DISSERTATIONES PSYCHOLOGICAE UNIVERSITATIS TARTUENSIS
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**MARGUS KANARIK**

# Inter-individual differences in vulnerability to depression: regional brain energy metabolism, serotonergic function and behaviour in animal models

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Supervisor: Jaanus Harro, MD, PhD, professor
University of Tartu, Estonia

Opponent: Gregers Wegener, MD, PhD, associate professor,
University of Aarhus, Denmark

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# LIST OF ORIGINAL PUBLICATIONS

**I** Kanarik, M., Matrov, D., Kõiv, K., Eller, M., Tõnissaar, M., Harro, J., 2008. Changes in regional long-term oxidative metabolism induced by partial serotonergic denervation and chronic variable stress in rat brain. Neurochemistry International 52, 432–437

**II** Kanarik, M., Alttoa, A., Matrov, D., Kõiv, K., Sharp, T., Panksepp, J., Harro, J., 2011. Brain responses to chronic social defeat stress: Effects on regional oxidative metabolism as a function of a hedonic trait, and gene expression in susceptible and resilient rats. European Neuropsychopharmacology 21, 92–107

**III** Harro, J., Kanarik, M., Matrov, D., Panksepp, J., 2011. Mapping patterns of depression-related brain regions with cytochrome oxidase histochemistry: Relevance of animal affective systems to human disorders, with a focus on resilience to adverse events. Neuroscience & Biobehavioural Reviews 35, 1876–1889.

**IV** Kanarik, M., Harro, J. Regional cerebral oxidative metabolism, sociability trait and intra-individual variability in social behaviour in rats (manuscript)

**V** Kanarik, M., Kaart, T., Matrov, D., Kõiv, K., Mällo, T., Tordera, R., Ramirez, J., Del Río, J., Harro, J. Revealing the brain regions and networks mediating vulnerability to depression: Oxidative metabolism mapping of the rat brain (manuscript)

Besides the aforementioned articles and manuscripts, unpublished results are included in the thesis: Regional neural activity in adult rats caused by neonatal maternal separation stress (Kanarik, M., Tordera, R., Ramirez, J., Del Rio, J., Harro, J.), hereafter **Study I;** Endocrine stress measures after chronic stress in rats vulnerable to depression (Kanarik, M., Kõiv, K., Matrov, D., Harro, J.), hereafter **Study II;** Stress-induced hyperthermia after chronic variable stress in rats with partial serotonergic lesion (Kanarik, M., Kõiv, K., Harro, J.), hereafter **Study III.**

The author of this thesis is the lead author of **Papers I, II, IV, V,** and **Studies I, II, III,** and wrote segments of **Paper III**; responsible for the design of studies included in **Papers II, IV, and Studies II and III** and was one of the investigators responsible for planning experiments in **Paper V**; carried out behavioural procedures reported in **Papers II, IV,** and was one of the researchers responsible for the behavioural procedures in **Studies II** and **III**; carried out laboratory work in **Papers I, II, IV** and **Studies I, II;** was responsible for data analysis in **Papers I, II, IV,** and **Studies I, II, III** and involved in analysis of data in **Paper V**.

# 1. INTRODUCTION

## 1.1. Why affective and stress-related disorders should be studied?

### 1.1.1 Symptomatology, prevalence, and impact on personal life

Depression is a highly prevalent psychopathological condition with the main symptoms of persistently lowered mood, loss of pleasure, and reduced energy, accompanied by changes in body weight, appetite, sleep rhythm, agitation or inhibition, feelings of worthlessness and guilt, distractibility, concentration difficulties and suicidal ideation (APA, 1994; WHO, 1992). Depression and anxiety disorders are two most prevalent mental health problems in industrial countries, with depression affecting 6.9% of European and 10% of the USA population, and anxiety disorders affecting 14% of European population in a 12-month period (Andrade et al., 2003; Wittchen et al., 2011). Lifetime prevalence of depression is the highest in the USA and Netherlands, affecting up to 16–17% of the population (Andrade et al., 2003). The point prevalence of major depressive disorder in Estonia – 5.6% – is higher than in other European countries (Kleinberg et al., 2010). Although older people are at higher risk to develop depression, data from younger cohorts indicate an increase in the prevalence of depression (Andrade et al., 2003; Wittchen et al., 2011). Epidemiological and clinical surveys have established that depression tends to progress chronically with recurrent episodes in up to 80% of people with lifetime history of depression and with each following episode further increasing the chance of recurrence (Andrade et al., 2003; Kessler et al., 1997; Maletic et al., 2007; Solomon et al., 2000). The mean duration of the longest lifetime depressive episode is from 3 to 4 months, though the median duration is remarkably shorter – 8 weeks (Andrade et al., 2003).

Besides major depression, a large number of people suffer from dysthymia – a disorder with lifetime prevalence of 2.8–7.2% according to different studies (Merikangas et al., 1996). Symptoms of dysthymic disorder – decreased mood for a majority of days and for most of the day, accompanied with changes in appetite and sleep, low energy and concentration, low self-esteem and hopelessness – are less severe than in depression. Dysthymic symptomatology is highly persistent, 2 years being the minimal duration for diagnosis (APA, 1994; WHO, 1992). Dysthymia increases the odds of having a major depressive episode, resulting in "double depression" and predicts depression recurrence, the comorbidity estimates for dysthymia and depression ranging from 40 to 80% (Griffiths et al., 2000).

Depressive symptomatology not fulfilling the criteria for major depression and dysthymia, designated the so-called minor depression and sub-threshold depression, is still a source of dysfunction and a good predictor of future major depressive disorder (Kessler et al., 1997). Minor/sub-threshold depression, a condition better characterised by affective and cognitive, as opposed to somatic, symptoms, that causes remarkable disability in everyday life, has a point

prevalence of up to 10% (Barbui et al., 2011; Meeks et al., 2011; Rapaport et al., 2002).

General anxiety disorder, frequently co-morbid with depression, has the lifetime prevalence of 5% and annual prevalence 1.5–3% in the USA (Michael and Margraf, 2004; Wittchen et al., 1994). General anxiety is described by impairing levels of uncontrollable worrying accompanied by somatic symptoms, concentration difficulties and irritability (APA, 1994; WHO, 1992). Social anxiety/phobia is yet another disorder co-occurring with depression. Social anxiety/phobia is characterised by impairing levels of unreasonable fear and avoidance of social situations and somatic stress symptoms that can reach the level of a panic attack (APA, 1994; WHO, 1992). The highest estimates for social phobia are 13% for lifetime and 8% for annual prevalence. This disorder impairs the person's ability to communicate and process socially relevant information, inclines the patient towards substance abuse and un-fulfillment of social transitions e.g., school graduation (Kessler, 2003; Michael and Margraf, 2004).

Depression has been predicted to become the second largest cause of economic burden on society by 2020 (Murray and Lopez, 1997). By 2009, major depression was already the third largest cause of economic burden, accounting for 7.2% of the years of life lost due to disorders (103.7 years lost per 10 000 persons) (WHO, 2009; Wittchen et al., 2011). The total annual cost due to depression is assessed to comprise 1% of the GDP of European countries (118 billion euros in 2004), this being more than one third of the cost of all mental disorders. Most of the cost derives from indirect/non-medical expenses, e.g., absenteeism, early retirement, premature death, reduced productivity (Sobocki et al., 2006).

Symptoms of depression have a profound effect on the individual's every-day life and coping and lower the quality of life of the affected people e.g., their subjective wellbeing and success in their social and professional life (Katschnig and Angermeyer, 1997; Lépine et al., 1997; Pyne et al., 1997). After remission, the quality of life is still poorer in formerly depressed but medicated individuals compared to general population, although their state is much improved compared to depressed patients (Angermeyer et al., 2002). Living together with or caring for a person with psychiatric problems can reduce the quality of life of family members, and the subjective wellbeing of the disordered person and the family members are highly correlated (Fujino and Okamura, 2009).

### 1.1.2. Inter-relations of affective and anxiety disorders

General anxiety disorder, frequently co-morbid with depression, has the lifetime prevalence of 5% and annual prevalence 1.5–3% in the USA (Michael and Margraf, 2004; Wittchen et al., 1994). General anxiety is described by impairing levels of uncontrollable worrying accompanied by somatic symptoms, concentration difficulties and irritability (APA, 1994; WHO, 1992).

Social anxiety/phobia is yet another disorder co-occurring with depression. Social anxiety/phobia is characterised by impairing levels of unreasonable fear and avoidance of social situations and somatic stress symptoms that can reach the level of a panic attack (APA, 1994; WHO, 1992). The highest estimates for social phobia are 13% for lifetime and 8% for annual prevalence. This disorder impairs the person's ability to communicate and process socially relevant information, and it inclines the patient towards substance abuse and unfulfillment of social transitions e.g., school graduation (Kessler, 2003; Michael and Margraf, 2004).

Approximately half of the patients with the history of depression also have at least one anxiety disorder (Andrade et al., 2003). Most often depression is comorbid with general anxiety disorder (odds ratio estimates ranging from 3.0 to 20.7), panic disorder (odds ratios from 2.7 to 30) or social phobia (odds ratios from 2.2 to 18.2), with the anxiety disorders usually preceding the depressive episode in illness history (Andrade et al., 2003; Merikangas et al., 1996). Comorbidity profiles of major depression and dysthymia differ: depression co-occurs more often with social anxiety whereas dysthymics have almost no social anxiety but extremely high co-morbidity with general anxiety disorder (65%) (Griffiths et al., 2000; Pini et al., 1997). Trait anxiety is proposed to be a risk factor for the development of depression (Sandi and Richter-Levin, 2009). Both depression and several anxiety disorders can be treated with the same classes of drugs – selective serotonin reuptake inhibitors and serotonin-noradrenaline reuptake inhibitors (Baldwin, 2008).

At a very general level behaviour/individual differences can be forced into broad and universal dimensions, and if depression is intrinsic to one of such dimensions and anxiety to another, then all individuals can be characterised by a combination of varying levels of depression and anxiety (Shankman and Klein, 2003). The tripartite model of anxiety and depression postulates that the dimension of negative affectivity (reminiscent of neuroticism) is common to both anxiety and depression, positive affectivity (similar to extraversion) discriminates depression from anxiety, as does autonomic arousal, and the combination of the these traits determines whether the person is prone to develop anxiety, depression or both (Anderson and Hope, 2008). The model does not explain the necessity and/or sufficiency of individual components in the development of pathology and overlooks differences in the subtypes of the two disorders (Anderson and Hope, 2008; Shankman and Klein, 2003). The approach/withdrawal system theory posits that individuals can be characterised by the activity of behavioural systems of approach to incentive and withdrawal from aversive stimuli. Active avoidance system is characteristic of anxiety and passive approach system of depression (Davidson, 1998; Shankman and Klein, 2003).

### 1.1.3. Theories of the adaptational value of depression and anxiety

When thinking about psychopathological conditions it is useful to keep in mind that these syndromes might have (or at least have had) associations with mechanisms that possess fitness increasing value – although anxiety and depression have deleterious effects on the behaviour of individual humans and other animals, they have not been selected out in evolution. Depressed or anxious behaviour might serve as parts of the species survival system with possible trade-off value (Nesse, 2001). The function of anxiety is seemingly more readily interpretable – anxiety within normal range serves as a protection against potentially harmful situations, and hypo- or hyperactive response of the fear and anxiety generating networks leads to pathological levels of anxiety (Marks and Nesse, 1994). In case of depression there is an ongoing debate whether depression is an exaggeration of the adaptive form of behaviour or whether depression is adaptive even in its full severity (Hendrie and Pickles, 2009; Nettle, 2004).

Several theories of the adaptive value of depression have been constructed around the notion of exploitation and conservation of resources – energetic, health-related or social (Allen and Badcock, 2006; Gilbert, 2006). The social navigation/bargaining hypotheses posit that depression helps to focus exclusively on one most relevant task and serves as a signal of need from partners with shared fitness (Hagen, 2002; Nettle, 2004). Depressive state can protect from harm in the pursuit of unrealistic goals by acceptance of lower social rank, and by reducing social conflict, depression is adaptive for the whole group with shared fitness (Nesse, 2000). Successful transition from dominant to submissive status may enhance reproductive success – individuals that become depressed during hierarchy transitions gain a reproductive advantage as they are not harmed or cast out of the social group. This mechanism assumes that males of higher status have better access to females, which might not be the case in rats and mice, the typical subjects of preclinical models (Hendrie and Pickles, 2009). As depression is frequently elicited by highly infectious situations e.g., fights or overcrowding, it is proposed that the physiological changes of depression can aid in avoiding of and coping with infections, and the inhibited behaviour is believed to reduce the spreading of infectious disease (Kinney and Tanaka, 2009).

### 1.1.4. Efficacy/effectiveness of available treatments

Lack of treatment or treatment discontinuation leads to impaired social and work-related life and decreased subjective well-being, and can lead to subsequent depressive episodes (Keller and Boland, 1998; Maletic et al., 2007). In Europe only 37% of people with clinical levels of depressive symptomatology and 26% of people with anxiety have had contact with healthcare professionals during the last year (Wittchen and Jacobi, 2005). Fortunately there are

several classes of monoaminergic antidepressant drugs available, but about 20% of the treated individuals do not respond to these drugs, furthermore, only 50% of the treated people achieve remission (Nestler et al., 2002). In the "real world", clinical outpatient population treatment with citalopram led to remission of approximately one third and response in half of the patients (Trivedi et al., 2006). Resistance to antidepressants and the chronicity of depression predict an especially unfavourable outcome on depression symptomatology and everyday functioning (Fekadu et al., 2009; Torpey and Klein, 2008). Recently critical views challenging the effect of selective serotonin reuptake inhibitors in mild/moderate depression have arisen that could be, leaving the misinterpretation of placebo response in these analyses aside, constructively interpreted as a problem of depression severity or sub-type/syndrome and treatment mismatch (Ghaemi, 2008; Kirsch et al., 2008). Taking all this together, there is a (growing) need for better treatment of depression. Current pharmacological interventions are of moderate efficacy and at least one fifth of depressed patients probably need other kind of drugs than currently available. In order to discover new drugs we probably must first develop new animal models to find new drug targets.

### 1.1.5. Why do we need yet other animal models of depression?

There is no prevailing theory of the neurobiological causes of depression, if not to consider the monoamine theory a coherent approach. Research in the last decades has implicated hundreds of new molecules in the development of depression. Although depression can be treated, and we know that initiation of the antidepressive effect may involve changes in monoamine systems, the exact mechanisms how higher serotonin or noradrenaline levels reverse depression or anxiety is not understood (Lee et al., 2010). Depression is probably not a unitary construct, thus modelling more specific dysfunctions of the disorder – endophenotypes – would be preferable. Endophenotype (or intermediate phenotype) is a measurable index somewhere between the (genetic) cause and the clinical manifestation of the disorder, that allows to target certain aspects of the disorder, and by this reduces variance and allows to avoid false negative results (Gottesman and Gould, 2003). As disorders have various symptoms, to characterise the full scope of the disorder several endophenotype based animal models are needed. Even within a single endophenotype there are several biological systems, and within the systems there are several nodes of any pathway that can dysfunction in depressed individuals and are also potential targets for treatment. Considering the diversity of potential targets, having different models increases the chance of revealing unknown neural mechanisms.

Inter-individual differences have for a long time been implicated in the aetiology of human psychopathological conditions (Bowlby, 1940; Eysenck et al., 1976; Horwood and Fergusson, 1986). Yet in preclinical models trait-like

constructs have come to be more widely used only during the last two decades, the oldest examples dating back to 1960ies (Broadhurst and Bignami, 1965; Henn et al., 1985; Piazza et al., 1989; Rägo et al., 1988). The search for the underlying pathology of depression presumes different models than those currently used for antidepressant screening or the measurement of antidepressive effect, because screening methods may not target the pathology at all and treatment does not necessarily reverse the neurobiological changes in depression, but can result from a new equilibrium in neural function. Behavioural models and tests used in depression research are largely validated with established antidepressants, thus making the finding of new targets with different mechanisms unlikely. Thus models based on inter-individual differences in affective processes can be useful in the search for neurobiology of depression.

## 1.2. The aetiopathogenesis of affective and anxiety disorders

### 1.2.1. Stress as the cause and/or trigger of affective disorders in humans

Stressful situations are inevitable in the lives of humans and other animals. Furthermore, the function of acute stress is to increase the adaptation of the organism to environmental change by recruiting energetic, metabolic, immunological, neurochemical and behavioural resources (McEwen, 2007). The persistence of the presence of stressors and the repeated occurrence of the acute reaction can however lead to behavioural and neurochemical changes that in turn can elicit depression and other adverse behavioural and health effects (McEwen, 2007).

Stressful life events are proposed to either cause or trigger a depressive episode. Clinical observations and epidemiological studies have demonstrated that in most cases a depressive episode is preceded by a stressful life event (Bedi, 1999; Brown, 1998; Hammen, 2005). Besides causing depression, adverse environment also affects the healing process – remission rates of depression are lower for people in aversive environment during the depressive episode and treatment (Brown et al., 2010). Events related to loss, either of a close person, or in more abstract form, e.g., loss of autonomy, idea or self-esteem, are particularly related to depression, while events related to danger predict anxiety disorders. The co-occurrence of the two classes of events is a risk factor for comorbid depression and anxiety (Brown, 1998). The most stressful properties of an event are the lack of sense of control, humiliation, defeat and feeling of entrapment (Brown, 1998). It is not merely the objective stressfulness of the stressor that determines its impact on development of depression, but matching the event to personal framework of comparison and life expectancies (Brown, 1998; Mossakowski, 2011).

Some authors propose that rare and extremely stressful life events are the cause, not merely the trigger, of depression (Brown, 1998). This line of evidence is supported by the temporal distance between extremely stressful events during childhood/adolescence and adulthood depression, and, to a lesser degree, generalised anxiety disorder (Brown, 1998; Pine et al., 2002). According to another view, though not mutually exclusive with the preceding notion, depression can be caused by an array of minor, but frequent, stressful life events – the so called “daily hassles” that occur relatively independently of major stressful life events, and have a cumulative effect on the development of depression (Harkness and Monroe, 2006). Separation of these two concepts could help to explain the different depression sub-types. Cumulation of the effects of minor life events can induce melancholic depression, the more severe form of depression with higher incidence of somatic complaints and motor agitation or retardation, whereas the non-melancholic depression is less dependent on minor stressful life events (Harkness and Monroe, 2006). While

stressful events with negative valence predict depression well, the positive stressful events interact with negative ones, and interestingly they do not necessarily protect, but can rather increase the risk for depression. Nevertheless, this should happen only at above a certain level of exposure to negative events (Overbeek et al., 2010).

In species with social cohabitation and hierarchies, social environment largely determines the behaviour. Two large classes of environments account for most of the social contacts in humans – family relations and workplace/ school environment. Family environment has a profound effect on children in the development of psychopathology, as childhood abuse and maltreatment are causal in the development of adulthood depression (Al-Modallal et al., 2008; Brown and Harris, 2008). Marital/partnership discord is both a result and a cause of depression, whereas social support decreases the chance of becoming depressed (Buu et al., 2011; Kouros and Cummings, 2011; Rehman et al., 2008). Adverse school and workplace environment exposes adolescents and adults frequently to stressful events, including falling victim of bullying, and can potentially lead to an increased risk for pathology, mainly affective, anxiety and conduct disorders (Barrett and Heubeck, 2000; Björkqvist, 2001). Thus in humans both single "catastrophic" events and the cumulation of minor stressors can lead to depressive symptomatology, and social environment is the largest source of such stressors.

### 1.2.2. Preclinical models of chronic stress

Preclinical models of chronic stress vary in the selection of stressors (social, systemic, processive), time of administration (prenatal, postnatal, adolescent, adult), length of the regimen (sub-chronic, chronic), ecological validity (e.g., predatory odour vs. formalin stress) and predictability (e.g., single vs. multiple stressors) (Anisman and Matheson, 2005). Rodent models of environmentally evoked depressive-like state have mostly adopted the idea that moderately stressful events occurring chronically contribute more toward construct validity than a single catastrophic event, as exposure to the latter has been proposed as a model of post-traumatic stress disorder (Pollak et al., 2010; Siegmund and Wotjak, 2006). Single stressors usually do not lead to depressive/anxious behaviour, with the possible exception of social defeat that does elicit long-lasting behavioural changes (Koolhaas et al., 1997; Meerlo et al., 1996). As we know from human studies, unpredictability and lack of control determine the severity of stress, and these features are also pursued in animal models of chronic stress (Anisman and Matheson, 2005; Brown, 1998).

Repeated exposure to a single stressor has long been one of the main chronic stress paradigms. Repeated restraint can induce several behavioural changes e.g., anxious behaviour in open field and elevated plus maze, diminished social interaction and preference, behavioural despair, anhedonia, and spatial learning deficits (Doremus-Fitzwater et al., 2009; Hayase, 2011; Klenerova et al., 2010; McEwen and Magarinos, 1997). Repeated restraint causes hippocampal damage

and affects dopaminergic, serotonergic and glutamatergic transmission (Browne et al., 2011; Cabib et al., 1998; Clement et al., 1998; McEwen and Magarinos, 1997; Quinton and Yamamoto, 2007). In another popular chronic stress paradigm foot-shocks are delivered repeatedly. In case of repeated foot-shock, habituation develops as a function of stress intensity – if high intensities are used, habituation does not occur (Rabasa et al., 2011). A recent interesting development is the so-called "emotional stress", where one rat is subjected to witness the other animal receive repeatedly foot-shocks but is free of any physical manipulations (Pijlman et al., 2003). Another important aspect in the development of depression – stressor controllability – can be integrated in rodent studies by using yoked pairs: two animals receive identical electric shocks but only one of them can control the current applied to both of them, thus making the psychological aspect, not merely physical pain, central in this paradigm (Tanaka, 1999).

Behavioural and endocrine stress response can desensitise/habituate when experiencing the same stressor, and therefore chronic single stressor paradigms might not be optimal for modelling depression. Thus depression-like behaviour in rodents should rather be modelled using paradigms that do not allow habituation.

#### 1.2.2.1. Chronic variable stress

A widely used chronic stress paradigm utilises a variety of stressors intermittently to mimic the unpleasant but not catastrophic events of everyday life in humans. Chronic variable stress, chronic mild stress and chronic unpredictable (mild) stress may tend to differ in the choice of stressors, but use the same general principles (Harro et al., 2001; Katz et al., 1981; Mao et al., 2010; Mineur et al., 2006; Qin et al., 2004; Willner, 1997; 2005). The ecological validity of chronic variable stress paradigms rests on the assumption that several stressors, not depressogenic if presented alone, act cumulatively and induce depressive behaviour, and by including a number of variable stressors in the regimen the risk of animals getting habituated with the procedure is minimised (Anisman and Matheson, 2005). The stressors used include both systemic (directly affecting homeostasis e.g., low temperature) and processive ones (involving higher order mental processes, e.g., novelty), short duration (e.g., tail pinch) and long duration (e.g., overnight illumination) impacts, and a number of social stressful conditions (overcrowding, social defeat) (Harro et al., 2001; Herman and Cullinan, 1997; Katz et al., 1981; Willner, 2005).

Behavioural effects brought about by chronic multiple stressor paradigms are manifold, including a decrease in hedonic behaviour as measured by sucrose preference tests and intracranial self-stimulation; reduction of active coping behaviour in the forced swimming test; decreased sexual and aggressive behaviour; disturbed sleep patterns (increased REM and fragmentation); and increased freezing when a conditioned fear stimulus is presented (Bekris et al., 2005; D'Aquila et al., 1994; Grønli et al., 2004; Henningsen et al., 2009; Willner, 1997; 2005).

The effect of chronic variable stress on novelty-related anxious behaviour is complex – both increases and decreases in horizontal and vertical activity have been reported, and chronic stress can decrease anxiety in the elevated plus-maze while causing social avoidance (Bondi et al., 2007; Cox et al., 2011; Gouirand and Matuszewich, 2005; Grønli et al., 2005; Kompagne et al., 2008; Luo et al., 2008; Matrov et al., 2011; McGuire et al., 2010; Willner, 1997; 2005). Chronic mild stress has been demonstrated to increase the time spent in the open arm of the elevated plus-maze, and this could be explained by stress-induced activation locomotive behaviour (McGuire et al., 2010; Strekalova et al., 2005). In most experiments, however, either anxiogenic effects of chronic stress have been reported or chronic stress has no effect on plus-maze behaviour (Bondi et al., 2007; Cox et al., 2011; Matuszewich et al., 2007). Rats that have undergone chronic mild stress are more prone to react fearfully during fear recall and the anxiogenic effects of chronic stress seem to grow with the increasing temporal interval between testing and stress, as indicated by the delay in the expression of increased defensive burying behaviour and decreased locomotion (Matuszewich et al., 2007; McGuire et al., 2010). Chronic variable stress has been shown to reduce immobility i.e., activate the coping behaviour compared to controls, especially on the first day, in the forced swimming test, and to reduce the active contact with another weight-matched unfamiliar rat in the social interaction test, thus having anxiogenic effects (Harro et al., 2001; Häidkind et al., 2004; Tõnissaar et al., 2008b). Altogether, chronic multi-stressor treatment does induce depressive/anxious behaviours in some specific tests; in the prototypical anxiety test, the elevated plus-maze, apparently contradictive results been reported.

Studies on the impact of chronic stress on learning behaviour have yielded contradicting results. Chronic stress can disrupt behaviour in tasks demanding attention, working memory and (re)-learning, object recognition and spatial allocentric learning, and some of these effects can appear independent of sucrose intake reduction (Bondi et al., 2007; Henningsen et al., 2009). Learning in the water maze can be facilitated in chronically stressed rats, possibly depending on the animals' sex (Bowman et al., 2003; Gouirand and Matuszewich, 2005; McFadden et al., 2011). Chronic stress may have a more clear disruptive effect on the more complicated tasks. In simpler spatial navigation tasks a facilitative effect of higher glucocorticoid levels can be assumed (Akirav et al., 2004). Another factor is probably the nature of the particular stressor and the protocol used, as three weeks of restraint for six hours a day seems to be highly effective in inducing spatial memory deficits (Wright and Conrad, 2005). The length of stress regimen and the severity of stress affect memory function probably via varying effects on circulating glucocorticoid levels (Coburn-Litvak et al., 2003).

Some of the neurochemical changes induced by chronic multi-stressor treatment include increased dopamine function in the frontal cortex and hypothalamus and decreased dopamine function in striatum; increased serotonin function in hypothalamus and hippocampus and decreased serotonin function in

frontal cortex; decreased hippocampal extracellular GABA and increased glutamate levels; and the impact of chronic stress can be sex-specific (Bekris et al., 2005; Bowman et al., 2003; Cox et al., 2011; Grønli et al., 2007; McEwen, 2007; Willner, 1997; 2005). Chronic mild stress can cause a 10–15% decrease in the hippocampal volume, though in case of transient reduction it is not the result of neuronal apoptotic processes, reduction in hippocampal neurogenesis, changes in hippocampal, amygdalar and prefrontal dendrite micro-structure, or changed hippocampal synaptic function as measured by decreased long-term potentiation (Krugers et al., 2010). Antidepressant treatment is efficient in normalizing many of the behavioural and neurochemical changes induced by chronic regimen with multiple stressors (Bekris et al., 2005; Bondi et al., 2007; Willner, 1997; 2005).

#### 1.2.2.2. Social defeat stress

In humans, other primates and rodents, the social environment, especially the social hierarchy in uncertain/changing conditions, is the greatest source of stressful stimulation (Bartolomucci et al., 2005). Social defeat is the most potent aversive event to rodents, when acute endocrine stress reaction is taken as a measure of stressor severity (Koolhaas et al., 2011). Laboratory animals can be subjected to chronic social stress in a number of ways. In recent years, the most widespread model has been the resident-intruder paradigm (Blanchard et al., 2001; Miczek, 1979; Miczek et al., 2008). In this model, one rat, the intruder, when placed repeatedly in the home cage of an aggressive animal, the resident, is attacked and defeated, leading to the development of depressive/anxious behaviour (Blanchard et al., 2001; Miczek et al., 2008; Rygula et al., 2005). Resident-intruder social defeat can however not be regarded as a unitary construct: there are several ways to inflict it, differing in the number and duration of aggressive encounters, and these lead to distinct biochemical /behavioural endpoints (Kroes et al., 2007; Miczek et al., 2008; Rygula et al., 2005).

Chronic social stress produces in rodents anhedonia and behavioural despair; diminished exploration and locomotion; social aversion; changes in circadian patterns; increased anxiety; hypersensitivity to non-social stressors; hippocampal dysfunction leading to cognitive impairment; increased HPA axis activity and a long-lasting decrease in glucocorticoid receptor levels and increased self-administration of drugs of abuse (Berton et al., 2006; Buwalda et al., 2005; Meerlo et al., 2009; Miczek et al., 2008; Rygula et al., 2006; Rygula et al., 2005; Rygula et al., 2008; Sterlemann et al., 2008; Sterlemann et al., 2010). The adverse effects of social stress on anhedonia, behavioural despair and locomotion can be reversed by chronic administration of antidepressants (Rygula et al., 2006; Rygula et al., 2008). Social stress-induced reduction in cyto- and neurogenesis is reversed by fluoxetine treatment, absence of long-term potentiation is reversed by antidepressants only partially, and changes in hippocampal microstructure are not responsive to antidepressive treatment (Czéh et al., 2010; Czéh et al., 2006; von Frijtag et al., 2001).

Social stress is considered to have a unique and strong impact on rodents, with even single exposure leading to long-lasting changes in brain function (Kavushansky et al., 2009; Koolhaas et al., 2011; Meerlo et al., 1996). Social defeat stress is also unique in a sense that there is limited habituation to defeat in social conflict in subordinate rats, whereas victorious dominant animals habituate to aggressive encounters (Sgoifo et al., 2005). Sgoifo and colleagues propose that it is the greater level of controllability that allows the victorious residents to habituate; on the other hand it should be noted that intruder rats do have some control over the situation as they can regulate the behaviour of aggressive residents by either submission, freezing or counterattack (Blanchard et al., 2002; Sgoifo et al., 2005).

Thus social defeat stress is a well validated model to induce depression-like and anxious behaviour in rodents.

## 1.2.3. Inter-individual differences in susceptibility to depression

### 1.2.3.1. Susceptibility to depression and personality dispositions in humans

Synthesis of the knowledge of heritability of depression ($h^2$=0.3 – 0.4) and the implication of stressful factors in the pathogenesis has led to a growing recognition of the interactive effect of inherent/individual properties and environmental stressors in depressogenesis (Bienvenu et al., 2011; Boardman et al., 2011; Kendler, 2001; Kendler et al., 2006; Schmidt et al., 2008; Wurtman, 2005). Results from epidemiological studies measuring both life events and genetic risk prove that most often depression is caused by an interaction between the two, though purely "reactive" and "endogenous" forms of depression exist at very low prevalence (Kessing, 2007). Some studies have indicated that genetic liability to depression or a vulnerability factor of neuroticism is a better predictor of depression than stressful life events, the best predictions though arise from the combination of the two factors (Kendler, 2001; Ormel et al., 2001). Neuroticism, as measured within the five-factor personality paradigm, is characterised by emotional instability, tension, and a general tendency to experience negative emotions more easily (McCrae and John, 1992). Depression is shown to be frequently comorbid with anxiety disorders, but pre-existing trait anxiety is considered to be a risk factor for developing depression (Sandi and Richter-Levin, 2009). As anxiety disorders comorbid with depression are present predominantly before the depressive episode, and neuroticism reflects to a great deal trait anxiety of an individual (correlations of the two constructs can be as high as 0.7), it is possible that anxiety can rather be the cause than the consequence of depression, or the prodromal state leading to the expression of depression (Harro and Oreland, 2001).

### 1.2.3.2. Animal models of individual susceptibility to depression

The interaction effect of stress and inherent properties on behaviour has generated a search in preclinical science for vulnerability factors to depression

(Harro, 2010). The most common vulnerability phenotypes include maternal or juvenile stress, neurotoxin treatment, behavioural selection (e.g., low vs. high exploration) and inbreeding of behavioural traits (e.g., trait anxiety) (Eiland and McEwen, in press; El Yacoubi and Vaugeois, 2007; Harro, 2010; Landgraf and Wigger, 2002; Mällo et al., 2007). Animals with vulnerability to depression may display some depressive/anxious behaviour persistently, but may also be vulnerable only when facing stressors. This approach assumes that the behavioural features selected for, or induced, have some temporal consistency – are trait-like. One of the most effective ways to achieve this is by inbreeding of animals with the extreme expression of certain behaviour. Based on anxious behaviour in the elevated plus maze, two lines of rats and mice with high and low anxiety levels have been bred. The two differ remarkably in behaviour in anxiety tests but also in behavioural and endocrine acute stress reactivity, inter-male aggression levels, coping in tests of depression, the latter being reversible by antidepressant treatment (Keck et al., 2005; Landgraf, 2003; Landgraf and Wigger, 2002; Neumann et al., 2011; Veenema et al., 2007). Neurochemically, the high anxiety animals show higher vasopressin levels in paraventricular hypothalamus, and reduced serotonergic transmission at pre- and postsynaptic level (Keck et al., 2005; Landgraf, 2003; Landgraf and Wigger, 2002). The Flinders Sensitive Line of rats, serendipitously discovered as a depression model, is characterised by passive coping in the forced swimming test and passive exploration of open field, both reversible by chronic antidepressant treatment, and an increased response to chronic stress, as indicated by anhedonia (Neumann et al., 2011).

