar in control and MS rats at the time of transmitter implantation (466±11 vs. 462±13 g) and sacrifice (457±9 and 460±11 g). Two-way ANOVA on body weight values revealed a significant effect of time ($F(5,110)=8.44$, $p<0.01$) due to post-surgery weight decline, that was similar in the two groups of animals. However, no differences were found between MS and control rats across the experimental protocol. In other words, maternal separation stress did not produce changes in body weight temporal dynamics, that were similar in control and MS rats both before and after IRS. However, maternally separated rats at sacrifice had significantly heavier adrenals compared to controls (MS: 20.99±1.18 mg/100g; control: 16.50±1.27 mg/100g) ($t(22)=2.60$, $p<0.05$). In addition, adrenals excised from 10 additional animals exposed only to early life manipulation were significantly larger than those of controls (n=10) (MS: 17.58±1.46 mg/100g; control: 13.38±1.13 mg/100g; $t(18)=2.27$, $p<0.05$).

3.5 Cardiac anatomy and myocardial structure

As shown in Table 3, slight differences were observed between MS and control rats as far as LV weight and LV linear parameters are concerned. LV weight and its ratio to heart weight were significantly lower in MS group, whereas RV weight was similar (Table 3). As a result of modest reductions of both LV length (-3%) and equatorial diameter (-6%), chamber volume was significantly smaller (-14%) in MS rats compared to control rats (Table 3). To determine whether these changes in gross cardiac anatomy were accompanied by changes in cardiomyocyte dimensions, myocyte cross sectional area (CSA) was measured. In the MS group, cardiomyocyte CSA was 5% larger than in the

controls (294.09±3.92 vs. 278.79±2.28 μm^2; t(22)=2.34, p<0.05), suggesting that maternal separation induced a mild hypertrophy of individual cells.

Table 3. Values (mean ± SEM) of gross cardiac characteristics, myocardial fibrosis in the left ventricle and vascular distribution in the left ventricle, in maternally separated (MS; n=12) and control (n=12) rats.

	Control	MS
LVW, mg	889.5± 22.08	818.8± 20.17 *****
RVW, mg	201.9 ± 7.01	201.8 ± 8.11
LVW/HW,mg/mg	0.908 ± 0.005	0.802 ± 0.006 *
RVW/HW, mg/mg	0.192 ± 0.005	0.198 ± 0.006
LV Chamber length, mm	14.58 ± 0.27	14.20 ± 0.23
LV chamber equatorial diameter, mm	5.32 ± 0.18	5.02 ± 0.15
LV wall thickness, mm	2.11 ± 0.06	2.14 ± 0.06
LV chamber volume, mm3	220 ± 15.67	189 ± 11.90 *****
Perivascular fibrosis, %	0.316 ± 0.047	0.225 ± 0.043 *****
Interstitial fibrosis, %	0.158 ± 0.060	0.243 ± 0.056 *****
Capillary density, n/mm2	132.2 ± 16.61	178.8 ± 11.42 *****
Venule density, n/mm2	6.31 ± 1.02	9.13 ± 2.54

LV: left ventricle; LVW: left ventricular weight; RVW: right ventricular weight; HW: heart weight.
*: significant differences between MS and control rats, p<0.05 (Student's "t" test).

The morphometric analysis of the myocardium documented no significant differences between the two groups of animals in terms of total amount of myocardial fibrosis in the LV myocardium (MS: 0.468±0.077 % vs control: 0.474±0.080 %, n.s.). The volume fraction of myocytes was also unaffected by the experimental conditions (MS: 91.41±0.55 % vs control: 91.38±0.70 %, n.s). Perivascular and interstitial fibrosis, including small foci of collagen accumulation distributed in the myocardium, were present in both MS and control hearts. Interestingly, these forms of collagen accumulation were differently expressed in

