UNIVERSITAøDEGLI STUDI DI PARMA

Dottorato di ricerca in Fisiopatologia Sistemica

Ciclo XXI

Cardiac Autonomic Stress Responsivity in rodents

Coordinatore:

Chiar.mo Prof. Ezio Musso

Tutor:

Chiar.mo Prof. Andrea Sgoifo

Dottoranda: Francesca Mastorci

Table of Contents

General Introduction

1. Stress and Cardiovascular dysfunction

1.1 Psychobiological mechanisms underlying cardiovascular disease

2. Stress and Depression

2.1 Neuroendocrine mechanisms in depressive disorder

2.1.1 Dysregulation of the Hypothalamicópituitaryóadrenal axis

2.1.2 Serotonin alterations in depression

3. Comorbidity between depression and cardiovascular dysfunction

3.1 Potential common mechanisms underlying depression and cardiovascular function

3.1.1 Stressor reactivity, depression and cardiovascular dysfunction

3.1.2 Neurohumoral and immune function

3.1.3 Autonomic nervous system dysfunction

3.1.4 The role of central neurotransmitter systems

4. Social stress

4.1 Acute social stress

4.2 Chronic social stress

4.3 Social stress and cardiovascular pathophysiology

Box: Cardiac sympathovagal balance explored via Heart Rate Variability

Time Domain Methods

Frequency Domain Analysis

Non-Linear Methods

5. Rodent models of depression

5.1 Learned Helplessness

5.2 Behavioral Despair Paradigm

5.3 Chronic Mild Stress

5.4 Prenatal stress as an animal model of depression

5.5 Depression induced via social stress

6. Neurobiology of cardiovascular response to stress

6.1 Key nuclei implicated in the central autonomic regulation of stress response

6.2 Role of 5-HT$_{1A}$ receptors in cardiovascular response to stress

Outline of this thesis

References

1. Stress and Cardiovascular dysfunction

Cardiovascular disease (CVD), including coronary heart disease (CHD), high blood pressure, and stroke, are the main causes of serious illness, and a major cause of death and reduced quality of life in developed countries (Domanski et al., 2002). Coronary heart disease, in particular, is the largest single cause of death in Europe, accounting for 21% of male and 22% of female deaths (Alleander et al., 2008). It is also the leading cause of death in people aged less than 75 years (20% male and 19% female death). However, death rates have been declining for the past three decades in many countries, due primarily to prevention by traditional risk factors, as cigarette smoking, hypertension, diabetes mellitus, dyslipidemia, family history of premature coronary disease, and sedentary lifestyle (Merz et al., 2002); and also to increased survival following acute coronary events. This means that the prevalence of diagnosed CHD in the population has been rising. Recent data from the USA indicate that 6.5% of the population have a history of heart attack (myocardial infarction) or angina pectoris (Anon, 2007). CVD is caused by the interaction of physiological, environmental, and behavioral variables, many of which are a function of individualøs lifestyle and can be controlled and modified by the individual.

The collection of contributors to cardiovascular disease suggests that behavioural factors play an important role in its aetiology; this has led to an interest in identifying lifestyle and psychological markers of increased risk.

An increasing body of evidence suggests that exposure to chronic and acute psychological stress may play a role in the development and/or triggering of cardiovascular pathology (Everson-Rose and Lewis, 2005; Kuper et al., 2005).

Pychosocial stress is a significant risk factor for cardiovascular disease in both patients with established disease and no-diseased individuals (Rozanski et al., 1999).

Although most of these studies appear to support a simple, direct relationship between psychosocial stress and CVD, the magnitude of risk varies considerably across studies (Rozanski et al., 2005).

Psychosocial factors that have been implicated as risk factors for developing heart disease include oneøs accessibility to social and economic resources, chronic stress exposure, especially in the work place, and aspects of the social environment such as social isolation and low social support.

In the same way, emotional factors linked to stress such as depression, anxiety, hostility and anger are thought to contribute to the development of cardiovascular disease (Suls and Bunde, 2005; Steptoe, 2006). More in details, depression is associated with increased cardiovascular morbidity and mortality, both aetiologically and in terms of prognosis, being a known risk factor for the development of cardiovascular disease. Hostility and anger are assumed to increase the risk of CVD through stress-induced cardiovascular and neuroendocrine hyperreactivity and health risk

behaviours (Boyle et al., 2004). In addition, lack of social support has been related to health-risk behaviour, psychological stress, cardiac symptoms, an increased risk of recurrent cardiac events and mortality (Barefoot et al., 2000). Lack of optimism (Shen et al., 2004) and perceived stress (Heslop et al., 2001) are also connected with increased cardiovascular mortality and morbidity. Aside from social factors, a strong association between CHD and low socioeconomic status (low education, low income level) has also been well established. Continuing socioeconomic disadvantage is linked to a higher risk of cardiovascular mortality and morbidity, as well as increased behavioural and medical risk factors, as smoking, excessive weight, sedentary lifestyle, heavy alcohol use, higher blood pressure and higher levels of cholesterol (Baker et al., 2002).

Rozanski and colleagues analyzed an extensive body of evidence from animal models (particularly the cynomolgous monkey) indicating that acute psychosocial stress can lead, possibly via a mechanism involving excessive sympathetic nervous system activation, to a variety of cardiovascular functional and structural effects, ranging from heart rate and blood pressure increase to direct effects on coronary vascular endothelium (see Figure 1). In fact, an increased sympathetic activity was shown to enhance the risk of severe or lethal cardiac tachyarrhythmias (such as ventricular tachycardia or ventricular fibrillation), particularly when acting on an altered cardiac substrate. Furthermore, hyperresponsivity of the sympathetic nervous system, manifested by exaggerated heart rate and blood pressure responses to stressors of different nature, is an intrinsic characteristic in some individuals (Schwartz and Priori, 1990). Clinical consequences of these effects include development of myocardial ischemia, cardiac arrhythmias, and fostering of more vulnerable coronary plaques and hemostatic changes. These alterations would form substrate for the development of acute myocardial infarction and sudden cardiac death (Rozanski et al., 1999).

In general, a confluence of pathophysiological and epidemiological studies established that both acute and chronic forms of psychosocial stress contribute to the pathogenesis of cardiovascular disease, such as coronary artery disease. In addition to directly promoting the pathogenesis of CVD, psychosocial stress factors also induce cardiovascular pathogenesis in two other basic ways: (i) they contribute to maintenance of unhealthy lifestyle behaviours that promote atherosclerosis, such as smoking and poor diet; and (ii) after the development of clinical disease, when targeted reduction of all unhealthy lifestyle behaviours becomes increasingly dominant, coexisting psychosocial stressors form an important barrier to successful modification of these lifestyle behaviours (Figure 2). On the other hand, amelioration of behavioural risk factors may also advance psychosocial well-being.
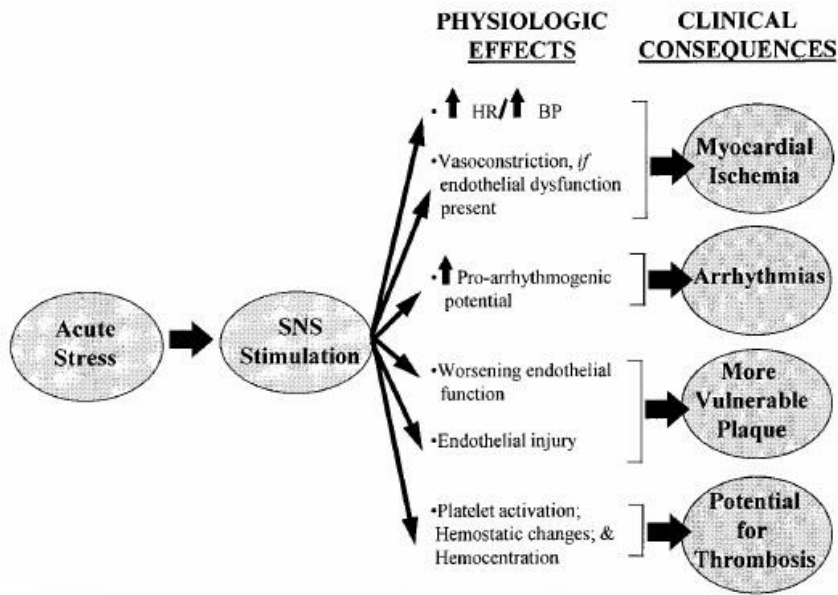
Figure 1. Schematic diagram of pathophysiological effects of acute psychosocial stress (from Rozanski et al. Circulation, 1999). HR: heart rate, BP: blood pressure, SNS: sympathetic nervous system
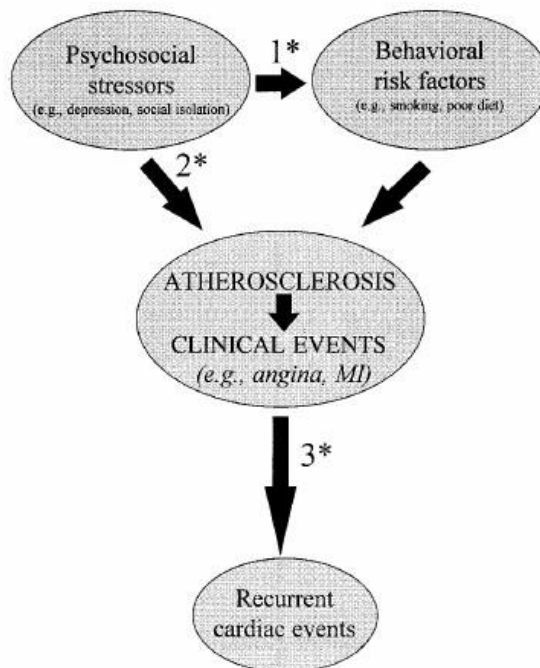

Figure 2. Schematic diagram of effects of psychosocial factors in pathogenesis of CVD (from Rozanski et al. Circulation, 1999).MI: myocardial infarction

1.1 Psychobiological mechanisms underlying cardiovascular disease

Psychological stress elicits a series of physiological responses that are potentially relevant to the triggering of CVD. Some of these responses occur both in healthy individuals and in patients with advanced coronary atherosclerosis, while others are present only in people with diseased coronary vessels. There are five categories of physiological responses to acute stressors which might have possible negative effects in people with advanced coronary artery disease.

First is the extensively studied haemodynamic response, which includes increases in blood pressure, heart rate and cardiac output, coupled with regional changes in blood flow that promote preferential energy supply to working muscle in the voluntary musculature and myocardium (Hjemdahl, 2007). Haemodynamic stress responses are stimulated in part through the second component, namely autonomic dysfunction. Both sympathetic activation and parasympathetic withdrawal not only influence pressor responses, but can also stimulate malignant arrhythmias. Reduced parasympathetic activity, indexed by decreased heart rate variability, is a predictor of CHD in population studies, death in patients following acute myocardial infarction (MI), and sudden cardiac death (Thayer and Lane, 2007). Heart rate variability is also reduced by acute mental stress, with more prolonged responses in people of lower socioeconomic status (Steptoe et al., 2002). Depression has been associated with reduced heart rate variability in patients with acute coronary syndromes, as MI and unstable angina (Carney et al., 2001). Autonomic dysfunction is closely associated with the third component, specifically neuroendocrine activation. Acute stress induces increases in hypothalamic-pituitary-adrenocortical activity and in catecholamine levels. There is a particularly large increase in noradrenaline overflow from the heart during mental stress, reflecting sympathetic stimulation of the myocardium (Esler et al., 1989).

The fourth component of the acute physiological stress responses is activation of inflammatory processes. Inflammatory markers such as IL-6 and tumour necrosis factor (TNF)   increase following emotional stress, although responses take 60-90 min to become measurable in the circulation (Steptoe et al., 2007).

Finally, platelets are potentially particularly important in the triggering process, in particular, platelet activation is increased during emotional stress, with more prolonged responses in patients with coronary artery disease compared to controls (Strike et al., 2004).

2. Stress and Depression

Depressive disorders are the second leading cause of disability worldwide, after ischemic heart disease, with the lifetime incidence of depression estimated at nearly 12% in men and 20% in women, respectively (Mathers and Loncar, 2006; Murray and Lopez, 1997).

In agreement with the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 1994) depression is a multifaceted psychological disorder characterized by behavioural, neuroendocrine, and physiological alterations. Depressed patients often experience anhedonia (a reduced responsiveness to pleasurable stimuli), difficulty in sleeping, appetite changes, fatigue, loss of the ability to experience pleasure in work, suicidal thoughts, slowing of speech and action (American Psychiatric Association, 1994). Depression is an extreme example of a group of behaviors which result in greatly reduced interaction with the environment. This psychological disorder may be accompanied by perturbations of most of the major physiological systems (Shively et al., 2009). In particular, depressive disorder has been shown to be characterized by monoamine dysregulation (serotonin and norepinephrine) (Meltzer, 1990), hypothalamic-pituitary-adrenal dysfunction (cortisol and corticotrophin-releasing hormone) (Asnis, 1987), and altered immune system components (proinflammatory cytokines) (Cunningham and De Souza, 1993).

It has long been recognized that depression can be precipitated in susceptible individuals by a series of stressful life events. Indeed, individuals that frequently experience severe stressful life events, for instance loss, humiliation or defeat, are more likely to develop major depression relative to individuals who do not experience such major stressful events (Heim and Nemeroff, 2001; Riso et al., 2002)

The link between stress and depression has long been observed, particularly at the clinical level, where exposure to stressful life events has been associated with the development of depressive symptoms in certain individuals, under certain conditions. This has been shown to depend on the characteristics of stressful life events and the psychological resources of each individual to cope with them. In fact, stress per se is not sufficient to cause depression. Most people do not become depressed after serious stressful experiences, whereas many who do become depressed do so after stress experiences which for most people are quite mild. Conversely, severe, dangerous stress, such as that experienced during combat, rape, or physical abuse, does not typically induce depression, but causes post-traumatic stress-disorders (PTSD) that is distinct from depression based on symptomatology, treatment, and longitudinal course of illness.

On this regard, both acute and chronic stress were associated with depression in adults and their effects were additive (Van De Willige et al., 1995; Ensel and Lin, 1996). Chronic stressors,

however, have a greater impact on depression than do acute events (McGonagle and Kessler, 1990) and not surprisingly, are found to be associated with depression over a longer 'at risk' period (Kendler and Prescott, 1998). I try to elucidate this association in **Chapter 3**.

This relationship has been clearly documented both in patient populations (Mazure et al., 2000) and in community samples (Mazure et al., 2000). Less certain, however, is the nature of the relationship between major depression and stressful life events. In particular, it remains unclear to what extent stressful life events cause subsequent onsets of depression and to what extent the occurrence of stressful life events and onsets of depression are correlated for other reasons. Initial attempts to predict depression by using multivariate models have found that stressful life events are a potent predictor of depression and that other risk factors indipendently contribute to the potential for depression. Such factors include previous history of depression (Kendler et al., 1993), gender (Lewinsohn et al., 1988), age (Lewinsohn et al., 1988), and genetic inheritance (Kendler et al., 1993).

Although concerns about a noncausal association between stressful life events and major depression have been expressed for a long time, two recent sets of findings have studied the salience of this relationship. First, numerous studies have now shown that exposure to stressful life events is substantially influenced by genetic factors (Plomin et al., 1990; Kendler et al., 1993; Foley et al., 1996). Individuals do not experience stressful life events at random; rather, some individuals have a stable tendency to select themselves into situations with a high probability of producing stressful life events. Second, the genetic risk factors for stressful life events are positively correlated with the genetic risk factors for major depression (Kendler et al., 1993; Kendler et al., 1997). That is, a genetically influenced set of traits both increases individuals' probability of selecting themselves into high-risk environments likely to produce stressful life events and increases their vulnerability to major depression. This underscores the view that depression in most people is caused by interactions between a genetic predisposition and some environmental factors, which makes the mechanisms of such interactions an important focus of investigation.

2.1 Neuroendocrine mechanisms in depressive disorder

2.1.1 Dysregulation of the Hypothalamicópituitaryóadrenal axis

A prominent mechanism by which the brain reacts to acute and chronic stressors is activation of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 3). Neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing factor (CRF), which stimulates the synthesis and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH then stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in

rodents) from the adrenal cortex. Glucocorticoids exert profound effects on general metabolism and also dramatically affect behavior via direct actions on numerous brain regions. The activity of the HPA axis is controlled by several brain pathways, including the hippocampus (which exerts an inhibitory influence on hypothalamic CRF-containing neurons via a polysynaptic circuit) and the amygdala (which exerts a direct excitatory influence) (Figure 3). Glucorticoids, by potently regulating hippocampal and PVN neurons, exert powerful feedback effects on the HPA axis. Levels of glucocorticoids that are seen under normal physiological circumstances seem to enhance hippocampal inhibition of HPA activity. They may also enhance hippocampal function in general and thereby promote certain cognitive abilities. However, sustained elevations of glucocorticoids, seen under conditions of prolonged and severe stress, may damage hippocampal neurons, particularly CA3 pyramidal neurons. The precise nature of this damage remains incompletely understood, but may involve a reduction in dendritic branching and a loss of the highly specialized dendritic spines where the neurons receive their glutamatergic synaptic inputs (McEwen 2000; Sapolsky 2000). Such a positive feedback process with pathological consequences has been implicated in a subset of depression. Abnormal, excessive activation of the HPA axis is observed in approximately half of individuals with depression, and these abnormalities are corrected by antidepressant treatment (Arborelius et al. 1999; Holsboer, 2001). Some patients exhibit increased cortisol production, as measured by increases in urinary free cortisol and decreased ability of the potent synthetic glucocorticoid, dexamethasone (see Figure 3), to suppress plasma levels of cortisol, ACTH, and -endorphin (which is derived from the same peptide precursor as ACTH). Dexamethasone is a synthetic glucocorticoid that mimics cortisol by inducing negative feedback to the pituitary, hypothalamus and hippocampus. The normal physiologic response to its administration is decreased cortisol secretion due to negative feedback influencing the HPA pathway. However, cortisol is not suppressed upon the administration of dexamethasone in approximately 50% of adult depressed patients (Asnis et al., 1987), indicating that this glucocorticoid may be hypersecreted in depressed individuals. There also is direct and indirect evidence for hypersecretion of CRF in some depressed patients (Arborelius et al. 1999; Holsboer, 2001; Kasckow et al. 2001). ACTH responses to intravenously administered CRF are blunted, and increased concentrations of CRF have been found in cerebrospinal fluid. A small number of postmortem studies of depressed individuals have reported increased levels of CRF in the PVN of the hypothalamus, whereas levels of CRF receptors are down-regulated perhaps as a response to elevated CRF transmission.
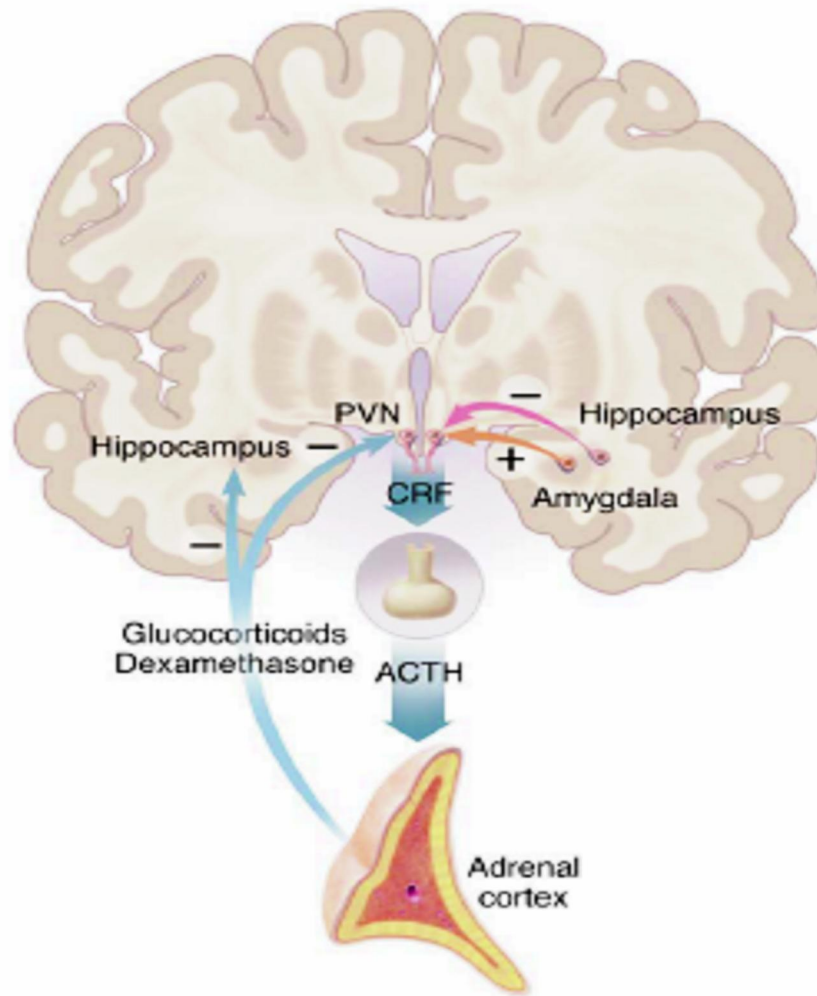
Figure 3. Regulation of the Hypothalamic-Pituitary-Adrenal Axis. CRF-containing parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) integrate information relevant to stress. Prominent neural inputs include excitatory afferents from the amygdala and inhibitory (polysynaptic) afferents from the hippocampus, as shown in the figure. Other important inputs are from ascending monoamine pathways (not shown). CRF is released by these neurons into the hypophyseal portal system and acts on the corticotrophs of the anterior pituitary to release ACTH. ACTH reaches the adrenal cortex via the bloodstream, where it stimulates the release of glucocorticoids. In addition to its many functions, glucocorticoids (including synthetic forms such as dexamethasone) repress CRF and ACTH synthesis and release. In this manner, glucocorticoids inhibit their own synthesis. At higher levels, glucocorticoids also impair, and may even damage, the hippocampus, which could initiate and maintain a hypercortisolemic state related to some cases of depression (From Lupien et al., Nat Rev Neurosci. 2009)

2.1.2 Serotonin alterations in depression

Serotonin (5-hydroxytryptamine, 5-HT), is a neurotransmitter involved in the regulation of emotion, mood, sleep and aggression, and it plays a key role in the onset and course of depression (Maes and Meltzer, 1995; Neumeister et al., 2004) (see Figure 4). In fact, the serotonin hypothesis of depression suggests that a deficiency of brain serotonergic activity increases vulnerability to depression (Maes and Meltzer, 1995). Evidence supporting reduced brain 5-HT function in depression comes from studies reporting lower plasma availability of the 5-HT precursor, tryptophan, for uptake into the brain, reduced cerebrospinal fluid (CSF) concentration of the serotonin metabolite and decreased platelet 5-HT uptake in depression (Maes and Meltzer, 1995; Neumeister et al., 2004). Together, these studies suggest diminished brain 5-HT uptake and metabolism in depressive patients.

Serotonin plays an important role in regulating HPA axis activity and stress coping and there are strong interrelationships between stress and serotonin function. In various animal models, it has been shown that serotonin is important in activating the HPA axis by stimulating CRF release, triggering ACTH release and stimulating corticosteroid secretion (Lefebvre et al., 1992; Fuller, 1996). The serotonergic activation of the HPA axis has also been suggested for humans (Dinan, 1996). Furthermore, in animals, it has been found that acute stress leads to a rise in brain 5-HT turnover by increasing tryptophan availability and stimulating tryptophan hydroxylase (De Kloet et al., 1982; De Kloet et al., 1983). The increased release of brain 5-HT is important because it enhances the negative feedback control of cortisol on the HPA axis, as a biological mechanism for stress adaptation (Van Praag, 2004). In animals, continued stress exposure or chronic stress is found to have a negative influence on the 5-HT system and may increase 5-HT sensitivity or vulnerability as a compensatory response (Adell et al., 1988). The existence of a clear mutual relationship between reduced 5-HT function, reduced stress adaptation and subsequent increased vulnerability to mood deterioration was further suggested by findings that increases in brain 5-HT improve stress coping and, subsequently, lead to reduced depressive mood in healthy stress-susceptible subjects but not in controls (Markus et al., 1998)

Another important aspect in the study of depression, and in particular, in the relationship between stress and depression, is a genetic predisposition to disease.

In this context, the serotonin transporter gene is the most studied in major depressive disorders (see Figure 4). This gene is of interest because it contains a polymorphism, i.e. variations in gene structure which give rise to 2 different alleles (long and short). People usually have 2 copies of each gene in their DNA; therefore, a person can be homozygous for the long allele, homozygous for the short allele or heterozygous. The short allele slows down the synthesis of the serotonin transporter. This is thought to reduce the speed with which serotonin neurons can adapt to changes in their

stimulation (Lesch et al., 1996). Given that an acute stressor increases serotonin release, the polymorphism may influence a person's sensitivity to stress. Based on these data, it is assumed that serotonergic vulnerability (defined as a serotonergic system which is more vulnerable or sensitive to serotonergic alterations or dysregulations), particularly under major or prolonged stress exposure, may constitute a risk factor for depression. This may be particularly relevant for individuals with a positive family history of depression in which a genetic predisposition, possibly related to allelic variations in genes that influence the serotonergic system, may promote the development of depression in response to severe and continued stress exposure. Despite the fact that this genetic vulnerability may promote the development of depression, the majority of individuals with a positive family history of depression do not develop depression (Sullivan et al., 2000). Gene–environment interactions have been suggested in the aetiology of depression. Because depression is often preceded by stress, researchers have hypothesized that a genetic vulnerability to depression, related to the serotonergic system, might be expressed only if an individual is exposed to stress (Van Praag, 2004). Hence, bidirectional interactions are proposed between the stress system and the serotonergic system. Ultimately, these interactions may induce serotonergic dysfunction and promote the development of a depressive disorder (Van Praag et al., 2004).
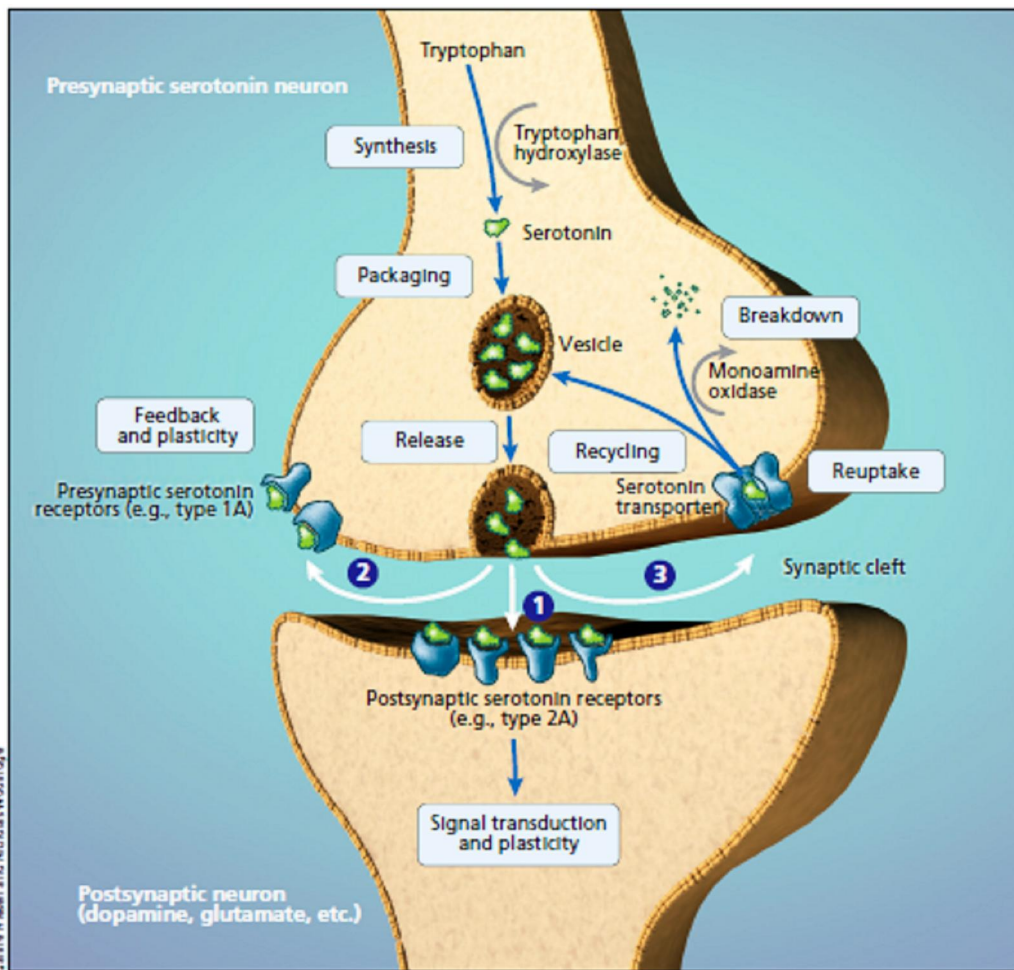
Figure 4. The serotonin synapse. Serotonin is synthesized from tryptophan by the enzyme tryptophan hydroxylase. Serotonin is then packaged into vesicles for release into the synaptic cleft, which occurs when there is sufficient stimulation of the neuron. Serotonin released from the serotonin neuron into the synaptic cleft has multiple actions. (1) Serotonin binds to its receptors on other neurons. Activation of postsynaptic receptors results in transduction of the signal that initially stimulated the serotonin neuron. (2) Serotonin also binds to presynaptic serotonin receptors on the neuron from which it was released, which provides feedback and regulates plasticity of the neuron. (3) Serotonin is taken up back into the presynaptic serotonin neuron by the serotonin transporter. Serotonin is then recycled for future release or broken down by monoamine oxidase and excreted in urine (From Root et al., CMAJ 2009)

3. Comorbidity between depression and cardiovascular dysfunction

Evidence obtained from epidemiological and clinical studies in humans and in non-human animals suggests that depression has significant adverse effects on the course of cardiovascular dysfunction. In particular, depressed patients are twice as likely as nondepressed patients to have a major cardiac event within 12 months of the diagnosis of cardiovascular pathology (Carney et al., 1988). Cardiovascular pathophysiology, such as coronary artery disease (CAD), myocardial infarction and congestive heart failure (CHF), is significantly related to altered mood states, on the other hand depressive syndromes are believed to be risk factors for cardiac morbidity and mortality (Pennix et al., 2001; Freedland et al., 2003; Glassman, 2007).

The fact that most coronary disease follows rather than precedes depression does not imply that depression causes coronary artery disease. It could be that some of the biological changes associated with depression increase the risk of coronary artery disease. However, it is also absolutely reasonable that preceding genetic or environmental events could lead to biological alterations conducive to both conditions. In addition, because mood may be influenced by health consequences of cardiovascular disease, it is possible that individuals with more severe disease are more likely to be depressed.

This risk is shown to be independent of traditional cardiovascular risk factors including smoking, hypertension, hypercholesterolemia, and increased body mass index (Wilson et al., 1998; Pennix et al., 2001; Barefoot and Schroll, 1996). Depressed individuals may be more likely than nondepressed individuals to have one or more of these risk factors, and therefore the link between depression and cardiovascular diseases may be, in part, due to risk factor clustering.

Previous studies have demonstrated that depression may predispose an individual to atherosclerosis, arrhythmias, myocardial infarction, heart failure and sudden death (Stewart et al., 2003). This association has been identified in current cardiac patients as well as individuals with no history of heart disease. Table 1 summarize the findings of the most important studies in this area.

Similar to cardiovascular disease, the prevalence of depression is also greater than average in other medical conditions such as cancer, erectile dysfunction and cerebrovascular diseases; however, unlike its relationship with CAD, a bi-directional relationship between depression and these conditions has not been found (Robinson et al., 2002; Roose, 2003).

Depression has been cited as a significant risk factor for recurrent cardiac events in patients with established cardiovascular disease. In fact, 20-50% of patients who die from myocardial infarction are thought to be significantly depressed prior to the infarction (Glassman and Shapiro, 1998). As compared to non-depressed individuals, patients with depression are at a much greater risk of death

due to cardiac related events for up to 10 years following the diagnosis of established CAD (Bareefoot and Schroll, 1996).

Importantly, depression is also associated with cardiovascular pathology in patients with no history of heart disease. Pratt and colleagues (1996) found that in people who were free of heart disease, those that had a history of depression were 4 times more likely to suffer a heart attack in the next 14 years than those who did not have the mood disorder.

The associated vulnerability between depression and cardiovascular dysregulation is not unidirectional. Depression can influence cardiovascular function, but cardiovascular diseases also influence affective states. While the prevalence of depression in the general polulation at any one point in time is 2-9% (American Psychiatric Association, 1994), it is currently estimated to be 45% among post-myocardial infarct patients (Schleifer et al., 1989). Cardiovascular disease-induced depression may be the result of psychological factors or physiological factors.

It is likely that a combination of both psychological and physiological mediators links cardiovascular disease and mood change. It is also possible that a common pathology may initiate both conditions. In general, despite the evidence taht heart disease and depression are epidemiologically linked, the mechanistic correlation between the two is not well understood. Depression, for example, is associated with changes in an individualøs health status that may influence the development and the course of cardiovascular disease, including the presence of cardiovascular risk factors such as smoking and hypertension. In addition, depression is associated with physiologic changes, including nervous system activation, cardiac rhythm disturbances, systemic and localized inflammation, and hypercoagulability, that negatively influence the cardiovascular system. Further, stress may be an underlying trigger that leads to the development of both depression and cardiovascular disease (Joynt et al., 2003).

Specific neurophysiological and behavioral abnormalities have been observed in depressed patients, which may account for the relationship between this disorder and cardiovascular illness.

In **Chapter 2 and 3** I attempt to study this relationship in rats underwent to different type of stress models, i.e. environmental, physical and emotional challenges, to induce depressive symptoms in foetal and adult age.

However, the cause or causes of the high co-morbidity of depression and heart disease are unknown. On the other hand, it seems reasonable to hypothesize that there are points of convergence in the aetiology or pathological progression of each illness which produce factors common for both disorders. The identification of mediators common to heart disease and depression can serve as an important source of hypotheses to generate research investigating why these disorders are so frequently co-morbid.

Table 1. Association between depression and cardiovascular diseases: results from the most important studies (from Lippi et al., Semin Thromb Hemost. 2009)

Study	Number of Patients	Results
Depression in patients without a preexisting CVD		
Ford et al, 1998[91]	1190	Clinical depression is an independent risk factor for incident CAD and MI (RR, 2.1)
Penninx et al, 1998[96]	3701 aged >70 years	Depressed older men are at increased risk for cardiovascular events (RR, 2.1) or cardiac mortality (RR, 1.8) than that of those without a history of depression
Ferketich et al, 2000[94]	5007 women/2886 men	Depression is associated with an increased risk of CHD in both men and women (RR, 1.7), as well as coronary heart disease mortality in men (RR, 2.3)
Penninx et al, 2001[92]	2397	Depression increases the risk for cardiac mortality in subjects with (RR 1.6 for MID and 3.0 for MAD) and without (RR 1.5 for MID and 3.9 for MAD) CAD at baseline
Rutledge et al, 2006[95]	505 women	Women with elevated depression symptoms showed an increased incidence of death and cardiac events (RR, 3.1)
Janszky et al, 2007[93]	799 cases/2339 controls	A significantly increased risk for MI was found among subjects hospitalized for depression (RR, 2.3)
Depression in patients with preexisting CVD		
Horsten et al, 2000[106]	292 women with CAD	Depressive symptoms independently predicted recurrent cardiac events in women with CAD (HR, 1.9)
de Jonge et al, 2006[107]	468 MI patients	Incident post-MI depression had an impaired cardiovascular prognosis
Whooley et al, 2008[10]	1017 with stable CAD	The association between depression and adverse cardiovascular events was largely explained by behavioral factors (HR, 1.05; 95% CI, 0.79 to 1.40; $p = 0.75$ after adjustment)

CAD, coronary artery disease; MI, myocardial infarction; RR, relative risk; MID, minor depression; MAD, major depression; HR, hazard ratio.

3.1 Potential common mechanisms underlying depression and cardiovascular function

In recent years, the literature has attempted to describe the pathophysiologic mechanisms relating depression and stress to heart failure. Common mechanisms involved in the link between depression and cardiovascular disease may include reactivity to exogenous stressors, alterations of neurohumoral, immune and autonomic regulation, and dysfunction of neurotrasmitter systems.

3.1.1 Stressor reactivity, depression and cardiovascular dysfunction

Exposure to environmental stressors has been shown to be responsible for influencing the development of both depression and cardiovascular diseases. The presence of stressors does not favor behavioral or physiological adaptation, and therefore may play an important role in the development of depressive signs and symptoms (Anisman and Matheson, 2005) and cardiovascular dysregulation (Sgoifo et al., 1999). Exposure to different types of stressors also contributes to cardiovascular diseases and their antecedent risk factors, such as hypertension, changes in vascular resistance, endothelial dysfunction, altered baroreceptor reflex function and ventricular arrhythmias (Johnson and Anderson, 1990; Sanders and Lawler, 1992; Bairey Merz et al., 2002; Schwartz et al.,

2003). Moreover, environmental stressors produce alterations in central processes, including changes in norepinephrine, dopamine, serotonin and corticotropin-releasing factor (CRF), as well as activation of neuroendocrine, immune and autonomic nervous systems (Herman et al., 1982; Joseph and Kennett, 1983; Adell et al., 1988; Vaidya, 2000; Anisman and Matheson, 2005), producing alterations in both mood and cardiac dysfunction.

3.1.2 Neurohumoral and immune function

It is possible that reactivity to exogenous stressors leads to altered mood and cardiovascular regulation via neuroendocrine or neuroimmune systems, or through a disruption of autonomic function. For example, the hypothalamicópituitaryóadrenal (HPA) axis is dysregulated in depressed individuals, including: (i) alterations of CRF, (ii) increases in circulating adrenocorticotropic hormone (ACTH), cortisol or corticosterone, and (iii) impaired feedback regulation of the axis (Carroll et al., 1976, Raadsheer et al., 1995, Maes et al., 1998; Weber et al., 2000); these changes are not dissimilar to those observed following exposure to chronic stressors. Cortisol promotes many functions that, in the short-term, may help the body to cope with a stressor. However, sustained high levels of circulating cortisol concentrations, in response to repeated or sustained stress, may cause changes in physiology that have long-term detrimental effects on health including the promotion of cardiovascular risk. In fact, cortisol may increase coronary heart disease risk by several mean. For instance, cortisol increases visceral fat deposition, and visceral obesity promotes inflammation, insulin resistance, hypertension, and hypercholesterolemia, all of which increase atherogenesis (Whitworth et al., 2005). Interactions between the HPA axis and the sympathetic nervous system may increase CHD risk in depression. These alterations provide evidence for neuroendocrine dysfunction in depression, which may influence cardiovascular regulation via several potential processes, such as activation of the immune system, release of humoral factors, or activation of the sympathetic nervous system.

The endocrine system interacts with the immune system both within the brain and in the periphery. Depression is associated with activation of immune system factors and disruption of immune processes (Maes, 1995; Dantzer, 2006). However, it is not clear whether this activation is a cause or a consequence of altered mood states. Alternatively, Smith proposed a macrophage theory of depression (1991), suggesting that excessive secretion of monokines such as interleukin (IL)-1, tumor necrosis factor (TNF)- and interferon, contribute to the pathophysiology of depression. While the precise causal mechanisms involving depression and immune system changes are not entirely elucidated, activation of the immune system is also associated with specific cardiovascular disorders. For instance, pro-inflammatory cytokines such as TNF- , IL-1 and IL-6 are released into the systemic circulation in congestive heart failure (Das, 2000). These have adverse effects on

the heart and circulation (Kapadia et al., 1998), and therefore may feed back to the central nervous system to induce directly signs and symptoms of depression.

In addition to the immune system, the renin–angiotensin–aldosterone system (RAAS) is activated in some forms of heart disease, resulting in high circulating levels of angiotensin II and aldosterone (Felder et al., 2001). Several of these factors interact in the central and peripheral nervous systems, which may influence the onset of depression and cardiovascular disease.

Neurohumoral activation, manifest in both generalized and specific HPA axis dysfunction, RAAS activation and altered immune function, interacts with autonomic regulation of the heart (Thayer and Sternberg, 2006).

3.1.3 Autonomic nervous system dysfunction

Autonomic nervous system dysfunction appears to be one of the most realistic mechanistic explanations for the association between depression and cardiovascular disease. Observation of either decreased parasympathetic activity, increased sympathetic activation, or both would be important as an imbalance between the sympathetic and parasympathetic systems may increase the risk for several types of adverse cardiac events. In this context, many studies examined changes in norepinephrine (NE) in individuals with depressive disorder, but no co-morbid medical conditions. Many early studies suggested increased urinary and plasma NE or NE metabolite levels in patients with depression resulting from increased systemic sympathetic activation (Veith et al., 1994).

In addition, patients with depression also exhibit increased sympathetic activation in response to acute stressors. Increased circulating NE is observed in response to physical (Carney et al., 1999) and psychological stressors (Mausbach et al., 2005). Other markers of sympathetic activity also suggest altered autonomic nervous system function in patients with depression. For examples, studies suggest that patients with depression have decreased heart rate variability (HRV) (Carney et al., 1995) which is knock to predict adverse outcomes in patients with heart disease. Additionally, a recent study by Carney and colleagues suggests that HRV may partially explain the increased risk of death following myocardial infarction in coronary artery disease patients with co-morbid depression (Carney et al., 2005).

Among other potential effects, altered autonomic nervous system function increases susceptility to ventricular arrhythmias triggered by inadequately opposed sympathetic stimulation (Podrid et al., 1990). As described, patients with depression show autonomic nervous system changes comparable to the changes observed in patients with heart failure (Dallack and Roose, 1990).

In general, depression may be characterized by changes in autonomic function, including activation of the sympathetic nervous system, withdrawal of vagal tone to the heart, elevations in heart rate, reductions in heart rate variability and altered baroreceptor reflex function (Carney et al., 1995;

Krittayaphong et al., 1997; Pitzalis et al., 2001; Barton et al., 2007). Similar autonomic changes are associated with cardiovascular risk factors such as hypertension, increased body mass index and increased blood glucose, and have been observed in both acute and chronic cardiovascular conditions including atherosclerosis, myocardial ischemia and arrhythmias (Carney et al., 1993; Kristal-Boneh et al., 1995; La Rovere et al., 1998; Esler and Kaye, 2000); all these changes can influence neurohumoral and neuroimmune activation.

3.1.4 The role of central neurotransmitter systems

Several central nervous system processes are altered in depression and cardiovascular disease, and can be affected by behavioral, physiological or other neural inputs. Disrupted monoamine function has been implicated in the pathophysiology of depression (Lambert et al., 2000). Evidence from pharmacological studies indicates that monoamine oxidase inhibitors and tricyclic antidepressants have been used as effective antidepressants in some patients (Garlow and Nemeroff, 2004). However, in the context of heart disease, these antidepressants have cardiotoxic effects, including pro-arrhythmic properties (Glassman, 1998). Consequently, research has focused more specifically on the role of the serotonergic system in depression (Maes and Meltzer, 1995; Lucki, 1998; Cryan et al., 2005). In particular, it has been reported that: (i) serotonin plays a significant role in behaviors that are disrupted in depression (e.g., mood, sleep, appetite), (ii) a decrease in brain 5-HT concentration can precipitate depression in recovering patients, (iii) depression is associated with several changes in central 5-HT and 5-HT receptors (e.g., decreased tryptophan concentrations, impaired 5-HT synthesis or release, changes in 5-hydroxyindoleacetic acid, malfunctions at postsynaptic 5-HT receptors, alterations in 5-HT transporter density), and (iv) pharmacological agents that alter 5-HT are effective antidepressants. In addition, changes in the function of 5-HT type 1A (5-HT$_{1A}$) receptors may play a role in the link between depression and cardiovascular disorders (Nalivaiko, 2006).

In summary, the association between cardiovascular disease and depression is multifaceted and likely bidirectional. Depression may perhaps be conceptualized to influence cardiovascular function via neuroimmunomodulatory autonomic dysregulation (see Figure 5). The central nervous system is proposed to play a major role in the aetiology of both depression and cardiovascular dysregulation. Peripheral nervous system changes are likely mediated by brain mechanisms that can lead to specific cardiac events. Feedback from the cardiovascular system to the brain perpetuates the cycle of dysregulation, and may also lead to depressive symptoms. The influence of exogenous stressors, either alone or coupled with a genetic or experiential predisposition, may play a role in the initiation of depression and in the pathogenesis of heart disease (Grippo and Johnson, 2002). Autonomic function is altered in depression, evidenced by reduced heart rate variability, impaired baroreflex sensitivity and changes in heart rate. These changes may influence the pathogenesis of diseases that affect the cardiovascular system. Several lines of evidence also point to a modification of immune function in depression. Pathology of the immune system is associated with several variables that influence both mood changes and heart disease. Immune dysfunction influences both central and peripheral components of the autonomic nervous system.
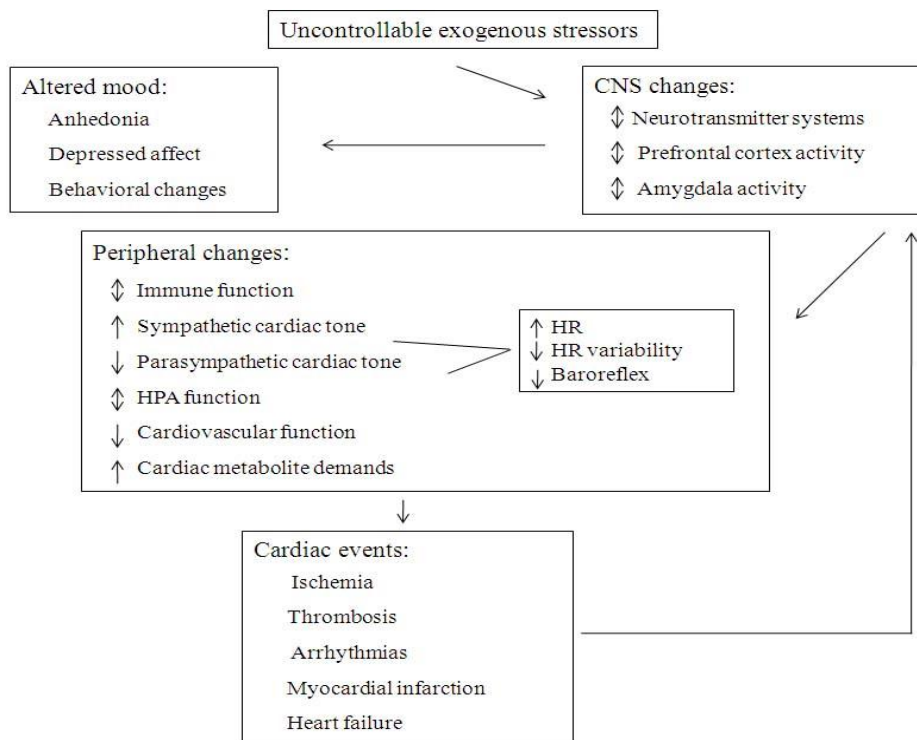

Figure 5. An integrative pathophysiologic model of the important pathways that may influence the association between depression and cardiovascular regulation (from Grippo and Johnson, Neuroscience and Biobehavioral Reviews, 2002). CNS: central nervous system; HR: heart rate

4. Social stress

Social stress factors could be defined as challenging stimuli originating from the interaction with conspecifics. It is a chronic or recurring factor in the life of virtually all mammalian species and is particularly relevant to species with a complex social organization; this suggests, that the social environment could be a relevant source of negative inputs for an individual (Sapolsky, 1992).

In fact, animal models that involve a social context seem to be more appropriate because they represent situations that individuals may meet in their everyday lives; indeed they likely provided much of the impetus for the evolution of stress response mechanisms (Blanchard et al., 2001).

In many animal species, social stress factors results from competition for resources such as space, access to a reproductive partner, food, or water, and involve agonistic behaviours with different degrees of aggression, which may result in wounding, exhaustion and sometimes even death.

In humans, social stress episodes do not necessarily imply overt aggressive acts; nevertheless, intraspecific interactions involve competitive/hostile behaviours, which represent a severe challenge to physiological and psychological homeostasis. For these reasons, social stress can be one of the most important sources of stress in human life and may also play a critical role in the development of stress-related disorders and disease. Understanding the mechanisms underlying stress-induced disturbances will ultimately allow for improved clinical therapies and possible preventive strategies to decrease the incidence of these disorders.

In this regard, a number of models have been developed to evaluate the natural tendency of different species to form social hierarchies when housed in groups, including rats (Fokkema et al., 1995), and mice (Ely and Henry, 1978). Establishing and maintaining dominance in a group setting is psychologically and physically stressful for all parties, including both the dominant and the subordinate animals. In addition, since animals are group-housed for extended period of time, the members of the group are constantly exposed to stress.

Increasingly, a greater amount of attention has been focused upon developing animal models that utilize more naturalistic experimental paradigms to model stress that is ethologically relevant to the model organism. Consequently, several animal models of social stress have been developed in order to clarify the mechanisms underlying human stress-pathologies (Koolhaas et al., 1995); furthermore, these paradigms are applied for basic research on the adaptative process to social environmental challenges.

Rodents and non-human primates are the most commonly used animals to model social stress; rodent models of social stress are generally used in the laboratory in part because of their short lifespam enabling researchers to conduct longitudinal studies in a short time span. In addition, they are relatively easy to maintain since they do not require the housing space and other resources as

primates and are thus also less costly. Because of the amount of research on rodents, there is a vast literature documenting the behaviour of rats and mice as well as some of their genetic correlates. This extensive database enables investigators to make direct genetic manipulations via knockout and transgenic technology furthering the understanding of stress disorders.

Non-human primates are highly dependent on social interaction and relationship and therefore are a useful model for studying social stress and the resulting effects. Their neuroanatomy and neurophysiology are also considered to be more analogous to those of humans contributing to construct validity of the models.

Animal models of social stress involve single, intermittent, or chronic exposure of a subject animal to a conspecific. The types of interactions that occur during conspecific encounters vary with the subject species, and the age, gender, and previous history of the individual, as well as the circumstances in which the exposure takes place. Most laboratory studies of social stress effects utilize rodents, typically laboratory rats or mice, and sometimes hamsters. Although other species, notably primates, also serve as subjects of laboratory investigation of social stress effects, their social and stress-related behaviours are more commonly observed under seminatural conditions, or in the wild. A number of different social stress situations are used in laboratory studies involving two or more animals in dyadic, group, or colony situations, respectively.

Exposure to acute and/or chronic social stress has been associated with the onset of several psychopathologies, including depression (Blanchard et al., 1995). Kind of stimulus, duration, intensity and predictability of the stressor applied are all relevant factors determining the development of a stress state (Koolhaas et al., 1997). The experiments included in this thesis are linked by a common threat, that is the exposure of rats and mice to adverse social conditions.

In **Chapter 2, 3, 4 and 5** the role of acute and chronic social stress in depressive symptomatology and cardiac function is examined.

4.1 Acute social stress

Animal models of social challenge are not only a naturalistic, biologically-relevant model for studying stress response and pathology. In fact, the majority of stress stimuli in humans that lead to psychopathology are of social nature (Brown and Prudo; 1981). Many studies have indicated that different types of social stress can also elicit qualitatively different patterns of behavioural and physiological stress response (Blanchard et al., 2001). For this reason, there has been extensive research on the mechanisms involved in stress-related disorders in animal models undergoing social stress.

In particular, a number of studies indicated that loss of environmental control is a major factor in inducing stress-related pathology; in a social context, this means loss of social control. On this regard, the most frequently used model for rodents is the social defeat paradigm; this experimental paradigm mimics threat to (or loss of) social control.

Social defeat in rats is obtained via the resident-intruder paradigm (Miczek, 1979). This method is based on the establishment of a territory by a male and its defence against unfamiliar male intruders. Usually, the experimental male (õthe intruderö) is introduced into the home cage of an aggressive male (õthe residentö), by what it is rapidly investigated, attacked and finally defeated (Figure 6). Rats and mice are naturally social animals, and this model is thus thought to be applicable to the study of mechanisms contributing to social stress-related disorders. However, it is important to note the caveat that the resident-intruder paradigm often does not reflect natural environmental conditions. Depending on the specific social defeat paradigm used, the experimenter can manipulate test conducting parameters to generate the desired outcome in resident-intruder models. For example, the resident is usually selected for high body weight and aggression to give it a greater advantage in defending territory. In many cases, the resident male is also housed with a female, a procedure that increases aggression in male rats (Flannelly and Lore, 1977). Residents are typically used repeatedly, thereby giving them experience of victory and further increasing their aggressiveness.

A number of studies performed in rats revealed that the exposure to social defeat induces significant acute and long-lasting physiological, neuroendocrine and behavioural changes (Koolhaas et al., 1997; Miczek et al., 1990; Sgoifo et al., 1996; Sgoifo et al., 1997). In particular, short-term effects include body temperature increases, robust activations of the sympatho-adrenomedullary system and the pituitary-adrenocortical axis (as shown by plasma levels of catecholamines, ACTH and corticosterone), shift of sympathovagal balance towards sympathetic prevalence and increased occurrence of cardiac arrhythmias (see Figure 7). Koolhaas and colleagues (1997) indicated that the temporal dynamics of these responses is differential, depending on investigated the parameter.

In particular, the onset of glucocorticoid responsivity is slower, but lasts longer as compared to catecholamine or heart rate; in fact, plasma corticosterone levels are still significantly elevated up to about 4 hours after the end of the stressor; on the contrary, plasma testosterone levels drop below baseline and may remain low for about two days.

In addition, several reports have documented long-lasting physiological and behavioral effects of a single episode of social defeat which can persist up to weeks, including changes in body weight, food intake and preference, circadian rhythmicity of heart rate, body temperature and physical activity, social and exploratory behavior (Koolhaas et al., 1997; Meerlo et al., 1999) (see Figure 8).

In addition, two social stress paradigms that are significantly different in conceptualization from the above, in that they deemphasize the role of agonistic behaviour, are crowding and social isolation. Properly speaking, crowding should refer only to studies in which animals are placed together in housing situations such that each has less than a standard amount of space. Since there is little information on what are the optimum or even reasonable space requirements for animals of most species, this is quite an arbitrary definition of crowding. Additionally, õcrowdingö measured as animals per unit area may be quite different than õcrowdingö as number of interacting animals per housing unit. Crowding also implies that the mechanism of social stress is proximity, rather than agonistic interaction *per se*, and crowding stress studies may or may not involve attempts to measure agonistic reactions and to identify dominant and subordinate animals within the groups.

The use of social isolation as a stressor seems odd in view of the extensive use of social encounters as stressors. However, differences in social organization between species may make one or the other condition, isolation or grouping, particularly stressful. Sex differences may also be a factor. For rats, social grouping appears to be more stressful for males while isolation is more stressful for females (Brown and Grunberg, 1995; Haller and Halasz, 1999).

a)

b)

c)

Figure 6. Illustrations of a) sensory contact phase, b) physical interaction during Social Defeat Paradigm; and c) a detail of physical interaction between resident (wild type) and intruder rats (wistar).
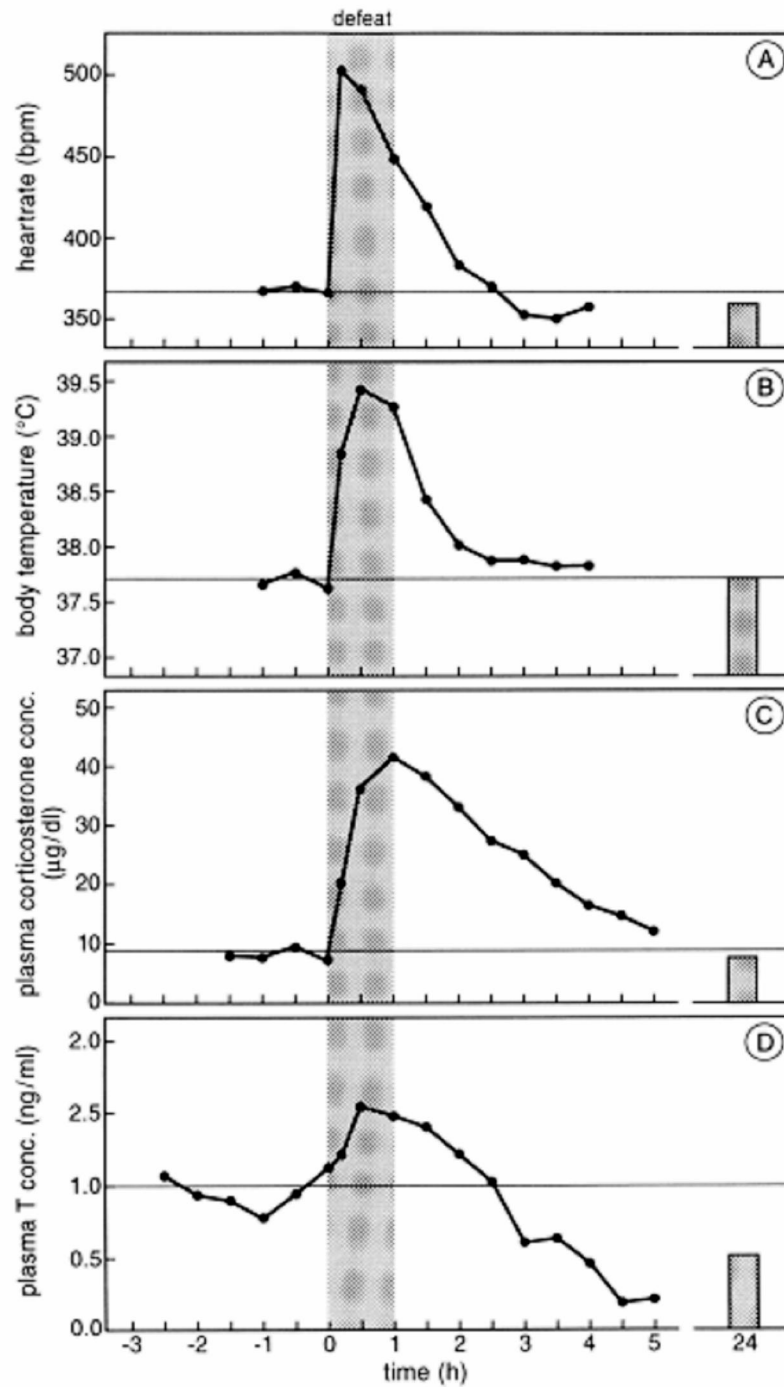
Figure 7. Time course of some physiological and neuroendocrine responses induced by a single episode of social defeat (A) Heart Rate, (B) Body temperature, (C) Plasma corticosterone; and (D) Plasma testosterone (from Koolhaas et al. Neuroscience and Biobehavioral Reviews, 1997).
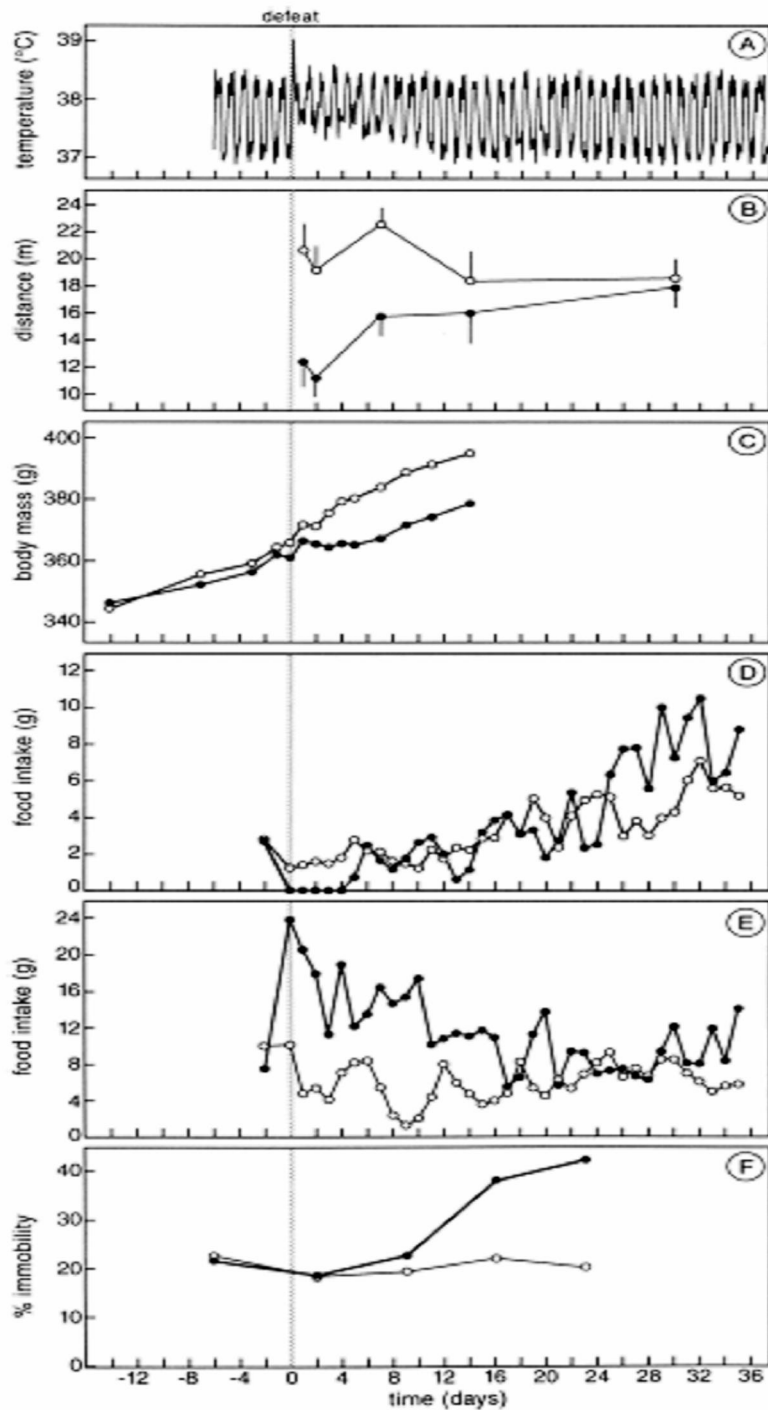
Figure 8. Time course of some physiological and neuroendocrine responses after a single episode of social defeat (A) Circadian variation in body temperature, (B) distance moved in open-field test, (C) body weight; (D) carbohydrate intake in a diet selection experiment, (E) fat intake in a diet selection experiment; and (F) behavioural reactivity in an orientation attention test (from Koolhaas et al. Neuroscience and Biobehavioral Reviews, 1997).