In another popular individual-differences based model rats are divided on the basis of a locomotor response in a novel open field (originally a circular runway) into individuals with high and low locomotor response (Kabbaj, 2004). Behaviourally, high responders are more responsive to psychostimulant effects on locomotion, more prone to self-administration of stimulant drugs, more motivated to explore novel environments and less anxious (Dellu et al., 1996; Kabbaj, 2004; Kabbaj et al., 2000). On the other hand, high responders are also more susceptible to stress – these animals have a passive coping strategy and lose their motivational and novelty-seeking superiority after social isolation stress or social defeat (Calvo et al., 2011; Dietz et al., 2008; Duclot et al., 2011; Kabbaj, 2004; Kabbaj and Akil, 2001; Kabbaj et al., 2001).

Based on the learning/anxious response in the shuttle-box, rats with inherently high and low avoidance have been bred. Low avoidance rats have a passive coping and anxious phenotype, show slower avoidance learning in the shuttle-box, increased stress induced freezing and endocrine response, and display anxiety in several behavioural tests (Steimer and Driscoll, 2003; 2005). Differences between animals with high and low avoidance trait in novelty induced behaviour are accompanied by increased short-term activation, as measured by immediate early gene expression, in rats with low avoidance in prefrontal cortex and amygdala (Meyza et al., 2009).

Some animals are more prone to develop helpless behaviour in unavoidable highly stressful situations. A line of congenitally helpless rats has been bred with severely diminished escape response from an avoidable shock (Henn et al., 1985; Shumake et al., 2005). Rats that failed to press the lever and avoid the shock in 2/3 of trials were considered helpless and were used for mating, and after the procedure was repeated for 25 generations, 95% of the rats could be regarded as congenitally helpless (Shumake et al., 2005). Congenitally helpless rats display less anxious behaviour – higher vertical and horizontal activity and preference for open test-compartments. On the other hand, these rats consume less sucrose solution, respond more to fear conditioning and form extremely stable fear memories, and cope passively in the forced swimming test. Some of these behaviours are brought to control levels by antidepressant treatment (Patel et al., 2004; Shumake et al., 2005; Vollmayr et al., 2004). Helplessness is accompanied by a specific neurometabolic pattern in frontal cortex, hypothalamic stress response circuits, dopaminergic reward circuits, and in habenula, a brain region involved in the regulation of the activity of both serotonergic and dopaminergic systems. Also increased glutamate/GABA ratios in hippocampus and frontal cortex have been reported (Sartorius et al., 2007; Shumake et al., 2003; Shumake and Gonzalez-Lima, 2003).

Besides using a priori defined factors, animals vulnerable to depression can be differentiated from resilient counterparts on the basis of post-stress behaviour. Development of anhedonia as measured by sucrose intake, submissive behaviour in social conflict, or basal corticosterone levels after the stress regimen distinguish between animals that are more resilient or susceptible to chronic stress (Panksepp et al., 2004; Schmidt et al., 2009; Strekalova and Steinbusch, 2010). Following social defeat stress, social preference of a neutral target mouse is indicative of depressive state – depression resilient mice behave in the social preference test identically with control animals (Krishnan et al., 2007). The virtue of such post hoc distinction of vulnerability is the flexibility to use any behavioural or biochemical end-point used in the experiment.

#### 1.2.3.2.1. Partial serotonergic denervation as a model of depression/anxiety vulnerability

Pathological changes involving the monoamine systems are involved in the development of depression, and due to the advance of selective serotonin reuptake inhibitors in the treatment of depression and anxiety, the serotonin system has received focused attention as a candidate in the aetiology of affective disorder (Chaouloff, 2000; Harro and Oreland, 2001). Administration of neurotoxins that specifically compromise serotonergic function leads to symptoms of affective and stress-related disorders (Cunningham et al., 2009; Leonard, 2000; Ludwig and Schwarting, 2007).

Many key proteins of the serotonergic system in different brain regions have been implicated in depressive symptomatology in both animal models and in human disorder. A few diverse examples of implications of serotonin in depression are: changes in serotonin and its metabolite levels/turnover or

release following chronic stress in different brain regions in animals; changes in serotonin receptor sensitivity in response to chronic stress; therapeutic effect of antidepressant treatment by a cascade of events starting with increasing the amount of serotonin in the synaptic cleft and resulting altered (auto)receptor sensitivity; depressogenic properties of acute dietary tryptophan (serotonin precursor) depletion and depressogenic effect of monoamine depletion by reserpine as an rodent model of depression and in people taking reserpine as hypertension treatment; more frequent occurrence of less functional allelic variations of tryptophan hydroxylase 1 and 2 genes among people with major depression and neurotic/anxious personality, and sensitivity of the serotonin transporter gene promoter region short allelic variant carriers to stress in the development of depression (Bekris et al., 2005; Bell et al., 2005; Elhwuegi, 2004; Gamaro et al., 2003; Jans et al., 2010; Karg et al., 2011; Leonard, 2005; Mangiavacchi et al., 2001; Nash, 2005; O'Neil and Moore, 2003; Piñeyro and Blier, 1999; Zhang, 2005). Conclusively, it is clear that the serotonergic system is malfunctioning in affective disorders and in animal models of depression, but there can be multiple simultaneous molecular mechanisms in a number of brain circuits that contribute to the depressive state.

The natural variability of the capacity of serotonin system is one of the factors that causes chronic stress to induce a mild to severe depression-like state in a number of rats while some animals remain unaffected (Chaouloff et al., 1999). Partial denervation of the serotonergic system with parachloroamphetamine allows to experimentally elicit a vulnerability that will render the animals more susceptible to environmental stressors and to control for the vulnerability/protective properties of a certain neurotransmitter system.

Parachloroamphetamine and other substituted amphetamines are potent neurotoxins with a multiphase, time dependent impact on neuronal functioning. The acute effect of parachloroamphetamine is a massive, dose dependent release of serotonin, resulting in a transient depletion followed within days by the onset of degeneration of axon terminals, especially in neocortex, striatum and thalamus, leaving the preterminal axons and cell bodies intact (Mamounas and Molliver, 1988; Wilson, 1993). The abovementioned studies used destruction of the serotonergic system as a tool to study morphology and morphological changes, but such a near-total lesion is of limited value from the behavioural viewpoint. It has been suggested that partial lesions with small toxin doses can be utilised to add ecological validity to animal experiments (Datla and Curzon, 1996).

It has been previously shown that small doses of parachloroamphetamine (2 mg/kg) that cause a restricted reduction of serotonin in frontal cortex, cerebral cortex, hippocampus, hypothalamus and cerebellum can induce behavioural alterations bearing similarities to the negative impact of chronic stress – increased anxiety in social interaction test and impulsivity as indicated by more active coping in the forced swimming test (Harro, 2002; Harro et al., 2001; Häidkind et al., 2004). Rats with partial serotonergic lesions who were submitted to chronic stress had higher sucrose solution intake and this was

decreased to control level by chronic citalopram treatment (Tõnissaar et al., 2008b). A partial serotonergic lesion caused by methylenedioxymeth-amphetamine similarly induces long-term changes in behavioural inhibition, as it facilitates aggression during social interactions and increases activity in the open field (Ando et al., 2006).

Thus partial serotonergic lesion has significant effects on behaviour and a potential to further change the impact of chronic stress.

#### 1.2.3.2.2. Affective disorders and social behaviour, sociability trait in rodents

The relationship between social behaviour and psychopathology is complex – social behaviour can be the cause (via e.g., exposure to social stress), consequence (e.g., deterioration of personal relationships) and, if altered, a symptom of several mental disorders, it can also moderate the remission and relapse (APA, 1994; Björkqvist, 2001; Kronmüller et al., 2011; McKee et al., 2011; WHO, 1992). Thus dysfunctional social relations resulting from mental disorders can themselves be a source of chronic stress, forming a vicious circle.

Disturbances in social behaviour are important symptoms in a number of psychopathological conditions such as schizophrenia, autism spectrum dis-orders, depression and social anxiety disorder, and social behaviour has served as the readout in corresponding animal models (APA, 1994; Berton et al., 1997; Dieckmann et al., 2007; Haller and Bakos, 2002; Haller and Kruk, 2006; Hammock and Young, 2006; Happe et al., 2006; WHO, 1992). There is a growing interest in targeting social dysfunction in mental disorders and reversal of social dysfunction is already considered a valid behavioural endpoint in interventions targeting depression (Novick, 2011; Vialou et al., 2010).

Sociability is a stable characteristic of an animal expressed behaviourally as proneness to initiate or actively accept social contact with a conspecific on neutral territory (Tõnissaar et al., 2008a; Tõnissaar et al., 2004). Elements of social behaviour are included in the personality models of humans, e.g., in the five factor model (McCrae and John, 1992). Sociability, as defined for the animal model in present study, would translationally reflect the drive for social contact included in the extraversion dimension, and the ability for non-agonistic social interactions included in the agreeableness dimension (McCrae and John, 1992). The trait-like nature of sociability suggests a strong genetic control of the disposition. The genetic component of social behaviour has been revealed by demonstrating different levels of social investigation or social avoidance in several inbred rat and mouse lines (Berton et al., 1997; Kantor et al., 2000; Moy et al., 2004; Moy et al., 2008; Moy et al., 2007). The highly genetic regulation of social behaviour can be mediated by activity in specific neurotransmitter systems. Pharmacological manipulations have revealed the role of dopamine, serotonin, GABA, glutamate and the neuropeptides oxytocin and vasopressin (Cho et al., 1999; Jenkins et al., 2008; Knutson et al., 1998; McGregor et al., 2008; Morley et al., 2005; Morley and McGregor, 2000; Rademacher et al., 2002; Silverman, 1965). Constitutional levels of a specific form of social behaviour in humans, affiliation, are proposed to rely on the interplay of

dopamine, opioids, glutamate, oxytocin and vasopressin (Depue and Collins, 1999; Depue and Morrone-Strupinsky, 2005). Sociability may be inversely associated with cortical serotonin metabolism in rats, but positive correlations between sociability trait and serotonin function have also been reported (Mehlman et al., 1995; Tõnissaar et al., 2004). Sociability levels mediate the response to chronic stress – rats with high sociability levels develop anhedonic behaviour more readily after chronic stress, whereas low sociability rats behave more actively in the forced swimming test (Tõnissaar et al., 2008a).

Thus sociability can be regarded as a stable trait-like phenotype and it mediates stress-vulnerability/resilience.

#### 1.2.3.2.3. Incentive behaviour in affective disorders and hedonic trait in rats

The incapability to experience pleasure is one of the core symptoms of depression (APA, 1994; WHO, 1992). In depressed humans, anhedonia usually manifests as retreating from activities formerly perceived as pleasurable. In animals, behavioural endpoints to reveal anhedonia often include measurement of intake or preference of palatable food. Sucrose solution is known to have highly rewarding properties in conditioning tasks, with rats behaving more actively with increasing concentrations of sucrose solution (Brennan et al., 2001). Changes in the consumption of palatable food/liquid is a wide-spread method to characterise the changes inflicted in rodents by chronic stress to indicate that the animal is in a "depressive" affective state (Strekalova and Steinbusch, 2010; Willner, 1997; 2005).

The observation of large inter-individual differences in sucrose consumption led to a thought that sucrose consumption can be regarded as a behavioural trait. Tõnissaar et al. demonstrated that sucrose intake is a stable characteristic of an animal, especially if measured during its active time – the dark period in the rat, and this trait correlates with the dopamine $D_2$ receptor function in the nucleus accumbens (Tõnissaar et al., 2006).

Preference for sweet taste seems to be under genetic control in humans and in rodents: mouse lines differ in sucrose consumption levels and rat lines with high and low saccharin intake have been bred (Gosnell and Krahn, 1992; Gosnell et al., 2010; Keskitalo et al., 2007a; Keskitalo et al., 2007b; Pothion et al., 2004). Animals that prefer sweet taste also prefer other substances with rewarding properties, like alcohol, and vice versa, alcohol-preferring animals also consume more sweet solutions (Gosnell and Krahn, 1992; Martinetti et al., 2007; Sinclair et al., 1992). Several neurotransmitter systems have been implicated in sucrose preference – most often dopamine and opioids but also oxytocin and serotonin (Amico et al., 2005; Berridge and Kringelbach, 2008; Brennan et al., 2001; Kranz et al., 2010; Le Merrer et al., 2009; Miedlar et al., 2007; Taha et al., 2006; Wise, 2008; Wise, 2005).

In pursuit of face validity for sucrose consumption/preference test used in rodents the translational value of the test can be questioned. Rodent anhedonia model does not directly imitate human food related motivation and shouldn't probably even be conceptualised as inability to feel pleasure, but should rather

be viewed as a broader construct reflecting general motivational dysfunction and assignment of value to stimuli (Berridge, 2007). Face validity (apparent validity) in this, and probably also in other cases, though attractively simple, bears risks of anthropomorphism and does not necessarily aid in developing valid models (Holmes, 2003).

Thus rats display a stable hedonic trait and sucrose intake relates to chronic stress response and the development of depressive-like behaviour.

#### 1.2.3.2.4. Maternal separation

The notion that newborns need constant physical nurture and bodily contact, besides nutrition and stable body temperature, was first coined in John Bowlby's influential work. He was also the first to introduce the term maternal separation as a source of distress (Duniec and Raz, 2011). The adverse impact of maternal separation is clearly measurable with ultrasonic separation distress vocalisations emitted by rat pups when separated from their mothers, and the brain mechanisms underlying separation distress are proposed to be among those from where depression originates (Panksepp and Watt, 2011). Maternal separation is an extremely potent stressor as a single 24 hour episode of separation can lead to adult behavioural disturbances in rodents (Lehmann et al., 1999). Several regimes differing in the number and duration of separation sessions are utilised, most models using repeated separations for a shorter period of time. A few days old rat pups are removed from their dam and kept in environment with controlled temperature usually for an hour a day for 2–3 weeks (Zimmerberg and Sageser, 2011). The dam is left waiting alone in the home cage, and one of the measures in this paradigm is the increased maternal care when the pups are returned to their home cage (Zimmerberg and Sageser, 2011).

Maternal separation causes changes in juvenile play behaviour (e.g., less boxing), greater anxiety in elevated plus maze, disturbed social recognition and spatial learning, more activity in the open field and exaggerated acute stress response (in heart rate and corticosterone levels), and passive coping style in the forced swim test (Eiland and McEwen, in press; Kalinichev et al., 2002; Lajud et al., in press; Lukas et al., 2011; Muhammad and Kolb, 2011; Sanders and Anticevic, 2007; Zimmerberg and Sageser, 2011). Higher baseline corticosterone levels but blunted acute stress-induced ACTH levels have also been reported in maternally separated rats (Lajud et al., in press; Marais et al., 2008). It must be noted that both spatial and fear learning deficits are more robust and more frequently found than anxious behaviour or increased stress reactivity after maternal separation (Hulshof et al., 2011; Lehmann et al., 1999). Greater immobility in the forced swimming test was found only in maternally separated animals that were subjected to chronic stress in adulthood (Marais et al., 2008). When using sub-chronic separation regimens, the effects of maternal separation on behaviour can depend on the time of applying the stressor, and spatial learning and fear conditioning seem to have different developmental windows (Lehmann et al., 1999).

Rats that were subject to maternal separation had lower brain weight as adults, though a small reduction in body weight was present as well (Muhammad and Kolb, 2011). Neonatal stress affects several neuronal systems implicated in affective disorders and learning in adulthood – increased spine density in medial prefrontal and orbital frontal cortices and nucleus accumbens, possibly a compensation for lighter brains; less vasopressin release in septum in response to a social stimulus; decreased noradrenaline and dopamine levels in the frontal cortex; decreased serotonin content in nucleus accumbens; decreased serotonin and dopamine content and metabolite levels in the amygdala; and elevated corticotropin-releasing factor receptor 1 baseline and stress induced levels in cerebral cortex, hypothalamus and hippocampus, and a huge increase in corticotropin-releasing factor receptor 2 in amygdala in maternally separated animals (Eiland and McEwen, in press; Lukas et al., 2011; Muhammad and Kolb, 2011; Niwa et al., 2011; O'Malley et al., 2011; Oreland et al., 2011). The impact of chronic ethanol intake on regional monoamine levels depended on the animals' previous exposure to maternal separation (Oreland et al., 2011). Interestingly the behavioural/physiological plasticity of the newborn rat allows the endocrine stress response to habituate during repeated separation (Daskalakis et al., 2011).

The maternal separation paradigm applied in the experiment reported in this thesis has yielded adult rats more passive on both trials in the forced swimming test; anhedonic, as indicated by lower sucrose preference; and more anxious in the elevated plus maze (Aisa et al., 2008; Aisa et al., 2007). Maternally separated rats had extremely high HPA axis reactivity to acute stress (6 fold difference compared to controls), a decrease in glucocorticoid receptor levels in hippocampus, and a learning deficit in Morris water maze (Aisa et al., 2008; Aisa et al., 2007).

Conclusively, repeated maternal separation is a chronic stressor that induces both immediate and postponed changes in animals.

#### 1.2.3.2.5. Exploratory behaviour and positive affectivity

The purpose of exploratory (or novelty-related) behaviour is gathering of and acting upon information in an unfamiliar environment, driven by conflicting motivations to explore the potential resources of novel environment or to stay within the secure and familiar surroundings (Harro, 1993). The role of novelty-related behaviour as one of the basic behavioural domains can be, with some additions, further extrapolated in human decision-making, as decisions depend on whether to exploit safe options with predictable results or to explore risky choices with possible gains (Cohen et al., 2007). The relevance of novelty-related behaviour to depression has been pointed out from another angle by Bevins and Besheer by using conditioned place preference for a novel object as a measure of drug-withdrawal induced anhedonia (Bevins and Besheer, 2005). In this approach the role of anxiety is diminished, as the anxiety-generating stimulus is not present at the time of behaviour measurement, and the test does not rely on locomotion (Bevins and Besheer, 2005).

Animals differ in their spontaneous and stimulated behavioural response to novel stimuli and, as novelty-related behaviour is implicated in psychopathology, these individual differences can be used to exemplify the neurobiology of these pathologies (Harro, 2010).

One of the more widely spread methods of measurement of novelty-related trait in rodents is spontaneous horizontal locomotor response to an unknown environment. Animals more active in such conditions are susceptible to depressive/anxious behaviour after social defeat and are prone to drug self-administration, probably due to sensation seeking (Blanchard et al., 2009; Duclot et al., 2011). Among other tests, spontaneous vertical activity (though rearing is more strongly associated with anxiety) and individual differences in activity caused by administration of a non-specific dopamine receptor agonist apomorphine have also been used to distinguish more novelty-seeking animals (Harro, 2010; Pawlak et al., 2008).

Novelty related behaviour, as measured in the exploration box test, is more complex, as it is measures open field activity, object exploration and it allows rats to refrain from the open field in a small shelter (Mällo et al., 2007b). The test measures two behaviourally unseparable constructs – anxiety caused by novelty and motivation to uncover the novel field (Mällo et al., 2007; Otter et al., 1997). This way two symptoms associated with depression are clustered, as animals with low exploratory activity (LE) have both high anxiety and low exploratory motivation whereas high-exploratory rats (HE) have high exploratory drive and low anxiety (Harro, 2010). HE-rats are more anxious in the elevated plus-maze, more passive in the forced swimming test and prone to more persistent fear memory than LE-animals (Mällo et al., 2007b). LE-rats have lower baseline and amphetamine-induced levels of striatal extracellular dopamine than HE-animals, and the same baseline level of serotonin as in HE-rats is achieved with higher reuptake activity (Mällo et al., 2008; Mällo et al., 2007).

Rats have complex social behaviour and, among other cues, ultrasonic vocalisations are used to convey information about the animals' condition and intentions (Brudzynski, 2005; Portfors, 2007). Ultrasonic vocalisations around the frequency of 50 kHz are emitted by juvenile rats during social play and by adult rats during highly rewarding stimuli (Knutson et al., 2002; Panksepp, 2007). In an attempt to mimic some of the behaviours eliciting 50 kHz ultrasonic vocalisations during play behaviour, Panksepp and colleagues stimulated the juvenile rat by mimicking rough and tumble play i.e., "tickled" the animal, and discovered that this artificial "social cue" provokes a similar response as play-fighting (Panksepp and Burgdorf, 2000). Fifty kHz ultrasonic vocalisations can be elicited by electric stimulation of the prefrontal cortex, nucleus accumbens, ventral pallidum, lateral preoptic area, lateral hypothalamus, ventral tegmental area, and raphe, areas involved in reward behaviour as revealed by self-stimulation studies (Burgdorf et al., 2007). An opioid agonist and amphetamine increased 50 kHz chirpings whereas dopamine

antagonism reduced this behaviour (Burgdorf et al., 2001; Burgdorf et al., 2007).

High-chirping rats tended to consume less sucrose, be more passive in the exploration test (females only) and show more passive coping style in the forced swimming test (females only) than low-chirping rats (Mällo et al., 2007b). On the other hand LC animals were more susceptible to chronic stress, as revealed by reduced 50 kHz ultrasonic vocalisations, slower weight gain, decreased sucrose consumption and more active exploratory response to novelty (Mällo et al., 2009).

Thus animals with persistently lower levels of both exploration and positive emotionality are more vulnerable to develop depressive symptomatology.

### 1.2.4. Neurochemical systems proposed to be involved depression

The monoamine hypothesis of depression has thus far been the most influential. The effect of most antidepressants is initiated by increasing monoamine levels but the original notion that lower levels of serotonin and/or noradrenaline are causing depression has evolved to more detailed hypotheses e.g., expression levels of receptors and serotonin transporter, dynamics of pre- and post-synaptic mechanisms, and time-dependent interactions of the monoamine systems (Boer, 2006; Harro and Oreland, 2001; Hirschfeld, 2000). Disruptions in the dopaminergic system are involved in the motivational deficits and insensitivity to reward – anhedonia, one of the core symptoms of depression (Nestler et al., 2002). Cholinergic system is also dysfunctional in depression and this probably accounts for some of the learning difficulties frequently seen in mood disorders, and serves as a target for cognitive enhancement (Dagyte et al., 2011).

Glutamate levels are increased and GABA levels are decreased in plasma and cerebrospinal fluid in depressed patients, allowing to construct a theoretical model that the ratio of inhibitory GABA and excitatory glutamate signal on the paraventricular hypothalamus is disrupted in depression (Alcaro et al., 2010; Gao and Bao, 2011). There is a reason to believe that the increase/decrease in glutamate and GABA levels is region-specific. Increased glutamate and glutamate/GABA ratio has been shown in anterior cingulate, amygdala and occipital cortex, and the reduction of glial cells/glial glutamate uptake can be involved in this (Kugaya and Sanacora, 2005). An interaction of monoamines, glutamate and GABA has been proposed, as noradrenaline hypoactivity could result from presynaptic GABAergic inhibition and serotonin hypoactivity from glutamatergic inhibition (Werner and Coveñas, 2010).

Neuropeptides e.g., the tachykinin family and galanin, are frequently reported to be dysregulated in depressive states. Galanin receptor activation subtype-specifically either increases or decreases depressive behaviour (Anisman et al., 2008; Kuteeva et al., 2008; Machado-Vieira et al., 2010). Depression is associated with unresponsive glucocorticoid feedback system and elevated

corticotrophin releasing factor and vasopressin levels in cerebrospinal fluid, plasma and brains of humans and rodents (Gao and Bao, 2011). Brain derived neurotrophic factor deficiency, especially in the hippocampus, is involved in vulnerability to depression. Brain derived neurotrophic factor levels are lower in ventral tegmental area after chronic stress in rodent models of depression, and normal levels are restored by antidepressant treatment; depressed patients have lower prefrontal cortex and hippocampal brain derived neurotrophic factor content as indicated by post-mortem measurement (Berton et al., 2006; Elfving et al., 2010; Lee et al., 2010). Adult age neurogenesis in hippocampus is suppressed by depression, chronic stress and by increased levels of circulating glucocorticoids, and is restored by antidepressant treatment (Sahay and Hen, 2007). The causal role of neurogenesis in depression is though questioned and the extensiveness of its role in antidepressant effect is not settled yet (Eisch et al., 2008; Sahay and Hen, 2007).

Examples of molecules outside the synaptic transmission system implied in depression are glycogen synthase kinase–3 and protein kinase C, modifiers of histone structure and proteins involved in apoptosis (Boer, 2006; Machado-Vieira et al., 2010). Recently theories linking depression with inflammation and mitochondrial defects have emerged, showing increases in oxidative and nitrosative stress, disturbed mitochondrial energy metabolism and more active inflammatory system e.g., higher levels of cytokines in depression (Anisman et al., 2008; Gardner and Boles, 2011; Leonard and Song, 1996; Maes et al., 2011). Depressive behaviour can be induced by administration of inflammatory agents, and drugs targeted at mitochondrial dysfunction mitigate depression (Gardner and Boles, 2011; Maes et al., 2011).

Conclusively, several neurochemical mechanisms, possibly simultaneously, lead to depression, the mechanisms can be specific to subtypes/syndromes and symptoms, and the mechanisms are probably not mutually exclusive.

## 1.2.5. How can the involvement of brain regions/networks in stress related behaviour be assessed?

### 1.2.5.1. Lesion studies

Anatomically precise lesions to a large variety of brain regions have implications on animal affective behaviour. Lesions of the bed nucleus of the stria terminalis and lateral septum increase “passive coping” – immobility in the forced swimming test – and disrupt normal fear-learning (Contreras et al., 1995; Hammack et al., 2004; Pezük et al., 2008; Pezük et al., 2006). Habenular lesions have a potentially “antidepressive” effect – in chronically stressed rodents, lesions of lateral habenula increase active coping in the forced swimming test, possibly via increased dorsal raphe serotonergic activity (Yang et al., 2008). Median raphe lesions can have anxiolytic effect via serotonergic mechanisms but by damaging the passing fibres via other neurotransmitter systems as well, and lesions to the cerebellum can relieve anxiety (Andrade et al., 2004; Bobée

et al., 2000; Konno et al., 2007). Medial prefrontal cortex lesion (including prelimbic, infralimbic and anterior cingulate) decreases anxious/fear related behaviour, and muscimol-induced inactivation of the infralimbic cortex reduces passive coping and reverses the anxious/depressive inbred phenotype in an animal model of depression (Shah and Treit, 2003; Slattery et al., in press). High-frequency stimulation of the medial prefrontal cortex, causing a transient functional deficit, effectively reduces depressive behaviour in the forced swimming test, especially if the electrodes are in the prelimbic area (Hamani et al., 2010a; Hamani et al., 2010b; Hamani and Nóbrega, 2010). Lesions specifically aimed at anterior cingulate cortex increase passive coping and anxious behaviour, and orbitofrontal lesions cause anxiety (Bissiere et al., 2006; Rudebeck et al., 2007). Basolateral amygdala inhibition via high-frequency stimulation reduces stress-related behaviour in an animal model of post-traumatic stress (Langevin et al., 2010). At present, data from humans and rodents on high-frequency stimulation-induced functional suppression for the same locus are scarce. In animals, the most promising candidate areas to mediate alleviation of a depressed state are frontal regions. In humans, several areas have been on trial – nucleus accumbens, habenula (case study) and subgenual anterior cingulate cortex (Hamani and Nóbrega, 2010). Electrical activation of ventral tegmental area leads to decreases in both anhedonia and passive coping in the forced swim test, and in humans a clinical trial with electrophysiological suppression of nucleus accumbens activity has reduced depressive symptoms (Bewernick et al., 2010; Friedman et al., 2008). High-frequency stimulation of the subthalamic nucleus in the rat, a treatment that is anti-parkinsonian humans, can be depressogenic, and it induces passive coping in forced swim test (Benabid et al., 2009; Temel et al., 2007).

### 1.2.5.2. Measurement of regional brain activation

#### 1.2.5.2.1. Immediate early gene expression and glucose utilisation in rodents

A histochemical tool that helped to reach a qualitatively new level of understanding of functional brain anatomy by enabling large-scale task-related activity mapping in rodents that relies on immediate early gene expression was invented in the early 1980ies (Kovács, 2008; Sagar et al., 1988). Activation of postsynaptic neurons induces signal transduction pathways that can, in turn, induce immediate early gene expression and neuronal depolarisation, thus allowing to draw conclusions about neuronal activity (Hoffman and Lyo, 2002). Immediate early gene mapping has been carried out in a number of animal models that are relevant for affective neuroscience, such as exposure to rough and tumble play, exposure to predators or predator odours, playback of ultrasonic vocalizations of conspecifics, comparison of animals with different strategies to control their environment, as well as changes in aggression and anxiety, with and without administration of various drugs (Comoli et al., 2003; Dielenberg et al., 2001; Gordon et al., 2002; Haller et al., 2006; Kroes et al., 2007; Sadananda et al., 2008; Singewald et al., 2003). As a gross generalisation, the aforementioned studies have most frequently revealed the involvement of

cerebral cortex, nuclei of the corpus amygdala, bed nucleus of stria terminalis, periaqueductal gray matter, septum, and various nuclei of hypothalamus to be activated in stressful circumstances. In addition, immediate early gene expression patterns have been found to be influenced by emotional arousal in olfactory bulbs, striatum and nucleus accumbens, hippocampus, nuclei of the thalamus, raphe and locus coeruleus. There is a large overlap between the regions activated by both negatively and positively valenced stimuli, suggesting that activity in amygdalar regions, bed nucleus of stria terminalis, several hypothalamic regions and periaqueductal gray might reflect an overall emotional response.

Glucose is the main source of energy in the brain and cells in more active brain regions incorporate more glucose. By adding a radiolabel to glucose with a substitution at C2 position, the molecule is transported in the cell, but does not undergo full glycolysis and thus accumulates in the cell (Sokoloff et al., 1977). A histochemical method for detecting acute neuronal activity via glucose ($^{14}$C–2-deoxyglucose) utilisation has been widely used, but chronic stress/ depression studies with this technique are scarce (Caldecott-Hazard et al., 1988; Sokoloff et al., 1977; Wree, 1990). It has been reported that acute stressors evoke rather a similar 2-deoxyglucose and immediate early gene regional activity pattern, though the two measures show a different response to acute antidepressant treatment (Duncan et al., 1993). Interestingly, chronic citalopram administration, that normalises the behavioural effects of olfactory bulbectomy, does not reverse the changes in brain glucose utilisation, but rather results in a new activity pattern (Skelin et al., 2009). Thus it might not be necessary to reverse the neurophysiological shift to alleviate depression, but maybe just to reach a new balance in chemical/electrical activity. Another 2-deoxyglucose study revealed elevated glucose utilisation in habenula and reduced brain activity in prefrontal cortical and anterior thalamic regions and in inferior colliculi in different animal models of depression (Caldecott-Hazard et al., 1988).

#### 1.2.5.2.2. Functional brain activity imaging in human depressed subjects

Several methods are available for human functional brain imaging – most commonly functional magnetic resonance imaging (fMRI) and positron emission tomography are used. fMRI assays the regional distribution of oxyhemoglobin rich blood and positron emission tomography is used to measure regional distribution of radiolabelled substrates/ligands, glucose among others. Both methods can give an indirect index of cerebral energy metabolism (Gupta et al., 2004). Abnormalities in ventromedial prefrontal cortex, lateral orbital prefrontal cortex, dorsolateral prefrontal cortex, anterior cingulated cortex, ventral striatum, amygdala, thalamus, hypothalamus and hippocampus are the most frequent findings distinguishing depressed patients from healthy controls, with neocortex and hippocampus assumed to be responsible for cognitive/learning difficulties, striatum for reduced incentive behaviour and affect-laden learning, and hypothalamus responsible for bodily activation/inhibition (Lee et al., 2010; Maletic et al., 2007; Nestler et al., 2002).

#### 1.2.5.2.3. Brain oxidative energy metabolism

Although the brain weight comprises only approximately 2% of the body mass of an average human, it consumes about 20% of the oxygen and 25% of the glucose of the whole organism (Erecinska et al., 2004; Magistretti et al., 1995). Cerebral energy metabolism depends on glucose that is mostly aerobically oxidised to $CO_2$ and $H_2O$. The ratio of oxygen consumption to $CO_2$ production, the respiratory quotient, is almost one, indicating that carbohydrates are the substrates for oxidative metabolism (156 μmol/100g tissue/min) (Magistretti et al., 1995). Cerebral function rests on continuous glucose and oxygen supply via bloodflow, proper oxygen tension, and intact mitochondria (Magistretti et al., 1995). Glucose permeates the blood-brain-barrier via facilitated diffusion and in normal conditions the amount of glucose entering extracellular matrix is much larger than needed, and some of it diffuses back into blood. Fifteen percent of glucose diffuses from arterial capillaries into extracellular space and one third of it will diffuse back to venous capillaries, thus net 10% of blood glucose is utilised in normal conditions (Paulson, 2002). Glucose is metabolised to pyruvate via glycolysis, yielding two adenosine triphosphate (ATP) molecules per glucose. Further, pyruvate can enter the citrate cycle and via oxidative phosphorylation yield thirty ATP molecules per glucose; 80% of glucose in the brain enters the aerobic pathway, and the rest will serve as a source of carbon skeletons and anaerobic metabolism (Magistretti et al., 1995). Changes in activity elicited by physical and mental processes are relatively small, present in distinct neuronal populations, and quite often increases in some areas are accompanied by decreases in others. Task-related changes in glucose utilisation do not exceed 5% to 10%, but the actual energetic changes depend on how much of glucose is directed to aerobic and anaerobic pathways (Buzsáki et al., 2007).