the experimental animals. Interstitial fibrosis was slightly larger in MS rats when the ventricular wall was considered as a whole (Table 3) and also when the 3 different layers where separately examined, i.e. endomyocardium, midmyocardium and epimyocardium (MS: 0.12±0.06 %, 0.42±0.16 % and 0.18±0.07 %, respectively) compared to control group (0.02±0.02 %, 0.35±0.12 and 0.10±0.04 %, respectively). Perivascular fibrosis was more abundant in the midmyocardium and epimyocardium of control hearts (0.29±0.10 % and 0.76±0.14 % vs. MS: 0.19±0.06 % and 0.44±0.09 %, respectively), resulting in an overall larger amount of this form of collagen deposition in these animals (Table 3) (figure 4, panel A).

The morphometric evaluation of vascular structures in the LV myocardium (Table 3) showed that capillary density was significantly larger in MS rats compared to control counterparts (figure 4, panel B). Although also venule density was 1.5-fold increased in MS rats, this value did not reach statistical significance (Table 3).

A B

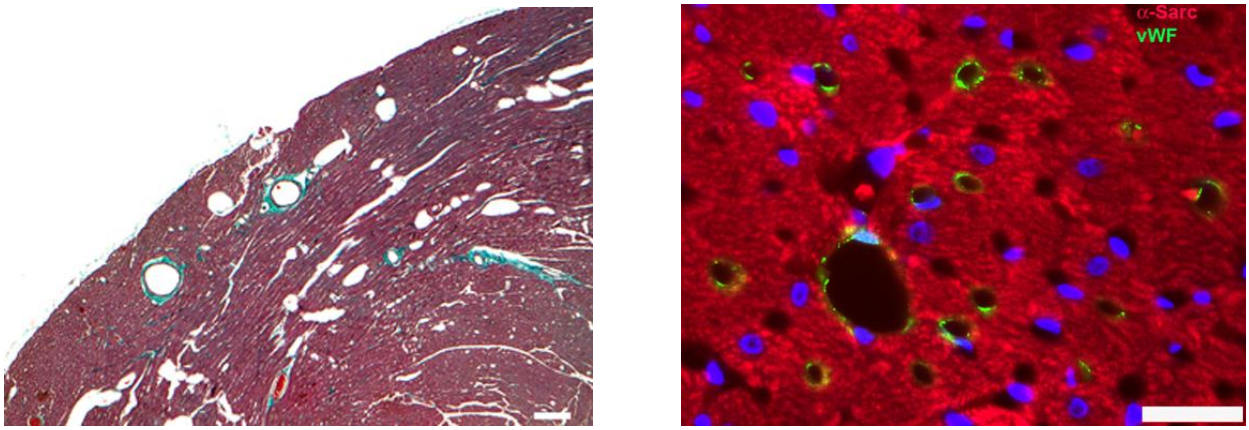

Figure 4. Panel A) Masson's Thrichrome staining of a cross section of the left ventricle from a control rat heart rat illustrating perivascular fibrosis (greeny blue) in the epi-midmyocardium. Interstitial fibrosis between muscle fibers (brownish) is virtually absent. Scale Bar=100 µm. Panel B) Immunofluorescence image of the myocardium of a MS rat heart documenting von Willebrand factor (vWF, green) labeling of capillary profiles flowing between cardiomyocytes recognized by the red fluorescence of α-sarcomerin actin (α-Sarc). Scale Bar=25 µm.

4. Discussion

The aims of this study were to investigate the long-term effects of early life adverse experience on (i) autonomic neural regulation of heart rate and (ii) cardiac morphological/morphometrical characteristics in male rats.

The results obtained suggest that maternal separation occurring in the first two weeks of neonatal life did not substantially alter adult cardiac sympathovagal balance, neither at rest nor under acute challenging conditions, namely restraint stress and pharmacological autonomic blockade. However, maternally-separated rats did not show the enduring vagal rebound exhibited by control counterparts in the days following adult intermittent stress, that involved both the night and light phase of the circadian rhythm. In addition, maternal

separation induced a few minor changes in cardiac anatomy, involving modest degrees of cardiomyocyte hypertrophy, increased angiogenesis, and myocardial damage.