4.2 Chronic social stress

Animal models of social stress involve single, intermittent, or chronic exposure of a subject animal to a conspecific. Exposure to chronic stress has been found to be detrimental to health; however, not all individuals exposed to chronic social stress develop psychopathologies. Consequently, the development of appropriate animal models to investigate the biological basis of individual differences in vulnerability to chronic social stress is a major challenge of bio-medical research.

Bartolomucci and colleagues have proposed an ethologically oriented model of chronic psychosocial stress, which is based on a natural behaviour of male mice (Bartolomucci et al., 2001). This model of chronic stress is modified from those previously developed with tree shrews and mice (Fuchs et al., 1996; Kudrayvtseva, 2000). In this paradigm, resident/intruder dyads live chronically in sensory contact and physically interact on a daily basis. The animals are individually housed for one week to permit the establishment of an individual territory. Each resident mouse receives an intruder mouse and the two animals are allowed to interact freely for a few minutes. After the interaction, the two animals are separated by means of a partition, which allows continuous sensory contact but no physical interaction. Then, the partition is removed daily at an unpredictable moment in the initial part of the light phase. At the beginning of the stress protocol, social relationships between the resident and the intruder mouse undergo dynamic changes, which then lead either the resident or the intruder to acquire the dominant social rank. Accordingly, individual animals subjected to this procedure of social stress can be divided in four behavioural categories: Resident Dominant, Resident Subordinate, Intruder Dominant and Intruder Subordinate. This model offers the opportunity to investigate whether territory ownership (being resident in a territory) and social status (being dominant or subordinate), as well as their interaction (e.g. a resident becoming dominant or subordinate) are factors affecting the individual vulnerability to stress exposure. In this paradigm, the physical component of the stress protocol is of minor relevance when compared to the psychological one, because it is reduced to a brief daily physical interaction that is interrupted as soon as fight escalates in order to prevent injuries. Therefore, the effects observed in this model at the physiological and behavioural level are much more likely due to the psychological perception the mice have of the stressful context.

Another paradigm of chronic social stress is the visible burrow system (VBS) model of social hierarchy. Briefly, the VBS model involves housing mixed-gender groups of rats in a semi-naturalistic burrow environment continuously for a number of weeks.

Within few days after colony formation, a dominance hierarchy forms among the males of the group resulting in 1 dominant and 3 or 4 subordinate animals (Blanchard et al., 1995).

The VBS model has the advantage of forming stable hierarchies over time; i.e. it has been used for investigating the effects of single as well as repeated exposure to social stress.

Other chronic stress paradigms usually involve repeated application of the same stressor and often at the same time of day, such that the animal may be able to learn to predict the timing of the stressor and thus facilitate physiological *habituation*. Habituation was shown to take place for various types of response (sympathetic-adrenomedullary and pituitary-adrenocortical) and it can be viewed as an adaptive mechanisms protecting from health-threatening repeated activations of the main stress response neuroendocrine systems (McCarty and Patak, 2000).

In general, exposure to chronic social stress results in a spectrum of physiological, neuroendocrine and behavioural changes were documented, which vary according to the social status and context considered (Bartolumucci et al., 2005). For instance, chronic social stress induced changes in motor activity (Fuchs and Flugge; 2002). More specifically, when chronically stressed mice were subsequently faced with a new environment, dominant, but not subordinate, showed behavioural hyperactivity and reduced depression-like behaviours (Bartolomucci et al., 2001).

One of the most consistent and pronounced effects in animals during and following social stress is a decrease in body weight. Subordination is a severe stressor in rats, usually resulting in weight loss or slowed weight gain that may persist for weeks even after the stressor has been terminated. This reduction in body weight could reflect reduced food intake and/or increased metabolic rate (Haller et al., 1999).

In addition to behavioural changes, activation of the HPA axis represents the hallmark of the stress-response and it has been repeatedly shown that chronic social stress results in elevated circulating adrenocorticotrophic hormone (ACTH) and glucocorticoids, and dysregulation of the HPA axis regulatory feedback (McEwen, 2000). In addition, mice under chronic stress develop a clear adrenal hyperactivity, likely due to an altered inhibitory feedback of the corticosterone on the hippocampus. Interestingly, HPA axis alterations developed in all stressed animals, independently of whether they were dominants, subordinates, residents or intruders (Keeney et al., 2006; Bartolomucci et al., 2001). Along with behavioral and neuroendocrine alterations, chronic social stressors can also have a strong impact on immune function. In particular, when rodents are exposed to chronic social defeat, it has been observed that there is a reduction in *in vitro* lymphocyte proliferation and natural killer cell activity (Stefanski, 2000).

Laboratory models of social stress have been used for many years to examine the behavioural and physiological mechanisms leading to disease conditions resulting from exposure to stressors. However, it is often the case that the social stress paradigm bears little resemblance to the natural conditions under which social stress-related pathologies develop. In fact, footshock, restraint, forced swim are commonly used to generate stress in the laboratory, but do not reliably resemble the

challenges which the animals normally face within their natural environment. In particular, these types of stressors may elicit behavioural and physiological responses different from those resulting from acute or chronic social stress (Koolhaas et al., 1997). In the last years, a greater amount of interest has been focused upon adopting animal models that utilize more naturalistic experimental paradigms to model stress that is ethologically relevant. As a result, several animal models of social stress have been developed in order to investigate questions related to the etiology, treatment, and prevention of stress-related pathologies. In particular, rodent models in which the social interactions are experimentally modified are becoming increasingly popular as realistic models of human disease.

4.3 Social stress and cardiovascular pathophysiology

Pathologies influencing the cardiovascular system are relatively frequent among stress related psychosomatic disorders. Specifically, social stressors seem to play an important role in the onset and progression of cardiocirculatory diseases, such as hypertension, atherosclerosis and cardiac arrhythmias (Henry and Grim, 1990; Folkow et al., 1997; Sgoifo et al., 1999a; Sgoifo et al., 1999b; Kaplan and Manuk, 1999). Experimental and clinical data have shown that the stimuli deriving from the social environment (work difficulties, mobbing, adverse family or partner relationship) are the most common stress factors in humans and may produce severe alterations of the cardiovascular system (Hemingway et al., 2001; OøKeefe et al., 2004). However, a number of reasons makes it rather difficult in humans to investigate the pathogenic role of social challenge on the cardiovascular system: (i) it is rather difficult to control for and standardize individual social history preceding laboratory assessement, (ii) obvious ethical limitations impede free manipulation of social environment, (iii) it is rather time/energy consuming to characterize the role of social factors because of the long time span of cardiovascular disease.

Human and animal data suggest that an important determinant of cardiovascular stress reactivity and morbidity is the individual behavioural strategy of coping with social challenges (Henry et al., 1986; Henry et al., 1993; Waldstein et al., 1997; Koolhaas et al., 1999). Indeed, cardiovascular pathophysiological consequences seem to depend tightly on what perception an individual has and what behavioural strategy he adopts when dealing with the challenge imposed by another member of the social group.

On this regard, Henry proposed that there are two innate response patterns, which might explain the differential sensitivity in developing hypertension (Henry and Grim, 1990). One pattern is related to dominance behaviour; it is characterized by behavioural arousal, high levels of aggression and territorial control, and is termed õactive copingö response pattern.

It is associated with increased cardiac output and redistribution of blood flow to the brain and skeletal muscles, mediated by a robust activation of the sympathetic-adrenomedullary system.

The other pattern is related to subordination, i.e. defeat or perception of a threat to or loss of control; it is characterized by a generalized behavioural inhibition (õpassive copingö) and a stronger activation of the HPA axis (Henry and Grim, 1990).

Human studies performed by Rosenman and Friedman defined the õtype Aö behavioural pattern as associated to a higher proness to develop coronary disease. Type A category includes subjects who are hostile, competitive, achievement oriented. The relative absence of type A characteristics defines õtype Bö, non-coronary-prone behavioural pattern (Rosenman and Friedman, 1974; Rosenman et al., 1975; Herd, 1991).

Glass and colleagues examined the relationship between type A behavioural pattern and cardiovascular/catecholaminergic responses to experimental conditions designed to induce hostility and competitiveness (Glass et al., 1980). Type A subjects exhibited significantly larger increments of systolic pressure, heart rate and plasma adrenaline as compared to type B counterparts.

Recently, Newton and Bane investigated the cardiovascular outcome of the exposure to social challenge in man: cardiovascular reactivity (heart rate and arterial pressure changes) was positively correlated with the level of dominance/hostility shown by the opponent in a dyadic interaction (Newton and Bane, 2001).

As preview mentioned, in man it is difficult to systematically control and standardize social stimuli; consequently, some animal models of social stress can be very useful to this purpose.

Monkeys and rodents, in particular, have been broadly used to mimic the aetiology and symptomatology of stress-related cardiovascular pathology in humans (Henry et al., 1993; Kaplan et al., 1982; Sgoifo et al., 1997; Sgoifo et al., 1998).

At this regard, Sgoifo and colleagues (1999a) have studied the acute consequences of a social aversive stimulus (social defeat) (Koolhaas et al., 1990; Miczek, 1979) on the autonomic control of the electrical activity of the heart and compared these effects with those observed in three non-social stress paradigms, specifically restraint (Parè and Glavin, 1986), shock-probe test (De Boer et al., 1990); and swimming (Scheurink et al., 1989). The heart rate and the heart rate variability data suggest that during defeat autonomic control was markedly shifted toward a sympathetic dominance; on the contrary, in rats exposed to non-social stressors, sympathovagal balance was substantially maintained. These differences in autonomic stress responsivity explain the different susceptibility to arrhythmias, and propose that a robust challenge of social nature can be far more detrimental for cardiac electrical stability than other non-social aversive stimuli (Sgoifo et al., 1997). In other words, the lack of control experienced by the subordinate when losing a social confrontation is characterized by a robust shift of sympathovagal balance towards sympathetic

dominance (Sgoifo et al., 1999a), a remarkable occurrence of cardiac arrhythmias (Sgoifo et al., 1997; Sgoifo et al., 1998); and significant blood pressure elevations (Fokkema et al., 1986). All these cardiocirculatory consequences, however, are resolved soon (within approximately 1 hour) after the end of the stimulus, and are generally considered part of an acute, adaptative stress response.

Besides these short-term activations, however, several reports have documented long-lasting effects of social challenge on cardiac autonomic balance. In particular, the loss of social status may produce long-lasting consequences on the circadian rhythmicity of heart rate (Meerlo et al. 1999).

Many studies suggest that the intermittent exposure to the same stressor can lead to a gradual reduction in physiological, neuroendocrine and behavioral responses (*habituation*). Sgoifo and colleagues (2001) have investigated possible habituation processes of cardiac autonomic responsiveness and susceptibility to cardiac arrhythmias in rats exposed to intermittent social victory or social defeat. This study showed a clear habituation profile of cardiac autonomic responsivity, both in terms of sympathovagal balance and susceptibility to cardiac tachyarrhythmias only in rats exposed to social victory, whereas no habituation was found in repeatedly defeat animals. In other words, this study suggested that habituation of cardiac autonomic responsiveness to an intermittent social challenge takes place only when the animal can exert a reasonable degree of control over the stressor.

Box 1:

Cardiac sympathovagal balance explored via Heart Rate Variability

The cardiovascular system, the heart and circulation, are mostly controlled by higher brain central and cardiovascular control areas in the brain stem through the activity of sympathetic and parasympathetic nerves (Hainsworth, 1998). Control is also influenced by baroreceptors, chemoreceptors, muscle afferent, local tissue metabolism and circulating hormones (Levy and Martin, 1979). The autonomic nervous system (SNA) includes sympathetic and parasympathetic nerves; both divisions contain both afferent and efferent nerves and both myelinated and non-myelinated fibres. In general, the effects of the two divisions are complementary, with activity in sympathetic nerves exciting the heart (increased heart rate), constricting blood vessels, decreasing gastrointestinal motility and constricting sphincters, while parasympathetic nerves inducing the opposite response. The autonomic system supplies both afferent and efferent nerves to the heart, with sympathetic nerve ending all over the myocardium, and parasympathetic on the sino-atrial node, on the atrial myocardium and atrio-ventricular node. These nerves not only control heart rate and force, but both sympathetic and parasympathetic nerves supply important reflexogenic areas in different parts of the heart (Persson, 1996). These neural pathways are also directly linked to baroreceptor reflex activity, with changes in blood pressure playing a key role in either increasing or decreasing activity of one or the other pathway. Normal heart-beat and blood pressure vary secondary to respiration, in response to physical, environmental, mental and multiple other factors and is characterized by a circadian variation. Both the basic heart rate and its modulation are primarily determined by alterations in autonomic activity. Increased parasympathetic nervous activity slows the heart rate and increased sympathetic activity increases the heart rate (Figure 9).

In a healthy individual, the role of ANS in the beat-to-beat adjustment of haemodynamics parameters is essential to adequate cardiovascular functioning. As a result, cardiovascular control, as expressed by the time-dependence of haemodynamic variables, is a direct reflection of autonomic activity. It may be a useful tool to examine autonomic fluctuations under different physiological situations (Akselrod et al., 1981). Autonomic nerves, consequently, have an essential role in the regulation of the cardiovascular system both in ensuring optimal function during various activities in health under varying physical conditions, and also in mediating several of the manifestations of cardiac diseases (Eckberg and Fritsch, 1991).

In this context, one of the most interesting non-invasive diagostic methods increasingly used in medicine is the analysis of heart rate variability (HRV), that allows insight into the neural control mechanisms of the heart (Aubert et al., 2003; Batin and Nolan, 1996; Hayano et al., 1991; Malik, 1990; Malliani et al., 1991; Task Force, 1996).

Alteration in the HRV pattern provides an early and sensitive indicator of compromised health (Dekker et al., 1997; Dekker et al., 2000; Liao et al., 1997; Liao et al., 1998). In fact, a high variability in heart rate is a sign of good adaptability; on the other hand, lower variability is often an indicator of abnormal and insufficient adaptability of the autonomic nervous system, implying the presence of a physiological malfunction in the individual for which additional investigations are required to yield a specific diagnosis, and reflecting the vital role the autonomic nervous system plays in maintaining health.

The variations in heart rate may be evaluated by a number of methods. In general, the first step for the analysis of HRV is obtaining high-quality ECG under stationary conditions (Figure 10). For frequency domain measurements, it is recommended that the duration of the recordings is at least two-times the wavelenght of the lowest frequency component. Consequently, the minumum duration for the assessment of the high frequency (HF) component (0.15 Hz) would be 13.3 seconds and for the low frequency (LF) component (0.04 Hz) 50 seconds. However, it is generally recommended to have minimum duration recordings of 5 minutes or even better 10 minutes. Moreover, in order to have a good time resolution and event definition, a sampling rate of at least 250 Hz and up to 1000 Hz is recommended. The second step is the recognition of the QRS complex. The result of analysis is a discrete, unevenly spaced time event series: the tachogram. It is essential that before processing, these signals are corrected for ectopic and missed beats (Aubert and Ramaekers, 1999; Pumprla et al., 2002). A final step is needed before spectral analysis can be performed.
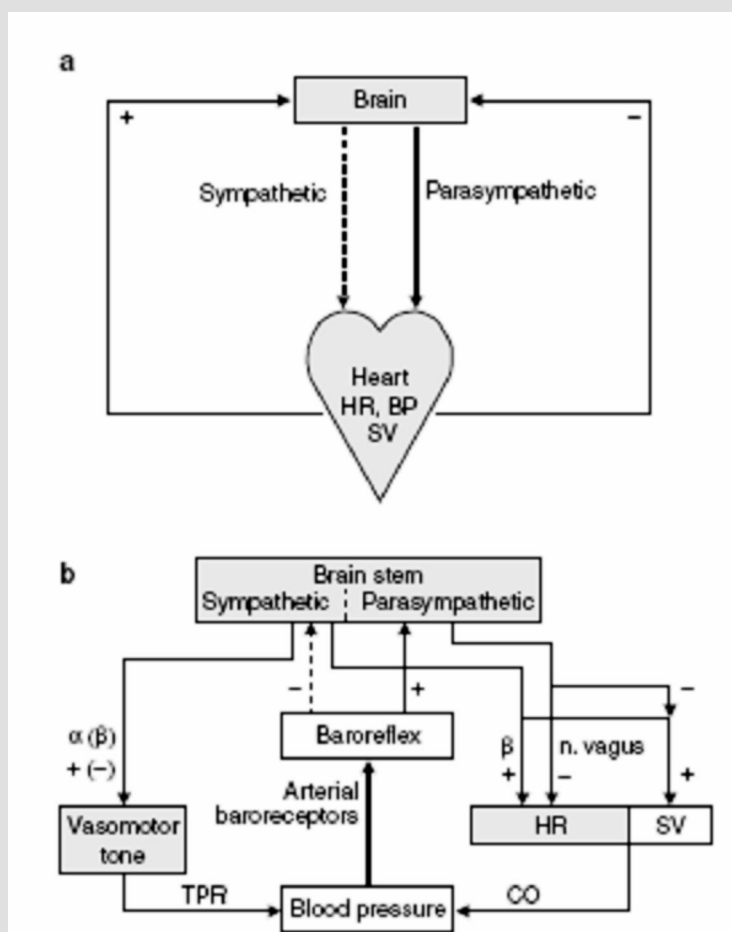
Figure 9. (a) A very simple model illustrating the influence of the sympathetic (increase in heart rate) and parasympathetic (decrease in heart rate) nervous activity on heart rate, the so called ‑balance modelø (b) A more elaborate working model of autonomic neural control mechanisms of HR, BP and the feedback mechanism from the baroreflex. This illustrates independent actions of the vagal, - and -sympathetic systems. Their action can be assessed by measuring heart rate variability, blood pressure variability and the baroreflex mechanism. The parasympathetic activity is responsible for the bradycardia accompanying baroreceptor stimulation and for the tachycardia accompanying baroreceptor deactivation, with the sympathetic nervous system also playing a minor role. BP = blood pressure; CO = cardiac output; HR = heart rate; n. vagus = nervus vagus; SV = stroke volume; TPR = total peripheral resistance; = -sympathetic system; = -sympathetic system. (from Aubert et al. Sports Medicine, 2003).
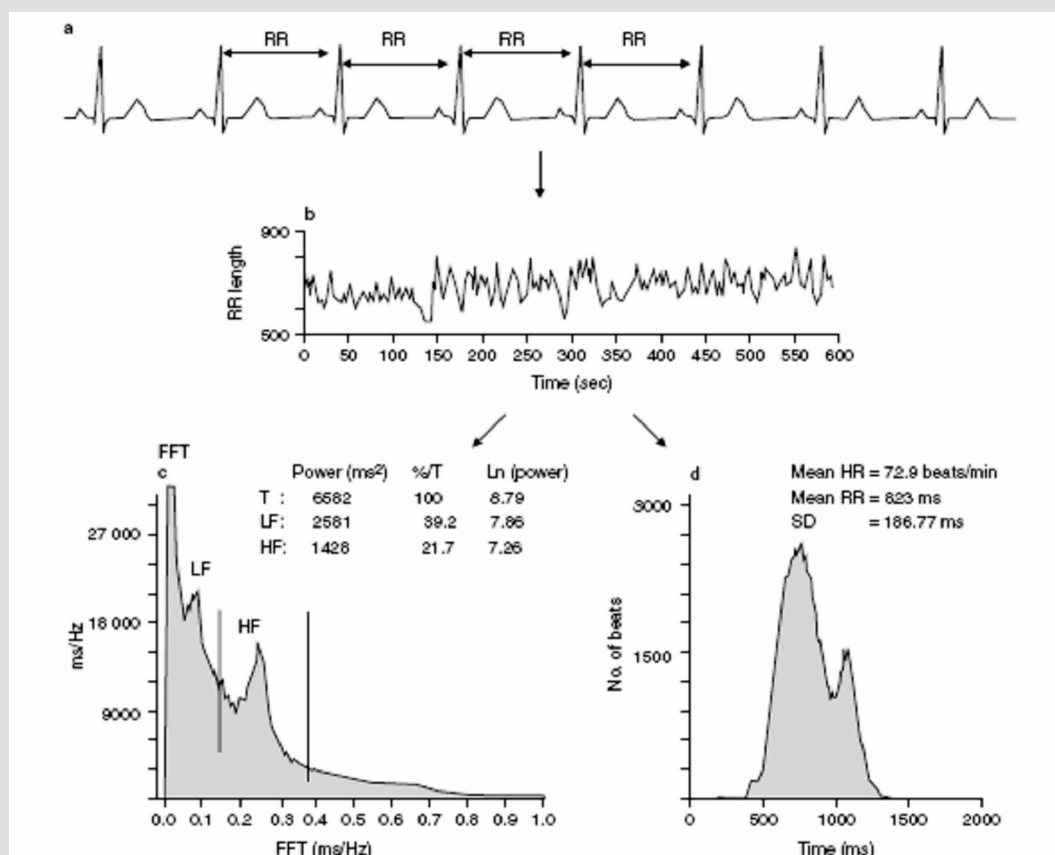
Figure 10. Analysis of heart rate variability. Calculation of consecutive RR intervals (a) on the ECG, results in the tachogram (b) that can be analysed in the frequency domain (c) and the time domain (d). The spectral analysis (c) and the histogram (d) here reported are results from a 24-hour Holter recording. The histogram shows two peaks: one is around 1100ms, which corresponds to mean heart rate at night, and the other is around 750ms, which corresponds to mean heart rate during the day. FFT = fast Fourier transform; HF = high frequency; HR = heart rate; LF = low frequency; Ln = natural logarithm; T = total (from Aubert et al. Sports Medicine, 2003).

Time Domain Methods

These methods are mathematically simple techniques to measure the amount of variability present in pre-specified time periods in a continuous electrocardiogram. Parameters in the time domain are easily computed with simple statistical analysis. References for a standardisation of valid parameters have been published (Task Force, 1996). The definitions for the most frequently used time domain parameters are listed as follows:

- *Standard deviation* (SD) of the RR interval (SD_{RR}, ms) over the recorded time interval (result from corrected signals for ectopic and missed beats by filtering and interpolation algorithms). Theoretically, SD_{RR} estimates overall heart rate variability and therefore includes the contribution of both branches of the autonomic nervous system to heart rate variations: it measures the state of the balance between the activities of the sympathetic component (low-frequency variations) and the parasympathetic branch (high-frequency variations).

- *The root mean square of successive RR interval differences* (r-MSSD, ms). The r-MSSD focuses on high-frequency, short-term variations of RR interval, which are mainly due to the activity of the parasympathetic nervous system (Malik and Camm, 1990; Stein et al., 1994; Sgoifo et al., 1997).

- *The percentage of successive interval differences larger than 50ms* (pNN50, %). pNN50 can be considered as vagal index because it quantifies the short-term variations of the RR interval, which are due to the activity of the parasympathetic nervous system (Stein et al., 1994).

A decrease of these parameters normally reflects a withdrawal of parasympathetic activity and a shift of autonomic balance towards a relative sympathetic prevalence (Kleiger et al., 1992). In addition, clinical studies reveal that a reduction of the values of these parameters is strongly associated with higher risk of cardiac death in cardiovascular and non-cardiovascular patients (Nolan et al., 1998; Thayer and Lane, 2007).

Frequency Domain Analysis

Spectral analysis is commonly used to investigate autonomic cardiovascular control in a variety of physiological and pathophysiological conditions, where heart period can be described as the sum of elementary oscillatory components, defined by their frequency and amplitude (i.e. in the frequency domain) (Task Force, 1996).

The oscillatory pattern which characterizes the spectral profile of heart rate short-term variability consists of two major components, at low (LF, 0.04ó0.15 Hz, with a central frequency around 0.1 Hz) and high (HF, 0.15ó0.4 Hz, with a central frequency at the respiratory rate around 0.25 Hz) frequency, respectively, related to vasomotor and respiratory activity.

Parasympathetic efferent activity was considered rsponsible for HF that is respiration-linked oscillation of HRV. Both parasympathetic and sympathetic outflows were considered to determine LF, together with other regulatory mechanisms such as the renin-angiotensin system and baroreflex. With this procedure the state of sympathovagal balance modulating sinus node pacemaker activity can be quantified in a variety of physiological and pathophysiological conditions. However, the use

of frequency domain methods is not free from restrictions. Especially, spectral methodology needs stationary conditions, not frequent in biological systems.

Non-Linear Methods

Nonlinear events are certainly involved in the origin of HRV. They are determined by complex interactions of hemodynamic, electrophysiological, and humoral variables as well as by the autonomic and central nervous regulations. It has been speculated that the analysis of HRV based on the methods of nonlinear dynamics might provide valuable information for the physiological interpretation of HRV and for the assessment of the risk of sudden cardiac death.

Methods related to the chaos theory are used to describe the nonlinear properties of heart rate fluctuation, as Poincarè plot. This method may give some additional information as compared to other traditional techniques. In particular, spectral analysis requires stationarity of the signal and is sensitive to artifacts; in contrast Poincarè plot is less sensitive to these prerequisites (Seely and Macklem, 2004). Other types of nonlinear methods used to complement the conventional measures of HRV are those based on fractal theory. Actually, the nonlinear methods for HRV analysis may provide a more sensitive way to characterise function or dysfunction of the control mechanism of the cardiovascular system.

In the last two decades, analysis of HRV has been extensively applied to the investigation of normal physiology. The use of HRV analysis has provided a simple reproducible method of non-invasive autonomic assessment. This has helped to clarify the role of the autonomic nervous system in regulating the cardiovascular response to change in posture (parasympathetic dominance when supine, sympathetic dominance when standing), or exposure to stressors (sympathetic dominance).

Measurement of HRV may be of prognostic value even in individuals who are free of overt clinical disease, with low values identifying those at increased risk of premature cardiac disease; in fact HRV analysis is increasingly used to measure autonomic dysfunction in different pathological states. Actually, there is growing evidence for the role of autonomic nervous system in a number of somatic and mental diseases (Thayer and Sternberg, 2006). In particular, the autonomic imbalance in which the sympathetic branch predominates, characterized by a reduction in HRV, is associated with various pathological conditions of either cardiac or non-cardiac origin, as diabetes mellitus (Ziegler, 1999) and psychopathology (Thayer et al., 1996).

5. Rodent models of depression

Animal models for psychopathology have become an invaluable instrument in the analysis of the causes, genetic, physiological, psychological, environmental or pharmacological, that can bring about symptoms homologous to those of depressed patients (Shekhar et al., 2001). Research with human subjects is useful for answering certain experimental questions; however, animal methods may allow for the direct study of the pathogenesis, biological mechanisms, and treatments for depression, and they are valuable tools for studying the interaction between psychological and physiological aspects. Animal models of depression are typically based on exposure of animals to a stressful condition (a potential or actual threatening situation) and a specific tests for measuring behavioural and physiological changes.

Currently, animal models are sought that have three types of validity: *i) Face validity*, where the model is phenotypically similar and implies that the response observed in the animal model should be identical to the behavioural and physiological responses observed in humans; *ii) Predictive validity* requires that the model should be sensitive to clinically effective pharmacological agents used in humans; *iii) Construct validity* relates to the similarity between the theoretical rationale underlying the animal model and human behavior. This requires that the aetiology of the behavioral and biological factors underlying the disorder is similar in animals and humans (Willner et al., 1997). In general, in an ideal model one would like to have identical causal conditions to the human disease state (etiological validity), identical symptom profiles to the illness state (face validity), and the same treatment responses to that seen in human depression, (predictive validity). The first condition is difficult to study since we do not understand the aetiology of depression in detail. Face validity for depression includes alterations in mood (difficult to test in animals), alteration in appetite and weight, changes in cognition, changes in the HPA axis, sleep disturbances, libido changes, often coupled with decreased pleasure seeking or anhedonia. Anhedonia is often measured as a decreased preference for sucrose solution, which is a drink that is usually preferred by rodents to water. The last condition, predictive validity, requires a behavioral response to antidepressant that involves a time delay onset of action and specificity for only clinically active antidepressants.

Several animal models of depression have been developed and reasonably well validated. These include the learned helplessness model created by Seligman (Seligman et al., 1980), the behavioral despair model developed by Porsolt (Porsolt et al., 1978), and the chronic mild stress model described by Willner and colleagues (Willner et al., 1997).While some animal models were mainly designed to have high predictive validity for the purpose of screening antidepressive drugs, others are high in both face and construct as well as predictive validity (Bourin et al., 2007).

5.1 Learned Helplessness

The term *'Learned helplessness'* refers to a constellation of behavioral changes that follows exposure to stressors that are not controllable by means of behavioral coping, but that fail to occur if the stressor is controllable (Maier and Watkins, 2005). A presumed state of depression is induced in animals by exposing them to aversive stimuli like shock under circumstances in which they cannot control or predict the onset or duration of these stimuli. This model derives from a cognitive vision of depression in which events are viewed negatively and interpreted as not controllable leading to feelings of anxiety and helplessness when dealing with them. Indeed, the cognitive theory of depression suggests that uncontrollable stress, which cannot be predicted, might lead to reactions and behaviors of vulnerability similar to those found in depression. On the other hand, the major criticism to this model concerns the lack of reliability in human specie, as well as the lack of concrete experimental evidence that learned helplessness is a behavioural process characterizing depressed individuals (Geyer and Markou, 1995). This procedure results in long-lasting deficits in the motivation and ability to escape in subsequent tests where escape is possible, and show behavioural alterations such as vocalizations and passivity, as well as alterations in sleep-wake patterns (Adrien et al., 1991). Pharmacological treatment with antidepressants such as imipramine reduces these behavioural changes (Besson et al., 1999). In addition, the fact that only a proportion of animals develop helplessness suggested that this model has a genetic component and is appropriate for studying the interaction of stress and genetic vulnerability (Vollmayr et al., 2004).

5.2 Behavioral Despair Paradigm

The ±Behavioral Despair Paradigmø (BDP; also named the forced swim test), is currently one of the most frequently used behavioral test for investigating antidepressant potential. (Petit-Demouliere et al., 2005; Borsini and Meli, 1988). This paradigm was developed using rats and then adapted to mice (Porsolt et al., 1978). In this procedure, rats or mice are forced to swim in a confined space on one or several successive occasions; the test is based on the observation that when placed in a cylinder containing water, rodents rapidly become immobile after unsuccessful attempts to escape. Antidepressants decrease the duration of immobility which is used as the main predictor of antidepressant-like activity. Despite its recognized predictive validity for antidepressants, the BDP has been criticized for relatively low sensitivity to antidepressants acting on serotonin and positive response to psychostimulants, with the latter considered to be devoid of real antidepressant activity (Borsini and Meli, 1988; Rupniak, 2003). In addition, although this model has one of the highest degrees of pharmacological predictive validity in terms of identifying antidepressants, a problem exists about the interpretation of immobility during forced swimming as reflecting failure to cope.

Instead the immobility might reflect a successful, adaptive strategy that conserves energy and permits the animal to float for prolonged periods of time, thus surviving longer (Geyer and Markou, 1995).

5.3 Chronic Mild Stress

Under circumstances other than natural disasters and war, humans are not normally exposed to brief but intense stressors. Rather, people are more likely to be subjected to periods of stress that wax and wane during their lifetime. Some people are less resilient to these stressors, and can be vulnerable to mild but prolonged stressors (Hammen, 2005; Kessler, 1997). A stress model of depression seeking to simulate this environmental condition was initially proposed and developed by Katz and his colleagues who subjected rats to a combination of various stressors, such as electrical shock, immersion in cold water, reversal of light/dark cycle, fasting, isolation, tail pinch, being shaken, moved from cage to cage over a period of 3 weeks. Following this induction period the rats were exposed to high intensity light and sound that provoked a reduced motility in the stressed animal when compared to non-stressed animals (Katz, 1981a; Katz et al., 1981b). Furthermore, chronically stressed animals reduced their intake of a palatable saccharine solution (Katz, 1982) suggesting that they were impaired in their capacity to derive pleasure from this solution, i.e., anhedonic.

The validity, reliability and utility of the chronic mild stress model (CMS) have been thoroughly described by Willner (1997), whereby rodents are subjected to similar but milder stressors to those used by Katz (1981a,b). The measure most commonly used to track CMS effects is a decrease in consumption of a palatable sweet solution. The CMS paradigm has been considered to provide a relative realistic animal model of anhedonia, the core symptom of depressive disorder (Willner, 1997). Consequently, in the beginning the behavioural read-out variable indicative of anhedonia in this CMS model was sucrose or saccharine intake (Muscat et al., 1992; Papp et al., 1991); however, this has been considered by some to be too variable and other indices of anhedonia-like behaviour such as novel object place conditioning (Anisman and Matheson, 2005; Barr and Phillips, 1998; Bevins and Besheers, 2005).

Recent studies with the CMS model have provided insight into potential behavioral and physiological processes that are dysregulated in depression. In a series of studies conducted by Grippo and coworkers (2002, 2003), adult, male rats were exposed to 4 weeks of CMS, which induced behavioral changes linked to depression syndromes. The procedure induces anhedonia (reduced responsiveness to a previously defined rewarding stimulus) in rats, as measured by changes in sucrose consumption (Willner et al., 1987) or behavioral responses to rewarding electrical brain stimulation (Moreau et al., 1992). Long-term disturbances in circadian rhythms of locomotor activity have also been demonstrated in rats exposed to 4 weeks of CMS (Gorka et al.

1996), as well as disrupted sexual behavior and sleep patterns (Cheeta et al., 1997; DøAquila et al., 1994).

Furthermore, CMS is also employed for studying the effects of depression on physiological and cardiovascular variables. In particular, rats that were previously exposed to CMS showed exaggerated pressor and heart rate reactivity when submitted to a novel acute stressor (Grippo et al., 2002). While the behavioral changes associated with CMS recover within a few weeks following cessation of the stressors, these cardiovascular disruptions persist across time, suggesting that simple remediation of the depressive signs is not associated with alleviation of the underlying cardiovascular pathophysiology.

5.4 Prenatal stress as an animal model of depression

Prenatal environment exerts profound influences on the development of an organism and stressful events during pregnancy can induce alterations in the foetal environment resulting in early and long-term structural and functional consequences (Maccari et al., 2003; Wadhwa et al., 2001; Weinstock, 1997, 2001). In this regard, prenatally stressed rats display biobehavioural alterations that can parallel to some extent indices of human depression.

In rat models of prenatal stress pregnant dams have been exposed to a variety of stressors, including saline injections (White and Birkle, 2001), immobilization (Ward and Stehm, 1991), hypoxia (Peyronnet et al., 2002), electric footshock (Weinstock et al., 1998), placental insufficiency (Alexander, 2003), and REM sleep deprivation (Suchecki and Palermo-Neto, 1991). Lately, a frequently used protocol is a modified version of Ward and Weisz model (Maccari et al., 1995; Ward and Weisz, 1984), consisting of restraining the mothers during the last week of pregnancy. This paradigm produces a robust psychoneuroendocrine stress activation in the mothers that interferes with the development of neural networks and neuroendocrine systems in the offspring, which in turn modulate behavioral and physiological stress responses in adulthood (Kofman, 2002).

Prenatally stressed rats exhibit prolonged adrenocortical stress responsivity, disturbed circadian rhythmicity of heart rate, body temperature, and physical activity, and increased adrenal weight when challenged in adulthood. This evidence supports the idea that prenatal stress *per se* does not change dramatically a given structure or function, but it affects resilience and renders the animal more susceptible to pathophysiological outcomes, as depression, when further insults occur during adulthood (Mastorci et al., 2009). In **Chapter 2** I made use of this paradigm to study the long-term effects of foetal manipulation, with special emphasis on depressive-like symptoms in adulthoodq1\.

5.5 Depression induced via social stress

It is generally acknowledged that the main source of stress stimuli in humans is of social nature and contributes to the development and expression of mood disorders and anxiety disturbances (Brown, 1993; Kessler, 1997). The most commonly used stressors in rats and mice studies include restraint stress, uncontrollable electric shocks, forced running, forced swim, and sequential exposure to a variety of severe or mild chronic mild stressors. Although effective and useful, these stressors are physical, potentially painful and they offer little face validity compared to social and psychological stressors that are of particular interest in humans. These conventional animal models of stress appear to be quite far from real life events, either for the experimental animal model or the human counterpart. In the social domain, the most frequently used model for rodents is acute or chronic social defeat, that induces depressive-like behavior in male animals (Fuchs and Flugge, 2002; Rygula et al., 2005). Acute or brief intermittent social defeat stress allowed the analysis of behavioral, endocrine, and brain system alterations, which were prevented by antidepressant drugs. Behavioral changes such as decreased social interaction (Meerlo et al., 1996) and anhedonia (von Frijtag et al., 2002), next to physiological (Meerlo et al., 1996), neuroendocrine (Blanchard et al., 1993) and neurobiological (Buwalda et al., 1999) consequences of social stress are interpreted as signs mimicking certain aspects of human depression.

In particular, in a study by Rygula and colleagues (2005), chronic social stress protocol resulted in a series of depressive-like symptoms: i) prolonged immobility time in the forced swimming test (symptomatic of decreased motivation and behavioral despair), ii) reduced preference for sweet sucrose solution (an indicator of anhedonia in rodents), and iii) reduced locomotor and exploratory activity (suggesting changes in incentive motivation). In agreement with these results, Nestler and colleagues (2002) assessed behavioral and neurochemical alterations following exposure of mice to social defeat for 10 consecutive days, and suggested that this paradigm could represent a good model of depression.

Becker and colleagues (2008) explored the validity of prolonged social defeat procedure as a model of depression in rats.

They haved found that repeated social defeat elicits changes that could be considered as behavioral and biological correlates of depressive symptoms in humans, such as a hyperactivity of HPA axis, increased immobility time in the forced swimming test, decrease of body weight and of sweet water consumption and reduction of hippocampal volume associated with a decreased cell proliferation in the dentate gyrus.

In general, most relevant studies have implicated chronic stress as a major factor in the onset of depression. The chronic character of stressors may result in various forms of stress pathology.

However, chronic stress is not a well-defined concept. Using a series of intermittent daily changing stressors rather than the continuous presence of a stressor has limited relevance to mimic the aetiology of depression (Koolhaas et al., 1997). Moreover, stressors must mimic the environmental challenges that an animal may meet in its everyday life in a natural environment with respect to the type, severity and frequency of the stressors (Blanchard et al., 2001). In this context, loss of environmental control (in a social setting, this might mean loss of social control, that is, social defeat) is a major factor in inducing stress pathology. For this reason, using an expanded (over 20 days) social stress episode repeated over 4 weeks in rats provides a realistic simulation of the aetiology of depression. In fact, repeated social defeat procedure resulted in profound long-term changes of behavioral and biological parameters in subordinate rats (Becker et al., 2008). In particular, this model produced a hyperactivity of HPA axis, as show by the increases in serum corticosterone levels and adrenal weight, and decreases of body weight and sweet water consumption. Another behavioural change that may be regarded as a relevat index of depression-like symptom is the prolonged immobility in the forced swim test. In **Chapter 3** I employed social defeat associated to isolation in rats to better understand cardiovascular, behavioural and hormonal alterations in depression.

6. Neurobiology of cardiovascular response to stress

Central cardiac control has been studied extensively and several reviews provide comprehensive coverage of relevant neurophysiological mechanisms (Dampney, 1994). Figure 11 summarizes current knowledge about brain areas involved in the regulation of cardiac function. The two basic principles of the neural control of the heart are its dual nature (vagal/sympathetic) and hierarchical organization (intrinsic cardiac network/intrathoracic ganglia/spinal cord/lower brain stem/upper brain stem/forebrain).
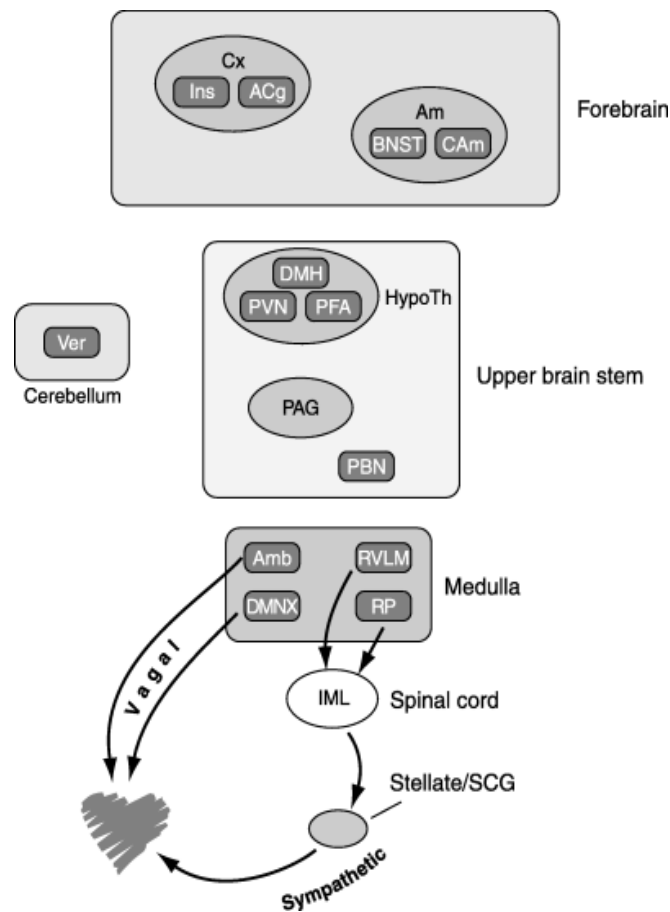

Figure 11. **Brain structures involved in the control of the heart.** ACg, anterior cingulate; Amb, nucleus abigous; BNST, bed nucleus of stria terminalis; CAm, central amygdala; DMH, dorsomedial hypothalamus; DMNX, dorsal motor nucleus of the vagus nerve; IML, intermediolateral column; Ins, insular cortex; PAG, periaqueductal grey; PBN, parabrachial nucleus; PFA, periphornical area; PVN, paraventricular nucleus; RP, raphe pallidus; RVLM, rostral ventro-lateral medulla; SCG, superior cervical ganglia; Ver, vermix (from Nalivaiko, Clin Exp Pharmacol Physiol. 2006)

Both human and animal studies demonstrate that sympathetic neural activity predisposes the heart to cardiac arrhythmias and increases the likelihood of ventricular fibrillation (Schwartz, 1996; Nalivaiko et al., 2004). Enhancing sympathetic activity by electrical stimulation of several brain areas or of the stellate ganglia or cardiac nerves (Priori et al., 1988) increases cardiac susceptibility to arrhythmias, as does exposure to sympathomimetic amines.

Conversely, pharmacological (Yusuf et al., 1996) or surgical (Schwartz et al., 1991) suppression of sympathetic activity to the heart appeared to be cardioprotective. At present, understanding of the brain pathways mediating stress-related cardiac events is limited. Clearly, cardiac effects induced by psychological stress are initiated in the forebrain. Stimulation of the insular cortex (Oppenheimer et al., 1991) the central amygdala (Markgraf and Kappor, 1988) and the dorsomedial hypothalamus (Poisson et al., 2000) elicits arrhythmias in experimental animals.

Vagal and sympathetic outflow to the heart constitute the two major efferent neural pathways. The lower brain stem contains integrative control centres that regulate these outflows. Cardiac vagal neural activity originates in the nucleus ambiguus and the dorsal vagal nucleus; whereas the rostral ventrolateral medulla (RVLM) was considered as a principal origin of sympathetic cardiac activity (Dampney, 1994; Campos and McAllen, 1999).

6.1 Key nuclei implicated in the central autonomic regulation of stress response

The dorsomedial hypothalamus (DMH) has been shown to be crucial in the control of the cardiovascular response during psychological stress exposure (DiMicco et al., 2002). Pharmacological stimulation of the DMH in anaesthetised rats causes tachycardia, pressor responses, and increased renal sympathetic nerve activity (Fontes et al., 2001; Horiuchi et al., 2004). In conscious rats stimulation of the DMH causes tachycardia and pressor responses (Goren et al., 2003), and the pharmacological inhibition of the DMH by local administration of the $GABA_A$ receptor agonist, muscimol, attenuates acute psychological stress-evoked tachycardia and pressor responses (Stotzpotter et al., 1996a; Stotzpotter et al., 1996b; McDougall et al., 2004). This indicates that the DMH may be ‐hard wiredø into the stress circuitry responsible for the regulation of the sympathoadrenal system (SAS) as the stressors used in the aforementioned studies were psychological in nature and the paradigms did not involve conditioning.

The DMH may mediate autonomic flow via a number of descending pathways. In anaesthetised animals the pressor responses elicited by pharmacological stimulation of the DMH can be attenuated by pharmacological inhibition of the RVLM and both the tachycardia and blood pressure responses can be blocked by inhibition of the rostral raphe pallidus (but overlying raphe magnus cannot be excluded due to the methodology of these experiments) (Fontes et al., 2001, Samuels et al., 2002; Horiuchi et al., 2004). Additionally, rabbits exposed to psychological stressors exhibit

cutaneous vasoconstriction as evidenced by reduced blood flow to their ear pinnae (Yu and Blessing, 1997; Nalivaiko and Blessing, 1999). A similar response can be evoked by electrical stimulation of the DMH region in anaesthetised rabbits, which was subsequently blocked by pharmacological inhibition of the rostral medullary raphe (Nalivaiko and Blessing, 2001). Indeed, pharmacological stimulation of the rostral medullary raphe in anaesthetised rats has been shown to increase cardiac sympathetic nerve activity and tachycardia, a response apparently independent of RVLM involvement (Cao and Morrison, 2003).

Additionally, the modulation of the cardiovascular system by the DMH has been shown to be mediated in part by the lateral/dorsolateral region of the periaquiductal grey (PAG) in conscious rats (da Silva et al., 2003). Thus, the DMH would seem to modulate sympathetic outflow by direct efferents to the raphe pallidus and the RVLM and indirectly via the PAG to raphe magnus (Farkas et al., 1998) and the RVLM during psychological stress exposure. Recently the role of the PAG with respect to generation of a stress response has been reviewed (Bandler et al., 2000; Keay and Bandler, 2001), where the dorsolateral and lateral columns of the PAG have been identified as central to ─fight/flightø responses. Additionally, lesions of the dorsal PAG in rats suggest an excitatory influence on the tachycardic response to cat odor exposure (Dielenberg et al., 2004), although no effect was observed with air-jet stress exposure (Lam et al., 1995). Meanwhile, recent studies focusing on the ventrolateral PAG have shown that inhibition of this region did not impact on the cardiovascular response evoked by conditioned fear, but did impede the return to resting (pre-stress) levels (Walker and Carrive, 2003). The PAG, therefore, would seem to play a role in the mediation of the cardiovascular response in both unconditioned and conditioned paradigms.

Another important area that links psychologically induced stress with the cardiovascular system (in particular, with the blood pressure-regulatory system) is the central nucleus of the amygdala (CeA). This is an integratory forebrain nucleus that receives input from higher centres in the forebrain and has extensive connections with the hypothalamus and the medulla oblongata, areas involved in the regulation of cardiovascular reflexes. Based on studies using electrical or chemical stimulation or electrolytic lesions of CeA, it has become clear that CeA plays an important role in the regulation of blood pressure in response to stressful or fearful stimuli.

A crucial medullary area known to receive projections from the CeA is the nucleus tractus solitarius (NTS). The NTS is the site of the first synapse for afferent fibres originating from baroreceptors, chemoreceptors and the heart. Another projection from the CeA to the brainstem that is believed to be important in blood pressure regulation is the projection to the RVLM (Wallace et al., 1992; Takayama et al., 1990). These RVLM neurons are the major source of descending input to the sympathetic vasomotor neurons in the spinal cord, which supply the heart and blood vessels and play a major role in the tonic and reflex control of blood pressure (Dampney, 1994). When activated

experimentally in anesthetized cats, these sympathetic premotor neurons drive the neural supplies to blood vessels, adrenal medulla, and heart but apparently not to other targets (McAllen, 1986).

A small population of amygdala efferents appear to form synapses with catecholaminergic neurons in the RVLM (Wallace et al., 1992). The C1 catecholamine cells are found in the pressor region of the RVLM and many of these C1 cells are known to project to the intermediolateral cell column (IML) in the spinal cord. In conclusion, the CeA is capable of modulating blood pressure and sympathetic outflow, at least in part, via its direct projections to medullary neurons. Through direct GABAergic projections to the NTS, the CeA may influence the activity of neurons that are involved in blood pressure regulation, such as neurons that participate in baroreceptor reflex circuits in the NTS. The direct projections from the CeA to RVLM baroreceptive neurons suggest that the CeA may also have a direct effect on sympathetic nerve activity and blood pressure.

6.2 Role of 5-HT$_{1A}$ receptors in cardiovascular response to stress

Serotonin (5-HT) has an extensive spectrum of physiological, behavioral and cognitive actions. These actions are mediated via at least 14 subtypes of 5-HT receptors (Hoyer et al., 2002). Structure, anatomical locations, pharmacology and cellular effects of these receptors are covered in a comprehensive review by Barnes and Sharp (1999). 5-HT was initially characterized as a vasoactive substance, and the knowledge about its cardiovascular effects continuously expands. The plasma level of 5-HT is very low, and its peripheral vascular effect occur when the substance is released from the platelets. Within the central nervous system, the two principal 5-HT receptor subtypes involved in modulation of cardiac and vascular control are 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors. Activations of central 5-HT$_{1A}$ receptors causes sympatho-inibitory effects (fall in arterial pressure AP and heart rate HR), while activation of 5-HT$_{2A}$ receptors induces sympatho-activatory actions (pressor, tachycardic, rise in lumbar sympathetic, renal and cardiac nerve discharge.

Psychological stressors facilitate serotonergic neurotransmission in the brain (Chaouloff et al., 1999). Most earlier information about cardiovascular effects of 5-HT receptor agonists and antagonists was collected either in anesthetized state or during quiet waking of experimental animals. It is only recently that several research groups focused on 5-HT receptor-mediated cardiovascular changes in conscious rats and rabbits undergoing stress exposure.

In two recent studies conducted in conscious rabbit and rats, Nalivaiko and colleagues found that systemic administration of 8-OH-DPAT (a selective 5-HT$_{1A}$ receptor agonist) suppressed tachycardic and pressor responses to psychological stressors (Nalivaiko et al., 2005; Ngampramuan et al., 2008). These results appeared in very good accord with the work of van den Buuse and Wegener (2005), who studied the effects of several 5-HT$_{1A}$ agonists on basal arterial pressure and

HR and on AP and HR changes elicited by the open field test. More recently, Vianna and Carrive (2008) confirmed Nalivaikoøs data in restrained rats, and extended the study to the conditioned fear paradigm where systemically administered 8-OH-DPAT also substantially and significantly attenuated pressor and tachycardic responses. Therefore, anti-tachycardic and/or anti-pressor effects of systemically administered 5-HT$_{1A}$ agonists during stress could be considered quite consistent as they were indipendently observed by three different groups in two mammalian species and in stress paradigms as different as airjet, restraint, novelty and conditioned fear.

Systemic administration of 8-OH-DPAT also reduced stress activated vagal withdrawal observed at the beginning of a restraint test (Ngampramuan et al., 2008). Possible mechanisms underlying this effect are covered by Jordan (2005), that suggests activations of cardiomotor vagal neurons. In fact, local application of 8-OH-DPAT activated cardiac vagal neurons, and this response was suppressed by WAY-100,635, a selective 5-HT$_{1A}$ receptor antagonist (Wang and Ramage, 2001). As 5-HT$_{1A}$ receptors are inhibitory ones, Jordan (2005) suggested that they could be located on the inhibitory GABA-ergic interneurons in the nucleus ambiguus. The source of serotonergic innervation of this nucleus is the medullary raphe.

In order to reveal the location of 5-HT$_{1A}$ receptors, Nalivaiko and coworkers performed brain microinjections of 8-OH-DPAT in rats during restraint and in rabbits during airjet stress. The target for the microinjections was the medullary raphe region as evidence accumulates that this could be a location of presympathetic cardiomotor neurons, that these neurons are silent at rest but could be activated in stress conditions (Samuels et al., 2002; Zaretsky et al., 2003), and that the activation of medullary 5-HT$_{1A}$ receptors attenuates centrally induced tachycardia in anesthetized rats (Morrison, 2004). Admistration of 8-OH-DPAT mimicked anti-tachycardic effects of systemically injected drug, supporting the idea that cardiac presympathetics are indeed located in the raphe (Nalivaiko et al., 2005; Ngampramuan et al., 2008). Conversely, intra-raphe microinjection of the drug did not prevent or reduce stress-induced rise in AP (Nalivaiko et al., 2005) suggesting that anti-pressor effect could be mediated by 5-HT$_{1A}$ receptors with different anatomical localization (possibly in the RVLM vasopressor region).

The medullary raphe/parapyramidal area is a major centre that integrates the neural control of several autonomic functions during stress. This brain stem region also represents the major source of descending 5-HT projections to the spinal sympathetic neurons. Many bulbospinal neurons located in the raphe/parapyramidal area are serotonergic and express inhibitory 5-HT$_{1A}$ autoreceptors on their perikarya and dendrites (Helke et al., 1997). Retrograde viral tracing revealed that this same area contains presympathetic cardiomotor neurons; their neurotransmitter phenotype is currently unknown. If all or some of these rapheóspinal cardiomotor neurons are serotonergic and express autoinhibitory 5-HT$_{1A}$ receptors, they may constitute our hypothetical cardiomotor brain

area involved in psychogenic cardiac disturbances. If this is so, a reduced function or density of inhibitory 5-HT$_{1A}$ receptors may lead to an increase of cardiac sympathetic nerve activity and, consequently, to increased release of noradrenaline in the myocardium.

Now, the source of 5-HT that activates 5-H$_{1A}$ receptors in the medullary raphe under natural circumstances is unknown, It may be that the neurotransmitter is released either from the neighbouring synaptic terminals or from the dendrites of serotonergic neurons themselves. In this context, Nalivaiko and coworkers (2006) suggest that the purpose of this serotonergic inhibition (or autoinhibition) is to limit excessive activity in the cardiac sympathetic nerves. The finding of involvement of medullary 5-HT$_{1A}$ receptors in cardiac control during stress, led us to the hypothesis that the missing link between mental and cardiac disorders may be associated with the function, or rather dysfunction, of the raphe neurons. A reduced density of 5-HT$_{1A}$ receptors in the raphe area (possibly occurring during chronic stress/depression) may lead to increased sympathetic outflow to the heart and, thus, may be a critical link between anxiety/depression and cardiac disorders (Figure 12). A reduced density of 5-HT$_{1A}$ receptors in the cingulate cortex, in the amygdala and possibly in hypothalamic regions may also lead to additional activation of presymapthetic cardiomotor neurons, via a descending activatory pathway to the medullary raphe.

I further examined the role of 5-HT$_{1A}$ receptors in cardiovascular response to stress in **Chapter 5**, by means of knockout mice model.
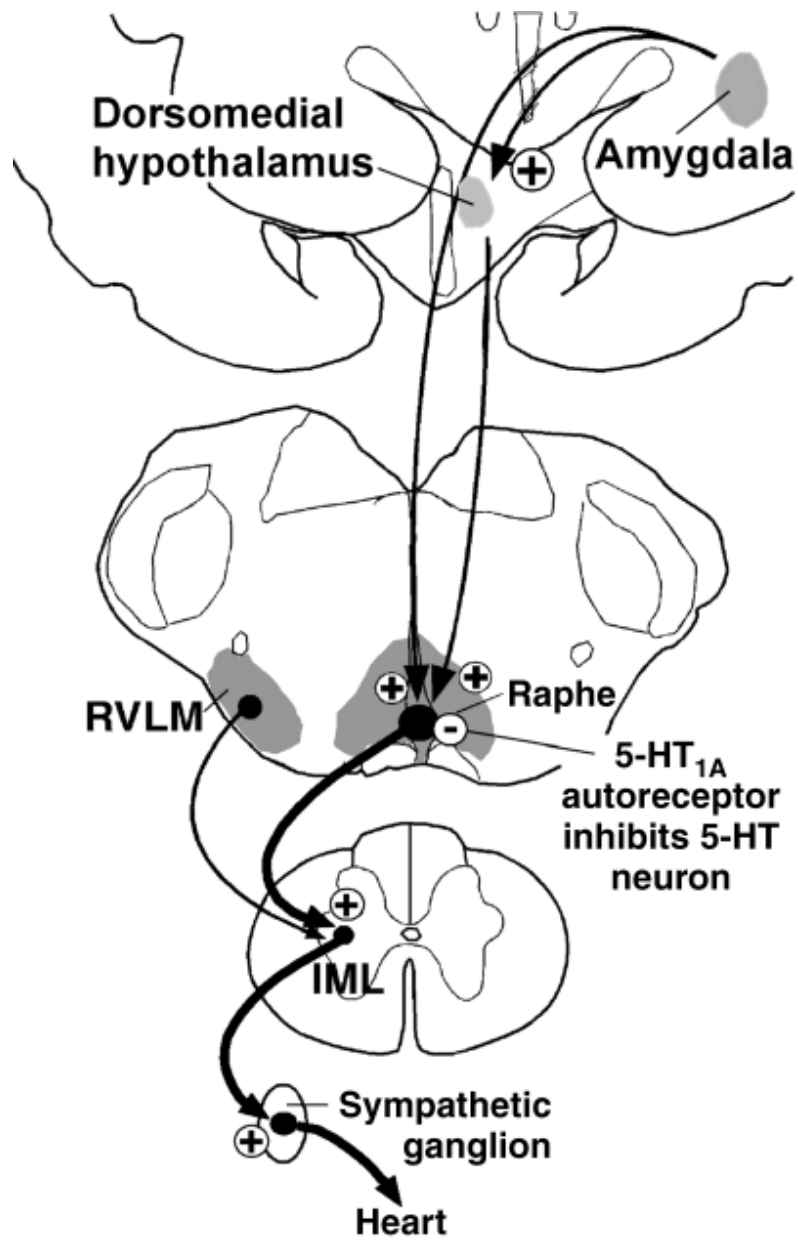
Figure 12. Hypothesis: reduction of 5-HT$_{1A}$ receptor function in the medullary raphe may lead to insufficient inhibition of rapheóspinal presympathetic cardiomotor neurons. This may result in the increase of cardiac sympathetic nerve activity (thick lines), leading to elevated noradrenaline release in the heart. IML, intermediolateral column of the spinal cord; RVLM, rostral ventrolateral medulla (from Nalivaiko, Clin Exp Pharmacol Physiol. 2006).

Outline of this thesis

The main aim of this thesis is to understand cardiac autonomic reactivity to stress in rodents. In particular, I study cardiovascular, neuroendocrine, and behavioral effects of exposure to acute or chronic, social or non-social stress in rats and mice. In the first time, I chose to investigate the long-term consequences of foetal manipulation, more specifically, I examine if exposition to stress during the last week of pregnancy can predispose to develop pathologies as depression in adult life. Subsequently, I studied, in adulthood, the role of an adverse stress episode such as social defeat followed by a prolonged period of isolation in producing effects which resemble some of the symptoms of depression in humans. The next step was tried the central mechanisms generating increases in cardiac sympathetic activity during stress, and for this aim I used systemic administration of 8-OH-DPAT, a 5-HT$_{1A}$ agonist possessing central sympatholytic properties. Finally, I chose to study the role of these receptors in cardiovascular stress response using mouse knockout for serotonin 1A receptors.