Processes that make up neuronal energy demand include the propagation and maintenance of membrane potentials (action potential, postsynaptic potential), synthesis of enzymes, vesicular transport and reuptake, with the electrochemical component demanding 40–60% of the energy, and even more at times of high activity (Erecinska et al., 2004). Among electrochemical processes maintaining membrane potential in dendrite arbor is most energy consuming, whereas axonal currents spend less energy (Erecinska et al., 2004). The energetic demand of glia can be larger than previously estimated, as the membrane potential of astrocytes has to be restored by $Na^+K^+$ATPase after glutamate and $K^+$ uptake from extracellular space, thus the proportion of energy metabolism of astrocytes can account for up to 30% of the whole brain (Hertz et al., 2006; Magistretti et al., 1995). Astrocytes are vital for the neuronal metabolism as revealed by studies of lactate shuttle mechanism and expression pattern of glycolytic enzymes and lactate dehydrogenase isoforms. In vivo studies support the hypothesis that glucose is first transported to astrocytes, and metabolised to lactate and pyruvate that in turn are transported to the neuron for oxidative phosphorylation with the yield of thirty six ATP molecules per pyruvate (Magistretti, 2009; Wyss et al., 2011).

Serotonin, noradrenaline, vasoactive intestinal peptide and dopamine are involved in the acute regulation of energy need by increasing glycogenolysis and blood flow, thus forming a prerequisite for an increase in oxidative metabolism and subsequent long-term increase in the expression of electron transport proteins (Magistretti et al., 1993; Poblete and Azmitia, 1995; Zauner and Muizelaar, 1997; Walls et al., 2009).

Mitochondria are the locus of energy production in the eukaryotic cell, where in the electron transport chain, energy from reducing equivalents, originating from aerobic metabolism of the glycolytically derived pyruvate, is used for adenosine triphosphate (ATP) synthesis. ATP is the universal source of energy responsible for supplying the biochemical reactions. Approximately 30 kJ of energy is generated hydrolysing one mole of ATP by releasing one of the mutually electrostatically repulsive inorganic phosphate groups. According to the chemiosmotic theory, the energy needed for ATP synthesis is generated by dislocation of H-ions from the mitochondrial inner matrix to the intermembrane space and encompassing H in the matrix to $O_2$, generating a protonmotive force that is resolved by allowing $H^+$ diffuse down the electrochemical gradient thus generating energy (Oster et al., 2000). In the citrate cycle energy-carrying reducing equivalents, 3 molecules of protonated nicotinamide adenine dinucleotide (NADH) and 1 protonated flavin adenine dinucleotide ($FADH_2$) molecule are produced, that are further used as proton donors in the respiratory chain, where oxygen is reduced to water (Richter and Ludwig, 2009).

There are four electron transferring complexes: NADH-coenzyme Q reductase (complex I), succinate-CoQ reductase (complex II), ubiquinol-cytochrome c reductase (complex III), and cytochrome c oxidase (complex IV). Only complexes I, III and IV add to the electrochemical gradient, whereas complex II functions as an electron transporter only (Lenaz and Genova, 2010). There is a reason to believe that the complexes are not randomly located in the membrane, but rather form supramolecular formations, as complexes I and III behave as a single unit in mammals (Lenaz and Genova, 2010). Electrons are mediated between them by the mobile carriers ubiquinone and cytochrome c. The latter is a molecule located on the external surface of the inner membrane. One pool of cytochrome c diffuses freely in the membrane, and another pool is more strongly anchored to the inner membrane and forms a complex with other enzymes of respiratory chain (Lenaz and Genova, 2010). Complex V is the FoF1 ATP synthase responsible for oxidative phosphorylation. The ion channel, the Fo part, allows $H^+$ ions to move along the electrochemical gradient generating a rotary torque that is used by the F1 portion of the enzyme to catalyse the attachment of $PO4^{3-}$ to ADP (Oster et al., 2000).

Complex IV, cytochrome (c) oxidase (EC 1.9.3.1), is a member of heme-copper oxygen reductase superfamily that catalyzes the reduction of oxygen to water and translocates protons across the mitochondrial membrane. Cytochrome oxidase transfers 4 electrons to water and translocates another 4 (Brzezinski and Gennis, 2008; Brunori et al., 2005; Lenaz and Genova, 2010). Mammalian cytochrome oxidase is a highly complicated and evolutionally conserved

structure consisting of 13 subunits. Three of the largest subunits, forming the catalytic site, are coded by the mitochondrial genome, the rest by the nuclear DNA (Herrmann and Funes, 2005).

As the most costly neural processes are involved in synaptic transmission, especially in the postsynaptic realm, and aerobic oxidative metabolism is the main source of energy in the brain, assessment of electron transport chain activity would be indicative of activity of neuronal populations.

#### 1.2.5.2.4. Cytochrome oxidase histochemistry

The methods used to detect regional brain activation that were described above – immediate early gene expression, glucose utilisation and fMRI – are useful for detecting short term changes as a response to stimulation, and it has been convincingly demonstrated that chronic stress exposure and behavioural phenotype affect cerebral responses to acute stressors. When the task is to find brain areas where baseline activity differentiates animals with a vulnerability phenotype and chronic stress exposure from controls, long-term changes in energy metabolism should be measured.

Enzyme histochemistry serves as a link between biochemistry and morphology, as two parameters are assessed – the activity of the enzyme and its anatomical distribution (Meier-Ruge and Bruder, 2008). Histochemical detection of cytochrome oxidase activity was adapted for brain energy research by Wong-Riley and colleagues, and has since been used as marker in functional anatomy, regional learning-related changes and in models of pathological changes in Parkinson's and Alzheimer's diseases and depression (Gonzalez-Lima and Cada, 1998; Hevner, 1998; Wong-Riley, 1979). In cytochrome oxidase histochemistry the fully functional enzyme in unfixed brain slices uses externally added proton donor, 3,3'-diaminobenzidine, as a substrate, and externally added cytochrome c to carry electrons from diaminobenzidine to cytochrome oxidase. One of the reaction products, the oxidised diaminobenzidine, forms a visible precipitate (Gonzalez-Lima and Cada, 1998). The enzyme activity measured by cytochrome oxidase histochemistry reflects the amount of cytochrome oxidase expressed in the mitochondrion (Wong-Riley et al., 1998).

Postsynaptic potentials cause the largest energy demand in neurons. Postsynaptic potentials are relatively small in amplitude (up to 10 mV) but have a high energetic impact as the graded potentials are temporally and spatially summated over a large dendrite arbour and cell soma, and energy is needed for restoration of membrane potential after postsynaptic signalling (Giuliodori and Zuccolilli, 2004). Synaptic transmission can change the potential of a small portion of postsynaptic membrane (by influx of $Na^+$ for excitatory depolarisation and the influx of $Cl^-$ and efflux of $K^+$ for inhibitory hyperpolarisation), but this area can depolarise/hyperpolarise the adjacent segment of the membrane and thus the temporally/spatially additive postsynaptic potential occurs (Khurana, 2005). Excitation of postsynaptic membranes is more energy-demanding, as the maximum change in voltage of the excitatory postsynaptic

potential is 8 mV, whereas that for the inhibitory postsynaptic potential is 4 mV (Khurana, 2005).

All electrical changes within the brain that rely on oxidative metabolism are reflected in cytochrome oxidase activity levels – both excitatory and inhibitory potentials. As dendrites, especially opposing glutamatergic axon terminals, have the highest cytochrome oxidase staining intensity, it can be concluded that excitatory postsynaptic processes mostly contribute to cytochrome oxidase expression levels (Wong-Riley et al., 1998). The simplest reason for the assumption of glutamate's huge impact is the sheer abundance of its signal – e.g., 85% cortical synapses are glutamatergic, and the majority of brain regions express at least a moderate level of glutamate receptors (Magistretti, 2009; Petralia et al., 1994). Furthermore, glutamate and cytochrome oxidase are subject to common genetic control mechanisms, as e.g., the expression of both the NMDA receptor subunit 1 and cytochrome oxidase subunits are initiated by nuclear respiratory factor 1 (Dhar et al., 2009; Dhar and Wong-Riley, 2009).

The overwhelming majority of neurons are glutamatergic, and of the remaining population, for example in hippocampus, 15%–20% are inhibitory GABA-ergic interneurons (Buzsáki et al., 2007). Postsynaptic GABA rich synapses have lower cytochrome oxidase expression levels compared to glutamate-rich synapses, probably because depolarisation is more energy costly than hyperpolarisation (Mjaatvedt and Wong-Riley, 1988; Wong-Riley et al., 1998). Nevertheless, the inhibitory signal contributes to baseline energy expenditure and changes in the GABA-ergic signal could lead to increased/decreased energy consumption. Ackermann and colleagues have presented the argument that inhibitory neural signal must increase energy expenditure rather than decrease, because hyperpolarisation of the target cells is the consequence of ionic movements through membranes, and such movements activate energy-requiring postsynaptic mechanisms restoring membrane potential by pumping ions against concentration gradients (Ackermann et al., 1984). To tests this, firing of hippocampal pyramidal cells was inhibited by low-frequency electric stimulation after $^{14}C$ deoxyglucose administration, and indeed the resulting GABA release was accompanied by increased glucose uptake in target cells depending on the duration of inhibition (Ackermann et al., 1984). Furthermore, excitatory and inhibitory postsynaptic potentials are additive, e.g., if a given area receives high level of both excitation and inhibition it could mean that action potentials are not generated but the energetic demand is still greater than in case of either excitation and inhibition alone (Giuliodori and Zuccolilli, 2004). The cost of both excitation and inhibition at the level of a single target neuron is increased if there is a temporal overlap between the two types of synaptic events (Buzsáki et al., 2007). The notion that receiving more frequent tonic input from GABA-ergic neurons increases metabolism in target areas is also supported by positron emission tomography with $^{18}F$-fluorodeoxyglucose (Buzsáki et al., 2007).

Cytochrome oxidase activity is affected by a variety of stimuli that depend on environmental factors and thus increase the variability in data. Besides the

slow regulation of energy production via cytochrome oxidase expression, conformational changes e.g., due to product feedback are responsible for faster changes in the activity of the enzyme (Beauvoit and Rigoulet, 2001). Cytochrome oxidase is directly inhibited by toxins such as cyanide and azide and by carbon monoxide as they block the binding of oxygen to cytochrome oxidase (Berndt et al., 2001; Cooper and Brown, 2008). Nitric oxide can, at low and physiologically relevant concentrations, potently and reversibly inhibit cytochrome oxidase activity, but at higher concentrations cause mitochondrial damage (Brunori et al., 2004). Higher levels of nitric oxide result from increased glutamatergic signalling that can lead to either transient hypo-energetic state by cytochrome oxidase blockage or to neuronal damage, as nitric oxide is one of the molecules proposed to mediate excitotoxicity (Brunori et al., 2004; Moncada and Bolaños, 2006). The tight coupling of energy metabolism, glutamate and nitric oxide function is regulated at transcriptional level, as expression of glutamate receptors, cytochrome oxidase subunits and neuronal nitric oxide synthase are activated by the same transcription factor, nuclear respiratory factor 1 (Dhar et al., 2009; Dhar and Wong-Riley, 2009).

Dopamine and serotonin both influence energy metabolism. Serotonin increases and dopamine inhibits axonal transport of mitochondria in hippocampal cultured cells (Chen et al., 2007; Chen et al., 2008). Dopamine and its metabolite 3,4-dihydrophenylacetic acid inhibit the activity of several mitochondrial electron transfer complexes, and chronic acetylcholine receptor activation by nicotine induces the expression of several genes of the electron transport chain proteins, including cytochrome oxidase subunit 1 (Gautam and Zeevalk, 2011; Wang et al., 2009).

Cytochrome oxidase activity slightly increases during wakefulness, but some regions, like the suprachiasmatic nucleus, are more active during the time of relative physical inactivity (Isobe et al., 2011; Nikonova et al., 2005). Although the number of mitochondria does not necessarily change with age, there is a clear decline in energetic function, as the levels of Complexes I and IV are reduced by approximately 30% in two-year old rats compared to young animals (Navarro and Boveris, 2004).

Several limitations of the cytochrome oxidase histochemistry approach and method should be considered in interpretation of results. The electro-physiological and neurochemical events that lead to changes in cytochrome oxidase activity are only partly known; only the basic excitatory and inhibitory neurotransmitters have been extensively studied in relation to cytochrome oxidase expression; the method describes brain activity in a cumulative way i.e., the balance in short-term increases and reductions must be in favour of one of these to reach a cumulative change, but short-term changes in the opposite direction are possible meanwhile; the activity of a brain region revealed by cytochrome oxidase histochemistry can result from secondary neural activity or behavioural activity accompanying the behaviours that the animals are selected for/induced (but this is common to all methods measuring neural activity); cytochrome oxidase histochemistry is informative only of region-specific

changes, direct information of activity changes in circuits can be obtained only via using additional methods (e.g., stimulation, lesions), as an indirect measure statistical connectivity analysis can performed; translational comparison with human data is complicated, since there are currently no in vivo imaging techniques of long-term neuronal activity in humans.

Conclusively, recognising the risk of over-simplification, as both excitatory and inhibitory input can increase postsynaptic activity and increase energy demand, we depart from the understanding that regions with higher synaptic activity express more cytochrome oxidase. Thus by determining which brain regions have constitutionally different neural activity depending on the trait or chronic treatment, we can elucidate the neuroanatomical substrate of the trait.

#### 1.2.5.2.5. Functional connectivity/ brain regional co-activation

In human fMRI studies functional connectivity is described as a temporal co-activation of brain regions (Greicius, 2008). The majority of functional connectivity models describe task related activity patterns, whereas recently also the resting state has been targeted to estimate basal neural connectivity of brain regions (Greicius, 2008).

It is proposed that disconnections between cortical and limbic areas are characteristic of depression. Pathways from integrative cortex (lateral, rostral and orbital prefrontal cortex) to emotional/visceral cortex (ventromedial prefrontal cortex, ventral anterior cingulate) and from cognitive/executive cortex (dorsolateral prefrontal and dorsal anterior cingulate cortices) to limbic areas (hippocampus, amygdala, nucleus accumbens) are hypoactive in depressed patients, resulting in less cortical control over limbic activity and henceforward an overactive autonomic and endocrine stress system (Greicius, 2008; Maletic et al., 2007).

In animal studies functional connectivity has been widely used in the immediate early gene and 2-deoxyglucose mapping. In this context functional connectivity is defined as inter-correlations of activities of brain regions, and it should be noted that this definition does not assume direct neuronal pathways between the areas, although hypotheses regarding well-defined networks can be proposed. The co-activation of brain regions can mean that there is a direct anatomical connection, there is an indirect anatomical connection via other nuclei, the regions are involved in separate functions that are both simultaneously activated to adapt to environment, or the activity of one region is caused by the behavioural actions initiated by another region.

### 1.2.6. Autonomic and HPA reactions to acute stress – stress-induced hyperthermia and endocrine function

Stress response is the mechanism by which organisms adapt to the demands of the changing environment and re-establish the challenged homeostasis (Kyrou and Tsigos, 2009). As a generalisation, there are two mechanisms by which stress response mediates the adaption of organisms to changing conditions – rapid activation of the sympathetic autonomic nervous system and a slower and more long-lasting activation of the hypothalamic-pituitary-adrenal (HPA) axis (Kyrou and Tsigos, 2009). Both of these mechanisms have a general activating impact on energy metabolism and expenditure and they support rapid action, the so called fight or flight response (McEwen and Wingfield, 2003; Vinkers et al., 2008). Stress and pathology are linked by two possible mechanisms, first, although adaptive in nature, repeated stress reactions have an adverse impact on health, including brain function, and secondly, there is a reason to suspect disruptions in stress response mechanisms in psychopathological conditions (Kyrou and Tsigos, 2009; McEwen, 2007). Higher baseline and stimulated levels of noradrenaline have been described in depressed patients and symptoms of autonomic activity form the core of anxiety disorders (Veith et al., 1994; Vinkers et al., 2008). Abnormal basal activity or reactivity of the HPA axis is linked to a variety of somatic and psychological pathologies e.g., depression, posttraumatic stress disorder and memory dysfunction (Kyrou and Tsigos, 2009; McEwen, 2007).

#### 1.2.6.1. Stress-induced hyperthermia

Stress-induced hyperthermia is a transient elevation in body temperature via autonomic activation after acute stress that can be viewed as an indicator of the level of stress-reactivity and anxiety (Bouwknecht et al., 2007; de Mooij-van Malsen et al., 2011). Stress-induced hyperthermia has been characterised in several rodent species, including rats and mice, in other mammals like foxes, rabbits and sheep, and in non-human primates (Bouwknecht et al., 2007; Vinkers et al., 2008). In humans, stressful situations like exams also result in core body temperature elevation (Briese, 1995; Marazziti et al., 1992). The fact that stress induces hyperthermia in several non-human animal species and in humans, and the role of autonomic reactivity in stress-related psychopathology e.g., anxiety, make it a method with good translational value (Friedman, 2007; Nordquist et al., 2008; Vinkers et al., 2008).

The biochemical mechanisms of stress-induced hyperthermia are at least partly and stressor-specifically different from infection-induced fever (Oka et al., 2001; Vinkers et al., 2009b). Neuroanatomically thermoregulation is controlled by hypothalamic preoptic area via raphe pallidus and dorsomedial hypothalamus (DiMicco and Zaretsky, 2007; Morrison, 2004; Oka et al., 2001). A large variety of stressors can induce thermogenic responses in rodents – including handling (e.g., the invasive temperature measurement itself), novel cage environment and behavioural testing (e.g., open field and light/dark test),

alarm pheromones, social conflict, restraint and confinement, maternal separation, cohort removal, social isolation, and even subtle manipulations as moving the cage of the animal (Bouwknecht et al., 2007; Dallmann et al., 2006; Gordon and Yang, 2001; Hennessy et al., 2010; Keeney et al., 2001; Kikusui et al., 2001; McGivern et al., 2009; Michel and Cabanac, 1999; Rodgers et al., 1994; Tornatzky and Miczek, 1994).

Chronic stress can either accentuate or diminish the effects of acute stress on body temperature, probably as a function of stressors and time between the two stresses. For some stressors e.g., social defeat, stress-induced hyperthermia still takes place without habituation despite repeated presentation (Bhatnagar et al., 2006; Chung et al., 1999a; Endo and Shiraki, 2000; Hayashida et al., 2010; Keeney et al., 2001; Rimondini et al., 2003).

The central coordination of stress-induced hyperthermia response largely depends on the serotonergic system. Serotonin 1a agonists can prevent stress-induced hyperthermia, as can serotonergic antidepressants after chronic administration (Conley and Hutson, 2007; Vinkers et al., 2010). There is large inter-individual variability in the properties of stress-induced hyperthermia in animals with different geno- and phenotype e.g., inbred mouse lines, genetically engineered rodents and depressive/anxious rodents display different autonomic activation in stressful cirumstances (Bouwknecht et al., 2007; Kõiv and Harro, 2010; Van Bogaert et al., 2006; Vinkers et al., 2008).

Thus testing the autonomic nervous system responsiveness to acute stress via hyperthermia would serve as a valid marker on anxiety.

#### 1.2.6.2. Endocrine stress response

The endocrine stress response is elicited when limbically controlled corticotropin-releasing factor and arginine vasopressin stimulate adrenocorticotropic hormone synthesis and release in the pituitary, and adrenocorticotropic hormone, when reaching adrenal glands through the blood-stream, induces synthesis and release of glucocorticoids – corticosterone in rats and cortisol in humans (de Kloet et al., 2008; McEwen and Wingfield, 2003). Glucocorticoids have a widespread target-specific action throughout the body, including the brain (McEwen, 2007). In normal conditions corticotropin-releasing factor is secreted in hypothalamus in a circadian burst pattern, with the highest values approximately at the time of awakening, and this rhythmicity can be affected in depressed patients (Gorwood, 2010; Kyrou and Tsigos, 2009). Corticosterone levels do not increase only in response to stress, but also in anticipation of change, either rewarding or not (Koolhaas et al., 2011). The highest acute glucocorticoid level increases are reported in social settings with sexual behaviour inducing the highest levels, followed by social defeat and social dominance (Koolhaas et al., 2011).

The central locus in the control of the HPA axis is the paraventricular hypothalamus, where corticotropin releasing factor and vasopressin are synthesised. Excitatory signal to paraventricular hypothalamus for complex, processive (i.e., non-systemic) stressors is conveyed from cortical integration areas (that also

receive serotonergic and noradrenergic input from dorsal raphe and locus coeruleus) via amygdala and septum, that rely to paraventricular hypothalamus through bed nuclei of the stria terminalis and hypothalamic nuclei (Ziegler and Herman, 2002). The top-down inhibition of this network is executed by medial prefrontal cortex, hippocampus and subiculum via bed nucleus of stria terminalis and hypothalamic nuclei (Ziegler and Herman, 2002).

Corticotropin releasing factor levels in plasma, cerebrospinal fluid and brain tissue are increased in depression and higher baseline and stress-induced glucocorticoid levels have been reported for depressed patients (Gao and Bao, 2011; Lopez-Duran et al., 2009). There is a possibility though that the HPA axis hyper-reactivity is not a feature of pure depression or pure anxiety, but rather caused by (or causing) the combination of the two – patients with primarily depressive or primarily anxious symptomatology had normal HPA axis reactivity whereas the co-morbidity of the two disorders yielded an hyper-active response to Trier Social Stress test (Cameron, 2006). Besides genetic control of the HPA axis, its reactivity is programmable by early experience – stressful events in childhood can amplify the adult response to stress (Mormede et al., 2011; Pariante and Lightman, 2008).

In normal individuals the HPA axis activity is controlled by a negative feedback system, both clinical and preclinical studies indicate defective HPA feedback in depression (Akil, 2005; Lopez-Duran et al., 2009). In normal conditions, increase in the HPA axis components operates as a signal to inhibit the system, whereas in chronically stressed rats the response to a novel stressor is increased. Thus corticosterone might facilitate the HPA activity in depression (Dallman et al., 2004). Boyle and colleagues have proposed a knockout mouse model for depression that lacks the glucocorticoid receptor in the forebrain (Boyle et al., 2005). Disrupting the negative feedback renders the HPA axis more active, induces depressive-like behaviour in the forced swimming, tail suspension and sucrose preference tests, and this condition is reversible by chronic imipramine treatment (Boyle et al., 2005).

Thus measurement of endocrine stress markers would be indicative of an anxious/depressive-like state.

# 2. AIMS AND RESEARCH QUESTIONS OF THE THESIS

The aim of the presented studies was the explorative mapping of brain regions that mediate chronic stress response and vulnerability to depression in rats, and to assess behaviour, autonomic and endocrine function of stress responses and vulnerability to depression, with a special focus on the serotonergic function. More specifically, the research questions are:

- What are the brain regions that display a change in long-term energy metabolism in response to chronic social stress and chronic variable stress?
- What are the brain regions that show different levels of oxidative energy metabolism in relation to vulnerability traits to depression – sociability trait, hedonic trait as measured by sucrose consumption, serotonergic dysfunction and adulthood depressive/anxious phenotype caused by neonatal maternal separation?
- What are the brain regions that display changed levels of oxidative energy metabolism in response to chronic social stress in rats with higher and lower levels of hedonic trait?
- What are the brain regions that display changed levels of oxidative energy metabolism in response to chronic variable stress in rats with partial serotonergic denervation?
- What are the brain regions that mediate vulnerability to depression in common to different animal models?
- How does chronic stress affect regional functional connectivity of the brain and what are the networks responsible for depression vulnerability?
- What is the effect of chronic social defeat in tests measuring depressive-like behaviour?
- Does stress-induced hyperthermia depend on the intactness of the serotonergic system and chronic variable stress experience?
- What is the effect of chronic stress on HPA axis function, as reflected in baseline and stress-induced levels of corticosterone and adrenal gland weight, in rats vulnerable to depression?

# 3. MATERIALS AND METHODS

## 3.1. Animals and general procedures

Except in **Study I,** all aspects of animal husbandry were identical throughout the experiments. Male Wistar rats were used in all experiments. Animals were purchased from Scanbur BK AB (Sollentuna, Sweden) and arrived in our animal house at the age of three weeks. Rats were kept in standard polypropylene cages in a light controlled room (12-h light/dark cycle; lights on at 8:30 a.m.) maintained at 22°C with food (Lactamin R35, Sweden) and water available ad libitum. Animals of the same experimental group were always housed together and experimental groups were weight-matched. All experiments were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the respective Ethics Committees. All efforts were made to minimize the number of animals used and their suffering.

In the experiments reported in **Paper I** and also included in the joint analysis in **Paper V,** 29 rats, weighing 260–332 g at the beginning of the experiment were housed four per cage. Animals were treated with parachloroamphetamine at the age of 2 months and 21 days and submitted to chronic variable stress at 3 months and sacrificed at 4 months of age, 4 days after the last stressful procedure.

In experiments reported in **Paper II and Study II,** also included in the analysis presented in **Paper V,** 70 rats, weighing 523–659 g at the beginning of the experiment, were housed three or four per cage. Animals were submitted to chronic social defeat stress at 7 months of age. Of the 70 animals, 48 were allocated into the social stress group (intruders) and 22 into the control group. Groups were matched for body weight and baseline sucrose intakes. These groups were further divided in two with half of the animals decapitated after chronic stress and used for histochemistry and the other half for behavioural experiments. An additional 48 male rats of the same age were singly-housed and used as aggressive residents, who were treated with apomorphine to evoke aggressive behaviour towards intruders.

In the experiments reported in **Paper IV,** 28 rats, weighing 370–435 g at the beginning of the experiment, were single-housed for ten days before the behavioural testing to increase social motivation. Animals were tested thrice in the social interaction test separated by 10 days. Rats were sacrificed and brains harvested at three months of age. Based on the mean social interaction in three tests with different weight-matched partners, animals with the highest (n=7), medium (n=8) and the lowest (n=8) sociability levels were chosen for histochemical analysis.

In experiments described in **Study III,** 40 rats, with the mean weight of 660 grams at the beginning of the experiment, were housed 4 animals per cage. Groups were matched by weight and body temperature during the first measurement. At the age of 7 months, rats received parachloroamphetamine

(2 mg/kg) injection and body temperature was measured after drug administration; 8 days later the second body temperature measurement took place, followed by 25 days of chronic variable stress. After the stress regimen body temperature was measured again, and this was followed by behavioural testing in the open field and light/dark box.

In the experiments reported in **Study I** and also included in the analysis of **Paper V,** timed-pregnant Wistar rats were provided on gestation day 16 from Charles River Laboratories (Barcelona, Spain), and individually housed in a temperature (21±1°C) and humidity (55±5%) controlled room on a 12-h light/dark cycle with food and water freely available. Five stress-group rats were subject to maternal separation from postnatal day 2–21, 5 control rats were kept in standard animal-house conditions. At postnatal day 75 animals were sacrificed and brains harvested.

In experiments described in **Study II,** 80 rats, with the mean weight of 660 grams at the beginning of the experiment, were housed 4 per cage. Rats received parachloroamphetamine (2 mg/kg) injection at 7 months of age, and after 8 days they were subjected to 25 days of chronic variable stress. After chronic stress rats were acutely stressed to induce corticosterone release, and immediately sacrificed for blood collection.

For the analyses presented in **Paper V,** data from 5 experiments using identical measurement protocol for cytochrome oxidase activity were pooled. Three of these experiments are reported in **Paper I**, **Paper II** and **Study I**. Data from another 2 experiments were included, and the general procedure is described in the following paragraphs. Of the 5 experiments included, 3 used a 2-factorial stress-diathesis model and 2 were based on predefined vulnerability phenotypes. In all experiments, animals were classified by two factors, pre-defined vulnerability phenotype and chronic stress, forming four groups: resilient/control, vulnerable/control, resilient/stressed and vulnerable/stressed. Of the studies using a stress/diathesis paradigm the following treatment groups were included in the analyses: chronic variable stress/serotonergic denervation; hedonic trait/chronic social stress; positive emotionality/chronic variable stress. Vulnerability phenotypes included in the study were post-maternal separation state and low exploratory behaviour.

In the analysis presented in **Paper V** the F1 generation from seven breeding pairs born in our animal house was used to study the positive emotinality effect (Mällo et al., 2009). Thirty three male pups were weaned at the age of 3 weeks and single-housed. “Tickling” sessions started the next day after single-housing – manual imitation of full body stimulation during social play was imitated. The animals were group-housed 4 rats per cage 2 weeks after the end of the tickling sessions, and remained so until the end of the experiment. The animals were divided into groups emitting high or low levels of 50 kHz USVs and were submitted to chronic stress at the age of 2 months, following the stress animals were sacrificed by decapitation and whole brains immediately frozen on dry ice.

In the analysis presented in **Paper VI,** another experiment is included: 42 10-week old rats with the average body weight of 303 g were tested in the

exploration box, as described before, and subsequently 10 animals with high exploratory phenotype and 10 animals with low exploratory phenotype were selected (Mällo et al., 2007a). Animals were housed 4 per cage and rats with different exploration group designations were housed randomly.

## 3.2. Chemicals and drug administration

The neurotoxin parachloroamphetamine was administered 9 days before the chronic variable stress regimen in all the studies reported in this thesis (**Paper I** and **Studies II** and **III**). Parachloroamphetamine (Sigma-Aldrich Co.) was dissolved in distilled water and injected in the dose of 2 mg/kg (expressed as for hydrochloride) intraperitoneally in a volume of 1 ml/kg. Control animals received a vehicle injection. Apomorphine (Reachim) was dissolved in 0.001% ascorbic acid solution and injected in the dose of 1 mg/kg subcutaneously in a volume of 1 ml/kg.

## 3.3. Biochemical assays

### 3.3.1. Cytochrome oxidase histochemistry and image analysis

Cytochrome oxidase activity was measured with identical protocols in all experiments presented in this thesis. Unanesthetised rats were decapitated, brains removed and immediately frozen on dry ice. Brains were stored at –80°C until coronally sectioned (thickness 40 μm) in a cryostat microtome at –20°C, slides with sectioned tissue were kept refrigerated at –80°C until stained. The staining procedure used is based on the protocol described by Gonzalez-Lima and Cada (1998) with minor modifications. The 0.1 M $Na_2HPO_4/NaH_2PO_4$ buffer solution adjusted to pH of 7.4 was used. Automatic agitation was used in all the steps of the protocol. First the refrigerated sections were fixed for 5 min in 0.125% glutaraldehyde (v/v) solution in cold buffer (4°C). Next the specimens were washed with four changes (5 min each) of 10% sucrose in the buffer solution at room temperature. To enhance staining intensity, the sections were pre-incubated for 10 min with 0.0275% cobalt chloride (w/v) and 0.5% dimethyl sulfoxide (DMSO, v/v) in 0.05 M Tris buffer with 10% sucrose (w/v) adjusted to pH to 7.4 with approximately 0.1% HCl (v/v). The metal ions included in the previous step were removed by a 5 min wash with the buffer solution. Thereafter the sections were stained for one hour at room temperature in an incubation solution consisting of 0.05% DAB (3,3′-diaminobenzidine tetrahydrochloride, AppliChem), 0.0075% cytochrome c (Sigma, prepared using TCA), 5% sucrose, 0.002% catalase (Sigma) and 0.25% DMSO (v/v) in sodium phosphate buffer. To avoid non-specific auto-oxidation the reaction was conducted in dark. Finally, the reaction was stopped by introducing the slides for 30 min to 3.5% formalin (v/v) and 10% sucrose in phosphate buffer.

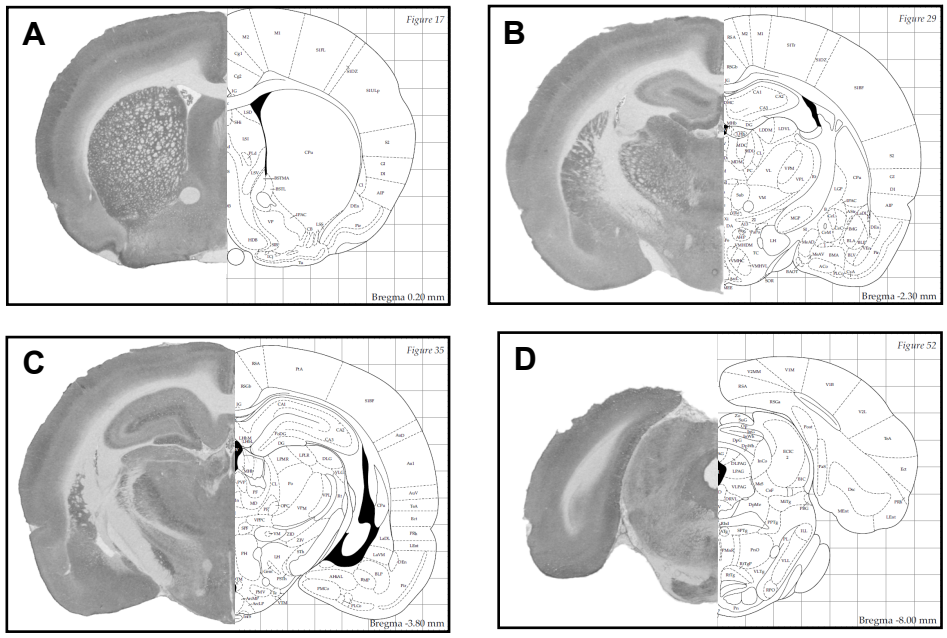


**Figure 1.** Examples of cytochrome oxidase stained brain sections. Regions of interest in these examples include: **A** – bed nucleus of stria terminalis, vertical and horizontal diagonal band, ventral pallidum and septum; **B** – hypothalamic areas, e.g., ventromedial hypothalamus, and amygdala; **C** – hippocampus, habenula and posterior paraventricular thalamus; **D** – pontine nuclei, raphe and periaqueductal gray.