In this study, early maternal separation was combined with a stress protocol based on intermittent restraint episodes in adulthood. Maternal separation did not change in a significant manner the acute response to a single restraint test and the habituation profile of cardiac autonomic response through repeated restraint episodes. Interestingly, in an experimental procedure resembling the present one (i.e. a 2-week daily maternal separation followed by intermittent restraint stress in adulthood), Hulshof and colleagues (2011) reported a similar lack of differences between control and MS rats in HPA axis stress reactivity and habituation (i.e. similar plasma ACTH and CORT levels).

In the present paper, cardiac autonomic balance was assessed by means of a large number of heart rate variability indexes, belonging to both time and frequency domains. During restraint test 1 and 5, MS and control animals exhibited a similar increase in cardiac chronotropism and an incomplete return to baseline heart rate values during the immediate post-test period. Concomitant, reduced values of time-domain HRV indexes and HF power point to a withdrawal of the parasympathetic drive to the heart (Kleiger et al., 1993), that did not recover to initial values until the end of the test. Accordingly, the increase of LF power and LF/HF ratio during both restraint and recovery period points to a shift of autonomic regulation towards a sympathetic prevalence (Aubert et al., 1999). Noteworthy, these HRV data also indicate that the dynamics of cardiac autonomic balance were similar across restraint episodes in MS and control rats, i.e. both groups did not exhibit habituation-like effects.

As expected (Aubert et al., 1999; Japundzic et al., 1990), the injection of a cholinergic muscarinic blocker (methylscopolamine) induced a robust increase of heart rate, accompanied by a clear reduction of the values of r-MSSD and pNN20. The decrease of

HF power was accompanied by a rise of LF power and LF/HF ratio. Subsequent injection of a beta-blocker (atenolol) caused the return of heart rate and spectral indexes of HRV to their basal values, whereas time domain parameters remained substantially suppressed. Overall, the results obtained with the double pharmacological autonomic blockade suggest that maternal separation had no substantial effects on cardiac pacemaker intrinsic activity, neither before nor after the intermittent restraint stress.

So far few studies investigated the long-lasting consequences of maternal separation on cardiovascular function and, to our knowledge, the present work is the first one that explored long-term cardiac autonomic effects through heart rate variability parameters. The lack of differences between maternally separated and control rats, both at rest and during acute environmental and pharmacological challenge, supports the idea that maternal separation per se does not bring about a cardiac autonomic pathophysiologic phenotype. In this regard, our findings are in agreement with a number of other studies that did not find any clear effect of maternal separation on adult baseline heart rate and arterial blood pressure (Loria et al., 2010a; Loria et al., 2010b; Sanders and Anticevic, 2007). However, in the study by Sanders and Anticevic on borderline hypertensive rats MS animals showed higher heart rate responsivity to restraint stress compared to control animals, despite blood pressure response was similar in the two groups. Although the maternal separation protocol in the latter study was similar to ours, it may well be that borderline hypertensive rats are more susceptible to environmental stressors and that different blood pressure phenotypes play a pivotal role in mediating the differences in autonomic regulation of cardiac stress response.

The recording of the daily rhythms of heart rate and cardiac vagal activity allowed to further explore the long-lasting effects of maternal separation on cardiac chronotropy and its autonomic neural regulation, before and after the period of adult intermittent stress.

Maternal separation and adult exposure to five restraint episodes did not affect the light-dark oscillation of heart rate. However, while autonomic control of cardiac chronotropy remained largely unchanged in MS rats, control counterparts showed a prolonged increase of cardiac parasympathetic drive following IRS, involving both the dark and light phases of the circadian rhythm. Since the average night and day values of heart rate were similar before and after the stress period, we hypothesize that the increase of parasympathetic drive observed in control rats following IRS was accompanied by a concomitant enhancement of sympathetic tone.