In particular, the goals of this thesis could be summarize in the following points:

- In **Chapter 2** I investigate possible long-term effects of prenatal stress on (i) acute adrenocortical and cardiac sympathovagal stress reactivity (ii) circadian rhythmicity of heart rate, body temperature, and physical activity, and (iii) myocardial and adrenal structure.

- In **Chapter 3** I implemented an animal model of depression based on the exposure to an adverse stress episode (*social defeat*) followed by prolonged social isolation and explored its effects on: (i) acute adrenocortical and cardiac sympathovagal stress reactivity (ii) sucrose intake (iii) circadian rhythmicity of heart rate, body temperature, and physical activity, and (iv) myocardial and adrenal structure.

- In **Chapter 4** I tested wheter ventricular arrhythmias precipitated by acute stressors could be suppressed by 8-OH-DPAT, a 5-HT1A agonist possessing central sympatholytic properties

- In **Chapter 5** I made use of a mouse knockout for serotonin 1A receptors and I explored the role of these receptors in the presympathetic modulation of acute and long-term cardiac stress responses to both brief and long-lasting challenges.

References

aan het Rot M., Mathew S.J., Charney D.S. Neurobiological mechanisms in major depressive disorder, *CMAJ* 180 (2009), 305-313.

Adell A., Garcia-Marquez C., Armario A., Gelpi E. Chronic stress increases serotonin and noradrenaline in rat brain and sensitizes their responses to a further acute stress, *J. Neurochem.* 50 (1988), 1678ó1681.

Adrien J., Dugovic C., Martin P. Sleep-wakefulness patterns in the helpless rat, *Physiol Behav.* 1991 (2), 257-62.

Akselrod S., Gordon D., Ubel F.A., Shannon D.C., Berger A.C., Cohen R.J. Power spectrum analysis of heart rate fluctuation: a quantitative probe of beat-to-beat cardiovascular control, Science 213 (1981), 220-222.

Alexander B.T. Placental insufficiency leads to development of hypertension in growth-restricted offspring, Hypertension 41 (2003), 457-462.

Allender S., Scarborough P., Peto V., Rayner M., Leal J., Luengo-Fernandez R., Gray A. European Cardiovascular Disease Statistics 2008 edition. *British Heart Foundation* 2008, London.

American Psychiatric Association. Diagnostic and statistical manual of mental disorders (4th ed.), *American Psychiatric Association* 1994, Washington, DC.

Anisman H., Matheson K. Stress, depression, and anhedonia: caveats concerning animal models, *Neurosci Biobehav Rev* 29 (2005), 525ó546.

Anon. Prevalence of heart disease-United States, *JAMA* 297 (2005), 1308-1309.

Arborelius L., Owens M.J., Plotsky P.M., Nemeroff C.B. The role of corticotropin-releasing factor in depression and anxiety disorders, *J Endocrinol.* 160 (1999), 1-12.

Asnis G.M., Halbreich U., Ryan N.D., Rabinowicz H., Puig-Antich J., Nelson B., Novacenko H., Friedman J.H. The relationship of the dexamethasone suppression test (1 mg and 2 mg) to basal plasma cortisol levels in endogenous depression, *Psychoneuroendocrinology* 12 (1987), 295ó301.

Aubert A.E., Ramaekers D. Neurocardiology: the benefits of irregularity. The basics of methodology, physiology and current clinical applications, *Acta Cardiol.* 54 (1999), 107-120.

Aubert A.E., Seps B., Beckers F. Heart rate variability in athletes, *Sports Med* 33 (2003), 889-919.

Bairey Merz C.N., Dwyer J., Nordstrom C.K., Walton K.G., Salerno J.W., Schneider R.H. Psychosocial stress and cardiovascular disease: pathophysiological links, *Behav. Med.* 27 (2002), 141‒147.

Baker D., Mead N., Campbell S. Inequalities in morbidity and consulting behaviour for socially vulnerable groups, *Br J Gen Pract* 52 (2002), 124-30.

Bandler R., Keay K.A., Floyd N., Price J. Central circuits mediating patterned autonomic activity during active vs. passive emotional coping, *Brain Res. Bull.* 53 (2000), 95‒104.

Barefoot J.C., Helms M.J., Mark D.B., Blumenthal J.A., Califf R.M., Haney T.L., O'Connor C.M., Siegler I.C., Williams R.B. Depression and long-term mortality risk in patients with coronary artery disease, *Am J Cardiol* 78 (1996), 613‒617.

Barefoot J.C., Brummett B.H., Clapp-Channing N.E., Siegler I.C., Vitaliano P.P., Williams R.B., Mark D.B. Moderators of the effect of social support on depressive symptoms in cardiac patients, *Am J Cardiol.* 86 (2000), 438-442.

Barnes N.M., Sharp T. A review of central 5-HT receptors and their function, *Neuropharmacology* 38 (1999), 1083‒1152.

Barr A.M., Phillips A.G. Chronic mild stress has no effect on responding by rats for sucrose under a progressive ratio schedule, *Physiol Behav* 64 (1998), 591‒597.

Bartolomucci A., Palanza P., Gaspani L., Limiroli E., Panerai A.E., Ceresini G., Poli M.D., Parmigiani S. Social status in mice: behavioral, endocrine and immune changes are context dependent, *Physiol Behav* 73 (2001), 401-410.

Bartolomucci A., Palanza P., Sacerdote P., Panerai A.E., Sgoifo A., Dantzer R., Parmigiani S. Social factors and individual vulnerability to chronic stress exposure, *Neurosci Biobehav Rev* 29 (2005), 67-81.

Barton D.A., Dawood T., Lambert E.A., Esler M.D., Haikerwal D., Brenchley C., Socratous F., Kaye D.M., Schlaich M.P., Hickie I., Lambert G.W. Sympathetic activity in major depressive disorder: identifying those at increased cardiac risk?, *J. Hypertens* 25 (2007), 2117‒2124.

Batin P.D., Nolan J. Assessment of autonomic function: reflex testing or variability analysis, *J Amb Mon* 9 (1996), 255‒273.

Becker C., Zeau B., Rivat C., Blugeot A., Hamon M., Benoliel J.J. Repeated social defeat-induced depression-like behavioral and biological alterations in rats: involvement of cholecystokinin, *Mol Psychiatry* 13 (2008), 1079-1092.

Besson A., Privat A.M., Eschalier A., Fialip J. Dopaminergic and opioidergic mediations of tricyclic antidepressants in the learned helplessness paradigm, *Pharmacol Biochem Behav* 64 (1999), 541-548.

Bevins R.A., Besheer J. Novelty reward as a measure of anhedonia, *Neurosci Biobehav Rev* 29 (2005), 707-714.

Blanchard D.C., Sakai R.R., McEwen B., Weiss S.M., Blanchard R.J. Subordination stress: behavioral, brain, and neuroendocrine correlates, *Behav Brain Res* 58 (1993), 113-121.

Blanchard D.C., Spencer R.L., Weiss S.M., Blanchard R.J., McEwen B., Sakai R.R. Visible burrow system as a model of chronic social stress: behavioral and neuroendocrine correlates. *Psychoneuroendocrinology* 20 (1995), 117-134.

Blanchard R.J., McKittrick C.R., Blanchard D.C. Animal models of social stress: effects on behavior and brain neurochemical systems, *Physiol Behav* 73 (2001), 261ó271.

Borsini F., Meli A. Is the forced swimming test a suitable model for revealing antidepressant activity?, *Psychopharmacology* 94 (1988), 147-160.

Bourin M., Petit-Demoulière B., Dhonnchadha B.N., Hascöet M. Animal models of anxiety in mice, *Fundam Clin Pharmacol* 21 (2007), 567-574.

Boyle S.H., Williams R.B., Mark D.B., Brummett B.H., Siegler I.C., Helms M.J., Barefoot J.C. Hostility as a predictor of survival in patients with coronary artery disease, *Psychosom Med* 66 (2004), 629-632.

Brown G.W., Prudo R. Psychiatric disorder in a rural and an urban population: 1. Aetiology of depression, *Psychol Med.* 11 (1981), 581-599.

Brown G.W. Life events and affective disorder: replications and limitations, *Psychosom Med* 55 (1993), 248-259.

Brown K.J., Grunberg N.E. Effects of housing on male and female rats: crowding stresses male but calm females, *Physiol Behav* 58 (1995), 1085-1089.

Buwalda B., de Boer S.F., Schmidt E.D., Felszeghy K., Nyakas C., Sgoifo A., Van der Vegt B.J., Tilders F.J., Bohus B., Koolhaas J.M. Long-lasting deficient dexamethasone suppression of hypothalamic-pituitary-adrenocortical activation following peripheral CRF challenge in socially defeated rats, *J Neuroendocrinol* 11 (1999), 513-520.

Campos R.R., McAllen R.M. Cardiac inotropic, chronotropic, and dromotropic actions of subretrofacial neurons of cat RVLM, *Am. J. Physiol* 276 (1999), 1102ó1111.

Cao W.H., Morrison S.F. Disinhibition of rostral raphe pallidus neurons increases cardiac sympathetic nerve activity and heart rate, *Brain Res* 980 (2003), 1ó10.

Carney R., Blumenthal J., Freeland K., Stein P., Howells W., Berkman L., Watkins L., Czajkowski S., Hayano J., Domitrovich P., Jaffe A. Low heart rate variability, the effect of depression on post-myocardial infarction mortality, *Arch Intern Med* 165 (2005), 1486ó1491.

Carney R., Freeland K., Veith C.P., Skala J., Lynch T., Jaffe A. Major depression, heart rate, plasma norepinephrine in patients with coronary heart disease, *Biol Psychiatry* 45 (1999), 458ó463.

Carney R., Saunders R., Freeland K., Stein P., Rich M., Jaffe A. Association of depression with reduced heart rate variability in coronary artery disease, *Am J Cardiol* 76 (1995), 562ó564.

Carney R.M., Blumenthal J.A., Stein P.K., Watkins L., Catellier D., Berkman L.F., Czajkowski S.M., OøConnor C., Stone P.H., Freedland K.E. Depression, heart rate variability, and acute myocardial infarction, *Circulation* 104 (2001), 2024ó2028.

Carney R.M., Freedland K.E., Rich M.W., Smith L.J., Jaffe A.S. Ventricular tachycardia and psychiatric depression in patients with coronary artery disease, *Am. J. Med.* 95 (1993), 23ó28.

Carney R.M., Rich M.W., Freedland K.E., Saini J., teVelde A., Simeone C., Clark K. Major depressive disorder predicts cardiac events in patients with coronary artery disease, *Psychosom Med* 50 (1988), 627-633.

Carroll B.J., Curtis G.C., Mendels J. Neuroendocrine regulation in depression. II. Discrimination of depressed from nondepressed patients, *Arch. Gen. Psychiatry* 33 (1976), 1051ó1058.

Chaouloff F., Berton O., Mormede P. Serotonin and stress, *Neuropsychopharmacology* 21 (1999), 28ó32.

Cheeta S., Ruigt G., van Proosdij J., Willner P. Changes in sleep architecture following chronic mild stress. *Biol Psychiatry* 41 (1997), 419-27.

Cryan J.F., Valentino R.J., Lucki I. Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test, *Neurosci Biobehav Rev* 29 (2005), 547-569.

Cunningham E.T. Jr, De Souza E.B. Interleukin 1 receptors in the brain and endocrine tissues, *Immunol Today* 14 (1993), 171-176.

da Silva L.G., de Menezes R.C., dos Santos R.A., Campagnole-Santos M.J., Fontes M.A. Role of periaqueductal gray on the cardiovascular response evoked by disinhibition of the dorsomedial hypothalamus, *Brain Res* 984 (2003), 206ó214.

Dallack G., Roose S. Perspectives on the relationship between cardiovascular disease and affective disorder, *J Clin Psychiatry* 51 (1990), 4ó9.

Dampney R.A. Functionalorganization of central pathways regulating the cardiovascular system, *Physiol Rev* 74 (1994) , 323-364.

Dantzer R. Cytokine, sickness behavior, and depression, *Neurol. Clin* 24 (2006), 441ó460.

D'Aquila P.S., Brain P., Willner P. Effects of chronic mild stress on performance in behavioural tests relevant to anxiety and depression. *Physiol Behav.* 56 (1994), 861-867.

Das U.N. Free radicals, cytokines and nitric oxide in cardiac failure and myocardial infarction, *Mol. Cell. Biochem* 215 (2000), 145ó152.

de Boer S.F., Slangen J.L., van der Gugten J. Plasma catecholamine and corticosterone levels during active and passive shock-prod avoidance behavior in rats: effects of chlordiazepoxide, *Physiol Behav* 47 (1990), 1089ó1098.

De Kloet E.R., Kovács G.L., Szabó G., Telegdy G., Bohus B., Versteeg D.H. Decreased serotonin turnover in the dorsal hippocampus of rat brain shortly after adrenalectomy: selective normalization after corticosterone substitution, *Brain Res* 239 (1982), 659-663.

De Kloet E.R., Versteeg D.H., Kovacs G.L. Aldosterone blocks the response to corticosterone in the raphe-hippocampal serotonin system, *Brain Res.* 264 (1983), 323-327.

Dekker J.M., Crow R.S., Folsom A.R. Low heart rate variability in a 2-min rhythm strip predicts risk of coronary heart disease and mortality from several causes. The ARIC Study, *Circulation* 102 (2000), 1239ó1244.

Dekker J.M., Schouten E.G., Klootwijk P., Pool J., Swenne C.A., Kromhout D. Heart rate variability from short electrocardiographic recordings predicts mortality from all causes in middle-aged and elderly men. The Zutphen Study, *Am J Epidemiol* 145 (1997), 899ó908.

Dielenberg R.A., Leman S., Carrive P. Effect of dorsal periaqueductal gray lesions on cardiovascular and behavioral responses to cat odor exposure in rats, *Behav. Brain Res* 153 (2004), 487ó496.

DiMicco J.A., Samuels B.C., Zaretskaia M.V., Zaretsky D.V. The dorsomedial hypothalamus and the response to stress: part renaissance, part revolution, *Pharmacol. Biochem. Behav* 71 (2002), 469ó480.

Dinan T.G. Serotonin and the regulation of hypothalamic-pituitary-adrenal axis function, *Life Sci* 58 (1996), 1683-1694.

Domanski M., Mitchell G., Pfeffer M., Neaton J.D., Norman J., Svendsen K., Grimm R., Cohen J., Stamler J. Pulse pressure and cardiovascular disease-related mortality: follow-up study of the Multiple Risk Factor Intervention Trial (MRFIT), *JAMA*. 287 (2002), 2677-2683.

Eckberg D.L., Fritsch J.M. Human autonomic responses to actual and simulated weightlessness, *J Clin Pharmacol* 31 (1991), 951-955.

Ely D.L., Henry J.P. Neuroendocrine response patterns in dominant and subordinate mice, *Horm Behav* 10 (1978), 156-169.

Ensel W., Lin N. Distal stressors and the life stress process, *Journal of Community Psychology* 24 (1996), 66 82.

Esler M., Jennings G., Lambert G. Measurement of overall and cardiac norepinephrine release into plasma during cognitive challenge, *Psychoneuroendocrinology* 14 (1989), 477ó481.

Esler M., Kaye D. Sympathetic nervous system activation in essential hypertension, cardiac failure and psychosomatic heart disease, *J. Cardiovasc. Pharmacol* 35 (2000), 1ó7.

Everson-Rose S.A., Lewis T.T. Psychosocial factors and cardiovascular diseases, *Annu Rev Public Health* 26 (2005), 469-500.

Farkas E., Jansen A.S., Loewy A.D. Periaqueductal gray matter input to cardiac-related sympathetic premotor neurons, *Brain Res* 792 (1998), 179ó192.

Felder R.B., Francis J., Weiss R.M., Zhang Z.H., Wei S.G., Johnson A.K. Neurohumoral regulation in ischemia-induced heart failure. Role of the forebrain, *Ann. N. Y. Acad. Sci* 940 (2001), 444ó453.

Flannelly K., Lore R.The influence of females upon aggression in domesticated male rats (Rattus norvegicus), *Anim Behav* 25 (1977), 654-659.

Fokkema D.S., Koolhaas J.M., van der Gugten J. Individual characteristics of behavior, blood pressure, and adrenal hormones in colony rats, *Physiol Behav* 57 (1995), 857-862.

Fokkema D.S., Koolhaas J.M., van der Meulen J., Schoemaker R. Social stress induced pressure breathing and consequent blood pressure oscillation, *Life Sci* 38 (1986), 569-575.

Foley D.L., Neale M.C., Kendler K.S. A longitudinal study of stressful life events assessed at interview with an epidemiological sample of adult twins: the basis of individual variation in event exposure, *Psychol Med* 26 (1996), 1239-1252.

Folkow B., Schmidt T., Uvnas-Moberg K. Stress, health and the social environment, *Acta Physiol Scand* 161 (1997), 16179.

Fontes M.A., Tagawa T., Polson J.W., Cavanagh S.J., Dampney R.A. Descending pathways mediating cardiovascular response from dorsomedial hypothalamic nucleus, *Am. J. Physiol* 280 (2001), 289162901.

Freedland K.E., Rich M.W., Skala J.A., Carney R.M., Dávila-Román V.G., Jaffe A.S. Prevalence of depression in hospitalized patients with congestive heart failure, *Psychosom Med* 65 (2003), 119-28.

Fuchs E., Flügge G. Social stress in tree shrews: effects on physiology, brain function, and behavior of subordinate individuals, *Pharmacol Biochem Behav* 73 (2002), 247-258.

Fuchs E., Kramer M, Hermes B, Netter P, Hiemke C. Psychosocial stress in tree shrews: clomipramine counteract behavioral and endocrine changes, *Pharmacol, Biochem Behav* 54 (1996), 2196228.

Fuller R.W. Serotonin receptors involved in regulation of pituitary-adrenocortical function in rats, *Behav Brain Res* 73 (1996), 215-219.

Garlow S.J., Nemeroff C.B. The neurochemistry of mood disorders: clinical studies. *In: D.S. Charney and E.J. Nestler, Editors, Neurobiology of Mental Illness, Oxford University Press, New York* (2004), 4406460.

Geyer M.A., Markou A. Animal models of psychiatric disorders. *In: F. Bloom and D. Kupfer, Editors, Psychopharmacology: the fourth generation of progress, Raven Press, New York* (1995), 7876798.

Glass D.C., Krakoff L.R., Contrada R., Hilton W.F., Kehoe K., Mannucci E.G., Collins C., Snow B., Elting E. Effect of harassment and competition upon cardiovascular and plasma catecholamine responses in type A and type B individuals, *Psychophysiology* 17 (1980), 4536463.

Glassman A.H. Depression and cardiovacular comorbidity, *Dialogues in clinical neuroscience* 9 (2007), 9-17.

Glassman A.H. Cardiovascular effects of antidepressant drugs: updated, *Int. Clin. Psychopharmacol* 13 (1998), 25630.

Glassman A.H., Shapiro P.A. Depression and the course of coronary artery disease, *Am J Psychiatry* 155 (1998), 4611.

Goren M.Z., Yananli H.R., Berkman K., Onat F., Aker R. The influence of dorsomedial hypothalamic nucleus on contralateral paraventricular nucleus in NMDA-mediated cardiovascular responses, *Brain Res* 968 (2003), 2196226.

Gorka Z., Moryl E., Papp M. Effect of chronic mild stress on circadian rhythms in the locomotor activity in rats, *Pharmacol Biochem Behav* 54 (1996), 229-234.

Grippo A.J., Beltz T.G., Johnson A.K. Behavioral and cardiovascular changes in the chronic mild stress model of depression, *Physiol Behav* 78 (2003), 703-710.

Grippo A.J., Johnson A.K. Biological mechanisms in the relationship between depression and heart disease, *Neurosci Biobehav Rev* 26 (2002), 941-962.

Hainsworth R. Physiology of the cardiac autonomic system, *In: Malik M, editor. Clinical guide to cardiac autonomic tests. Dordrecht: Kluwer Academic Publishers*, 1998.

Haller J., Fuchs E., Halász J., Makara G.B. Defeat is a major stressor in males while social instability is stressful mainly in females: towards the development of a social stress model in female rats, *Brain Res Bull* 50 (1999), 33-39.

Haller J., Halász J. Mild social stress abolishes the effects of isolation on anxiety and chlordiazepoxide reactivity, *Psychopharmacology* 144 (1999), 311-315.

Hammen C. Stress and depression, *Annu Rev Clin Psychol* 1 (2005), 293ó319.

Hayano J., Sakakibara Y., Yamada A. Accuracy of assessment of cardiac vagal tone by heart rate variability in normal subjects, *Am J Cardiol* 67 (1991), 199ó204.

Heim C., Nemeroff C.B. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies, *Biol Psychiatry* 49 (2001), 1023-1039.

Helke C.J., Capuano S., Tran N., Zhuo H. Immunocytochemical studies of the 5-HT(1A) receptor in ventral medullary neurons that project to the intermediolateral cell column and contain serotonin or tyrosine hydroxylase immunoreactivity, *J. Comp. Neurol* 379 (1997), 261ó270.

Hemingway H., Malik M., Marmot M. Social and psychosocial influences on sudden cardiac death, ventricular arrhythmia and cardiac autonomic function, *Eur Heart J* 22 (2001), 1082ó1101.

Henry J.P., Liu Y.Y., Nadra W.E., Oian C.G., Mormede P., Lemaire V., Ely D., Hendley E.D. Psychosocial stress can induce chronic hypertension in normotensive strains of rats, *Hypertension* 21 (1993), 714ó723.

Henry J.P., Stephens P.M., Ely D.L. Psychosocial hypertension and the defence and defeat reactions, *J Hypertens* 4 (1986), 687ó697.

Henry J.P., Grim C.E. Psychosocial mechanisms of primary hypertension, *J Hypertens* 8 (1990), 783-793.

Herd J.A. Cardiovascular response to stress, *Physiol Rev* 71 (1991), 305ӗ330.

Herman J.P., Guillonneau D., Dantzer R., Scatton B., Semerdjian-Rouquier L., LeMoal M. Differential effects of inescapable footshocks and of stimuli previously paired with inescapable footshocks on dopamine turnover in cortical and limbic areas of the rat, *Life Sci* 30 (1982), 2207ӗ2214.

Heslop P., Smith G.D., Carroll D., Macleod J., Hyland F., Hart C. Perceived stress and coronary heart disease risk factors: the contribution of socio-economic position, *Br J Health Psychol* 6 (2001), 167-178.

Hjemdahl P. Cardiovascular system and stress (2nd ed.), *In: G. Fink, Editor, Encyclopedia of Stress vol. 1, Academic Press, Oxford* (2007), 396ӗ409.

Holsboer F. Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy, *J Affect Disord* 62 (2001), 77-91.

Horiuchi J., McAllen R.M., Allen A.M., Killinger S., Fontes M.A., Dampney R.A. Descending vasomotor pathways from the dorsomedial hypothalamic nucleus: role of medullary raphe and RVLM, *Am. J. Physiol* 287 (2004), 824ӗ832.

Hoyer D., Hannon J.P., Martin G.R. Molecular, pharmacological and functional diversity of 5-HT receptors, *Pharmacol. Biochem. Behav* 71 (2002), 533ӗ554.

Johnson A.K., Anderson E.A. Stress and arousal. *In: J.T. Cacioppo and L.G. Tassinary, Editors, Principles of Psychophysiology: Physical, Social, and Inferential Elements, Cambridge University Press, Cambridge* (1990), 216ӗ252.

Jordan D. Vagal control of the heart: central serotonergic (5-HT) mechanisms, *Exp. Physiol* 90 (2005), 175ӗ181.

Joseph M.H., Kennett G.A. Stress-induced release of 5-HT in the hippocampus and its dependence on increased tryptophan availability: an in vivo electrochemical study, *Brain Res.* 270 (1983), 251ӗ257.

Joynt K.E., Whellan D.J., O'Connor C.M. Depression and cardiovascular disease: mechanisms of interaction, *Biol Psychiatry* 54 (2003), 248-261.

Kapadia S., Dibbs Z., Kurrelmeyer K., Karla D., Seta Y., Wang F., Bozkurt B., Oral H., Sivasubramanian N., Mann D.L. The role of cytokines in the failing human heart, *Cardiol. Clin.* 16 (1998), 645ӗ656.

Kaplan J.R., Manuck S.B. Status, stress, and atherosclerosis: the role of environment and individual behavior, *Ann NY Acad Sci* 851 (1999), 19ӗ627.

Kaplan J.R., Manuck S.B., Clarkson T.B., Lusso F.M., Taub D.M. Social status, environment, and atherosclerosis in cynomolgus monkeys, *Arteriosclerosis* 2 (1982), 359-368.

Kasckow J.W., Baker D., Geracioti T.D. Jr. Corticotropin-releasing hormone in depression and post-traumatic stress disorder, Peptides 22 (2001), 845-851.

Katz R.J. Animal model of depression: effects of electroconvulsive shock therapy, *Neurosci Biochem Rev* 5 (1981a), 2736277.

Katz R.J., Roth K.A., Carroll B.J. Acute and chronic stress effects on open field activity in the rat: implications for a model of depression, *Neurosci Biochem Rev* 5 (1981b), 2476251.

Katz R.J. Animal model of depression: pharmacological sensitivity of a hedonic deficit. Pharmacol Biochem Behav. 16 (1982), 965-968.

Keay K.A., Bandler R. Parallel circuits mediating distinct emotional coping reactions to different types of stress, *Neurosci. Biobehav. Rev* 25 (2001), 6696678.

Keeney A., Jessop D.S., Harbuz M.S., Marsden C.A., Hogg S., Blackburn-Munro R.E. Differential effects of acute and chronic social defeat stress on hypothalamic-pituitary-adrenal axis function and hippocampal serotonin release in mice, *J Neuroendocrinol* 18 (2006), 330-338.

Kendler K., Prescott C. Stressful life events and major depression: risk period, long-term contextual threat, and diagnostic specificity, *Journal of Nervous and Mental Disorders* 186 (1998), 661 669.

Kendler K.S., Karkowski-Shuman L. Stressful life events and genetic liability to major depression: genetic control of exposure to the environment?, *Psychol Med.* 27 (1997), 539-547.

Kendler K.S., Kessler R.C., Neale M.C., Heath A.C., Eaves L.J: The prediction of major depression in women: toward an integrated etiologic model, *Am J Psychiatry* 150 (1993), 113961148.

Kessler R.C. The effects of stressful life events on depression, *Annu Rev Psychol* 48 (1997), 191-214.

Kleiger R.E., Stein P.K., Bosner M.S., Rottman J.N. Time domain measurements of heart rate variability, *Cardiol Clin.* 10 (1992), 487-498.

Kofman O. The role of prenatal stress in the etiology of developmental behavioural disorders, *Neurosci Biobehav Rev* 26 (2002), 457-470.

Koolhaas J.M., Hermann P.M., Kemperman C., Bohus B., van den Hoofdakker R.H., Beersma D.G.M. Single social defeat in male rats induces a gradual but long lasting behavioral change: a model of depression?, *Neurosci Res Comm* 7 (1990), 35ó41.

Koolhaas J.M., Korte S.M., de Boer S.F., van der Vegt B.J., van Reenen C.G., Hopster H., de Jong I.C., Ruis M.A., Blokhuis H.J. Coping styles in animals: current status in behavior and stress physiology, *Neurosci Biobehav Rev* 23 (1999), 925ó935.

Koolhaas J.M., Meerlo P., de Boer S.F., Strubbe J.H., Bohus B. Social stress in rats: An animal model of depression?, *Acta Neuropsychiat* 7 (1995), 27ó29.

Koolhaas J.M., Meerlo P., de Boer S.F., Strubbe J.H., Bohus B. The temporal dynamics of the stress response, *Neurosci Biobehav Rev* 21 (1997), 775ó782.

Kristal-Boneh E., Raifel M., Froom P., Ribak J. Heart rate variability in health and disease, *Scand. J. Work Environ. Health* 21 (1995), 85ó95.

Krittayaphong R., Cascio W., Light K., Sheffield D., Golden R., Finkel J.B., Glekas G., Koch G., Sheps D. Heart rate variability in patients with coronary artery disease: differences in patients with higher and lower depression scores, *Psychosom Med* 59 (1997),231ó235.

Kundryavsteva N.N. A sensory contact model for the study of aggressive and submissive behavior in male mice, *Aggressive Behav* 17 (1991), 285ó291.

Kuper H., Marmot M., Hemingway H. Systematic review of prospective cohort studies of psychosocial factors in the etiology and prognosis of coronary heart disease, *Semin Vasc Med* 2 (2002), 267-314.

La Rovere M.T., Bigger Jr. J.T., Marcus F.I., Mortara A., Schwartz P.J. Baroreflex sensitivity and heart-rate variability in prediction of total cardiac mortality after myocardial infarction, *Lancet* 351 (1998), 478ó484.

Lam W., Louis W.J., Verberne A.J. Effect of dorsal periaqueductal grey lesion on baroreflex and cardiovascular response to air-jet stress, *J. Auton. Nerv. Syst* 53 (1995), 35ó42.

Lambert G., Johansson M., Ågren H., Friberg P. Reduced brain norepinephrine and dopamine release in treatment-refractory depressive illness: evidence in support of the catecholamine hypothesis of mood disorders, *Arch. Gen. Psychiatry* 57 (2000), 787ó793.

Lefebvre H., Contesse V., Delarue C., Feuilloley M., Hery F., Grise P., Raynaud G., Verhofstad A.A., Wolf L.M., Vaudry H. Serotonin-induced stimulation of cortisol secretion from human adrenocortical tissue is mediated through activation of a serotonin4 receptor subtype, *Neuroscience* 47 (1992), 999-1007.

Lesch K.P., Bengel D., Heils A., Sabol S.Z., Greenberg B.D., Petri S., Benjamin J., Müller C.R., Hamer D.H., Murphy D.L. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region, *Science* 274 (1996), 1527-1531.

Levy M.N., Martin P.J. Neural control of the heart, I*n: Berne RM, editor. Handbook of physiology. Bethesda (MD): American Physiological Society*, 1979: 581-620.

Lewinsohn P.M., Hoberman H.H., Rosenbaum M. A prospective study of risk factors for unipolar depression, *J Abnorm Psychol* 97, (1988), 251ó264.

Liao D., Cai J., Rosamond W.D. Cardiac autonomic function and incident coronary heart disease. A population-based case-cohort study. The ARIC study, *Am J Epidemiol* 145 (1997), 696ó706.

Liao D., Sloan R.P., Cascio W.E. Multiple metabolic syndrome is associated with lower heart rate variability: The Atherosclerosis Risk in Communities Study, *Diabetes Care* 21 (1998), 2116ó2122.

Lippi G., Montagnana M., Favaloro E.J., Franchini M. Mental depression and cardiovascular disease: a multifaceted, bidirectional association, *Semin Thromb Hemost* 35 (2009), 325-336.

Lucki I. The spectrum of behaviors influenced by serotonin, *Biol. Psychiatry* 44 (1998), 151ó162.

Lupien S.J., McEwen B.S., Gunnar M.R., Heim C. Effects of stress throughout the lifespan on the brain, behaviour and cognition, *Nat Rev Neurosci.* 10 (2009), 434-445.

Maccari S., Darnaudery M., Morley-Fletcher S., Zuena A.R., Cinque C., Van Reeth O. Prenatal stress and long-term consequences: implications of glucocorticoid hormones. *Neurosci Biobehav Rev.* 27 (2003), 119-127.

Maccari S., Piazza P.V., Kabbaj M., Barbazanges A., Simon H., Le Moal M. Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress, *J Neurosci*, 15 (1995), 110-116.

Maes M. Evidence for an immune response in major depression: a review and hypothesis, *Prog. Neuropsychopharmacol. Biol. Psychiatry* 19 (1995), 11ó38.

Maes M., Lin A., Bonaccorso S., van Hunsel F., Van Gastel A., Delmeire L., Biondi M., Bosmans E., Kenis G., Scharpe S. Increased 24-h uninary cortisol excretion in patients with post-traumatic stress disorder and patients with major depression, but not in patients with fibromyalgia, *Acta Psychiatr. Scand.* 98 (1998), 328ó335.

Maes M., Meltzer H.Y. The serotonin hypothesis of major depression. *In: F.E. Bloom and D.J. Kupfer, Editors, Psychopharmacology: The Fourth Generation of Progress, Raven Press, New York* (1995), 933ó 944.

Maier S.F., Watkins L.R. Stressor controllability and learned helplessness: the roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor, *Neurosci Biobehav Rev* 29 (2005), 829-841.

Malik M., Camm A.J. Heart rate variability. *Clin Cardiol*. 13 (1990), 570-576.

Malliani A., Pagani M., Lombardi F., Cerutti S. Cardiovascular neural regulation explored in the frequency domain, Circulation 84 (1991), 482ó492.

Markgraf C.G., Kapp B.S. Neurobehavioral contributions to cardiac arrhythmias during aversive pavlovian conditioning in the rabbit receiving digitalis. *J. Auton. Nerv. Syst* 23 (1988), 35ó46.

Markus C.R., Panhuysen G., Tuiten A., Koppeschaar H., Fekkes D., Peters M.L. Does carbohydrate-rich, protein-poor food prevent a deterioration of mood and cognitive performance of stress-prone subjects when subjected to a stressful task?, *Appetite* 31 (1998), 49-65.

Mastorci F., Vicentini M., Viltart O., Manghi M., Graiani G., Quaini F., Meerlo P., Nalivaiko E., Maccari S., Sgoifo A. Long-term effects of prenatal stress: changes in adult cardiovascular regulation and sensitivity to stress, *Neurosci Biobehav Rev* 33 (2009), 191-203.

Mathers, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030, *PLoS Med* 3 (2006), 442.

Mausbach B., Dimsdale J., Ziegler M., Ancolo-Isreal S.M., Patterson T., Grant I. Depressive symptoms predict norepinephrine response to a psychological stressor task in Alzheimerøs caregivers, *Psychosom Med* 67 (2005), 638ó642.

Mazure C.M., Bruce M.L., Maciejewski P.K., Jacobs S.C. Adverse life events and cognitive-personality characteristics in the prediction of major depression and antidepressant response, *Am J Psychiatry* 157 (2000), 896-903.

McAllen R.M. Location of neurones with cardiovascular and respiratory function, at the ventral surface of the cat's medulla, *Neuroscience* 18 (1986), 43-49.

McCarty R., Patak K. Alarm phase and general adaptation syndrome. *In: Encyclopedia of Stress Vol.1, edited by Fink G. Sasn Diego: Academic Press*, 2000, 126-130.

McDougall S.J., Widdop R.E., Lawrence A.J. Medial prefrontal cortical integration of psychological stress in rats, *Eur. J. Neurosci* 20 (2004), 2430ó2440.

McEwen B.S. The neurobiology of stress: from serendipity to clinical relevance, *Brain Res* 886 (2000), 172-189.

McGonagle K., Kessler R. Chronic stress, acute stress, and depressive symptoms. *American Journal of Community Psycholology* 18 (1990), 681 706.

Meerlo P., de Boer S.F., Koolhaas J.M., Daan S., Van den Hoofdakker R.H. Changes in daily rhythms of body temperature and activity after a single social defeat in rats, *Physiol Behav* 59 (1996), 735-739.

Meerlo P., Sgoifo A., de Boer S.F., Koolhaas J.M. Long-lasting consequences of a social conflict in rats: behavior during the interaction predicts subsequent changes in daily rhythms of heart rate, temperature, and activity, *Behav Neurosci.* 113 (1999), 1283-1290.

Meerlo P., Sgoifo A., de Boer S.F., Koolhaas J.M. Daily rhythms of heart rate, temperature and activity after social conflict in rats. Determinants of long-term consequences of aggressive interactions, *Behav Neurosci* 113 (1999), 1283ó1290.

Meltzer H.Y. Role of serotonin in depression, *Ann N Y Acad Sci* 600 (1990), 486-500.

Merz C.N., Buse J.B., Tuncer D., Twillman G.B. Physician attitudes and practices and patient awareness of the cardiovascular complications of diabetes, *J Am Coll Cardiol* 40 (2002), 1877-1881.

Miczek K.A. A new test for aggression in rats without aversive stimulation: differential effects of d-amphetamine and cocaine, *Psychopharmacology* 60 (1979), 253ó259.

Miczek K.A., Thompson M.L., Tomatzky W., Oliverio A. Short and long term physiological and neurochemical adaptations to social conflict. *In: S. Puglisi-Allegra, Editor, NATO ASI Series D: Behavioural and Social Sciences, Kluwer, Dordrecht* (1990), 15ó30.

Moreau JL, Jenck F, Martin JR, Mortas P, Haefely WE. Antidepressant treatment prevents chronic unpredictable mild stress-induced anhedonia as assessed by ventral tegmentum self-stimulation behavior in rats. Eur Neuropsychopharmacol. 1992 Mar;2(1):43-9.

Morrison S.F. Activation of 5-HT1A receptors in raphe pallidus inhibits leptin-evoked increases in brown adipose tissue thermogenesis, *Am. J. Physiol* 286 (2004), 832ó837.

Murray C.J., Lopez A.D. Alternative projections of mortality and disability by cause 1990ó2020: Global Burden of Disease Study, *Lancet* 349 (1997), 1498ó1504.

Muscat P., Papp M., Willner P. Reversal of stress-induced anhaedonia by the atypical antidepressants, fluoxetine and maprotiline, *Psychopharmacology* 109 (1992), 433ó438.

Nalivaiko E., De Pasquale C.G., Blessing W.W. Ventricular arrhythmias triggered by alerting stimuli in conscious rabbits pre-treated with dofetilide. *Basic Res. Cardiol*. 99 (2004), 142ó151.

Nalivaiko E. 5-HT(1A) receptors in stress-induced cardiac changes: a possible link between mental and cardiac disorders, *Clin Exp Pharmacol Physiol* 33 (2006), 1259-1264.

Nalivaiko E., Blessing W.W. Raphe region mediates changes in cutaneous vascular tone elicited by stimulation of amygdala and hypothalamus in rabbits, *Brain Res* 891 (2001), 130ó137.

Nalivaiko E., Blessing W.W. Synchronous changes in ear and tail blood flow following salient and noxious stimuli in rabbits, *Brain Res* 847 (1999), 343ó346.

Nalivaiko E., Ootsuka Y., Blessing W.W. Activation of 5-HT1A receptors in the medullary raphe reduces cardiovascular changes elicited by acute psychological and inflammatory stresses in rabbits, *Am. J. Physiol* 289 (2005), 596ó604.

Nestler E.J., Gould E., Manji H., Buncan M., Duman R.S., Greshenfeld H.K. Preclinical models: status of basic research in depression, *Biol Psychiatry* 52 (2002), 503ó528.

Neumeister A., Bain E., Nugent A.C., Carson R.E., Bonne O., Luckenbaugh D.A., Eckelman W., Herscovitch P., Charney D.S., Drevets W.C. Reduced serotonin type 1A receptor binding in panic disorder, *J Neurosci* 24 (2004), 589-591.

Newton T.L., Bane C.M.H. Cardiovascular correlates of behavioral dominance and hostility during dyadic interaction, *Int J Psychophysiol* 40 (2001), 33ó46.

Ngampramuan S., Baumert M.I.B., Kotchabhakdi N., Nalivaiko E. Activation of 5-HT1A receptors attenuates tachycardia induced by restraint stress in rats, *Am. J. Physiol* 294 (2008), 132ó141.

Nolan J., Batin P.D., Andrews R., Lindsay S.J., Brooksby P., Mullen M., Baig W., Flapan A.D., Cowley A., Prescott R.J., Neilson J.M., Fox K.A. Prospective study of heart rate variability and mortality in chronic heart failure: results of the United Kingdom heart failure evaluation and assessment of risk trial (UK-heart), *Circulation* 98 (1998), 1510-1516.

O'Keefe J.H. Jr, Poston W.S., Haddock C.K., Moe R.M., Harris W. Psychosocial stress and cardiovascular disease: how to heal a broken heart, *Compr Ther* 30 (2004), 37-43.

Oppenheimer S.M., Wilson J.X., Guiraudon C., Cechetto D.F. Insular cortex stimulation produces lethal cardiac arrhythmias: A mechanism of sudden death?, *Brain Res* 550 (1991), 115ó121.

Papp M., Willner P., Muscat R. An animal model of anhedonia: attenuation of sucrose consumption and place preference conditioning by chronic unpredictable mild stress, *Psychopharmacology* 104 (1991), 255ó259.

Penninx J.H., Beekman A.T.F., Honig A., Deeg D.J.H., Schoevers R.A., van Eijk J.T.M., van Tilburg W. Depression and cardiac mortality: results from a community-based longitudinal study, *Arch Gen Psychiatry* 58 (2001), , 221ó227.

Persson P.B. Modulation of cardiovascular control mechanism and their interaction, *Phys Rev A* 76 (1996), 193-244.

Petit-Demouliere B., Chenu F., Bourin M. Forced swimming test in mice: a review of antidepressant activity, *Psychopharmacology* 177 (2005), 245-255.

Peyronnet J., Dalmaz Y., Ehrström M., Mamet J., Roux J.C., Pequignot J.M., Thorén H.P., Lagercrantz H. Long-lasting adverse effects of prenatal hypoxia on developing autonomic nervous system and cardiovascular parameters in rats, *Pflugers Arch* 443 (2002), 858-865.

Pitzalis M.V., Iacoviello M., Todarello O., Fioretti A., Guida P., Massari F., Mastropasqua F., Russo G.D., Rizzon P. Depression but not anxiety influences the autonomic control of heart rate after myocardial infarction, *Am. Heart J* 141 (2001), 765ó771.

Plomin R., Lichtenstein P., Pedersen N.L., McClearn G.E., Nesselroade J.R. Genetic influence on life events during the last half of the life span, *Psychol Aging* 5 (1990), 25-30.

Podrid P., Fuchs T., Candinas R. Role of sympathetic nervous system in the fenesis of ventricular arrhythmia, *Circulation* 82 (1990), 103ó110.

Poisson D., Christen M.O., Sannajust F. Protective effects of the antihypertensive agent moxonidine against neurogenic cardiac arrhythmias in halothane-anesthetized rabbits, *J. Pharmacol. Exp. Ther* 293 (2000), 929ó938.

Porsolt R.D., Anton G., Blavet N., Jalfre M. Behavioral despair in rats: a new model sensitive to antidepressant treatments, *Eur J Pharmacol* 47 (1978), 379ó391.

Pratt L.A., Ford D.E., Crum R.M., Armenian H.K., Gallo J.J., Eaton W.W. Depression, psychotropic medication, and risk of myocardial infarction. Prospective data from the Baltimore ECA follow-up, *Circulation* 94 (1996), 3123-3129.

Priori S.G., Mantica M., Schwartz P.J. Delayed afterdepolarizations elicited in vivo by left stellate ganglion stimulation, *Circulation* 78 (1988), 178ó185.

Pumprla J., Howorka K., Groves D., Chester M., Nolan J. Functional assessment of heart rate variability: physiological basis and practical applications, *Int J Cardiol* 84 (2002), 1-14.

Raadsheer F.C., van Heerikhuize J.J., Lucassen P.J., Hoogendijk W.J.G., Tilders F.J.H., Swaab D.F. Corticotropin-releasing hormone mRNA levels in the paraventricular nucleus of patients with Alzheimer's disease and depression, *Am. J. Psychiatry* 152 (1995), 1372ó1376.

Riso L.P., Miyatake R.K., Thase M.E. The search for determinants of chronic depression: a review of six factors, *J. Affect Disord.* 70 (2002), 103ó115.

Robinson R.G., Krishnan K.R.R. Depression and the medically ill. *In: K.L. Davis, D. Charney, J.T. Coyle and C. Nemeroff, Editors, Neuropsychopharmacology: the fifth generation of progress, Lippincott Williams & Wilkins, Philadelphia* (2002), 1179ó1185.

Roose S.P. Depression: links with ischemic heart disease and erectile dysfunction, *J Clin Psychiatry* 64 (2003), 26-30.

Rosenman R.H., Brand R.J., Jenkins D., Friedman M., Straus R., Wurm M. Coronary heart disease in Western Collaborative Group Study. Final follow-up experience of 8½ years, *JAMA* 233 (1975), 872ó877.

Rosenman R.H., Friedman M. Neurogenic factors in pathogenesis of coronary heart disease, *Med Clin North Am* 58 (1974), 269ó279.

Rozanski A., Blumenthal J.A., Davidson K.W., Saab P.G., Kubzansky L. The epidemiology, pathophysiology, and management of psychosocial risk factors in cardiac practice: the emerging field of behavioral cardiology, *J Am Coll Cardiol* 45 (2005), 637-651.

Rozanski A., Blumenthal J.A., Kaplan J. Impact of psychological factors on the pathogenesis of cardiovascular disease and implications for therapy, *Circulation* 99 (1999), 2192-2217.

Rupniak N.M.J. Animal models of depression: challenges from drug development, *Behav Pharmacol* 14 (2003), 385ó390.

Rygula R., Abumaria N., Flügge G., Fuchs E., Rüther E., Havemann-Reinecke U. Anhedonia and motivational deficits in rats: impact of chronic social stress, *Behav Brain Res.* 162 (2005), 127-134.

Samuels B.C., Zaretsky D.V., DiMicco J.A. Tachycardia evoked by disinhibition of the dorsomedial hypothalamus in rats is mediated through medullary raphe, *J. Physiol* 538 (2002), 941ó946.

Sanders B.J., Lawler J.E. The borderline hypertensive rat (BHR) as a model for environmentally induced hypertension: a review and update, *Neurosci. Biobehav. Rev* 16 (1992), 207–217.

Sapolsky R.M. Neuroendocrinology of the stress-response. *In: J.B. Becker, S.M. Breedlove and D. Crews, Editors, Behavioral endocrinology, The Mit Press, Cambridge* (1992), 287–324.

Scheurink A.J., Steffens A.B., Bouritius H., Dreteler G.H., Bruntink R., Remie R., Zaagsma J. Adrenal and sympathetic catecholamines in exercising rats, *Am J Physiol* 256 (1989), 155-160.

Schleifer S.J., Macari-Hinson M.M., Coyle D.A., Slater W.R., Kahn M., Gorlin R., Zucker H.D. The nature and course of depression following myocardial infarction, *Arch Intern Med* 149 (1989), 1785-1789.

Schwartz A.R., Gerin W., Davidson K.W., Pickering T.G., Brosschot J.F., Thayer J.F., Christenfeld N., Linden W. Toward a causal model of cardiovascular responses to stress and the development of cardiovascular disease, *Psychosom. Med* 65 (2003), 22–35.

Schwartz P.J., Locati E.H., Moss A.J., Crampton R.S., Trazzi R., Ruberti U. Left cardiac sympathetic denervation in the therapy of congenital long QT syndrome. A worldwide report, *Circulation* 84 (1991), 503–511

Schwartz P.J., Priori S.G. Sympathetic nervous system and cardiac arrhythmias. *In: Zipes DP, Jalife J, editor. Cardiac electrophysiology. From cell to bedside, Philadelphia, PA: Saunders*, 1990, 330-343.

Schwartz P.J. The autonomic nervous system and life-threatening arrhythmias. *In: Shepherd JT, Vatner SF. Nervous Control of the Heart. Harwood Academic Publishers, Amsterdam*, 1996, 329–356.

Seely A.J., Macklem P.T. Complex systems and the technology of variability analysis, *Crit Care*. 8 (2004), 367-384.

Seligman M.E., Weiss J., Weinraub M., Schulman A. Coping behavior: learned helplessness, physiological change and learned inactivity, *Behav Res Ther* 18 (1980), 459-512.

Sgoifo A., de Boer S.F., Buwalda B., Korte-Bouws G., Tuma J., Bohus B., Zaagsma J., Koolhaas J.M. Vulnerability to arrhythmias during social stress in rats with different sympathovagal balance, *Am J Physiol* 275 (1998), 460-466.

Sgoifo A., de Boer S.F., Haller J., Koolhaas J.M. Individual differences in plasma catecholamine and corticosterone stress responses of wild-type rats: relationship with aggression, *Physiol Behav* 60 (1996), 1403-1407.

Sgoifo A., de Boer S.F., Westenbroek C., Maes F., Beldhuis H., Suzuki T., Koolhaas J. Incidence of arrhythmias and heart rate variability in wild-type rats exposed to social stress, *Am J Physiol* 273 (1997), 1754ϳ1760.

Sgoifo A., Koolhaas J.M., Musso E., de Boer S.F. Different sympathovagal modulation of heart rate during social and non-social stress episodes in wild-type rats, *Physiol Behav* 67 (1999a), 733ϳ738.

Sgoifo A., Koolhaas J.M., de Boer S.F., Musso E., Stilli D., Buwalda B., Meerlo P. Social stress, autonomic neural activation, and cardiac activity in rats, *Neurosci Biobehav Rev* 23 (1999b), 915ϳ923.

Sgoifo A., Pozzato C., Costoli T., Manghi M., Stilli D., Ferrari P.F., Ceresini G., Musso E. Cardiac autonomic responses to intermittent social conflict in rats, *Physiol Behav* 73 (2001), 343ϳ349.

Shekhar A., McCann U.D., Meaney M.J., Blanchard D.C., Davis M., Frey K.A., Liberzon I., Overall K.L., Shear M.K., Tecott L.H., Winsky L. Summary of a National Institute of Mental Health workshop: developing animal models of anxiety disorders, *Psychopharmacology* 157 (2001), 327-339.

Shen B.J., McCreary C.P., Myers H.F. Independent and mediated contributions of personality, coping, social support, and depressive symptoms to physical functioning outcome among patients in cardiac rehabilitation, *J Behav Med* 27 (2004), 39-62.

Shively C.A., Musselman D.L., Willard S.L. Stress, depression, and coronary artery disease: modeling comorbidity in female primates, *Neurosci Biobehav Rev* 33 (2009), 133-144.

Smith R.S. The macrophage theory of depression, *Med. Hypotheses* 35 (1991), 298ϳ306.

Stefanski V. Social stress in laboratory rats: hormonal responses and immune cell distribution, *Psychoneuroendocrinology* 25 (2000), 389-406.

Stein P.K., Bosner M.S., Kleiger R.E., Conger B.M. Heart rate variability: a measure of cardiac autonomic tone, *Am Heart J* 127(1994), 1376-1381.

Steptoe A. Depression and the development of coronary heart disease. *In: Steptoe A. (Ed.) Depression and Physical Illness. Cambridge University Press, Cambridge,* 2006, 53-86.

Steptoe A., Feldman P.M., Kunz S., Owen N., Willemsen G., Marmot M. Stress responsivity and socioeconomic status: a mechanism for increased cardiovascular disease risk?, *Eur. Heart J.* 23 (2002), 1757ϳ1763.

Steptoe A., Hamer M., Chida Y. The effects of acute psychological stress on circulating inflammatory factors in humans: a review and meta-analysis, *Brain Behav. Immun.* 21 (2007), 901ϳ912.

Stewart R.A., North F.M., West T.M., Sharples K.J., Simes R.J., Colquhoun D.M., White H.D., Tonkin A.M. Long-Term Intervention With Pravastatin in Ischaemic Disease (LIPID) Study Investigators. Depression and cardiovascular morbidity and mortality: cause or consequence?, *Eur Heart J* 24 (2003), 2027-2037.

Stotzpotter E.H., Morin S.M., Dimicco J.A. Effect of microinjection of muscimol into the dorsomedial or paraventricular hypothalamic nucleus on air stress-induced neuroendocrine and cardiovascular changes in rats, *Brain Res*. 742 (1996a), 219ó224.

Stotzpotter E.H., Willis L.R., Dimicco J.A. Muscimol acts in dorsomedial but not paraventricular hypothalamic nucleus to suppress cardiovascular effects of stress, *J. Neurosci*. 16 (1996b), 1173ó1179.

Strike P.C., Magid K., Brydon L., Edwards S., McEwan J.R., Steptoe A. Exaggerated platelet and hemodynamic reactivity to mental stress in men with coronary artery disease., *Psychosom Med*. 66 (2004), 492-500.

Suchecki D., Palermo Neto J. Prenatal stress and emotional response of adult offspring. Physiol Behav. 49 (1991), 423-426.

Sullivan E.M., Griffiths R.I., Frank R.G., Strauss M.J., Herbert R.J., Clouse J., Goldman H.H. One-year costs of second-line therapies for depression, *J Clin Psychiatry* 61 (2000), 290-298.

Suls J., Bunde J. Anger, anxiety, and depression as risk factors for cardiovascular disease: the problems and implications of overlapping affective dispositions, *Psychol Bull* 131 (2005), 260-300.

Takayama K., Okada J., Miura M. Evidence that neurons of the central amygdaloid nucleus directly project to the site concerned with circulatory and respiratory regulation in the ventrolateral nucleus of the cat: A WGA-HRP study, *Neurosci. Lett* 109 (1990), 241ó246.

Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, Heart rate variabilityô standards of measurement, physiological interpretation, and clinical use, *Circulation* 93 (1996), 1043ó1065.

Thayer J.F., Sternberg E. Beyond heart rate variability: vagal regulation of allostatic systems, *Ann. N. Y. Acad. Sci*. 1088 (2006), 361ó372.

Thayer J.F., Lane R.D. The role of vagal function in the risk for cardiovascular disease and mortality, *Biol Psychol* 74 (2007), 224-242.

Vaidya V.A. Stress, depression, and hippocampal damage, *J. Biosci*. 25 (2000), 123ó124.

Van De Willige G., Ormel J., Giel R. Etiological meaning of severe life events and long-term difficulties in the onset of depression and anxiety. *Tijdschrift Voor Psychiatrie* 37 (1995), 689 703.

van den Buuse M., Wegener N. Involvement of serotonin1A receptors in cardiovascular responses to stress: a radio-telemetry study in four rat strains, *Eur. J. Pharmacol.* 507 (2005), 187ó198.

van Praag H.M. Can stress cause depression?, *Prog Neuropsychopharmacol Biol Psychiatry* 28 (2004), 891-907.

Veith R., Lewis N., Linares O., Barnes R., Raskind M., Villacres E., Murburg M., Ashleigh E., Castillo S., Peskind E., Pascualy M., Halter J. Sympathetic nervous system activity in major depression, *Arch Gen Psychiatry* 51 (1994), 411ó422.

Vianna D., Carrive P. 8-OH-DPAT attenuates cardiovascular responses to conditioned fear and restraint stresses, *Proc. Aust. Neurosci. Soc.* 18 (2008), 81.

Vollmayr B., Bachteler D., Vengeliene V., Gass P., Spanagel R., Henn F. Rats with congenital learned helplessness respond less to sucrose but show no deficits in activity or learning, *Behav Brain Res* 150 (2004), 217-221.

Von Frijtag J.C., Van den Bos R., Spruijt B.M. Imipramine restores the long-term impairment of appetitive behavior in socially stressed rats, *Psychopharmacology* 162 (2002), 232-238.

Wadhwa P.D., Sandman C.A., Garite T.J. The neurobiology of stress in human pregnancy: implications for prematurity and development of the fetal central nervous system, *Prog Brain Res* 133 (2001), 131-142.

Waldstein S.R., Bachen E.A., Manuck S.B. Active coping and cardiovascular reactivity: a multiplicity of influences, *Psychosom Med* 59 (1997), 620ó625.

Walker P., Carrive P. Role of ventrolateral periaqueductal gray neurons in the behavioral and cardiovascular responses to contextual conditioned fear and poststress recovery, *Neuroscience* 116 (2003), 897ó912.

Wallace D.M., Magnuson D.J., Gray T.S. Organization of amygdaloid projections to brainstem dopaminergic, noradrenergic and adrenergic cell groups in the rat, *Brain Res. Bull* 28 (1992), 447ó454.

Wang Y., Ramage A.G. The role of central 5-HT(1A) receptors in the control of B-fibre cardiac and bronchoconstrictor vagal preganglionic neurones in anaesthetized cats, *J. Physiol* 536 (2001), 753ó767.

Ward I.L., Stehm K.E. Prenatal stress feminizes juvenile play patterns in male rats, *Physiol Behav* 50 (1991), 601-605.

Ward I.L., Weisz J. Differential effects of maternal stress on circulating levels of corticosterone, progesterone, and testosterone in male and female rat fetuses and their mothers, *Endocrinology* 114 (1984), 1635-1644.

Weber B., Lewicka S., Deuschle M., Colla M., Vecsei P., Heuser I. Increased diurnal plasma concentrations of cortisone in depressed patients, *J. Clin. Endocrinol. Metab.* 85 (2000), 1133ó1136.

Weinstock M., Poltyrev T., Schorer-Apelbaum D., Men D., McCarty R. Effect of prenatal stress on plasma corticosterone and catecholamines in response to footshock in rats, *Physiol Behav* 64 (1998), 439-444.

Weinstock M. Alterations induced by gestational stress in brain morphology and behaviour of the offspring, *Prog Neurobiol* 65 (2001), 427-451.

Weinstock M. Does prenatal stress impair coping and regulation of hypothalamic-pituitary-adrenal axis?, *Neurosci Biobehav Rev* 21 (1997), 1-10.

White D.A., Birkle D.L. The differential effects of prenatal stress in rats on the acoustic startle reflex under baseline conditions and in response to anxiogenic drugs, *Psychopharmacology* 154 (2001), 169-176.

Whitworth J.A., Williamson P.M., Mangos G., Kelly J.J. Cardiovascular consequences of cortisol excess, *Vasc Health Risk Manag.* 1 (2005), 291-299.

Willner P., Towell A., Sampson D., Sophokleous S., Muscat R. Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant, *Psychopharmacology* 93 (1987), 358-364.

Willner P. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation, *Psychopharmacology* 134 (1997), 319ó329.

Wilson S., Johnston A., Robson J., Poulter N., Collier D., Feder G., Caulfield M.J. Comparison of methods to identify individuals at increased risk of coronary disease from the general population, *BMJ.* 326 (2003), 1436.

Yu Y.H., Blessing W.W. Cutaneous vasoconstriction in conscious rabbits during alerting responses detected by hippocampal theta-rhythm, *Am. J. Physiol* 272 (1997), 2086216.

Yusuf S., Anand S., Avezum Jr A., Flather M., Coutinho M. Treatment for acute myocardial infarction. Overview of randomized clinical trials, *Eur. Heart J* 17 (1996), 16ó29.

Zaretsky D.V., Zaretskaia M.V., Samuels B.C., Cluxton L.K., DiMicco J.A. Microinjection of muscimol into raphe pallidus suppresses tachycardia associated with air stress in conscious rats, *J. Physiol* 546 (2003), 243ó 250.

Ziegler D. Cardiovascular autonomic neuropathy: clinical manifestations and measurement, *Diabetes Rev* 7 (1999), 342-357.

CHAPTER 2

Long-term effects of prenatal stress: Changes in adult cardiovascular regulation and sensitivity to stress

Francesca Mastorci[a], Massimo Vicentini[a], Odile Viltart[b], Massimo Manghi[a], Gallia Graiani[c], Federico Quaini[c], Peter Meerlo[d], Eugene Nalivaiko[e], Stefania Maccari[f] and Andrea Sgoifo[a]

[a]Stress Physiology Lab., Dept. of Evolutionary and Functional Biology, University of Parma, Italy
[b]NeuroImmunoEndocrinology Lab., Pasteur Institute of Lille, France
[c]Dept. of Internal Medicine, University of Parma, Italy
[d]Dept. of Molecular Neurobiology, University of Groningen, Haren, The Netherlands
[e]Neurocardiology Lab., School of Biomedical Sciences, University of Newcastle, Australia
[f]Perinatal Stress Team, University of Lille 1, Villeneuve dÆAscq, France

Chapter 2

1. Introduction

2. Animal models of prenatal stress

2.1 Primate models

2.2 Rodent models

3. Prenatal stress: neuroendocrine and behavioral consequences

3.1 Neurotransmitter systems

3.2 Neuroendocrine function

3.3 Behavior

3.4 Maternal glucocorticoids as a possible mechanism

4. Adverse prenatal conditions influence adult cardiovascular function and structure

4.1 Epidemiological evidence

4.2 Prenatal stress, the sympathetic-adrenomedullary system, and long-term cardiovascular implications

4.3 Prenatal stress, the renin–angiotensin system and adult cardiovascular function

4.4 Two major approaches to the study of the relationship between prenatal stress and later cardiovascular disease

4.5 Intrauterine growth restriction and later cardiovascular effects

5. Repeated restraint stress in rat pregnant rats: long-term neuroendocrine, autonomic, and cardiac effects in the offspring

5.1 Aims

5.2 Methods

5.3 Results

5.4 Discussion

6. Conclusions

References

ABSTRACT

Prenatal environment exerts profound influences on the development of an organism and stressful events during pregnancy can bring about long-term physiological/behavioral alterations in the offspring. Epidemiological evidence points to a relationship between intrauterine growth restriction (IUGR), body weight at birth, and adult cardiovascular disease. Experimental research employed different models of IUGR, including altered maternal nutrition, exposure to elevated glucocorticoids, and reduced placental perfusion, all of which can program, when acting during sensitive temporal windows of foetal life, alterations in cardiovascular regulation and stress sensitivity. Original data are presented indicating that prenatal psychological stress (intermittent restraint) does not induce in the rat adult offspring changes of plasma corticosterone levels, cardiac autonomic modulation, and circadian rhythmicity of heart rate (HR), body temperature (T) and physical activity (Act) at rest. However, prenatally stressed rats ó when further stimulated in adulthood ó exhibit prolonged adrenocortical stress responsivity, disturbed circadian rhythmicity of HR, T, and Act, and increased adrenal weight. This evidence supports the idea that prenatal stress *per se* does not change dramatically a given structure or function, but it affects resilience and renders the animal more susceptible to pathophysiological outcomes when further insults occur during adulthood.