The sections were dehydrated in ethanol, cleared in xylene and coverslipped. Regions of interest to be compared in data analysis were stained in the same incubation medium.

Stained and coverslipped sections were digitized and saved in a non-compressed format. Image analysis was conducted using the Image J 1.34 s freeware on the blue channel (resulting from a RGB split) of the background-subtracted image (**Figure 1**). Regions of interest were detected from the stained images with the help of rat brain atlas (Paxinos and Watson, 1986). Grayscale values were transformed to optical density values with the help of Kodak grayscale tablet with known grayscale and optical density values. Optical density of any given region was sampled from three consecutive slices in one brain and averaged. The optical density value was sampled randomly from right or left hemisphere of different animals. Regions of interest were selected with a freehand selection tool covering the whole brain region, leaving out defected areas. In the analysis reported in **Paper I** the obtained optical density values were transformed to standard scores (T-scores), in **Papers II** and **IV** and in **Study I** the optical density values were transformed to enzyme activity units (μmol/min/g tissue) using external standards: 40 μm sections of rat brain homogenate, with cytochrome oxidase activity previously spectrophotometrical-

ly measured, were included in each staining bath, their optical density was measured and used to calculate enzyme activity (μmol/min/g tissue). The number of regions of interest included in the analyses was 89 in **Paper I**, 86 in **Paper II**, 87 in **Paper IV**, 67 in **Paper V** and 99 in **Study I**.

### 3.3.2. Corticosterone measurement

Corticosterone levels are reported in **Study II**. Rats for the analysis of corticosterone levels were all sacrificed approximately within 80 min. Trunk blood was collected into cooled vials containing $K_3$EDTA and was kept on ice. After blood collection from 4 animals (in approximately 15 min), blood samples were centrifuged (4000 g for 10 minutes at room temperature), plasma was pipetted into vials, frozen and kept at –80°C. Before corticosterone levels were assayed, the samples were defrosted on ice. Measurement was performed according to manufacturer's guidelines with an immuno-enzymatic assay (Correlate EIA TM, Assay Design Inc., Ann Arbor, MI, USA). After carrying out the reactions the 96-well plate was read with a Multiskan FC (Thermo Scientific).

## 3.4. Behavioural procedures and tests

### 3.4.1. Stress

#### 3.4.1.1. Chronic variable stress

The chronic variable stress procedure of the experiment reported in **Paper I** also included in **Paper V**, was essentially conducted as previously described (Harro et al., 2001; Tõnissaar et al., 2008)**.** Rats allocated to the stress group were submitted to the chronic variable stress procedure in a separate room during 21 days. Various stressors of different duration were applied one by one every day, each one thrice altogether. The stressors, in the order of presentation, included: (1) movement restriction in a small cage (11 cm × 16 cm × 7 cm for 2 h), (2) cage tilt at 45° (for 24 h), (3) tail pinch with a clothes-pin placed 1 cm distal from the base of tail (5 min), (4) cold (4°C) water and wet bedding (initially, 400 ml of water was poured on a rat, and the sawdust bedding was kept wet for the following 22 h), (5) forced swimming (5 min at room temperature), (6) strong illumination (900 lx) during the predicted dark phase (for 12 h) and (7) stroboscopic light (for 14 h, 10 Hz).

In the chronic variable stress procedure of another experiment included in **Paper V**, the chronic variable stress lasted for 4 weeks and comprised of seven different stressors that were intermittently used once every week: (1) imitation of intraperitoneal injection using a special glove and syringe without needle being pressed to the animal's body for several seconds; (2) stroboscopic light (for 14 h, 10 Hz, 2 lx); (3) tail-pinch with a clothes-pin placed 1 cm distal from the base of tail (5 min); (4) cage tilt at 45° (for 24 h); (5) strong illumination

(900 lx) during predicted dark phase (for 12 h); (6) cold (4°C) water and wet bedding (400 ml of water was poured on the rats, and the sawdust bedding was kept wet for the following 22 h); (7) movement restriction in a small cage (11×16×7 cm) for 2 h. The stressors were administered during the light phase of the cycle (except for the stressors that lasted overnight).

Chronic variable stress procedure used in the experiment reported in **Studies II** and **III** was also carried out with some modifications. Chronic stress lasted for 25 days, and 12 stressors were presented. During the stress period there were two stress-free days and on two occasions two stressors were presented during one day. The stressors were: 1) strong illumination (900 lx) during the predicted dark phase (for 12 h); 2) forced swimming (5 min at room temperature); 3) cage tilt at 45° (for 24 h); 4) movement restriction in a plastic tube (diameter 8 cm, length 15 cm) for 2 h; 5) cold (4°) water and wet bedding (400 ml of water was poured on a rat and bedding, bedding was kept wet for the following 24 h); 6) momentary mild electric shock (1.5 mA, 5 times); 7) overcrowding – 8 rats from different home cages were placed in a standard group-housing cage (60x38x20 cm) for 12 hours of dark phase; 8) stroboscopic light (for 12 h, 10 Hz); 9) cold environment (4°C) for 1 hour; 10) tail pinch with a clothes-pin placed 1 cm distal from the base of tail (5 min); 11) water in the cage up to the rats sternum (12 hours); 12) short immobilisation (the rat was firmly fixated in hand and turned on its back as if during intraperitoneal injections for 5 s, three times in a row).

#### 3.4.1.2. Chronic social defeat stress

The chronic social defeat stress regimen used in **Paper II** and **Study II,** also included **in Paper V**, was conducted as follows: Resident rats were selected on the basis of their body weight (greater than the weight of intruder animals) from the same cohort as intruder rats. To further promote stable aggressiveness and reduce inter-individual and inter-trial variability in aggressive encounters, the non-specific dopamine receptor agonist, apomorphine, was used to facilitate aggression as described elsewhere (Pruus et al., 2000; Rudissaar et al., 2008). Specifically, two weeks prior to social stress the residents received daily subcutaneous injections of apomorphine (1 mg/kg dissolved in 0.001% ascorbic acid). Every third day the residents were allowed to fight each other for 15 min as the pro-aggressive properties of apomorphine only appear if the animal has experienced aggressive encounters (Rudissaar et al., 2008).

The chronic social defeat stress regimen was based on the resident-intruder paradigm. Single-housed residents were taken to the experimental room where they received an injection of 1 mg/kg of apomorphine and were returned to their home cages for 5 min. During this time the group-housed intruders were brought to the experimental room in their home cages, weighed and introduced to the residents' cages. One session lasted for one hour, during this time the intruders were repeatedly defeated by the residents. Altogether 15 defeat sessions were conducted. The resident–intruder pairs were formed so that each intruder encountered each of the residents only once, helping to assure that

learned inter-individual behaviour patterns were minimized. Control animals spent the same amount of time alone in a novel cage in a separate room. Half of the intruders and half of the controls were sacrificed one day after the chronic stress regimen ended, the other half was used for behavioural testing.

#### 3.4.1.3. Maternal separation

The maternal separation protocol used in **Study I** and also included in **Paper V** was the following: As previously described (Aisa et al., 2007), on postnatal day 2, all pups were randomly assigned to the control group (animal facility rearing) or the maternal separation group. Control pups were only briefly manipulated to change the bedding in their cages once weekly, the separation group pups were separated from their dam for 180 min from postnatal day 2 to 21 inclusive. Before manipulation of the maternal separation pups, the dam was removed from her home cage and placed in an adjacent cage. The pups were removed as complete litters, placed in an empty cage with standard bedding material and transferred to an incubator in an adjacent room. To compensate for the mother's body heat, the temperature of the incubator was adjusted to the age of the neonates: 32 ± 0.5°C (postnatal day 2–5), 30 ± 0.5ºC (postnatal day 6–14) or 28 ± 0.5ºC (postnatal day 15–21). Rats were weaned on postnatal day 23 and only males were chosen for the present work. Animals were sacrificed and brains harvested in adulthood (postnatal day 75). In the analysis of cytochrome oxidase activity in medial amygdala and lateral anterior olfactory bulb one animal, with oxidative metabolism differing 1.7 standard deviations from the mean, was excluded.

#### 3.4.1.4. Acute stress

In **Study II,** acute elevation stress was used for the provocation of endocrine stress response. Rats were individually placed on elevated platforms (platform height 1 m from ground, diameter 10 cm) for 20 min immediately prior to sacrifice, and trunk blood collected for corticosterone measurement.

#### 3.4.1.5. Measurement of body temperature and stress-induced hyperthermia

Stress-induced hyperthermia (**Study III**) was induced and measured as described before (Kõiv and Harro, 2010). Body temperature measurement took place at 3 sessions – after parachloroamphetamine administration, and before and after chronic variable stress. During each session, 3 measurements were performed: the first measurement (T0) reflected the stress-free baseline body temperature and the measurement itself served as an acute stressor; 30 minutes later (T30) the acute stress effect was measured, and 2 hours after the acute stress (T120) recovery of body temperature was recorded. Animals were transported to a separate room 15 min before the measurement. A lubricated flexible probe was rectally inserted 5 cm deep and 1 min later the reading was taken. After that rats were returned to their home cage and kept in the same room until the second measurement in 30 min (T30). After T30, rats were

returned to the animal room and after 90 min the third measurement was carried out in the testing environment.

## 3.4.2. Behavioural tests

### 3.4.2.1. Sucrose consumption test and the hedonic trait assignment

Sucrose consumption measurement was reported in **Paper II**. The results of three consecutive sucrose consumption tests at the beginning of the experiment were averaged and used as a baseline value. After every five social stress episodes sucrose consumption tests were performed to assess any changing hedonic responsiveness of the animals. Sucrose consumption tests were carried out during the dark phase and lasted for 12 h each. Two bottles were used, one filled with 1% sucrose solution and the other with water. As the animals were group-housed during the stress regimen, they were placed into individual cages 2 h before the testing and group-housed again at the beginning of the light phase. Animals continued to have free food access throughout each sucrose test. If sucrose intake was measured during chronic social stress regimen, the scheduled stressor was omitted on the day of testing. Sucrose and water consumption were measured by weighing the bottles at the beginning and the end of the test. Groups with high and low sucrose consumption (HS- and LS-rats) were formed by a median split of the baseline consumption.

### 3.4.2.2. Social interaction test and the sociability trait assignment

The sociability trait was measured in the experiments reported in **Paper IV**. Behaviour of rats was measured in the social interaction test on three occasions separated by ten days as previously described (File and Hyde, 1978; Tõnissaar et al., 2004). Sociability levels were based on the mean time in three tests spent in non-aggressive active social contact (allogrooming, sniffing the partner, crawling under and over, following) during a 10 min session with a weight-matched strange rat on a 30x30 cm well lit arena. Based on the mean social interaction score, animals with the highest (HS, n=7), medium (MS, n=8) and the lowest (LS, n=8) sociability levels were chosen for histochemical analysis. Assessment of intra-individual variability of social interaction was based on the relative standard deviation of three social interaction tests (standard deviation/mean of tests x 100).

### 3.4.2.3. Exploration box test and the exploratory trait assignment

Exploratory behaviour was measured in an experiment included in **Paper V**. The exploration box test was carried out as described before (Mällo et al., 2007a). The exploration box was made of metal and consisted of an open area 0.5 m × 1 m (height of side walls 40 cm) with a small compartment 20 cm × 20 cm × 20 cm attached to one of the shorter sides of the open area. The open area was divided into eight squares of equal size. In the open area, four objects, three unfamiliar and one familiar (a glass jar, a cardboard box, a wooden handle and a

food pellet) were situated in certain places (which remained the same throughout the experiment). The small compartment, which had its floor covered with wood shavings, was directly linked to the open area through an opening (size 20 cm × 20 cm). The apparatus was cleaned with dampened laboratory tissue after each animal. The exploration test was initiated by placing a rat into the small compartment, which was then covered with a lid. The following measures were taken by an observer: (a) latency of entering the open area with all four paws on it; (b) entries into the open area; (c) line crossings, (d) rearings; (e) exploration of the three unfamiliar objects in the open area; (f) the time spent exploring the open area. To provide an index of exploration considering both the elements of inquisitive and inspective exploration, the scores of line crossing, rearing and object investigation were summed for each animal. A single test session lasted 15 min and experiments were carried out under dim light conditions (3–7 lx in the open area). The activity on the first testing session does not correlate highly with the following tests, but already the second test session, carried out 24 h after the first, gives a good prediction of activity levels on the consecutive test sessions (Mällo et al., 2007a). Therefore, the rats were tested in the exploration box for two consecutive days to determine their stable exploratory activity levels and were assigned to the low exploratory and high exploratory activity groups on the basis of the sum of exploratory activity during the second testing session. Low exploratory rats exhibited near zero activity scores and high exploratory rats scored 100 or more exploratory events.

#### 3.4.2.4. Elicitation and measurement of 50 kHz ultrasound vocalisations and the positive emotionality trait assignment

Fifty kHz ultrasonic vocalisation was measured in an experiment included in **Paper V** (Mällo et al., 2009). Rats were given daily sessions of tickling-like stimulation that mimics natural rough-and-tumble play in juvenile rats and elicits high levels of 50-kHz chirping (Burgdorf and Panksepp, 2001). Tickling sessions lasted for 14 days as we have previously shown that by this period the animals develop a stable level of ultrasonic vocalisation response that enables the classification of animals into high-chirping and low-chirping rats (Mällo et al., 2007b). The procedure was carried out as previously described (Mällo et al., 2007b): animals were individually removed from their home cage into a smaller cage and given 15 s for habituation, followed by 15 s of tickling by experimenter. Altogether, four 15 s sessions of stimulation were given over 2 min, after which animals were returned to home cage and test cage cleaned. A microphone was located about 20 cm from the floor of the tickling cage, recording high frequency audio files which were later analyzed with the Avisoft SASLab Pro (Avisoft Bioacoustics, Berlin, Germany) software, creating spectrograms from which 50-kHz USVs were manually counted. The animals were divided into groups emitting high or low levels of 50 kHz USVs by the median split of the average response on days 12–14 of tickling.

#### 3.4.2.5. Forced swimming test

The forced swimming test results were reported in **Paper II**. The forced swimming test was carried out after the modified Porsolt test (Porsolt et al., 1978) as described previously (Häidkind et al., 2004; Porsolt et al., 1978). The measurements were based on the behavioural categories described by Armario et al. (1988). A rat was judged to be immobile when it remained floating in the water with all limbs motionless or made minimal movements in order to maintain its head above the water. The rat was judged to struggle whenever it made intense movements of all the four limbs with the two front paws breaking the surface of the water or "climbing" the walls of the tank.

## 3.5. Adrenal gland weight

Adrenal weight was measured in experiments reported in **Study II**. After decapitation of animals, both adrenal glands were removed, frozen and weighed separately. If the difference between the weight of the left and right adrenal was greater than 1.5-fold, inaccurate dissection was assumed and both results were omitted from analysis.

## 3.6. Data analysis

In analyses in **Papers I–IV** and **Studies I-III,** groups were compared with t-tests or factorial and repeated measures ANOVA, and where appropriate, Fisher's LSD was used for post hoc comparisons. Cytochrome oxidase activity was compared independently in all brain areas. Pearson correlation was used for associations. Normality of the distribution was assessed with the Shapiro-Wilk's test. Group parameters presented in the text and figures are mean ± standard error of measurement (Mean ± SEM).

In the analyses presented in **Paper V,** data from 5 different experiments were pooled after region-wise standardisation of data within each experiment. Only brain regions where cytochrome oxidase activity was measured in all 5 experiments were included. Missing values were replaced with an experiment-, region- and treatment group-specific mean to avoid data loss in matrix analysis. For group mean analysis, all brain regions were analysed independently with general linear models with brain region as the dependent and vulnerability, stress and experiment as categorical factors. Four between group effects were assessed: effects of the experiment, stress, vulnerability, and the interaction of stress and vulnerability with Fisher's LSD test for group-wise comparisons. For comparison of two independent correlation coefficients the z-statistic was calculated and its two-tailed significance assessed. To study the similarities and dissimilarities among brain activity correlations of different experimental groups the differences between correlation matrixes were calculated. The multidimensional scaling with R-software was applied to project these patterns of differences into 2-dimensional space.

# 4. RESULTS AND DISCUSSION

## 4.1. Higher levels of a hedonic trait render animals behaviourally more reactive to chronic social stress

In this experiment (**Paper II**) animals with high and low levels of sucrose intake were socially defeated in fifteen resident-intruder stress sessions, and their sucrose intake during the stress and behaviour in forced swimming test after the stress regimen was measured.

Individual differences in sucrose intake were fairly large, the baseline for animals with high sucrose intake (HS-animals, 88.9 ± 3.7) was twice as high as for animals with low sucrose intake (LS-animals, 43.7 ± 2.4 g), and the animals did not differ in water intake or body weight. The extensive group differences are even more impressive when considering that the method for group assignment, the median split, leaves a large number of animals with values close to median in both groups. The individual stability of sucrose solution consumption during the three baseline tests was high, correlations between tests ranging from 0.75 – 0.78, and the average baseline sucrose consumption correlated highly with the sucrose test performed six weeks later ($r = 0.71$; $p<0.0001$).

The results of the present investigation are in general accord with previous data from this laboratory, demonstrating that sucrose consumption and preference in rats exhibits remarkable stability (Tõnissaar et al., 2006). One methodological difference with the previous work was that overnight sucrose testing was used instead of an one-hour test. The advantage of the twelve-hour test was thought to be the absence of interference of novelty/environmental change with sucrose consumption, which did seem to be a factor in e.g., association between sucrose intake and dopamine $D_2$ receptor activation in striatum (Tõnissaar et al., 2006). During the overnight test all animals had the possibility to habituate to the situation, and the results should give a better estimate of their sucrose intake in low anxiety conditions. In this study we also demonstrated the stability of the trait in longer perspective, as the correlations between tests performed on consecutive days and after six weeks were virtually equal. In the present study we also show that HS-rats have higher metabolic activity in the limbic forebrain and more stress-reactive mesolimbic dopamine system (see section 4.3.2. and 4.3.5.). Large individual stability, and the fact that mouse lines differing in the amount of sucrose intake and rat lines with either high or low preference for sweet taste have been bred successfully, indicates high genetic control over the trait (Gosnell and Krahn, 1992; Gosnell et al., 2010; Pothion et al., 2004). Large evolutionary conservation of behaviours related to taste preference is revealed by a similar behaviour of tongue protrusion in response to sweet taste in different mammalian species (Berridge, 2000). Sucrose preference is also a stable variable in humans independent of the technique of measurement, and it seems to have a genetic component (Keskitalo et al., 2007a; Keskitalo et al., 2007b). Nevertheless, translation from humans to rodent is not free of contra-

diction: in humans depression can also lead to increased food intake whereas in rodents a decrease in sweet solutions is usually the intended endpoint.

Rats prefer more concentrated sucrose solutions to weaker ones, and intracranial self-stimulation and sucrose intake are responsive to similar environmental and drug treatments. Rats with higher basal sucrose intake self-administer amphetamine more willingly, and rats with higher sucrose intake trait are willing to work more for sugar reward in a progressive ratio experiment (Avena et al., 2008; Brennan et al., 2001; DeSousa et al., 2000; Spector and Smith, 1984; Willner, 2005). Thus, sucrose intake, psychostimulant drug self-administration and intracranial self-stimulation may share some neurobiological substrates. Similarly to drugs with rewarding properties, sucrose ingestion induces release of some neurotransmitters e.g., increasing accumbal dopamine and opioid levels (Avena et al., 2008).

The effect of chronic social stress was indicated in decreased and eventually negative weight gain that was not dependent on the hedonic trait, and an increase in adrenal gland weight. Overall, chronic social stress had no clear and robust effect on sucrose intake. Stressed animals started to consume more sucrose solution during the stress regimen, and this increase was significant only in HS-animals. This was probably a result of baseline difference (though statistically insignificant) between the stress and control animals in the HS-group, reaching the canonical levels of statistical significance due to a minor increase.

One of the symptoms of human depression, and also in animal models, is a change in body weight. In humans both steep increases and decreases can occur in depression (APA, 1994; WHO, 1992). However, most animal depression models have consistently yielded weight losses or diminished weight gains e.g., (Rygula et al., 2008; Willner, 1997), while this can be because the experiments have been designed to achieve it. In the present study the body weight of chronically stressed animals dropped below pre-stress values whereas control animals progressively gained weight throughout the same period. This verifies that our stress regimen did have a strong physiological impact in the expected direction. Body weight change in rodents is an indirect measure of the severity of chronic stress and conclusions about the direction of the change should be avoided, as body weight change is species specific – depressed hamsters, for example, gain weight (Foster et al., 2006).

Sucrose preference/consumption is currently one of the standard measures of depressive affect following chronic stress. Lower sucrose intake is usually interpreted to reflect anhedonia. Anhedonia has been demonstrated in many models involving chronic stress, such as chronic variable stress, social defeat and maternal separation (Aisa et al., 2008; Rygula et al., 2005; Willner, 2005). However, we did not observe ”anhedonia” in this study, but rather found slight elevations in sucrose intake after stress, largely because HS-animals consumed more sucrose following social defeat than in control conditions. The difference in sucrose intake caused by chronic social stress was statistically significantly different in HS-rats, but since HS-animals belonging to the stress-group had

somewhat higher sucrose intake prior to the stress regimen than HS-rats of the control group, this difference can not be interpreted as valid. Thus sucrose consumption did not differentiate rats with depression-like behaviour from controls. Despite of this, energy metabolism in both ventral tegmental area and nucleus accumbens was affected by chronic social stress in this study (see section 4.2.2.). We have previously observed that chronic variable stress has distinct effects on sucrose intake dependent on a number of factors, and can indeed even increase sucrose intake if in condition of partial denervation of the serotonin system (Harro et al., 2001; Tõnissaar et al., 2008b). As this effect was prevented by chronic citalopram treatment, it seems possible to model in certain conditions the carbohydrate-craving atypical depression in the rat by means of applying chronic stress (Tõnissaar et al., 2008b). Various methodological differences might account for the variance in sucrose intake test. For example, as quite commonly access to food is restricted prior to the sucrose tests, the higher hunger response in controls might facilitate the observation of anhedonic behaviour in stressed animals that may have developed a different metabolic regulation (Reid et al., 1997). It has been demonstrated that 30–50% of mice do not develop anhedonia after chronic stress and such animals can be used as an internal control (Strekalova and Steinbusch, 2010).

Chronic social stress and the hedonic trait had an interactive impact on both the time spent struggling and the time spent immobile in the forced swimming test, while significant differences were found only on the second day of the test (**Figure 2**). On the second day of the forced swimming test stressed HS-rats struggled less than HS-controls. While on the first day of forced swimming test all animals behaved almost identically, on the second day struggling of the HS-controls increased as compared to the first day, and struggling of HS-stressed animals remained the same as on the first day, resulting in a marked difference between stressed and control HS-group.

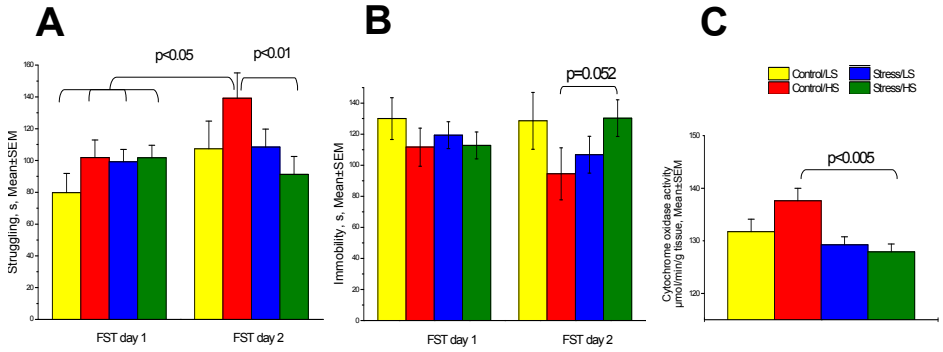


**Figure 2.** Groupwise, the effect of chronic social stress in rats with high and low sucrose intake (HS and LS) results in a similar pattern in the second day response to forced swimming (FST) and in the average cytochrome oxidase activity. **A** – struggling in the FST, **B** – immobility in the FST and **C** – average cytochrome oxidase activity of all measured brain areas. For group-wise comparisons LSD post hoc test was used.

These findings were paralleled, though with less statistical power, in the lower immobility time of HS-controls compared to stressed HS-rats on the second day of the forced swimming test. Thus animals with higher levels of the hedonic trait displayed more depression-like behaviour after stress, as indicated by this passive coping behaviour.

The results of the forced swimming test should be interpreted with caution as the test was not initially meant for measuring differences in phenotypes, but to be used as a screening tool for antidepressants, emphasizing the predictive validity. However, it is often held that rats displaying more immobility in the test, especially on the second day, are more behaviourally despaired/helpless and in a depression-like state (Porsolt et al., 1978; Yan et al., 2010). The difference of this test from learned helplessness, where animals are rendered inactive/helpless, lies in the energetic demand of the environment: one cannot rule out that immobile and energy conserving floating is the adaptive behaviour in the Porsolt's test (West, 1990). Another possible interpretation arises from the activating effect of serotonergic denervation in the forced swimming test. Serotonergic lesions induce more struggling, as do antidepressant drugs though their neurobiological impact is opposite, thus the struggling in forced swimming test can be a marker of several behaviours – antidepressive effect in case of antidepressants and possibly impulsivity in conditions of serotonergic depletion (Harro, 2002). In the present study the possibility that HS-animals are more impulsive is probably minimal. Impulsive struggling should be more obvious on the first day of the test as an acute reaction to stressful stimulus (Harro, 2002), but in the present study, the effect in HS-rats rather appeared as a result of learning, because their behaviour changed on the second day of the test. Conservatively, the forced swimming test results would lead to a conclusion that rats with higher sucrose intake are more reactive to environmental changes. Whether the reactions of HS-animals are indicative of lack of adaptive energy saving, increased impulsivity, behavioural despair or less efficient learning of the inevitability of confinement remains to be elucidated. The high reactivity of HS-rats is however evident – first, they respond to forced swimming test procedure, as HS-controls show a marked increase in the second day compared to the first, and secondly, HS-animals were the only group affected by chronic social stress in the forced swimming test. The interaction between vulnerability phenotypes and behavioural response to stress has been demonstrated before, as rats that are subjected to maternal separation have been shown to display passive coping behaviour in the forced swimming test in adulthood only after chronic stress (Marais et al., 2008). Vulnerability phenotype has also been shown to affect the impact of single stressor, as chronic maternal gestational stress rendered the animals more reactive to a single adulthood stressor, as indicated by increased startle response (Hougaard et al., 2011; Kjær et al., 2010).

Conclusively, sucrose intake is a stable trait, and rats with higher sucrose intake are more vulnerable to chronic stress as revealed by passive coping in the forced swimming test.

## 4.2. What are the brain regions that display a change in long-term level of energy metabolism in response to chronic stress?

### 4.2.1. Chronic variable stress increases long-term neural activity in hypothalamus and amygdala

In order to find out which brain regions are activated/inhibited by stress, rats underwent chronic variable stress and subsequently cerebral energy metabolism was assessed with cytochrome oxidase histochemistry **(Paper I).**

Chronic variable stress had an activating impact on brain energy metabolism in medial preoptic area, and cortical and medial amygdala (**Figure 3**).

These regions that displayed long-term activation after chronic stress in our experiment have also all been implicated in acute stress response (Herman and Cullinan, 1997; Jankord and Herman, 2008; Tomasz, 1997). Medial preoptic area and medial amygdala are in particular involved in the regulation of paraventricular hypothalamus, and by this means the endocrine stress response (Ziegler and Herman, 2002). Medial amygdala controls the activity of paraventricular hypothalamus both directly and indirectly, via a number of nuclei, among others the medial preoptic area (Herman et al., 2005). Amygdalar control of medial preoptic area function is confirmed by showing that medial amygdala lesions block acute restraint-induced immediate early gene activation in preoptic neurons (Lin et al., 2011b). Amygdala activity mainly results in increased endocrine response, and this mechanism is proposed to be defective in depressed patients (Gao and Bao, 2011; Ziegler and Herman, 2002). Both medial and cortical amygdala are involved in olfactory and pheromone signal processing, both serving as the primary sensory systems in rodents, and these regions have reciprocal connections and share numerous efferent and afferent targets (Kang et al., 2011; Meurisse et al., 2009; Sosulski et al., 2011).

Besides the control of paraventricular hypothalamus, medial preoptic area also is one of the regions involved in autonomic stress response and body temperature regulation (Oka et al., 2001). Chronic stress can affect thermo-regulation and as we report in **Study III,** chronically stressed animals have higher baseline body temperature. Inhibition of medial preoptic area by the GABA agonist muscimol leads to increased autonomic nervous system activity, including increased body temperature, whereas activation of medial preoptic area by GABA receptor blockage with bicuculline leads to defective temperature control (Ishiwata et al., 2005). Thus both inhibition and release from inhibition can lead to defective thermoregulation. The present data indicate higher activity in both medial amygdala and medial preoptic area after stress. Thus, when proposing that GABA-ergic medial amygdala activity leads to higher medial preoptic area activity, one must assume that excitatory and inhibitory signals that converge in the medial preoptic area sum up to a larger energetic need of the nucleus.

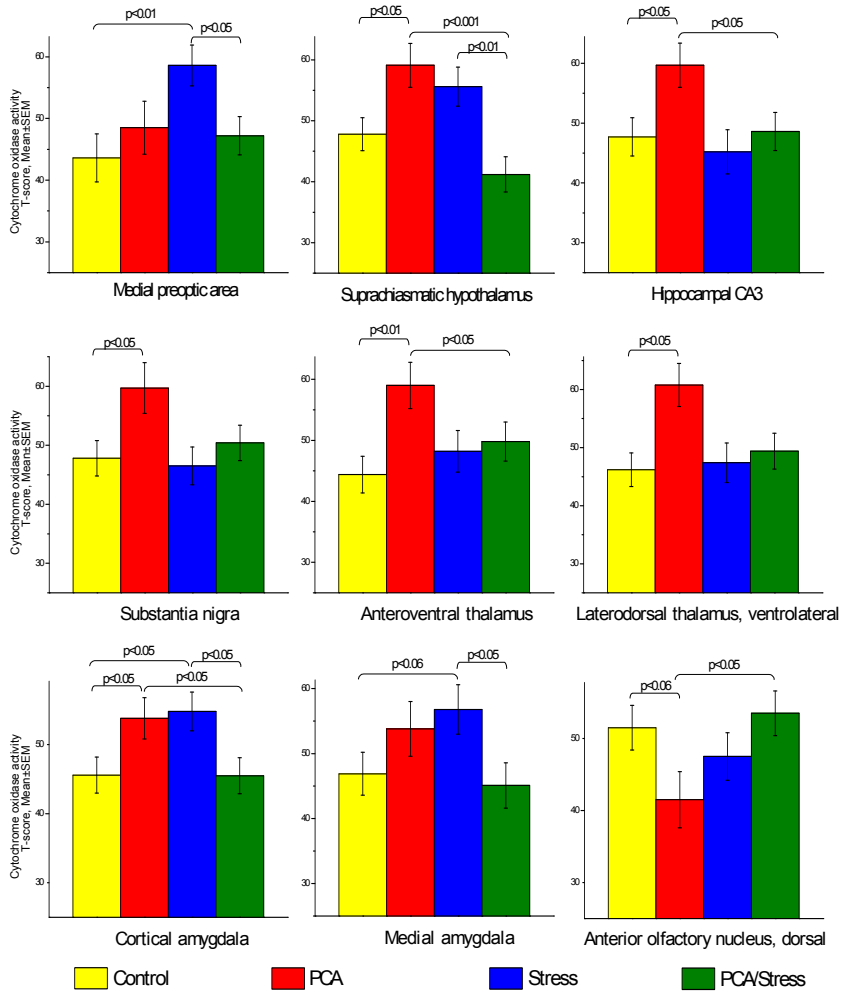


**Figure 3.** Cytochrome oxidase activity (standard T-score, mean±SEM) after chronic variable stress in animals with serotonergic lesions induced by parachloroamphetamine (PCA, 2 mg/kg). For group-wise comparisons LSD post hoc test was used.

On the other hand, it is also known that pathways from the medial amygdala induce medial preoptic area activity via glutamate release (Dominguez and Hull, 2001; Hull and Dominguez, 2006). Increased medial preoptic area glutamate levels (300% of baseline) have been measured in males during copulation (Dominguez et al., 2006). Combining this with the fact that sexual activity produces extremely high stress axis response, we can propose that activity in this region is responsive to environmental challenges and mediated by glutamate (Koolhaas et al., 2011). As glutamatergic signal contributes most to the cytochrome oxidase activity, it could be assumed that repetitive stress had

induced higher cytochrome oxidase activity levels in this region via glutamatergic mechanisms.