This peculiar phenomenon, that was observed in control but not in maternally separated rats, might be called “enduring vagal rebound” or “persistent vagal rebound”. In the literature, “vagal rebound” usually has a rather different meaning; it refers to short-term, briefly-lasting vagal hyperactivity following a stressor, a sympathetic overdrive or reperfusion after acute myocardial infarction (Chiladakis et al., 2001; Mezzacappa et al., 2001). In this paper, it is a relatively persistent, long-term consequence of an intermittent stressor and can be viewed as an adaptive response of the organism. In fact, the increase of vagal activity that often occurs to counterbalance stress-induced sympathetic activation is known to be associated with a reduced risk of cardiovascular disease and mortality (La Rovere et al., 1998).

If this point of view is correct, one may infer that cardiac autonomic neural control of adult animals that experienced early life challenge was less stress-responsive (i.e. less flexible ?) compared to control counterparts, thus less favourable in terms of resilience. On the other hand, one may also maintain that the lack of changes in autonomic modulation observed in MS rats was the consequence of a shift of the regulatory range, that implied a reduced sensitivity to adult intermittent stress exposure (Koolhaas et al., 2011). Indeed, several studies support the view that early maternal environment prepares the regulatory

range of the offspring for the conditions it may have to cope with in adulthood (Champagne et al., 2008; Gluckman et al., 2007; Kaiser and Sachser, 2005). An example of this is given in a recent study in mice by Heiming and co-workers: they showed that mice that were raised in a threatening environment exhibited less anxiety and more exploratory behavior as adults when confronted with challenging situations than animals that were raised in a stable environment (Heiming et al., 2009).

One may question that the data collected following scopolamine administration do not support the observed raise in vagal activity emerging from post-stress rhythm analysis. Indeed one may expect a somewhat larger tachycardic response to scopolamine in control animals after IRS. Likely, the reason for this discrepancy is that the second pharmacological challenge was performed during the light phase of the day after the end of rhythm recordings (day 31 in figure 1). If one looks at figure 2 (panel D), r-MSSD values of control rats on the last light phase (l7) were not significantly different anymore from corresponding MS values. In other words, the pharmacological challenge was performed when the “vagal difference” between control and MS rats was already gone.

In the current study, animals exposed to maternal separation and adult intermittent stress had heavier adrenal glands compared to control rats, that may reflect either an enhanced steroidogenesis in the cortical area or an increased catecholamine biosynthetic activity in the adrenal medulla (or both). However, increased adrenal weight in maternally separated rats that were not exposed to adult manipulations suggests that maternal separation per se is able to induce adrenal enlargement. As a matter of fact, the available data from the literature on the effects of postnatal stress on adrenal gland weight are not consistent. Some authors showed that 24-h maternal deprivation induces significantly heavier adrenals (Husum et al., 2002; Rots et al., 1996), while others reported no adrenal enlargement (Wigger and Neumann, 1999) or even lighter glands (Slotten et al., 2006).

Unfortunately, we did not determine plasma corticosterone concentrations in this study; therefore, any attempt to relate cardiac changes to increased corticosterone secretion would be far too speculative in this context.