1. Introduction

Cardiovascular diseases such as hypertension and ischemic cardiomyopathy are the leading causes of death in western countries (Wilson et al., 1998). Smoking, exposure to tobacco smoke, lack of physical activity, obesity, high cholesterol or abnormal blood lipids, diabetes and emotional stress are well recognized risk factors for cardiovascular morbidity and mortality (Khot et al., 2003). Although it has been clearly shown that some individuals are genetically prone to develop cardiovascular diseases, genetic background does not seem to account for all pathophysiological outcomes. More recently, a growing body of literature underlined the role of an additional risk factor: prenatal programming. Programming results from adaptive changes in gene expression patterns that occur in response to stressors leading to altered growth of specific organs and systems during their most critical time of development (Barker, 1998b). Indeed, prenatal environment exerts profound influences on the development of an organism and stressful events during pregnancy can induce alterations in the foetal environment resulting in early and long-term structural and functional consequences (Maccari et al., 2003; Wadhwa et al., 2001; Weinstock, 1997; Weinstock, 2001). The nature and severity of prenatal stress effects seem to be influenced by the timing of the stressors intervening during gestation. In fact, a large number of scientific reports support the idea that prenatal development is characterized by sensitive periods or developmental windows when organisms are more vulnerable to stressors (Rice and Barone, 2000; Seckl, 1998; Symonds et al., 2007). Human studies reveal that offspring from mothers that suffered from adverse conditions during their first trimester display modest effects, while babies whose mothers were exposed to stress during the third trimester exhibit long-lasting consequences such as low birth weight, heart malformations, hearing loss, and skeletal abnormalities (Talge et al., 2007). In spite of the wealth of literature on the short- and long-term neuronal, neuroendocrine and behavioral consequences of prenatal stress in humans and animals, there is less information regarding the effects of stressors occurring during pregnancy on the adult cardiovascular system. Providing current update for this issue is the major aim of our review.

Firstly, we summarize the evidence for neuroendocrine and behavioral consequences of prenatal stressors, features which have been widely described in the literature; this part is deliberately concise and refers to the special issue õPrenatal programming of behaviour, physiology and cognitionö (vol. 29, number 2, pp. 207ó384, 2005) of Neuroscience & Biobehavioral Reviews for an exhaustive view on the topic. Then, the review briefly lists different animal models of prenatal stress, with special emphasis on those non-human primate and rodent models which have been used to study long-term cardiovascular implications. Afterwards, the effects of prenatal stress on cardiovascular function and structure are thoroughly reviewed, with reference to epidemiological

evidence, the role of the sympathetic-adrenomedullary and reninóangiotensin systems, and the two major approaches to the study of the relationship between adverse prenatal environment and cardiovascular (patho)physiology. Finally, original experimental results from our laboratory are presented, where cardiac, autonomic, and neuroendocrine parameters were collected altogether, in adult rats born to mothers which were exposed to repeated restraint stress during pregnancy

2. Animal models of prenatal stress

Animal models have been widely used to investigate the relationship between adverse environment during foetal life and vulnerability to psychosomatic/psychological disorders in adulthood, as they offer the opportunity to separate the role of prenatal stress from other morbidity risk factors. In addition, animal models allow to focus on specific periods of pregnancy and to highlight the different impact of a stressor according to differences in foetal age.

2.1 Primate models

Although most prenatal stress studies have been conducted in rodents, non-human primate models are particularly valuable because of their slow-paced foetal growth rates, long gestations, enriched placental nourishment, and single births (Newell-Morris and Fahrenbruch, 1985). An example of prenatal manipulation in non-human primates involves removing pregnant monkeys from their cages and subsequently exposing them to uncontrollable noise burst. In a study by Clarke et al. (1994) on rhesus monkeys this manipulation was applied once per day, 5 days a week, for 25% of gestation period. Another type of prenatal stress manipulation in non-human primates involves injecting pregnant females with the synthetic glucocorticoid analog dexamethasone, usually during the fourth month postconception of their 5.5-month-long gestation (Coe and Lubach, 2005; Uno et al., 1990).

2.2 Rodent models

Among rodents, guinea pigs represent a valid animal model given that the landmarks of brain and neuroendocrine growth are well characterized in this species (Dobbing and Sands, 1970). They were used to explore the long-term brain, endocrine, autonomic, and behavioral effects in the offpsring born to pregnant females which were exposed to unstable social environment (Kaiser and Sachser, 1998), strobe light (Kapoor and Matthews, 2005), or synthetic glucocorticoids (Banjanin et al., 2004).

In rat models of prenatal stress pregnant dams have been exposed to a variety of stressors, including saline injections (White and Birkle, 2001), immobilization (Ward and Stehm, 1991), hypoxia (Peyronnet et al., 2002), electric footshock (Weinstock et al., 1998), placental insufficiency (Alexander, 2003), and REM sleep deprivation (Suchecki and Palermo-Neto, 1991).

Lately, a frequently used protocol is a modified version of Ward and Weisz model (Maccari et al., 1995; Ward and Weisz, 1984), consisting of restraining the mothers during the last week of pregnancy. This paradigm produces a robust psychoneuroendocrine stress activation in the mothers that interferes with the development of neural networks and neuroendocrine systems in the offspring, which in turn modulate behavioral and physiological stress responses in adulthood (Kofman, 2002; Sternberg and Ridgway, 2003).

The long-term effects of different prenatal manipulations in rodents and primates are detailed in the following chapters.

3. Prenatal stress: neuroendocrine and behavioral consequences

Early stress has been linked to many changes in neurotransmitter systems, neuroendocrine function and behavior which become evident at different life ages, from neonatal stage to adulthood.

3.1 Neurotransmitter systems

Mild to severe prenatal stressors have been shown to affect adult brain receptor functions, including monoaminergic (Griffin et al., 2005; Takahashi et al., 1992; Viltart et al., 2006), and glucocorticoid receptor systems (Wilcoxon and Redei, 2007). In particular, alterations in norepinephrine (NE) and dopamine (DA) turnover were observed after foetal stress, including reduced levels of NE in the cerebral cortex and both NE and DA in the locus coeruleus (Takahashi et al., 1992). Prenatal stress has been shown to affect also the serotonergic system in the hippocampus of both adolescent and adult rat offspring (Hayashi et al., 1998; Morley-Fletcher et al., 2004; Peters, 1990), and this would predispose to the development of mood disorders in later life. In fact, disturbances of the serotonergic and noradrenergic system functioning are known to play an important role in the pathophysiology of anxiety and depressive disorders (Ressler and Nemeroff, 2000).

3.2 Neuroendocrine function

The most often reported neuroendocrine consequences of exposure to adverse environmental stimuli during foetal development are changes in the activity of the adult hypothalamic-pituitary-adrenocortical (HPA) axis (Henry et al., 1994; Maccari et al., 1995). Prenatal stress in rodents

causes an increased level of pituitaryóadrenal (re-)activity in later life (Koenig et al., 2005; Weinstock, 2005). Adult prenatally stressed rats show higher plasma corticosterone concentrations at baseline (Henry et al., 1994; Ward et al., 2000), as well as larger release of pituitary and adrenal hormones in response to stress episodes (Henry et al., 1994; Weinstock et al., 1992; Weinstock et al., 1998). However, an early study in guinea pigs demonstated that a single maternal exposure (3 h) to a strobe light stressor on day 60 of gestation (term 70 days) results in lowered pituitary-adrenocortical stress responsivity in the adult male offspring (Cadet et al., 1986). A more recent study confirms that male adult guinea pigs whose mothers had been nutrient restricted exhibit reduced basal ACTH and cortisol levels, although female offspring show unchanged basal plasma ACTH and elevated cortisol concentrations (Lingas and Matthews, 2001).

In humans, gestational stress does not only activate the maternal pituitary-adrenocortical axis but it can also cause an increased release of corticotropin releasing hormone (CRH) from the placenta by catecholamines and cortisol (Petraglia et al., 1996) as well as by foetal hypoxia (Sug-Tang et al., 1992). In contrast to the negative feedback control that it exerts on the release of hypothalamic CRH, cortisol stimulates the release of the peptide from the placenta resulting in a positive feedback. In rats, the foetus has been shown to respond to maternal stress by releasing CRH from the hypothalamus during the late gestational period. In addition, a 30-min restraint stress in the mother on gestational days 15ó17 increased the expression of CRH mRNA in the foetal paraventricular nucleus (Fujioka et al., 1999). A recent study has reported a significant increase in hypothalamic CRH mRNA in both unrestrained and restraint-stressed adult offspring born to mothers who were exposed to dexamethasone during pregnancy (Shoener et al., 2006; Welberg et al., 2001). Although altered mRNA expression does not consistently predict the magnitude or functionality of corresponding protein products, the increase in CRH mRNA was associated with a significantly higher level of serum corticotropin and corticosterone.

Adult rats born to mothers which were stressed during the last week of pregnancy also exhibit lower expression of glucocorticoid (GR) and mineralocorticoid (MR) receptors in the hippocampus (Henry et al., 1994; Maccari et al., 1995; Weinstock et al., 1992). This reduced GR and MR expression leads to attenuated negative feedback control and may explain the larger and longer-lasting increments of plasma corticosterone levels following the exposure to an acute stressor.

Taken together, these data demonstrate that prenatal stress is associated with persistent changes in adult HPA axis activity and/or stress reactivity at each level of the axis (hypothalamic, pituitary and adrenal), although there is not full consensus on the direction and intensity of such changes.

3.3 Behavior

Also the behavioral phenotype in both infancy and adulthood appears to be modulated by adverse events occurring during foetal life (Darnaudery and Maccari, 2008; Weinstock, 2001; Welberg et al., 2001). In general, there is good agreement from human and animal studies that stressful events during foetal development are associated with late adverse neurobehavioral outcomes, including socioemotional and cognitive alterations during childhood and adulthood (Buitelaar et al., 2003). The exposure of women to psychological stressors during pregnancy increases the likelihood that their children develop psychopathologies later in life (Brown et al., 1996; Wadhwa et al., 2001; Weinstock, 2001). Davis et al. (2007) found that scores of anxiety and depression inventories obtained from mothers during the third trimester of gestation predicted the susceptibility to these pathologies by offspring in adulthood. In addition, a few studies have explored the relationship between prenatal stress and neurobehavioral alterations during the neonatal period. For example, Field et al. (2003) reported that newborns of mothers with high levels of anxiety display significantly greater right frontal brain activation, spend more time in deep sleep, and show more state changes and poorer performance in a standard test of neurobehavioral assessment of motor maturity. Interestingly, this neurophysiological profile also appears to be associated with some symptoms of depression later in infancy and adulthood (Davidson, 1998).

A consistent finding in the non-human primate research is that stressing the mother during pregnancy has long-term unfavourable effects on attention, neuromotor functions, and adaptability to novel and stressful situations in the offspring (Schneider et al., 1999). No differences in gestational length were observed as a function of prenatal stress exposure, although offspring tended to be smaller at birth (Schneider et al., 2002). Again, neurobehavioral consequences seem to depend on the timing of the manipulation during pregnancy. In fact, infant monkeys whose mothers were exposed to stress early in gestation performed more poorly in attention and motor maturity tests than those whose mothers were exposed during mid to late gestation (Schneider et al., 1999). The effects of prenatal manipulation in non-human primates were also examined when monkeys were 3ó4 years of age, a period considered to be analogous to human adolescence. Assessments at this stage demonstrated that foetal challenges elicit more locomotion following separation from cagemates and group formation test, but less exploratory behavior in an unfamiliar environment (Clarke and Schneider, 1997).

Studies in rodents revealed a clear relationship between stressful pregnancy and alterations in the behavior of the offspring (Kofman, 2002; Weinstock, 2005). As compared to control rats, adult offsprings of rat dams subjected to stressors during gestation display an increase in anxiety-related

behaviors, including decreased exploration and increased freezing when exposed to novelty (Vallée et al., 1997), suppressed open arm exploration in the elevated plus maze (Zimmerberg and Blaskey, 1998), and increased defensive withdrawal (Ward et al., 2000). Indeed, prenatally stressed rats tend to develop higher emotional reactivity, higher levels of anxiety, and depression-like behaviors in adulthood (Abe et al., 2007; Bhatnagar et al., 2005).

3.4 Maternal glucocorticoids as a possible mechanism

The mechanisms by which adverse maternal conditions affect offspring development may be diverse. Most studies investigated the impact of increased levels of maternal stress hormones, with special focus on the role of maternal glucocorticoid response to stress in the programming of the offspring HPA axis activity. Barbazanges et al. (1996) suggested that stress-induced increase in maternal glucocorticoids may be a mechanism by which prenatal stress impairs the development of the adult offspring's glucocorticoid stress response in rats. Other studies have directly investigated the effects of stress hormone exposure, for example by implanting glucocorticoid releasing pellets or exposure to synthetic glucocorticoids. Nevertheless, these studies together do not seem to provide a consistent picture. Offspring of female rats that were implanted with corticosterone pellets during the last third of pregnancy showed increased spontaneous ambulation, motility and rearing compared to the placebo treated group (Diaz et al., 1995). However, female rats that received dexamethasone during the same gestational period produced adult offspring with reduced exploratory behavior in the open-field test and reduced exploration in the elevated plus-maze (Welberg and Seckl, 2001).

Foetal exposure to synthetic glucocorticoid during gestation has profound effects on the offspring HPA activity in a number of species. In humans, this is clinically relevant because a large number of pregnant women have been treated with synthetic glucocorticoids, especially betamethasone and dexamethasone. Although there have been no reports of adverse effects of a single dose of antenatal glucocorticoid treatment in children, evidence is emerging that multiple courses may have negative neurobiological outcomes later in postnatal life (Andrews and Matthews, 2003). In particular, the exposure of foetuses to synthetic glucocorticoids in late gestation permanently alters HPA function in pre-pubertal, post-pubertal, and aging offspring. Prenatal glucocorticoid exposure also leads to modification of HPA-associated behaviours and organ morphology, as well as altered regulation of other neuroendocrine systems (Matthews et al., 2002; Matthews et al., 2004). Permanent changes in HPA function have a long-term impact on health, since elevated cumulative exposure to endogenous glucocorticoid has been linked to the premature onset of pathologies associated with aging. On this regard, it is important to note that there are significant differences between foetal

exposure to increased endogenous glucocorticoid and synthetic glucocorticoids such as dexamethasone. Endogenous glucocorticoids bind to both GR and MR receptors and the effects on the foetal brain are likely mediated by both of these receptors. In contrast, synthetic glucocorticoids bind predominantly to GRs, with MRs showing low affinity for these compounds (Kliewer et al., 1998); moreover they easily pass across the placenta and reach the foetus. As demonstrated by Miller et al. (1992) in a study on rats, different doses of dexamethasone led to significant GR receptor activation in the pituitary, whereas only an exceedingly high dexamethasone dose activated GR receptors in the hippocampus and hypothalamus.

Clearly, glucocorticoids represent a crucial factor by which maternal stress affects offspring development, although it does not seem to explain all the long-term effects. For a more comprehensive review on the neuroendocrine/behavioral consequences of prenatal stress and the possible underlying mechanisms refer to the special issue õPrenatal programming of behaviour, physiology and cognitionö (vol. 29, number 2, pp. 207ó384, 2005) of Neuroscience & Biobehavioral Reviews.

4. Adverse prenatal conditions influence adult cardiovascular function and structure

4.1 Epidemiological evidence

In the past 20 years, epidemiological evidence has suggested that a suboptimal intrauterine environment resulting in impaired foetal growth is a very important risk factor for cardiovascular and metabolic disease in adult life (Barker, 2002; Curhan et al., 1996; Dodic et al., 1999). In particular, these studies have demonstrated an association between low birth weight and the subsequent development of hypertension, atherosclerosis, coronary heart disease, and stroke (Barker et al., 1993). In addition, numerous metabolic disorders can be programmed by adverse intrauterine conditions, including insulin resistance, type 2 diabetes, dyslipidemia and obesity (Drake and Walker, 2004). Jones et al. (2007) provided clear evidence that growth retardation *in utero* (as reflected by small size at birth) is linked to altered autonomic cardiovascular control, involving modulation of both sympathetic and parasympathetic function, although in a gender-specific manner. Women (but not men) who were small at birth exhibited increased levels of low-frequency blood pressure variability at rest and during stress, reduced levels of high frequency heart period variability, and reduced baroreflex sensitivity. Similarly, Ward et al. (2004) showed that the magnitude of tachycardic and pressor responses to psychological stressors in adulthood is inversely correlated with birth weight. Evidence that coronary heart disease, hypertension, and diabetes are prenatally programmed was strenghtened by longitudinal studies on large populations in the United

Kingdom and Finland (Forsen et al., 2004; Godfrey and Barker, 2000). In the first one, subjects that were underweight at birth had high rates of coronary heart disease, high blood pressure, high cholesterol concentrations, and abnormal glucoseóinsulin metabolism. The authors suggested that such relationships between foetal development and related cardiovascular implications in adulthood were the consequence of high carbohydrate intake in early pregnancy and low protein intake in late pregnancy. These relations were independent from the length of gestation, suggesting that cardiovascular disease is related to foetal growth restriction rather than premature birth. Although key factors that interfere with foetal development and program adult cardiovascular disease remain uncertain, there are strong pointers to the importance of the foetal adaptations invoked when the maternal placental nutrient supply does not match the foetal demand (Godfrey and Barker, 2000). Forsen et al. (2004) followed up the path of growth of girls and boys in Finland, who later developed coronary heart disease. The authors observed that, though broadly similar, the paths of growth associated with the later development of coronary heart disease differed between girls and boys. In comparison with boys, the girls were short at birth, rather than thin, had compensatory growth in height during infancy, became thin, and thereafter had a rapid increase in weight and body mass index. The authors suggested that girls are less vulnerable to undernutrition *in utero* and are better able to sustain postnatal growth in an adverse environment.

In disagreement, Lucas and Morley (1994) provided human data which do not support the hypothesis that high blood pressure has early nutritional origins; in fact, they did not find any association between low ponderal index at birth (at full term) and increased later blood pressure at 7ó8 years of age.

4.2 Prenatal stress, the sympathetic-adrenomedullary system, and long-term cardiovascular implications

As detailed in Section 3 of this review, a large body of literature indicates that an adverse foetal environment is able to produce notable changes in biobehavioral responsivity to stressors during adult life, with heightened (re)activity of the classical neuroendocrine mediators of the stress response, i.e. the HPA axis and the sympathetic-adrenomedullary system. Because the hormonal mediators of the stress response (glucocorticoids and catecholamines) are important modulators of metabolism and cardiocirculatory function, it is reasonable to hypothesize an important role of these allostatic systems in mediating the long-term pathophysiologic outcome of an altered early environment (McEwen, 1998; Phillips, 2007). As far as the sympathetic-adrenomedullary system is concerned, evidence suggests that the activity of central monoaminergic areas in the brain can be permanently modified by foetal exposure to stressors (Hayashi et al., 1998). In addition, the

sympathetic innervation of peripheral tissues and the responsiveness of sympathetic nerves and adrenal medulla to standard stimuli are susceptible to modifications by exposure to early life stressors (Young, 2002). Since catecholaminergic and serotonergic neurons constitute the essential components of central neural cardiovascular control, alterations in their metabolism/activity during foetal life may bring about significant consequences on cardiovascular physiology in adulthood (Dampney, 1994). The exposure of pregnant rats to noise and light stress has been shown to sensitize plasma catecholamine responsivity to footshock in the adult offspring (Weinstock et al., 1998). In addition, when pregnant rats were exposed to hypoxia the levels and utilization of catecholamines were reduced in sympathetic ganglia, in target organs, in adrenals and in the rostral part of the A2 cell group in the nucleus tractus solitarius of the offspring at postnatal week 1, 3, and 9. In the 12-week-old offspring, the lowered sympathetic nervous activity was restricted to the stellate ganglion, heart and adrenals (Peyronnet et al., 2002).

Igosheva et al. (2004) focused on long-term cardiovascular effects of repeated prenatal stress (restraint test combined with heat and light stress) occurring in the third week of rat pregnancy. The pattern of blood pressure and heart rate response to adult restraint stress was changed if they were subjected to prenatal stress. Specifically, higher and longer-lasting heart rate and systolic pressure elevations were observed during stress; during the recovery phase, elevated heart rate, systolic and diastolic blood pressure were documented. Interestingly, female offspring appeared to be more sensitive to prenatal stress as compared to male counterparts. Another study by the same research group (Igosheva et al., 2007) confirmed that repeated prenatal restraint stress produces long-lasting effects on blood pressure responsiveness to acute stress and delayed post-stress recovery. In addition, vascular reactivity to neuropeptide Y and electrical field stimulation in mesenteric arteries was significantly increased in adult, prenatally stressed animals. The authors proposed that maternal stress in pregnancy may affect the NPY pathway and that persistently elevated vascular sensitivity to NPY may account for stress-induced systemic hypertension.

4.3 Prenatal stress, the renin–angiotensin system and adult cardiovascular function

The renin–angiotensin system (RAS), a regulatory system important in the long-term control of blood pressure, may be programmed *in utero* and may contribute to the pathogenesis of undernutrition programmed hypertension (Langley-Evans, 2001). Suppression of the RAS observed at birth may lead to permanent structural changes associated with the pathogenesis of hypertension in rat offspring from protein-restricted dams (von Lutterotti et al., 1991). The strongest evidence for the involvement of the RAS in hypertension programmed by maternal protein restriction is the normalization of blood pressure in the hypertensive rat offspring by angiotensin-converting enzyme

(ACE) inhibition (Langley-Evans and Jackson, 1995). Administration of the ACE inhibitor captopril to programmed offspring between 2 and 4 weeks of age exerted long-term antihypertensive effects, so that low-protein-exposed rats had similar blood pressure to normotensive rats 2 months after cessation of captopril (Sherman and Langley-Evans, 1998). Upregulation of the renal angiotensin type 1 receptor (AT1R) following late gestational protein restriction was observed in rats at 4 weeks of age by some investigators (Manning and Vehaskari, 2001), but not others (McMullen et al., 2003). More importantly, the critical role of RAS in the aetiology of hypertension programmed by protocols of *in utero* protein restriction is indicated by RAS blockade studies (Hall et al., 1990; Sahajpal and Ashton, 2003).

Furthermore, alterations in the RAS may also be responsible for marked increases in blood pressure programmed by prenatal exposure to glucocorticoids (OøRegan et al., 2004; Peers et al., 2001), indicating that different foetal insults lead to similar pathways of programmed hypertension. The mechanisms underlying glucocorticoid programming of hypertension remain unknown, although some data in sheep have implicated the RAS (Dodic et al., 2002; Moritz et al., 2002). Importantly, in adult rats, glucocorticoids regulate all the main components of RAS, including renin secretion, angiotensinogen synthesis (Bunnemann et al., 1993), ANG-converting enzyme activity, ANG II (Sato et al., 1994), and mineralocorticoid receptor expression.

In conclusion, prenatal glucocorticoid administration in the final week of gestation results in reduced birth weight and subsequent abnormalities in cardiovascular and metabolic physiology. Alterations within the RAS may, in part, underlie the hypertension associated with prenatal glucocorticoid treatment.

4.4 Two major approaches to the study of the relationship between prenatal stress and later cardiovascular disease

Different hypotheses have been proposed to explain the nature of the link between disturbed prenatal life and cardiovascular pathology in adulthood. One point of view maintains that prenatal environment exerts its effects mostly through maternal stress hormones (i.e. glucocorticoids). Another set of studies underlines the role of undernutrition and malnourishment. The two explanations are not mutually exclusive, rather they complement each other, since alterations of both endocrine and nutritional factors bring about unbalanced foetal development (Barker, 2001; Nyirenda and Seckl, 1998). For example, it has been hypothesized that the mechanism of maternal protein restriction programmed hypertension is dependent on maternal glucocorticoid production (Langley-Evans, 2001). Nutritionally induced hypertension and glucocorticoid programmed

hypertension share many characteristics and may employ common mechanisms. In particular, treatment of pregnant protein-restricted rats with metyrapone, an inhibitor of glucocorticoid synthesis, completely prevented hypertension development in the offspring (Langley-Evans, 2001).

Foetal exposure to increased maternal glucocorticoids during sensitive temporal windows appears to play a key role in programming the offspring to hypertension (Benediktsson et al., 1993). In normal conditions, the foetus is protected from maternal glucocorticoids, which are inactivated by the placental enzyme 11 -hydroxysteroid dehydrogenase type 2 (11 -HSD2). Under stress conditions, maternal glucocorticoid levels are significantly higher, increasing the chance that the foetus is exposed to their multiple actions. In support of this hypothesis, administration of dexamethasone to pregnant rats generates offspring with low birth weight that develop hypertension later in life (Ortiz et al., 2003). Since prenatal glucocorticoid exposure was shown to be associated with low birth weight and high blood pressure in humans and animals (Doyle et al., 2000; Newnham and Moss, 2001), elevated glucocorticoid levels might be an important mediator of both reduced neonatal birth size and adult hypertension in the offspring (Mairesse et al., 2007).

Another group of studies showed that foetal programming of adult cardiovascular disease is linked to low birth weight from intrauterine growth restriction due to undernutrition (Barker, 1998a). Significant cardiac functional changes have been demonstrated in the offspring of rats treated with a low-protein diet, including reduced cardiac output, increased end diastolic pressure, and reduced ventricular contraction and relaxation rates (Cheema et al., 2005). Maternal protein deprivation causes the pup's hearts to be more prone to developing arrhythmias and increased susceptibility to myocardial ischemic insults (Hu et al., 2000; Xu et al., 2006).

4.5 Intrauterine growth restriction and later cardiovascular effects

Epidemiological studies by Barker et al. (1993) and Barker (1998b) clearly showed that there is a relationship between intrauterine growth restriction (IUGR), body weight at birth and later lifetime incidence of hypertension and coronary heart disease. Since then, several experimental models of intrauterine growth restriction have been developed to foster the understanding of foetal programming of cardiovascular disease. Most of these can be grouped in three categories: altered maternal nutrition, exposure to elevated levels of glucocorticoids, and reduced placenta perfusion. Protein malnutrition in rats during the intrauterine period results in profound intrauterine growth retardation, that is associated with raised diastolic blood pressure and increased predisposition to cardiac arrhythmias in later life (Hu et al., 2000). These results are consistent with epidemiological observations made in humans, suggesting that intrauterine protein deprivation is a useful model for

the understanding of the mechanisms involved in the pathophysiology of cardiac disease. Elevated glucocorticoid levels in the third week of rat pregnancy also induce intrauterine growth retardation, that is associated with hyperinsulinaemia, hyperleptinaemia, and hypertension in adult life (Sugden et al., 2001). On the other hand, uteroplacental dysfunction induced by bilateral uterine artery ligation also produces intrauterine growth restriction in rats. This restriction was shown to be associated with raised baseline blood pressure, increasing pulse pressure with age, and an altered response (higher peak of systolic blood pressure and delayed heart rate recovery) following ammonia-induced olfactory stress (Schreuder et al., 2006a; Schreuder et al., 2006b).

Besides the effects on adult heart rate and blood pressure, a number of elegant studies also documented the long-term consequences of an adverse early environment on endothelial function and cardiac structure. Impaired endothelium-dependent vasodilation, that is known to contribute to the development of cardiovascular disease, has been shown in infants, children, and adults, all of whom were underweight at birth (Leeson et al., 2001; Louey and Thornburg, 2005; Martin et al., 2000a; Martin et al., 2000b).

In rat studies, adult offspring from protein-restricted dams showed an impairment in endothelium-dependent and -independent relaxation (Brawley et al., 2003); similarly, adult rats born to dams with placental insufficiency exhibited depressed endothelial function in the aorta (Payne et al., 2003). Zhang research group showed that rats undergoing prenatal hypoxia in the last days of pregnancy had a number of cardiac structural consequences in adulthood (Zhang, 2005). These consequences included greater proportion of myocites undergoing apoptosis following ischemia reperfusion compared with controls (Li et al., 2003), failure to show an increase in protective proteins (such as HSP70) in response to heat stress (Li et al., 2004), a greater proportion of non-proliferating binucleate cardiomyocites, such binucleate cells being larger than those from aged-matched controls (Bae et al., 2003).

Altogether, the available rodent literature confirms that prenatal stress manipulations are associated with a wide array of long-term consequences on the cardiocirculatory system. Similarities between physiological and structural consequences of malnutrition, prenatal hypoxia and excess glucocorticoid exposure suggest that there might be common underlying mechanisms which mediate the effects of prenatal environment on the adult cardiovascular system, possibly involving alterations of the hypothalamic-pituitary-adrenocortical axis (Igosheva et al., 2004). However, it is still rather difficult to detail the actual biological substrates linking maternal neuroendocrine environment, nutritional factor and oxygen supply to the foetus, its development and birth weight, and cardiovascular pathophysiology in adulthood (Louey and Thornburg, 2005).

5. Repeated restraint stress in rat pregnant rats: long-term neuroendocrine, autonomic, and cardiac effects in the offspring

5.1 Aims

In spite of the abundant literature based on experimental malnutrition, hypoxia or excess glucocorticoids, there is little information on the effects of psychological prenatal stressors on adult cardiac function at rest and during an acute challenge in rats. In addition, there are no studies taking into account the impact of this type of prenatal stressors on cardiac tissue anatomy. In the study described herewith, we examined cardiac functional and structural consequences and related them to HPA axis (re-)activity, in adult offspring of mothers repeatedly exposed to restraint test in late pregnancy.

In the present study, rat dams were repeatedly exposed to restraint stress in the last week of pregnancy, according to an experimental procedure previously described by Maccari et al. (1995). The aim of this study was to examine long-term effects on (i) heart rate and cardiac vagal activity in baseline conditions and in response to acute challenges, (ii) circadian rhythmicity of heart rate, body temperature, and physical activity, (iii) (re)activity of the hypothalamic-pituitary-adrenocortical axis, and (iv) myocardial and adrenal structure.

5.2 Methods

Wild-type-Groningen rats were used (*Rattus norvegicus*, WTG strain), originally derived from the Department of Behavioral Physiology (University of Groningen, The Netherlands). This strain is known to be highly stress reactive; we previously showed that males exposed to a brief social challenge (defeat) react with a higher sympathetic activation, a lower parasympathetic antagonism, and a higher incidence of ventricular arrhythmias as compared to Wistar strain rats (Sgoifo et al., 1998). At 20 weeks of age, females were housed with a male partner and, after assessment of the pregnant status, individually housed and randomly assigned to prenatal stress (PNS, *n*=16) and control (CTR, *n*=14) groups. PNS mothers were stressed daily from day 14 to day 21 of pregnancy (Morley-Fletcher et al., 2004). Briefly, pregnant females were restrained for 45 min (three times per day between 9 a.m. and 5 p.m.) in a plastic cylinder (8 cm diameter, 32 cm long) in a lighted environment. CTR mothers were left undisturbed for all pregnancy duration. When the offspring reached the age of 17 weeks, blood samples were collected from 39 PNS and 41 CTR males, in order to determine plasma corticosterone levels immediately after introduction into a restraint tube (min 0), at min 30 (at the end of the restraint test), and at min 60 and 120. At 21 weeks of age, 12

PNS and 10 CTR rats were implanted with radiotelemetry transmitters for electrocardiogram (ECG), body temperature (*T*), and physical activity (Act) recording (Sgoifo et al., 1996). After transmitter implantation, the animals were allowed 2 weeks for post-surgery recovery. Each instrumented rat was then exposed to three stress episodes (open field, water immersion, social defeat) (De Boer et al., 1990; Sgoifo et al., 2002), with 48-h time interval between each other. Continuous ECG recordings were obtained in baseline conditions (15 min), during each stress episode (15 min) and immediately after the stressor (30 min). From ECG recordings, heart rate (expressed as the average inter-beat-interval or RR, ms) and a time-domain index of vagal input to the heart (r-MSSD, ms) were quantified (Sgoifo et al., 1998). Before (pre-stress period, 3 days) and after (post-stress period, 9 days) the three challenges, heart rate (HR, beats/min), *T* (°C), and Act (counts/min) were sampled around-the-clock for 60 s every 60 min, in order to assess the daily rhythmicity and long-lasting effects of the stressors. Subsequently, the three parameters were quantified as mean values for the 12-h dark phase (activity phase) and the 12-h light phase (resting phase). For each individual rat, the daily amplitudes of the rhythms of HR, *T*, and Act were calculated as the difference between average activity and resting phase values, respectively (Meerlo et al., 1999)

At the end of the experiment, the animals were sacrificed and the hearts removed for morphometric analysis, in order to evaluate the total amount of interstitial and reparative fibrosis in the three layers of the left ventricular myocardium (subepicardium, midmyocardium, and subendocardium). The ventricles were separated and fixed in paraformaldeyde (4%) and 1-mm-thick slices were transversely cut from the left ventricle and embedded in paraffin. A 5- m thick section obtained from one of the two intermediate rings was stained with hematoxylinóeosin and analyzed at optical microscopy (magnification 250×). According to a procedure previously described (Capasso et al., 1990), for each section, a quantitative evaluation of the fibrotic tissue was performed in 60 randomly selected fields from sub-endocardium, mid-myocardium and sub-epicardium, 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 overall volume fraction of reparative and interstitial fibrosis in each of the three layers of the left ventricular wall, the number of points overlying myocardial scarrings were counted and expressed as percentage of the total number of points explored. In addition, adrenal glands were removed, carefully trimmed, and weighed to evaluate possible hypertrophic and/or hyperplastic effects due to prenatal stress and/or adult challenges.

5.3 Results

All the data reported below are expressed as means±S.E.M. Two-way ANOVA for repeated measures on baseline and stress corticosterone concentrations revealed a significant effect of time (F=126.59, $p<0.001$). Plasma corticosterone levels in prenatally stressed males were significantly higher at min 120 after the onset of the stressor, as compared to CTR counterparts (313.56±8.25 vs. 289.16±9.8; t=2.12, $p<0.05$) (Fig. 1).
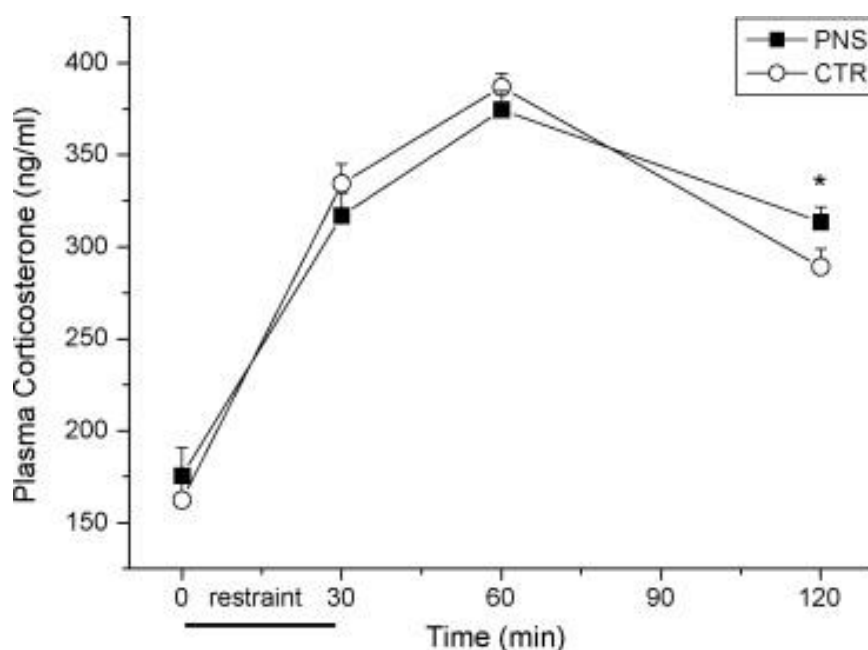

Figure 1. Time-course of plasma corticosterone levels during and after a 30 min restraint stress (as indicated by the solid line) in prenatally stressed (PNS, n=39) and control rats (CTR, n=41). Values are means±S.E.M. *Significantly different ($p<0.05$, Student õ*t*ö test) from control corresponding value.

Conversely, prenatal stress did not induce significant changes in the baseline values of heart rate parameters (RR and r-MSSD) (Table 1). Also the exposure to the three acute challenges (open field, water immersion, social defeat) produced similar overall changes for RR and r-MSSD in the two experimental groups, quantified as the area comprised between the baseline and the response time curve (AUC, Table 1). Prenatal stress did not induce significant alterations of baseline circadian rhythmicity of HR, *T*, and Act in adulthood. Indeed, the amplitude of the daily rhythms before the occurrence of adult challenges was not different between PNS and CTR rats (Fig. 2aóc). However, the series of subsequent stressors caused a reduction in the circadian rhythm amplitude for several

days, particularly in the PNS group (Fig. 2aóc). Two-way ANOVA for repeated measures was applied to the delta values of the rhythm amplitude relative to baseline over the post-stress period and revealed significant effects of (i) time, group, and time×group interaction for HR ($F=25.32$, $p<0.01$; $F=4.83$, $p<0.05$; $F=4.51$, $p<0.05$; respectively), (ii) time for Act ($F=6.89$, $p<0.05$), and (iii) time for T ($F=3.89$, $p<0.05$).

Table 1.

Baseline and area comprised between the response time curve and the baseline (AUC) for RR and r-MSSD during Open Field, Water Immersion, and Social Defeat test, in prenatally stressed (PNS, $n=12$) and control (CTR, $n=10$) rats implanted with radiotelemetry transmitters

	PNS		CTR	
	Baseline (ms)	AUC (ms min)	Baseline (ms)	AUC (ms min)
RR				
Open Field	189.2 ± 3.8	1924.2 ± 168.9	193.8 ± 2.1	2196.9 ± 116.3
Water Immersion	196.7 ± 5.6	2709.1 ± 234.4	203.8 ± 5.3	2945.8 ± 200.9
Social Defeat	195.8 ± 3.6	2320.3 ± 132.9	197.5 ± 5.2	2509.7 ± 199.2
r-MSSD				
Open Field	2.7 ± 0.3	43.2 ± 10.9	2.6 ± 0.2	35.5 ± 8.4
Water Immersion	3.1 ± 0.4	55.5 ± 17.3	3.2 ± 0.3	59.2 ± 14.4
Social Defeat	2.8 ± 0.3	39.8 ± 12.1	3.0 ± 0.3	58.2 ± 9.1

Values are means ± S.E.M.

RR, average RóR interval; r-MSSD, root mean square of successive RóR interval differences.

Post hoc Student's õ*t*ö test (CTR vs. PNS)ô *Open Field*. RR baseline: $t=1.45$, $p=0.16$; r-MSSD baseline: $t=0.95$, $p=0.35$; RR AUC: $t=1.28$, $p=0.22$; r-MSSD AUC: $t=0.54$, $p=0.59$. *Water Immersion*. RR baseline: $t=0.93$, $p=0.36$; r-MSSD baseline: $t=0.95$, $p=0.35$; RR AUC: $t=0.75$, $p=0.46$; r-MSSD AUC: $t=0.16$, $p=0.87$. *Social Defeat*. RR baseline: $t=0.29$, $p=0.78$; r-MSSD baseline: $t=0.61$, $p=0.55$; RR AUC: $t=0.81$, $p=0.42$; r-MSSD AUC: $t=1.17$, $p=0.26$.
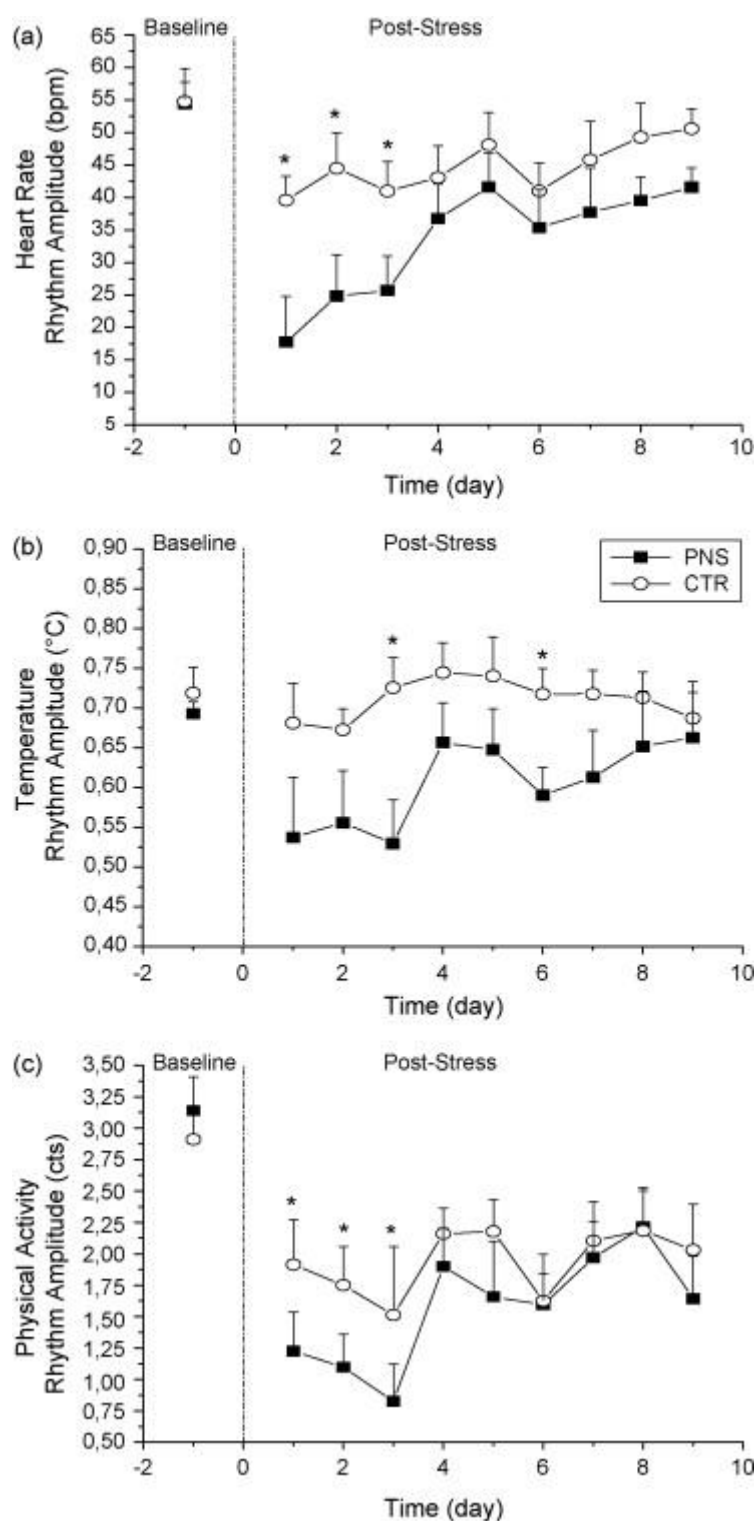
Figure 2. Time course of the amplitude of the daily rhythm for heart rate (panel a), body temperature (panel b) and physical activity (panel c), in baseline and post-stress period (before and after the adult challenges, respectively), in prenatally stressed (PNS, *n*=12) and control (CTR, *n*=10) rats implanted with radiotelemetry transmitters. Values are means±S.E.M. *Significantly different (*p*<0.05, Student õ*t*ö test) from control corresponding value.

The spatial distribution and morphologic features of myocardial fibrosis in the left ventricle were evaluated in the two experimental groups, and consisted of interstitial collagen deposition and small number of microscopic foci of scar distributed in the subepi-, mid- and subendo-myocardial layers of the left ventricle. Altogether, the amount of cardiac damage was modest (<1%) (Table 2). Although PNS rats exhibited, on average, a 30% larger score of mean fibrosis in the total wall, this difference did not reach significance due to large individual variability, neither within each single layer nor when the left ventricular wall was considered as a whole (Table 2).

Table 2.

Volume fraction (%) of fibrosis (interstitial+reparative) in the whole left ventricular wall and in each of the three wall layers, in prenatally stressed (PNS, n=12) and control (CTR, n=10) rats implanted with radiotelemetry transmitters

	Subepicardium	Midmyocardium	Subendocardium	Total wall
PNS	0.92 ± 0.23	0.56 ± 0.14	0.28 ± 0.11	0.61 ± 0.11
CTR	0.51 ± 0.18	0.83 ± 0.20	0.06 ± 0.04	0.46 ± 0.10

Values are means±S.E.M. Post hoc Student's õ*t*ö testô subepicardium: t=1.34, p=0.19; midmyocardium: t=1.14, p=0.27; subendocardium: t=1.79, p=0.09; total wall: t=0.95, p=0.35.

Adrenal weight (normalized for animal body weight) was similar in PNS and CTR rats that did not undergo transmitter implantation and acute challenges in adulthood (t=1.74, p=0.09) (Fig. 3). However, when we considered animals that experienced surgery and adult stressors, PNS rats had significantly heavier adrenals as compared to CTR counterparts (t=3.86, p<0.001) (Fig. 3).
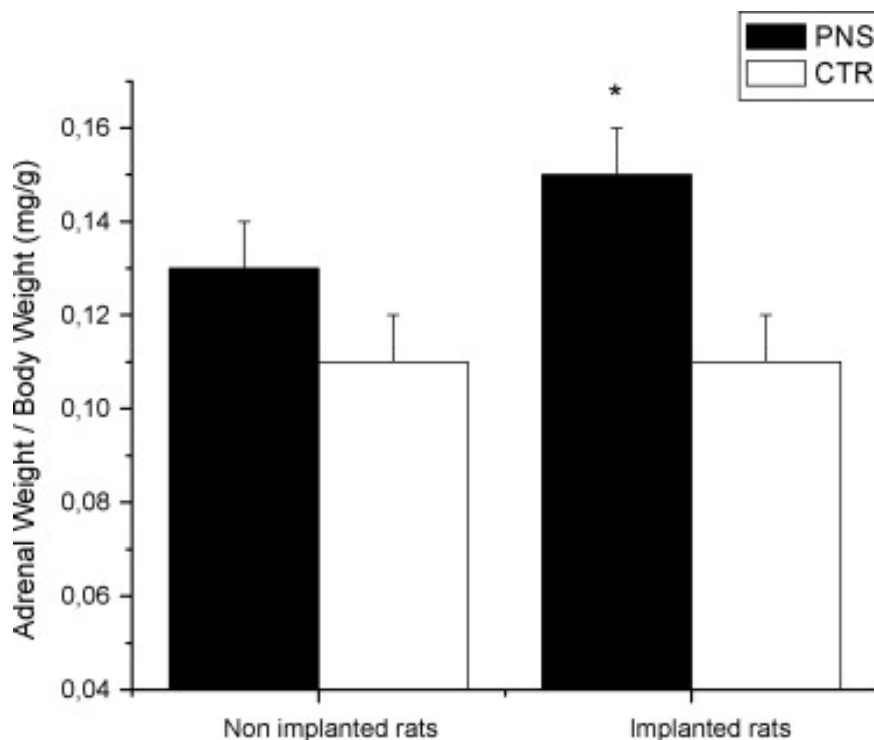

Figure 3.Adrenal weight expressed as a ratio to body weight (mg/g) in prenatally stressed (PNS: implanted n=12, non-implanted n=27) and control rats (CTR: implanted n=10, non-implanted n=31). Values are means±S.E.M. *Significantly different (p<0.01, Student õ*t*ö test) from control corresponding value.

Finally, correlation analysis performed in rats which underwent prenatal stress but did not undergo transmitter implantation and stress episodes in adulthood revealed a significant, negative association (R= 0.728, p<0.01) between body weight at 1 month of age and adrenal weight at sacrifice (Fig. 4a). In the same group of rats, there was a significant, positive correlation (R=0.433, p<0.05) between the peak of corticosterone response to restraint (t=60 min) and adrenal weight at sacrifice (Fig. 4b).
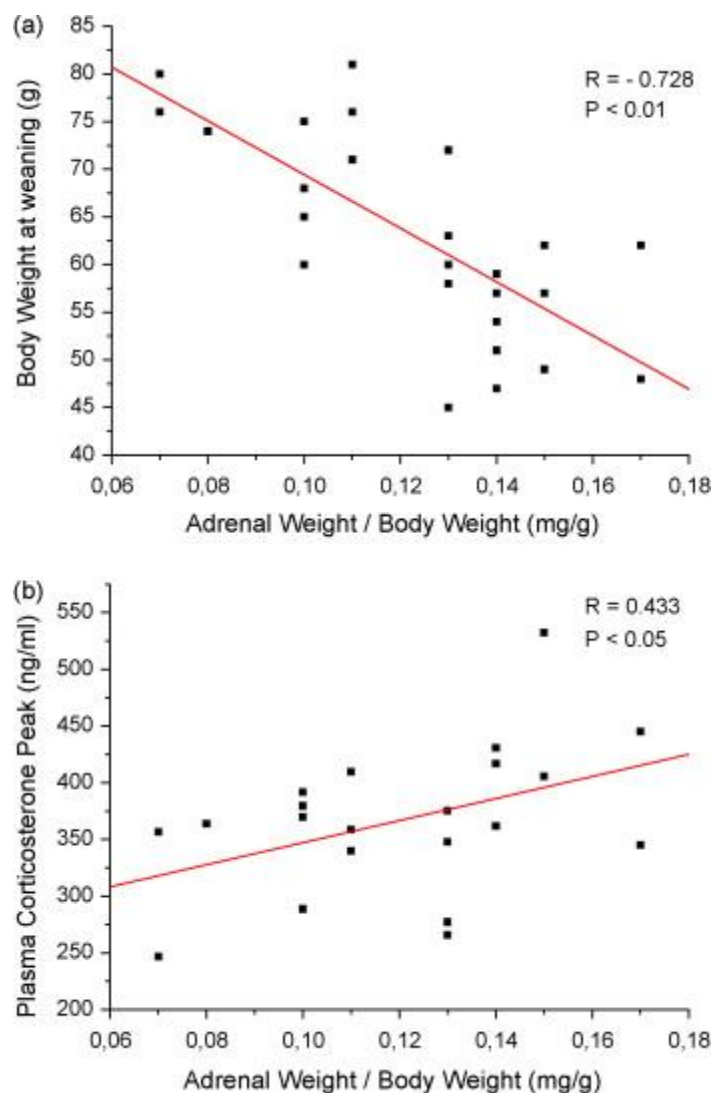
Figure 4. Correlations (Pearson's correlation coefficient) in prenatally stressed, non-implanted rats (*n*=27) between adrenal weight at sacrifice (ratio to the animal body weight, mg/g) and (a) body weight (g) at weaning; and (b) corticosterone peak during restraint test (*t*=60 min, ng/ml).

5.4 Discussion

The original data presented here clearly support a general trend that emerges from most of previous studies which focussed on enduring effects of prenatal stress. Normal physiological conditions at rest in adulthood do not exclude long-term implications of low-quality prenatal environment. Rather, it is often under stress conditions that functional consequences of an adverse prenatal environment become apparent in adult subjects (Peyronnet et al., 2002; Igosheva et al., 2004).

Plasma corticosterone levels in adult male rats born to dams repeatedly stressed in the last week of pregnancy were significantly higher 2 h after the onset of an acute stressor as compared to CTR counterparts. This evidence confirms previous results obtained by Maccari's group and points to a prolonged activation (or retarded inactivation) of brain circuitry responsible for stress-induced ACTH and corticosterone secretion in the offspring of stressed pregnant mothers (Barbazanges et al., 1996; Maccari et al., 1995; Maccari and Morley-Fletcher, 2007).

Conversely, repeated exposure of the mothers to restraint stress in late pregnancy did not induce significant alterations of heart rate and vagal input to the heart in the adult offspring, neither at rest nor in response to different acute challenges. This evidence is not in line with previous results obtained in rats by other research groups, especially as far as stress reactivity is concerned. For instance, protein malnutrition in pregnant rats was shown to produce in the adult offspring significantly larger increments in systolic and diastolic blood pressure following an olfactory stressor, as compared to control rats (Tonkiss et al., 1998). Similarly, prolonged prenatal hypoxia determined much larger increments of mean arterial pressure and its variability in the adult offspring during the exposure to a jet stream of high-pressure air (Peyronnet et al., 2002). When the prenatal stressor was represented by glucocorticoid (dexamethasone) exposure, the long-term cardiovascular effect was gender specific, with only females exhibiting hypertension and hyperactivity of the renin–angiotensin system at rest (O∂Regan et al., 2004). In the case of repeated restraint stress occurring in late pregnancy, an altered pattern of blood pressure and heart rate response to adult restraint stress was observed in the offspring, with higher and longer-lasting heart rate and systolic pressure elevations during the test and recovery phase; nevertheless, these effects were much more robust in the female offspring as compared to male counterparts (Igosheva et al., 2004). The discrepancy between these and more data from the literature and those obtained in our study is not too surprising, given that at least four factors might have contributed: (i) different types of prenatal challenge (protein malnutrition, glucocorticoid treatment, or intermittent restraint stress) and adult stressor used (olfactory stimulus, jet stream of air, restraint, open field, water immersion, or social defeat), (ii) different rat strains were used (Sprague–Dawley, Wistar, or Wild-type Groningen), (iii) different parameter were measured (systolic/diastolic and mean blood pressure, standard deviation of blood pressure, heart rate, or r-MSSD), and (iv) the recording means (telemetric or non-telemetric; femoral arterial/aortic externalized catheters or tail-cuff plethysmography). In addition, the effects documented by the above mentioned authors (specifically, those of O∂Regan and colleagues and Igosheva and colleagues) were quite gender specific, with females exhibiting a much clearer sensitivity as compared to males.

The effects of prenatal stress on adult biological rhythms have received much less attention in the past years, and the available studies mostly focussed on locomotor activity rhythms as a marker of the functional output of the biological clock. For instance, it was demonstrated that prenatal hypoxia impairs circadian synchronisation and response of the biological clock to light in adult rats (Joseph et al., 2002). Our study shows that circadian rhythms of heart rate, body temperature and locomotor activity of rats born to mothers undergoing repeated restraint stress during gestation were unchanged in baseline conditions. In contrast, these animals exhibited a clear reduction in the daily amplitude of the rhythms following the three adult-life stress episodes. This means that the association between prenatal manipulation and adult challenges caused changes in the circadian rhythmicity of these parameters that were larger than those produced by adult challenges alone.

The heart structure was not significantly affected by neither adult challenges alone nor adult challenges following prenatal stress (overall fibrosis <1% of the total left ventricular tissue). However, the average 30% larger score of myocardial fibrosis observed in the latter (non-statistically significant, though), allows to hypothesize that a stronger (or longer lasting) insult during foetal life might be able to sensitize to more robust structural damages at the heart level.

Prenatal stress *per se* did not induce significant changes of adrenal weight, but its association with stressors occurring in adulthood rendered the animals prone to increased adrenal enlargement. Of course, our data do not allow evaluating the differential role of hypertrophy and hyperplasia in determining increased adrenal volume. In animal studies, authors usually ascribe adrenal enlargement to hypertrophic processes (e.g. Llorente et al., 2002; Ward et al., 2000), though no systematic histological analyses of the adrenals have been provided so far. However, it is conceivable that glomerulosa cells are responsible for hyperplasia and a source for progenitor cells; thus, we tend to believe that short-term increase in adrenal volume to meet systemic need may be accomplished by cellular hypertrophy, whereas long-term conditions likely activate both hypertrophy and hyperplasia to maintain adrenal homeostasis.

Although prenatal stress *per se* did not seem to affect in a significant manner none of the parameters measured, we were interested in testing individual vulnerability to prenatal stress. For this purpose, we checked possible relationships between adrenal weight at sacrifice and (i) body weight at weaning and (ii) peak plasma corticosterone following acute restraint stress, in animals which underwent only prenatal stress without further challenges in adulthood. In other words, the questions we wanted to answer were (i) is proneness to higher HPA axis stress responsiveness an early marker of risk for adrenal enlargement in animals that suffered prenatal stress alone? (ii) similarly, is body weight at weaning a reliable marker of subsequent risk of adrenal enlargement in

rats that suffered prenatal stress only?ö We found a negative association between adrenal weight at sacrifice and body weight at 1 month of age, i.e. the lighter the animal at weaning the higher the susceptibility to adrenal hypertrophy and/or hyperplasia in adulthood. This result supports the general view on the usefulness of early body weight measurements as a marker of increased risk of adult structural change or disease. Moreover, in the same group of rats, there was a significant positive correlation between adrenal weight at sacrifice and the peak of corticosterone levels in response to restraint, i.e. the higher the adrenocortical stress responsivity, the larger the risk of adrenal enlargement in adulthood.

6. Conclusions

Adverse life events experienced by the pregnant mother and her reactions to them can produce alterations in the foetal environment, which in turn may have profound, long-term effects on the offspring physiology and behavior. The nature and magnitude of these effects depend on when the stressors take place during gestation, with clear windows of higher susceptibility. The available data from clinical and experimental studies suggest that some effects are highly species-specific and/or gender-dependent. The mechanisms by which prenatal environment influences the basal activity and stress reactivity of different neuronal and endocrine systems in adulthood are only partly understood. A large body of literature supports the mediating role of variations in the mother HPA axis function which have a number of rapid effects in the foetal brain, including modification of neurotransmitter systems and transcriptional machinery. In addition, it is quite clear that the amount of oxygen and the amount/type of nutrients that a foetus receives are major determinants of body size at term and health later in life.

Autonomic functions and cardiovascular physiology appear to be programmed by the intrauterine environment. Low birth weight and other indices of reduced foetal growth, induced by prenatal perturbations such as maternal undernutrition, excess glucocorticoids or placental insufficiency, are associated with an increased prevalence of cardiovascular and metabolic disease in adult life. Hypertension, cardiac autonomic imbalance and susceptibility to cardiac arrhythmias have been documented, both in baseline conditions and as a response to adult stressors. The use of appropriate experimental paradigms of psychological prenatal stress, like the repeated restraint test in rats, suggests that stressors of psychological nature that occur during pregnancy can affect in the long run the performance and adaptiveness of the offspring cardiovascular system.

The new data by our group reported here suggest that repeated psychological stress acting on mothers in the last third of pregnancy *per se* do not produce long-lasting changes in (i) cardiac

autonomic (re-)activity, (ii) baseline values of plasma corticosterone and heart rate, body temperature and physical activity rhythms, and (iii) adrenal weight and myocardial structure. However, they clearly indicate that repeated prenatal manipulations induce heightened sensitivity to acute stressors occurring in adulthood. In particular, when exposed to physical and emotional challenges in adult age, prenatally stressed male rats exhibited longer-lasting adrenocortical stress responsivity, larger and longer-lasting disturbances of circadian rhythmicity of heart rate, body temperature and physical activity, and increased adrenal weight, as compared to controls. Indeed, this appears to be the most recurrent evidence in the literature on prenatal stress, regardless the type of behavioral and/or physiological target examined: prenatal stress by itself does not appear to change dramatically a given structure or function, but it affects resilience and renders the animal more susceptible to further insults occurring in adulthood.

Acknowledgements

References

Abe H., Hidaka N., Kawagoe C., Odagiri K., Watanabe Y., Ikeda T., Ishizuka Y., Hashiguchi H., Takeda R., Nishimori T., Ishida Y. Prenatal psychological stress causes higher emotionality, depression-like behavior, and elevated activity in the hypothalamo-pituitary-adrenal axis, *Neurosci. Res.* 59 (2007), pp. 145ó151.

Alexander B.T. Placental insufficiency leads to development of hypertension in growth-restricted offspring, *Hypertension* 41 (2003), pp. 457ó462.

Andrews M.H., Matthews S.G. Antenatal glucocorticoids: is there cause for concern, *Fetal Matern. Med. Rev.* 14 (2003), pp. 329ó354-

Bae S., Xiao Y., Li G., Casiano C.A., Zhang L. Effect of maternal chronic hypoxic exposure during gestation on apoptosis in fetal rat heart, *Am. J. Physiol. Heart Circ. Physiol.* 285 (2003), pp. H983óH990.

Banjanin S., Kapoor A., Matthews S.G. Prenatal glucocorticoid exposure alters hypothalamicópituitaryó adrenal function and blood pressure in mature male guinea pigs, *J. Physiol.* 558 (2004), pp. 305ó318.