Another explanation to the increased activity in medial amygdala and medial preoptic area would be that higher cytochrome oxidase activity in these regions was reached via independent routes. Medial amygdala and medial preoptic area have a multiplicity of efferents and afferents, as these regions are very actively incorporated into behavioural control. The HPA axis control of medial preoptic area is executed via reciprocal connections with numerous brain regions including other hypothalamic areas, raphe nuclei, septum, bed nucleus of stria terminalis, periaqueductal central gray and thalamus (Chiba and Murata, 1985). Medial amygdala has an even more diverse function as it sends axons to hypothalamus, hippocampus, septum, striatum, olfactory bulbi, bed nucleus of the stria terminalis, thalamus and ventral pallidum, and receives signal from insular cortex, hypothalamus, bed nucleus of the stria terminalis, and other amygdalar regions, mainly cortical amygdala (Canteras et al., 1995; Meurisse et al., 2009).

The activation of medial preoptic area also suggests a change in the regulation of sex hormones, as chronic stress increases gonadotropine-releasing hormone positive cell counts in medial preoptic area, and cells in this area control the gonadal axis and respond to circulating testosterone hormone levels (Gray et al., 2010). It is also intriguing in this context that while medial preoptic area is strongly implicated in sexual behaviour, medial amygdala is significantly contributing to the anger/rage emotive system and aggressive behaviour (Panksepp, 1998).

Taken together, in three brain regions, namely the medial preoptic area, and cortical and medial amygdala, chronic stress increased oxidative activity, and all these regions are implicated in the acute stress response.

### 4.2.2. Chronic social stress inhibits long term neural activity

In this experiment (reported in **Paper II**) animals were socially defeated in fifteen sessions in the resident-intruder paradigm settings and after the stress regimen brains were harvested for cytochrome oxidase measurement.

Chronic social stress decreased oxidative energy metabolism in temporal and granular retrosplenial cortices, hippocampal CA3 and dentate gyrus, mammillary bodies, paraventricular thalamus, substantia nigra pars compacta, ventral tegmental area and laterodorsal segment of periaqueductal gray (see **Figure 4** for a selection of results). In the present study chronic social defeat stress decreased energy metabolism in all affected brain regions. The majority of chronic stress studies investigating regional changes in brain activity, in both humans and rodents, have found region-specific activation/inhibition – elevated activity in brain areas related to stress responses/anxiety and decreased activity in brain areas involved in hedonia/motivation (Stone et al., 2008). The one study where only decreased energy metabolism was demonstrated used

cytochrome oxidase histochemistry as a method and was conducted on newborn rats of the congenitally helpless line (Shumake et al., 2004). In adult animals of the same line, however, region-specific increases and decreases in cytochrome oxidase were found (Shumake and Gonzalez-Lima, 2003).

Chronic social defeat reduced cytochrome oxidase activity in hippocampal CA3 area in the presented study. The impact of chronic stress on hippocampal macro- and microstructure is well established in several paradigms, including chronic social stress (Blanchard et al., 2001; Czèh et al., 2001; McEwen, 2001). Chronic stress leads to structural changes (dendrite atrophy, loss of mossy fibre axon terminal, terminal loss of synapses) in both CA1 and CA3 areas of hippocampus, and this is accompanied by deficits in spatial learning (Sousa et al., 2000). The involvement of hippocampal function in both acute and chronic stress is also demonstrated by a decrease in immediate early gene expression in hippocampus after chronic social stress in mice (Singewald et al., 2009). Hippocampus is also an important site of glucocorticoid action and the HPA axis feedback regulation via the levels of glucocorticoid receptors, and acute activation of hippocampus and dentate gyrus after single social defeat can be blocked by glucocorticoid (but not mineralocorticoid) receptor antagonists (Calfa et al., 2007). Increase in glucocorticoid levels leads to increased glutamatergic signalling in the hippocampus, and higher glutamate levels are frequently reported in depressed patients (Gao and Bao, 2011; Kugaya and Sanacora, 2005; McEwen, 2001). Thus, simplistically, an increase in energy expenditure would be expected after social defeat, but a decrease in cytochrome oxidase activity was observed in our study. Although glutamate signalling increases during/after chronic stress, there is a possibility that this leads to lower levels of cytochrome oxidase activity as glutamate is shown in vitro to substantially decrease the function of all complexes of the respiratory chain (Rego et al., 2000). If glutamatergic signalling is prolonged, increased nitric oxide levels can either transiently inhibit energy metabolism, or in the long-term, excitotoxic events followed by apoptosis in the postsynaptic cell will occur, leading again to overall reduced metabolic activity of the brain region (Brown, 2010).

Temporal cortex, a region where chronic social defeat reduced energy metabolism in the presented study, is also implicated in learning, as demonstrated using animal models of ageing and Alzheimer disease, and in patients with post-traumatic stress disorder (Lanius et al., 2006; Miller and McEwen, 2006; Wang et al., 2010). Chronic stress causes non-neuronal apoptosis in this region that can be reversed by tianeptine treatment (Lucassen et al., 2004). Temporal cortex is responsive to chronic stress in humans, as in adults with childhood neglect, temporal cortex displays lower baseline activity (Chugani et al., 2001).

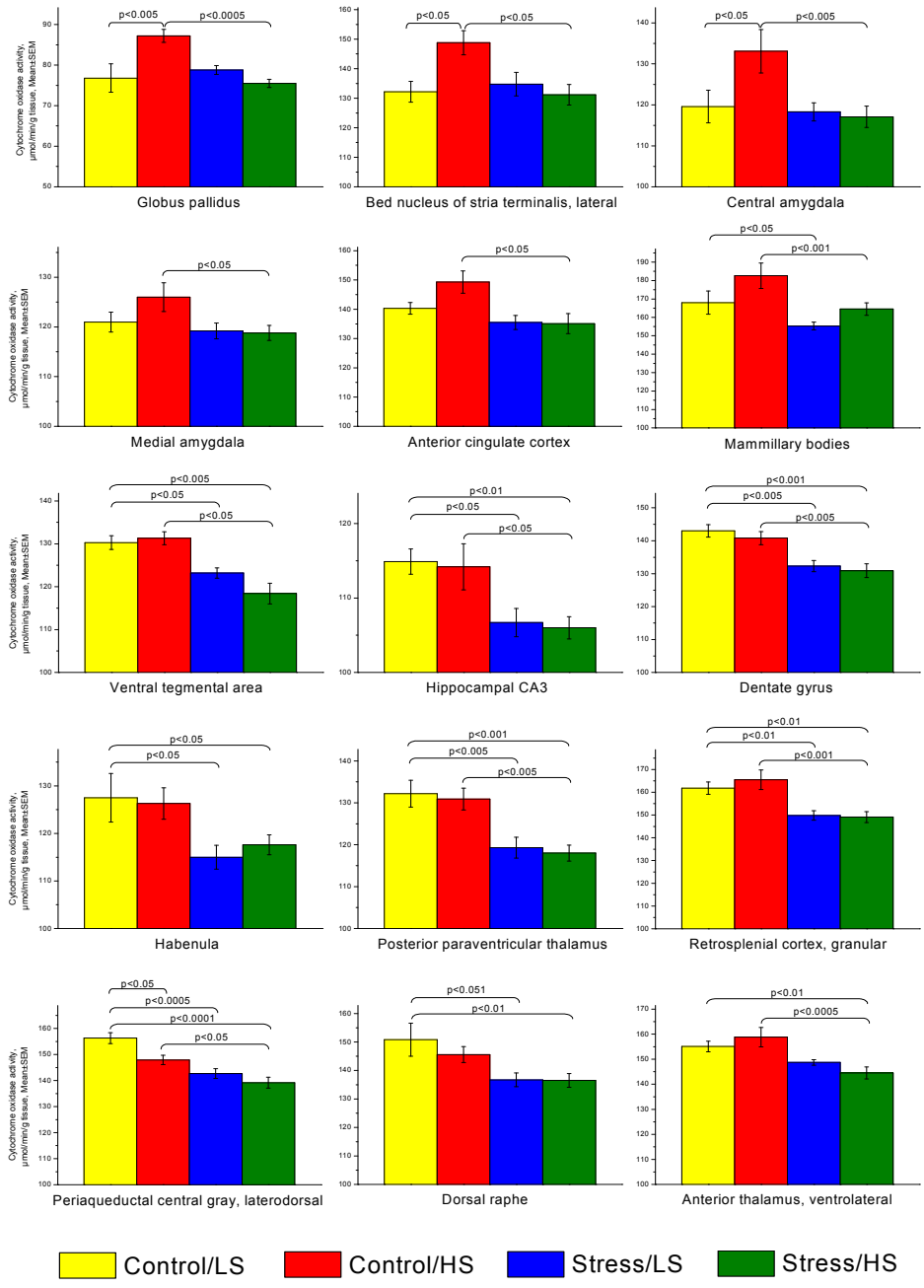


**Figure 4.** Cytochrome oxidase activity (µmol/min/gram tissue, mean±SEM) in response to chronic social defeat stress in animals with high and low sucrose consumption (HS and LS). For group-wise comparisons LSD post hoc test was used.

Cytochrome oxidase activity was decreased after chronic social stress in retrosplenial cortex, a region related also to memory and learning impairment. Hypoactivity of this region leads specifically to amnesia, but hyperactivity is found in stress-related disorders with a high component of learning, such as post-traumatic stress disorder (Lanius et al., 2006; Vann et al., 2009). In human brain-imaging studies this region is implicated in emotional responses to stimuli (Maddock, 1999). In rodents retrosplenial lesions usually result in spatial memory deficits, but this area is also involved in contextual fear learning and active avoidance learning: lesions of retrosplenial cortex impair the formation of contextual fear and increase response latency in active avoidance test (Keene and Bucci, 2008; Lukoyanov and Lukoyanova, 2006). Animals with retrosplenial lesions are less active during the first exposure to open field (Lukoyanov and Lukoyanova, 2006). The retrieval of fear-related memories is dependent on glutamatergic signalling, as retrosplenial NMDA receptor blockade abolished fear-conditioned freezing in mice (Corcoran et al., 2011). The exposure to social defeat does probably involve some learning about the environmental cues, and certainly about the resident's behaviour. Mammillary bodies, that have also been implicated in learning, displayed a similar decrease in metabolic activity due to chronic stress as retrosplenial cortex (Conejo et al., 2004; Tsanov et al., 2011).

In posterior paraventricular thalamus chronic social stress decreased cytochrome oxidase activity. Posterior paraventricular thalamus expresses mineralocorticoid and glucocorticoid receptors and the activation of either during repeated stress exposure enhances, and blockage decreases, habituation to homotypic stressors. Posterior paraventricular thalamus lesions abolish habituation of the HPA axis (Bhatnagar et al., 2002; Jaferi and Bhatnagar, 2006). Besides the endocrine response, posterior paraventricular thalamus lesions can affect stress-related behaviour as chronically stressed rats with lesions of posterior paraventricular thalamus display more vigorous defensive burying behaviour to a heterotypic stressor (Bhatnagar et al., 2003). Acute stress induces short-term activation in paraventricular thalamus and in its target areas, such as the medial prefrontal cortex, nucleus accumbens, and central and basolateral amygdala. Acute stress in rats with thalamic lesions facilitates central amygdala response to acute stressors (Bubser and Deutch, 1999; Spencer et al., 2004).

Two dopaminergic nuclei, ventral tegmental area and substantia nigra pars compacta, displayed a decrease in metabolic activity after chronic social stress. Ventral tegmental area, along with paraventricular thalamus, is the main effector of nucleus accumbens (Charney, 2004; Moga et al., 1995). The mesolimbic dopamine pathway plays a major role in reward-related and motivated behaviour and there is ample evidence that chronic stress in rodents and depression in humans affects reward/motivation (Charney, 2004; Willner, 2005). Chronic stress sensitises the dopaminergic response to acute stress – chronic variable stress did not increase baseline dopamine levels on its own, but doubled acute stress induced extracellular dopamine levels in frontal cortex.

This increase was reversed by antidepressants to control levels, whereas antidepressant treatment did not affect dopamine levels on its own (Cuadra et al., 2001). Ventral tegmental area function affects forebrain, especially accumbal, dopamine levels, and in a genetic rat model of depression, the Flinders Sensitive Line, dopamine levels were decreased in nucleus accumbens, and this effect could be reversed with antidepressant treatment (Friedman et al., 2008). Depressive phenotype is also associated with the firing rate pattern of neurons in ventral tegmental area as the frequency of bursts is decreased and the number of electrically active cells is markedly decreased (Friedman et al., 2008; Moore et al., 2001). Antidepressant administration to rats increases spontaneous firing in ventral tegmental area, and electroconvulsive shock increases both spontaneous firing and burst-firing (West and Weiss, 2011). The relationship between dopamine and reward is more complex, though, as this network is also involved in aversion learning, and re-exposing previously defeated animals to social threat increases accumbal dopamine levels (Blanchard et al., 2001). Although rats in our experiment did not develop decreased sucrose preference, it is probably rather the question of test methodology, as body weight reduction indicated a large impact of stress.

Substantia nigra pars compacta gives rise to the nigro-striatal dopaminergic circuit and has been foremost implicated in voluntary motor function and sleep rhythms, both of which are severely disturbed in people with Parkinson's disease (Lima et al., 2009). Parkinson's disease, caused by apoptosis of substantia nigra pars compacta dopaminergic cells, is highly comorbid with depression, but the causal mechanism of depression in patients with Parkinson's disease is thought not to involve substantia nigra (Lieberman, 2006). Even though substantia nigra pars compacta is not directly implicated in the genesis of depression, it receives input from nuclei related to depressed behaviour, such as raphe and locus coeruleus, and could mediate the affective state and motor response (Guatteo et al., 2009). There is one study where substantia nigra compacta, as well as ventral tegmental area, lesions induced helplessness, and this effect could be reversed by antidepressant treatment (Winter et al., 2007). The involvement of dopamine in the neurobiology depression can also be deduced from the efficacious antidepressive treatment with the dopaminergic drug bupropion, a dopamine and noradrenaline reuptake inhibitor (Dhillon et al., 2008).

Chronic social stress decreased cytochrome oxidase activity in periaqueductal gray matter. During the confrontation, socially defeated rats either attempt to escape from the dominant animal or, more frequently, submit and freeze. Both of these behaviours are initiated by the periaqueductal gray area activity. Laterodorsal part of periaqueductal gray is implicated in behavioural activation/inhibition during extremely high stress (Brandão et al., 2008). Escape and freezing can be elicited by a different intensity of electrical current or different concentrations of a NMDA receptor agonist applied to exactly the same location in periaqueductal gray. These different stimulations induce a very different profile of activation in target brain structures (Borelli et

al., 2005; Ferreira-Netto et al., 2005; Vianna et al., 2003). Interestingly, periaqueductal gray area was the only region that displayed a short-term increase in immediate early gene expression after a mild stressor in animals submitted to chronic social stress (Singewald et al., 2009). Chronic variable stress has been shown to increase cytochrome oxidase activity in periaqueductal gray whereas in the present experiment social defeat reduced the long-term activity of periaqueductal gray, suggesting qualitatively different impact of these chronic stress protocols (Matrov et al., 2011).

Thus chronic social stress decreases neuronal activity in several regions that control acute stress reactions, and are involved in memory/learning, motivational functions and coping.

## 4.3. What brain regions mediate traits of depression vulnerability?

### 4.3.1. Partial serotonergic lesion increases cerebral long-term activity

Serotonergic lesion by parachloroamphetamine generally led to increased neural activity **(Paper I)**. Significantly higher cytochrome oxidase levels were found in suprachiasmatic nucleus, hippocampal CA3, substantia nigra, anteroventral and ventrolateral laterodorsal thalamus and cortical amygdala. In contrast, cytochrome oxidase activity in dorsal anterior olfactory nucleus was decreased (**Figure 3**).

Altogether it appears that the long-term effect of parachloroamphetamine induced lesion is to release the serotonergic target areas from inhibition, as revealed by increased oxidative activity in several brain regions. Recognising the extensive effect of glutamate on cerebral energy demand, the regional activity pattern observed could be caused by diminished serotonergic inhibition of glutamate signalling (Wong-Riley et al., 1998). In rats with extensive serotonin lesions induced by 5,7-dihydroxytryptamine, baseline extracellular glutamate levels increased over 300%, suggesting a tonic control of serotonin over glutamate (Di Cara et al., 2001). In several areas of the rodent thalamus, administration of serotonin or its agonists has been found to cause inhibition of the firing rate of the target neurons (Grasso et al., 2006). Dorsal raphe lesions resulted in enhanced excitation in target areas (Blasiak et al., 2006; Grasso et al., 2006). The excitation/inhibition evoked by serotonin seems, however, to be dependent on serotonin receptor types predominantly expressed in a given region (Di Mauro et al., 2003; Panksepp, 1998; Stanford et al., 2005).

Serotonergic lesions caused an increase in oxidative energy metabolism in suprachiasmatic hypothalamus. This nucleus receives both serotonergic and glutamatergic efferents, and serotonin strongly inhibits the excitation caused by glutamate, thus a serotonergic lesion would lead to higher activation of the nucleus (Jiang et al., 2000; Quintero and McMahon, 1999). Suprachiasmatic nucleus is the brain region controlling circadian activity patterns by inherent and environmental cues. Glutamatergic retino-hypothalamic tract and the serotonergic input from midbrain raphe nuclei probably convey both complementary and competitive signals involved in circadian behaviour, photic regulation is mediated mainly by glutamate and non-photic by serotonin (Glass et al., 2003; Yamakawa and Antle, 2010). In nocturnal rodents cytochrome oxidase activity levels in the suprachiasmatic nucleus have been shown to be the highest during the light period i.e., during the relatively passive time (Isobe et al., 2011; Nikonova et al., 2005; Ximenes da Silva et al., 2000).

Partial serotonergic lesion caused a decrease in cytochrome oxidase activity in the dorsal anterior olfactory nucleus. Olfactory bulbi receive a strong serotonergic innervation from dorsal and median raphe, but whether a subregion is inhibited by GABA or excitated by serotonin depends on the specific

olfactory stimuli (Hardy et al., 2005; McLean and Shipley, 1987). Serotonin levels are decreased in forebrain and midbrain after olfactory bulbectomy, the destruction of olfactory nuclei that is used to model symptoms of depression (Song and Leonard, 2005). Why serotonergic denervation decreases energy metabolism in the dorsal anterior olfactory nuclei, and why the effect is present in only one of the many anterior olfactory nuclei, remains unanswered at present, and as many comparisons were made could also be a chance finding.

Oxidative energy metabolism increased due to a partial serotonergic lesion in hippocampal CA3 area. Hippocampus receives both dense glutamatergic and serotonergic innervations, and the serotonergic inhibition is exercised through GABA-ergic interneurons (Freund et al., 1990; Petralia et al., 1994). Thus it is reasonable to suggest that reducing serotonergic innervation by neurotoxin treatment would shift the activity in brain metabolism toward higher levels. Besides this, an additional possible mediating mechanism contributing to elevated hippocampal activity after serotonergic denervation is the involvement of glucocorticoids. Serotonergic lesions can increase blood corticosterone levels in rats, corticosterone induces an increase in glutamate release and glutamatergic signalling in hippocampus, thus serotonergic denervation can lead to increased hippocampal activity via peripheral endocrine hyperactivity (Chung et al., 1999b; Karst and Joels, 2005; McEwen, 2007).

Substantia nigra was also metabolically activated by the serotonergic lesion. Although parachloroamphetamine acutely induces dopamine as well as serotonin release, long-term effect of parachloroamphetamine is proposed to be specific to serotonin in the rat (Wilson, 1993). Nevertheless, there are extensive pathways from dorsal raphe to dopaminergic neurons, and serotonergic control in substantia nigra is probably inhibitory in nature, thus serotonergic lesion is expected to increase activity in this region (Gervais and Rouillard, 2000). In a way contrary to our results serotonergic lesion induced by 3,4-methylenedioxymethamphetamine decreased glucose utilisation in hippocampal CA3 and substantia nigra, but this decrease was relative to animals with intact serotonin system after acute stress (Ando et al., 2006). Amygdala receives a dense innervation from raphe nuclei, as up to 10% of neurons in the dorsal raphe nuclei project to amygdala (Azmitia and Whitaker-Azmitia, 1995; Ma et al., 1991). Serotonin has a predominantly inhibitory role in amygdala, and higher levels of available serotonin inhibit amygdala reactivity to fearful stimuli (Eidelberg et al., 1967; Sheline et al., 2001). Whether the decreased cytochrome oxidase activity is the direct result of the serotonergic lesion or a secondary effect is not obvious, as this brain region receives extensive glutamatergic and GABA-ergic innervation (Davis and Myers, 2002).

Taken together, partial serotonergic lesion activates brain energy metabolism in hippocampus, hypothalamus, thalamus and amygdala.

### 4.3.2. Rats with higher expression of a hedonic trait have increased levels of neuronal long-term activity

In the experiment reported in **Paper II**, regional neuronal long-term activity was assessed in rats selected for high and low sucrose intake (HS- and LS-rats) reflecting the hedonic/motivational trait.

The scope of brain regions involved, according to the present findings, in the hedonic behaviour is surprisingly wide, ranging from prefrontal areas to basal ganglia and midbrain periaqueductal gray. In dorsomedial frontal cortex, bed nucleus of stria terminalis, globus pallidus, nucleus of the diagonal band, nucleus basalis, substantia innominata, central amygdala, supraoptic hypothalamus, lateroanterior hypothalamus and ventromedial hypothalamus the HS-animals had higher cytochrome oxidase activity, whereas in dorsal and laterodorsal parts of periaqueductal gray the LS-rats displayed higher energy metabolism (**Figure 4**).

Dorsomedial frontal cortex was among the regions where HS-animals had higher cytochrome oxidase activity. In humans, dorsomedial prefrontal cortex is involved in complex functions, as this area activates during self-focused tasks, has a constantly high baseline activation caused by stimulus-independent introspective mental activities, and is deactivated by focusing on specific tasks (Gusnard and Raichle, 2001). Dorsomedial prefrontal cortex, along with midline frontal regions, is proposed to control affective behaviour via self-regulation and introspection (Northoff, 2005). In rats this area mediates the goal-directed learning and is activated following erroneous behaviour in a conditioned task (de Wit et al., 2006; Narayanan and Laubach, 2008). More relevant for the present study, dorsomedial cortex is part of the network including amygdala and hippocampus that is responsible for contextual conditioning of reward (Fuchs et al., 2004). Furthermore, dorsomedial frontal cortex innervates the core regions of nucleus accumbens with glutamatergic projections. Inactivation of dorsomedial cortex by GABA receptor agonists decreases accumbal neuronal firing and behavioural response to a cue conditioned to sugar reward (Ishikawa et al., 2008). Glutamatergic signal in dorsomedial frontal cortex and ventral tegmental area participates in the forming of drug-reward learning, and drug withdrawal is accompanied by glutamate receptor-specific increases and decreases in postsynaptic neurons of these regions (Ghasemzadeh et al., 2011). The presented findings suggest that not only is context-dependent learning about rewards associated with activity in dorsomedial frontal cortex, but animals more sensitive to reward have persistently higher neuronal activity in this area. Interestingly, stress had no impact on the metabolic activity in this area, though chronic stress is a known factor in several aspects of drug-related behaviour (Miczek et al., 2008).

Lateral bed nucleus of the stria terminalis is implicated foremost in the regulation of anxiety as it mediates signals from basolateral and central amygdala to hypothalamic nuclei controlling the endocrine stress response (Davis et al., 2009). Bed nucleus of the stria terminalis receives a signal from

amygdalar nuclei via central amygdala, and the function of the bed nucleus of the stria terminalis is also controlled by hippocampal subiculum and infralimbic cortex (Georges and Aston-Jones, 2002; Jalabert et al., 2009). Bed nucleus of the stria terminalis sends efferents, besides hypothalamus, to ventral tegmental area, and thus the activity of mesolimbic dopamine system, responsible for incentive/motivated behaviour, is partially controlled by the glutamatergic signal from the bed nucleus of the stria terminalis (Jalabert et al., 2009). Administration of rewarding drugs causes a dose-dependent increase in bed nucleus of the stria terminalis dopamine levels. The bed nucleus of the stria terminalis and central amygdala display immediate early gene expression to food reward in animals with reduced motivational state (Carboni et al., 2000; Harris and Aston-Jones, 2007). Central amygdala targeted injections of μ-opioid agonists can add further incentive value to rewarded conditioned stimuli, and increased central amygdalar activity during conditioning is proposed to be the mechanism whereby conditioned stimuli can evoke as large a response as the unconditioned reward (Mahler and Berridge, 2009). Besides that, administration of opioid agonists in central amygdala increases the consummatory behaviour towards the unconditioned stimulus (Mahler and Berridge, 2009).

Substantia innominata and nucleus basalis, both showing increased activity in HS-rats, are considered part of ventral striato-pallidal portion of the basal forebrain, and these regions receive innervation, among other regions, from central amygdala, ventral tegmental area and bed nucleus of the stria terminalis (Grove, 1988; Heimer, 2003; Holland, 2007). These basal forebrain structures are responsible for food-related motivational behaviour, as lesions to substantia innominata/ventral pallidum do not just block the hedonic response to sweet tastes, but also induce aversive reactions (Cromwell and Berridge, 1993). A more detailed analysis indicates that substantia innominata contributes only to consummatory aspects of food intake and not the hedonic reaction to food (Smith and Berridge, 2005). Substantia innominata, along with nucleus accumbens, is also involved in a more specific behaviour, as these regions mediate motivation for mating behaviour (Sakuma, 2008). Medial forebrain bundle stimulation, a rewarding treatment for the animal, induces immediate early gene expression in reward-related areas including substantia innominata (Nakahara et al., 1999). Globus pallidus, an output structure of basal ganglia usually associated with motor behaviour, is implicated in sending reward-related signals to habenula, a nucleus modifying the function of dopaminergic and serotonergic systems (Bianco and Wilson, 2009; Hong and Hikosaka, 2008). Globus pallidus is rendered less reactive to rewarding stimuli in adults with a history of childhood maltreatment (Dillon et al., 2009). In the nucleus of the diagonal band, a region where HS-animals had higher cytochrome oxidase activity but stress had no effect, activation following extended intracranial self-stimulation of lateral hypothalamus has been demonstrated by measuring glycogenolysis (Konkle et al., 1999).

Hypothalamic regions associated with the expression of aggressive and reproductive behaviour, i.e., ventromedial and lateral anterior hypothalamus,

displayed higher metabolic activity in HS-rats (Carrillo et al., 2011; Lin et al., 2011a). Interestingly, ventromedial hypothalamus also regulates glucose metabolism via autonomic nervous system and noradrenaline release in hypoglycemic conditions (Barnes et al., in press). Besides the hypothalamic areas mediating agonistic behaviour, supraoptic hypothalamus, a nucleus involved in affiliative behaviour, displayed increased energy metabolism in animals with higher levels of the hedonic trait. Supraoptic nucleus is one of the sources of oxytocin and vasopressin in brain and periphery, and is implicated in pathologies concerning social behaviour, e.g., autism (Cheng et al., 2008; Marazziti and Catena Dell'osso, 2008; McGregor et al., 2008; Neumann, 2007; Rosen et al., 2007). The only two regions where rats with low sucrose intake had higher cytochrome oxidase activity were dorsal and laterodorsal periaqueductal gray nuclei. These nuclei are highly conserved and serve survival function in social environment, mediating escape and freezing related behaviour (Ferreira-Netto et al., 2005).

### 4.3.3. Neonatal maternal separation predominantly decreases adulthood regional cerebral metabolic activity

In this experiment (reported in **Study I**) cerebral long-term neuronal activity was measured in adult rats that were submitted to maternal separation as neonates.

Neonatal maternal separation increased cytochrome oxidase activity in ventral pallidum, anterior medial thalamus, medial amygdala, mammillary bodies, lateral part of the anterior olfactory bulb and interpeduncular nuclei, whereas a decrease was found in lateral preoptic area (**Figure 5**).

Ventral pallidum, part of the ventral striatopallidal system of the basal forebrain, is a region designated as one of the "hedonic hot-spots" of the brain, mediating pleasurable sensations and motivation (Heimer, 2003; Smith and Berridge, 2005). A pleasurable sensory sensation, sweet taste, causes a highly evolutionarily conserved tongue protrusion behavioural in mammals, and this behaviour can be also elicited by pallidal opioid receptor agonist microinjections (Peciña et al., 2006). Disruption in reward-directed behaviour, anhedonia, has been evident in several animal stress models, including rats submitted to maternal separation protocol (Aisa et al., 2008). Nucleus accumbens, another brain area mediating hedonic reactions, functions interactively with the ventral pallidum, as the activity of both of these nuclei potentiates the action of the other (Peciña et al., 2006).

In the lateral preoptic area, a decrease in oxidative metabolism in response to maternal separation was found. Activity of this area has been described to respond to acute restraint and immobilisation, whereas exposure to a novel environment induced only a marginal change (Briski and Gillen, 2001; Stamp and Herbert, 2001). Possibly the novel environment was not stressful enough to induce the activation of this region, but surprisingly the severity of restraint did not affect the immediate early gene expression in this region, as it was equal in

response to moderate restraint and total immobilisation (Briski and Gillen, 2001). Involvement of lateral preoptic area in stress response is demonstrated by immediate early gene expression caused by the administration of the glucucorticoid receptor agonist dexamethasone (Briski et al., 1997). The role of lateral preoptic area in stress/depression-related networks is further suggested by its efferent connections to habenula, a brain region relaying signal to midbrain serotonergic and dopaminergic nuclei (Kowski et al., 2008). Self-stimulation of the medial forebrain bundle and prefrontal cortex increases immediate early gene expression in several reward-related brain areas including the lateral preoptic area (Arvanitogiannis et al., 2000; Nakahara et al., 1999). A characteristic feature of lateral preoptic response is its relative lack of habituation to acute activation by repeated stressor presentation and repeated self-stimulation (Nakahara et al., 1999; Stamp and Herbert, 2001).

Anterior medial thalamus receives its main input from mammillary bodies, and both of these regions had elevated cytochrome oxidase activity in maternally separated animals (van Groen et al., 1999). Relevantly for the present study, anterior medial thalamus sends axons to retrosplenial cortex, and both these nuclei are part of the Papez' circuit (van Groen et al., 1999). Although in recent years the anterior thalamic nuclei have more often been associated with learning/memory function, there is one case study demonstrating anterior medial thalamic involvement in affective processes, as a bilateral lesion of this region led to agitated, treatment-resistant depression (Aggleton et al., 1996; Mark et al., 1970). Mammillary bodies are also implicated in memory, with prolonged training in the Morris water maze increasing their neuronal oxidative metabolic activity, and maternal separation is known to induce memory deficits (Aggleton et al., 2010; Aisa et al., 2007; Conejo et al., 2004).

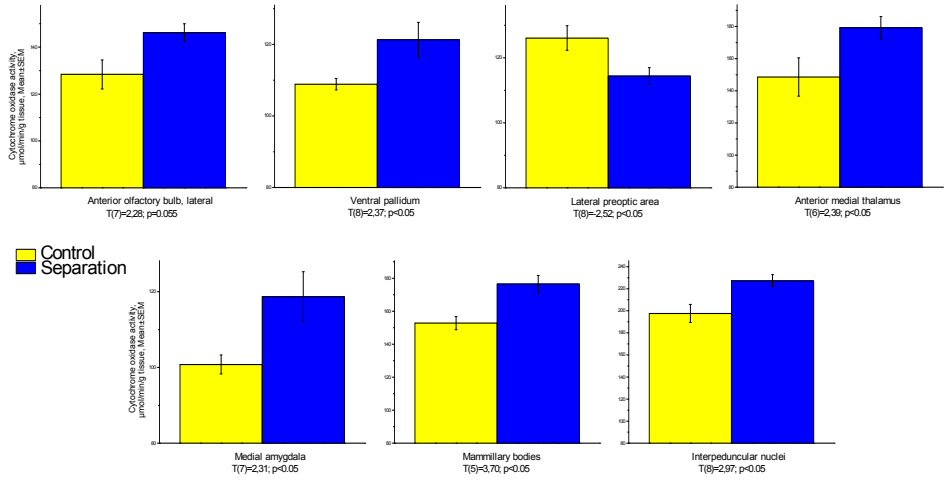


**Figure 5.** Cytochrome oxidase activity (µmol/min/gram tissue, mean±SEM) in adult rats submitted to neonatal maternal separation. For comparison of groups T-test was used.

Besides spatial learning, mammillary bodies have been implicated in contextual fear-conditioning (Célérier et al., 2004). Glucocorticoid receptor levels were decreased in mammillary bodies after chronic variable stress in rats previously subjected to maternal separation as neonates, and these animals also had a persistently high immediate early gene expression in this region (Rivarola and Suárez, 2009). As describer earlier, cytochrome oxidase activity of mammillary bodies was also affected by chronic social stress, but contrary to an increase caused by maternal separation, social stress reduced metabolic activity in this region (Paper II).