The evaluation of the effects of maternal separation on cardiac architecture indicated a moderate reduction in LV weight and chamber dimensions in the absence of clear changes in myocardial structural composition. Myocardial fibrosis was minimally affected by MS and only interstitial deposition of collagen was moderately increased. These gross anatomical changes, in association with modest signs of cellular hypertrophy, likely reflect a physiological adaptation to maternal stress of the growing heart during early postnatal development. The possibility cannot be excluded that a stressor imposed during a delicate phase of cardiac development in which remarkable cellular changes occur may structurally affect the heart. It is well established that a switch from a prevailing hyperplastic to a predominant hypertrophic growth (Anversa et al., 1980) as well as apoptotic death of cardiomyocytes (Kajstura et al., 1995) all rapidly interplay within 3 weeks after birth in the rat heart. Whether MS-driven neurohormonal factors, implicated in physiologic and pathologic processes of the heart may alter cardiomyocyte turnover resulting in cardiac structural remodelling following animal growth is more than speculative. Slight hypertrophy of cardiomyocytes was observed here in the LV in association with a reduced mass and modest collagen deposition, suggesting that precocious cell loss and reactive hypertrophy may have occurred early during post-natal cardiac development. Interestingly, all these events are known to be evoked in the heart by the renin-angiotensin system (Sadoshima and Izumo, 1993), which in turn has been implicated in MS (Loria et al., 2010b).

The early postnatal period is also characterized by a dramatic growth of the coronary vascular bed and the functional capacity of the myocardium reaches adult values at approximately 16 days of age (Hopkins et al., 1973). The mean transmural number of

capillaries undergoes four-fold increase in the left ventricle from postnatal day 1 to 11 and the aggregate ventricular capillary length more than doubles between postnatal day 5 and 11 (Olivetti et al., 1980). Our data point to an increased capillarization of the left ventricular myocardium in maternally separated animals. The detected increase in the density of vascular structures is relevant under the contention that enlargement of cardiomyocytes, as shown in the myocardium of MS rats, would result in reduction in the number of capillaries per unit area (n/mm^2) according to morphometric principles. However, why promotion of angiogenesis or lack of regression of capillary formation should occur in the heart under this experimental conditions requires further investigation.

In conclusion, the present study on rats shows that daily 3-h maternal separation during the first two weeks of postnatal life did not affect in a significant manner adult intrinsic heart rate and acute cardiac autonomic responsivity to a psychological challenge. However, maternally-separated rats did not exhibit the enduring vagal rebound observed in control counterparts after adult intermittent stress, that might be viewed as an adaptive response aiming at favouring cardiovascular stress resilience. Moreover, maternal separation induced some – although minor – changes in cardiac anatomy, involving modest degrees of cardiomyocyte hypertrophy, increased angiogenesis, and myocardial damage. Whether these functional and structural changes could have evolved to a more pathologic cardiac phenotype later in life is plausible, but remains to be determined.

The overall mildness of cardiac functional and structural effects of maternal separation reported in this paper suggests that this approach might not be the best preclinical tool for modeling early-age, stress-induced cardiac disease in humans.

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CHAPTER 6

SUMMARY AND DISCUSSION

1. Summary of the results

The main scope of this thesis was the investigation of the pathophysiological mechanisms linking psychopathologies and cardiovascular disease. In particular, the studies here reported focused on the exploration of cardiac autonomic impairment, that seems to play a key role in the link between psychiatric and cardiovascular pathologies. To reach this purpose, different rodent models of psychological-cardiovascular comorbidity were implemented. A brief summary of the results is reported below.

In Chapter 2 and Chapter 3 the relationship between depression and cardiovascular dysfunction was analyzed in two different rodent models based on social stress. In Chapter 2 an experimental paradigm based on the exposure of adult rats to an adverse stress event (social defeat) followed by a prolonged period of social isolation was implemented. The aims of this study were: first, to confirm the efficacy of the stress procedure applied in determining a depressive-like state; second, to verify its impact on cardiac function and structure. Animals subjected to both social defeat and isolation displayed many physiological and behavioral depressive-like symptoms, including HPA axis negative feedback dysfunction, adrenal gland enlargement, altered circadian rhythmicity of body temperature and locomotor activity, reduced preference for sucrose consumption, and increased anxiety in the elevated plus maze. However, the depression-like state induced via this protocol was associated with only modest cardiac changes, including transitory alteration of heart rate circadian rhythm, lack of habituation of cardiac autonomic responsivity to an acute stressor, and moderate hypertrophy affecting the right ventricle of the heart.