Barbazanges A., Piazza P.V., Le Moal M., Maccari S. Maternal glucocorticoid secretion mediates long-term effects of prenatal stress, *J. Neurosci.* 16 (1996), pp. 3943ó3949.

Barker D.J., Gluckman P.D., Godfrey K.M., Harding J.E., Owens J.A., Robinson J.S. Fetal nutrition and cardiovascular disease in adult life, *Lancet* 341 (1993), pp. 938ó941.

Barker D.J. Mothers, Babies, and Health in Later Life, Churchill Livingstone, Edinburgh (1998a).

Barker D.J. In utero programming of chronic disease, *Clin. Sci.* 95 (1998b), pp. 115ó128.

Barker D.J. The malnourished baby and infant, *Br. Med. Bull.* 60 (2001), pp. 69ó88.

Barker D.J. Fetal programming of coronary heart disease, *Trends Endocrinol. Metab.* 13 (2002), pp. 364ó 368.

Benediktsson R., Lindsay R.S., Noble J., Seckl J.R., Edwards C.R. Glucocorticoid exposure in utero: new model for adult hypertension, *Lancet* 341 (1993), pp. 339ó341.

Bhatnagar S., Lee T.M., Vining C. Prenatal stress differentially affects habituation of corticosterone responses to repeated stress in adult male and female rats, *Horm. Behav.* 47 (2005), pp. 430ó438

Brawley L., Itoh S., Torrens C., Barker A., Bertram C., Poston L., Hanson M. Dietary protein restriction in pregnancy induces hypertension and vascular defects in rat male offspring, *Pediatr. Res.* 54 (2003), pp. 83ó 90.

Brown A.S., Susser E.S., Butler P.D., Richardson Andrews R., Kaufmann C.A., Gorman J.M. Neurobiological plausibility of prenatal nutritional deprivation as a risk factor for schizophrenia, *J. Nerv. Ment. Dis.* 184 (1996), pp. 71ó85.

Buitelaar J.K., Huizink A.C., Mulder E.J., de Medina P.G., Visser G.H. Prenatal stress and cognitive development and temperament in infants, *Neurobiol. Aging* 24 (2003), pp. 53ó60.

Bunnemann B., Lippoldt A., Aguirre J.A., Cintra A., Metzger R. Glucocorticoid regulation of angiotensinogen gene expression in discrete areas of the male rat brain. An in situ hybridization study, *Neuroendocrinology* 57 (1993), pp. 856ó862

Cadet R., Pradier P., Dalle M., Delost P. Effects of prenatal maternal stress on the pituitary adrenocortical reactivity in guinea-pig pups, *J. Dev. Physiol.* 8 (1986), pp. 467ó475.

Capasso J.M., Palackal T., Olivetti G., Anversa P. Left ventricular failure induced by hypertension in rats, *Circ. Res.* 66 (1990), pp. 1400ó1412.

Cheema K., Dent M., Saini H., Aroutiounova N., Tappia P. Prenatal exposure to maternal undernutrition induces adult cardiac dysfunction, *Br. J. Nutr.* 93 (2005), pp. 471ó477

Clarke A.S., Wittwer D.J., Abbott D.H., Schneider M.L. Long-term effects of prenatal stress on HPA axis activity in juvenile rhesus monkeys, *Dev. Psychobiol.* 27 (1994), pp. 257ó269.

Clarke A.S., Schneider M.L. Effects of prenatal stress on behavior in adolescent rhesus monkeys, *Ann. N.Y. Acad. Sci.* 807 (1997), pp. 490ó491.

Coe C.L., Lubach G.R. Developmental consequences of antenatal dexamethasone treatment in nonhuman primates, *Neurosci. Biobehav. Rev.* 29 (2005), pp. 227ó235

Curhan G.C., Willett W.C., Rimm E.B., Spiegelman D., Ascherio A.L., Stampfer M.J. Birth weight and adult hypertension, diabetes mellitus, and obesity in US men, *Circulation* 94 (1996), pp. 3246ó3250.

Dampney R.A. Functional organization of central pathways regulating the cardiovascular system, *Physiol. Rev.* 74 (1994), pp. 323ó364.

Darnaudery M., Maccari S. Epigenetic programming of the stress response in male and female rats by prenatal restraint stress, *Brain Res. Rev.* 57 (2008), pp. 571ó585.

Davidson R.J. Affective style and affective disorders: perspectives from affective neuroscience, *Cogn. Emotion* 12 (1998), pp. 307ó330.

Davis E.P., Glynn L.M., Schetter C.D., Hobel C., Chicz-Demet A., Sandman C.A. Prenatal exposure to maternal depression and cortisol influences infant temperament, *J. Am. Acad. Child Adolesc. Psychiatry* 46 (2007), pp. 737ó746

De Boer S.F., Koopmans S.J., Slangen J.L., Van der Gugten J. Plasma catecholamine, corticosterone and glucose responses to repeated stress in rats: effect of interstressor interval length, *Physiol. Behav.* 47 (1990), pp. 1117ó1124.

Diaz R., Ogren S.O., Blum M., Fuxe K. Prenatal corticosterone increases spontaneous and d-amphetamine induced locomotor activity and brain dopamine metabolism in prepubertal male and female rats, *Neuroscience* 66 (1995), pp. 467ó473.

Dobbing J., Sands J. Growth and development of the brain and spinal cord of the guinea pig, *Brain Res.* 17 (1970), pp. 115ó123.

Dodic M., Peers A., Coghlan J.P., M. Wintour. Can excess glucocorticoid, predispose to cardiovascular and metabolic diseases in middle age? *Trends Endocrinol. Metab.* 10 (1999), pp. 86ó91.

Dodic M., Hantzis V., Duncan J., Rees S., Koukoulas I., Johnson K., Wintour E.M., Moritz K. Programming effects of short prenatal exposure to cortisol, *FASEB J.* 16 (2002), pp. 1017ó1026.

Doyle L.W., Morley C.J., Halliday J. Prediction of survival for preterm births. Data on the quality of survival are needed, *BMJ* 320 (2000), p. 648.

Drake A.J., Walker B.R. The intergenerational effects of fetal programming: non-genomic mechanisms for the inheritance of low birth weight and cardiovascular risk, *J. Endocrinol.* 180 (2004), pp. 1ó16.

Field T., Diego M., Hernandez-Reif M., Schanberg S., Kuhn C., Yando R., Bendell D. Pregnancy anxiety and comorbid depression and anger: effects on the fetus and neonate, *Depress. Anxiety* 17 (2003), pp. 140ó151

Forsen T., Osmond C., Eriksson J.G., Barker D.J. Growth of girls who later develop coronary heart disease, *Heart* 90 (2004), pp. 20ó24.

Fujioka T., Sakata Y., Yamaguchi K., Shibasaki T., Kato H., S. Nakamura. The effects of prenatal stress on the development of hypothalamic paraventricular neurons in fetal rats, *Neuroscience* 92 (1999), pp. 1079ó1088.

Godfrey K.M., Barker D.J. Fetal nutrition and adult disease, *Am. J. Clin. Nutr.* 71 (2000), pp. 1344ó1352.

Griffin W.C., Skinner H.D., Birkle D.L. Prenatal stress influences 8-OH-DPAT modulated startle responding and [3H]-8-OH-DPAT binding in rats, *Pharmacol. Biochem. Behav.* 81 (2005), pp. 601ó607.

Hall J.E., Guyton A.C., Mizelle H.L. Role of the reninóangiotensin system in control of sodium excretion and arterial pressure, *Acta Physiol. Scand.* 591 (1990), pp. 48ó62.

Hayashi A., Nagaoka M., Yamada K., Ichitani Y., Miake Y., Okado N. Maternal stress induces synaptic loss and developmental disabilities of offspring, *Int. J. Dev. Neurosci.* 16 (1998), pp. 209ó216.

Henry C., Kabbaj M., Simon H. Prenatal stress increases the hypothalamic-pituitary-adrenal axis response in young and adult rats, *J. Neuroendocrinol.* 6 (1994), pp. 341ó345.

Hu X.W., Levy A., Hart E.J., Nolan L.A., Dalton G.R., Levi A.J. Intra-uterine growth retardation results in increased cardiac arrhythmias and raised diastolic blood pressure in adult rats, *Cardiovasc. Res.* 48 (2000), pp. 233ó243.

Igosheva N., Klimova O., Anishchenko T., Glover V. Prenatal stress alters cardiovascular responses in adult rats, *J. Physiol.* 557 (2004), pp. 273ó285.

Igosheva N., Taylor P.D., Poston L., Glover V. Prenatal stress in the rat results in increased blood pressure responsiveness to stress and enhanced arterial reactivity to neuropeptide Y in adulthood, *J. Physiol.* 582 (2007), pp. 665ó674.

Jones A., Beda A., Ward A.M., Osmond C., Phillips D.I., Moore V.M., Simpson D.M. Size at birth and autonomic function during psychological stress, *Hypertension* 49 (2007), pp. 548ó555.

Joseph V., Mamet J., Lee F., Dalmaz Y., Van Reeth O. Prenatal hypoxia impairs circadian synchronisation and response of the biological clock to light in adult rats, *J. Physiol.* 543 (2002), pp. 387ó395.

Kaiser S., Sachser N. The social environment during pregnancy and lactation affects the female offspringsø endocrine status and behaviour in guinea pigs, *Physiol. Behav.* 63 (1998), pp. 361ó366.

Kapoor A., Matthews S.G. Short periods of prenatal stress affect growth, behaviour and hypothalamo-pituitary-adrenal axis activity in male guinea pig offspring, *J. Physiol.* 566 (2005), pp. 967ó977.

Khot U.N., Khot M.B., Bajzer C.T., Sapp S.K., Ohman E.M., Brener S.J., Ellis S.G., Lincoff A.M., Topol E.J. Prevalence of conventional risk factors in patients with coronary heart disease, *JAMA* 290 (2003), pp. 898ó904.

Kliewer S.A., Moore J.T., Wade L., Staudinger J.L., Watson M.A., Jones S.A., McKee D.D., Oliver B.B., Willson T.M., Zetterström R.H., Perlmann T., Lehmann J.M. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway, *Cell* 92 (1998), pp. 73ó82.

Koenig J.I., Elmer G.I., Shepard P.D., Lee P.R., Mayo C., Joy B., Hercher E., Brady D.L. Prenatal exposure to a repeated variable stress paradigm elicits behavioral and neuroendocrinological changes in the adult offspring: potential relevance to schizophrenia, *Behav. Brain Res.* 156 (2005), pp. 251ó261.

Kofman O. The role of prenatal stress in the etiology of development behavioural disorders, *Neurosci. Biobehav. Rev.* 26 (2002), pp. 457ó470.

Langley-Evans S.C., Jackson A.A. Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets, *Comp. Biochem. Physiol. A. Physiol.* 110 (1995), pp. 223ó228.

Langley-Evans S.C. Fetal programming of cardiovascular function through exposure to maternal undernutrition, *Proc. Nutr. Soc.* 60 (2001), pp. 505ó513.

Leeson C.P., Kattenhorn M., Morley R., Lucas A., Deanfield J.E. Impact of low birth weight and cardiovascular risk factors on endothelial function in early adult life, *Circulation* 103 (2001), pp. 1264ó1268.

Li G., Xiao Y., Estrella J.L., Ducsay C.A., Gilbert R.D., Zhang L. Effects of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rats, *J. Soc. Gynecol. Invest.* 10 (2003), pp. 265ó274.

Li G., Bae S., Zhang L. Effect of prenatal hypoxia on heat stress-mediated cardioprotection in adult rat heart, *Am. J. Physiol. Heart Circ. Physiol.* 286 (2004), pp. H1712ó H1719.

Lingas R.I., Matthews S.G. A short period of maternal nutrient restriction in late gestation modifies pituitary-adrenal function in adult guinea pig offspring, *Neuroendocrinology* 73 (2001), pp. 302ó311.

Llorente E., Brito M.L., Machado P., González M.C. Effect of prenatal stress on the hormonal response to acute and chronic stress and on immune parameters in the offspring, *J. Physiol. Biochem.* 58 (2002), pp. 143ó149.

Louey S., Thornburg K.L. The prenatal environment and later cardiovascular disease, *Early Hum. Dev.* 81 (2005), pp. 745ó751

Lucas A., Morley R. Does early nutrition in infancy born before term programme later blood pressure?, *BMJ* 309 (1994), pp. 304ó308.

Maccari S., Piazza P.V., Kabbaj M., Barbazanges A., Simon H., Le Moal M. Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress, *J. Neurosci.* 15 (1995), pp. 110ó116.

Maccari S., Darnaudery M., Morley-Fletcher S., Zuena A.R., Cinque C., Van Reeth O. Prenatal stress and long-term consequences: implications of glucocorticoid hormones, *Neurosci. Biobehav. Rev.* 27 (2003), pp. 119ó127.

Maccari S., Morley-Fletcher S. Effects of prenatal restraint stress on the hypothalamus-pituitary-adrenal axis and related behavioural and neurobiological alterations, *Psychoneuroendocrinology* 32 (2007), pp. 10ó15.

Mairesse J., Lesage J., Breton C., Bréant B., Hahn T., Darnaudéry M., Dickson S.L., Seckl J., Blondeau B., Vieau D., Maccari S., Viltart O. Maternal stress alters endocrine function of the feto-placental unit in rats, *Am. J. Physiol. Endocrinol. Metab.* 292 (2007), pp. 1526ó1533.

Manning J., Vehaskari V.M. Low birth weight-associated adult hypertension in the rat, *Pediatr. Nephrol.* 16 (2001), pp. 417ó422.

Martin H., Gazelius B., Norman M. Impaired acetylcholine-induced vascular relaxation in low birth weight infants: implications for adult hypertension?, *Pediatr. Res.* 47 (2000a), pp. 457ó462.

Martin H., Hu J., Gennser G., Norman M. Impaired endothelial function and increased carotid stiffness in 9-year-old children with low birth weight, *Circulation* 102 (2000b), pp. 2739ó2744.

Matthews S.G., Owen D., Banjanin S., Andrews M.H. Glucocorticoids, hypothalamo-pituitary adrenal (HPA) development, and life after birth, *Endocr. Res.* 28 (2002), pp. 709ó718.

Matthews S.G., Owen D., Kalabis G., Banjanin S., Setiawan E.B., Dunn E.A., Andrews M.H. Fetal glucocorticoid exposure and hypothalamo-pituitary-adrenal (HPA) function after birth, *Endocr. Res.* 30 (2004), pp. 827ó836

McEwen B.S. Protective and damaging effects of stress mediators, *N. Engl. J. Med.* 338 (1998), pp. 171ó179.

McMullen S., Gardner D.S., Langley-Evans S.E. Prenatal programming of angiotensin II type 2 receptor expression in the rat, *Br. J. Nutr.* 91 (2003), pp. 133ó140.

Meerlo P., Sgoifo A., de Boer S.F., Koolhaas J.M. Long-lasting consequences of a social conflict in rats: behavior during the interaction predicts subsequent changes in daily rhythms of heart rate, temperature, and activity, *Behav. Neurosci.* 113 (1999), pp. 1283ó1290.

Miller A.H., Spencer R.L., Pulera M., Kang S., McEwen B.S., Stein M. Adrenal steroid receptor activation in rat brain and pituitary following dexamethasone: implications for the dexamethasone suppression test, *Biol. Psychiatry* 32 (1992), pp. 850ó869.

Moritz K.M., Johnson K., Douglas-Denton R., Wintour E.M., Dodic M. Maternal glucocorticoid treatment programs alterations in the reninóangiotensin system of the ovine fetal kidney, *Endocrinology* 143 (2002), pp. 4455ó4463.

Morley-Fletcher S., Darnaudéry M., Mocaer E., Froger N., Lanfumey L., Laviola G., Casolini P., Zuena A.R., Marzano L., Hamon M., Maccari S. Chronic treatment with imipramine reverses immobility behaviour, hippocampal corticosteroid receptors and cortical 5-HT(1A) receptor mRNA in prenatally stressed rats, *Neuropharmacology* 47 (2004), pp. 841ó847

Newell-Morris L., Fahrenbruch C. Practical and evolutionary considerations for use of the nonhuman primate model in prenatal research. In: E.S. Watts, Editor, *Nonhuman Primate Models for Human Growth and Development*, Alan R. Liss, New York (1985), pp. 9ó40.

Newnham J.P., Moss T.J. Antenatal glucocorticoids and growth: single versus multiple doses in animal and human studies, *Semin. Neonatol.* 6 (2001), pp. 285ó292.

Nyirenda M.J., Seckl J.R. Intrauterine events and the programming of adulthood disease: the role of fetal glucocorticoid exposure, *Int. J. Mol. Med.* 2 (1998), pp. 607ó614.

OøRegan D., Kenyon C.J., Seckl J.R., Holmes M.C. Glucocorticoid exposure in late gestation in the rat permanently programs gender-specific differences in adult cardiovascular and metabolic physiology, *Am. J. Physiol. Endocrinol. Metab.* 287 (2004), pp. E863óE870.

Ortiz L.A., Quan A., Zarzar F., Weinberg A., Baum M. Prenatal dexamethasone programs hypertension and renal injury in the rat, *Hypertension* 41 (2003), pp. 328ó334.

Payne J.A., Alexander B.T., Khalil R.A. Reduced endothelial vascular relaxation in growth-restricted offspring of pregnant rats with reduced uterine perfusion, *Hypertension* 42 (2003), pp. 768ó774.

Peers A., Campbell D.J., Wintour E.M., Dodic M. The peripheral reninóangiotensin system is not involved in the hypertension of sheep exposed to prenatal dexamethasone, *Clin. Exp. Pharmacol. Physiol.* 28 (2001), pp. 306ó311.

Peters D.A. Maternal stress increases fetal brain and neonatal cerebral cortex 5-hydroxytryptamine synthesis in rats: a possible mechanism by which stress influences brain development, *Pharmacol. Biochem. Behav.* 35 (1990), pp. 943ó947.

Petraglia F., Florio P., Nappi C., Genazzani A.R. Peptide signaling in human placenta and membranes: autocrine, paracrine, and endocrine mechanisms, *Endocr. Rev.* 17 (1996), pp. 156ó186.

Peyronnet J., Dalmaz Y., Ehrstrom M., Mamet J., Roux J.C., Pequignot J.M., Thoren H.P., Lagercrantz H. Long-lasting adverse effects of prenatal hypoxia on developing autonomic nervous system and cardiovascular parameters in rats, *Pflugers Arch.* 443 (2002), pp. 858ó865.

Phillips D.I. Programming of the stress response: a fundamental mechanism underlying the long-term effects of the fetal environment?, *J. Intern. Med.* 261 (2007), pp. 453ó460.

Ressler K.J., Nemeroff C.B. Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders, *Depress. Anxiety* 12 (2000), pp. 2ó19

Rice D., Barone Jr S. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models, *Environ. Health Perspect.* 108 (2000), pp. 511ó533.

Sahajpal V., Ashton N. Renal function and angiotensin AT1 receptor expression in young rats following intrauterine exposure to maternal low-protein diet, *Clin. Sci.* 104 (2003), pp. 607ó614.

Sato A., Suzuki H., Nakazato Y., Shibata H., Inagami T., Saruta T. Increased expression of vascular angiotensin II type 1A receptor gene in glucocorticoid-induced hypertension, *J. Hypertens.* 12 (1994), pp. 511ó516.

Schneider M.L., Roughton E.C., Koehler A.J., Lubach G.R. Growth and development following prenatal stress exposure in primates: an examination of ontogenetic vulnerability, *Child. Dev.* 70 (1999), pp. 263ó274.

Schneider M.L., Moore C.F., Kraemer G.W., Roberts A.D., De Jesus O.T. The impact of prenatal stress, fetal alcohol exposure, or both on development: perspectives from a primate model, *Psychoneuroendocrinology* 27 (2002), pp. 285ó298.

Schreuder M.F., van Wijk J.A., Delemarre-van de Waal H.A. Intrauterine growth restriction increases blood pressure and central pulse pressure measured with telemetry in aging rats, *J. Hypertens.* 24 (2006a), pp. 1337ó1343.

Schreuder M.F., Fodor M., van Wijk J.A., Delemarre-van de Waal H.A. Association of birth weight with cardiovascular parameters in adult rats during baseline and stressed conditions, *Pediatr. Res.* 59 (2006b), pp. 126ó130.

Seckl J.R. Physiologic programming of the fetus, *Clin. Perinatol.* 25 (1998), pp. 939ó962.

Sgoifo A., Stilli D., Medici D., Gallo P., Aimi B., Musso E. Electrode positioning for reliable telemetry ECG recordings during social stress in unrestrained rats, *Physiol. Behav.* 60 (1996), pp. 1397ᴓ1401

Sgoifo A., de Boer S.F., Buwalda B., Korte-Bouws G., Tuma J., Bohus B., Zaagsma J., Koolhaas J.M. Vulnerability to arrhythmias during social stress in rats with different sympathovagal balance, *Am. J. Physiol. (Heart Circ. Physiol.)* 275 (1998), pp. 460ᴓ466.

Sgoifo A., Pozzato C., Meerlo P., Costoli T., Manghi M., Stilli D., Olivetti G., Musso E. Intermittent exposure to social defeat and open-field test in rats: acute and long-term effects on ECG, body temperature and physical activity, *Stress* 5 (2002), pp. 23ᴓ35.

Sherman R.C., Langley-Evans S.C. Early administration of angiotensin-converting enzyme inhibitor captopril, prevents the development of hypertension programmed by intrauterine exposure to a maternal low-protein diet in the rat, *Clin. Sci. (Lond)* 94 (1998), pp. 373ᴓ381.

Shoener J.A., Baig R., Page K.C. Prenatal exposure to dexamethasone alters hippocampal drive on hypothalamic-pituitary-adrenal axis activity in adult male rats, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290 (2006), pp. 1366ᴓ1373.

Sternberg W.F., Ridgway C.G. Effects of gestational stress and neonatal handling on pain, analgesia, and stress behavior of adult mice, *Physiol. Behav.* 78 (2003), pp. 375ᴓ383.

Suchecki D., Palermo-Neto J. Prenatal stress and emotional response of adult offspring, *Physiol. Behav.* 49 (1991), pp. 423ᴓ426.

Sugden M.C., Langdown M.L., Munns M.J., Holness M.J. Maternal glucocorticoid treatment modulates placental leptin and leptin receptor expression and materno-fetal leptin physiology during late pregnancy, and elicits hypertension associated with hyperleptinaemia in the early-growth-retarded adult offspring, *Eur. J. Endocrinol.* 145 (2001), pp. 529ᴓ539.

Sug-Tang, Bocking A.D., Brooks A.N., Hooper S., White S.E., Jacobs R.A., Fraher L.J., Challis J.R. Effects of restricting uteroplacental blood flow on concentrations of corticotrophin-releasing hormone, adrenocorticotrophin, cortisol, and prostaglandin E2 in the sheep fetus during late pregnancy, *Can. J. Physiol. Pharmacol.* 70 (1992), pp. 1396ᴓ1402.

Symonds M.E., Stephenson T., Gardner D.S., Budge H. Long-term effects of nutritional programming of the embryo and fetus: mechanisms and critical windows, *Reprod. Fertil. Dev.* 19 (2007), pp. 53ᴓ63.

Takahashi L.K., Turner J.G., Kalin N.H. Prenatal stress alters brain catecholaminergic activity and potentiates stress-induced behavior in adult rats, *Brain Res.* 574 (1992), pp. 131ᴓ137.

Talge N.M., Neal C., Glover V. Antenatal maternal stress and long-term effects on child neurodevelopment: how and why?, *J. Child. Psychol. Psychiatry* 48 (2007), pp. 245‒261.

Tonkiss J., Trzcinska M., Galler J.R., Ruiz-Opazo N., Herrera V.L. Prenatal malnutrition-induced changes in blood pressure: dissociation of stress and nonstress responses using radiotelemetry, *Hypertension* 32 (1998), pp. 108‒114

Uno H., Lohmiller L., Thieme C., Kemnitz J.W., Engle M.J., Roecker E.B., Farrell P.M. Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques. I. Hippocampus, *Brain Res. Dev.* 53 (1990), pp. 157‒167.

Vallée M., Mayo W., Dellu F., Le Moal M., Simon H., Maccari S. Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: correlation with stress-induced corticosterone secretion, *J. Neurosci.* 17 (1997), pp. 2626‒2636.

Viltart O., Mairesse J., Darnaudéry M., Louvart H., Vanbesien-Mailliot C., Catalani A., Maccari S. Prenatal stress alters Fos protein expression in hippocampus and locus coeruleus stress-related brain structures, *Psychoneuroendocrinology* 31 (2006), pp. 769‒780.

von Lutterotti N., Camargo M.J., Mueller F.B., Timmermans P.B., Laragh J.H. Angiotensin II receptor antagonist markedly reduces mortality in salt-loaded Dahl S rats, *Am. J. Hypertens.* 4 (1991), pp. 346‒349.

Wadhwa P.D., Sandman C.A., Garite T.J. The neurobiology of stress in human pregnancy: implications for prematurity and development of the fetal central nervous system, *Prog. Brain Res.* 133 (2001), pp. 131‒142.

Ward A.M., Moore V., Steptoe A., Cockington R., Robinson J.S., Phillips D.I. Size at birth and cardiovascular responses to psychological stressors: evidence for fetal programming in women, *J. Hypertens.* 22 (2004), pp. 2295‒2301.

Ward H.E., Johnson E.A., Salm A.K., Birkle D.L. Effects of prenatal stress on defensive withdrawal behavior and corticotropin releasing factor systems in rat brain, *Physiol. Behav.* 70 (2000), pp. 359‒366.

Ward I.L., Weisz J. Differential effects of maternal stress on circulating levels of corticosterone, progesterone and testosterone in male and female rat fetus and their mothers, *Endocrinology* 84 (1984), pp. 1135‒1145.

Ward I.L., Stehm K.E. Prenatal stress feminizes juvenile play patterns in male rats, *Physiol. Behav.* 50 (1991), pp. 601‒605.

Weinstock M., Matlina E., Maor G.I., Rosen H., McEwen B.S. Prenatal stress selectively alters the reactivity of the hypothalamic-pituitary-adrenal system in the female rats, *Brain Res.* 595 (1992), pp. 195‒200.

Weinstock M. Does prenatal stress impair coping and regulation of hypothalamic-pituitary-adrenal axis?, *Neurosci. Biobehav. Rev.* 21 (1997), pp. 1ɓ10.

Weinstock M., Poltyrev T., Schorer-Apelbaum D., Men D., McCarty R. Effect of prenatal stress on plasma corticosterone and catecholamines in response to footshock in rats, *Physiol. Behav.* 64 (1998), pp. 439ɓ444.

Weinstock M. Alterations induced by gestational stress in brain morphology and behavior of the offspring, *Prog. Neurobiol.* 65 (2001), pp. 427ɓ451.

Weinstock M. The potential influence of maternal stress hormones on development and mental health of the offspring, *Brain Behav. Immun.* 19 (2005), pp. 296ɓ308.

Welberg L.A., Seckl J.R. Prenatal stress, glucocorticoids and the programming of the brain, *J. Neuroendocrinol.* 13 (2001), pp. 113ɓ128.

Welberg L.A., Seckl J.R., Holmes M.C. Prenatal glucocorticoid programming of brain corticosteroid receptors and corticotropin-releasing-hormone: possible implications for behaviour, *Neuroscience* 104 (2001), pp. 71ɓ79.

White D.A., Birkle D.L. The differential effects of prenatal stress in rats on the acoustic startle reflex under baseline conditions and in response to anxiogenic drugs, *Psychopharmacology* 154 (2001), pp. 169ɓ176.

Wilcoxon J.S., Redei E.E. Maternal glucocorticoid deficit affects hypothalamic-pituitary-adrenal function and behavior of rat offspring, *Horm. Behav.* 51 (2007), pp. 321ɓ327.

Wilson P.W., DøAgostino R.B., Levy D., Belanger A.M., Silbershatz H., Kannel W.B. Prediction of coronary heart disease using risk factor categories, *Circulation* 12 (1998), pp. 1837ɓ1847.

Xu Y., Williams S., OøBrien D., Davidge S. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring, *FASEB J.* 8 (2006), pp. 1251ɓ1253

Young J.B. Programming of sympathoadrenal function, *Trends Endocrinol. Metab.* 13 (2002), pp. 381ɓ385.

Zhang L. Prenatal hypoxia and cardiac programming, *J. Soc. Gynecol. Investig.* 12 (2005), pp. 2ɓ13.

Zimmerberg B., Blaskey L.G. Prenatal stress effects are partially ameliorated by prenatal administration of the neurosteroid allopregnanolone, *Pharmacol. Biochem. Behav.* 59 (1998), pp. 819ɓ827.

CHAPTER 3

Social defeat and isolation: depression and cardiovascular function in rats

Francesca Mastorci[a], Gallia Graiani[b], Maria Ida Razzoli[c], Roberto Arban[c],
Federico Quaini[b], Andrea Sgoifo[a]

[a]Stress Physiology Lab., Dept. of Evolutionary and Functional Biology, University of Parma, Italy
[b]Dept. of Internal Medicine, University of Parma, Italy
[c]Neurosciences CEDD, GlaxoSmithKline Medicine Research Centre, Verona, Italy.

Chapter 3

1. Introduction

2. Methods

2.1 Animals and housing

2.2 Radiotelemetry system

2.3 Surgery: transmitter implantation

2.4 Experimental protocol

2.5 Specific experimental procedures

2.5.1 Social Defeat

2.5.2 Elevated plus-maze test

2.5.3 Open field test

2.5.4 Dexamethasone Suppression Test

2.5.5 Sucrose preference test

2.6 Corticosterone assay

2.7 Electrocardiographic data acquisition and analysis

2.8 Around-the-clock-heart rate, body temperature, and physical activity sampling and processing

2.9 Post-mortem measurements

2.10 Statistical Analysis

3. Results

3.1 Body Weight

3.2 Adrenocortical response to dexamethasone suppression test and adrenal weight

3.3 Sucrose Preference Test

3.4 Long-term chronobiological effects of social defeat and isolation

3.5 Physiological response to Open Field test

3.6 Behavioral data

3.6.1 Open field test

3.6.2 Elevated plus maze test

3.7 Morphometrical analysis of the myocardium

4. Discussion

References

ABSTRACT

Stressful life events increase the risk of psychopathologies such as depression. This psychological disorder is characterized by physiological, neuroendocrine, and behavioral alterations and is considered an indipendent risk factor in the onset and progression of cardiovascular disease. In this study we implemented an animal model of depression based on the exposure to an adverse stress episode (social defeat) followed by prolonged social isolation and explored its effects on: (i) acute adrenocortical and cardiac sympathovagal stress reactivity (ii) sucrose intake (iii) circadian rhythmicity of heart rate, body temperature, and physical activity, and (iv) myocardial and adrenal structure. Adult male wild-type rats were implanted with telemetry transmitters for ECG, temperature (T) and activity (Act) recordings. Treated animals (TREAT, n=22) were exposed to a social defeat episode followed by 4-week isolation, while controls (CTR, n=22) remained undisturbed with their female partners. During the isolation period, cardiac sympathovagal balance (heart rate variability measurements) at rest, hypothalamic-pituitary-adrenal (HPA) axis reactivity to a dexamethasone suppression test (determination of plasma corticosterone levels), and anhedonia (% sucrose solution consumption) were assessed. Before and after defeat, heart rate (HR), T, and Act were sampled around-the-clock to assess their circadian rhythmicity. At sacrifice, adrenal glands were weighed and the hearts removed for morphometrical analysis. A significant effect of defeat+isolation was observed for HPA axis reactivity to the dexamethasone challenge, i.e., stressed rats had higher corticosterone concentrations as compared to controls.. In addition, treated rats exhibited a significant reduction of sucrose solution consumption in the third week of isolation. Moreover, the amplitude of HR circadian rhythm was significantly smaller in TREAT compared to CTR rats, in the 6 days following social defeat, while activity rhythm amplitude was significantly reduced all throughout the isolation phase. An habituation-like effect for heart rate (RR) and vagal activity (r-MSSD) response to repeated open-field test was observed in control rats, which was not observed in treated counterparts. At sacrifice, TREAT rats had a significantly larger adrenal gland weight as compared to CTRs. The two groups exhibited a similar, modest amount of cardiac structural damage, as expressed by the volume fraction of myocardial fibrosis in the left ventricular wall. Social defeat and a prolonged period of isolation produced some structural, physiological, and behavioral effects in rats, which resemble those observed in depressed and chronically stressed subjects. These effects mainly consisted in HPA axis negative feedback dysfunction, adrenal gland enlargement, biological rhythm alterations, and the establishment of an anhedonic condition.

1. Introduction

Depressive disorders are the second leading cause of disability worldwide, after ischemic heart disease, with the lifetime incidence of depression estimated at nearly 12% in men and 20% in women, respectively (Mathers and Loncar, 2006; Murray and Lopez, 1997).

However, in spite of intensive research during the past decades, critical risk factors involved in the onset or development of depression have not been defined.

Depression is characterized by significantly depressed mood and the reduced responsiveness to pleasurable stimuli (anhedonia) coupled with behavioural and cognitive changes such as sleep alterations, weight gain or weight loss, and difficulty concentrating and making decisions (American Psychiatry Association, 1994). This psychological disorder is multifaceted, and it significantly affects an individual's mental and physical health. Several studies, including both cross-sectional and prospective analyses, have demonstrated an extensive co-morbidity of depression and cardiovascular disease (Barefoot et al., 1996; Frasure-Smith et al., 1993). Depression is a recognized risk factor for cardiovascular disease; this risk is shown to be independent of traditional cardiovascular risk factors such as hypertension, high cholesterol and increased body mass index (Barefoot and Schroll, 1996; Penninx et al., 2001). Previous research has demonstrated that depression may predispose an individual to atherosclerosis, arrhythmias, myocardial infarction, heart failure and sudden death. This association has been identified in current cardiac patients as well as individuals with no history of heart disease. Although a large number of studies have indicated the importance of the association between mood disorders and heart disease, the precise pathophysiological mechanisms underlying this association remain unclear.

In addition, numerous investigations have found a correlation between the occurrence of stressful life events and the subsequent onset of an episode of major depression (Kendler et al., 1999). Indeed, individuals that frequently experience severe stressful life events, for instance loss, humiliation or defeat, are more likely to develop major depression relative to individuals who do not experience such major stressful events (Heim and Nemeroff, 2001). Generally, stressful life events have been shown to influence the pathogenesis of depressive disorders. However, less certain is the nature of the relationship between depression and stressful life events. In particular, it remains unclear to what extent stressful life events cause subsequent beginning of depression and to what extent the occurrence of stressful life events and onset of depression are correlated for other reasons. Environmental stress can lead to altered neurochemical function, such as changes in the utilization and synthesis of norepinephrine, changes in dopamine activity and enhanced synthesis of serotonin (Ressler and Nemeroff, 2000). Stressors such as marital conflicts, health problems and work overload have been shown to be associated with both unipolar and bipolar depression (Arean

and Reynolds, 2005). Several behavioral changes, including those in escape performance, appetitive responses and exploration, have been observed in rats following exposure to stressors (Anisman and Zacharko, 1992). Furthermore, it has been suggested that chronic stressors, which do not favour the development of adaptation, are likely to be associated with depressive symptoms (Anisman and Zacharko, 1990).

Animal models for psychopathology have become an invaluable instrument in the analysis of the causes, genetic, physiological, psychological, environmental or pharmacological, that can bring about symptoms homologous to those of patients with a specific disorder, as depression (Shekhar et al., 2001). Research with human subjects is useful for answering certain experimental questions; however, animal methods may allow for the direct study of the pathogenesis, biological mechanisms, and treatments for depression, and they are valuable tools for studying the interaction between psychological and physiological aspects. Animal models of depression are typically based on exposure of animals to a stressful condition (a potential or actual threatening situation) and a specific test for measuring behavioural and physiological symptoms. For example, footshook, restraint, forced swim, as well as typical animal model of depression such as *Learned Helplessness* and *Chronic Mild Stress* are commonly used to generate stress in the laboratory but do not reliably resemble challenges animals are normally faced within their natural environment and may elicit behavioural and physiological responses different from those resulting from social or psychological stressors (Herman and Cullinan, 1997; Koolhaas et al., 1997). Although effective and useful, these stressors are rather physical, potentially painful and they offer little face validity compared to social and psychological stressors that are of particular interest in humans. These conventional animal models of stress appear to be quite far from real life events, either for the experimental animal model or the human counterpart. Currently, a greater amount of attention has been focused upon developing animal models of depression that utilize more naturalistic experimental paradigms to model stress that is ethologically relevant to the model organism. Consequently, several animal models of social stress have been developed in order to investigate questions related to the etiology, treatment, and prevention of stress-related disorders. The most frequently used models for rodents is the acute or chronic social defeat that induces depressive-like behavior in male animals (Fuchs and Flugge, 2002; Rygula et al., 2005). Social defeat in rats is a natural stressor that is known to induce a classical stress response, with an acute and strong cardiovascular and neuroendocrine activation, hypertermia, and behavioral reaction (Miczek et al., 1990; Tornatzky and Miczek, 1994). Most of these responses diminish within a few hours after termination of social interaction. However, a number of reports have indicated much longer lasting effects on various physiological and behavioral parameters (Koolhaas et al., 1990; Meerlo et al., 1996a). Behavioral changes, like a decreased social interaction (Meerlo et al., 1996a), and anhedonia (von Frijtag et al., 2002), next to

physiological (Meerlo et al., 1996a), neuroendocrine (Blanchard et al., 1993), and neurobiological (Buwalda et al., 1999) consequences of social stress are interpreted as signs mimicking certain aspects of human depression.

The aim of this study was apply a biologically relevant model of social challenge (social defeat followed by isolation) and verify whether it produced alterations in acute adrenocortical and cardiac sympathovagal stress reactivity, in sucrose intake, in circadian rhythmicity of heart rate, body temperature, and physical activity, and myocardial and adrenal structure which may reflect depressive symptoms.

2. Methods

All experimental procedures in this study were 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).

2.1 Animals and housing

Fourty-four wild-type groningen male and fourty-four Wild-Type Groningen female rats (Rattus norvegicus, WTG strain) were used, originally derived from the University of Groningen (The Nederlands) and bred in our department under conventionally clean conditions. Animals were housed in unisexual groups of 4 individuals, from weaning until the onset of experiments (3 months of age), in clear Plexiglas cage measuring 60x40x20 cm.

At the age of 15 weeks males were transfered to the experimental room, and coupled with sterilized females in cages measuring 40x30x15 cm. Twenty additional Wistar males were used as intruders in the õresident-intruder testö (see section õSocial Defeatö for details). Before and during the experimental treatment, all the animals were constantly kept in rooms with controlled temperature (22±2°C) and lighting (light on 17:00 to 05:00 hr). The bedding of the cages consisted of wood shavings, and food and water were freely available.

2.2 Radiotelemetry system

The radiotelemetry system employed in this study enabled the recording of electrocardiogram (ECG), body temperature (T), and physical activity (Act) from freely-moving and freely-behaving animals. Radiotelemetry systems for measuring physiological parameters represent a basic

refinement in the study of cardiovascular changes during stress exposure. It consisted of flat transmitters measuring 25x15x8 mm (TA11CTA-F40, Data Sciences Int., St. Paul, MN, USA) and platform receivers measuring 32x22x3 mm (RPC-1; Data Sciences International). Telemetry signals were fed to a PC containing õDataquest Art 2.2ö data acquisition system (Data Sciences Int., St. Paul, MN, USA). ECG, T, and Act data were acquired following the procedures described below in the section õElectrocardiographic data acquisition and analysisö.

2.3 Surgery: transmitter implantation

The transmitter was chronically implanted in 44 animals 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 in the abdominal cavity and the two electrodes (wire loops) were fixed respectively to the dorsal surface of the xiphoid process and in the anterior mediastinum close to the right atrium. The rats were anesthetized with Zoletil 100 (200 mg/Kg, SC, Virba). Subsequently, the animals were prophylactically injected with gentamicina sulfate (50 mg x 1 ml of solution) (Aagent, Fatro, 0.2 ml/Kg, SC) and individually housed in clear Plexiglas cages. After the transmitter was implanted and before any measurement was started, the animals were allowed 10 days for recovery of body weight and circadian rhythmicity of heart rate.

2.4 Experimental protocol

The rats were chronically implanted with a transmitter and randomly assigned to two experimental groups: Treated (TREAT; n=22) and Control counterparts (CTR; n=22). During post-surgery period, each animal was maintained in a favourable social environment with its female partner. Subsequently, treated animals were exposed to a social defeat episode (Miczek, 1979; Sgoifo et al., 1997) followed by 4-week isolation, while controls remained undisturbed with their female partners. During the isolation period, rats were exposed to different challenges in order to quantify a number of physiological-neuroendocrine and behavioral markers of depression. In particular, rats were submitted to the elevated plus maze, open field, dexamethasone suppression test, and sucrose preference test. These challenges were adopted to evaluate cardiac sympathovagal balance, hypothalamic-pituitary-adrenocortical (HPA) axis reactivity, level of perceived anxiety, and establishment of an anhedonic state. Before and after social defeat, heart rate (HR, bpm), body temperature (T, °C), and physical activity (Act, cpm) were sampled around-the-clock for 60s every 60 min, to assess their circadian rhythmicity. At sacrifice, the adrenal glands and heart were removed for morphometrical analysis. The experimental design is demonstrated in Figure1.

2.5 Specific experimental procedures

2.5.1 Social Defeat

The social stress episode defeat consisted of a õresident-intruderö test (Miczek, 1978; Sgoifo et al., 1996). The experimental animal was introduced for 30 min into the home cage (60x95x55 cm) of a highly aggressive conspecific male (trained fighter), after temporary removal of the female partner, where it was vigorously attacked and finally defeated. After physical interaction, the two animals were separated with a bulkhead that allowed olfactory, acoustic, and visual contact, but impeded physical interaction for additional 30 min.

After the test, each animal was returned to its own cage, from where the female partner had already been removed and the 4-week isolation period started.

2.5.2 Elevated plus-maze test

The apparatus (Handley and Mithani, 1984) consisted of four 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 40 cm high walls), and two being open, having at their intersection a central square platform (10x10 cm) which gave access to all four arms. All floor surfaces were black and made of polyvinyl carbonate. Under dim red light condition each rat was placed on the central platform facing one closed arm. The ratøs behavior during 5 min of test was recorded using a video camera positioned above the maze. The variable recorded were: number of entries and time spent inside each arm or the central platform, open arm entries as percentage of all entries (open+closed arms), time spent in open arms as percentage of total time spent in all arms (open+closed arms). The plus-maze was carefully cleaned after each test.

2.5.3 Open field test

The open field test evaluates the general locomotor and exploratory behavior of rats (Kennet 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 in 16 sectors by black lines. Each rat was placed in the centre of the open field and behaved freely for 15 min (Sgoifo et al., 2002); its behavior was recorded by a video camera above the maze. During this period, radiotelemetric parameters were recorded via eight receivers positioned under the apparatus. Immediately after the challenge, the animals were reintroduced in their own home cage for recovery recording. The behavioral variables recorded were: number of entries and percentage of time spent in outer squares, number of entries and time spent in inner squares, as well as time spent moving (sec), mean time per entry (sec) and locomotion (number of lines crossed) for each of both categories. After each test, the apparatus was carefully cleaned.

2.5.4 Dexamethasone Suppression Test

Hypothalamic-pituitary-adrenocortical (HPA) axis reactivity was assessed to a dexamethasone suppression test via plasma corticosterone level determinations. Dexamethasone is a synthetic glucocorticoid that mimics corticosterone by inducing negative feedback to the pituitary, hypothalamus, and hippocampus. Both groups of animals were treated with dexamethasone at dose of (30 g/Kg, SC). Plasma corticosterone level determinations were assessed 240 minutes after drug injection, just after introduction of the animal in a restraint tube (8 cm diameter and 25 cm length). Blood samples (0.5 ml) were taken from the tail vein within 3 min after the onset of the restraint.

2.5.5 Sucrose preference test

Sucrose preference test was employed to operationally define anhedonia. Anhedonia was specifically defined as a reduction in absolute sucrose intake and sucrose preference relative to a control group and pre-established baseline values. First, animals were trained to freely choose between water or sucrose solution (1%). The sucrose preference test consisted of first removing the fluids (at 5 PM) from each rat's cage for a period of 20 h. At 1 PM the next day, water and 1% sucrose were placed back in the cages in pre-weighed glass bottles, and rats were allowed to consume the fluids for 1 h. The bottles were then removed and weighed. To prevent possible effects of side preference in drinking, the position of the bottles was switched after 30 min. One preference test was conducted before social defeat (baseline) and at 1-week interval throughout the isolation period. The preference for sucrose was calculated from the amount of sucrose solution consumed, expressed as percentage of the total amount of liquid drunk and was used as a measure for rats sensitivity to reward (Katz, 1982)
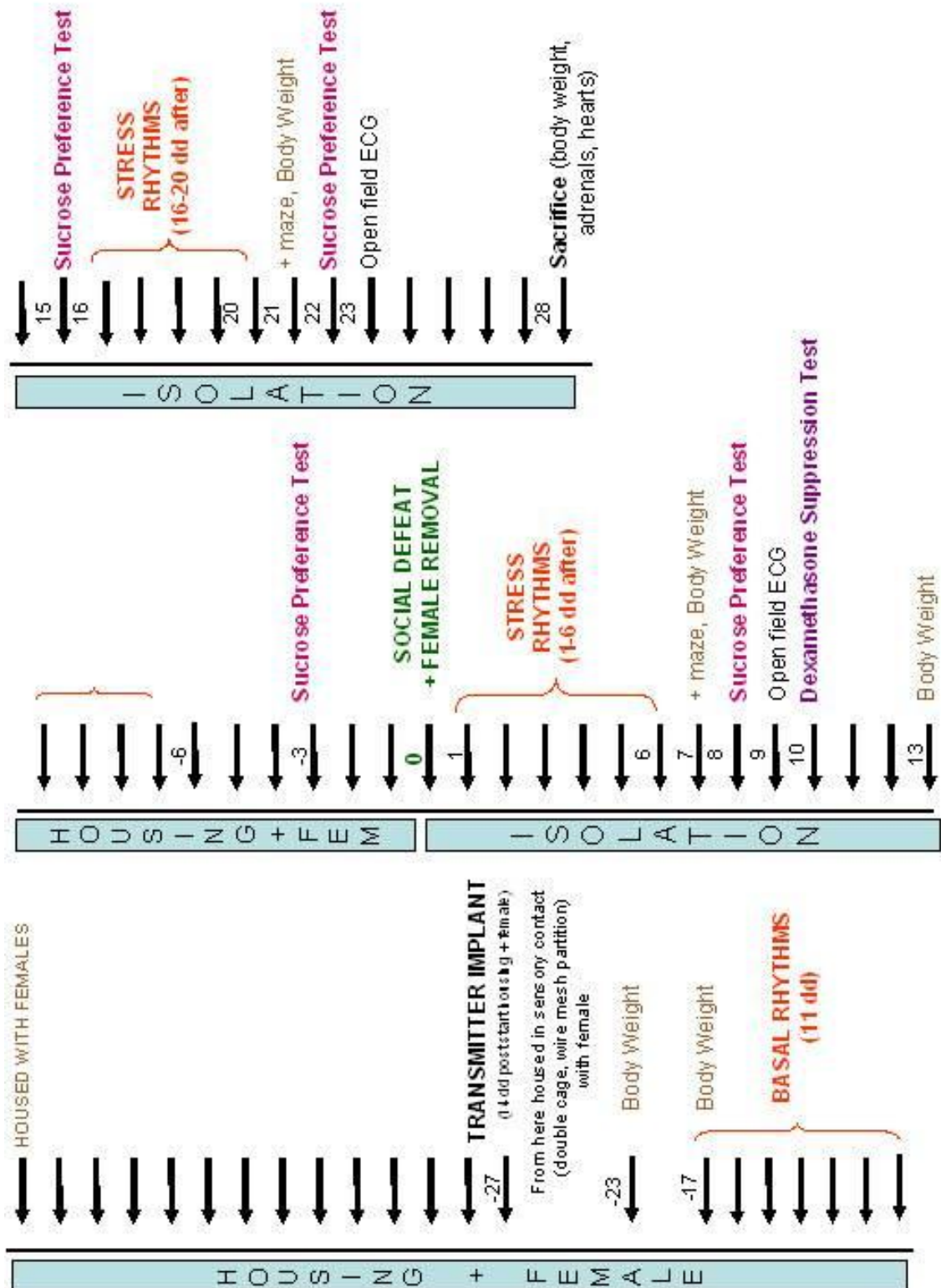
Figure 1. Timeline of procedures used in the current study.

2.6 Corticosterone assay

Tail blood was collected in chilled tubes containing EDTA for determination of corticosterone levels. Blood 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) following the manufacturer's instructions.

2.7 Electrocardiographic data acquisition and analysis

Continuous ECG recordings (sampling frequency: 1000 Hz) were performed at both open field test episodes, in three recording periods: baseline (animals undisturbed in their home cage), test (open field), and recovery (animals returned to their own cages), each lasting 10 minutes.

ECG signals were fed to a PC containing õDataquest Art 2.2ö data acquisition system (Data Sciences Int., St Paul, Minnesota, Usa), for monitoring and acquisition of ECG waves. Offline analysis was performed by means of a software package developed in our lab (XRRECG) (Sgoifo et al., 1999) for quantification of time-domain indexes of heart rate variability (Stein et al., 1994).

The following ECG 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). RR represents an instantaneous measure of heart rate, the r-MSSD focuses on high-frequency, short-term variations of RR interval, which are due to the activity of the parasympathetic nervous system on the heart (Sgoifo et al., 1997; Stein et al., 1994; Task Force, 1996). Generally speaking, reductions in the value of these variability indexes (as compared to baseline) reflect shifts of the autonomic balance towards sympathetic dominance, while increased values of such parameters indicate a shift of sympathovagal balance towards parasympathetic prevalence (Stein et al., 1994).

2.8 Around-the-clock-heart rate (HR), body temperature (T), and physical activity (Act) sampling and processing

Heart rate (expressed as beats/min), body temperature (expressed as °C), and physical activity (expressed as counts) were recorded in the three following phases: (i) Baseline, 11 days, starting 11 days after transmitter implantation and with the animal in its own home cage with a female, (ii) Stress, 6 days, between the 1st and the 6th day after social defeat; and (iii) Stress 2, 5 days, between the 16th and the 20th day after social defeat. HR, T, Act were sampled around-the-clock for 60 s every 60 min, in order to assess their circadian rhythmicity. The three parameters were quantified as means of 12-h light phase (light), and 12-h dark phase (dark). For each individual rat, the daily

amplitudes of the rhythms of heart rate, temperature, and activity were calculated as the difference between average activity and resting phase values, respectively (Meerlo et al., 1999).

2.9 Post-mortem measurements

At the end of the experimental protocol, all animals were killed for post-mortem measures, namely for weight measurement of adrenals and for myocardial morphometrical analysis.

Adrenal glands were removed to evaluate possible hypertrophic effects due to social defeat and isolation. Glandular weight was expressed as ratio to the animal body weight.

The hearts were arrested in diastole with cadmium chloride solution (100 mM, IV), and rapidly removed and weighed. Then, the ventricles were separated and fixed in paraformaldeyde (4%). Ten 1-mm thick slices were transversely cut from the left ventricle and embedded in paraffin. A 5-µm thick section obtained from one of the two intermediate rings was stained with hematoxylin-eosin and used for morphometrical analysis. The section was analyzed at optical microscopy (magnification 250X) in order to evaluate the total amount of interstitial and reparative fibrosis in the left ventricular myocardium. Reparative fibrosis describes discrete areas (foci) of myocardial scarring resulting from focal myocyte cell loss, while interstitial fibrosis corresponds to widening of interstitial space due to collagen accumulation in the absence of apparent focal cell death (Beltrami et al., 1994). According to a procedure previously described (Capasso et al., 1990), for each section a quantitative evaluation of the fibrotic tissue was performed in 60 randomly selected fields from sub-endocardium, mid-myocardium and sub-epicardium, 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 mm2. 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 percentage of the total number of points explored.

2.10 Statistical Analysis

Data were analyzed by means of SPSS 12.0 statistical package (SPSS, Chicago, IL, USA). All parameters were expressed as means ± SEM. Statistical significance was set at $p < 0.05$.

Body weight, quantified from surgery to sacrifice, was evaluated as area under the response time curve (AUC) with pre-stress weight as reference basal value. The comparison between the two groups was performed by means of Studentøs T test.

ECGs were continuously recorded during the open field test. RR and r-MSSD were quantified as means of each 10-min recording phase (baseline, test, and recovery). A quantitative overall evaluation of RR and r-MSSD responsiveness during the two stress episodes was obtained by

computing the area comprised between the response time curve and the baseline (AUC; including all time points – minutes – of the test and recovery periods). The mean value of the ten 1-min time points of the baseline was considered as the reference basal value. The AUC of delta values (AUC $_{open\ field\ 2}$ – AUC $_{open\ field\ 1}$) were statistically analyzed by means of 1-Way ANOVA, in order to compare the effects of the group (TREAT vs CTR).

Mean values of the daily amplitude of the rhythms of HR, T, and Act during the isolation period, (namely days 1-6 and 16-20 after defeat), were analyzed by means of 2-Way ANOVA for repeated measures, with õgroupö as between-subject factor (2 levels: TREAT and CTR) and õrecording dayö as within-subject factor (11 levels: 6 stress, 5 stress 2), and õgroup × recording dayö interaction.

The percentage of sucrose preference was measured at 8, 15 and 22 days after defeat. Two-way ANOVA for repeated measures was applied with õgroupö as between-subject factor (2 levels: TREAT and CTR), õrecording periodö as within-subject factor (4 levels: 1 baseline, 3 after defeat), and õgroup × recording periodö interaction.

The comparison between TREAT and CTR rats for the plasma corticosterone levels measured 10 days after social defeat, and for the weight of adrenal glands was performed by means of Studentøs T test.

Two-Way ANOVA was used for the statistical treatment of morphometrical data, with the õmyocardium layerö as within-subject factor (3 levels: sub-epicardium, mid-myocardial and sub-endocardium) and the õgroupö as between-subject factor (2 levels: TREAT and CTR), and õmyocardium layer × groupö interaction.

After ANOVA, post-hoc analysis was applied when appropriate by means of õtö test.

3. Results

3.1 Body Weight

The Figure 2 displays the time course of body weight measured once a week from surgery to sacrifice. An overall evaluation of the effects of defeat and isolation was obtained by means of AUC (area comprised between the response time curve and the baseline). AUC was significantly higher in control rats as compared to treated animals (CTR: 340.4 ± 81.4 gr vs TREAT: -14.7 ± 139.9; $t=2.19$, $p<0.05$), suggesting that social challenge was associated with reduction of body weight.
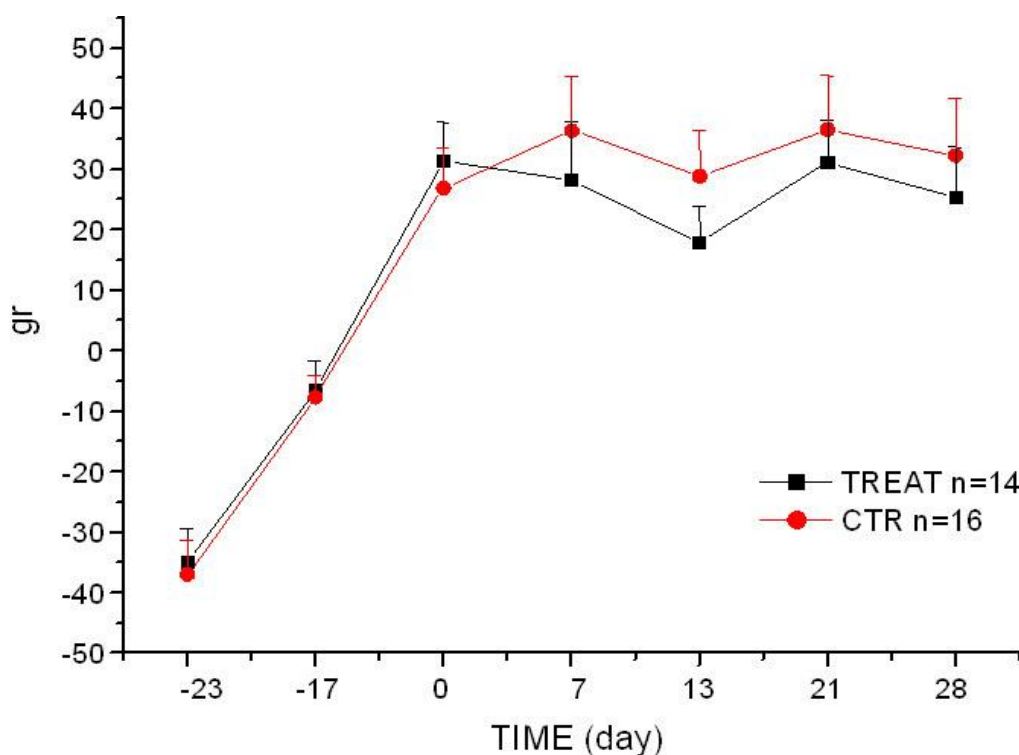
Figure 2. Time course of delta body weight from 4 days after surgery to sacrifice.

3.2 Adrenocortical response to dexamethasone suppression test and adrenal weight

Plasma corticosterone levels in response to Dexamethasone Suppression Test assessed 10 days after social defeat episode, were significantly higher in treated rats as compared to control counterparts (TREAT: 14.3 ± 3.5 ng/ml vs CTR: 6.9 ± 0.5; t=2.09, p<0.05).

At sacrifice, animals submitted to social defeat and isolation exhibited significantly heavier adrenals than CTR counterparts (TREAT: 0.1190 ± 0.01 mg/g vs CTR: 0.1022 ± 0.01; t=3.16, p<0.01).

3.3 Sucrose Preference Test

Two-way ANOVA identified a significant effect of the õgroup × recording periodö interaction (F=4.10, p<0.05). Post-hoc analysis showed that at week 1 (day 8) and 2 (day 15) after defeat, TREAT and CTR animals had developed a similar, gradual increase in the preference for sucrose solution. However, at week 3 TREAT rats exhibited a significantly lower value of sucrose consumption as compared to CTR counterparts (TREAT: 59.9 ± 5.4 % vs CTR: 73.0 ± 2.6; t=2.17, p<0.05).
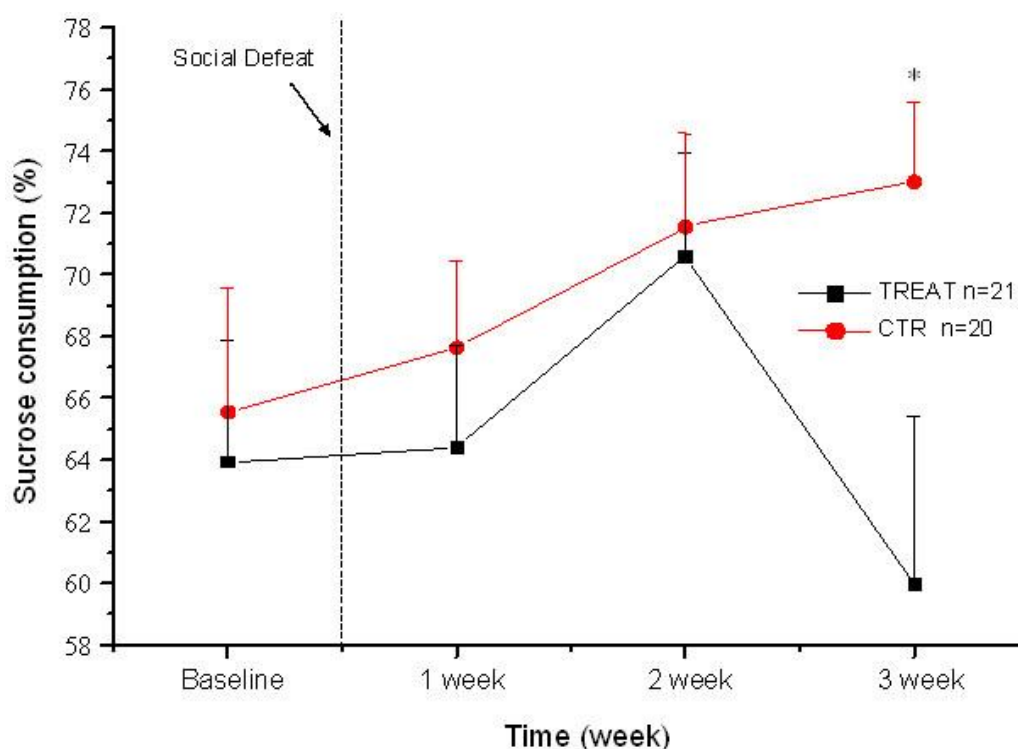
Figure 3. Sucrose consumption (%) at baseline and after social defeat episode in both groups. *Significantly different (*p*<0.05, Student õ*t*ö test) from control corresponding value.