In medial amygdala an increase in metabolism was observed in response to maternal separation. Medial amygdala is a region that projects both directly and indirectly, through bed nucleus of the stria terminalis and hypothalamic nuclei, to paraventricular hypothalamus, and is involved in the modulation of stress response (Paper I; Ziegler and Herman, 2002). Social defeat substantially increases the expression of immediate early genes in medial amygdala and increases corticotropin-releasing factor receptor mRNA levels in this region (Fekete et al., 2009). Besides stress, activation due to reproductive behaviour induces immediate early gene expression in medial amygdala (Sudo et al., 1997). Within a single laboratory, chronic variable stress can increase but chronic social stress decrease cytochrome oxidase activity in this area (Paper I; Paper II; Mällo et al., 2009).

In the lateral part of the anterior olfactory bulb, maternal separation caused an increase in oxidative activity. The involvement of olfactory nuclei in depression-like behaviour is suggested by the effects of olfactory bulbectomy that are reversible by chronic antidepressant treatment (Song and Leonard, 2005). As the olfactory system is highly interconnected with the limbic system, adaptive changes in limbic areas, following the destruction of bulbi, can cause several symptoms reminiscent of depression (Song and Leonard, 2005). Chronic variable stress, contrary to the present results, decreased oxidative energy metabolism in olfactory bulbi (Paper I).

Maternal separation caused an increase in cytochrome oxidase activity in the interpeduncular nuclei. Cytochrome oxidase activity was also found to be increased in this area in congenitally helpless rats, a genetic model of depression (Shumake et al., 2003). Interpeduncular nuclei together with habenula are assumed to form a pathway between the rostral and caudal parts of the brain, involved in a number of different functions ranging from more simple behaviours e.g., nociception and feeding behaviour, to more complex processes e.g., affective behaviour and learning (Hikosaka, 2010; Klemm, 2004).

### 4.3.4. Long-term neural activity is predominantly non-linearly associated with the sociability trait

In this experiment, reported in **Paper IV**, rats were tested repeatedly in the social interaction test to differentiate animals on the basis of the sociability trait.

Sociability does not relate linearly to successful coping in social settings, and an optimal level, rather than the extremes, ensures a better outcome. On the one hand, diminished sociability is intrinsic to autistic spectrum disorders, social behaviour is inhibited in depression and premorbid low sociability/shyness in childhood predicts future schizophrenia (Goldberg and Schmidt, 2001; Sacco et al., 2010). On the other hand, a specific genetic defect causing the Williams syndrome increases sociability to a dysfunctional level, and inappropriate social behaviour is frequently manifested in bipolar patients during the manic phase (APA, 1994; Martens et al., 2008). When conducting the social interaction test, the observer can note that rats may consistently reject the approaches of an overtly "hypersocial" partner. Thus we decided to divide animals into three groups with high (HS), medium (MS) and low sociability (LS).

The mean scores of the social interaction test were 46±5.7, 87±3.0 and 113±5.3 (s, M±SEM) for LS-, MS- and HS-animals, respectively. LS-rats had significantly higher cytochrome oxidase activity than HS-animals in dorsomedial caudate putamen, with MS-rats displaying intermediate values (**Figure 6**). In median preoptic nucleus, posterior paraventricular thalamic nucleus and median raphe the relationship between sociability and oxidative metabolism was nonlinear: MS-rats had higher cytochrome oxidase activity than LS- and HS-animals. In supraoptic hypothalamic nucleus there was also a non-linear relation, but in this brain region MS-rats had lower cytochrome oxidase levels than HS- and LS-animals.

We also calculated the intra-individual variance of social behaviour to provide an index of individual stability of the expression of the trait. Probably rats with higher stability are less affected by environmental factors, whereas in other animals social behaviour is more partner or context dependent. The intra-individual variance in sociability, expressed as relative standard deviation, correlated positively with cytochrome oxidase activity in the prelimbic cortex ($r=0.50$, $p<0.05$), bed nucleus of stria terminalis ($r=0.45$, $p<0.05$), and caudate putamen ($r=0.43$, $p<0.05$). There was a negative correlation between variance in sociability and oxidative activity in the nucleus accumbens core ($r=–0.42$) $p<0.05$). One of the main findings of this experiment is the non-linear relationship between sociability levels and long-term neuronal activity, animals with both high and low sociability levels being more similar in oxidative metabolism and differing from rats with medium sociability levels. Nonlinear relations between behaviour and physiology are not uncommon – for example, the classic psycho-physiological theory proposes an inverted U-shaped relation between arousal levels and cognitive performance in difficult tasks (Yerkes and Dodson, 1908). The theory that the optimal state of vigilance, produced either by exogenous administration or endogenous release of glucocorticoids, should lead to best performances in complex hippocampus-dependent tasks, has been, at least partially, confirmed in humans and laboratory animals (Lupien et al., 2007; Zoladz and Diamond, 2008). Nonlinear relations are important in stress adaptation, as both excessive and insufficient hormonal reactions to stress are disadvantageous for adaptation (McEwen and Wingfield, 2003).

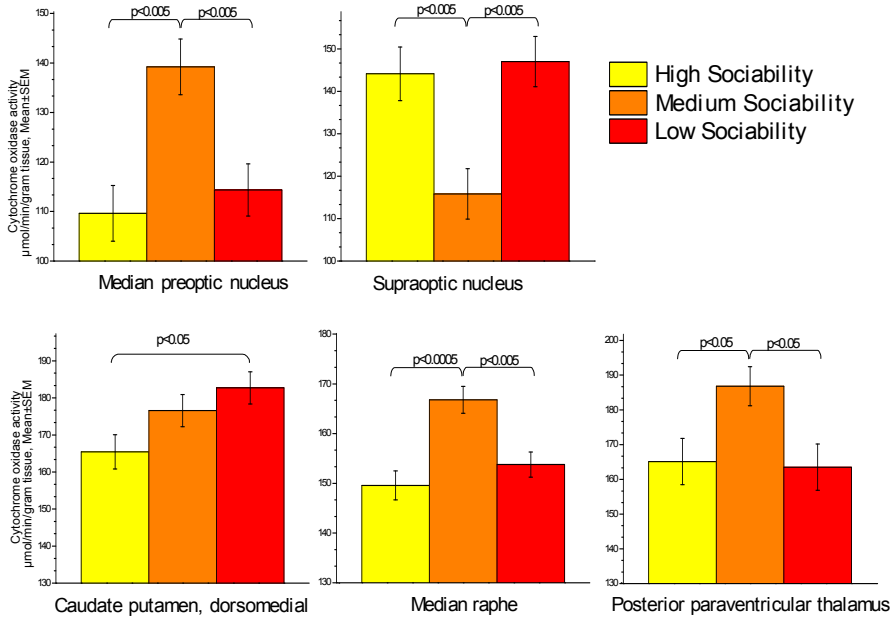


**Figure 6.** Cytochrome oxidase activity (µmol/min/g tissue, mean±SEM) in animals with high, low and medium sociability levels. For group-wise comparisons LSD post hoc test was used.

Dorsomedial caudate putamen was the only region with a clear linear relation between sociability and cytochrome oxidase activity. This area is involved in spatial learning, learning related flexibility and impulse control and has been implicated in motivational behaviour along with ventral striatum (Eagle and Baunez, 2010; Fasano and Brambilla, 2002; Palmiter, 2008; Ragozzino, 2003). Elevations of dopamine levels in the dorsal striatum are more frequent during social contact as compared to solitude and this region is acutely activated in response to social play in juvenile rats (Gordon et al., 2002; Robinson et al., 2002). Social contact has rewarding value in humans and in rats (Douglas et al., 2004; Insel, 2003; Krach et al., 2010; Panksepp, 1998; Van Loo et al., 2004). The involvement of dorsomedial caudate putamen in motivational processes and the rewarding quality of social contact implies that the animal's sociability levels could partly be the result of the motivational function of dorsomedial caudate putamen. Resting state low frequency oscillations in the nucleus caudatus and putamen are positively correlated with the extraversion trait in humans, and extraverted people seek and enjoy social contacts more (Kunisato et al., 2011; McCrae and John, 1992).

Dorsal striatum receives input from paraventricular thalamus, especially the posterior division, a region where MS-animals had higher cytochrome oxidase activity than the extremes (Vertes and Hoover, 2008). Posterior paraventricular

thalamus receives serotonergic afferents and is acutely activated by the serotonergic/dopaminergic drug 3,4-methylenedioxymethamphetamine that can induce pro-social behaviour (Hargreaves et al., 2007; Vertes et al., 2010). Posterior paraventricular thalamus mediates adaptive habituation to repeated stress and we have previously shown inhibition of the posterior paraventricular thalamus after chronic social stress and behavioural vulnerability of HS-rats to chronic stress (Paper II; Jaferi and Bhatnagar, 2006; Tõnissaar et al., 2008a). Thus posterior paraventricular thalamus can be proposed to be involved in the mediation of both the sociability trait and vulnerability to depression.

Intact median raphe is necessary in the development of aversive/anxious behaviour and in fear-related learning (Andrade et al., 2004; Dos Santos et al., 2005; Ohmura et al., 2008; Silva et al., 2004; Vicente et al., 2008) and thus it is possible that median raphe oxidative metabolism in this study characterises the contributions of anxiety to the sociability model. A specific type of social behaviour, aggression, is proposed to be controlled, at least partially, by the serotonergic median raphe nucleus in rodents, with deficient raphe functioning leading to heightened aggression and more vigorous resistance to social defeat (Ferris et al., 1999; File et al., 1979). Thus higher activity in median raphe could be implicated in non-agonistic social interaction that requires the suppression of aggressive behaviour. Immediate early gene expression in median raphe has been detected in rats in response to gamma-hydroxybutyrate, a drug that promotes pro-social behaviour (Chadman, 2010; McGregor et al., 2008; van Nieuwenhuijzen et al., 2009). The association of serotonin with social behaviour is region-specific, as in the frontal cortex the serotonin metabolite content correlates negatively with the level of sociability, and septal serotonin levels decrease due to acute social behaviour (Tõnissaar et al., 2004).

In the supraoptic hypothalamic nucleus, oxidative metabolism was higher in HS- and LS-rats compared to MS-rats. Supraoptic nucleus is one of the main sources of oxytocin and vasopressin innervation in several rodent species and oxytocin is implicated in social behaviour and, among other pathologies, in autism (Cheng et al., 2008; Marazziti and Catena Dell'osso, 2008; McGregor et al., 2008; Neumann, 2007; Rosen et al., 2007). The pro-social feeling induced by 3,4-methylenedioxymethamphetamine in humans is accompanied by a robust elevation in peripheral oxytocin levels and 3,4-methylenedioxymethamphetamine induced social huddling behaviour in rodents is mediated by oxytocin (Dumont et al., 2009; McGregor et al., 2008). The central and peripheral action of supraoptic oxytocin can be dissociated in case of an aversive social contact – in response to social defeat oxytocin release in supraoptic nucleus increases, but blood levels do not (Engelmann et al., 1999). A traditional view holds that the central oxytocin projections arise from the paraventricular hypothalamus, and supraoptic nucleus is involved in peripheral release, but recent data shows that supraoptic nucleus also projects to extra-hypothalamic regions (Ross and Young, 2009). Considering that we also found the involvement of median raphe in sociability, it is interesting that the pro-social drug 3,4-methylenedioxymethamphetamine induces the activation of oxytocinergic neurons in the

supraoptic nucleus (Hargreaves et al., 2007; Thompson et al., 2007). Another neuropeptide frequently implicated in social behaviour and social cognition is vasopressin, and vasopressinergic neurons in the supraoptic nucleus are activated during play-fighting in hamsters (Caldwell et al., 2008; Cheng et al., 2008; Engelmann and Landgraf, 1994; Hammock and Young, 2006). In some species of rodents, like the highly social naked mole rat, median preoptic nucleus contains vasopressinergic neurons. This is one of the neurochemical/ anatomical features that distinguishes them from other rodents with less social lifestyles (Rosen et al., 2007).

The variance in social interaction scores observed with repeated measurement is likely to derive from several sources, being probably mainly dependent on the extent to which the social activity of an animal is influenced by the behaviour of its partner animal. Two of the regions where cytochrome oxidase activity correlated with the stability of sociability trait, prelimbic cortex and bed nucleus of the stria terminalis, control the activity of several limbic nuclei. Prelimbic cortex controls the "prelimbic circuit" involving thalamus, amygdala, hippocampus, and nucleus accumbens, and bed nucleus of the stria terminalis has reciprocal connections with amygdala and is one of the main relays in the descending endocrine stress axis control (Ziegler and Herman, 2002; Walker et al., 2003; Vertes, 2006). Intact prelimbic cortex is necessary for regulation of anxiety, and the acute inactivation of both prelimbic cortex and bed nucleus of the stria terminalis decreases the impact of acute stress (Crestani et al., 2010; Scopinho et al., 2010; Stern et al., 2010). Thus it can be suggested that animals with higher constitutional activity in prelimbic cortex and in the bed nucleus of the stria terminalis are more susceptible to external stressful stimuli, and therefore have a larger fluctuation in test results and lower stability of the trait.

In nucleus accumbens core, a region most often associated with rewarding and incentive behaviour and pleasure, cytochrome oxidase activity correlated negatively with individual stability of the sociability trait (Peciña et al., 2006; Wise, 2005). Conversely, in the dorsomedial caudate putamen, a region that among several other functions is also implicated in reward and goal-directed behaviour, intra-individual variance in social interaction was positively correlated with energy metabolism (Palmiter, 2008). This discrepancy may be related to the possibly larger contribution of the former brain region to the assessment of the incentive "value" of the social contact, and to goal-directed locomotor activity of the latter.

Conclusively, rats with different sociability levels were distinguished by the long-term energy metabolism in several nuclei that are implicated in motivational and pro-social behaviour and stress response; intra-individual variability in social interaction was related to brain areas controlling motivation, stress reactivity and anxiety; and the relationship between energy metabolism and sociability was predominantly nonlinear – rats with high and low levels of sociability differed from rats with medium sociability levels.

### 4.3.5. Higher expression of a hedonic trait renders the regional long-term neural activity more responsive to chronic social stress

In this experiment (reported in **Paper II**) a stress-diathesis model based on a hedonic trait and social defeat was used to study long-term regional activity of the brain. To examine the effect of social stress on rats with different levels of expression of a hedonic and motivational trait, animals were tested in the sucrose consumption test and divided into groups with high and low sucrose intake (HS- and LS-rats). Thereafter rats were submitted to fifteen one-hour sessions of chronic social defeat stress in the resident-intruder paradigm.

Both the hedonic trait level and chronic stress had an independent impact on regional energy metabolism (see Section 4.2.2 and 4.3.2), but the dominant finding was the interaction of the two factors. Social defeat stress decreased cytochrome oxidase activity in all the affected regions. Overall, the HS-animals had higher levels of metabolic activity that in some regions reached statistical significance, whereas chronic social stress mostly reduced the cytochrome oxidase activity in HS-animals to control-like or even slightly lower levels. If the average metabolic activity of all eighty six regions was calculated, the decrease induced by chronic stress was apparent only in the HS-animals (**Figure 2**). Thus, just like in the case of behavioural data presented above (see section 4.1.), rats with high sucrose intake were more reactive to stress. Chronic social defeat significantly decreased oxidative metabolism only in HS-rats in various brain regions, such as anterior cingulate cortex, retrosplenial agranular cortex, nucleus accumbens core, claustrum, lateral bed nucleus of stria terminalis, globus pallidus, horizontal nucleus of the diagonal band, ventral caudate putamen, nucleus basalis of Meynert, substantia innominata, central and medial amygdala, magnocellular preoptic nucleus, lateroanterior hypothalamic nucleus, anterior hypothalamic area, ventromedial hypothalamus, anteroventral and mediodorsal thalamus and red nucleus. There were, however, some areas where thr LS-rats displayed higher reactivity to chronic social stress. These included hippocampal CA1, habenula, periaqueductal central gray, dorsal raphe and cerebellar vermis (**Figure 4**).

The two distinctive findings in this study are the extensive interaction between the vulnerability phenotype and chronic stress, and the exclusively inhibitory effect of defeat stress on energy metabolism. One possible explanation for the overall reduction in energy metabolism after social defeat stress is the severity of the stress. Social defeat may by nature be more stressful than chronic variable/mild stress protocol, and here we employed a relatively stressful protocol even if compared to several other social defeat methods, using more repetitions of the one-hour sessions and not shielding the intruder during the session to prevent direct physical contact. The chronic stress-induced overall reduction in brain energy metabolism found in this study concurs with several recently advanced hypotheses about psychiatric disorders and metabolic dysfunction (Gardner and Boles, 2011). Rezin and colleagues have drawn

attention to the possibility that energy metabolism dysfunctions are present in several psychiatric conditions and the neurochemistry of depression bears resemblance to metabolic encephalopathy (Harvey, 2008; Rezin et al., 2009a). A hypothesis about the stress-hormone induced translocation of cytochrome c from the mitochondrial membrane into the cytoplasm resulting in apoptosis has been put forward by Zhang and co-workers (Zhang et al., 2006). On the other hand, we have found mainly increases in the activity of several brain regions in response to chronic variable stress (Paper 1; Mällo et al., 2009).

Chronic social stress decreased, in some regions interactively with the hedonic trait and in others independently, the oxidative metabolism in ventral tegmental area, lateral hypothalamus, mammillary bodies, hippocampus, amygdala, bed nucleus of the stria terminalis, anterior cingulate, and nucleus accumbens. All these regions are considered as part of the extended mesolimbic dopamine circuit (Alcaro et al., 2007; Berridge and Kringelbach, 2008). Nucleus accumbens, receiving dopaminergic input from ventral tegmental area, is implicated in several central aspects of behaviour: in the control of appetitive and/or hedonic processes, possibly in the aetiology of depressive disorders, and in aversion learning as well (Carlezon and Thomas, 2009; Nestler and Carlezon, 2006; Wise, 2008). It seems that the mesolimbic dopamine pathway/targets are central in the neurobiological basis of response to chronic social stress, given the number of nuclei in this circuit affected. Reduction of oxidative metabolism in dopamine-dependent motor nuclei of the nigrostriatal pathway – substantia nigra and caudate putamen — puts the whole dopamine system in the centre of chronic stress response (Guatteo et al., 2009; Lima et al., 2009). This is further corroborated as the mesolimbic dopaminergic system is also proposed to be the neurobiological circuit responsible for inherent helpless behaviour and normalisation of ventral tegmental energetic function co-occurs with the reduction of depression-like behaviour (Shumake et al., 2010; Shumake and Gonzalez-Lima, 2003). A dopaminergic nucleus showing a distinctively strong reduction only in HS-animals was the core of nucleus accumbens, one of the main regions responsible for hedonic aspects of motivation, though this function may not be mediated via dopamine (Berridge and Kringelbach, 2008).

Regions most often discussed in the context of memory and learning — hippocampal CA1, CA3 and dentate gyrus — had a marked reduction in cytochrome oxidase activity in response to chronic social defeat stress. Of these areas CA1 seemed to be more reactive in LS-animals, but the difference between HS- and LS-rats was marginal, so overall hippocampal cytochrome oxidase levels were affected by stress irrespective of the hedonic trait.

All metabolic mapping studies performed on models of depression/ vulnerability to depression performed by our group have indicated involvement of the anterior thalamic nuclei in response to stress (Paper I; Matrov et al., 2011; Mällo et al., 2009). A marked decrease in oxidative metabolism in response to stress was found in ventrolateral and dorsomedial nuclei of antero-ventral thalamus selectively in the high sucrose consuming group. Anterior thalamus, a region receiving input from hippocampus, may be one of the brain

regions mediating learning deficits co-occurring with chronic stress (Aggleton et al., 2009; Tsanov et al., 2011; van Groen et al., 2002). The hippocampal input to anterior thalamus has two functionally separate routes: besides the direct connection, the hippocampus also has an effect on anterior thalamus indirectly through mammillary bodies (Tsanov et al., 2011), which was another region where chronic stress reduced cytochrome oxidase activity in the present study. In rats, anterior thalamic lesions have been shown to lead to hypoactivity in hippocampus, retrosplenial and anterior cingulate cortex – areas that in animals with intact thalamus are activated during orientation in novel environment (Jenkins et al., 2004). There is also some preliminary evidence that the anterior thalamic – mammillary – retrosplenial circuit is involved in fear learning (Conejo et al., 2007; Hart et al., 1997; Stolar et al., 1989; Vertes et al., 2001).

Several cortical areas, including anterior cingulate cortex and retrosplenial cortex, had reduced activity in HS-rats after chronic social stress. Both of these cortical areas are proposed to be involved in the innate vulnerability to depression in rats and humans (Shumake and Gonzalez-Lima, 2003). A region closely associated with anterior cingulate – the dorsomedial frontal cortex – also displayed elevated energy metabolism in high sucrose-intake animals. Dorso-medial cortex is one of the few brain areas that had increased cytochrome oxidase activity after antidepressant treatment that also reversed the depressive behavioural profile in congenitally helpless rats (Shumake et al., 2010). Retrosplenial cortical areas are hypothesised to be involved in the processing of emotionally laden stimuli in humans, possibly mediating the link between memory and emotion (Maddock, 1999). Some subregions of the retrosplenial cortex are also proposed to be involved in spatial memory (Pothuizen et al., 2010; van Groen et al., 2004). Interestingly this region has functional reciprocal connections to anterior thalamus, where defeat stress in the present study also reduced energy metabolism (Garden et al., 2009; Jenkins et al., 2004).

The energy metabolism of two interconnected nuclei in the extended amygdala, associated with the control of stress reactions, was also sensitive to chronic social stress in HS-animals. The medial part of the central amygdala relays fast control over hypothalamus, whereas the lateral part of central amygdala promotes a slower reaction through the lateral division of bed nucleus of stria terminalis (Walker and Davis, 2008). In the present study both central amygdala and lateral bed nucleus of the stria terminalis displayed higher oxidative metabolism in HS-animals than in LS-rats, but this difference was diminished by the defeat stress. Central amygdala has been considered as a passive output relay of fear-learning signals from other amygdalar areas to nuclei controlling behavioural responses, but recent evidence shows the involvement of central amygdala in the expression, encoding and consolidation of fear-related memories (Ciocchi et al., 2010; Wilensky et al., 2006).

In periaqueductal central gray, cytochrome oxidase activity decreased in response to defeat stress and this effect was more robust in rats with low sucrose intake. Dorsal and laterodorsal divisions of central gray area are responsible for pain, fear-related freezing and defensive/escape behaviour and

distress vocalisation (Behbehani, 1995; Ferreira-Netto et al., 2005; Kroes et al., 2007). Periaqueductal central gray activation that induces escape behaviour, activates, among other nuclei, the ventromedial hypothalamus, a region most often implicated in aggressive behaviour (Vianna et al., 2003). It is not surprising that social defeat affects the activity of central gray areas, as during defeat the animals almost continuously emit 22 kHz ultrasonic vocalisations and have to balance freezing and escape behaviour to protect themselves from the aggressive resident.

The oxidative activity of the dorsal raphe, the origin of ascending serotonergic fibres, was also more stress-reactive in LS-animals (Lowry et al., 2008). Dorsal raphe has widespread anatomical and functional connections with much of the forebrain, and also the nuclei of the reward system (Kranz et al., 2010), and this region exhibited a marked reduction in energy metabolism in this study. Dorsal raphe is proposed to be activated both by continuous and repeated uncontrollable social stress. However, the involvement of this nucleus may not be specific to social stress, as changes in the function of this area have been shown with other stress regimes (Miczek et al., 2008; Savitz et al., 2009). The changes in dorsal raphe were dissociated from median raphe in this study as the latter displayed no response to stress, but this laboratory has previously shown the involvement of median raphe in chronic variable stress (Matrov et al., 2011; Mällo et al., 2009).

For their congenital helplessness model of depression vulnerability, Shumake and colleagues assigned a central role to the habenula, a region which in the present study had decreased oxidative metabolism in response to social stress. Habenula is proposed to be the region inhibiting the global dopaminergic activity via the ventral tegmentum and the serotonin system via dorsal raphe (Hikosaka, 2010; Shumake and Gonzalez-Lima, 2003). Habenula is also a candidate for the site of antidepressive effect as the activity of this region is normalised by antidepressant treatment (Shumake et al., 2010). Habenula seems to play a crucial role in the chronic stress response as glucose uptake in this region was elevated in three different depression models (Caldecott-Hazard et al., 1988). Both midbrain target areas of habenula, ventral tegmentum and dorsal raphe, had decreased oxidative metabolism after chronic stress in the presented study.

The habenula is the target of a distinct group of neurons from the globus pallidus, a region where oxidative metabolism was decreased by stress and where rats with high sucrose intake had higher metabolic activity. This connection is proposed to relay reward expectancy related information in rhesus monkeys, and in humans, pallidal defects have been shown to lead to motivational deficits/anhedonia (Hikosaka, 2010; Hong and Hikosaka, 2008; Miller et al., 2006).

The mediodorsal thalamus, another region where stress caused a decrease in energy metabolism, is implicated in the generation of impulsive behaviour. Inhibition in this area by high-frequency stimulation is accompanied by worsened impulse control, and causes a decrease in the immediate early gene

expression of another area with lowered metabolism in response to stress in this study – the cerebellum (Moers-Hornikx et al., 2009). Chronic stress-induced dysfunction in cerebellar electron transport chain has been repeatedly demonstrated, and this dysfunction was found to be reversed by ketamine treatment (Rezin et al., 2008; Rezin et al., 2009b). The ventromedial hypothalamus, a region with lowered cytochrome oxidase activity after chronic stress in the present study, has classically been considered the neural substrate of aggressive behaviour, with the anterior portion involved in defensive and posterior in offensive aggression (Olivier, 1977). This area has been shown to display short-term activation in response to repeated social defeat without habituation (Miczek et al., 2008). It can be noted that in the course of the one-hour defeat session even the most submissive rats did demonstrate some active defence (e.g., boxing). Another role attributed to ventromedial hypothalamus is the control on food intake/satiety, with the destruction of the area leading to augmented intake (King, 2006). Interestingly, in the present study, animals with higher sucrose preference had elevated energy metabolism in this area but sucrose consumption trait did not influence weight gain.

Although chronic defeat stress increased adrenal gland weight in rats with low sucrose intake, the main effector in the acute and chronic hormonal stress response system, the paraventricular hypothalamus, did not show any change in long-term oxidative metabolism (Jankord and Herman, 2008; Shumake et al., 2003). It is possible that after adrenal hypertrophy and hyperplasia have been caused by elevated paraventricular hypothalamic function in the beginning of stress regimen, the same amount of adrenocorticotropic hormone can cause a larger release of glucocorticoids (Ulrich-Lai et al., 2006). It has been shown that the short-term activation of paraventricular hypothalamus habituates during repeated social stress, although stress hormone levels can be elevated for a longer period of time (Miczek et al., 2008; Rygula et al., 2008).

A number of nuclei involved in the control of movement had decreased energy metabolism after chronic social defeat stress: globus pallidus, ventral caudate putamen (with accumbens excluded), substantia nigra, red nucleus, and cerebellar vermis (DeLong, 1971; Guatteo et al., 2009; Lima et al., 2009; Strick et al., 2009). In globus pallidus, caudate putamen and red nucleus the stress-induced decrease in energy metabolism was detected in rats with high levels of the hedonic trait, whereas in cerebellum the decrease was more pronounced in rats with low levels of hedonic trait.

In conclusion, major responses to chronic social stress were observed in brain regions representing three major functional groups: regions involved in emotional/motivational aspects of behaviour, regions involved in memory processes, and central motor systems. Data from the present investigation do not indicate if the observed change in energy metabolism in a given region reflects brain malfunction in response to stress or adaptive changes to sustain adequate behaviour in conditions of chronic stress.

### 4.3.6. Serotonergic denervation and chronic variable stress cancel out each other's effect on long-term neural activity

In this experiment, reported in **Paper I**, we again utilised a stress-diathesis model combining the vulnerability experimentally caused by partial serotonergic denervation with parachloroamphetamine administration to emulate an inherent serotonergic deficiency, and chronic variable stress.

Both chronic variable stress and serotonergic deficiency had an impact of their own in distinct brain regions (see Sections 4.2.1 and 4.3.1), but the main finding of this study is the interaction between the two factors. Chronic variable stress and serotonergic denervation, when co-applied, resulted in cytochrome oxidase activity levels identical to control rats. The regions where oxidative energy metabolism was significantly affected by the combination of serotonergic lesion and chronic stress were medial preoptic area, suprachiasmatic hypothalamic nucleus, hippocampal CA3, anteroventral thalamus, cortical and medial amygdala, and dorsal anterior olfactory nucleus (**Figure 3**).

In case of some brain regions it appeared that in animals with serotonergic denervation the regional energy metabolism was not reactive to stress, possibly because the pathways that are activated during stress had been lesioned. In medial preoptic area, and cortical and medial amygdala, the serotonergic lesion prevented the potential increase in oxidative metabolism induced by stress. As response to stress is adaptive in essence, it can be suggested that the activation of serotonergic nuclei can be of benefit to the animal (Panksepp, 1998). If the serotonergic system is defective, these adaptive reactions can be compromised, leaving the animal more vulnerable to stress. There is evidence that rats with defective serotonin system have altered adaptive behavioural responses – animals pre-treated with the neurotoxin 5,7-dihydroxytryptamine did not display the typical freezing behaviour in a social defeat situation (Chung et al., 1999b). Expression of immediate early genes in medial amygdala was more pronounced in stressed animals with serotonergic lesions (Chung et al., 1999a). Medial preoptic area is known to receive strong input from serotonergic dorsal raphe fibres (Malinina et al., 2005). Thus, by decreasing serotonergic control over medial preoptic area, the pathway normally active during stress is rendered inactive.

There also were several brain regions where stress had an impact only on animals with serotonergic lesions, decreasing the oxidative activity to control levels. These regions included the suprachiasmatic hypothalamus, anteroventral thalamus, hippocampal CA3 and cortical amygdala. Though stress alone had no effect on oxidative activity of these regions, it did block the increase in metabolic activity in serotonin deficient rats. Serotonergic lesions could release the target areas from inhibition, resulting in elevated metabolic activity. In line with this suggestion, the activation of dorsal raphe by corticotropin-releasing factor during stress could increase the output of this nucleus and decrease metabolic activity in target areas (Leonard, 2005).

Chronic stress or depression can alter the daily rhythms in rodents. For example, chronic mild stress exerts disturbances on the diurnal and circadian rhythms of the locomotor activity and circadian changes in body temperature in the rat (Gorka et al., 1996; Meerlo et al., 1996; Ushijima et al., 2006). The relationship of circadian activity rhythms and chronic stress was further indirectly confirmed by this study: stress had an impact on oxidative activity of suprachiasmatic nucleus, the internal pacemaker of the organism, but only in animals with serotonergic lesions. It is possible, though, that the photic stressors specifically (stroboscope and light during the habitual dark phase) had affected the function of the suprachiasmatic nucleus of the stressed animals (Glass et al., 2003; Mistlberger, 2006). There is also evidence that serotonergic lesions caused by parachloroamphetamine or another serotonergic neurotoxin, 3,4-methylenedioxymethamphetamine, modulate circadian rhythm changes in rodents (Morin and Allen, 2006; Penev et al., 1995). Our results suggest that from a long-term metabolic perspective, stressors could have more marked effect on suprachiasmatic function in animals with serotonergic deficit.

Anteroventral thalamus, a region where stress eliminated the effect of serotonergic lesions, but had no effect on its own, has usually been linked to spatial learning and memory processes (van Groen et al., 2002). It is known that chronic stress has a strong impact on spatial memory formation in Y-maze and Morris water maze (Kleen et al., 2006; Song et al., 2006). It could be hypothesized that the adverse impact of chronic stress on memory can partially be mediated by alterations in the anteroventral thalamus, at least in animals with inferior capacity of the serotonergic system. Anterior thalamic nuclei are considered a part of the limbic Papez' circuit controlling affective/memory processes, and there are some indications of anterior thalamic involvement in fear learning as this area is acutely activated during fear recollection (Aggleton and Brown, 1999; Conejo et al., 2007; Vertes et al., 2001). Anterior, along with mediodorsal thalamus is part of the "limbic thalamus", and lesions targeted at this region disturb the acquisition of discriminative fear-learning in a more complex task that involves reacting to a conditioned stimulus and ignoring another similar unconditioned stimulus of the same class, and is dynamically co-activated with hippocampus (Hart et al., 1997; Stolar et al., 1989). The impact of serotonergic lesion and stress on another region of the brain extensively associated with memory, hippocampal CA3, was identical: chronic stress had an effect only after induced serotonergic dysfunction. The effect of chronic stress on hippocampal function is well established and chronic variable stress has been shown to decrease the activity of respiratory complexes, including cytochrome oxidase, in this region (McEwen, 2001; Tagliari et al., 2010).

Chronic variable stress had a reducing effect on cytochrome oxidase activity in the dorsal division of the anterior olfactory bulb, though once again only in animals with the serotonergic dysfunction. Dorsal division of the anterior part of the olfactory bulb has numerous reciprocal connections with the limbic forebrain, including the cortical amygdala (Song and Leonard, 2005). This

system, olfactory bulbs and amygdaloid complex, has previously been described as part of a possible neural substrate of response to chronic stress, and displayed long-lasting immediate early gene expression in response to chronic stressors (Matsuda et al., 1996).