The study described in Chapter 3 was aimed at verifying the effects of chronic social stress on cardiac autonomic regulation, electrophysiological properties and structural

characteristics. The results obtained suggest that a protocol based on repeated adverse social episodes (social defeat) represents a valid rat model for mimicking some features of human depression. Rats exposed to 12 social defeat sessions over a period of 4 weeks displayed physiological and behavioral symptoms of depression: decreased body weight gain, reduction of the circadian amplitude of heart rate and body temperature rhythms, functional alteration of the HPA axis, adrenal enlargement, onset of an anhedonic state, and increased immobility in the forced swimming test. After the chronic social stress period, the exposure of the rats to an acute stressor (restraint stress) failed to show modifications of cardiac autonomic stress reactivity explored via HRV analysis. In stressed animals, high-definition potential epicardial mapping performed before sacrifice revealed alterations of cardiac electrophysiological properties, including significant decrease in transversal conduction velocity, shortening of the effective refractory period, and increase in myocardial excitability. These data suggest that a depression-like state induced via chronic social stress is associated with altered myocardial electrical stability in a potentially pro-arrhythmic manner.

In Chapter 4 cardiac autonomic regulation was evaluated in a rodent model of anxiety based on the selective breeding of rats characterized by opposite levels of anxiety-related behavior (HAB and LAB). The main goal was to give further insights into the pathophysiological mechanisms linking anxiety and cardiovascular dysfunction. Rats with high anxiety showed lower heart rate and decreased HRV in baseline conditions, but also lower intrinsic heart rate which was associated to a shift of cardiac sympatovagal balance towards a sympathetic prevalence at rest. Conversely, less anxious animals were characterized by higher basal heart rate and HRV and elevated cardiac parasympathetic control. Cardiac stress response, i.e. tachycardia and vagal withdrawal, differed between the two rat lines, with anxious animals showing less responsivity to both social and non-

social stressors. Finally, the assessment of cardiac morphological and structural characteristics did not reveal significant differences between the two rat strains.

In Chapter 5 a rodent model of psychopathology based on early life stress was applied. In particular, the study focused on the long-term effects of a protocol of maternal separation (occurring during weaning) on cardiac autonomic regulation and cardiac structure in adult rats. The data suggest that a daily 3-h period of separation from the dam during the first two weeks of post-natal life did not alter autonomic regulation of heart rate in baseline conditions and in response to a pharmacological challenge, i.e. the pharmacological blockade of both branches of the ANS. In addition, no differences were detected in cardiac autonomic responsivity to an intermittent stress protocol in adulthood, i.e. 5 consecutive days of restraint stress. However, maternally separated rats failed to show the transient increase in parasympathetic drive after stress exposure, which characterized both the light and dark phase of the daily rhythm of control animals. Furthermore, stress experienced during the early phases of postnatal period induced some modest alterations of cardiac structure, involving cardiomyocyte hypertrophy, increased density of vascular structures, and myocardial fibrosis.

2. Heart rate variability in rodent models of psychopathology for studying cardiovascular comorbidity

The studies presented in this thesis share the common goal of evaluating cardiac autonomic regulation via heart rate variability analysis in different rodent models of psychopathology. HRV analysis was performed using many parameters belonging to both time and frequency domain (Task Force, 1996). Altogether, the results obtained suggest that HRV analysis can be a valid and reliable tool for investigating autonomic function in rats exposed to a variety of conditions and support the use of both time and frequency domain measures in order to get further insights into the neural mechanisms controlling heart rate. Time domain parameters are generally considered to be easily applicable to a variety of different experimental conditions because they do not need strict mathematical criteria. On the other hand, the application of frequency domain measures may be limited because the signal should satisfy several requirements in order to obtain a reliable spectral estimation (Task Force, 1996). From the data collected it is possible to maintain that not only time domain measurements, but also spectral measures represent a reliable option for the analysis of signals recorded from rats freely moving and freely behaving.