3.4 Long-term chronobiological effects of social defeat and isolation

In rats exposed to defeat episode and isolation, the amplitude of the daily rhythm of heart rate (HR) was significantly reduced as compared to CTR counterparts in the first 6 days and on day 18 after social defeat (Fig.4a). The amplitude of the rhythm of body temperature (T) was also reduced, although significant effects were limited to the 1st day after social stress (Fig.4b).

Physical activity (Act) was also affected by social defeat and isolation: the amplitude of the rhythm was significantly smaller in TREAT as compared to CTR rats in both blocks of sampling days (1-6 and 16-20 after defeat) (Fig.4c).

ANOVAs: Significant effect of õrecording dayö (Act: F=15.72, p<0.01), significant effect of õgroupö (HR: F=15.3, p<0.01; Act: F=30.36, p<0.01) and õgroup × recording dayö interaction (Act: F=4.87, p<0.05).

Post-hoc. Comparison between TREAT and CTR: (*Day 1* HR: t=3.01, p<0.01; T: t=2.13, p<0.05; Act: t=4.33, p<0.01) - (*Day 2* HR: t=3.13, p<0.01; Act: t=3.04, p<0.01) - (*Day 3* HR: t=2.72, p<0.01; Act: t=2.57, p<0.05) - (*Day 4* HR: t=2.68, p<0.05; Act: t=3.55, p<0.01) - (*Day 5* HR: t=2.87, p<0.01; Act: t=2.25, p<0.05) - (*Day 6* HR: t=2.54, p<0.05; Act: t=2.83, p<0.01) - (*Day 16* Act: t=3.07, p<0.01) - (*Day 17* Act: t=3.19, p<0.01) (*Day 18* HR: t=2.33, p<0.05; Act: t=2.85, p<0.01) - (*Day 19* Act: t=3.89, p<0.01) - (*Day 20* Act: t=2.08, p<0.05).
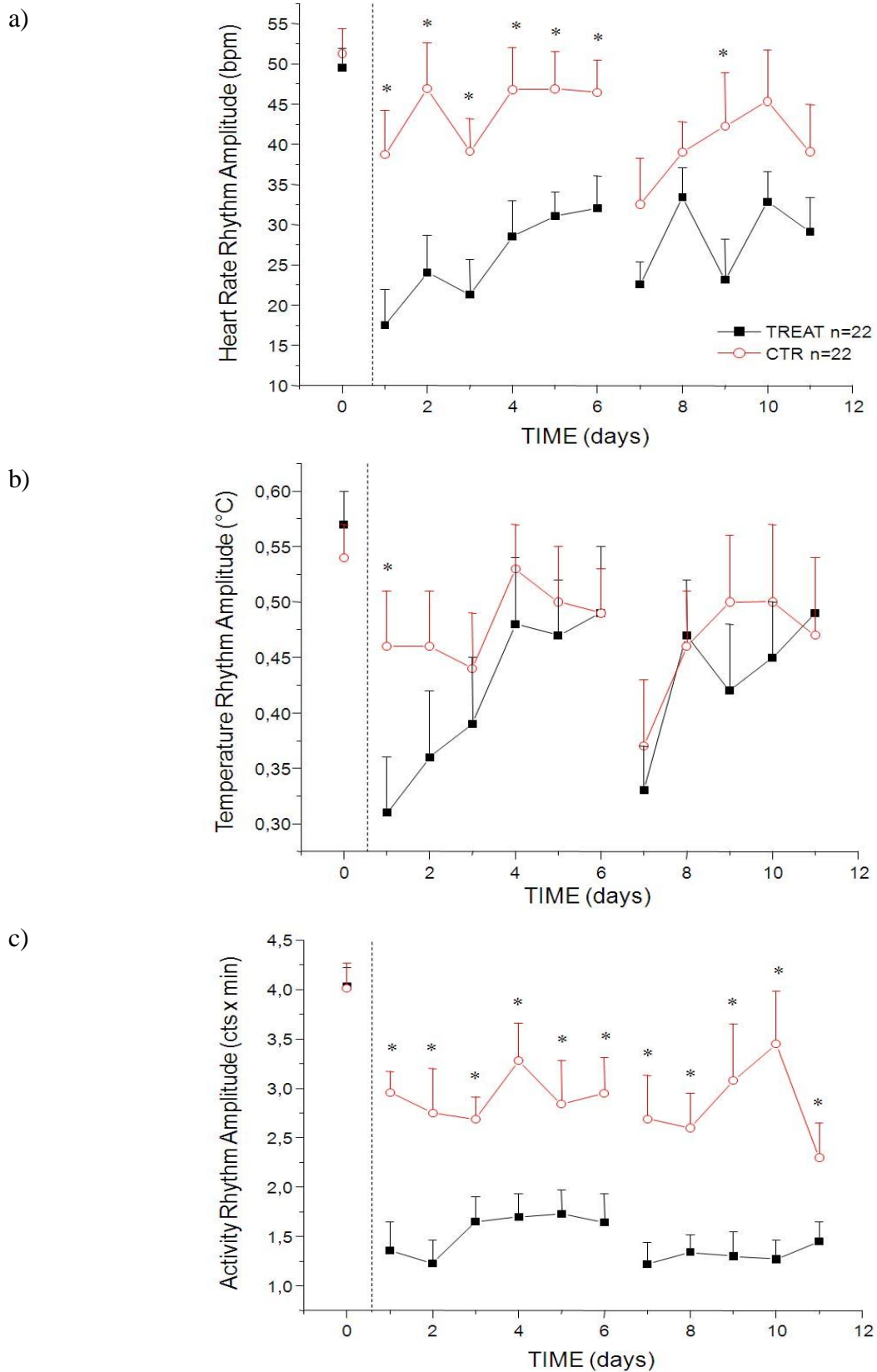
a)

b)

c)

Figure 4. Time course of the daily amplitude (night minus corresponding day value) of the rhythm of heart rate, body temperature, and physical activity, during the first and the third week of isolation period after social defeat, for TREAT and CTR rats.

3.5 Physiological response to open field test

A quantitative overall comparison between the two open field test was obtained by computing the area comprised between the response time curve and the baseline (AUC), for both electrographic parameters (Table 1). In particular, delta AUC values (AUC $_{open\ field\ 2}$ ó AUC $_{open\ field\ 1}$) indicated an habituation-like effect for RR interval and for vagal withdrawal response (r-MSSD) to repeated open field test in control rats, which was not observed in treated counterparts.

On the contrary, there were no significant differences between groups for body temperature and physical activity parameters.

Comparison performed via **1-way ANOVA**: Delta AUC RR ó (TREAT: -79.91±66.28 ms×min vs CTR: 131.26±66.69; t=2.24, p<0.05) - Delta AUC rMSSD ó (TREAT: -7.92±7.39 vs CTR: 15.09±5.19; t=2.49, p<0.05).

Table 1. Area comprised between the response time curve and the baseline (AUC) for RR, r-MSSD, body temperature, and physical activity during the open field test, respectively 9 and 23 days after social defeat

	TREAT (n=22) Open Field 1	TREAT (n=22) Open Field 2	CTR (n=21) Open Field 1	CTR (n=21) Open Field 2
AUC RR (ms×min)	758.45±61.81	775.38±91.13	797.71±76.74	651.77±94.85
AUC rMSSD (ms×min)	8.89±2.94	16.8±7.72	24.65±4.55	9.56±2.78
AUC T (°C×min)	6.86±1.02	6.69±1.41	11.59±1.70	8.61±2.36
AUC Act (cts×min)	474.63±21.99	268.04±36.51	479.95±39.92	316.8±42.18

Values are means ± standard error of the mean (SEM).

3.6 Behavioral data

3.6.1 Open field test

Table 2 summarizes the values of frequency, total duration, latency of entries in the different zones of the apparatus together with the total distance moved and velocity in the open field test at 9 and 23 days after defeat. None of these parameters exhibited significant differences between rats exposed to social defeat and control counterparts.

Table 2. Values of the parameters analyzed during Open Field Test 1 (9 days after Social Defeat) and Open Field Test 2 (23 days after Social Defeat) in treated and control rats.

		TREAT (n=22) Open Field 1	TREAT (n=22) Open Field 2	CTR (n=21) Open Field 1	CTR (n=21) Open Field 2
Frequency (n)	Corner	27.81±2.21	27.33±5.66	27.45±1.72	26.85±4.80
	Center	3.60±0.56	1.30±0.30	3.70±0.68	1.50±0.25
	Periphery	9.60±1.74	14.50±5.96	11.16±2.22	18.30±6.39
Tot. Duration (s)	Corner	361.29±36.94	463.65±28.29	347.59±42.44	471.77±28.24
	Center	15.10±2.72	6.56±1.33	17.60±4.31	5.41±0.76
	Periphery	534.42±30.24	557.37±19.51	429.49±49.13	519.32±30.42
Latency (s)	Corner	4.10±0.66	2.16±0.54	3.72±0.95	2.14±0.58
	Periphery	1.46±0.35	0.84±0.27	1.21±0.36	0.43±0.16
Dist. moved (cm)		3193.34±122.58	2497.97±160.04	3033.33±103.73	2486.37±171.95
Velocity (cm/s)		5.28±0.22	4.16±0.27	5.12±0.17	4.14±0.29

Values are means ± standard error of the mean (SEM).

3.6.2 Elevated plus maze

Table 3 summarizes the values of total number, latency, total duration of entries, and head dip during elevated plus maze 7 and 21 days after defeat. No differences in the mean values of these parameters were found between the two groups.

Table 3. Total number (number of events), latency (sec), and total duration (sec) of entries in the centre, in the open and closed arms of Elevated Plus Maze, 7 (EPM1) and 21 (EPM2) days after Social Defeat.

		TREAT (n=22) EPM 1	TREAT (n=22) EPM 2	CTR (n=21) EPM 1	CTR (n=21) EPM 2
Total Number (n)	Center	5.82±0.67	5.14±0.63	6.91±0.74	6.05±0.76
	Open	1.31±0.41	0.52±0.24	2.45±0.67	1.55±0.63
	Closed	17.54±1.1	17.36±1.39	17.05±0.92	16.66±1.20
	Head Dip	4.71±0.75	2.59±0.69	6.91±0.93	2.35±0.58
Latency (s)	Center	0	0	0	0
	Open	170.67±32.15	241.87±47.90	134.44±24.21	145.41±38.34
	Closed	20.48±4.45	12.99±2.54	29.97±6.94	8.91±1.52
	Head Dip	42.64±7.40	77.53±15.13	30.16±5.24	71.98±15.36
Tot. Duration (s)	Center	30.88±4.82	17.84±3.07	40.54±5.56	19.02±4.06
	Open	10.45±3.27	2.56±1.39	20.23±5.48	8.89±3.63
	Closed	253.53±15.31	271.23±21.04	238.89±16.27	262.74±20.92

Values are means ± standard error of the mean (SEM).

3.7 Morphometrical analysis of the myocardium

The two groups exhibited a similar, modest amount of cardiac structural damage, as expressed by the volume fraction of myocardial fibrosis in the left ventricular wall (Table 4).

Table 4. Myocardial fibrosis in the three layers of the left ventricular myocardium, and in the total wall in TREAT and CTR groups.

Volume fraction of fibrosis (%) in the left ventricular myocardium				
	Epicardium	**Middle**	**Endocardium**	**Total wall**
TREAT (n=22)	1.38±0.28	1.53±0.21	0.69±0.28	1.21±0.08
CTR (n=22)	1.68±0.45	1.83±0.36	0.40±0.14	1.32±0.27

Values are means ± standard error of the mean (SEM).

4. Discussion

The present study analyzed short- and long-term patho-physiological effects of the exposure to an adverse stress episode followed by prolonged social isolation. In particular, this study was aimed at investigating whether these challenges may produce physiological, behavioral, and structural consequences, which resemble some of the symptoms of chronic stress and depression in humans. In order to study these issues, we exposed rats to a social defeat episode followed by four-week isolation. During this period of individual housing the animals were submitted to a number of acute tests in order to evaluate possible changes of: (i) cardiac sympathovagal balance and explorative behavior during an open field test, (ii) HPA axis reactivity to a dexamethasone suppression test, (iii) edhonic behavior in a sucrose preference test, (iv) anxiety in a plus maze test. The study of long-term effects took into account the time evolution of the circadian rhythms of heart rate, body temperature and physical activity, together with the consequences on body weight, myocardial structure and adrenals. For this purpose, a wild-type strain of rats was used, which is characterized by a remarkable autonomic/neuroendocrine stress reactivity (Sgoifo et al., 1996; Sgoifo et al., 1998).

We found a habituation-like effect for RR interval and vagal index (rMSSD) mean values in the control group, i.e. a gradual reduction in the acute cardiac autonomic responsiveness upon repetition of the open field test. In other words, the shift of sympathovagal balance towards a sympathetic dominance was gradually reduced from the first to the second acute challenge. Interestingly, when autonomic responses to the 1st and 2nd test were compared in the treated group no differences could be found suggesting a lack of adaptation to a novel stressor. Our result are line with the idea that depression is associated with a reduced heart rate variability. On this regard, Grippo and colleagues (2003) have studied the cardiovascular alterations in the rats exposed to chronic mild stress, and have found that these animals after 4 week of chronic stress exhibited specific changes in cardiovascular function. Stressed rats displayed significantly elevated heart rate and significantly reduced heart rate variability as compared to control counterparts. Interestingly, as in our case, these changes were observed at the conclusion of the protocol. The cardiovascular changes observed in our model are similar to changes observed in human depression. In fact, depression appears to be associated with a reduced heart rate variability, which in turn can influence cardiovascular regulation. In general decreased heart rate variability is a risk factor for negative cardiovascular events and mortality in cardiac subjects. A high level of variability is common in normal cardiovascular activity (Bigger et al., 1988), whereas devreased vardiability is found in patients with severe cardiovascular diseases (Kristal-Bonet et al., 1995).

As for long-term consequences on chronobiological parameters average day values of HR rose in the first six days following the social defeat episode, and were associated with a significant reduction in the amplitude of day-night oscillation as compared to control counterparts. In addition, in the rats exposed to social defeat, the daily amplitude of the rhythm for physical activity was significantly depressed both in the first and third week of the isolation period. On the contrary, circadian rhythms of body temperature are not sensitive to negative social episodes. Changes in the daily amplitude of the rhythm for heart rate and physical activity, while the body temperature is maintained similar between grours, depends on the different nature of these parameters. Indeed, body temperature is a homeostatic parameter, a component of the internal milieu, that is truly essential for life and is, therefore maintained over a narrow range, as a result of your critical role in survival (McEwen, 2000). In contrast, there are allostatic parameters, i.e. systems that show õvariation to meet perceived/anticipated demandsö (Sterling and Eyer, 1988) characterizes the state of the organism in a changing world and reflects the operation of most body systems in meeting environmental challenges, as heart rate, blood pressure, and other tissue mediators like neurotransmitters and hormones.In other words, allostasis is the process that keeps the organism alive and functioning, maintaining homeostasis or õmaintaining stability through changeö (McEwen, 1998).

This condition may represent a marker of imbalance between normally precisely coordinated physiological and behavioral processes and may constitute a risk factor for the development of disease (Meerlo et al., 2002). Disturbances in circadian rhythms, though by themselves neither sufficient nor necessary to induce pathological changes in behavior and mood, may well contribute to the imbalance between neurochemical and neuroendocrine systems, and thus sensitize an individual to pathological mood alterations (Meerlo et al., 1997).

Rat subjected to social defeat and isolation showed a decrease in sucrose preference in a time-dependent manner. Reduced preference for the sucrose solution is interpreted as an analog of anhedonia, a condition in which the capacity to experience pleasure is totally or partially lost, identified as a core feature of depression (Loas, 1996). In particular, we found that rats submitted to an adverse social episode associated to prolonged period of isolation showed a reduced sucrose intake 3 weeks after defeat. These data are consistent with previous ones obtained in the chronic mild stress paradigm (Grippo et al., 2003). In fact, Grippo and colleagues have found that animals exposed to chronic mild stress exhibited a reduced sucrose intake relative to baseline values and the sucrose intake of the control group; the same result was shown by Rygula (2005) who found a reduced preference for sucrose solution 3 weeks after a social defeat episode. Recently it has been highlighted how divergent anhedonic consequences could originate depending upon the duration of the social defeat experience (Miczek et al., 2008). Specifically, intermittent exposure to brief defeat

episodes would produce behavioral sensitization contributing to the increase of drug abuse, while only continuous subordination stress would lead to blunted response to sweet rewards (Miczek et al., 2005).

In addition, overall analysis of body weight change across the isolation period revealed that treated rats exhibited a reduced weight gain as compared to control counterparts. Our results are in line with results from previous studies (Andre et al., 2005; Rygula et al., 2008; Razzoli et al., 2009), where a decrease of body weight was observed.

In terms of neuroendocrine activation, plasma corticosterone levels in response to the Dexamethasone Suppression Test, were significantly higher in stressed rats as compared to CTRs 10 days after the agonistic episode. Research regarding the dexamethasone suppression test provided evidence that depression is often associated with dysfunction of the HPA system. Dexamethasone is a synthetic glucocorticoid that mimics corticosterone by inducing negative feedback to the pituitary, hypothalamus and hippocampus. The normal physiologic response to its administration is decreased corticosterone secretion due to negative feedback influencing the HPA pathway. However, corticosterone is not suppressed upon the administration of dexamethasone in depressed individuals (Asnis et al., 1987), indicating that glucocorticoids may be hypersecreted in depressed patients. Therefore our results are in line with the idea that a social defeat episode followed by isolation is able to induce a neuroendocrine change that resembles human depression.

In addition, the social challenge determined an increased adrenal weight. Certainly, our data do not allow evaluating the differential role of hypertrophy and hyperplasia in determining this increase in adrenal volume. Usually authors ascribe adrenal enlargement to hypertrophic processes (Llorent et al., 2002; Ward et al., 2000) though no systematic histological analyses of the adrenals have been provided so far.

In order to evaluate possible change in explorative behavior and anxiety behavior, we submitted rats to the open field test and elevated plus maze respectively. There were no significant differences between groups in the expression of behavioral coping strategy in the two tests. However, treated rats, in the last week of isolation, exhibited a slight reduction in open field activity, as compared to control counterparts. This result is in line with a previous study (Meerlo et al., 1996b), where social defeat in rats resulted in reduction in locomotion in the open field.

Morphometric measurements indicated that exposue to a social defeat episode followed by isolation not induced cardiac structural alterations. On this regard, there are some controversies regarding the effects of social stress on the structure of the heart. Sanghez and colleagues (2002) asserted that intermale aggressive confrontations should not significantly affect heart structure, as shown by the lack of increase in creatine kinase activity and the ratio between aspartato transaminasi and alanine transaminase activity. On the contrary, Andrews and coworkers (2003) reported that isolation

followed by teritorial stress (housing in an unstable social environment) induced myocardial fibrosis, coronary collagen deposition, increase in coronary wall-to-lumen ratio, and coronary collagen-to-vessel ratio.

In summary, this study suggests that an adverse stress episode such as social defeat followed by a prolonged period of isolation produces a few physiological, behavioral and structural effects in rats, which resemble some of the symptoms of chronic stress and depression in humans. These effects mainly consist in: (i) changes of circadian rhythmicity of HR and Act (reduced amplitude of the daily rhythm), from the first day up to the end of the third week for activity and limited to the first week after defeat for heart rate; (ii) an habituation-like effect for RR interval and for vagal withdrawal response to repeated open field test in control rats, and a lack of adaptation in treated animals, (iii) onset of an anhedonic condition (reduced intake of sucrose), taking place about three weeks after the adverse social episode; (iii) higher adrenocortical response to dexamethasone suppression test, (iv) a rediction in body weight, and (v) increased weight of the adrenal glands.

References

American Psychiatric Association. Diagnostic and statistical manual of mental disorders (4th ed.), *American Psychiatric Association*, Washington, DC (1994).

Andre J, Zeau B, Pohl M, Cesselin F, Benoliel JJ, Becker C. Involvement of cholecystokininergic systems in anxiety-induced hyperalgesia in male rats: behavioral and biochemical studies, *J Neurosci*, 25 (2005), 7896-7904.

Andrews E, Jenkins C, Seachrist D, Dunphy G, and Ely D. Social stress increases blood pressure and cardiovascular pathology in a normotensive rat model, *Clin Exp Hypertens* 25 (2003), 856101.

Anisman H, Zacharko RM. Depression as a consequence of inadequate neurochemical adaptation in response to stressors, *Br J Psychiatry* 15 (1992), 36-43.

Anisman H, Zacharko RM. Multiple neurochemical and behavioral consequences of stressors: implications for depression, *Pharmacol Ther* 46 (1990), 119-36.

Areán PA, Reynolds CF. The impact of psychosocial factors on late-life depression, *Biol Psychiatry* 58 (2005), 277-282.

Asnis GM, Halbreich U, Ryan ND, Rabinowicz H, Puig-Antich J, Nelson B, Novacenko H, Friedman JH. The relationship of the dexamethasone suppression test (1mg and 2 mg) to basal plasma cortisol levels in endogenous depression, *Psychoneuroendocrinology* 12 (1987), 295-301.

Barefoot JC, Helms MJ, Mark DB, Blumenthal JA, Califf RM, Haney TL, O'Connor CM, Siegler IC, Williams RB. Depression and long-term mortality risk in patients with coronary artery disease, *Am J Cardiol* 78 (1996), 613-617.

Barefoot JC, Schroll M. Symptoms of depression, acute myocardial infarction, and total mortality in a community sample, *Circulation* 93 (1996), 1976-1980.

Beltrami CA, Finato N, Rocco M, Feruglio GA, Puricelli C, Cigola E, Quaini F, Sonnenblinck EH, Olivetti G, Anversa P. Structural basis of end-stage failure in ischemic cardiomyopathy in humans, *Circulation* 89 (1994), 151-163.

Bigger JT, Kleiger RE, Fleiss JL, Rolnitzky LM, Steinman RC, Miller JP. Components of heart rate variability measured during healing of acute myocardial infarction, *Am J Cardiol* 61 (1988), 208-215.

Capasso JM, Palackal T, Olivetti G, Anversa P. Left ventricular failure induced by hypertension in rats, *Circ Res* 66 (1990), 1400-1412.

Catalano JT. Guide to ECG analysis, *Philadelphia:J.B. Lippincott*, 1993.

J Buwalda B, de Boer SF, Schmidt ED, Felszeghy K, Nyakas C, Sgoifo A, Van der Vegt BJ, Tilders FJ, Bohus B, Koolhaas JM. Long-lasting deficient dexamethasone suppression of hypothalamic-pituitary-adrenocortical activation following peripheral CRF challenge in socially defeated rats, *Neuroendocrinol.* 11 (1999), 513-520.

Frasure-Smith N, Lespérance F, Talajic M. Depression following myocardial infarction. Impact on 6-month survival, *JAMA* 270 (1993), 1819-1825.

Fuchs E, Flügge G. Social stress in tree shrews: effects on physiology, brain function, and behavior of subordinate individuals, *Pharmacol Biochem Behav* 73 (2002), 247-258.

Grippo AJ, Beltz TG, Johnson AK. Behavioral and cardiovascular changes in the chronic mild stress model of depression, *Physiol Behav*, 78 (2003), 703-710.

Handley SL, Mithani S. Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour, *Naunyn Schmiedebergs Arch Pharmacol* 327 (1984), 1-5.

Heim C, Nemeroff CB. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies, *Biol Psychiatry* 49 (2001), 1023-1039.

Herman JP, Cullinan WE. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis, *Trends Neurosci* 20 (1997), 78-84.

Katz RJ. Animal model of depression: pharmacological sensitivity of a hedonic deficit, *Pharmacol Biochem Behav* 16 (1982), 965-968.

Kendler KS, Karkowski LM, Prescott CA. Causal relationship between stressful life events and the onset of major depression, *Am J Psychiatry* 156 (1999), 837-841.

Kennett GA, Dickinson SL, Curzon G. Enhancement of some 5-HT-dependent behavioural responses following repeated immobilization in rats, *Brain Res* 330 (1985), 253-263.

Koolhaas JM, De Boer SF, De Rutter AJ, Meerlo P, Sgoifo A. Social stress in rats and mice, *Acta Physiol Scand Suppl* 640 (1997), 69-72.

Koolhaas JM, Hermann PM, Kemperman C, Bohus B, van den Hoofdakker RH, Beersma DG. Single social defeat in male rats induces a graduated but not long lasting behavioral change: a model of depression?, *Neurosci Res Comm* 7 (1990), 35-41.

Kristal-Bonet E, Raifel M, Froom P, Ribak J. Heart rate variabilità in health and disease, *Scand J Work Environ Health* 21 (1995), 85-95.

Llorente E, Brito ML, Machado P, Gonzalez MC. Effect of prenatal stress on the hormonal response to acute and chronic stress and on immune parameters in the offspring, *J. Physiol. Biochem* 58 (2002), 143-149.

Loas G, Noisette C, Legrand A, Boyer P. Anhedonia, depression and the deficit syndrome of schizophrenia, *Acta Psychiatr Scand* 94 *(*1996), 477-479.

Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030, *PLoS Med.* 3 (2006), e442.

McEwen B.S. Protective and damaging effects of stress mediators, *New Eng. J. Med.* 338 (1998), 171ó179.

McEwen BS. The neurobiology of stress: from serendipity to clinical relevance, *Brain Res* 886 (2000), 172-189.

Meerlo P, De Boer SF, Koolhaas JM, Daan S, Van den Hoofdakker RH. Changes in daily rhythms of body temperature and activity after a single social defeat in rats, *Physiol Behav 59* (1996), 735-739.

Meerlo P, Overkamp GJ, Benning MA, Koolhaas JM, Van den Hoofdakker RH. Long-term changes in open field behaviour following a single social defeat in rats can be reversed by sleep deprivation, *Physiol Behav* 60 (1996), 115-119.

Meerlo P, Sgoifo A, De Boer SF, Koolhaas JM. Long-lasting consequences of a social conflict in rats: behavior during the interaction predicts subsequent changes in daily rhythms of heart rate, temperature, and activity, *Behav Neurosci* 113 (1999), 1283-1290.

Meerlo P, Sgoifo A, Turek FW. The effects of social defeat and other stressors on the expression of circadian rhythms, *Stress* 5 (2002), 15-22.

Meerlo P, van den Hoofdakker, Koolhaas JM, Daan S. Stress-induces changes in circadian rhythms of body temperature and activity in rats are not caused by pacemaker changes, *J Biol Rhythms* 12 (1997), 80-92.

Miczek KA., Thompson ML, Tornatzky W. Short and long term physiological and neurochemical adaptations to social conflict. In: Puglisi-Allegra, S.; Oliverio, A., eds. *Psychobiology of stress* Dordrecht: Kluwer, 1990: 15-30.

Miczek KA. A new test for aggression in rats without aversive stimulation: differential effects of d-amphetamine and cocaine, *Psychopharmacology* 60 (1979), 253-259.

Miczek KA. Delta-9-tetrahydrocannabiol: antiaggressive effects in mice, rats and squirrel monkeys, *Science* 199 (1978), 1459-1461.

Miczek K.A., Covington H.E., Yap J.J., Nikulina E.M. Emergence of anhedonia in laboratory rats during continuous subordination stress: relevance to antidepressants and stimulant abuse, *Neuropsychopharmacology* 30 (2005), S182.

Miczek K.A., Yap J.J., Covington H.E. Social stress, therapeutics and drug abuse: preclinical models of escalated and depressed intake, *Pharmacol. Ther.* 120 (2008), 102ó128.

Murray CJ, Lopez AD. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study, *Lancet* 349 (1997), 1436-1442.

Penninx BW, Beekman AT, Honig A, Deeg DJ, Schoevers RA, van Eijk JT, van Tilburg W. Depression and cardiac mortality: results from a community-based longitudinal study, *Arch Gen Psychiatry* 58 (2001), 221-227.

Razzoli M, Carboni L, Arban R. Alterations of behavioral and endocrinological reactivity induced by 3 brief social defeats in rats: relevance to human psychopathology, *Psychoneuroendocrinology* 34 (2009), 1405-1416.

Ressler K.J., Nemeroff C.B. Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders, *Depress Anxiety* 12 (2000), 2-19.

Rygula R, Abumaria N, Flügge G, Fuchs E, Rüther E, Havemann-Reinecke U. Anhedonia and motivational deficits in rats: impact of chronic social stress, *Behav Brain Res* 162 (2005), 127-34.

Rygula R, Abumaria N, Havemann-Reinecke U, Rüther E, Hiemke C, Zernig G, Fuchs E, Flügge G. Pharmacological validation of a chronic social stress model of depression in rats: effects of reboxetine, haloperidol and diazepam, *Behav Pharmacol* 19 (2008), 183-196.

Sanchez O, Arnau A, Pareja M, Poch E, Ramirez I, and Soley M. Acute stress-induced tissue injury in mice: differences between emotional and social stress, *Cell Stress Chaperones* 7 (2002), 36ó46.

Sgoifo A, Buwalda B, Roos M, Costoli T, Merati G, Meerlo P. Effects of sleep deprivation on cardiac autonomic and pituitary-adrenocortical stress reactivity in rats, *Psychoneuroendocrinology* 31 (2006), 197-208.

Sgoifo A, de Boer SF, Haller J, Koolhaas JM. Individual differences in plasma catecholamine and corticosterone stress responses of wild-type rats: relationship with aggression. *Physiol Behav*. 1996 Dec;60(6):1403-7.

Sgoifo A, De Boer SF, Westenbroek C, Maes FW, Beldhuis H, Suzuki T, Koolhaas JM. Incidence of arrhythmias and heart rate variability in wild-type rats exposed to social stress, *Am J Physiol* 273 (1997), 1754-1760.

Sgoifo A, Koolhaas JM, Musso E, De Boer SF. Different sympathovagal modulation of heart rate during social and non-social stress episodes in wild-type rats, *Physiol Behav* 67 (1999), 733-738.

Sgoifo A, Pozzato C, Meerlo P, Costoli T, Manghi M, Stilli D, Olivetti G, Musso E. Intermittent exposure to social defeat and open-field tests in rats: acute and long term effects on ECG, body temperature and physical activity, *Stress* 5 (2002), 23-35.

Sgoifo A, Stilli D, Medici D, Gallo P, Aimi B, Musso E. Electrode positioning for reliable telemetry ECG recordings during social stress in unrestrained rats, *Physiol Behav* 60 (1996), 1397-1401.

Sgoifo A., S.F. de Boer, C. Westenbroek, F.W. Maes, H. Beldhuis, T. Suzuki and J.M. Koolhaas, Incidence of arrhythmias and heart rate variability in wild-type rats exposed to social stress, *Am J Physiol* 273 (1997), 1754ó1760.

Sgoifo A, De Boer SF, Buwalda B, Korte-Bouws G, Tuma J, Bohus B, Zaagsma J, Koolhaas JM. Vulnerability to arrhythmias during social stress in rats with different sympathovagal balance, Am *J Physiol* 275 (1998), 460-466.

Shekhar A, McCann UD, Meaney MJ, Blanchard DC, Davis M, Frey KA, Liberzon I, Overall KL, Shear MK, Tecott LH, Winsky L. Summary of a National Institute of Mental Health workshop: developing animal models of anxiety disorders, *Psychopharmacology* 157 (2001), 327-339.

Stein PK,Bosner MS, Kleiger RE, Conger BM. Heart rate variability: a measure of cardiac autonomic tone, *Am Heart J* 127 (1994), 1376-1381.

Sterling P., J. Eyer, Allostasis: a new paradigm to explain arousal pathology. In: S. Fisher and J. Reason, Editors, *Handbook of Life Stress, Cognition and Health*, John Wiley & Sons, New York (1988), 629ó649.

Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology. Heart Rate Variability: Standards of Measurement, Physiological Interpretation, and Clinical Use, *Circulation* 93 (1996), 1043-1065.

Tornatzky W, Miczek KA. Behavioral and autonomic responses to intermittent social stress: differential protection by clonidine and metoprolol, *Psychopharmacology* 116 (1994), 346-356.

Von Frijtag JC, Van den Bos R, Spruijt BM. Imipramine restores the long-term impairment of appetitive behavior in socially stressed rats, *Psychopharmacology* 162 (2002), 232-238.

Walker MJ, Curtis MJ, Hearse DJ, Campbell RW, Janse MJ, Yellon DM, Cobbe SM, Coker SJ, Harness JB, Harron DW, Higgins AJ, Jiulia DG, Lab MJ, Manning AS, Northover BJ, Parrat JR, Riemersma RA, Riva E, Russel DC, Sheridan DJ, Winslow E, Woodward B. The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia, infarction, and reperfusion, *Cardiovasc Res* 22 (1988), 447-455.

Ward HE, Johnson EA, Salm AK, Birkle DL. Effects of prenatal stress on defensive withdrawal behaviour and corticotrophin releasing factor systems in rat brain, *Physiol Behav* 70 (2000), 359-366.

CHAPTER 4

8-OH-DPAT prevents cardiac arrhythmias and attenuates tachycardia during social stress in rats

Eugene Nalivaiko[a], Francesca Mastorci[b] and Andrea Sgoifo[b]

[a]School of Biomedical Sciences, University of Newcastle, Newcastle, Australia
[b]Stress Physiology Lab., Dept. of Evolutionary and Functional Biology, University of Parma, Italy

Chapter 4

1. Introduction

2. Materials and methods

2.1 Animals and housing

2.2 Preliminary surgery

2.3 Experimental protocol

2.3.1 Experiment 1: effects of 8-OH-DPAT on cardiac, thermal and locomotor response to social defeat in animals with a normal, unaltered heart rate

2.3.2 Experiment 2: effects of 8-OH-DPAT on cardiac, thermal and locomotor response to social defeat in animals with zatebradine-induced bradycardia

2.4 Data acquisition and analysis

2.5 Statistical analysis

3. Results

3.1 Degree of aggression during social defeat

3.2 Experiment 1

3.3 Experiment 2

4. Discussion

4.1 Arrhythmia-potentiating effect of zatebradine

4.2 Anti-arrhythmic effect of 8-OH-DPAT: mechanism and location

4.3 Effects of 8-OH-DPAT on other physiological variables

4.4 Significance and perspectives

References

ABSTRACT

The aim of this study was to apply a behavioural stress paradigm for studying the neural mechanisms underlying stress-induced arrhythmias, and to test whether such arrhythmias could be suppressed by systemic administration of 8-OH-DPAT, a 5-HT1A agonist possessing central sympatholytic properties. The study was conducted on adult male rats instrumented for telemetric recordings of ECG, body temperature and locomotor activity. In the first experiment, rats were subjected to social defeat after either 8-OH-DPAT (100 µg/kg s.c.) or vehicle injection. In the second experiment, prior to vehicle/8-OH-DPAT administration, animals were pre-treated with zatebradine, a blocker of the pacemaker current. 8-OH-DPAT caused prolongation of basal RR interval, increase in locomotion and hypothermia. Subjecting vehicle-treated animals to social defeat caused shortening in RR interval, increase in locomotor activity and hyperthermia, and provoked the occurrence of premature ventricular and supraventricular beats; all these effects were substantially attenuated by 8-OH-DPAT. Zatebradine caused prolongation of RR interval. In zatebradine/vehicle-treated rats, the incidence of ventricular and supraventricular premature beats during defeat increased 2.5-fold and 3.5-fold, respectively. 8-OH-DPAT administered after zatebradine significantly reduced these stress-induced arrhythmias. We conclude that: i) pharmacologically induced prolongation of RR interval may contribute to an increased susceptibility to stress-induced cardiac arrhythmias, possibly due to the prolongation of the ventricular diastolic period with restored excitability; and ii) systemic administration of 8-OH-DPAT abolishes these arrhythmic events, likely by suppressing stress-induced cardiac sympathetic outflow.

1. Introduction

Acute psychological stressors, apart from behavioural and neuroendocrine reactions, provoke a constellation of autonomic responses. With regard to the heart, stress-evoked changes could range from mild sinus tachycardia to ventricular arrhythmia, fibrillation and ultimately to sudden cardiac death. Increase in cardiac sympathetic nerve activity is the crucial pathogenetic link in a chain of events leading to ventricular tachyarrhythmias (Lown et al., 1999). Mechanisms underlying cardiac morbidity associated with chronic psycho-emotional stress, anxiety and depression may also include elevated cardiac sympathetic tone (Rozanski et al., 1999) and (Barton et al., 2007). At present, the only class of pharmacological agents used for preventing consequences of this sympathetic overactivity is -blockers acting directly on the heart. Since these drugs have a number of side effects and counter-indications, the ability to suppress potentially deleterious increase in cardiac sympathetic activity at its origin, in the brain, would be a valuable alternative.

So far, few attempts have been made to reach this goal, mainly due to lack of knowledge of pharmacological sensitivity of presympathetic cardiomotor neurons. A group of such neurons responsible for the increase in cardiac sympathetic nerve activity during stress is located in the medullary raphe/parapyramidal region (Zaretsky et al., 2003). We have recently demonstrated, in rats and rabbits, that activation of serotonin-1A (5-HT$_{1A}$) receptors in this area suppresses tachycardia elicited by psychological stressors (Nalivaiko et al., 2005; Ngampraumuan et al., 2008). Heart rate however is not an adequate index of pro-arrhythmic neural influences; in fact, it could be quite misleading. We have presented evidence, in both humans (Nalivaiko et al., 2007) and animals (Braga et al., 2007; Nalivaiko et al., 2003; Nalivaiko et al., 2004) that the sino-atrial node, cardiac conducting system and ventricular myocardium may be controlled independently, and that sympathetic and parasympathetic outflows controlling different cardiac functions could be activated simultaneously. Furthermore, tachycardia by itself is cardioprotective as it diminishes the duration of the ventricular diastolic period with restored excitability (also called õventricular vulnerable periodö). This emphasized the necessity of assessing effects of central sympatholytic agents on ventricular indices, preferentially directly on the incidence of arrhythmic events.

One difficulty in designing such studies is that experimental animals with healthy hearts usually do not develop ventricular arrhythmias. In our previous experiments, where we used relatively mild stressors (restraint in rats and air-jet stress in rabbits) (Nalivaiko et al., 2005; Ngampraumuan et al., 2008), ventricular premature beats (VPBs) were noted only occasionally. So far, the only behavioural animal stress model where VPBs could be consistently triggered is the social defeat paradigm applied to wild-type Norway rats (Sgoifo et al., 1998). Using this model, we tested

whether ventricular arrhythmias precipitated by acute stressors could be suppressed by systemic administration of 8-OH-DPAT, a 5-HT$_{1A}$ agonist possessing central sympatholytic properties. We also tested whether increase of interbeat interval would facilitate arrhythmogenesis; for this purpose we used zatebradine [1,3,4,5-tetrahydro-7,8-dimethoxy-3-[3-][2-(3,4-dimethoxyphenyl)ethyl] methylimino]propyl]-2H-3-benzazepin-2-on hydrochloride], a blocker of I_f pacemaker current DiFrancesco, 1994) that does not affect ventricular function.

2. Materials and methods

2.1 Animals and housing

Thirty-three adult male rats (*Rattus norvegicus*, Wild type Groningen strain) 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. Rats were housed in groups of 4 individuals from weaning until the onset of experiments (25 weeks of age; body weight: 481.2±5.9g). Before and during the experiments, the animals were kept in rooms with controlled temperature (22±2 C) and reversed-cycle lighting (lights on from 17:00 to 05:00h). Food and water were freely available.

2.2 Preliminary surgery

Three weeks prior to the recording sessions, rats were anesthetized with tiletamine hydrochloride+zolazepam hydrochloride (Zoletil, 200mg/kg, s.c.). Radiotelemetry transmitters (TA11CTA-F40, Data Sciences Int., St. Paul, MN, USA) were implanted according to a surgical procedure that guarantees high quality ECG recordings even during sustained physical activity (Sgoifo et al., 1996). 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 individually housed and injected for 2 days with gentamicine sulfate (Aagent, Fatro, 0.2 ml/kg, s.c.).

2.3 Experimental protocol

Animals were randomly assigned to 4 experimental groups. Groups 1 and 2 were used in Experiment 1, and Groups 3 and 4 were used in Experiment 2. Each animal was used for just one experimental procedure.

2.3.1 Experiment 1: effects of 8-OH-DPAT on cardiac, thermal and locomotor response to social defeat in animals with a normal, unaltered heart rate

Baseline parameters were recorded in the home cages for 15 min. Subsequently animals from Group 1 received an injection of the selective 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (100 µg/kg, s.c; group DPAT, *n*=9), and animals from Group 2 were injected with vehicle (Ringer's solution, s.c.; group SAL, *n*=6). The volume of all injections was 1 ml/kg. Fifteen min after the injection, animals of both groups were exposed for 15 min to the social stressor. The stress episode consisted of a classical õresidentóintruderö test (Miczek, 1979): the experimental animal (intruder) was introduced into the home cage of a dominant male (resident), after temporary removal of its female partner. There, it was vigorously attacked and finally subordinated by the resident animal (social defeat) (Buwalda et al., 2005).

2.3.2 Experiment 2: effects of 8-OH-DPAT on cardiac, thermal and locomotor response to social defeat in animals with zatebradine-induced bradycardia

Baseline parameters were recorded in the home cages for 15 min. Subsequently animals from Group 3 (ZAT-DPAT, *n*=9) were injected with zatebradine, a blocker of the pacemaker current I_f (2 mg/kg, s.c.) followed 30 min later by an injection of 8-OH-DPAT (100 µg/kg, s.c.) and, 15 min later, by the exposure to 15-min social defeat. Group 4 rats (ZAT-SAL, *n*=9) underwent the same experimental procedure, but instead of the serotonin agonist they received an injection of vehicle.

In both experiments, the number of attacks and the latency to first attack (in seconds) by resident rats were quantified in order to assess the intensity of the social aggression received by intruder rats during the defeat test. At the end of the test, experimental animals were returned to their home cages, and recordings were continued for 15 min (post-test period). All experiments were performed during the dark phase (between 9 a.m. and 1 p.m.). All recordings took place with the animal in its home cage, except for the test period. 8-OH-DPAT was purchased from Sigma (MN, USA); the dose of the drug was selected based on our previous experiments (Ngampramuan et al., 2008). Zatebradine was a generous gift from Boehringer Ingelheim (CT, USA). Both drugs were dissolved in sterile Ringer's solution just prior to injection.

2.4 Data acquisition and analysis

The radiotelemetry system employed in this study enabled recording of the electrocardiogram (ECG), body temperature (*T*, °C) and locomotor activity (Act, cpm, i.e. counts/min) in freely-moving animals. ECG analysis was performed using a software package developed in our lab for quantification of time-domain indexes of heart rate variability (Sgoifo et al., 2001). The following parameters were quantified: (i) the mean *RóR* interval duration (RR, ms), and (ii) the root-mean-

square of successive *RóR* interval differences (r-MSSD, ms). R-MSSD reflects the average 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. 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., 1988).

2.5 Statistical analysis

Initially, values of RR, r-MSSD, *T* and Act were provided as the mean of each 1-min time period in baseline, post-injection, test, and post-test periods. Then, an average value was calculated for each 15-min recording period. Differences between groups (DPAT vs. SAL, ZAT-DPAT vs. ZAT-SAL) for RR, r-MSSD, temperature, and locomotor activity were determined by comparing corresponding 15-min average values in the various recording periods. Initially, two-way ANOVA for repeated measures was applied, with group as between-subject factor (2 levels: DPAT and SAL for Experiment 1, ZAT-DPAT and ZAT-SAL for Experiment 2), time as within-subject factor (4 time points for Experiment 1: 15-min basal, 15-min post-DPAT or post-saline injection, 15-min stress test, and 15-min post-test; 6 time points for Experiment 2: 15-min basal, first 15-min post-zatebradine injection, second 15-min post-zatebradine injection, 15-min post-DPAT or post-saline injection, 15-min stress test, 15-min post-test), and group×time interaction.

The occurrence of ventricular and supraventricular premature beats (VPBs and SPBs, respectively) was separately analyzed by means of two-way ANOVA for repeated measures, with the same between-subject and within-subject criteria above described for the other parameters.

After ANOVAs, post-hoc analysis was performed when appropriate with Student *t*-test, after controlling for the homogeneity of variances via Levene test. The same post-hoc test was also applied to compare the effects of zatebradine and saline on the incidence of arrhythmias, again preceded by Levene test. Statistical significance for all tests was set at $p<0.05$. All parameters in figures and tables are expressed as mean±SEM (standard error of the mean).

3. Results

3.1 Degree of aggression during social defeat

Rats injected with either 8-OH-DPAT or vehicle received a similar number of attacks during the social defeat test (10.8±0.9 vs. 8.27±0.5, respectively). Similarly, the latency to first attack by resident rats did not differ in the two groups of animals (63.7±7.5 vs. 59.8±10.3 s, respectively). Therefore, the quantification of these two classical behavioural parameters assessing the intensity of resident‒intruder test demonstrated that the two groups of rats (i.e.: injected with either saline or 8-OH-DPAT) received a similar amount of aggression.

3.2 Experiment 1

Baseline values of all parameters were similar between the two groups of rats (Fig. 1 and Fig. 3 and Table 1). Vehicle injection provoked a short-lasting transient tachycardia, with return of the RR to baseline values within 10‒15 min. After 8-OH-DPAT administration, tachycardia quickly reverted to bradycardia associated with a significant increase in r-MSSD values. Subjecting animals to social stress resulted in sustained tachycardia and reduction of r-MSSD values. As depicted in Fig. 1 and detailed in Table 1, values of RR and r-MSSD were significantly higher in animals injected with the serotonin-1A agonist before (pre-test), during (test), and after (post-test) the adverse social episode (SAL vs. DPAT, $p<0.05$).
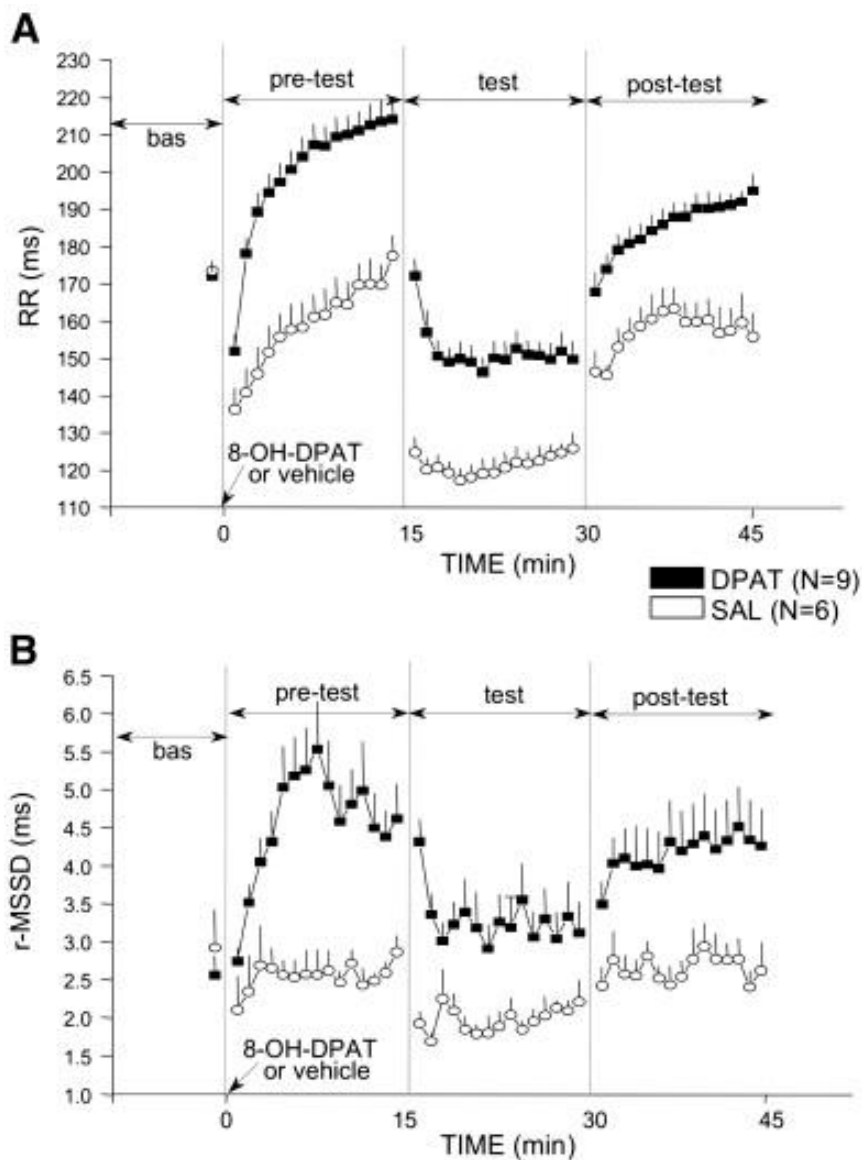
Figure 1. Time course of changes in *RóR* interval (RR, panel A) and root-mean-square of successive *RóR* interval differences (r-MSSD, panel B) in baseline conditions (bas), after drug injection (pre-test), during social defeat (test), and after defeat (post-test), in rats injected with either 8-OH-DPAT (DPAT) or saline solution (SAL). Baseline reference value (bas) is the mean value of the fifteen 1-min time points in resting conditions.

Table 1.

Fifteen-min mean values of average *RóR* interval (RR), root-mean-square of successive *RóR* interval differences (r-MSSD), body temperature (*T*) and locomotor activity (Act) at baseline, after drug injection (pre-test), during social defeat (test), and after defeat (post-test), in rats injected with either 8-OH-DPAT (DPAT) or saline solution (SAL).

		Baseline	Pre-test	Test	Post-test
RR (ms)	SAL	173±3	159±6	121±3^{bd}	157±5
	DPAT	172±4	200±5^{ab}	152±4^{abd}	185±4^{ac}
r-MSSD (ms)	SAL	2.9±0.5	2.5±0.2	2.0±0.1^{ce}	2.6±0.2
	DPAT	2.6±0.3	4.6±0.3^{ab}	3.3±0.3^{ace}	4.2±0.5^{a}
T (°C)	SAL	38.2±0.1	37.9±0.2	38.6±0.6^{cd}	39.0±0.1^{bd}
	DPAT	38.0±0.2	36.6±0.2^{ab}	35.8±0.2^{abe}	35.9±0.2^{ab}
Act (counts/min)	SAL	5.3±1.6	9.6±1.8	35.1±3.8^{bd}	10.0±1.2
	DPAT	4.6±1.0	18.2±2.2^{ab}	25.8±2.2^{abe}	23.7±4.1^{ab}

Data presented as mean±SEM. [a] ô significantly different from the corresponding value of SAL group ($p<0.05$); [b] and [c] ô significantly different from the basal value ($p<0.01$ and $p<0.05$, respectively); [d] and [e] ô significantly different from the post-injection pre-test value ($p<0.01$ and $p<0.05$, respectively). Results of ANOVA: (i) for RR interval ô significant effects of group ($F=33.17$, $p<0.01$), time ($F=10.25$, $p<0.01$), and group×time interaction ($F=6.97$, $p<0.05$); (ii) for r-MSSD ô significant effects of group ($F=10.82$, $p<0.01$), and group×time interaction ($F=5.13$, $p<0.05$); (iii) for body temperature ô significant effects of group ($F=168.58$, $p<0.01$), time ($F=11.41$, $p<0.01$), and group×time interaction ($F=67.04$, $p<0.01$); (iv) for locomotor activity ô significant effect of time ($F=25.38$, $p<0.01$).

Prior to stress, we observed on average no more than one supraventricular (Fig. 2B) or ventricular (Fig. 2C) premature beat per 15-min recording period, in the animals belonging to both experimental groups. During social defeat, the incidence of these events increased substantially in saline-treated animals, whereas the injection of 8-OH-DPAT significantly reduced the incidence of SPBs during defeat (Fig. 3A; SAL=3.8±0.9 vs. DPAT=1.2±0.4 events/15-min; $p<0.05$) and tended to reduce the incidence of VPBs (Fig. 3B). ANOVAs: SPBs ô significant effect of group ($F=8.5$, $p<0.05$); VPBs ô significant effect of time ($F=14.5$, $p<0.01$).
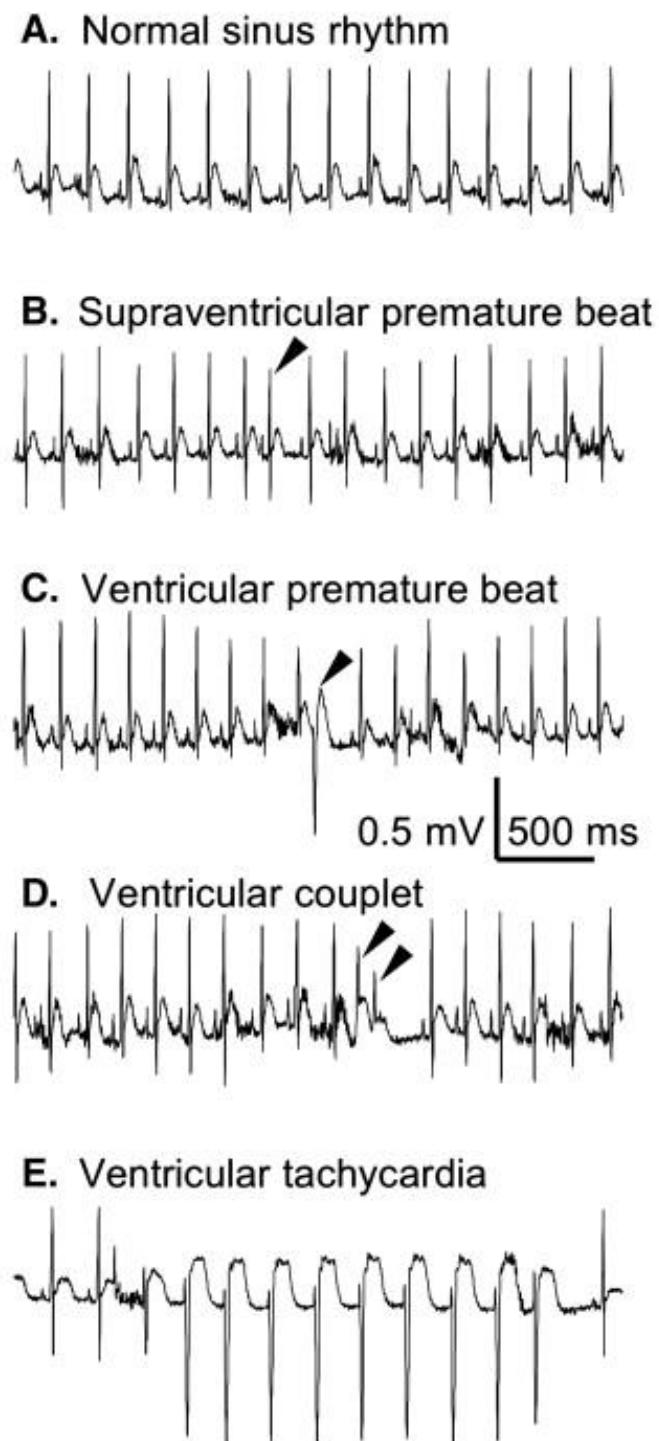
Figure 2. Telemetry electrocardiographic tracings with examples of different arrhythmic events observed during social defeat in rats.
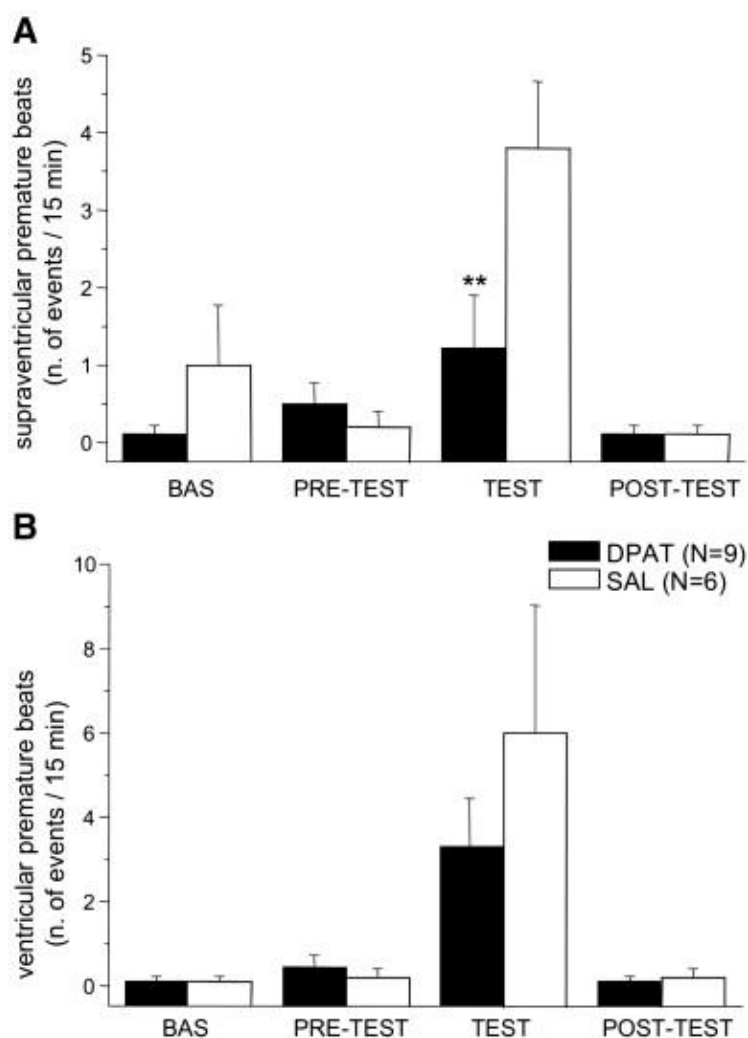
Figure 3. Incidence (number of events per 15-min recording period) of supraventricular (SPBs, panel A) and ventricular premature beats (VPBs, panel B) in baseline conditions (bas), after drug injection, during social defeat (test), and after defeat (post-test), in rats injected with either 8-OH-DPAT (DPAT) or saline solution (SAL). **ô significantly different from SAL corresponding value ($p<0.01$).

Social stress elicited a hyperthermic response in SAL rats that extended beyond the test period (Table 1). 8-OH-DPAT reduced core body temperature during all post-injection recording periods, and completely prevented stress-induced hyperthermia. Locomotor activity was also affected by the drug. Rats injected with 8-OH-DPAT showed significantly higher values of activity before and after the stress episode, but lower values during the social challenge as compared to saline-injected rats. Mean values and results of ANOVA and post-hoc tests for these variables are presented in Table 1.