The present results indicate that vulnerability caused by serotonergic lesions interacts with the effect of chronic stress, and this interactions more often than not renders regional energy metabolism to control levels. The interaction of vulnerability phenotype, caused by chronic prenatal stress, and chronic variable stress, on the level of serotonergic system was recently demonstrated: maternal stress and chronic variable stress decreased tryptophan hydroxylase 2 levels, whereas the combination of the two stresses led to tryptophane hydroxylase levels equal to controls (Leibold et al., 2011).

In conclusion, partial serotonergic denervation by parachloroamphetamine administration and chronic variable stress had an interactive impact on energy metabolism, the combination of manipulations resulting in oxidative energy metabolism comparable to control animals.

### 4.3.7. General conclusions from regional metabolic mapping of different models of depression

Behavioural phenotypes that either are directly representing depressive-like states or render animals more susceptible to the adverse effects of chronic stress display specific patterns of long-term neuronal activity, but there is very little overlap in regional energy-metabolism across depression-related phenotypes. The simplest reason for this is that the differences in the activity of oxidative metabolism in many instances are small compared to the normal variation, and any findings may represent either Type 1 or Type 2 errors. These models in question, partial serotonergic lesion, low sociability trait, high hedonic trait and neonatal maternal separation, have however different behavioural profiles and thus a large divergence in neuronal activity patterns is expected. Nevertheless, also a final common path to link the different vulnerabilities to depressive behaviour would be expected. Since all these models have the potential to render an animal more susceptible to the effects of chronic stress in common, it can be concluded that, as far as brain activity is concerned, they mediate vulnerability via different routes.

Studies on the neural substrate of personality in humans have also mostly revealed different and non-overlapping anatomical regions associated with personality traits that contribute to the genetic underpinnings of depression. Extraversion and neuroticism correlate with cortical thickness in distinct non-overlapping areas (Wright et al., 2006). Resting state regional cerebral glucose utilisation yields distinct and non-overlapping correlates for extraversion and neuroticism (Deckersbach et al., 2006; Kim et al., 2008). Extraversion and neuroticism also correlate with regional activation caused by anticipation of neutral, positive or negative stimuli in different brain areas (Brühl et al., 2011).

In a study associating all of the five-factor personality model traits with cerebral anatomy, the traits had almost completely distinct associations with cerebral regional volume (DeYoung et al., 2010). Spontaneous low frequency oscillations in the resting state give somewhat more overlapping results between the five factor personality traits, but the overlap is restricted to frontal cortical areas and precuneus (Kunisato et al., 2011). Thus, in humans, different basic personality dimensions rely mostly on independent neural substrate, and their associations with depression should thus be manifold and at least partly distinct.

Data on baseline/non-stimulated cerebral activity in rodents with different phenotypes is scarce, as the most often used method for brain activity detection, the immediate early gene c-fos immunocytochemistry, does not usually indicate baseline differences. In one study, though, inter-individual differences in anxiety caused by higher age were accompanied by higher basal immediate early gene expression in amygdala, and in general with increased hypothalamic and decreased amygdalar stress-induced immediate early gene expression (Meyza et al., 2011). Thus, there is no data in animals to indicate how large an overlap in the functional anatomy of different behavioural traits should be expected. There is also no comprehensive concept of the structure of behavioural dimensions in the rat, thus no knowledge of how interconnected the traits we measured should theoretically be. Looking at the factor-structure of behavioural test results measured in the same animals, the clearest clusters are usually formed by different behaviours within the same test, e.g., social interaction scores differing clearly from novelty-induced anxiety measures (Berton et al., 1997; Ramos et al., 1997). Serotonergic lesions do not affect sucrose consumption, but there is a modest decrease in social interaction after serotonergic denervation (Harro et al., 2001; Tõnissaar et al., 2008b).

The only brain region implicated in the two different vulnerability phenotypes is the supraoptic hypothalamic nucleus. In the present experiments the supraoptic nucleus was not reactive to stress but did differentiate between animals with high and low hedonic trait and between animals with medium and high/low sociability. This region has sparsely been associated with stress reactivity before, but is known to regulate several highly motivated behaviours, most notably maternal behaviour via peptidergic mechanisms (Martinez et al., 2002; Neumann, 2007; 2008; Ross and Young, 2009).

Neither did the two different chronic stress models utilised in the studies presented in this thesis elicit overlapping changes in regional neural activity patterns when applied to animals with no previous vulnerability. However, when stress-diathesis models were utilised, several commonalities in cerebral activation patterns emerged. Hippocampus was equally reactive to chronic social stress in rats with high and low levels of a hedonic trait, but more responsive in animals with serotonergic lesions if submitted to chronic variable stress. One could thus speculate that chronic social defeat stress that appears as more stressful is eliciting a functional down-regulation of the serotonergic system. Anterior thalamic nuclei were only activated by chronic social or variable stress if vulnerability factors were present – either a serotonergic lesion

or higher levels of the hedonic trait. Medial amygdala also displayed neural activation in response to chronic variable or chronic social stress only if vulnerability factors were present. Thus, when a stress-diathesis model was utilised and chronic stress was applied to rats with vulnerability to depression three important brain systems were affected. The extended amygdala is associated with the control of fear and stress reactions, and is implicated in human depression vulnerability by functional imaging studies (Herman et al., 2005; Sheline et al., 2001; Walker et al., 2003). A region most often associated with memory and learning, hippocampus, is also implicated in several dysfunctions in depressive states, e.g., inhibited neurogenesis, incomplete long-term potentiation, and reduction in glucocorticoid receptors, contributing to dysfunctional endocrine stress-response and behavioural deficits in depression (Joels et al., 2009; Wolf, 2009). The effect of chronic stress on hippocampal function is well established and chronic variable stress has been shown to decrease the activity of respiratory complexes, including cytochrome oxidase, in this region (McEwen, 2001; Tagliari et al., 2010). Anteroventral thalamus has usually been linked to spatial learning and memory, and since we know that chronic stress impairs learning, we can assume that the adverse impact of chronic stress on memory can partially be mediated by alterations in the anteroventral thalamus, and partially by hippocampus, as anterior thalamus has strong reciprocal connections with hippocampus (Kleen et al., 2006; Song et al., 2006; van Groen et al., 2002). Besides spatial learning, anterior thalamic nuclei are also involved in fear learning, as this area is acutely activated during fear recollection (Aggleton and Brown, 1999; Hart et al., 1997; Stolar et al., 1989; Vertes et al., 2001). Hippocampal CA3 and medial amygdala are frequently found to express immediate early genes as a response to acute and repeated stressful stimuli, and amygdalar and hippocampal acute stress response depends on the phenotype, as more anxious animals tend to show a larger response (Lehner et al., 2008; Martinez et al., 2002; Meyza et al., 2009; Singewald, 2007). These two areas implicated are also proposed to be dysfunctional in humans during depression, hippocampal gray matter volume and cell count is reduced in affective disorders, and in amygdala several markers of function and volume are decreased during depression (Drevets et al., 2008a). The function of medial amygdala in the mediation of chronic and acute stress effects is complicated, though. Medial amygdala controls the activity of the acute endocrine stress response, but chronic stress does not affect this specific function. On the other hand, decrease in weight gain during chronic stress, one of the most robust effects of stress, is exaggerated by medial amygdala lesions (Solomon et al., 2010). Medial amygdala is also implicated in the formation of conditioned fear response, as inhibition of this area by GABA-ergic agonists reduces conditioned fear, though it must be noted that suppression of protein synthesis in medial amygdala does not interfere with fear learning (Markham and Huhman, 2008).

Conclusively, commonalities in regional brain neural activity between different vulnerability phenotypes and chronic stress regimens emerge only when a stress-diathesis model is utilised.

When comparing the impact of chronic variable and social stress it is obvious that variable stress increased whereas social stress decreased regional metabolic activity in several brain regions. On the other hand, variable stress decreased activity in animals with vulnerability phenotype. Thus we propose that social stress is a more potent stressor that can have a substantial inhibitory impact on brain activity even without the vulnerability factors present. Social stress has several unique features: it is not subject to habituation; a single social stress session is enough to cause long-term behavioural and neurochemical changes; and acute social stress has the highest mobilising effect on the endocrine system (Koolhaas et al., 2011; Koolhaas et al., 1997; Meerlo et al., 1996). The vast impact of social stress was well characterised in a study by Matsuda and colleagues, where twenty four hours after the last session of repeated chronic defeat all forty nine studied brain regions displayed a persistent c-fos expression, sometimes reaching over twenty times the activity of controls, whereas mice that had received a single defeat were indistinguishable from controls twenty four hours after stress (Matsuda et al., 1996). Repeated exposure to non-social stressors, either restraint or variable stress regimen, results in habituation and inhibition in immediate early gene expression (Ostrander et al., 2009; Umemoto et al., 1994).

A highly speculative hypothesis can be put forward explain the larger impact of stronger stressors on cerebral cytochrome oxidase activity: moderate chronic stress activates several brain areas as an adaptive response to changed environment. Cytochrome oxidase histochemistry is most sensitive to changes in the glutamatergic system, due to its vast postsynaptic energy demand (Wong-Riley et al., 1998). Central stress reaction is coordinated, along with steroid hormones and neuropeptides, by glutamate, as stress induces increased glutamate release, and glutamate levels are persistently higher in chronically stressed rodents and depressed patients, and the dysbalance of excitation/ inhibition is recognized as one of the key elements in depression (Alcaro et al., 2010; Gao and Bao, 2011; Kugaya and Sanacora, 2005; Miczek et al., 2008; Reznikov et al., 2009; Steciuk et al., 2000; Takeda and Tamano, 2010). The vulnerability factors, on the other hand, can also be hypothesised to be associated with increased glutamatergic signalling. In animals with serotonergic lesions the release of glutamatergic signal from serotonergic inhibition has been demonstrated, and maternal separation/postnatal stress leads to adulthood changes in glutamate receptor and transporter levels, as during the first postnatal days glutamatergic system is remodelled (Di Cara et al., 2001; Pickering et al., 2006; Zhao et al., 2009). A dysfunction in social behaviour – social phobia – is associated with decreased GABA and increased glutamate signalling (Pollack et al., 2008). When moderate chronic stress is applied to animals with a pre-existing vulnerability to depression, the glutamatergic signals increase additively, and this can be hypothesised to lead to stimulation

of comparable intensity with chronic social stress. Increased glutamatergic signal causes excitotoxic damage and leads to apoptosis, this would lead to less cytochrome oxidase staining in a given region (Wolkowitz et al., 2010). Due to the neurodegeneration and energetic dysfunction, depression has been compared to metabolic encephalopathy (Harvey, 2008).

Stone and colleagues have proposed that depression is a state of hypoactivity of the brain's positive motivational network and hyperactivity of the stress network (Stone et al., 2008). Several regions proposed to be involved in the motivational network also mediate chronic stress and vulnerability phenotypes – medial preoptic area, mammillary bodies, substantia nigra and ventral tegmental area. The regions of this network mediate reward and approach, goal-directed behaviour, learning, and finally motor embodiment of the ideation (Stone et al., 2008). A number of regions proposed to form the stress network were also differentially activated in animals with the vulnerability phenotype or subjected to chronic stress – bed nuclei of the stria terminalis, central amygdala, dorsal raphe and periaqueductal gray (Stone et al., 2008). Although the major nodes of both networks are implicated in the presented depression/vulnerability models, the hypo/hyperactivity in these systems does not differentiate the depressed and vulnerable animals from control and resilient groups. This discrepancy might result from the fact that what Stone and colleagues term hypo- or hyperactivity, may be in fact hypo- or hyperreactivity, as a vast amount of data regarding the behavioural function of these nuclei derives from studies on human and animal short-term neuronal activity; thus we know these regions are involved in motivational/stress function, but we cannot infer baseline differences of neural activity from it. Another possibility to reconcile the discrepancy is to consider the methodological issues of cytochrome oxidase histochemistry. Cytochrome oxidase activity reflects the energy expenditure of the cell, the main energy demand coming from postsynaptic dendritic currents (Wong-Riley et al., 1998). Excitatory postsynaptic processes are most energy consuming, but inhibitory processes do not decrease, but rather increase energy demand, thus a change in cytochrome oxidase activity can refer to amount of control by its effector areas (Ackermann et al., 1984).

In several regions implicated in chronic stress/vulnerability to depression by differences in cytochrome oxidase activity, the activity has been shown to be reversible by antidepresssant treatment. Thus, in hippocampal CA3 and dentate gyrus, habenula, ventral tegmental area and cerebellum, this change in brain oxidative metabolism parallels the decrease of depression-like behaviour and physiological markers (Padilla et al., 2011; Rezin et al., 2009b; Shumake et al., 2010).

The results of metabolic mapping of different vulnerability phenotypes and chronic stress regimens suggest several functional systems for further, more detailed neurochemical and electrophysiological studies. The neural substrate of acute stress response has been proposed to be dysfunctional in depressive states (Gold and Chrousos, 2002), this is further corroborated by our results, showing the involvement of amygdala, bed nucleus of stria terminalis, and raphe nuclei

in the development of depression. Several regions belonging to the basal forebrain network are affected by stress or differentially active in vulnerable/ resilient animals – globus pallidus, ventral pallidum, nucleus accumbens, substantia innominata, nuclei of the diagonal band, and the network of extended amygdala, these regions forming the signal controlling cingulate and hippocampal function (Heimer, 2003). Alcaro and colleagues have emphasised the role excitatory/inhibitory balance of the midline brain regions in depression in humans and animal models (Alcaro et al., 2010). This approach is an accord with our results, as, firstly, the changes measured by cytochrome oxidase reflect to a large extent the glutamatergic input to nuclei, and secondly, the metabolic activity of midline regions like periaqueductal gray, raphe nuclei, paraventricular thalamus, habenula, anterior cingulate and posterior cingulate depends on the animals vulnerability phenotype and stress exposure. Another network that reflects the vulnerability to and impact of chronic stress includes anterior thalamic regions, mammillary bodies, hippocampus and both the anterior and posterior divisions of cingulate cortex. Interestingly the function of the anterior thalamic nuclei and retrosplenial cortices are shown to have highly specific functions in memory and learning, but these regions are most robustly implicated in several depression models in our laboratory (Aggleton et al., 1996; Aggleton et al., 2010; Aggleton et al., 2009; Matrov et al., 2007; Mällo et al., 2009; Pothuizen et al., 2010; Tsanov et al., 2011).

### 4.3.8. Brain regions mediating vulnerability to depression in common to several depression models

In this analysis (**Paper V**) data from five different oxidative energy metabolism mapping studies of depression models were pooled and analysed jointly. The models including five vulnerability models and two chronic stress paradigms are represented in the dataset. Rats with persistently higher level of expression of a hedonic trait, as measured by sucrose preference, have been found more responsive to chronic social defeat stress, as measured in the forced swimming test (Paper II). Rats with lower levels of spontaneous exploratory activity have an anxious/depressed phenotype in the elevated plus maze and forced swimming test, respectively, and these animals also lose body weight more readily during chronic stress (Matrov et al., 2011; Mällo et al., 2007a). Partial serotonergic denervation by parachloroamphetamine treatment (2 mg/kg) can induce behavioural changes comparable to chronic stress in social interaction test and forced swimming test (Harro et al., 2001). Maternal separation of rat pups causes a depressive-like phenotype in adulthood, as indicated by anhedonia and passive coping in the forced swimming test, an exaggerated endocrine stress response, and increased reactivity to adulthood chronic restraint, as indicated by increased anxiety in the elevated plus maze (Aisa et al., 2007; Eiland and McEwen, in press). Rats with lower levels of positive emotionality, measured as a lower level of emitting 50 kHz ultrasonic

vocalisations during a stimulation resembling play-behaviour, are more susceptible to chronic stress, as indicated by symptoms of anhedonia and anxiety (Mällo et al., 2009). In three of these studies, chronic stress was applied to both the vulnerability group and the "resilient" control group. Chronic social stress was applied in the study on the significance of the hedonic trait in vulnerability, and chronic variable stress was used in studies on the association of partial serotonergic denervation and low positive affect with depression (Paper I; Paper II; Mällo et al., 2009). In each of these three models, chronic stress had led to a distinct behavioural phenotype in the vulnerable animals as compared to the resilient controls (Paper II; Tõnissaar et al., 2008; Mällo et al., 2009).

Overall, vulnerability tended to be associated with higher cytochrome oxidase activity. This was statistically significant in reticular thalamus, hippocampal CA3, granular and agranular retrosplenial cortices and pontine nuclei (**Figure 7**). In reticular thalamus and granular retrosplenial cortex, the higher cytochrome oxidase activity in vulnerable rats was decreased by chronic stress to levels comparable with resilient control animals. In substantia nigra pars reticularis and temporal cortex resilient animals had higher cytochrome oxidase activity, but this difference was only observed after chronic stress. Habenula was the only region reactive to stress in resilient animals: chronic stress decreased energy metabolism in this area. Median raphe was the only nucleus where the combination of vulnerability and stress induced a large increase in metabolic activity.

Vulnerability to depression in general tended to be associated with higher levels of long-term neural activity, and in the reticular thalamus and granular retrosplenial cortex this increase was reversed by chronic stress. Reticular thalamus is considered to be involved in the regulation of arousal levels and filtering sensory input to thalamus, but has not usually been implicated in depression (McAlonan and Brown, 2002). A region where resilient rats had lower metabolic activity after stress, reticular substantia nigra, is implicated in attentional processes via connections to reticular thalamus (Pare et al., 1990).

The reticular thalamic nucleus has been shown to have functional connections with retrosplenial cortex, and this network has been proposed to be involved in attentional gating (Knyihar-Csillik et al., 2005). Retrosplenial cortex has also been related to memory and learning disturbances, as hypoactivity or lesions of this region lead to amnesia, while hyperactivity in this brain region has been demonstrated in disorders with a specific component of learning – post-traumatic stress disorder (Lanius et al., 2006; Vann et al., 2009). This region has also been implicated in emotional responses to stimuli in human brain-imaging studies (Maddock, 1999). In rodents, retrosplenial dysfunction usually results in spatial memory deficits, but effects on fear-learning and diminished novelty related locomotion have also been shown (Corcoran et al., 2011; Keene and Bucci, 2008; Lukoyanov and Lukoyanova, 2006).

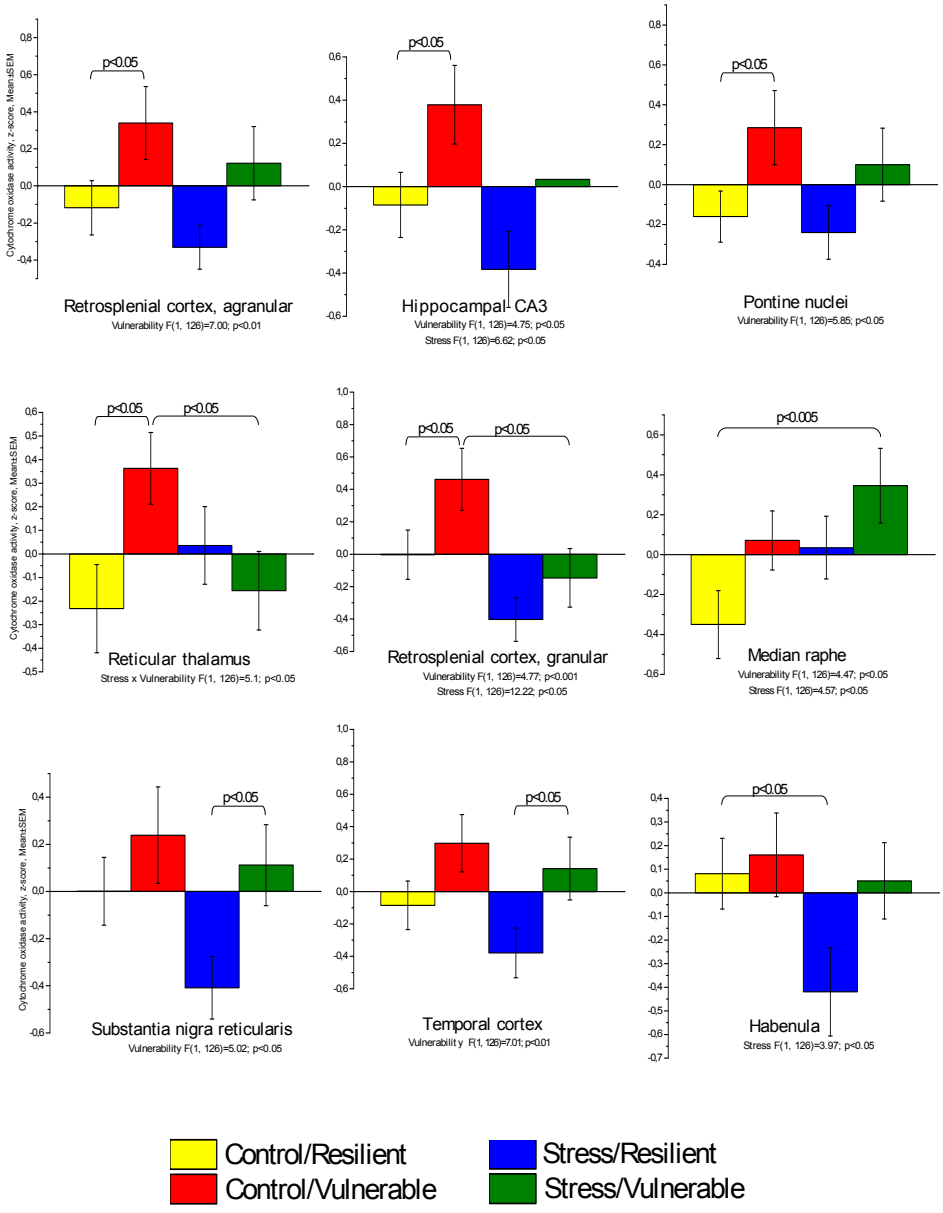


**Figure 7.** Cytochrome oxidase activity (standard z-score, mean±SEM) after chronic stress in rats with resilience or vulnerability to depression. Data from five cytochrome oxidase mapping studies were collapsed and analysed jointly. For group-wise comparisons LSD post hoc test was used.

Hippocampal CA3 area was found to have higher oxidative metabolism in vulnerability to depression. Although hippocampal involvement has often been indicated in depressogenesis, e.g., mediating the impact of early life adversity and depressive behaviour, this has been based on associations with a decrease in hippocampal volume or function (Blugeot et al., 2011; Rao et al., 2010), and it is not entirely clear whether hippocampal alterations are the substrate of vulnerability or the consequence of adverse events. If loss of hippocampal volume and plasticity were regarded as vulnerability markers for depression (Blugeot et al., 2011), then the increase in cytochrome oxidase activity could be a compensatory mechanism to ensure function. In non-human primates, though, hyper-metabolism in hippocampus has been shown in animals with higher trait anxiety, and there is some evidence of increased activity in hippocampal function in depressed humans (Small et al., 2011).

Habenula was the only brain region that represented a decrease in energy metabolism after chronic stress in resilient animals. This nucleus mediates signals from hypothalamic and limbic areas to serotonergic and dopaminergic neurons in raphe nuclei and ventral tegmental area (Hikosaka, 2010). Contrary to the present results, elevated cytochrome oxidase activity in habenula has been demonstrated in congenitally helpless rats, and higher levels of glucose utilisation in habenula have been shown in several animal models of depression (Caldecott-Hazard et al., 1988; Shumake et al., 2003). Chronic stress also precipitated differences in cytochrome oxidase activity between vulnerability phenotypes in the substantia nigra pars reticularis and temporal cortex. Temporal cortex has most often been shown to be involved in learning, possibly also fear learning (Lanius et al., 2006; Miller and McEwen, 2006; Wang et al., 2010).

In the hindbrain, median raphe showed increased cytochrome oxidase activity levels in vulnerable and stressed animals, as compared to resilient controls. The activity of raphe nuclei can be associated with anxious behaviour frequently seen in both animal models and depressed humans (Dos Santos et al., 2005; Vicente et al., 2008; Wittchen et al., 1994). Hindbrain pontine nuclei were more metabolically active in vulnerable animals, this region has been shown to be responsible for autonomic stress-related activation, as well as responsive to different acute stressors as indicated by immediate early gene activation (Palkovits et al., 1997; Pissiota et al., 2002). Thus the increased metabolic activity of pons in rats with vulnerability phenotype, described in the presented results, can reflect the higher stress reactivity of these animals.

Conclusively, in this joint analysis of data derived from distinct models, vulnerability was associated with higher oxidative energy metabolism in two inter-connected brain regions, the retrosplenial cortex and reticular thalamus, and chronic stress eliminated this difference. Energy metabolism in habenula was reduced by chronic stress. In addition, chronic stress increased cytochrome oxidase activity in median raphe in rats with higher vulnerability to depression.

### 4.3.9. Brain functional networks implicated in vulnerability to depression in common to several depression models

To reveal the brain functional networks involved in the development of depression, data pooled from five cytochrome oxidase mapping studies were subjected to analysis of inter-regional correlations (Paper V).

Correlation plots of regional energy metabolism indicating the associations between cytochrome oxidase activity in all brain regions are presented in **Figure 8**. The presence of notable positive correlations is the highest in rats with vulnerability phenotypes, and lowest (and more fragmented, i.e., forming clusters of brain regions interconnected) in animals who were subjected to chronic stress. Vulnerable animals differed from others especially clearly with regard to moderately high correlations of the activity of ventral tegmental area, substantia nigra reticularis and compacta as compared to other brain regions. These correlations were weaker in control animals and almost absent in both stressed groups. For example, the correlation of ventral tegmental area cytochrome oxidase activity with prelimbic cortex activity was 0.28 ($p>0.05$) for control/resilient animals, 0.13 ($p>0.05$) for stressed/resilient animals, 0.48 ($p<0.005$) for control/vulnerable rats, and 0.03 ($p>0.05$) for stressed/vulnerable rats.

The activity of dorsal, lateral-dorsal and lateral-ventral central gray, dorsal and median raphe, pontine nuclei, vermis and locus coeruleus did not correlate well with the activity of other brain regions, and even negative correlations were found in control/vulnerable animals between median raphe and olfactory bulbi, septum and caudete putamen. In vulnerable rats subjected to stress some moderate correlations between mid- and hindbrain regions vs. thalamic and hypothalamic areas were found. For example, dorsal periaqueductal gray activity correlated with lateral hypothalamic activity 0.15 ($p>0.05$) for control/resilient animals, 0.01 ($p>0.05$) for stressed/resilient animals, 0.18 ($p>0.05$) for control/vulnerable rats, and 0.40 ($p<0.05$) for stressed/vulnerable rats.

In vulnerable rats submitted to stress the activity of the posterior cingulate – agranular and granular retrosplenial cortex – did not correlate with the activity of forebrain and hypothalamic areas. For example, the correlations between the activities of granular retrosplenial and anterior cingulate cortices were 0.58 ($p<0.005$) for control/resilient animals, 0.54 ($p<0.005$) for stressed/resilient animals, 0.54 ($p<0.005$) for control/vulnerable rats, and –0.14 ($p>0.05$) for stressed/vulnerable rats.

Stressed rats were distinct from control animals as the correlations of the activity of hypothalamic areas – supraoptic and suprachiasmatic nuclei, lateral-anterior, anterior and lateral hypothalamus, to other brain regions was low. The weak association of hypothalamic areas with the rest of the brain after stress was, however, less general in rats with the vulnerability phenotype, being limited mainly to forebrain regions. For example the correlations of anterior hypothalamic area with ventral bed nucleus of the stria terminalis were 0.45

($p<0.005$) for control/resilient animals, 0.07 ($p>0.05$) for stressed/resilient animals, 0.48 ($p<0.005$) for control/vulnerable rats, and –0.11 ($p>0.05$) for stressed/vulnerable rats.

In stressed rats the activity of olfactory system – anterior olfactory nuclei, did not correlate well with rest of the brain areas, especially forebrain. For example the correlations for medial anterior olfactory nucleus with medial septum were 0.37 ($p<0.05$) for control/resilient animals, –0.07 ($p>0.05$) for stressed/resilient animals, 0.39 ($p<0.05$) for control/vulnerable rats, and –0.11 ($p>0.05$) for stressed/vulnerable rats. Stress tended to disrupt the intra-hypothalamic functional connectivity in resilient rats and vulnerable rats subjected to stress showed the most coherent correlation pattern between hindbrain regions.

The analysis of inter-correlations between all brain areas demonstrated that animals vulnerable to depression have the highest regional coherence in neuronal activity, whereas after chronic stress this coherence is much reduced. In human brain imaging studies it has been shown that depressed patients have increased intrinsic neuronal recruitment in frontal and cortical regions during a resting-state, and this leads to a bias towards more negatively valenced information processing (Zhou et al., 2010). In another study the higher frontal-limbic and frontal-striatal functional connectivity was reduced by antidepressant treatment (McCabe and Mishor, 2011). Thus persistently higher co-activity, at least in restricted areas, has been shown to be associated with depressive behaviour in humans, whereas the present results suggest that high regional correlations are intrinsic for animals vulnerable to depression. The majority of the correlations between brain regions in this study were positive, and a question may arise how only positive correlations can result from neural networks with inhibitory and excitatory processes. Cytochrome oxidase histochemistry measures mainly postsynaptic dendritic energy expenditure that is comprised mostly of postsynaptic currents, both inhibitory and excitatory. Thus both inhibition and excitation increase the energy demand of the cell, thus increasing cytochrome oxidase expression, and resulting in only positive correlations.

In the multidimensional scaling analysis, first, the pair-wise differences between brain region correlation matrices of experimental groups were calculated, and secondly, the inter-relations of the differences in correlation coefficients were calculated with multidimensional scaling. A two-dimensional model was employed in this analysis because adding the third dimension did not add to the badness-of-fit criterion. This criterion, ranging from 0 to 1, with 0 reflecting a perfect fit, describes how well the two-dimensional model reproduces the distance structure of the input data. The distance between any two brain regions along the two dimensions is a relative indicator of their functional connectivity.

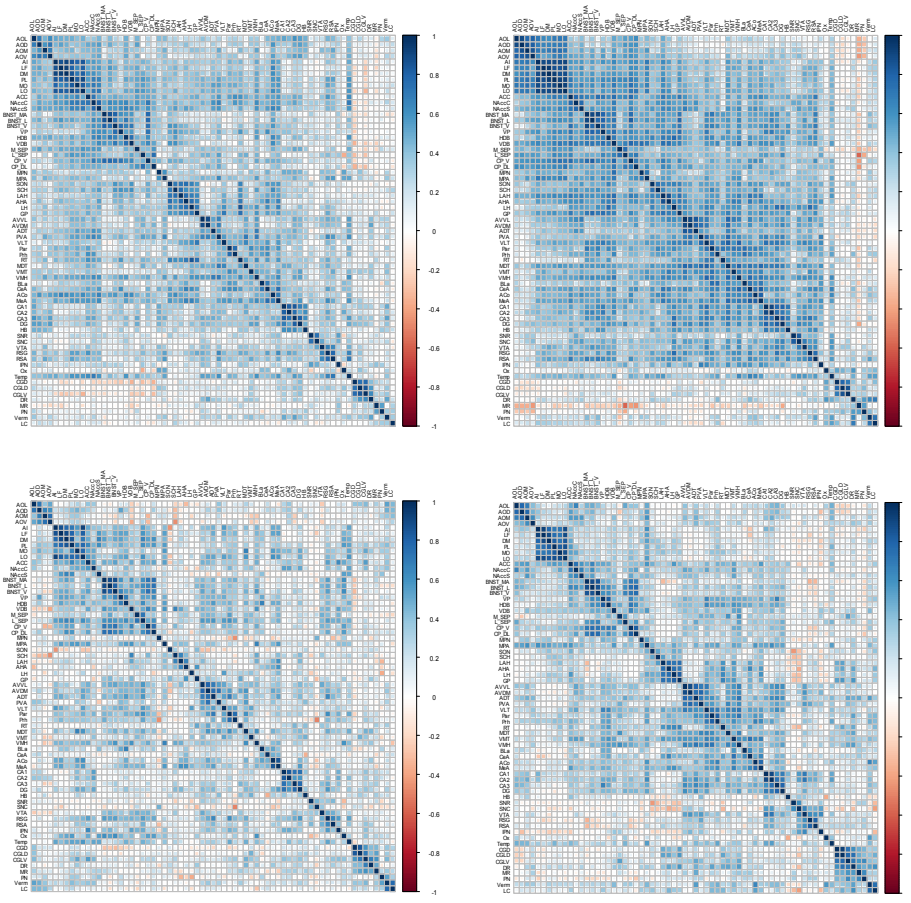

**Figure 8**. Inter-correlations of the metabolic activity of brain regions as measured by cytochrome oxidase histochemistry (on both axes of the figure) in control animals (upper left), in rats with vulnerability phenotype (upper right), in chronically stressed rats (lower left) and in vulnerable animals submitted to chronic stress (lower right). The colour at the convergence of a line and a column reflects the direction and strength of the correlation, as indicated on the calibration bar on the right.