Both time and frequency domain indexes were able to detect the changes in autonomic regulation of heart rate when the animals were exposed to different challenges, namely stressful stimuli and pharmacological manipulation. In addition, the application of a number of measures in the same study was able to give additional information on the level of activity of the two branches of the ANS. The time domain parameters r-MSSD, pNN20 and pNN10 were confirmed to be able to detect the changes of parasympathetic activity (Stein et al., 1994). The spectral indexes allowed a more precise evaluation of the direction and

magnitude of the changes in sympathovagal balance, because they add information about the level of activity of the sympathetic system (Montano et al., 2009).

All the HRV parameters considered in this thesis were able to identify the autonomic variations that characterize stress response. All the stressors employed (open field, restraint stress, social stress) induced a vagal withdrawal confirmed by the reduction of the time domain parameters and of the power of the HF spectral component (Kleiger et al., 1993), which tended to return to basal values during the recovery period. In addition, the increase of LF power and LF/HF ratio occurring during stress response pointed to a shift of autonomic regulation towards a sympathetic prevalence (Aubert et al., 1999).

HRV analysis was found to be a valid tool also for detecting the variations due to pharmacological autonomic challenges, namely concomitant sympatho-vagal blockade and sympathetic stimulation and blockade. As expected (Aubert et al., 1999; Japundzic et al., 1990), the injection of a cholinergic muscarinic blocker (methylscopolamine) induced a robust increase of heart rate, accompanied by a clear reduction of the time domain indexes. Spectral analysis revealed the decrease of HF power associated with a concomitant rise of LF power and LF/HF ratio. Subsequent injection of a beta-blocker (atenolol) caused the return of heart rate and spectral indexes to their basal values, whereas time domain parameters remained substantially suppressed. Furthermore, the administration of a beta-adrenergic agonist (isoproterenol) induced a pronounced rise of heart rate accompanied by reduction of the vagal indexes and increase of LF power and LF/HF ratio (Whalen and Lewis, 1999).

3. Cardiovascular dysfunction in rodent models of depression

Major life stressful events are widely recognized contributors to the development of depression in humans (Kendler et al., 1999). Animal models based on the exposure to uncontrollable and unpredictable stressors represent one of the most used strategies to mimic physiological and behavioral correlates of human depression (Anisman and Matheson, 2005). The paradigms used to generate stress in laboratory conditions often do not resemble the challenges which animals normally face within their natural environment and may therefore induce scarcely reliable physiological and behavioral responses. Since social stress is a chronic recurring factor in the everyday lives of all mammal species (Blanchard et al., 2001), animal models that involve some sort of social challenge seem to be more appropriate. In this regard, social defeat in rodents is an experimental paradigm which mimic loss of social control and it is associated with the onset of many stress-related pathologies (Koolhaas et al., 1997).

The data reported in Chapter 2 and Chapter 3 support the idea that exposure to adverse social events may sensitize to the subsequent development of a depressive-like state. Both stress protocols applied, i.e. a single episode of social defeat followed by a prolonged period of social isolation and exposure to repeated social defeat events, were able to determine physiological and behavioral depressive-like symptoms in rats, according to previous experimental evidences (Becker et al., 2008; Rygula et al., 2005). The use of a variety of parameters for evaluating the onset of depression strongly confirmed the validity of both experimental procedures as models of depression in rats. Body weight alterations, biological rhythms disturbances and HPA axis dysfunction constitute some of the most prevalent physiological signs of depressive illness (American Psychiatric Association, 2000) and were detected in rats exposed to both social stress protocols applied. In