3.3 Experiment 2

Fig. 4 depicts changes in RR interval and r-MSSD values following the injection of zatebradine, of 8-OH-DPAT or saline, and following the exposure to a social defeat episode. As expected, zatebradine produced a large increase of RR interval that was accompanied by a substantial increase in r-MSSD values (Table 2). Subsequent injection of 8-OH-DPAT per se did not produce further increments of RR or r-MSSD, but significantly attenuated stress-induced tachycardia and entirely prevented stress-induced fall in r-MSSD values (Table 2).
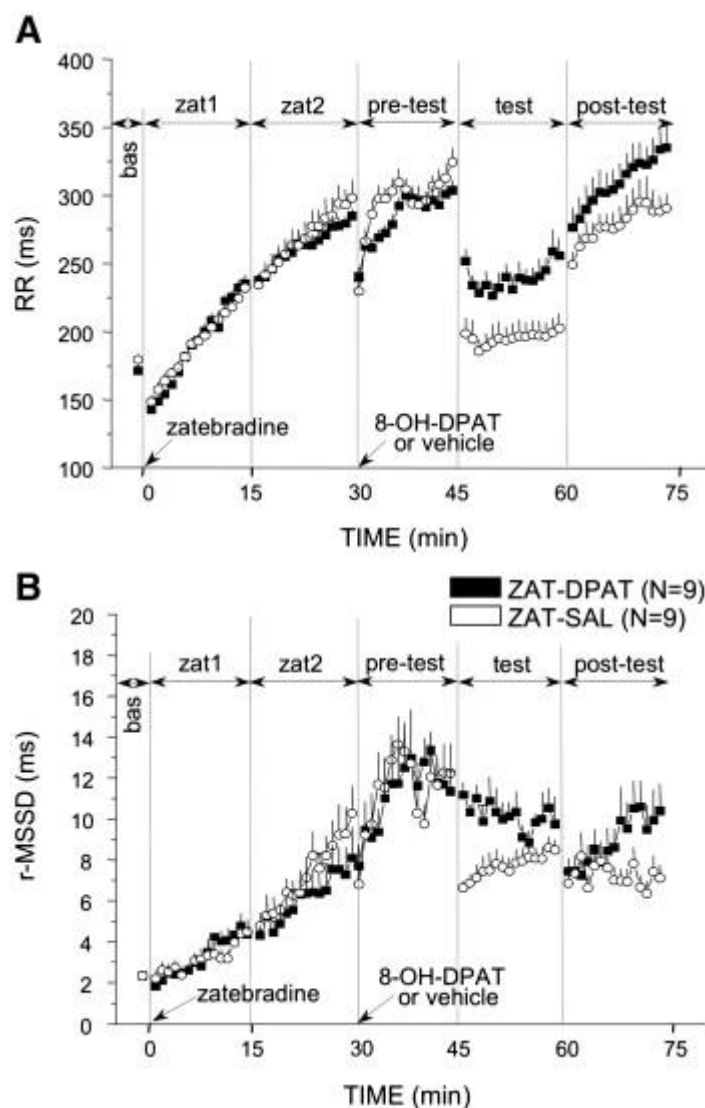
Fig. 4. Time course of changes in *RóR* interval (RR, panel A) and root-mean-square of successive *RóR* interval differences (r-MSSD, panel B) in baseline conditions (bas), after zatebradine injection, after drug injection (pre-test), during social defeat (test), and after defeat (post-test), in rats injected with either 8-OH-DPAT (ZAT-DPAT) or saline solution (ZAT-SAL). Baseline reference value (bas) is the mean value of the fifteen 1-min time points in resting conditions.**Table 2.**

Fifteen-min mean values of average *R-R* interval (RR), root-mean-square of successive *R-R* interval differences (r-MSSD), body temperature (*T*) and locomotor activity (Act) in baseline conditions (bas), after zatebradine injection (ZAT1 and ZAT2 for 0–15 and 16–30 min, respectively), after drug injection (pre-test), during social defeat (test), and after defeat (post-test), in rats injected with either 8-OH-DPAT (DPAT) or saline solution (SAL)

		Baseline	Post-ZAT1	Post-ZAT2	Pre-test	Test	Post-test
RR (ms)	SAL	179±4	192±4[c]	269±10[b]	295±7[b]	195±10[d]	279±11[b]
	DPAT	172±4	191±3[b]	263±6[b]	283±6[b]	239±10[abd]	309±15[b]
r-MSSD (ms)	SAL	2.3±0.2	3.2±0.3[c]	7.2±0.7[b]	11.3±1.0[b]	7.8±0.5[bd]	7.5±0.6[b]
	DPAT	2.3±0.2	3.3±0.2[b]	6.1±0.7[b]	11.1±1.0[b]	10.0±0.6[ab]	8.9±1.1[b]
T (°C)	SAL	37.8±0.1	37.9±0.1	37.8±0.1	37.4±0.1	37.7±0.2	38.0±0.2[e]
	DPAT	38.0±0.1	37.7±0.2	37.7±0.1	36.7±0.1[ab]	35.6±0.1[abd]	35.2±0.2[abd]
Act (counts/min)	SAL	3.8±1.2	10.6±2.0	4.9±1.2	5.2±1.0	34.0±3.6[b]	8.2±1.7[c]
	DPAT	6.8±1.6	12.8±2.2	5.2±0.8	22.5±1.6[ab]	21.5±3.4[ab]	14.8±2.2[ace]

Data presented as mean±SEM. [a] – significantly different from the corresponding value of SAL group ($p<0.05$); [b] and [c] – significantly different from the basal value ($p<0.01$ and $p<0.05$, respectively); [d] and [e] – significantly different from the post-injection pre-stress value ($p<0.01$ and $p<0.05$, respectively). Results of ANOVA: (i) for RR interval – significant effects of group ($F=5.14$, $p<0.05$), time ($F=127.10$, $p<0.01$), and group×time interaction ($F=9.77$, $p<0.01$); (ii) for r-MSSD: significant effect of time ($F=173.53$, $p<0.01$); (iii) for body temperature: significant effects of group ($F=72.23$, $p<0.01$), time ($F=259.25$, $p<0.01$), and group×time interaction ($F=216.57$, $p<0.01$); and (iv) for locomotor activity – significant effect of time ($F=47.22$, $p<0.01$).

Similar to the previous experiment, the incidence of ectopic beats was very low outside the stress period (Fig. 5). During defeat, there was a dramatic increase of these events in the zatebradine/saline group: the incidence of both supraventricular and ventricular premature beats was significantly higher than in animals that were not pre-treated with zatebradine (i.e. SAL group from Experiment 1; $p<0.05$; compare Fig. 3 and Fig. 5). Furthermore, in 3 saline-treated rats we also observed a small number of more complex ventricular arrhythmic events (7 couplets/triplets and one case of ventricular tachycardia; see Fig. 2D and E). The incidence of both SPBs and VPBs during defeat was significantly lower in rats injected with 8-OH-DPAT (SPBs: SAL=15±4 vs. DPAT=3±0.6 events/15 min, $p<0.05$; VPBs: SAL=14±2 vs. DPAT=2±1 events/15 min, $p<0.01$). ANOVAs: SPBs – significant effect of time ($F=19.9$, $p<0.01$) and group×time interaction ($F = 4.5$, $p<0.05$); VPBs – significant effect of group ($F=54.7$, $p<0.01$), time ($F=101.1$, $p<0.01$), and group×time interaction ($F=63.8$, $p<0.01$). In 8-OH-DPAT-treated animals no ventricular tachycardias occurred, and only one ventricular couplet was observed in one rat.
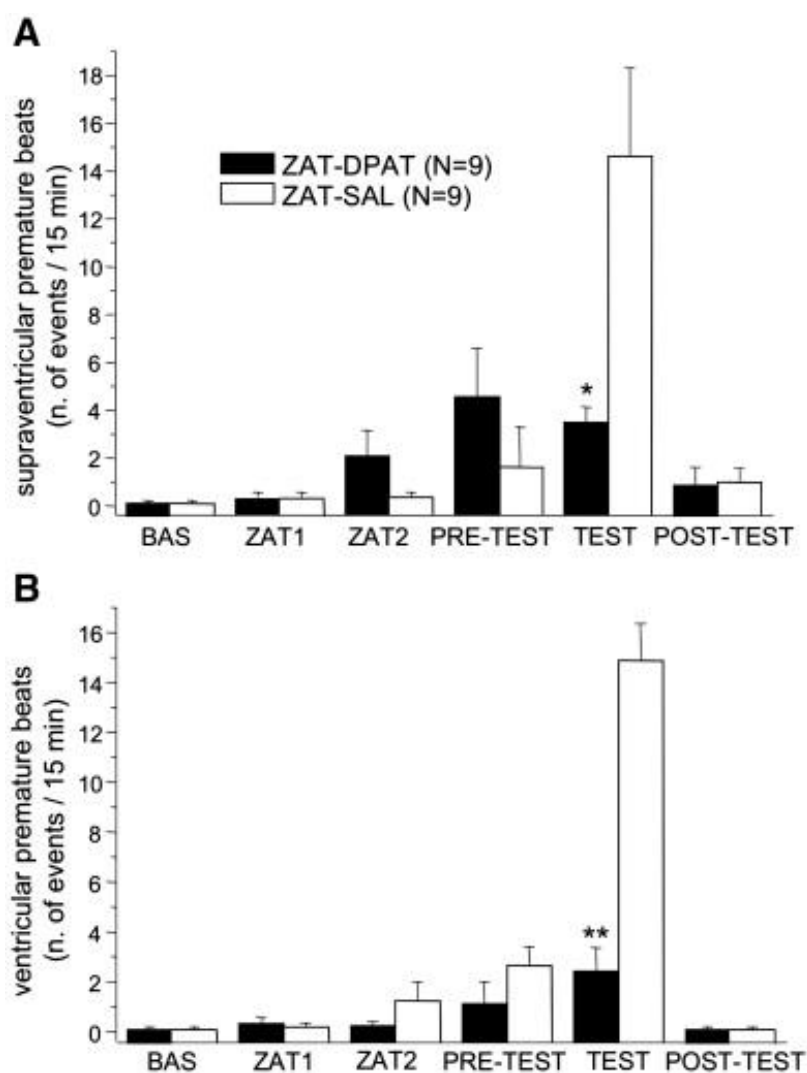
Figure 5. Incidence (number of events per 15-min recording period) of supraventricular (SPBs, panel A) and ventricular premature beats (VPBs, panel B) in baseline conditions (bas), after zatebradine injection (ZAT1 and ZAT2), after drug injection (PRE-TEST), during social defeat (test), and after defeat (post-test), in rats injected with either 8-OH-DPAT (DPAT) or saline solution (SAL). *and **ô significantly different from SAL corresponding value ($p<0.05$ and $p<0.01$, respectively).

Zatebradine did not affect body temperature and locomotor activity (Table 2). In contrast, rats showed significantly lower values of body temperature after the injection of the serotonin-1A agonist. After zatebradine, social stress caused a rise in body temperature (that became significant only during the post-test period this time), and 8-OH-DPAT prevented this hyperthermic response. As compared to the controls, rats injected with 8-OH-DPAT showed significantly larger amounts of locomotor activity before and after the stress episode, but lower values during the social challenge, in agreement with findings of Experiment 1. Mean values and results of ANOVA and post-hoc tests for these variables are presented in Table 2.

4. Discussion

Our major novel findings are that pharmacologically induced prolongation of RR interval results in an increased susceptibility to stress-induced cardiac arrhythmias, and that systemic administration of 5-HT$_{1A}$ receptor agonist 8-OH-DPAT reduces significantly the number and complexity of these arrhythmic events. We made our conclusions by measuring the incidence of stress-elicited ventricular premature beats. While the latter are considered relatively benign, they often precede more malignant tachycardias. In addition, it must be recognized that evoking even such minor arrhythmic effects as reported here is not easy in healthy rat hearts. We thus considered applying a biologically-relevant stress model (Koolhaas et al., 1997), whereby effects of an acute stressor on neurally-induced changes in myocardial excitability could be readily assessed as an additional strength of our present paper.

4.1 Arrhythmia-potentiating effect of zatebradine

Zatebradine is a specific bradycardic agent blocking the slowly depolarizing current (I_f) in the pacemaker region of the heart (DiFrancesco, 1994). The rationale for using it in our study was the expectation that the prolongation of ventricular diastolic period with restored excitability in the myocardium (õventricular vulnerable periodö) would enhance the incidence of stress-induced arrhythmic events, providing a good background for testing anti-arrhythmic effects of 8-OH-DPAT. Indeed, we found that pretreatment with zatebradine resulted in about 3.5-fold increase in supraventricular premature beats and in about 2.5-fold increase in ventricular premature beats during the stress episode. Interestingly, in previous animal experiments zatebradine was found to be either anti-arrhythmic, if ventricular arrhythmias were induced by myocardial ischemia (Naito et al., 2000) or ouabain intoxication (Furukaka et al., 1996), or it was without effect, in arrhythmias precipitated by reperfusion (Naito et al., 2000) or epinephrine administration (Furukaka et al.,

1996). It is thus likely that pro- or anti-arrhythmic properties of the drug depend on the pathogenesis of arrhythmias, so that the drug is clearly beneficial when the reduction of the metabolic demand during hypoxia is a key issue. This is supported by the observation that ischemia-induced ventricular arrhythmia depends on the heart rate (Bernier et al., 1989). In contrast, a combination of excessive catecholamine release from cardiac sympathetic terminals and the adrenal medulla (as happens during stress) with prolonged diastole may provide favourable conditions for the development of early or late after depolarization and for subsequent reentry. Arrhythmogenesis in this situation could be further facilitated by modest inhibitory effects of zatebradine on the delayed rectifier potassium current (I_{Kr}) (BoSmith et al., 1993). These speculations however do not explain why the drug did not potentiate arrhythmogenesis when it was co-administered with epinephrine in anesthetized dogs (Furukaka et al., 1996). A number of reasons ó from interspecies differences to differences in action between endogenously released and exogenously administered adrenergic agonists ó could be the cause of this discordance.

4.2 Anti-arrhythmic effect of 8-OH-DPAT: mechanism and location

We have recently demonstrated that 8-OH-DPAT substantially attenuates tachycardia in rats and rabbits subjected to psychological stressors, that this anti-tachycardic effect is mediated via 5-HT1A receptors, and that the effect is predominantly due to the suppression of cardiac sympathetic drive (Nalivaiko et al., 2005; Ngampramuan et al., 2008). As ventricular arrhythmias were not noted in those experiments, we failed to answer an important question ô whether 8-OH-DPAT possesses antiarrhythmic properties. The present study was specifically designed to address this issue, and our major technical challenge was to employ an animal stress model with substantial proarrhythmic effect. This has been achieved by using wild-type Groningen rats that are the only strain prone to high incidence of stress-induced arrhythmic events (Sgoifo et al., 1998; Sgoifo et al., 1997), by exposing them to a severe challenge such as social defeat (Gudelsky et al.,1986), and by prolonging the diastolic period with zatebradine (see above).

Systemic administration of 8-OH-DPAT dramatically reduced the incidence of stress-induced ventricular and supraventricular premature beats, and reduced the complexity of arrhythmic events. The drug also substantially and significantly reduced stress-induced tachycardia in zatebradine-treated animals. The apparent lack of the anti-tachycardic effect in the first experiment, when the drug was given alone, is likely due to the fact that post-vehicle, stress-induced tachycardia reached saturation (minimal possible cycle length), so that comparing deltas is not a valid approach here.

The principal pathogenetic link whereby psychological stressors trigger cardiac arrhythmias is an excessive release of norepinephrine from sympathetic terminals, due to an increase in cardiac sympathetic activity. As noted above, we found in our previous study that the anti-tachycardic effect of 8-OH-DPAT in rats subjected to restraint stress was mediated by the inhibition of this cardiac sympathetic outflow (Ngampramuan et al., 2008). Similar anti-tachycardic effect was observed in our current experiments, and we thus suggest that the anti-arrhythmic effect of the drug is due to the suppression of stress-elicited elevation of sympathetic outflow to the ventricular myocardium. It is most likely that both outflows were attenuated by the same mechanism ô i.e. the activation by 8-OH-DPAT of inhibitory $5-HT_{1A}$ receptors located on presympathetic cardiomotor neurons in the medullary raphe-parapyramidal area (Thor et al., 1990). It may be that antiarrhythmic/anti-tachycardic effects of the drug were also mediated by action on other brain structures ô for example by reducing ascending serotonergic influences from the dorsal raphe (Charara et al., 1994; Gonzalo-Ruiz et al., 1995) That cardiovascular effects of 8-OH-DPAT are not peripheral has been convincingly documented by Fozard et al. (1987). While our results suggest that 8-OH-DPAT is a potent inhibitor of sympathetic outflow to both the sino-atrial node and the ventricular myocardium, they do not allow to answer the intriguing question of whether both outflows are controlled by the same or by different neuronal pools.

8-OH-DPAT quickly and efficiently reduced basal heart rate when it was administered alone. In contrast, when the drug was delivered after lowering heart rate with zatebradine, it was without effect. This is fully consistent with the idea of central sympatholytic action of $5-HT_{1A}$ agonists: as norepinephrine exerts its cardiac chronotropic action largely by activating I_f current (Sawaki et al., 1995), the blockade of this current would mask any 8-OH-DPAT-mediated sympathetic withdrawal.

We also assessed the effects of the drugs and the stressor on the r-MSSD ô a time-domain index of heart rate variability reflecting short-term variations of *RôR* interval. These variations are mainly due to the vagal influences at the sino-atrial node (Stein et al., 1994). It is rarely acknowledged that the rise in r-MSSD could be also merely a result of the reduced heart rate; this was likely the case after zatebradine, where the drug target was downstream from the vagal postsynaptic action. An interesting finding of the present study is that 8-OH-DPAT prevented the r-MSSD reduction elicited by social defeat, when post-8-OH-DPAT stress-induced tachycardia was still quite substantial (see Fig. 4A and B). This may reflect suppression by 8-OH-DPAT of stress-induced vagal withdrawal ô a phenomenon previously observed in rats during restraint stress (Ngampramuan et al., 2008). In accordance with previous studies on the same strain of rats exposed to the same social challenge (Sgoifo et al., 1998; DiFrancesco, 1994) the present study underlines the association between larger values of r-MSSD and a lower incidence of ventricular arrhythmias.

4.3 Effects of 8-OH-DPAT on other physiological variables

Paradoxically, 8-OH-DPAT-induced bradycardia was associated with an *increase* in locomotor activity. The latter was likely a manifestation of behavioural serotonin syndrome ô a combination of hyperlocomotion, head weaving, flat body posture and reciprocal forepaw treading (Tricklebank et al., 1984; Blanchard et al., 1993). Goodwin et al. (1987) presented evidence that these behavioural responses are mediated by postsynaptic $5-HT_{1A}$ receptors, in contrast to the $5-HT_{1A}$ autoreceptors-mediated hypothermia. Our previous findings indicate that anti-tachycardic effects of 8-OH-DPAT could be also due to the activation of these autoreceptors located in the medullary raphe (Nalivaiko et al., 2005; Ngampramuan et al., 2008). As the location of $5-HT_{1A}$ receptors responsible for behavioural effects must be different, it is not particularly surprising that we observed apparently contrasting effects on behaviour and heart rate. We suggest that sympatholytic effect of 8-OH-DPAT overcame locomotion-induced cardiac sympathetic activation. It remains unclear why the drug reduced locomotor response during the social defeat phase.

The rationale for measuring body temperature in our study was to use it as a positive control for the efficacy of 8-OH-DPAT injections, as this drug has well-known hypothermic properties (Gudelsky et al., 1986). Indeed, we did find that the drug not only produced hypothermia, but also entirely prevented the hyperthermic response typically elicited by social defeat in rats (Koolhaas et al., 1997). It is achieved primarily by sympathetically-induced activation of thermogenesis in the brown adipose tissue and by sympathetically-mediated vasoconstriction in the cutaneous vascular bed (Ootsuka et al., 2007), the latter preventing heat dissipation. Interestingly, the brainstem location of presympathetic neurons that control both these processes is the same as that of presympathetic cardiomotor neurons ô i.e. the medullary raphe/parapyramidal region (Morrison, 2001; Blessing and Nalivaiko, 2001). Furthermore, similar to cardiomotor neurons, both these neuronal subpopulations could be inhibited by $5-HT_{1A}$ agonists (Morrison, 2004; Ootsuka et al., 2006), and this was most likely the mechanism underlying the hypothermic and anti-hyperthermic effect of 8-OH-DPAT reported above. As 8-OH-DPAT is a mixed $5-HT_{1A}/5-HT_7$ receptor agonist, its hypothermic effect could be at least in part mediated via $5-HT_7$ receptors.

4.4 Significance and perspectives

As far as results of animal studies could be applied to human cardiac pathophysiology, we believe that our findings are important in two aspects. First, they clearly demonstrate the viability of the idea of central cardiac sympatholytic action as a novel strategy for anti-arrhythmic therapy. At present, very little is known about cardiac effects of $5-HT_{1A}$ agonists in humans, and future

experiments are necessary to estimate whether this class of drugs has substantial potential. Of interest here are the results of two human studies of urapidil, a hypotensive drug (acting by post-synaptic blockade of a1-adrenoceptors) with central agonistic action at 5-HT$_{1A}$ receptors. In both instances authors noted that the hypotensive effect of urapidil was not associated with commonly observed reflexively-mediated tachycardia, and suggested that this was due to the central inhibition of cardiac sympathetic tone (Van der Stroom et al., 1994; Stoschitzky et al., 2007).

Secondly, the arrhythmia-potentiating effect of zatebradine reported here should be certainly taken into account, as much expectation is now placed on selective I_f blockers for treating patients with stable coronary artery disease and left ventricular systolic dysfunction. Chronic heart failure is associated with increased levels of norepinephrine in the myocardium (Kaye et al., 1995; Hasking et al., 1986), and it may be that prolongation of the diastolic period in this setting is potentially dangerous. Recent clinical study reported that mortality in patients with angina pectoris was 0.32%, 0.63% and 0.95% following treatment with atenolol, ivabradine 7.5 mg and ivabradine 10 mg, respectively (Tardif et al., 2005) (ivabradine is also a I_f blocker). While differences were not significant, the trend is clearly alerting. Hopefully, forthcoming publication of the outcome of the much larger international multi-centre study of ivabradine effects (Fox et al., 2006) will clarify this safety issue.

References

Barton D.A., Dawood T., Lambert E.A., Esler M.D., Haikerwal D., Brenchley C., Socratous F., Kaye D.M., Schlaich M.P., Hickie I., Lambert G.W. Sympathetic activity in major depressive disorder: identifying those at increased cardiac risk?, *J Hypertens* 25 (2007), pp. 2117‐2124.

Bernier M., Curtis M.J., Hearse D.J. Ischemia-induced and reperfusion-induced arrhythmias: importance of heart rate, *Am J Physiol* 256 (1989), pp. H21‐31.

Blanchard R.J., Shepherd J.K., Armstrong J., Tsuda S.F., Blanchard D.C. An ethopharmacological analysis of the behavioral effects of 8-OH-DPAT, *Psychopharmacol* 112 (1993), pp. 55‐63.

Blessing W.W., Nalivaiko E. Raphe magnus/pallidus neurons regulate tail but not mesenteric arterial blood flow in rats, *Neuroscience* 105 (2001), pp. 923‐929.

BoSmith R.E., Briggs I., Sturgess N.C. Inhibitory actions of ZENECA ZD7288 on whole-cell hyperpolarization activated inward current (If) in guinea-pig dissociated sinoatrial node cells, *Br J Pharmacol* 110 (1993), pp. 343‐349.

Braga V.A., Zoccal D.B., Soriano R.N., Antunes V.R., Paton J.F., Machado B.H., Nalivaiko E. Activation of peripheral chemoreceptors causes positive inotropic effects in a working heart‐brainstem preparation of the rat, *Clin Exp Pharmacol Physiol* 34 (2007), pp. 1156‐1159

Buwalda B., Kole M.H., Veenema A.H., Huininga M., de Boer S.F., Korte S.M., Koolhaas J.M. Long-term effects of social stress on brain and behavior: a focus on hippocampal functioning, *Neurosci Biobehal Rev* 29 (2005), pp. 83‐97

Charara A., Parent A. Brainstem dopaminergic, cholinergic and serotoninergic afferents to the pallidum in the squirrel monkey, *Brain Res* 640 (1994), pp. 155‐170.

DiFrancesco D. Some properties of the UL-FS 49 block of the hyperpolarization-activated current (i(f)) in sino-atrial node myocytes, *Pflug Arch* 427 (1994), pp. 64‐70

Fox K., Ferrari R., Tendera M., Steg P.G., Ford I. Committee, Rationale and design of a randomized, double-blind, placebo-controlled trial of ivabradine in patients with stable coronary artery disease and left ventricular systolic dysfunction: the morBidity‐mortality evAlUaTion of the I(f) inhibitor ivabradine in patients with coronary disease and left ventricULar dysfunction (BEAUTIFUL) study, *Am Heart J* 152 (2006), pp. 860‐866.

Fozard J.R., Mir A.K., Middlemiss D.N. Cardiovascular response to 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) in the rat: site of action and pharmacological analysis, *J Cardiovasc Pharmacol* 9 (1987), pp. 328ᴓ347.

Furukawa Y., Xue Y.X., Chiba S., Hashimoto K. Effects of zatebradine on ouabain-, two-stage coronary ligation- and epinephrine-induced ventricular tachyarrhythmias, *Eur J Pharmacol* 300 (1996), pp. 203ᴓ210.

Gonzalo-Ruiz A., Lieberman A.R., Sanz-Anquela J.M. Organization of serotoninergic projections from the raphé nuclei to the anterior thalamic nuclei in the rat: a combined retrograde tracing and 5-HT immunohistochemical study, *J Chem Neuroanat* 8 (1995), pp. 103ᴓ115

Goodwin G.M., DeSouza P.J., Green A.R., Head D.J. The pharmacology of the behavioural and hypothermic responses of rats to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), *Psychopharmacol* 91 (1987), pp. 506ᴓ511

Gudelsky G.A., Koenig J.I., Meltzer H.Y. Thermoregulatory responses to serotonin (5-HT) receptor stimulation in the rat. Evidence for opposing roles of 5-HT2 and 5-HT1A receptors, *Neuropharmacol* 25 (1986), pp. 1307ᴓ1313.

Hasking G.J., Esler M.D., Jennings G.L., Burton D., Johns J.A., Korner P.I. Norepinephrine spillover to plasma in patients with congestive heart failure: evidence of increased overall and cardiorenal sympathetic nervous activity, *Circulation* 73 (1986), pp. 615ᴓ621

Kaye D.M., Lefkovits J., Jennings G.L., Bergin P., Broughton A., Esler M.D. Adverse consequences of high sympathetic nervous activity in the failing human heart, *J Am Coll Cardiol* 26 (1995), pp. 1257ᴓ1263.

Koolhaas J.M., Meerlo P., De Boer S.F., Strubbe J.H., Bohus B. The temporal dynamics of the stress response, *Neurosci Biobehav Rev* 21 (1997), pp. 775ᴓ782.

Lown B., Verrier R.L, Rabinowitz S.H. Neural and psychologic mechanisms and the problem of sudden cardiac death, *Am J Cardiol* 39 (1977), pp. 890ᴓ902.

Miczek K.A. A new test for aggression in rats without aversive stimulation: differential effects of d-amphetamine and cocaine, *Psychopharmacol* 60 (1979), pp. 253ᴓ259

Morrison S.F. Activation of 5-HT1A receptors in raphe pallidus inhibits leptin-evoked increases in brown adipose tissue thermogenesis, *Am J Physiol* 286 (2004), pp. R832ᴓR837

Morrison S.F. Differential regulation of sympathetic outflows to vasoconstrictor and thermoregulatory effectors, *Ann N Y Acad Sci* 940 (2001), pp. 286ᴓ298.

Naito H., Furukawa Y., Chino D., Yamada C., Hashimoto K. Effects of zatebradine and propranolol on canine ischemia and reperfusion-induced arrhythmias, *Eur J Pharmacol* 388 (2000), pp. 171ó176.

Nalivaiko E., Catcheside P.G., Adams A., Jordan A.S., Eckert D.J., McEvoy R.D. Cardiac changes during arousals from non-REM sleep in healthy volunteers, *Am J Physiol* 292 (2007), pp. R1320óR1327.

Nalivaiko E., Ootsuka Y., Blessing W.W. Activation of 5-HT1A receptors in the medullary raphe reduces cardiovascular changes elicited by acute psychological and inflammatory stresses in rabbits, *Am J Physiol* 289 (2005), pp. R596óR604.

Nalivaiko E., De Pasquale C.G., Blessing W.W. Ventricular arrhythmias triggered by alerting stimuli in conscious rabbits pre-treated with dofetilide, *Basic Res Cardiol* 99 (2004), pp. 142ó151.

Nalivaiko E., De Pasquale C.G., Blessing W.W. Electrocardiographic changes associated with the nasopharyngeal reflex in conscious rabbits: vago-sympathetic co-activation, *Autonom Neurosci* 105 (2003), pp. 101ó104.

Ngampramuan S., Baumert B., Kotchabhakdi N., Nalivaiko E. Activation of 5-HT1A receptors attenuates tachycardia induced by restraint stress in rats, *Am J Physiol* 294 (2008), pp. R132óR141.

Ootsuka Y., Heidbreder C.A., Hagan J.J., Blessing W.W. Dopamine D2 receptor stimulation inhibits cold-initiated thermogenesis in brown adipose tissue in conscious rats, *Neuroscience* 147 (2007), pp. 127ó135.

Ootsuka Y., Blessing W.W. Activation of 5-HT1A receptors in rostral medullary raphe inhibits cutaneous vasoconstriction elicited by cold exposure in rabbits, *Brain Res* 1073ó1074 (2006), pp. 252ó261

Rozanski A., Blumenthal J.A., Kaplan J. Impact of psychological factors on the pathogenesis of cardiovascular disease and implications for therapy, *Circulation* 99 (1999), pp. 2192ó2217.

Sawaki S., Furukawa Y., Inoue Y., Oguchi T., Chiba S. Zatebradine attenuates cyclic AMP-related positive chronotropic but not inotropic responses in isolated, perfused right atria of the dog, *Clin Exp Pharmacol Physiol* 22 (1995), pp. 29ó34.

Sgoifo A., Pozzato C., Costoli T., Manghi M., Stilli D., Ferrari P.F., Ceresini G., Musso E. Cardiac autonomic responses to intermittent social conflict in rats, *Physiol Behav* 73 (2001), pp. 343ó349

Sgoifo A., De Boer S.F., Buwalda B., Korte-Bouws G., Tuma J., Bohus B., Zaagsma J., Koolhaas J.M. Vulnerability to arrhythmias during social stress in rats with different sympathovagal balance, *Am J Physiol* 275 (1998), pp. H460óH466.

Sgoifo A., de Boer S.F., Westenbroek C., Maes F.W., Beldhuis H., Suzuki T., Koolhaas J.M. Incidence of arrhythmias and heart rate variability in wild-type rats exposed to social stress, *Am J Physiol* 273 (1997), pp. H1754ó H1760.

Sgoifo A., Stilli D., Medici D., Gallo P., Aimi B., Musso E. Electrode positioning for reliable telemetry ECG recordings during social stress in unrestrained rats, *Physiol Behav* 60 (1996), pp. 1397ó1401.

Stein P., Bosner M., Kleiger R., Conger G. Heart rate variability: a measure of cardiac autonomic tone, *Am Heart J* 127 (1994), pp. 1376ó1381.

Stoschitzky K., Stoschitzky G., Wonisch M., Brussee H. Differential effects of urapidil and doxazosin on heart rate, *Eur J Clin Pharmacol* 63 (2007), pp. 259ó262.

Tardif J.C., Ford I., Tendera M., Bourassa M.G., Fox K., Investigators I. Efficacy of ivabradine, a new selective I(f) inhibitor, compared with atenolol in patients with chronic stable angina.[see comment], *Eur Heart J* 26 (2005), pp. 2529ó2536

Thor K.B., Blitz-Siebert A., Helke C.J. Discrete localization of high-density 5-HT1A binding sites in the midline raphe and parapyramidal region of the ventral medulla oblongata of the rat, *Neurosci Lett* 108 (1990), pp. 249ó254.

Tricklebank M., Forler C., Fozard J. The involvement of subtypes of the 5-HT1 receptor and of catecholaminergic systems in the behavioural response to 8-hydroxy-2-(di-n-propylamino)tetralin in the rat., *Eur J Pharmacol* 106 (1984), pp. 271ó282.

Van der Stroom J.G., Van Wezel H.B., Koolen J.J., Visser C.A., Van Zwieten P.A. Comparison of the effects of urapidil and nitroprusside on hemodynamics and myocardial function in hypertension following cardiac surgery, *Blood Pressure Suppl* 4 (1994), pp. 31ó38.

Walker M.J., Curtis M.J., Hearse D.J., Campbell R.W., Janse M.J., Yellon D.M., Cobbe S.M., Coker S.J., Harness J.B., Harron D.W. The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion, *Cardiovascular Research* 22 (1988), pp. 447ó455

Zaretsky D.V., Zaretskaia M.V., Samuels B.C., Cluxton L.K., DiMicco J.A. Microinjection of muscimol into raphe pallidus suppresses tachycardia associated with air stress in conscious rats, *J Physiol* 546 (2003), pp. 243ó250

CHAPTER 5

Cardiac stress responsivity in mice lacking 5-HT$_{1A}$ receptors

Francesca Mastorci[1], Luca Carnevali[1], Enrica Audero[2], Alessandro Bartolomucci[3], Cornelius Gross[2], Andrea Sgoifo[1]

[1] Stress Physiology Lab., Dept. of Evolutionary and Functional Biology, University of Parma, Italy
[2] Mouse Biology Unit, European Molecular Biology Laboratory (EMBL), Rome, Italy
[3] Dept. of Evolutionary and Functional Biology, University of Parma, Italy

Chapter 5

1. Introduction

2. Methods

2.1 Animals and housing

2.2 Radiotelemetry system

2.3 Surgery: transmitter implantation

2.4 Experimental protocol

2.4.1 Chronic Psychosocial Challenge

2.5 Acquisition and analysis of heart rate, body temperature, and physical activity data

2.6 Long-term effects on heart rate, body temperature, and physical activity

2.7 Adrenal weight

2.8 Statistical analysis

3. Results

3.1 Administration of 5-HT$_{1A}$ agonist (8-OH-DPAT)

3.2 Acute cardiac stress reactivity during Restraint test before and after Chronic Psychosocial Stress

3.2.1 Restraint test associated to saline injection

3.2.2 Restraint test associated to atenolol injection

3.2.3 Restraint test associated to scopolamine injection

3.3 Acute cardiac stress reactivity on the 1st, 8th, and 15th day of Chronic Psychosocial Stress

3.4 Chronobiological effects of Chronic Psychosocial Stress

3.5 Sporadic autonomic crises in Knockout mice

3.6 Sucrose Preference Test

4. Discussion

References

ABSTRACT

Stress-induced sympathetic activation may contribute significantly to cardiac morbidity. The central mechanisms linking stress, sympathetic hyperactivity and cardiac dysfunction are not fully understood, although abnormal functioning of serotonin 5-HT$_{1A}$ somatodendritic receptors in the lower brain stem seems to represent an important pathophysiological substrate. Previous studies in rats indicated that the activation of these receptors in the brain stem attenuates heart rate and blood pressure response to stressors, as well as cardiac arrhythmia susceptibility, supporting their crucial role in moderating stress-induced sympathetic activation. The present study made use of a mouse knockout for serotonin 1A receptors and was aimed at further exploring the role of these receptors in the presympathetic modulation of acute and long-term cardiac stress responses to both brief and long-lasting challenges. Male KO (n=22) and WT (n=17) mice were implanted with transmitters for ECG, temperature (T) and activity (Act) recordings and subjected to chronic social stress (CSS), consisting in 15 consecutive days of adverse sensory contact with an aggressive male and 5-min defeat episodes on day 1, 2, 3, 8, and 15. Before CSS, mice were injected with 8-OH-DPAT, a 5-HT$_{1A}$ receptor agonist and cardiac autonomic responsivity examined. Before and after CSS, all mice were submitted to (i) a sucrose preference test to establish edhonic behavior, (ii) a restraint test associated with vehicle injection and sympathetic or vagal pharmacological blockade to evaluate sympathovagal balance. Heart rate (HR), T, and Act were sampled around-the-clock in order to assess their circadian rhythmicity, before and during the CSS. From ECGs, cardiac autonomic balance was quantified via simple indexes of heart rate variability (average R-R interval and r-MSSD). At sacrifice, the adrenals were weighed and the hearts removed for morphometrical analysis. Mice lacking 5-HT$_{1A}$ receptors did not differ from WT counterparts in cardiac stress reactivity during agonistic interactions 1, 8 and 15. However, KO mice showed a higher tachycardic stress response and a larger reduction of vagal modulation during the restraint test associated with vehicle injection, both before and after psychosocial stress. In addition, following 8-OH-DPAT administration, KOs exhibited higher heart rates and lower values of vagal activity index. No significant differences were observed for circadian rhythmicity of HR, T, and Act in baseline conditions. However, CSS determined a reduction in the amplitude of HR rhythm that was larger in KO mice during the second week as compared to WTs. These results suggest that the deletion of 5-HT$_{1A}$ receptors enhances stress-induced tachycardia and reduces vagal modulation of heart rate; in addition, it increases the susceptibility to alterations in circadian rhythmicity of heart rate during a chronic psychosocial challenge.

1. Introduction

Recent studies provide clear and convincing evidence that psychosocial factors contribute significantly to cardiac morbidity and mortality (Rozanski et al., 1999). In particular, psychological stress elicits sympathetically mediated tachycardic response, but the central mechanisms generating increases in cardiac sympathetic activity remain partly uncertain (Dampney et al., 2002). It is of potential clinical relevance to suppress deleterious increases in cardiac sympathetic activity at its origin, in the brain. Few attempts have been made to reach this aim, mainly due to lack of knowledge of the localization and pharmacological sensitivity of presympathetic cardiomotor neurons. Recent evidences indicated that the final medullary relay for the descending pathways that activate the heart during stress is located in the raphe/parapyramidal area and that relevant cardiomotor neurons are sensitive to, and could be inhibited by, serotonin-1A (5-HT$_{1A}$) receptor agonists (Nalivaiko et al., 2005; Zaretsky et al., 2003). In fact, an involvement of 5-HT$_{1A}$ receptors in cardiovascular control is well documented, and the consensus is that their activation results in central sympatholitic effects (McCall and Clement, 1994; Ramage, 2001). In order to reveal location of 5-HT$_{1A}$ receptors, Nalivaiko and coworkers (2005) performed brain microinjections of 8-OH-DPAT (a selective 5-HT$_{1A}$ agonist) in rats during restraint and in rabbits during airjet stress. The target for the microinjection was the medullary raphe region as evidence accumulates that this could be a location of presympathetic cardiomotor neurons, that these neurons are silent at rest but could be activated by stressors (Samuels et al., 2002; Zaretsky et al., 2003). Actually, psychological stressors facilitate serotonergic neurotrasmission in the brain (Chauloff et al., 1999). Most earlier information about cardiovascular effects of 5-HT receptor agonists and antagonist was collected either in anesthetized state or during quiet waking of experimental animals. It is only recently that several research groups focussed on 5-HT receptor-mediated cardiovascular changes in rats and rabbits undergoing stressful challenges.

In this context, two recent studies have demonstrated that the activation of 5-HT$_{1A}$ receptors attenuates cardiovascular changes elicited by psychological stresses (Nalivaiko et al., 2005; Ngampramuan et al., 2008). They found that activation of 5-HT$_{1A}$ receptors in the medullary raphe/parapyramidal region reduced tachycardic and pressor responses to psychological stressors, such as airjet stress in rabbit and restraint stress in rats.

More recently, Nalivaiko and colleagues (2009) (see chapter 4 of this thesis) established that 8-OH-DPAT is very efficient in suppressing not only tachycardia but also cardiac arrhythmias in rats subjected to social defeat stress.

In agreement with pharmacological studies, stressful stimuli (footshock and novel environment) produced larger tachycardic responses in 5-HT$_{1A}$ receptor-knockout mice compared to wild-type counterparts (Gross et al., 2000; Pattij et al., 2002). These data affirm that mice lacking the 5-HT$_{1A}$ receptor not showed changes in baseline heart rate as compared to wild type. Conversely, after a mild stressor, like a subcutaneous injection, heart rate values increased more in KO mice compared to WT mice. In addition, when these animals were exposed to a novel environment, exhibited a strong tachycardic response, with an increase of approximately 250 bpm immediately at the start of the experiment.

The present study made use of a mouse knockout for serotonin 1A receptors and was aimed at further exploring the role of these receptors in the presympathetic modulation of acute and long-term cardiac stress responses to both brief and long-lasting challenges.

2. Methods

All experimental procedures in this study were 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).

2.1 Animals and housing

We used 3-month-old male mice lacking 5-HT$_{1A}$ receptor (KO, n=22) and their corresponding wild-type controls (WT, n=17). 5-HT$_{1A}$ KOs were obtained from the Mouse Biology Unit (European Molecular Biology Laboratory, Monterotondo) and their production has been described previously (Gross, 2000; Audero et al., 2009).

Mice were housed in unisexual groups of five individuals, from weaning until the onset of the experiment, in Plexiglas cages measuring 39×23×15 cm. Before and during the experimental treatment, all the animals were kept in rooms with controlled temperature (22±2°C) and lighting (light on from 07.00 to 19.00 h). The bedding of the cages consisted of wood shavings, and food and water were freely available.

2.2 Radiotelemetry system

In the present study, radiotelemetry devices were used to record the electrocardiogram (ECG), heart rate and physical activity. The system consisted of flat transmitters measuring 20 x 10 x 8 mm (TA10ETA-F20, Data Sciences Int., St. Paul, MN, USA) and platform receivers measuring 32 x 22 x 3 cm (RPC-1, Data Sciences Int.). Telemetry signals were fed to a PC containing ART-Silver 1.10 data acquisition system (Data Sciences Int.).

2.3 Surgery: transmitter implantation

The transmitter was implanted in animals partially 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 subcutaneously in the dorsal intrascapular region and the two electrodes (wire loops) were fixed, respectively, to the dorsal surface of the xiphoid process and in the anterior mediastinum close to the right atrium. The mice were anesthetized with 2,2,2-tribromoethanol (250 mg/Kg, IP). Subsequently, the animals were individually housed in clear Plexiglas cages measuring 39 x 23 x 15 cm. After the transmitter was implanted and before any measurement was started, the mice were allowed 10 days for recovery of body weight and circadian rhythmicity of heart rate.

2.4 Experimental protocol

Adult male, Knockout (KO, n=22) and Wild Type (WT, n=17) mice were implanted with telemetry transmitters for ECG, temperature (T) and activity (Act) recordings and starting from 32 days after surgery were subjected to chronic psychosocial stress, consisting in 15 consecutive days of adverse sensory contact with an aggressive male and 5-min defeat episode on day 1, 2, 3, 8, and 15. During the pre-stress period the animals were injected with 8-OH-DPAT (0.25 mg/Kg, i.p.), a 5-HT$_{1A}$ agonist possessing central sympatholytic properties. Before and after chronic stress, mice were submitted to (i) a sucrose preference test for anhedonia, (ii) a restraint test associated with vehicle as well as metylscopolamine (0.1 mg/Kg, i.p.) or atenolol (1 mg/Kg, i.p.). At sacrifice, adrenal glands were removed and weighed. The experimental design is demonstrated in Figure1.

2.4.1 Chronic Psychosocial Stress

The procedure is a modified version of the standard procedure described by Bartolomucci et al. (2001). On the first day of chronic psychosocial stress each instrumented mouse played the role of the intruder and was placed into the cage of a resident male having equal or larger body weight. The residents were housed with a female and for one week were trained to aggressively defend their own territory. After the interaction, the two animals were divided by means of a perforated polystyreneómetal partition allowing a continuous visualóolfactoryóacoustic contact, but hampering agonistic interaction. The partition was again removed for 5 min on day 1, 2, 3, 8, and 15 at an unpredictable time between 09:00 and 12:00 h, i.e. in the initial part of the light phase. The interaction lasted 5 min starting from the first attack by the resident animal.
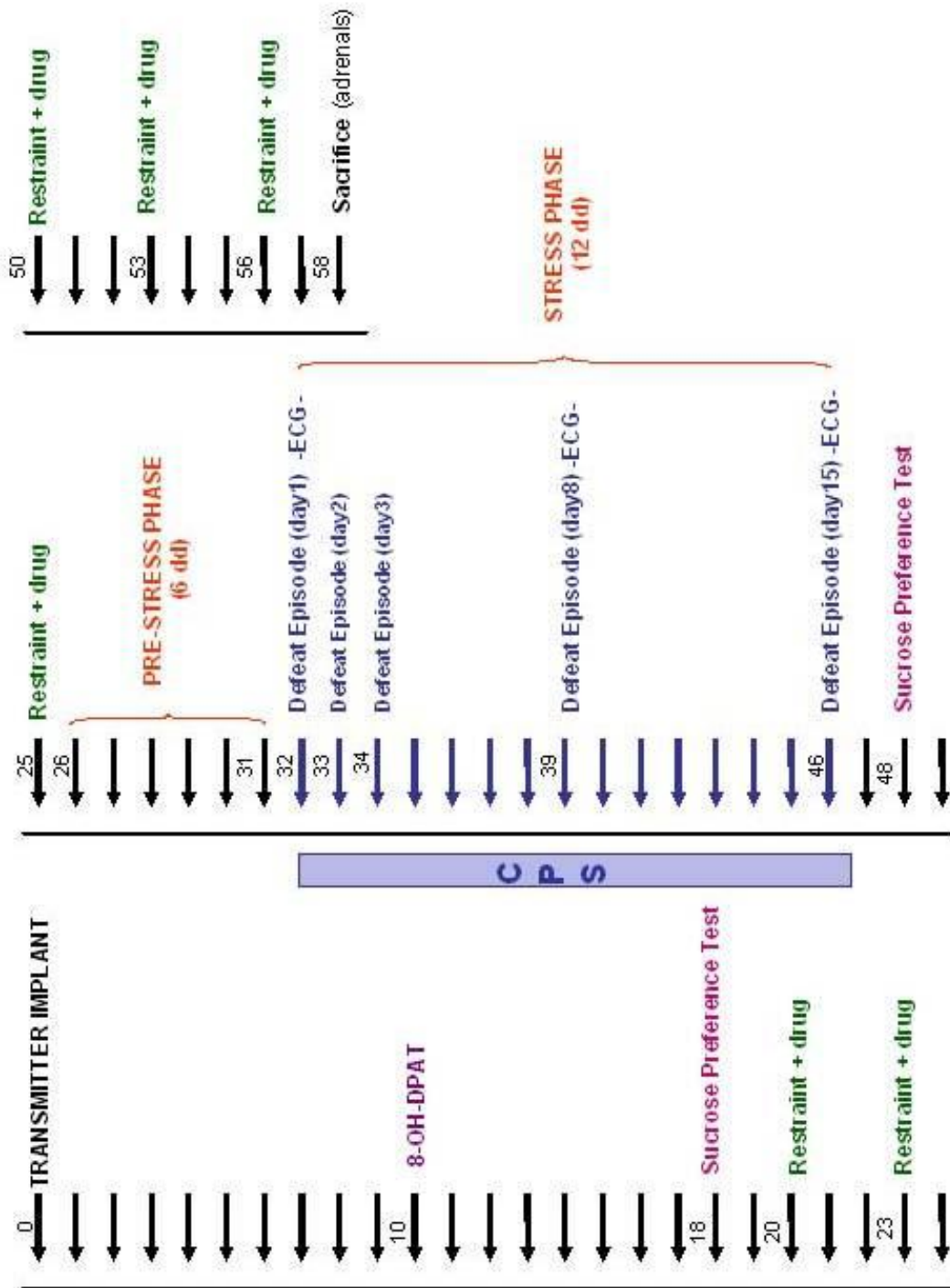
Figure 1. Timeline of procedures used in the current study. CPS: Chronic Psychosocial Stress

2.5 Acquisition and analysis of heart rate, body temperature, and physical activity data

Electrocardiograms, body temperature, and physical activity were recorded during:

(i) Day 1, 8 and 15 defeat episodes in three recording periods: Baseline (15 min, animals undisturbed in their home cages before partition removal), Test (5 min, after partition removal), and Post-test (15 min, animals again separated by the partition).

(ii) Restraint test associated with vehicle injection as well as sympathetic or vagal pharmacological blockade in four periods*:* Baseline (30 min), Post injection (15 min), Restraint (15 min), and Post-Test (30 min)

(iii) 8-OH-DPAT in two periods: Baseline (30 min) and Post Injection (45 min)

Offline analysis was performed by means of a software package developed in our lab (XRRECG) for quantification of time-domain indexes of heart rate variability (Sgoifo et al., 2001). The following parameters were quantified: (i) the mean RóR interval duration as a measure of heart rate (RR, ms), and (ii) the root-mean-square of successive RóR interval differences (r-MSSD, ms). R-MSSD reflects the average 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.

2.6 Long-term effects on heart rate, temperature, and physical activity

HR (expressed as bpm), body temperature (°C) and locomotor activity (counts/min, cpm) were sampled around-the-clock for 120 s every 60 min in the two following phases : (i) Pre-stress ó starting 6 days before chronic psychosocial stress with the animal in its own home cage, (ii) Stress ó 15 days ó between the 1st and the 15th day of CPS, with the animal in the residentøs cage.

For each individual mouse the daily amplitude of the rhythm of HR, T, and physical activity was calculated as the difference between average 12-hr dark and 12-hr light values, i.e. values for circadian activity and resting phases, respectively (Meerlo et al., 1999).

2.7 Adrenal weight

At the end of the experimental protocol, mice were anesthetized with 2,2,2-tribromoethanol (as previously described), and their adrenal glands rapidly removed and weighed.

Of course, this procedure does not allow to evaluate the differential role of hypertrophy and hyperplasia in determining increased adrenal volume. In animal studies, authors usually ascribe adrenal enlargement to hypertrophic processes (Llorente et al., 2002), though no systematic histological analyses of the adrenals have been provided so far. However, it is conceivable that

glomerulosa cells are responsible for hyperplasia and a source for progenitor cells; thus, we tend to believe that short-term increase in adrenal volume to meet systemic need may be accomplished by cellular hypertrophy, whereas long-term conditions likely activate both hypertrophy and hyperplasia to maintain adrenal homeostasis.

2.8 Statistical analysis

Data were analyzed by means of SPSS 15.0 statistical package (SPSS, Chicago, IL, USA).

First, average R-R interval, r-MSSD, T and Act were quantified as:

(i) mean of each recording period during defeat episodes: Baseline (15 min), Test (5 min), and Post-test (15 min).

(ii) mean of each 15-min recording period during restraint test: Baseline (15 min), Post injection (15 min), Restraint (15 min), Post-Test1 (15 min), Post-Test2 (15 min)

(iii) mean of each 15-min recording period during 8-OH-DPAT test: Baseline (15 min), first 15-min period post-8-OH-DPAT injection, second 15-min period post-8-OH-DPAT injection, third 15- min period post-8-OH-DPAT injection

Two-way ANOVA for repeated measures was applied to 8-OH-DPAT and restraint+drugs recordings with õgroupö as between-subject factor (2 levels: knockout and wild type), õrecording periodö as within-subject factor (8-OH-DPAT- 5 levels: 1 baseline, 5 post injection; Restraint+drugs- 5 levels: 1 baseline, 1 post injection, 1 restraint, 2 recovery periods), and õgroup × recording periodö interaction. After ANOVAs, post-hoc analysis was performed when appropriate with Student t-test, after controlling for the homogeneity of variances via Levene test.

Mean values of the daily amplitude of HR, T, and Act during chronic psychosocial stress (namely days 2-7 and 9-14), were analyzed by means of 2-Way ANOVA for repeated measures, with õgroupö as between-subject factor (2 levels: KO and WT) and õrecording dayö as within-subject factors (12 levels), and õgroup × recording dayö interaction.

The percentage of sucrose preference was expressed as value before and after Chronic Psychosocial Stress. Two-way ANOVA for repeated measures was applied with õgroupö as between-subject factor (2 levels: knockout and wild type), õrecording periodö as within-subject factor (2 levels: before and after chronic stress), and õgroup × recording periodö interaction.

The comparison between KO and WT for the weight of adrenal glands, expressed as a ratio to body weight (mg/g), was performed by means of Studentøs T test.

Statistical significance for all tests was set at $p<0.05$. All parameters in figures were expressed as mean ± SEM (standard error of the mean).

3. Results

3.1 Administration of 5-HT$_{1A}$ agonist (8-OH-DPAT)

Figure 2 depicts the temporal dynamics of average R-R interval and r-MSSD values before and after injection of 8-OH-DPAT. Baseline reference value is the mean value of the fifteen 1-min time points in resting conditions, while each time point on the graphs of post injection period represents the mean value of 1-min periods.

In the first 15 minutes following 8-OH-DPAT administration, knockout mice exhibited significantly higher values of heart rate (Fig.2a) and lower values of vagal activity index (Fig.2b), as compared to WT counterparts. In fact, following the injection of 8-OH-DPAT, tachycardia quickly reverted to bradycardia associated with a significant increase in r-MSSD values in WT mice, whereas heart rate acceleration and vagal withdrawal lasted in this first 15-min post-injection period in KO mice.

a)

b)
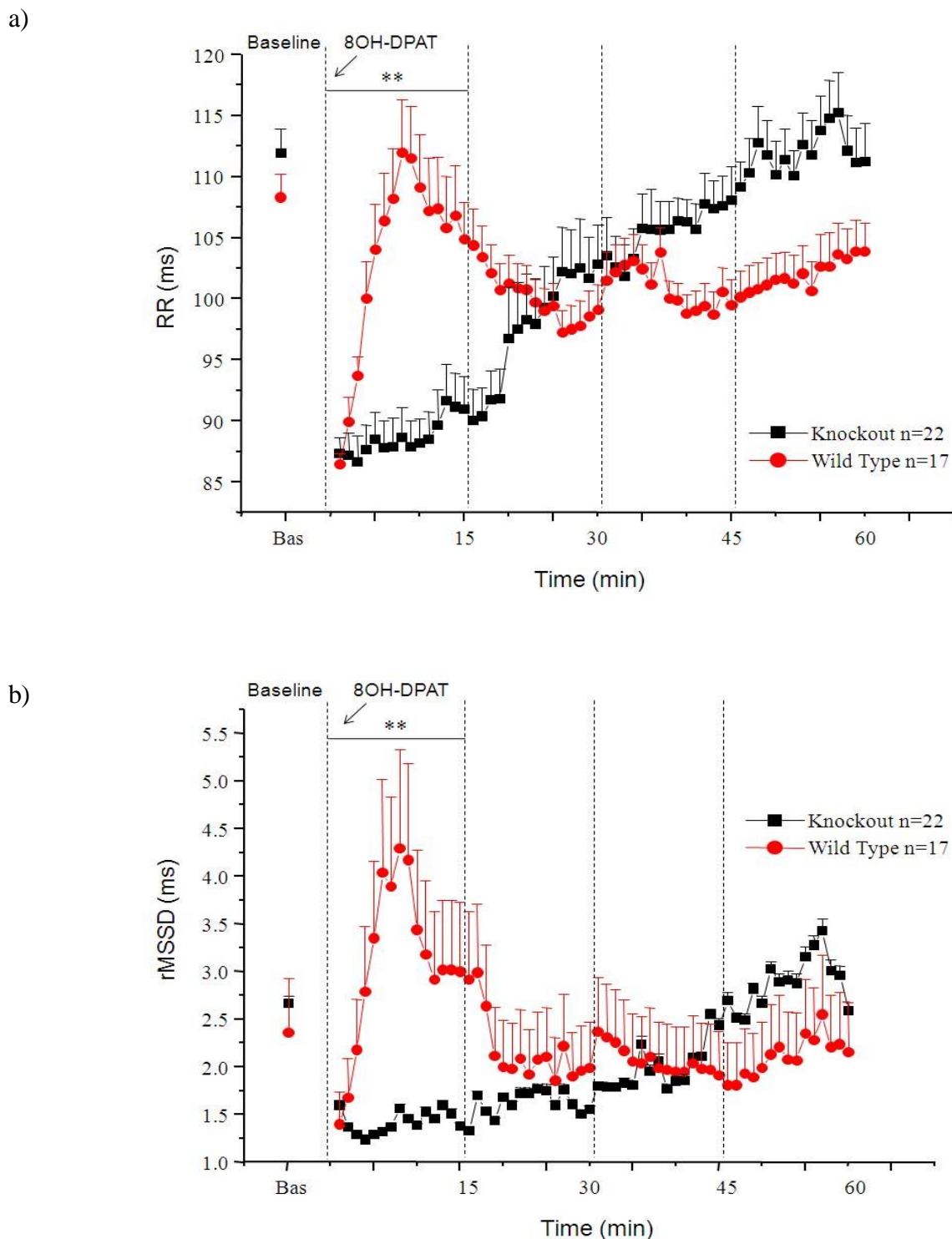
Figure 2. Time course of changes in RóR interval (RR, panel A) and root-mean-square of successive RóR interval differences (r-MSSD, panel B) in baseline conditions and after 8-OH-DPAT injection in knockout and wild type mice. Baseline reference value is the mean value of the fifteen 1-min time points in resting conditions - Statistical analysis on average values of 15-min recording periods - ** = KO significantly different from WT corresponding values (p<0.01)
ANOVAs: Effect of õrecording periodö x õgroupö interaction (RR: F=11.062, p<0.01; rMSSD: F=3.304, p=0.078) *Post-hoc*. Comparison between knockout and wild type: KO vs. WT ó RR first 15-min: t=-3.387, p<0.01; rMSSD first 15-min: t=-3.343, p<0.01.

The min-by-min time evolution of body temperature (T) values in mice exposed to 8-OH-DPAT injection is illustrated in Figure 3. Values of T were significantly higher in animals lacking 5-HT$_{1A}$ receptors in the first 30-min after agonist administration, as compared to WT counterparts. In other words, the lack of 5-HT$_{1A}$ receptors hampered the capacity of 8-OH-DPAT to prevent stress-induced hyperthermia.
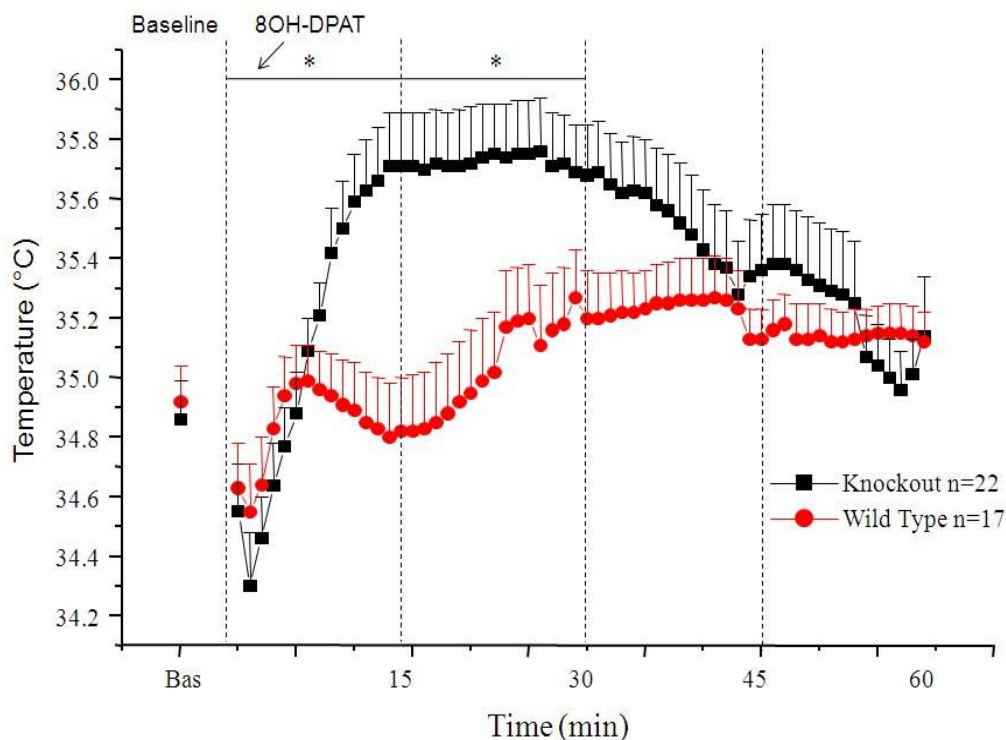
Figure 3. Time course of body temperature (T) in baseline conditions and after 8-OH DPAT injection in knockout and wild type mice. Baseline reference value is the mean value of the fifteen 1-min time points in resting conditions - Statistical analysis on average values of 15-min recording periods - * = KO significantly different from WT corresponding values (p<0.05)
ANOVAs: Significant effect of õrecording periodö (T: F=15.888, p<0.01) ***Post-hoc***. Comparison between knockout and wild type: KO vs. WT ó T first 15-min: t=2.148, p<0.05; second 15-min: t=2.631, p<0.05.

3.2 Acute cardiac stress reactivity during Restraint test before and after Chronic Psychosocial Stress

3.2.1 Restraint test associated to saline injection

Figure 4 depicts min x min time evolution of average R-R interval, r-MSSD, and body temperature during restraint test associated with saline injection before chronic psychosocial stress.

Baseline values of all parameters were not significantly different between the two groups of mice (Fig.4). Vehicle injection provoked a short-lasting tachycardia, especially in KO mice that had significantly lower values of RR in the first 5-min after injection (Fig.4a). In KO mice the rMSSD values were significantly reduced throughout the post injection period (Fig.4b). Knockout mice did not differ from wild type counterparts during restraint test; however, during first 15-min of post-test (post-test 1), mice lacking 5-HT_{1A} receptors exhibited significantly lower r-MSSD values as compared to WTs.

The exposure to restraint test induced significantly larger values of body temperature in knockout compared to wild type mice, involving the restraint and post-test 1 period (Fig.4c).

Figure 5 depicts min x min time evolution of average R-R interval, r-MSSD, and boby temperature during restraint stress associated with saline injection after chronic psychosocial stress.

Baseline values of the two parameters were similar between the two groups of mice (Fig.5); however, vehicle injection induced significantly lower values of RR (Fig.5a) and rMSSD (Fig.5b). In addition, values of both parameters in KO mice were significantly lower compared to WT corresponding values during the test and first 15-min of Post-Test period.