addition, stressed rats displayed the onset of anhedonic behavior, learned helplessness and elevated levels of anxiety, which are considered reliable behavioral correlates of a depressive-like state (American Psychiatric Association, 2000; Loas, 1996; Porsolt, 1978). The results described in this thesis suggest that the depressive-like state induced via social stress was associated with some alterations of cardiovascular function, although further studies are required to confirm the validity of these rat models of depression as valid preclinical approaches for the comprehension of the biological substrates underlying depression-cardiovascular comorbidity. Our findings support the previous evidences highlighted by Grippo and colleagues, who found that chronic mild stress was associated with several cardiac alterations, including elevated resting heart rate, reduced heart rate variability and increased risk of arrhythmic events (Grippo et al., 2002; Grippo et al., 2003; Grippo et al., 2004). Although our results do not support a critical impairment of cardiac autonomic function, they indicate that chronic social stress was able to induce alterations of cardiac electrophysiological properties 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.

4. Cardiovascular dysfunction in a rodent model of trait anxiety

The results obtained in Chapter 4 shed further light into the pathophysiological mechanisms linking anxiety and cardiovascular disorder, focusing in particular on the role of autonomic cardiac control. To our knowledge, this is the first study that extensively investigates autonomic properties in a model of rats selectively bred for opposite levels of trait anxiety. Up to now, only few studies examined the vegetative characteristics of these two rat strains and stress reactivity was mostly evaluated via behavioral and neuroendocrine measurements (Landgraf et al., 1999; Landgraf and Wigger, 2002; Liebsch et al., 1998).

In Chapter 4 the characteristics of ANS activity in a variety of conditions were explored, i.e. baseline, during and after stress exposure and under pharmacological challenges. In order to better understand the role of the sympathetic and parasympathetic branches in controlling heart rate, ECG signals were analyzed and values of different HRV indexes were calculated. The most important finding of this study is that rats with opposite levels of anxiety-related behavior showed different cardiac autonomic regulation both in baseline conditions and in response to stressful stimuli. In addition, they differed in the value of intrinsic heart rate, suggesting that the two rat lines were probably characterized not only by different levels of autonomic control, but also by distinct properties of the cardiac pacemaker.

From the data collected it is possible to infer that the autonomic properties associated with the presence of high levels of anxiety may be considered as less adaptive for the individual when confronted with challenging situations. However, further studies are needed in order to confirm this hypothesis.

5. Cardiovascular dysfunction in a rodent model of early life stress

A growing body of literature highlighted that early adverse events experienced in the postnatal period may increase the susceptibility to exaggerated stress reactivity and psychopathologies throughout life (Cirulli et al., 2009). In addition, there is also epidemiological evidence linking early life stress and the development of cardiovascular diseases in adulthood (Dong et al., 2004), although a reliable causal relationship is not yet established. Animal models of early adverse experiences, in particular maternal separation, may help in understanding the direct effects of postnatal stress on the heart. Until now, only few studies have investigated this link and the data obtained are not consistent. Also the data reported in Chapter 5 did not clarify whether postnatal stress, i.e. maternal separation, seriously affects cardiovascular function.

During early postnatal period the heart undergoes a series of modifications fundamental for its complete development, regarding in particular cardiomyocyte turnover (Kajstura et al., 1995), ANS fibers distribution to myocardial tissue (Tucker and Johnson, 1984) and vascularization (Olivetti et al., 1980). The data obtained in this study suggest that the daily separation from the dam during the first weeks of postnatal period did not significantly affect the correct development of the heart. Actually, maternal separation did not induce adult alterations of cardiac autonomic balance neither in baseline conditions nor during and after stress exposure. In addition, maternally separated rats showed only modest structural cardiac alterations, including cardiomyocyte hypertrophy, increased density of vascular structures, and slight degree of myocardial fibrosis.

The mildness of cardiac functional and structural effects of maternal separation found in this study suggests that this model of early life stress may not represent the best pre-clinical tool for modeling early-age, stress-induced cardiac disease in humans.

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