On the contrary, there were no significant differences between groups for body temperature (Fig.5c).
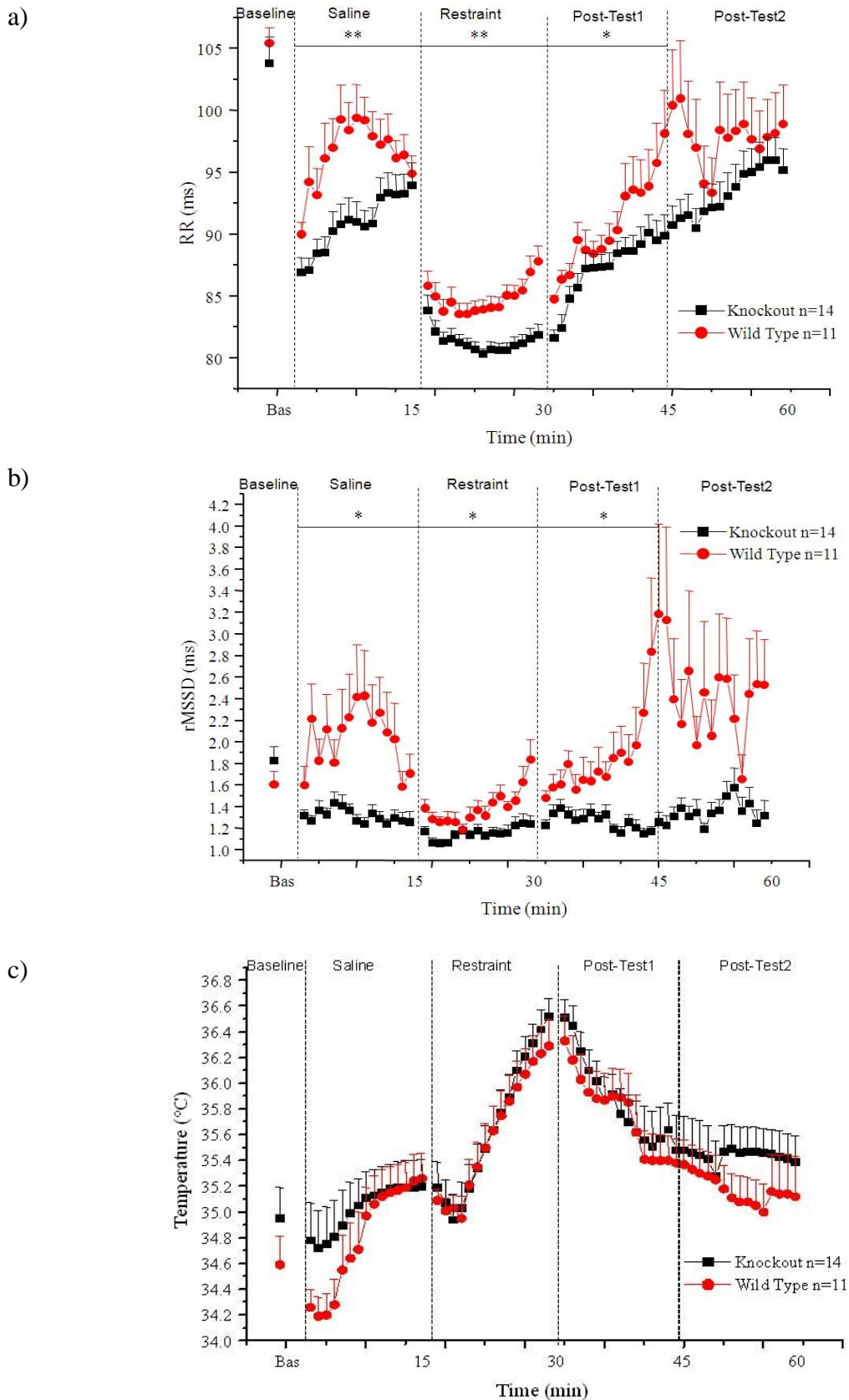
a)

b)

c)

Figure 4. Time course of changes in RóR interval (RR, panel A), root-mean-square of successive RóR interval differences (r-MSSD, panel B), and body temperature (panel C) in baseline conditions, after drug injection (saline), during restraint test (restraint), and after stress (Post-Test1 and Post-Test2) in ko and wt

184

mice before Chronic Psychosocial Stress. Baseline reference value is the mean value of the fifteen 1-min time points in resting conditions- Statistical analysis on average values of 15-min recording periods - * = KO significantly different from WT corresponding values (p<0.01)

ANOVAs: Significant effects of õrecording periodö (RR: F=16.713, p<0.01) õgroupö (RR: F=5.173, p<0.05), and õrecording period x groupö interaction (rMSSD: F=12.006, p<0.01)

Post-hoc. Comparison between knockout and wild type: KO vs. WT ó Post Injection: RR: t=-2.840, p<0.01; rMSSD: t=-2735, p<0.05; Restraint: RR: t=-3.797, p<0.01, rMSSD: =-2.558, p<0.05; Post-Test1: RR: t=-2.152, p<0.05; rMSSD: t=-2.945, p<0.05

a)

b)

c)

Figure 5. Time course of changes in RóR interval (RR, panel A), root-mean-square of successive RóR interval differences (r-MSSD, panel B), and body temperature (panel C) in baseline conditions, after drug injection (saline), during restraint test (restraint), and after stress (Post-Test1 and Post-Test2) in ko and wt mice after Chronic Psychosocial Stress. Baseline reference value is the mean value of the fifteen 1-min time

points in resting conditions - Statistical analysis on average values of 15-min recording periods - * = KO significantly different from WT corresponding values (*=p<0.05; **=p<0.01)

ANOVAs: Significant effects of õrecording periodö (RR: F=16.713, p<0.01) õgroupö (RR: F=5.173, p<0.05), and õrecording period x groupö interaction (rMSSD: F=12.006, p<0.01)

Post-hoc. Comparison between knockout and wild type: KO vs. WT ó Post Injection: RR: t=-2.840, p<0.01; rMSSD: t=-2.735, p<0.05; Restraint: RR: t=-3.797, p<0.01, rMSSD: =-2.558, p<0.05; Post-Test1: RR: t=-2.152, p<0.05; rMSSD: t=-2.945, p<0.05.

3.2.2 Restraint test associated to atenolol injection

Figure 6 displays min x min time evolution of average R-R interval, r-MSSD, and body temperature during restraint test associated with atenolol injection before chronic psychosocial stress.

Baseline values of all parameters were not significantly different between the two groups of mice (Fig.6). After atenolol injection, KO mice exhibited lower values of RR (Fig.6a) and rMSSD (Fig.6b) which lasted throughout the post injection period. During restraint test Knockout mice did not differ from wild type counterparts during restraint test; however, during first 5-min, mice lacking 5-HT$_{1A}$ receptors exhibited significantly lower RR values as compared to WTs.

Apart from very few time point, the exposure to restraint test associated with sympathetic blockade, induced significantly larger values of body temperature in knockout compared to wild type mice, involving the post injection, restraint, and post-test 2 periods (Fig.6c).

Figure 7 describes min x min time evolution of average R-R interval, r-MSSD, and body temperature during restraint test associated with sympathetic blockade after chronic psychosocial stress.

Again, baseline values of all parameters were similar between the two groups of mice (Fig.7); also, except a slight tendency during post-test 1 where KO mice showed lower values of RR (Fig.7a), there were no significant differences between groups in all parameters considered (Fig.7a, b, c).
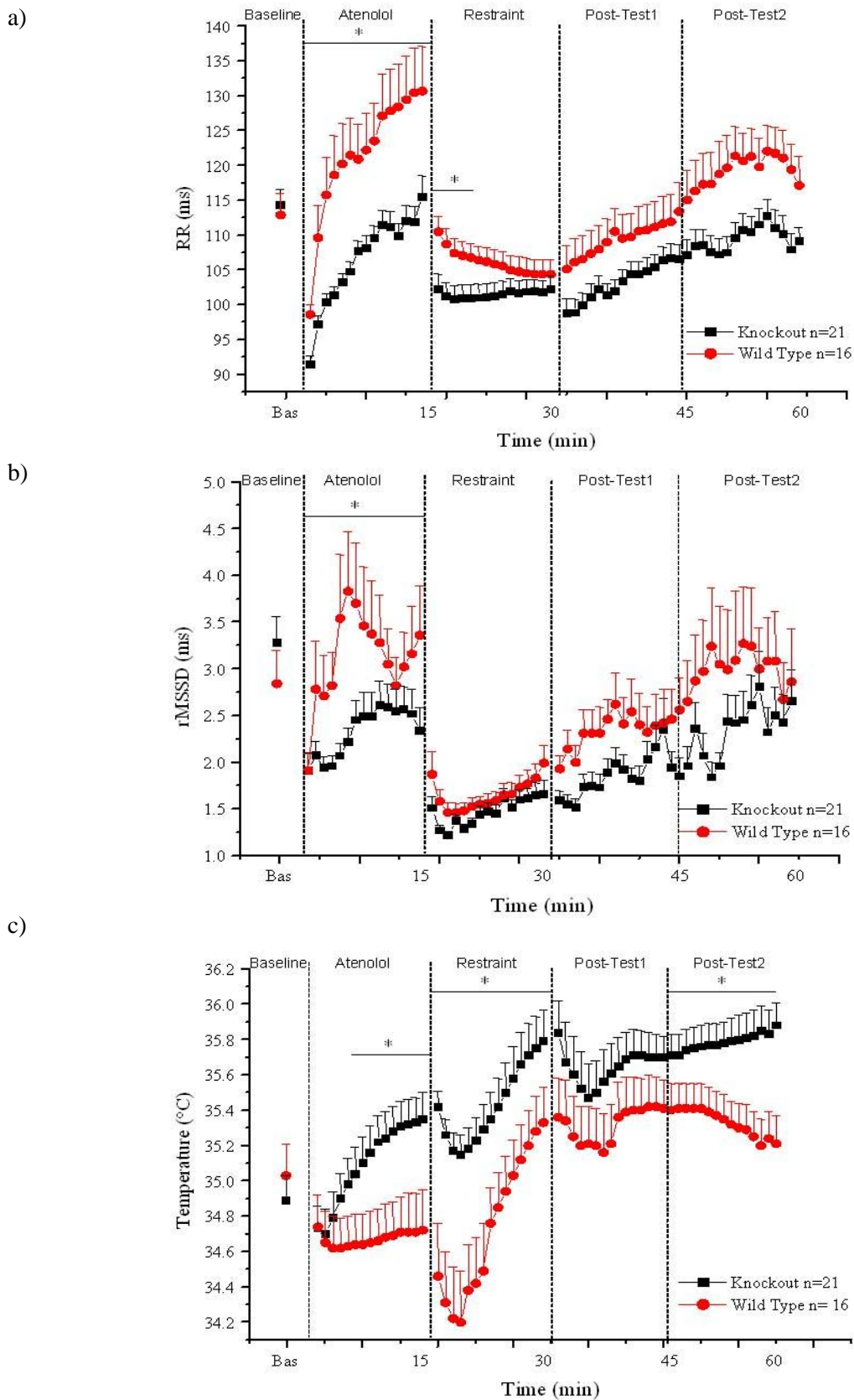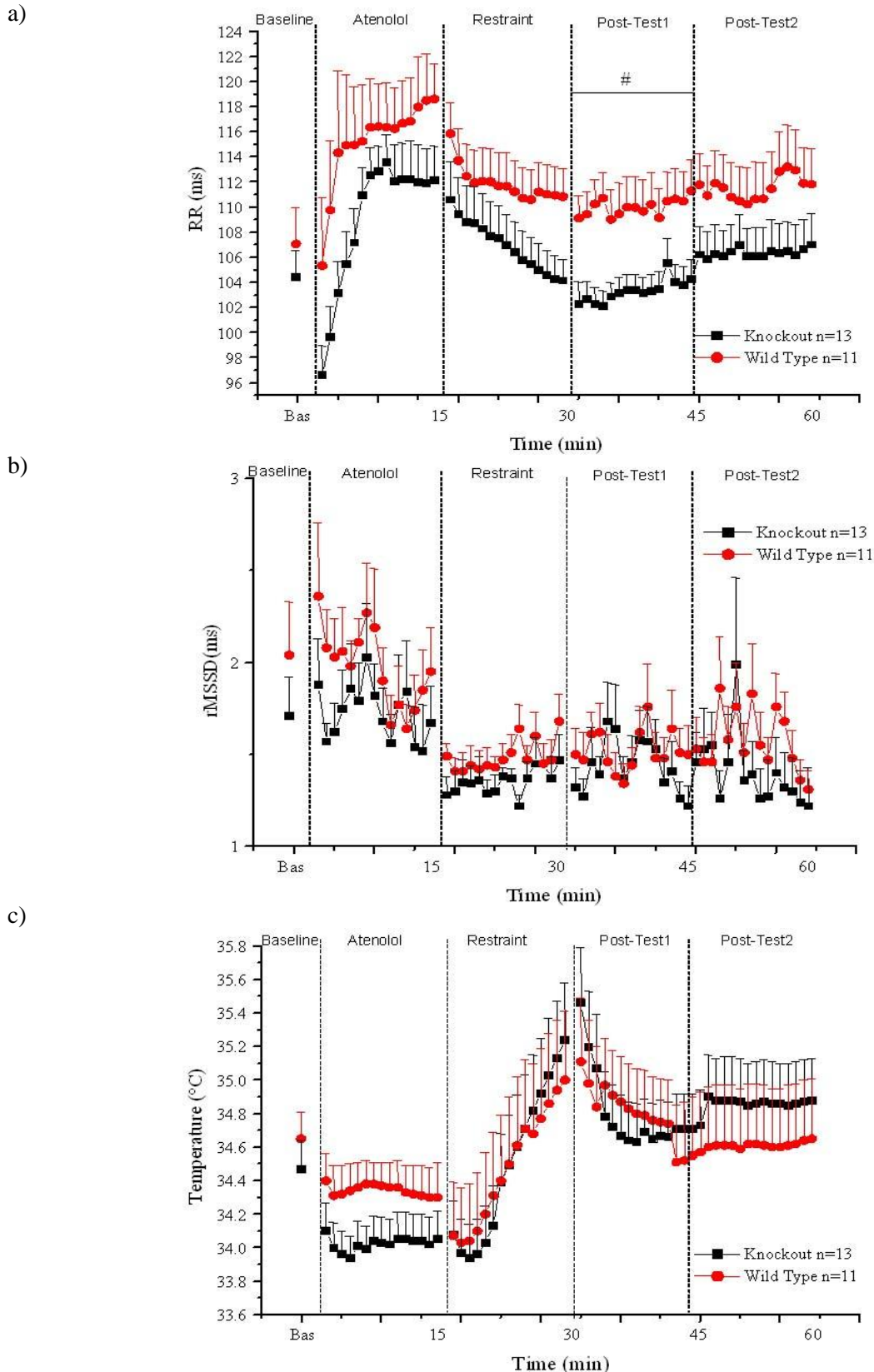
a)

b)

c)

Figure 6. Time course of changes in RóR interval (RR, panel A), root-mean-square of successive RóR interval differences (r-MSSD, panel B), and body temperature (panel C) in baseline conditions, after drug injection (atenolol), during restraint test (restraint), and after stress (Post-Test1 and Post-Test2) in ko and wt mice before Chronic Psychosocial Stress. Baseline reference value is the mean value of the fifteen 1-min

time points in resting conditions- Statistical analysis on average values of 15-min recording periods - * = KO significantly different from WT corresponding values (p<0.05)

ANOVAs: Effects of õrecording periodö (RR: F=3.524, p=0.070; T: F=48.663, p<0.01) õgroupö (T: F=3.538, p=0.068), and õrecording period x groupö interaction (rMSSD: F=3.629, p=0.066)

Post-hoc. Comparison between knockout and wild type: KO vs. WT ó Post Injection: RR: t=-2.680, p<0.05; rMSSD: t=-2.259, p<0.05; Restraint: RR first 5-min: t=-3.002, p<0.01, T: =2.789, p<0.05; Post-Test2: T: t=-2.315, p<0.05.
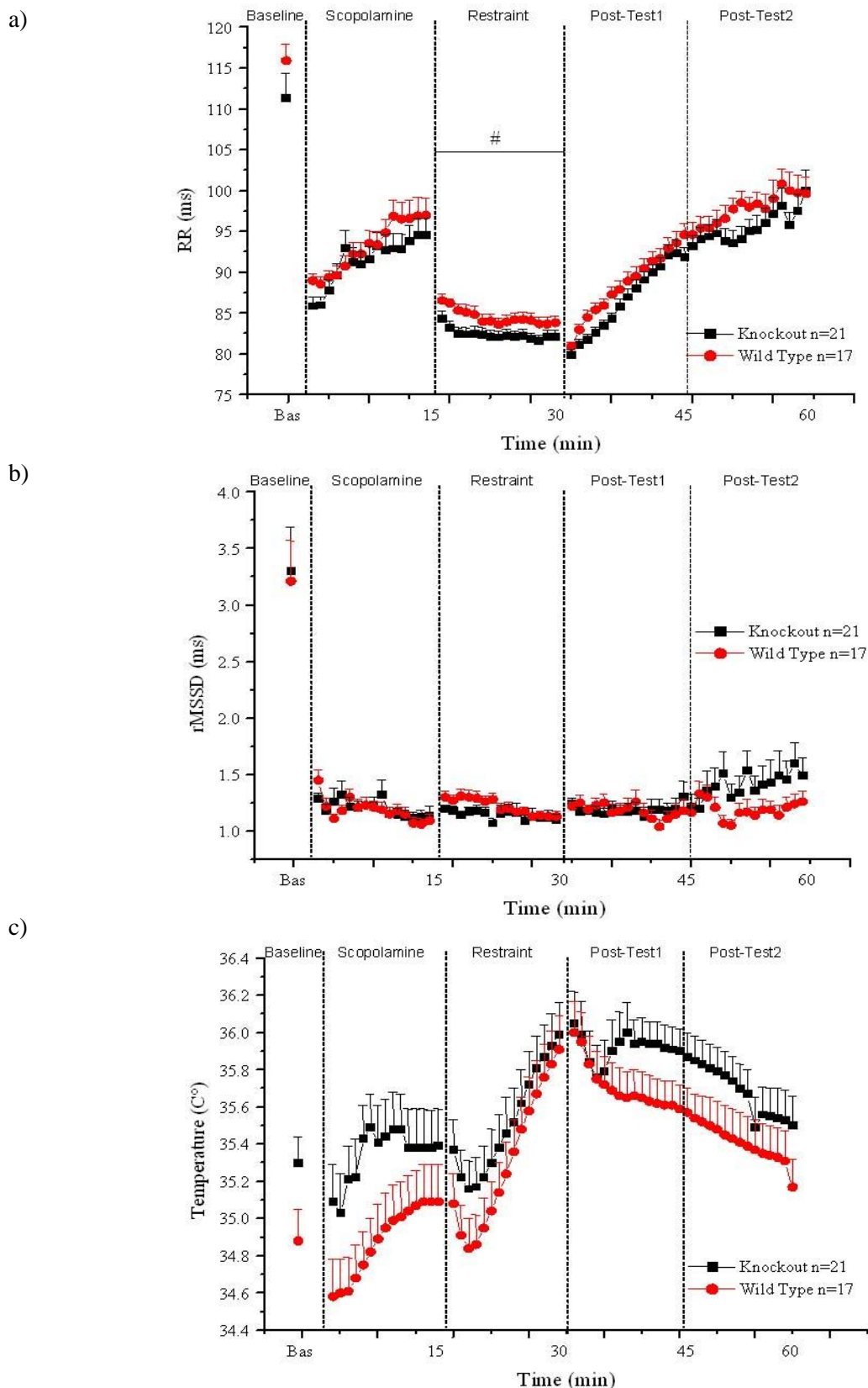
a)

b)

c)

Figure 7. Time course of changes in RóR interval (RR, panel A), root-mean-square of successive RóR interval differences (r-MSSD, panel B), and body temperature (panel C) in baseline conditions, after drug injection (atenolol), during restraint test (restraint), and after stress (Post-Test1 and Post-Test2) in ko and wt mice after Chronic Psychosocial Stress. Baseline reference value is the mean value of the fifteen 1-min time points in resting conditions- Statistical analysis on average values of 15-min recording periods - # = KO significantly different from WT corresponding values (p=0.060)

3.2.3 Restraint test associated to scopolamine injection

Figure 8 illustrates min x min time evolution of average R-R interval, r-MSSD, and body temperature during restraint test associated with vagal blockade before chronic psychosocial stress.

In general, values of all parameters were not significantly different between the two groups of mice (Fig.8a,b,c) in all recording periods, except for a slight tendency during restraint test for RR values (Fig.8a).

Figure 9 depicts min x min time evolution of average R-R interval, r-MSSD, and body temperature during restraint stress associated with scopolamine injection after chronic psychosocial stress.

Baseline values of all parameters were not significantly different between the two groups of mice (Fig.9). After injection of vagal blockade, both groups had developed a similar gradual reduction of RR (Fig.9a) and rMSSD values (Fig.9b). However, during recovery period (Post-test1 and Post-Test2) KO mice exhibited significantly lower values of RR (Fig.9a).

As expected, stress injection and restraint test caused an increase in body temperature values, but no significant differences between groups were found (Fig.9c).
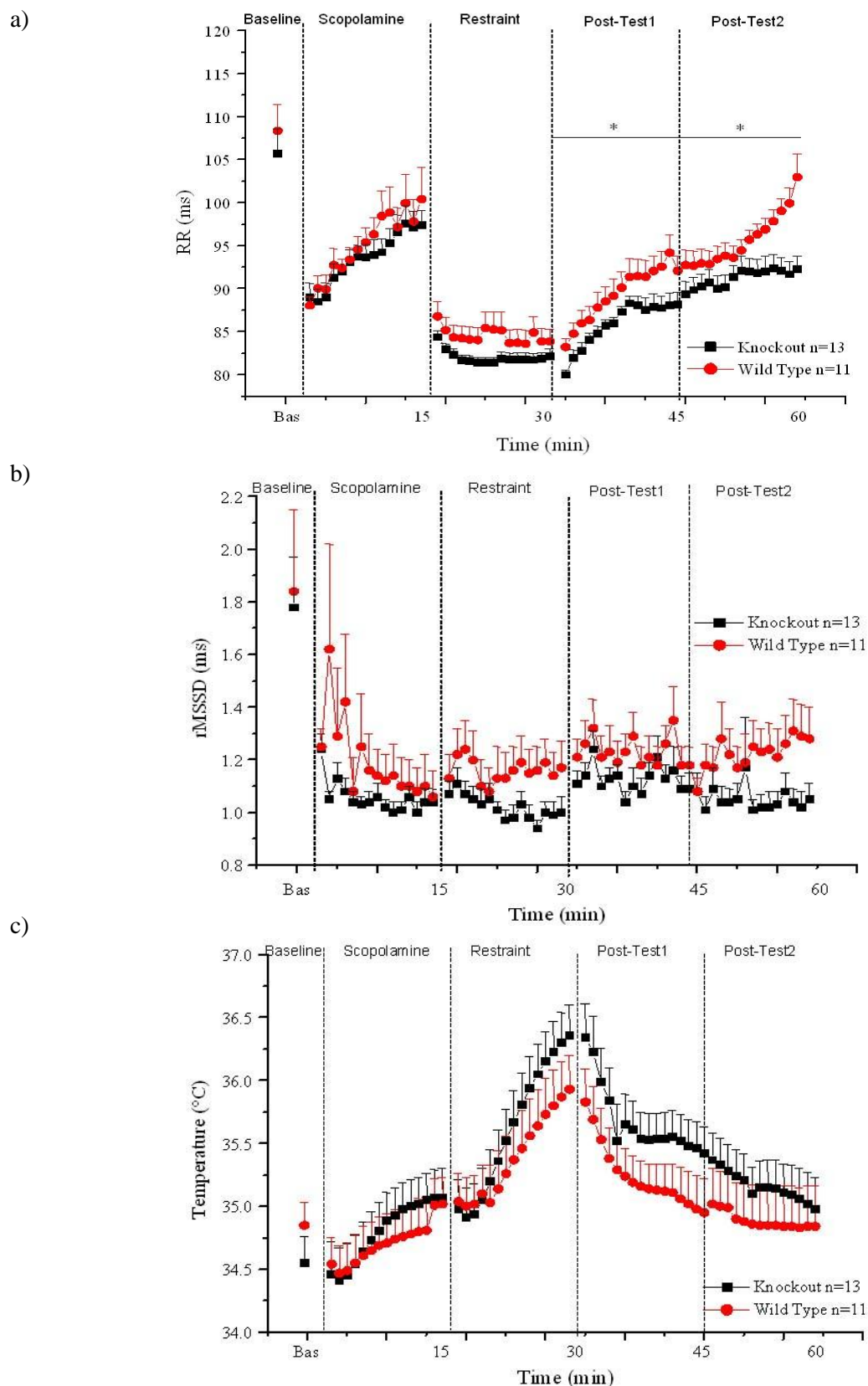
a)

b)

c)

Figure 8. Time course of changes in RóR interval (RR, panel A), root-mean-square of successive RóR interval differences (r-MSSD, panel B), and body temperature (panel C) in baseline conditions, after drug injection (scopolamine), during restraint test (restraint), and after stress (Post-Test1 and Post-Test2) in ko and wt mice before Chronic Psychosocial Stress. Baseline reference value is the mean value of the fifteen 1-min time points in resting conditions- Statistical analysis on average values of 15-min recording periods - # = KO significantly different from WT corresponding values (p=0.056)

ANOVAs: Effects of õrecording periodö (RR: F=95.244, p<0.01; rMSSD: F=47.603, p<0.01; T: F=24.749, p<0.01)
Post-hoc. Comparison between knockout and wild type: KO vs. WT ó Restraint: RR: t=-1.978, p=0.056

a)

b)

c)

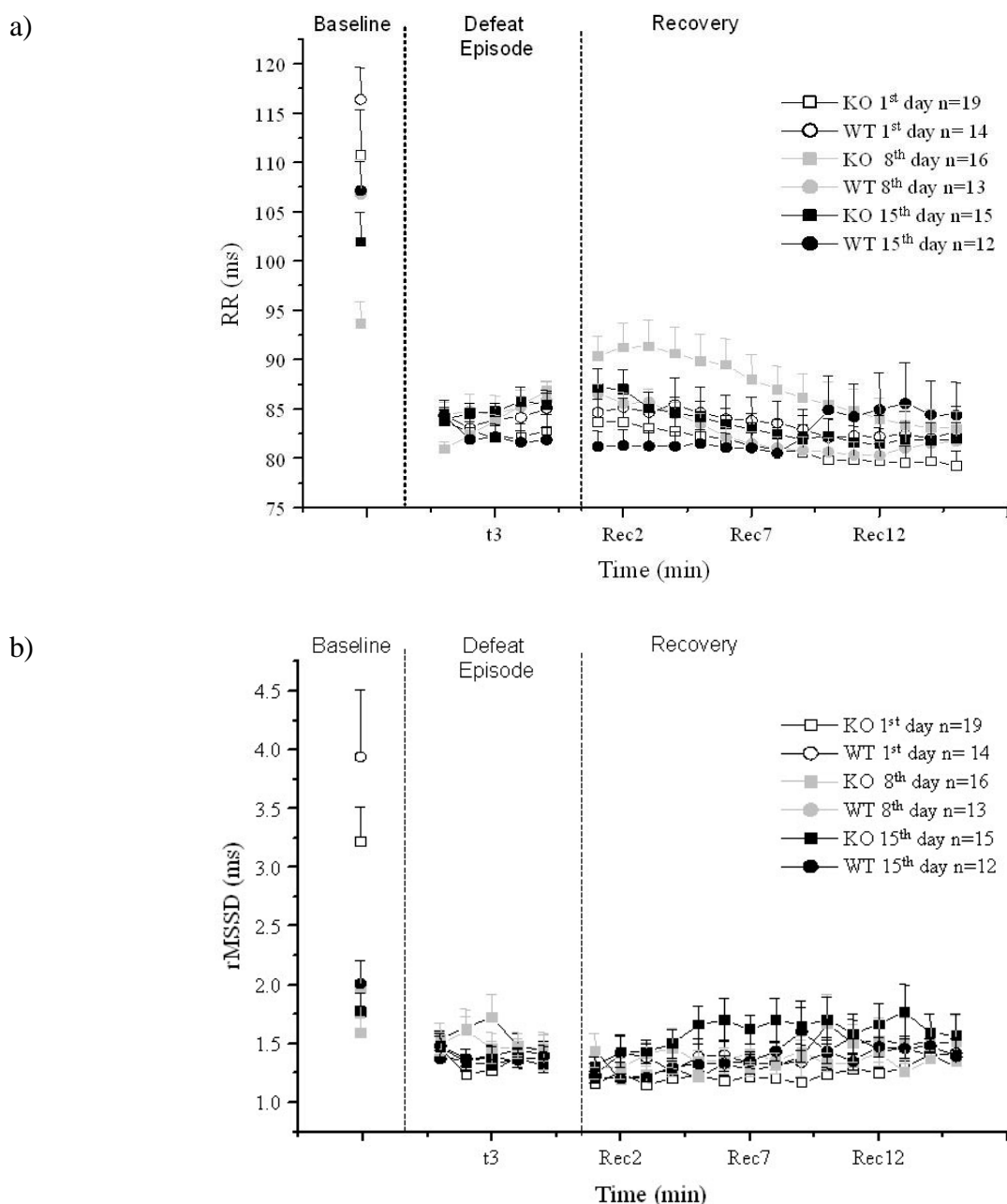**Figure 9.** Time course of changes in RóR interval (RR, panel A), root-mean-square of successive RóR interval differences (r-MSSD, panel B), and body temperature (panel C) in baseline conditions, after drug injection (scopolamine), during restraint test (restraint), and after stress (Post-Test1 and Post-Test2) in ko and wt mice after Chronic Psychosocial Stress. Baseline reference value is the mean value of the fifteen 1-min time points in resting conditions- Statistical analysis on average values of 15-min recording periods - * = KO significantly different from WT corresponding values (p<0.05)

ANOVAs: Significant effects of õrecording periodö (RR: F=36.781, p<0.01; rMSSD: F=10.150, p<0.01; T: F=9.882, p<0.01)
Post-hoc. Comparison between knockout and wild type: KO vs. WT ó Post-Test1: RR: t=-2.250, p<0.05; Post-Test2: RR: t=-2.316, p<0.05

3.3 Acute cardiac stress reactivity on the 1st, 8th, and 15th day of Chronic Psychosocial Stress

Figure 10 reports the time evolution of average R-R interval (Fig.10a) and R-R interval variability parameter (r-MSSD) (Fig.10b) on 1st, 8th, and 15th day of Chronic Psychosocial Stress. No significant differences between knockout and wild type mice were found for cardiac reactivity during defeat episodes occurring on day 1, 8, and 15 of chronic stress.

Figure 10. Time course of changes in R-R interval (RR, panel A) and root-mean-square of successive R-R interval differences (r-MSSD, panel B) during the defeat episode on day 1, 8, and 15 in KO and WT mice. Baseline reference value is the mean value of the fifteen 1-min time points in resting conditions.

3.4 Chronobiological effects of Chronic Psychosocial Stress

During Chronic Psychosocial Stress, knockout mice exhibited reduced daily amplitude of the rhythm of heart rate (HR) and body temperature (T) as compared to wild type counterparts and Pre-Stress phase (Fig.11a, b). In particular, the comparison between the two groups revealed that the amplitude of HR and T rhythms was significantly lower in KOs in the second week of chronic social stress (between defeat episodes occurring on day 8 and 15).
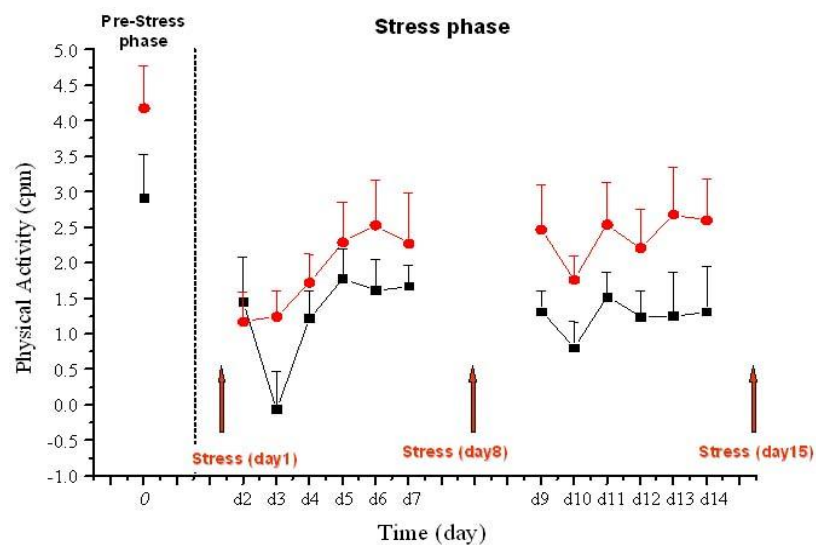
a)
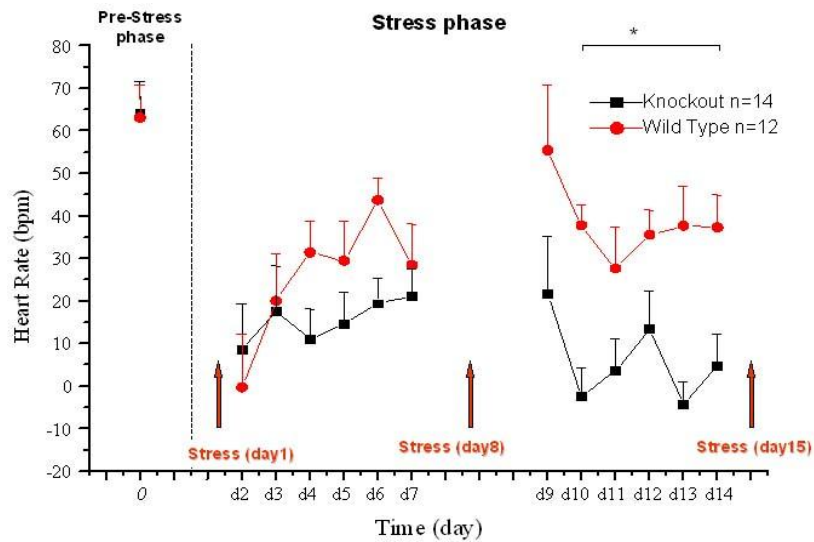
b)
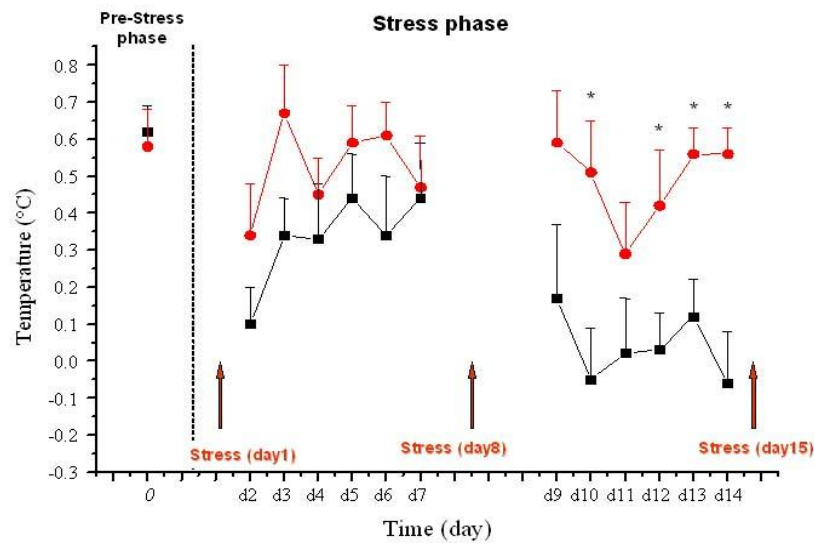
c)

Figure 11. Time course of the amplitude of the daily rhythm for heart rate (panel a), body temperature (panel b), and physical activity (panel c) in Pre-Stress phase and during Chronic Psychosocial Stress in KO and WT mice. Pre-stress phase value is the mean value of 6-days before CPS - Statistical analysis on average values

of 12-hour dark and light phases - * KO significantly different from WT corresponding values ($p<0.05$, Student õ*t*ö test).

Post-hoc. Comparison between knockout and wild type: KO vs. WT ó Day 10: HR: t=-4.515, p<0.05; T: t=-2.822, p<0.05; Day 11: HR: t=-1.981, p=0.059; Day 12: HR: t=-2.020, p=0.055; T: =-2.143, p<0.05; Day 13: HR: t=-3.779, p<0.05; T: =-3.515, p<0.05; Day 14: HR: t=-3.008, p<0.05; T: =-3.880, p<0.05.

3.5 Sporadic autonomic crises in Knockout mice

During Chronic Psychosocial Stress, knockout mice showed sporadic autonomic crises characterized by decreases in heart rate and body temperature that persisted for several hours and frequently recovered only after several hours (Fig. 12 a, b). No crises were observed in wild type counterparts. In particular, in some of the examined crises severe bradycardia and hypothermia were irreversible and progressed to death (Fig. 13 a, b).
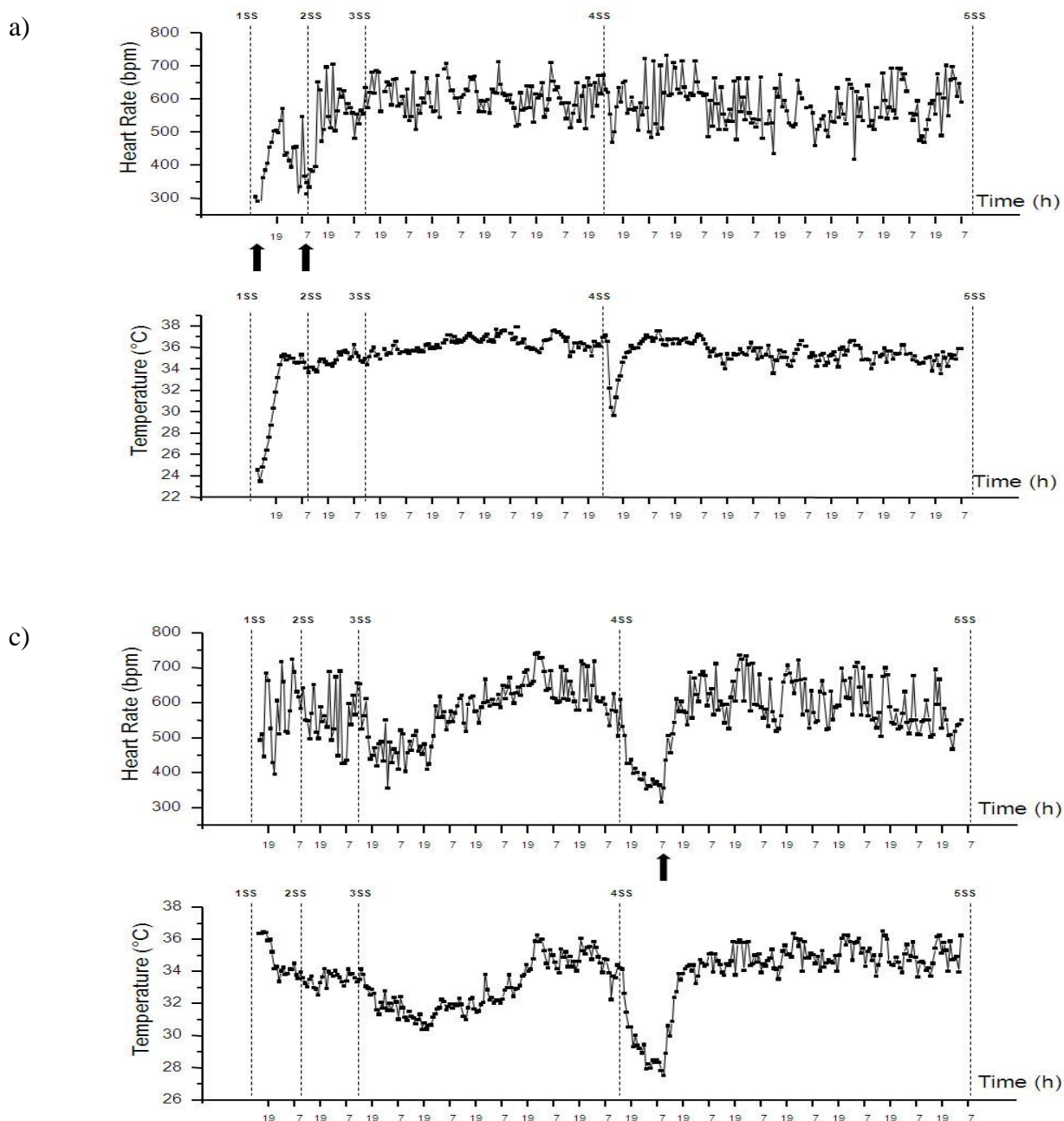

Figure 12. Representative animals of sporadic autonomic crises in 5-HT$_{1A}$ knockout mice. Crises were characterized by hypothermia and bradycardia that in these cases resolved (a, b).
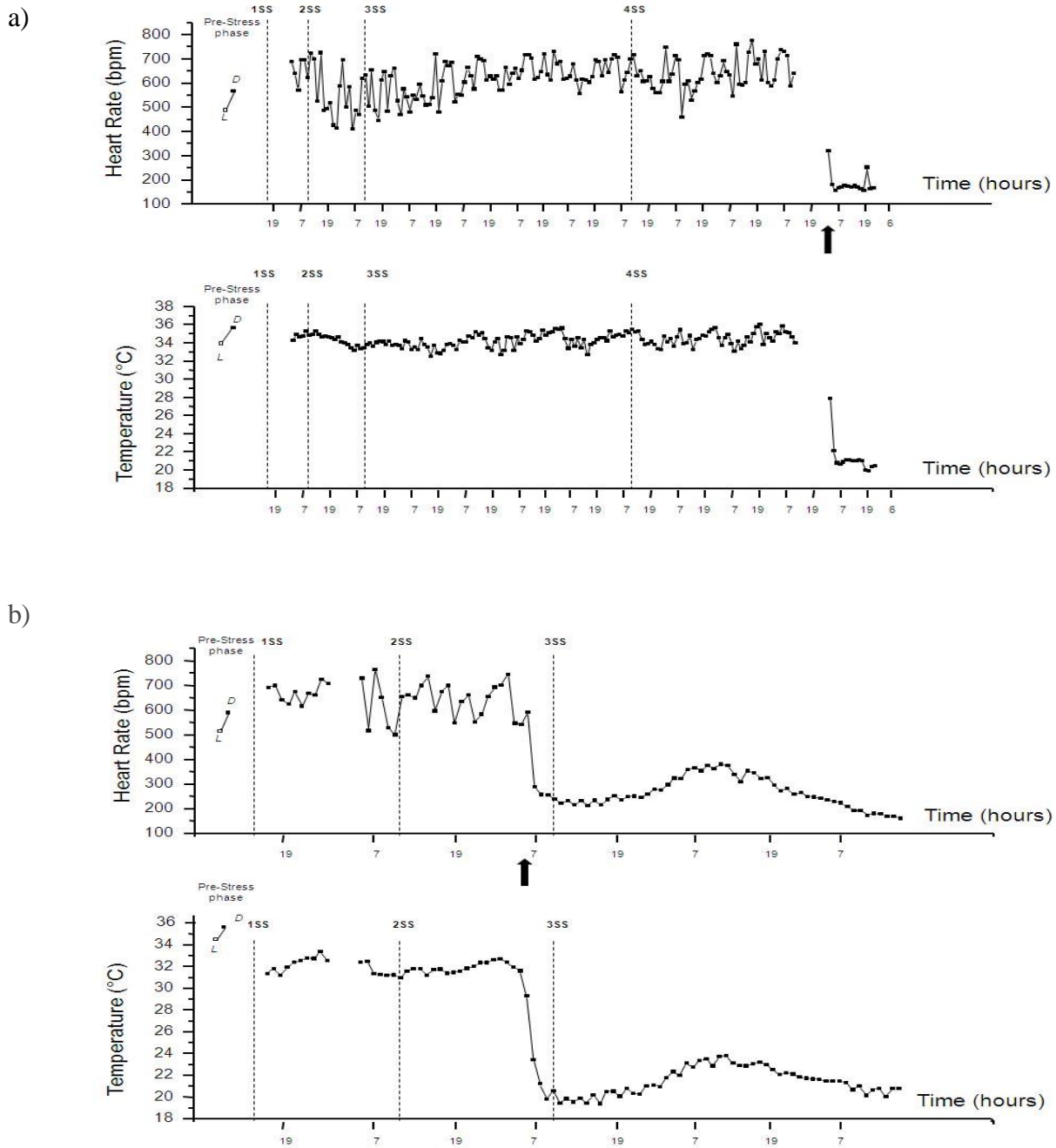
a)

b)

Figure 13. Representative animals of sporadic autonomic crises in 5-HT$_{1A}$ knockout mice. Crises were characterized by hypothermia and bradycardia that progressed to death (a, b).

3.6 Sucrose Preference Test

Figure 14 displays percent sucrose solution consumption in both groups before and after Chronic Psychosocial Stress. In baseline conditions, KO mice exhibited a lower consumption of palatable sucrose solution as compared to WTs. Such difference was not significant anymore following CPS.
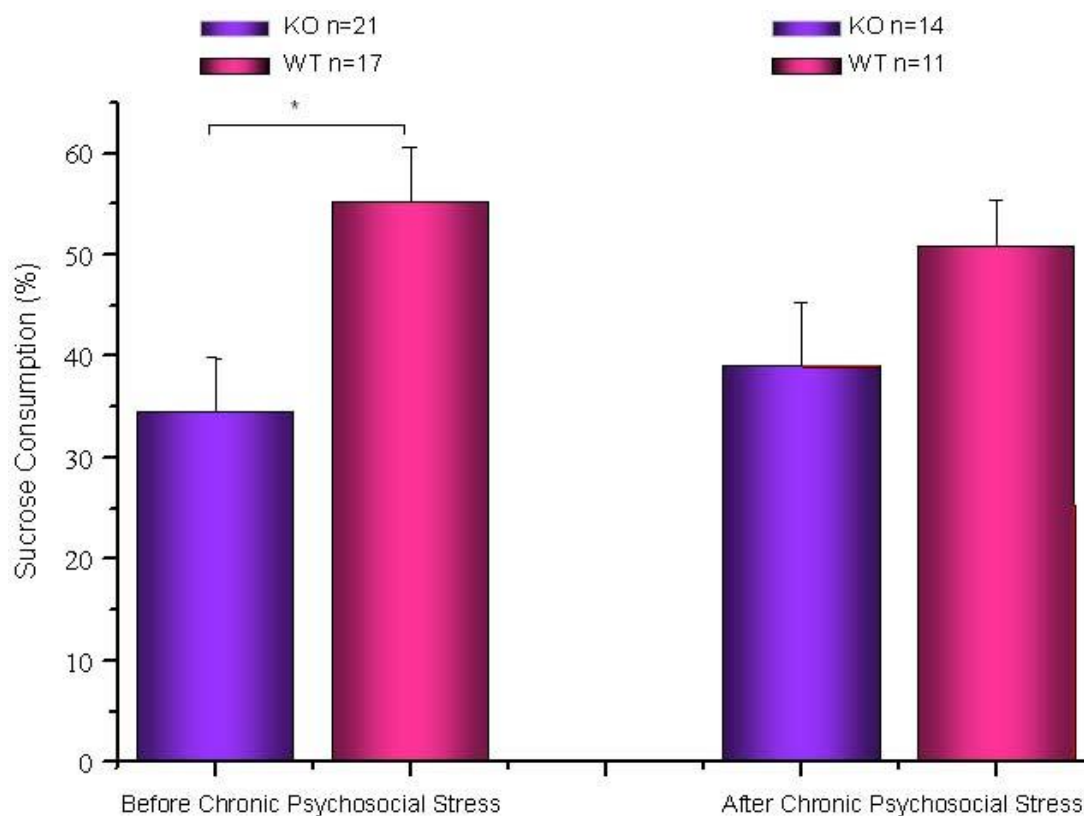

Figure 14. Percent preference for sucrose (relative to total fluid intake) during a 48-h sucrose preference test before and after chronic psychosocial stress in knockout and wild type mice.
- Statistical analysis on average values before and after CPS - * KO significantly different from WT corresponding values ($p<0.01$, Student õ*t*ö test). ***Post-hoc***. Comparison between knockout and wild type: KO vs. WT t=-2.746, p<0.01

4. Discussion

This study analyzed the short- and long-term patho-physiological effects of both acute and chronic challenges in male mouse knockout for serotonin 1A receptors. In particular, this study was aimed at further exploring the role of these receptors in the presympathetic modulation of cardiovascular stress responsivity. We evaluated the acute changes in heart rate and sympathovagal balance in response to systemic administration of 8-OH-DPAT, a 5-HT$_{1A}$ agonist possessing central sympatholytic properties, and during restraint test associated with saline, atenolol and scopolamine injections. This study assessed also the effects of chronic psychosocial stress, consisting in 15-daysø adverse sensory contact with an aggressive male and brief defeat episodes on day 1, 2, 3, 8, and 15. The study of long-term effects of chronic psychosocial challenge, took into account the time evolution of the circadian rhythms of heart rate, body temperature and physical activity, together with the consequences on sucrose intake and adrenal weights.

In agreement with Nalivaiko and colleagues (Nalivaiko et al., 2005; Ngampramuan et al., 2008; Nalivaiko et al., 2009), in response to serotonergic agonist, knockout mice exhibited a heightened tachycardic response and a dampened vagal modulation of heart rate. In fact, it was recently demonstrated that 8-OH-DPAT substantially attenuates tachycardia in rats and rabbits subjected to psychological stressors, and that the effect is predominantly due to the suppression of cardiac sympathetic drive. This is the first study, to our knowledge, that investigate the effects of systemic administration of this drug in 5-HT$_{1A}$ knockout mice.

Another interesting result of this study regards the cardiovascular response to restraint test. The mice lacking 5-HT$_{1A}$ receptors were characterized by larger sensitivity to restraint-induced heart rate acceleration following chronic psychosocial stress. In agreement, the level of vagal activity (as indicated by the values of r-MSSD) was significantly lower as compared to wild type mice, both before and after chronic stress.

As far as long-term consequences of chronic social stress on chronobiological parameters, and of possible role of 5-HT$_{1A}$ receptors in the presympathetic modulation, the amplitude of the daily rhythm of heart rate and body temperature appeared to be reduced in the second week of chronic social stress, as compared to pre-stress phase only in knockout mice. This condition represents an imbalance between normally correctly orchestrated physiological and behavioral processes and may constitute a risk factor for the development of disease (Meerlo et al., 2002). The reduction of the amplitude of the day-night oscillation of physiological parameters such as heart rate and body temperature is considered a marker of stress-related maladaptation (Meerlo et al., 1999).

It is interesting to note that during the chronic psychosocial stress, knockout mice showed sporadic autonomic crises characterized by decreased in heart rate and body temperature, and in some of the examined crises severe bradycardia and hypothermia were irreversible and progressed to death.

In conclusion, this study further supports the modulating role of somatodendritic 5-HT$_{1A}$ receptors on stress-induced cardiac sympathetic activation.

Indeed, mice lacking these receptors exhibited: (i) heightened tachycardic response and dampened vagal modulation of heart rate during a pharmacological (8-OH-DPAT) challenge, (ii) larger sensitivity to restraint-induced heart rate acceleration and vagal withdrawal became more evident after chronic psychosocial stress, and (iii) increased susceptibility to alterations in circadian rhythmicity of heart rate and body temperature during chronic social stress.

References

Audero E, Coppi E, Mlinar B, Rossetti T, Caprioli A, Banchaabouchi MA, Corradetti R, Gross C. Sporadic autonomic dysregulation and death associated with excessive serotonin autoinhibition, *Science* 321 (2008), 130-133.

Bartolomucci A, Palanza P, Gaspani L, Limiroli E, Panerai AE, Ceresini G, Poli MD, Parmigiani S. Social status in mice: behavioral, endocrine and immune changes are context dependent, *Physiol Behav* 73 (2001), 401-410.

Beltrami CA, Finato N, Rocco M, Feruglio GA, Puricelli C, Cigola E, Quaini F, Sonnenblick EH, Olivetti G, Anversa P. Structural basis of end-stage failure in ischemic cardiomyopathy in humans, *Circulation* 89 (1994), 151-163.

C. Gross, L. Santarelli, D. Brunner, X. Zhuang and R. Hen, Altered fear circuits in 5-HT(1A) receptor KO mice, Biol. Psychiatry 48 (2000), 1157–1163.

Capasso JM, Palackal T, Olivetti G, Anversa P. Left ventricular failure induced by long-term hypertension in rats, *Circ Res* 66 (1990), 1400-1412.

Costoli T, Bartolomucci A, Graiani G, Stilli D, Laviola G, Sgoifo A. Effects of chronic psychosocial stress on cardiac autonomic responsiveness and myocardial structure in mice, *Am J Physiol Heart Circ Physiol* 286 (2004), 2133-2140.

Dampney RA, Coleman MJ, Fontes MA, Hirooka Y, Horiuchi J, Li YW, Polson JW, Potts PD, Tagawa T. Central mechanisms underlying short- and long-term regulation of the cardiovascular system, *Clin Exp Pharmacol Physiol* 29 (2002), 261-268.

F. Chaouloff, O. Berton and P. Mormede, Serotonin and stress, *Neuropsychopharmacology* 21 (1999), 28–32.

Koolhaas JM, Meerlo P, De Boer SF, Strubbe JH, Bohus B. The temporal dynamics of the stress response, *Neurosci Biobehav Rev* 21 (1997), 775-782.

Llorente E, Brito ML, Machado P, Gonzalez MC. Effect of prenatal stress on the hormonal response to acute and chronic stress and on immune parameters in the offspring, *J. Physiol. Biochem* 58 (2002), 143-149.

McCall RB, Clement ME. Role of serotonin1A and serotonin2 receptors in the central regulation of the cardiovascular system, *Pharmacol Rev* 46 (1994), 231-243.

Meerlo P, Sgoifo A, De Boer SF, Koolhaas JM. Long-lasting consequences of a social conflict in rats: behavior during the interaction predicts subsequent changes in daily rhythms of heart rate, temperature, and activity, *Behav Neurosci* 113 (1999), 1283-1290.

Meerlo P, Sgoifo A, Turek FW. The effects of social defeat and other stressors on the expression of circadian rhythms, *Stress* 5 (2002), 15-22.

Nalivaiko E, Mastorci F, Sgoifo A. 8-OH-DPAT prevents cardiac arrhythmias and attenuates tachycardia during social stress in rats, *Physiol Behav* 96 (2009), 320-327.

Nalivaiko E, Ootsuka Y, Blessing WW. Activation of 5-HT1A receptors in the medullary raphe reduces cardiovascular changes elicited by acute psychological and inflammatory stresses in rabbits, *Am J Physiol Regul Integr Comp Physiol* 289 (2005), 596-604.

Ngampramuan S, Baumert M, Beig MI, Kotchabhakdi N, Nalivaiko E. Activation of 5-HT$_{1A}$ receptors attenuates tachycardia induced by restraint stress in rats, *Am J Physiol Regul Integr Comp Physiol* 294 (2008), 132-141.

Ramage AG. Central cardiovascular regulation and 5-hydroxytryptamine receptors, *Brain Res Bull* 56 (2001), 425-439.

Rozanski A, Blumenthal JA, Kaplan J. Impact of psychological factors on the pathogenesis of cardiovascular disease and implications for therapy, *Circulation* 99 (1999), 2192-2217.

Samuels BC, Zaretsky DV, DiMicco JA. Tachycardia evoked by disinhibition of the dorsomedial hypothalamus in rats is mediated through medullary raphe, *J Physiol* 538 (2002), 941-946.

Sgoifo A, de Boer SF, Westenbroek C, Maes FW, Beldhuis H, Suzuki T, Koolhaas JM. Incidence of arrhythmias and heart rate variability in wild-type rats exposed to social stress, *Am J Physiol* 273 (1997), 1754-1760.

Sgoifo A, Koolhaas JM, Musso E, De Boer SF. Different sympathovagal modulation of heart rate during social and nonsocial stress episodes in wild-type rats, *Physiol Behav* 67 (1999), 733-738.

Sgoifo A, Pozzato C, Costoli T, Manghi M, Stilli D, Ferrari PF, Ceresini G, Musso E. Cardiac autonomic responses to intermittent social conflict in rats, *Physiol Behav* 73 (2001), 343-349.

Sgoifo A, Pozzato C, Meerlo P, Costoli T, Manghi M, Stilli D, Olivetti G, Musso E. Intermittent exposure to social defeat and open-field test in rats: acute and long-term effects on ECG, body temperature and physical activity, *Stress*. 5 (2002), 23-35.

Sgoifo A, Stilli D, Medici D, Gallo P, Aimi B, Musso E. Electrode positioning for reliable telemetry ECG recordings during social stress in unrestrained rats. *Physiol Behav*. 1996 Dec;60(6):1397-401.

Stein PK, Bosner MS, Kleiger RE, Conger BM.Heart rate variability: a measure of cardiac autonomic tone. *Am Heart J*. 1994 May;127(5):1376-81.

T. Pattij, L. Groenink, T.H. Hijzen, R.S. Oosting, R.A. Maes, J. van der Gugten and B. Olivier, Autonomic changes associated with enhanced anxiety in 5-HT(1A) receptor knockout mice, *Neuropsychopharmacology* 27 (2002), pp. 380ó390.

Walker MJ, Curtis MJ, Hearse DJ, Campbell RW, Janse MJ, Yellon DM, Cobbe SM, Coker SJ, Harness JB, Harron DW. The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion. *Cardiovasc Res*. 1988 Jul;22(7):447-55.

Zaretsky DV, Zaretskaia MV, DiMicco JA. Stimulation and blockade of GABA(A) receptors in the raphe pallidus: effects on body temperature, heart rate, and blood pressure in conscious rats. *Am J Physiol Regul Integr Comp Physiol.* 2003 Jul;285(1):R110-6.

CHAPTER 6

Summary

Summary of the results

In this thesis I studied the effects of stress on cardiac sympathovagal balance, HPA axis function, and behavioral parameters on different animal models consisting of male rats and male mice lacking the 5-HT$_{1A}$ receptors. Here I present an overview of the results.

In **Chapter 2** I examined the long-term effects of foetal manipulation, in particular I used an experimental paradigm of psychological prenatal stress, namely the repeated restraint test on mothers in the last week of pregnancy. There is a growing body of evidence that demonstates that adverse life events experienced by the pregnant mother and her reactions to them can produce alterations in the foetal environment, which in turn may have profound, long-term effects on the offspring physiology and behaviour. The new data reported in this thesis suggests that prenatal stress induces heightened sensitivity to acute stressors occurring in adulthood. In particular, when exposed to environmental challenges in adult age, prenatally stressed rats showed depressive-like symptoms. Specifically, these rats exhibit longer-lasting adrenocortical stress responsivity, disturbances of circadian rhythmicity of heart rate, body temperature and physical activity, and increased adrenal weight as compared to controls.

The following step was to study the impact of adult stressors in the development of depression syndrome. At difference with Chapter 2, in **Chapter 3** I implemented an animal model of depression in adulthood. In particular, animals were exposed to an adverse stress episode (social defeat) followed by prolonged social isolation. I explored the effects of such an experimental paradigm on acute adrenocortical and cardiac sympathovagal stress reactivity, sucrose intake, circadian rhythmicity of heart rate, body temperature, and physical activity, myocardial and adrenal structure. The results support the current view that a significant social challenge can induce depression. Social defeat and a prolonged period of isolation produced some structural, physiological, and behavioral effects in rats, which resemble those observed in depressed and chronically stressed subjects. These effects mainly consisted in HPA axis negative feedback dysfunction, adrenal gland enlargement, biological rhythm alterations, and the establishment of an anhedonic condition.

In **Chapter 4** I applied again the social defeat model, for studying the neural mechanisms underlying stress induced arrthythmias, and I tested whether such arrthythmias could be suppressed by systemic administration of 8-OH-DPAT, a 5-HT1A agonist possessing central sympatholytic properties. Before the administration of 8-OH-DPAT, male rats were pre-treated with zatebradine, a pacemaker current blocker, in order to prolong the dyastolic period and therefore increase the risk

for stress-induced cardiac arrthythmias. The injection of the serotonergic agonist attenuated the tachycardia and hyperthermia induced by acute social stress. Interestingly, the systemic administration of the agonist almost abolished arrhythmic events, likely by suppressing stress-induced cardiac sympathetic outflow in the medullary raphe.

As suggested by previous studies and by the results reported in Chapter 4, the activation of central 5-HT$_{1A}$ receptors has sympatholytic effects, with attenuation of stress-induced heart rate and blood pressure raises. In **Chapter 5** I further evaluated cardiac sympathovagal modulation in mice lacking 5-HT$_{1A}$ receptors and exposed to chronic psychosocial stress. The results confirmed the role of these receptors in the cardiovascular control during stress. In particular, data suggest that the deletion of 5-HT$_{1A}$ receptors hampers the modulating role of these receptors on stress-induced tachycardia and vagal withdrawal.