Abbreviations: ACC – Anterior cingulate cortex; ACo - Cortical amygdala; ADT – Anterodorsal thalamic nucleus; AHA – Anterior hypothalamic area, anterior; AI - Agranular insular cortex; AOD - anterior olfactory nucleus, dorsal; AOL – Anterior olfactory nucleus, lateral; AOM - Anterior olfactory nucleus, medial; AOV - Anterior olfactory nucleus, ventral; AVDM – Anteroventral thalamic nucleus, dorsomedial division; AVVL – Anteroventral thalamic nucleus, ventrolateral division; BLa - Basolateral amygdala, anterior; BNST-L - Bed nucleus of stria terminalis, lateral; BNST-MA - Bed nucleus of stria terminalis, medial anterior; BNST-V - Bed nucleus of stria terminalis, ventral; CA1 - Cornu ammonis 1; CA2 – Cornu ammonis 2; CA3 - Cornu ammonis 3; CeA - Central amygdala; CGD - Central gray, dorsal; CGLD - Central gray, laterodorsal; CGLV - Central gray, lateroventral; CP-DL - Caudate putamen, dorsolateral; CP-V - Caudate putamen, ventral; DG - Dentate gyrus; DM – Dorsomedial frontal cortex; DR – Dorsal raphe; GP - Globus pallidus; HB - Habenula; HDB – Nucleus of diagonal band, vertical; IPN - Interpeduncular nuclei; L-SEP – Lateral septum; LAH - Lateroanterior hypothalamic nucleus; LC - Locus coeruleus; LF – Lateral frontal cortex; LH – Lateral hypothalamic area; LO – Lateral orbital cortex; M-SEP – Medial septum; MDT - Mediodorsal thalamic nucleus; MeA – Medial amygdala; MO – Medial orbital cortex; MPA - Medial preoptic area; MPN - Median preoptic nucleus; MR – Median raphe; NAccC – Nucleus accumbens core; NAccS – Nucleus accumbens shell; Occ - Occipital cortex; Par - Parietal cortex; PL - Prelimbic cortex; PN - Pontine nuclei; Prh - Perirhinal cortex; PVA - Paraventricular thalamic nucleus, anterior; PVP - Paraventricular thalamic nucleus, posterior; RSA - Retrosplenial cortex, agranular; RSG - Retrosplenial cortex., granular; RT - Reticular thalamic nucleus; SCH - Suprachiasmatic nucleus.; SNC - Substantia nigra pars compacta; SNR - Substantia nigra pars reticularis; SON - Supraoptic nucleus; Temp - Temporal cortex; VDB – Nucleus of diagonal band, horizontal; Verm - Cerebellar vermis; VLT - Ventrolateral thalamic nucleus; VMH - Ventromedial hypothalamic nucleus; VMT - Ventromedial thalamic nucleus; VP – Ventral pallidum; VTA - Ventral tegmental area

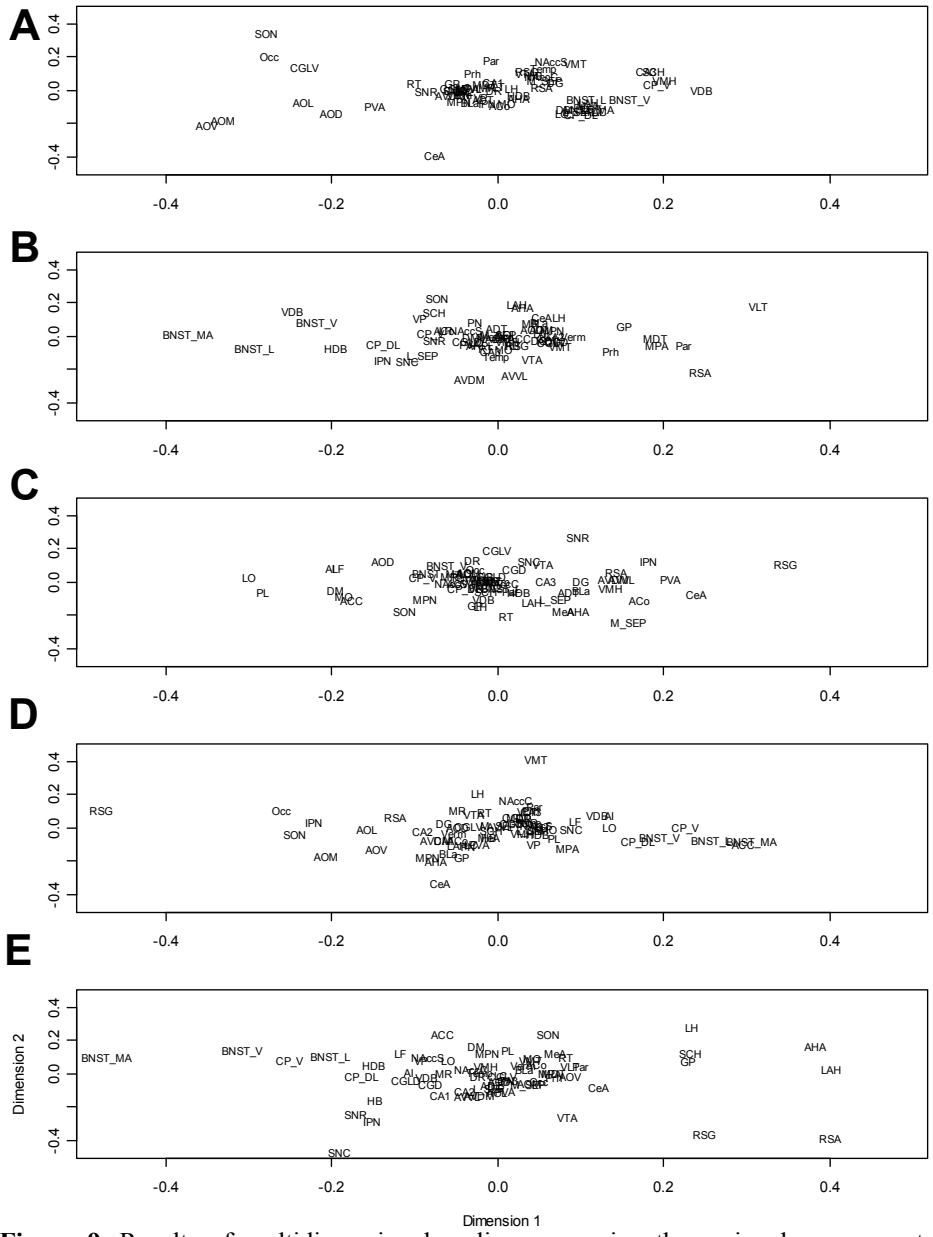


**Figure 9.** Results of multidimensional scaling comparing the regional energy metabolism correlation patterns of **A** – stressed/resilient vs. control/resilient rats; **B** – control/vulnerable vs. control/resilient animals; **C** – stressed/vulnerable vs. control/resilient; **D** – stressed/vulnerable vs. stressed/resilient animals; and **E** – stressed/vulnerable vs. control/vulnerable animals. Interrelations of differences in correlations are fitted in two-dimensional space represented by the axes of the figure. The distance between any two brain regions, each represented by its abbreviation, is a relative indicator of their functional inter-relatedness in terms of association strength. See Figure 8 for abbreviations

Comparing resilient groups and thus examining the "independent" effect of chronic stress (badness-of-fit criterion for the two-dimensional model 0.45), it was found that the stress effect was to lead to deviation of the activity of several olfactory nuclei, most notably the ventral and medial, but also lateral and dorsal anterior olfactory nuclei, supraoptic nucleus, occipital cortex, lateral-ventral region of the central gray and central amygdala from the activity of other brain regions (**Figure 9A**). In rats vulnerable to depression the activity of several nuclei deviated from other brain areas (badness-of-fit 0.50). These regions included the medial-anterior, lateral and ventral sub-regions of the bed nucleus of the stria terminalis; the horizontal and vertical diagonal band; ventrolateral and dorsomedial anteroventral thalamus and ventrolateral thalamus; agranular retrosplenial cortex, parietal cortex, medial preoptic area, mediodorsal thalamus, globus pallidus and perirhinal cortex (**Figure 9B**). Comparison of chronic stress/vulnerability group with the control/resilient animals (badness-of-fit 0.49), which is the most direct test of the stress-diathesis model, resulted in deviant activity in cortical regions, most clearly in the lateral orbital and prelimbic frontal cortices, but also agranular insular, dorsomedial, medial orbital and lateral frontal cortices as compared to other regions. The activity of both anterior and posterior parts of cingulate (anterior cingulate cortex and granular retrosplenial cortex) also differed from other brain areas in stressed rats with vulnerability phenotype (**Figure 9C**). When assessing the impact of chronic stress in vulnerable vs. resilient rats (badness-of-fit 0.48), the most deviant brain regions were granular retrosplenial, anterior cingulate and occipital cortices, supraoptic nucleus, interpeduncular nucleus, medial division of the anterior olfactory nucleus, bed nucleus of stria terminalis, caudate putamen, ventromedial thalamus and central amygdala (**Figure 9D**). The effect of stress specifically on vulnerable rats (badness-of-fit 0.43) resulted in a loss of connectivity between hypothalamic regions (anterior hypothalamic area, lateral hypothalamus, lateral anterior hypothalamus, suprachiasmatic hypothalamus), agranular and granular retrosplenial cortices, bed nucleus of stria terminalis, substantia nigra compacta and globus pallidus with the bulk of the brain areas (**Figure 9E**).

Multidimensional scaling is a method that allows assaying of the general pattern of inter-connectedness of all brain regions simultaneously. All experimental groups exhibited a unique profile of regional inter-connectivity as demonstrated by two-dimensional scaling of differences between correlations of metabolic activity in the brain regions. The differences in regional connectivity present in condition of chronic stress were most notable in the diminished connectivity of the olfactory system with the rest of the brain areas. Olfactory system is an important channel of information in rats as it guides the highly motivated aggressive and mating behaviours (Guillot and Chapouthier, 1996; Keller et al., 2010), but also all novelty-related, exploratory activities. Olfactory bulbs are interconnected with the limbic system and lesions of the olfactory system lead to depressive-like behaviour in rodents (Kang et al., 2011; Song

and Leonard, 2005; Vinkers et al., 2009a). Depressive behaviour has been associated with loss of bulbar volume and olfactory function in both rodents and humans (Negoias et al., 2010; Pause et al., 2001; Yang et al., 2011).

Vulnerable animals were distinguished by the relative dysconnection of the activity of the bed nucleus of stria terminalis from the rest of the regions. Bed nucleus of stria terminalis receives a signal from amygdalar nuclei via central amygdala, the function of bed nucleus of stria terminalis is also controlled by hippocampal subiculum and infralimbic cortex, and this region is implicated foremost in the regulation of anxiety, fear and stress response (Davis et al., 2009; Georges and Aston-Jones, 2002; Ziegler and Herman, 2002). Bed nucleus of stria terminalis sends efferents, besides hypothalamus, to ventral tegmental area and thus the activity of mesolimbic dopamine system responsible for incentive/motivated behaviour is partially controlled by the signal from the bed nucleus of stria terminalis (Alcaro et al., 2007; Georges and Aston-Jones, 2002; Jalabert et al., 2009). Via hypothalamic targets bed nucleus of stria terminalis mediates the limbic regulation of hypothalamic stress-hormone releasing regions (Ziegler and Herman, 2002). It thus appears that the bed nucleus of stria terminalis is a major regulator that by synchronization of activities in these brain regions is vital in maintaining emotional and motivational balance.

In vulnerable animals, frontal and retrosplenial cortical regions were relatively dysconnected from other brain areas after exposure to chronic stress in terms of long-term neuronal activity. Dysfunction in intra-cortical and cortico-limbic pathways is among the most frequent findings in human depression, and inhibition of the activity of anterior cingulate reduces depression (Bissiere et al., 2006; Drevets et al., 2008b; Ressler and Mayberg, 2007). In stressed animals with the vulnerability phenotype the connections of retrosplenial cortex with other brain regions were strongly diminished. Retrosplenial cortex, as mentioned above, has been implicated foremost in learning, but there is some evidence indicating the role of retrosplenial cortices in affective information processing (Alcaro et al., 2010; Maddock, 1999; Vann et al., 2009). The most characteristic feature of the impact of stress in vulnerable animals was the decreased connectivity of hypothalamus to other brain regions. Hypothalamic nuclei control homeostasis and endocrine function, including stress response, feeding and sexual behaviour (Abizaid and Horvath, 2008; Adamantidis and de Lecea, 2008; Charlton, 2008; Ziegler and Herman, 2002). Several hypothalamic nuclei relay inhibitory and excitatory signals from limbic and forebrain areas to paraventricular region, thus regulating the stress-induced corticotropin-releasing factor and vasopressin release (Jankord and Herman, 2008).

To investigate the regional connectivity in more detail, differences in correlation coefficients were assessed in four major brain circuits implicated in affective and stress-related disorders (**Table 1**). The neural circuit consisting of basal forebrain and hypothalamic nuclei controlling midbrain regions via habenula has been frequently implicated in chronic stress/depression (Caldecott-Hazard et al., 1988; Hikosaka, 2010; Sartorius and Henn, 2007; Shumake et al., 2003; Yang et al., 2008).

**Table 1.** Significant differences in correlations of regional cytochrome oxidase activities in animals with either vulnerability or resilient phenotype submitted to chronic stress. C/R – $p<0.05$, vs. Control/Resilient; C/V – $p<0.05$ vs. Control/Vulnerable; S/R – $p<0.05$ vs. Stress/Resilient; bold font $p<0.005$

| **Habenular circuit** | Control/ Resilient | Stress/ Resilient | | Control/ Vulnerable | | Stress/ Vulnerable | |
|---|---|---|---|---|---|---|---|
| | r | r | | r | | r | |
| **N. of the diagonal band, ventral vs.** | | | | | | | |
| dorsal raphe | -.133 | .189 | | .235 | | .379 | C/R |
| subst. nigra, compacta | .177 | -.168 | | .491 | | -.044 | C/V |
| **Medial septum vs.** | | | | | | | |
| dorsal raphe | -.086 | -.120 | | -.214 | | .398 | C/V |
| median raphe | -.198 | -.049 | | -.342 | | .239 | C/V |
| **Lateral septum vs.** | | | | | | | |
| median raphe | .022 | .114 | | -.588 | C/R | .161 | C/V |
| **Lateral anterior hypothalamus vs.** | | | | | | | |
| habenula | .176 | .146 | | .371 | | -.189 | C/V |
| subst. nigra, reticularis | .259 | -.082 | | .339 | | -.229 | C/V |
| subst. nigra, compacta | .109 | -.012 | | .427 | | -.265 | C/V |
| interpeduncular n. | .096 | .112 | | .418 | | -.134 | C/V |
| **Lateral hypothalamus vs.** | | | | | | | |
| subst. nigra, reticularis | .216 | -.246 | | .461 | | -.279 | **C/V** |
| subst. nigra, compacta | .040 | -.138 | | .616 | | -.304 | **C/V** |
| ventral tegmental a. | .333 | .005 | | .541 | | .013 | C/V |
| interpeduncular n. | .209 | .131 | | .369 | | -.192 | C/V |
| **Globus Pallidus vs.** | | | | | | | |
| caudate putamen, ventr. | .369 | .186 | | .641 | | .230 | C/V |
| habenula | .325 | .315 | | .583 | | .074 | C/V |
| subst. nigra, compacta | -.047 | -.301 | | .347 | | -.279 | C/V |
| interpeduncular n. | .180 | .163 | | .347 | | -.174 | C/V |
| dorsal raphe | .085 | .361 | | .193 | | .581 | C/R |
| **Interpeduncular nuclei vs.** | | | | | | | |
| Medial septum | -.023 | .462 | C/R | .431 | | .352 | |
| subst. nigra, reticularis | .543 | .065 | C/R | .254 | | .365 | |
| subst. nigra, compacta | .486 | -.052 | C/R | .291 | | .532 | |
| ventral tegmental area | .304 | .343 | | .211 | | .636 | C/V |
| median raphe | .407 | .181 | | -.114 | C/R | .111 | |
| **Extended amygdala and central stress response circuits** | Control/ Resilient | Stress/ Resilient | | Control/ Vulnerable | | Stress/ Vulnerable | |
| | r | r | | r | | r | |
| **Bed n. of stria terminalis, medial anterior vs.** | | | | | | | |
| lateral ant. hypothalamus | .258 | .128 | | .572 | | -.315 | C/R; **C/V** |
| lateral hypothalamus | .130 | .089 | | .525 | | -.174 | **C/V** |
| medial preoptic area | .077 | .264 | | .517 | C/R | .254 | |
| suprachiasmatic nucleus | .353 | .101 | | .570 | | -.088 | C/V |
| central amygdala | .260 | -.130 | | .512 | | .022 | C/V |
| ventral tegmental area | .135 | .406 | | .487 | | -.060 | C/V |
| **Bed n. of stria terminalis, lateral vs.** | | | | | | | |
| lateral ant. hypothalamus | .189 | .148 | | .605 | C/R | .006 | C/V |
| suprachiasmatic nucleus | .322 | .091 | | .568 | | .059 | C/V |
| ventral tegmental area | .125 | .458 | | .5871 | C/R | .027 | C/V |

| | Control/ Resilient | Stress/ Resilient | | Control/ Vulnerable | | Stress/ Vulnerable | |
|---|---|---|---|---|---|---|---|
| | r | r | | r | | r | |
| **Bed n. of stria terminalis, ventral vs.** | | | | | | | |
| lateral ant. hypothalamus | .473 | .204 | | .562 | | -.106 | C/V |
| lateral hypothalamus | .321 | .232 | | .597 | | -.026 | C/V |
| suprachiasmatic nucleus | .570 | .196 | | .609 | | .078 | C/R; C/V |
| ventral tegmental area | .096 | .387 | | .550 | C/R | -.054 | C/V |
| **Central amygdala vs.** | | | | | | | |
| lateral hypothalamus | .620 | .179 | C/R | .522 | | .459 | |
| **Cortical amygdala vs.** | | | | | | | |
| suprachiasmatic nucleus | .627 | .198 | C/R | .611 | | .354 | |
| **Cingulate-thalamic-hippocampal circuit** | Control/ Resilient | Stress/ Resilient | | Control/ Vulnerable | | Stress/ Vulnerable | |
| | r | r | | r | | r | |
| **Prelimbic cortex vs.** | | | | | | | |
| anterior thalamus, ventrolateral | .479 | .207 | | .431 | | -.065 | C/R; C/V |
| anterior thalamus, dorsomedial | .417 | .247 | | .366 | | -.117 | C/R |
| dentate gyrus | .288 | .321 | | .454 | | -.133 | C/V |
| retrosplenial cortex, granular | .534 | .381 | | .418 | | -.10 | C/R; C/V |
| retrosplenial cortex, agranular | .531 | .162 | | .494 | | .011 | C/R; C/V |
| **Anterior cingulate vs.** | | | | | | | |
| reticular thalamus | .591 | .110 | C/R | .224 | | .453 | |
| retrosplenial cortex, granular | .584 | .542 | | .537 | | -.139 | C/R |
| retrosplenial cortex, agranular | .452 | .332 | | .692 | | -.062 | C/R; C/V |
| **Hippocampal CA3 vs.** | | | | | | | |
| retrosplenial cortex, granular | .485 | .490 | | .726 | | .321 | C/V |
| **Intra-hippocampal circuit** | Control/ Resilient | Stress/ Resilient | | Control/ Vulnerable | | Stress/ Vulnerable | |
| | r | r | | r | | r | |
| **Hippocampal CA1 vs.** | | | | | | | |
| CA3 | .847 | .738 | | .770 | | .609 | C/R |
| **Mesolimbic dopamine circuit** | Control/ Resilient | Stress/ Resilient | | Control/ Vulnerable | | Stress/ Vulnerable | |
| | r | r | | r | | r | |
| **Ventral tegmental area vs**. | | | | | | | |
| anterior cingulate cortex | .401 | .103 | | .508 | | -.021 | C/V |
| CA1 | .252 | .210 | | .589 | | .703 | C/R; S/R |
| CA2 | .336 | .123 | | .549 | | .728 | C/R; S/R |
| bed n. of stria terminalis, medial anterior | see extended amygdala network above | | | | | | |
| bed n. of stria terminalis, lateral | see extended amygdala network above | | | | | | |
| bed n. of stria terminalis, ventral | see extended amygdala network above | | | | | | |
| lateral hypothalamus | see habenular circuit above | | | | | | |

For the presented analyses the network was delineated after Hikosaka (2010) and included the input and target regions of habenula: lateral and medial septum, horizontal and vertical nuclei of the diagonal band, lateroanterior hypothalamic nucleus, lateral hypothalamic area, ventral and dorsolateral caudate putamen, globus pallidus, substantia nigra reticularis and compacta, ventral tegmental area, interpeduncular nuclei, median and dorsal raphe. The most prominent pattern of results in this circuit is the impact of chronic stress on regional co-activation in vulnerable animals. Moderately high positive correlations in vulnerable rats between substantia nigra vs. nucleus of the diagonal band, lateral hypothalamus vs. ventral tegmental area, and globus pallidus vs. habenula were abolished by stress. Negative correlations between medial septum and raphe nuclei in vulnerable animals were reversed by stress. Positive correlations between lateral hypothalamus vs. habenula, substantia nigra, ventral tegmentum and interpeduncular nuclei were reversed to negative after stress. Connectivity of dorsal raphe with both nucleus of the diagonal band and globus pallidus was the lowest in resilient controls and the highest in stressed and vulnerable animals. The connection between interpeduncular nuclei and substantia nigra was abolished by chronic stress whereas stress increased the correlation between cytochrome oxidase activities in interpeduncular nuclei and medial septum. Median raphe had a higher negative correlation with lateral septum in vulnerable animals, and this association was abolished by stress. The connectivity of median raphe with interpeduncular nuclei was, conversely, high in control/resilient rats, but nonexistent in vulnerable rats.

The connectivity patterns of the neural circuits of the extended amygdala and brain regions controlling the stress reaction were also assessed. Analysed regions included the basolateral, central, medial and cortical amygdala, medial anterior, ventral and lateral bed nuclei of the stria terminalis, lateroanterior hypothalamic nucleus, lateral hypothalamic area, ventral tegmental area, locus coeruleus, dorsal, latero-ventral and latero-dorsal periaqueductal gray (Davis et al., 2009; Ziegler and Herman, 2002). The main finding for this network was also the stress-sensitivity of regional connectivity in vulnerable animals. The connections between bed nuclei of stria terminalis with lateral and suprachiasmatic hypothalamus, central amygdala and ventral tegmental area were the highest in vulnerable animals, and were abolished by chronic stress. The correlations of central amygdala to lateral hypothalamus and cortical amygdala to suprachiasmatic nucleus were highest in resilient control animals but abolished by stress.

Analysis of the network of cingulate cortices, thalamic regions and hippocampus included such areas as the ventrolateral and dorsomedial parts of the anteroventral thalamus, anterodorsal and reticular thalamus, prelimbic cortex, anterior cingulate cortex, agranular and granular retrosplenial cortex, hippocampal CA1, CA2, CA3 and dentate gyrus (Aggleton et al., 2010; Gonzalo-Ruiz and Lieberman, 1995). Relatively high correlations between prelimbic cortex and hippocampus with anterior thalamic and posterior cingulate areas were seen in both resilient and vulnerable controls compared to stressed and

vulnerable rats. The moderately high correlation between anterior cingulate cortex and reticular thalamus was abolished by stress. The correlation between hippocampal CA1 and CA3 regions was the highest in resilient controls and was decreased in stressed and vulnerable animals.

The correlations of the target areas of the mesolimbic dopamine system with ventral tegmental area were also considered, including the lateroanterior hypothalamic nucleus, lateral hypothalamic area, lateral septum, medial anterior, ventral and lateral bed nuclei of the stria terminalis, medial and central amygdala, hippocampal CA1, CA2, CA3 and dentate gyrus, nucleus accumbens shell and core and ventral pallidum (Alcaro et al., 2007). Metabolic activity in ventral tegmental area was moderately correlated with the activity of anterior cingulate in vulnerable animals, but this association was completely abolished by stress. The highest co-activation of hippocampal CA1 and CA2 with the ventral tegmental area was induced by the application of stress in vulnerable animals. The co-activity pattern of ventral tegmentum with bed nuclei of the stria terminalis was described above in the paragraph on the extended amygdala connectivity.

Analysis of the connectivity of brain regions forming functional networks suggests that several basic behavioural functions may be different in animals with higher vulnerability, and affected by chronic stress. The behavioural systems that are altered include the endocrine stress response, fear and anxiety, learning and motivated behaviour. Connectivity of hypothalamic and basal forebrain with midbrain nuclei via habenula is more reactive to chronic stress in vulnerable animals. Hyperactive habenular function may lead to serotonergic and dopaminergic dysfunction, as inhibition of habenular electrical activity alleviates depression (Bianco and Wilson, 2009; Sartorius and Henn, 2007; Shumake et al., 2003). The connectivity of bed nuclei of the stria terminals to its afferent and efferent structures is also stress responsive in vulnerable animals, and this can lead to dysfunctions in the activation of the mesolimbic motivational circuit and endocrine stress response (Alcaro et al., 2007; Ziegler and Herman, 2002). Loss of connectivity due to chronic stress in the prelimbic-cingulate-thalamic-hippocampal circuit suggests that learning difficulties in depression may arise from the dysfunction of this network (Aggleton et al., 2010; Pothuizen et al., 2010). Functional connectivity between ventral tegmental area and hippocampus was increased in vulnerable/stressed animals. Stress is known to increase drug-seeking and consummatory behaviours and the dynamics of dependence development, and the involvement of hippocampus in drug intake/seeking via context driven motivation processes has been demonstrated (Luo et al., 2011; Miczek et al., 2008; Schwabe et al., 2011; Sinha, 2008).

In conclusion, functional connectivity with other brain regions was low in olfactory system of vulnerable rats, in bed nucleus of stria terminalis of chronically stressed rats, and in frontal cortex of stressed and vulnerable rats. Inter-connectivity between brain regions across the whole brain was the highest in vulnerable rats and lowest in stressed animals.

## 4.4. Serotonergic lesion attenuates stress-induced hyperthermia in rats submitted to chronic variable stress

In **Study III** rats with partial serotonergic lesion, induced by para-chloroamphetamine (2 mg/kg), were submitted to chronic variable stress and their baseline body temperature and stress-induced hyperthermia were measured after parachloroamphetamine administration, and before and after chronic variable stress. During each session body temperature was measured three times: the first measurement (T0) was followed by the second 30 minutes later (T30) and the final measurement took place two hours after the first (T120).

Parachloroamphetamine (PCA) administration induced a large increase in body temperature (PCA main effect, $F(1, 39)=21.4$, $p<0.0001$; repeated measurement, $F(2, 39)=102.1$, $p<0.0001$, repeated measurement x PCA, $F(2, 39)=48.0$, $p<0.0001$). Thirty minutes after parachloroamphetamine administration (T30) body temperature had increased due to injection and measurement procedures, as both groups displayed a similar increase from T0 to T30 (T0 36.65±0.08 and T30 37.59±0.08 for PCA-treated rats; and T0 36.49±0.08 and T30 37.94±0.08 for control rats; $p<0.0001$), although control rats had slightly higher body temperature ($p<0.05$). Hyperthermia at T120 was induced by parachloroamphetamine as the treated group showed an increase, whereas controls a decrease from T30 to T120 (T120 38.68±0.15 for PCA-treated rats and 37.1±0.15 for control animals; $p<0.0001$), resulting in a large difference in body temperature between parachloroamphetamine and control animals at T120 ($p<0.0001$). Control animals, though, did not reach baseline levels after 120 minutes of recovery.

Stress-induced hyperthermia measurement before the chronic variable stress regimen indicated equal body temperature changes in all groups (repeated measurement $F(2, 39)=136.1$, $p<0.0001$). There was a steep increase from T0 to T30 (T0 36.54±0.12; T30 37.69±0.12; T120 37.03±0.11, average of all groups; $p<0.0001$) and a large decrease from T30 to T120 ($p<0.0005$), though still not reaching baseline levels. Stress-induced hyperthermia measurement after chronic variable stress (CVS) showed an increase in body temperature from T0 to T30 and a decrease from T30 to T120 in all groups (CVS main effect $F(1, 39)=10.5$, $p<0.005$; repeated measurement, $F(2, 39)=151.3$, $p<0.0001$; repeated measurement x PCA, $F(2, 39)=6.2$, $p<0.005$; **Figure 10A**). Body temperature was still higher than baseline at T120 in all groups, except for chronically stressed animals with serotonergic lesions. Serotonergic deficiency and chronic variable stress led to higher baseline body temperature at T0 compared to control rats with intact serotonergic system. At T30 serotonergic lesions decreased stress-induced hyperthermia in chronically stressed rats, and chronic stress increased stress-induced hyperthermia in animals with intact serotonergic system. At T120 chronic stress still caused higher body temperature in rats with intact serotonergic terminals. When analysing percentual change, serotonergic denervation in chronically stressed rats increased baseline body temperature (T0) compared to both controls with intact

and deficient serotonin systems (CVS main effect, $F(1, 39)=12.7$; $p<0.001$; **Figure 10B**). At T30 chronic stress increased stress-induced hyperthermia in animals with intact serotonin system.

Parachloroamphetamine-induced vast increase in body temperature is consistent with previously reported results, though the temporal dynamic is different probably due to dose used (Colado et al., 1997). Thirty minutes after administration of vehicle or parachloroamphetamine, the vehicle-treated rats had higher body temperature. It can be hypothesised that at that time serotonin levels had not yet reached the levels to induce hyperthermia, but the higher body temperature in controls remains unexplained at the moment.

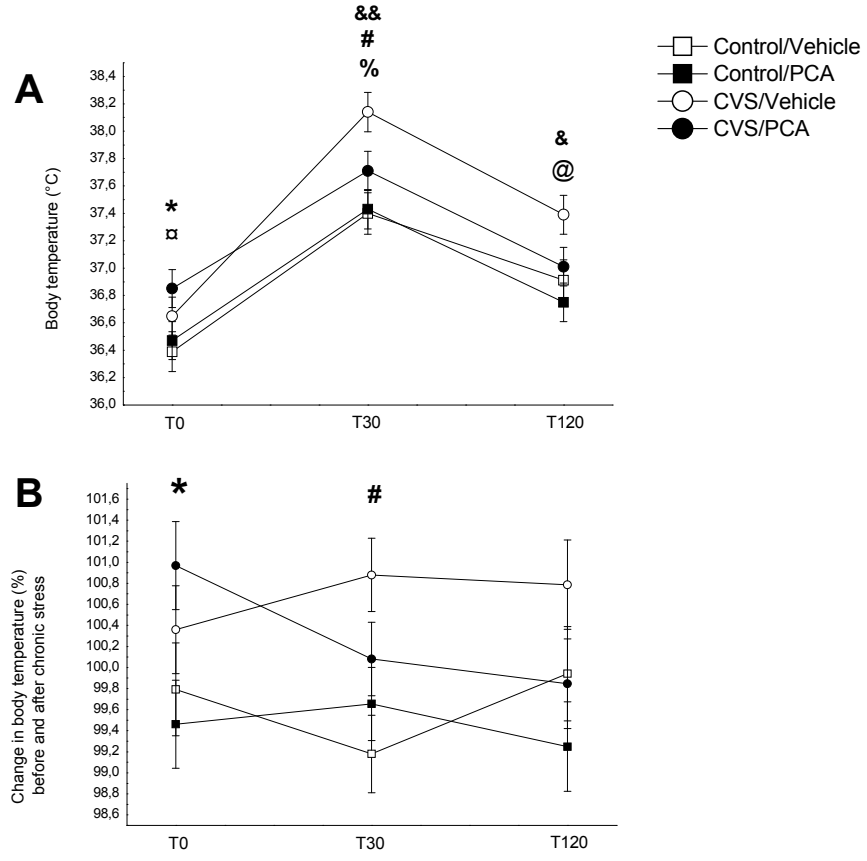


**Figure 10.** Body temperature and stress-induced hyperthermia in rats with parachloroamphetamine (PCA) induced serotonergic lesions submitted to chronic variable stress (CVS). **A** – Body temperature and stress-induced hyperthermia after chronic variable stress ¤ $p<0.0001$ T0 vs. T30; % $p<0.0005$ T30 vs. T 120; @ $p<0.05$ all groups except CVS/PCA at T0 vs. T120; * $p<0.05$ CVS/PCA vs. Control/Vehicle; # $p<0.05$ CVS/PCA vs. CVS/Vehicle; && $p<0.001$ CVS/Vehicle vs. Control/Vehicle; & $p<0.05$ CVS/Vehicle vs. Control/Vehicle. **B –** Percentual change in body temperature and stress-induced hyperthermia before and after chronic variable stress in rats treated with parachloroamphetamine (PCA). * $p<0.05$ CVS/PCA vs. Control/PCA and Control/Vehicle; # $p<0.01$ Control/Vehicle vs. CVS/Vehicle

Chronic stress increased basal body temperature in serotonin-deficient rats. Baseline levels of body temperature are proposed to reflect a depression-like state whereas stress-induced hyperthermia is considered to be an index of anxiety, the two mechanisms having a separate neural substrate (Hayashida et al., 2010; Olivier et al., 2008a). In accordance with our results, higher baseline body temperature has been demonstrated in rodents subjected to chronic social stress and olfactory bulbectomy (Bhatnagar et al., 2006; Hayashida et al., 2010; Keeney et al., 2001; Vinkers et al., 2009a). Interestingly chronic emotional stress can have a more pronounced effect than physical stress – witnessing another rats get foot-shocked for twelwe weeks increased baseline body temperature three months after the stress regimen ended, whereas foot-shock itself did not (Endo and Shiraki, 2000). Serotonin transporter knock-out mice, that have increased intra- and extracellular serotonin levels and behave anxiously, have higher basal body temperature (Holmes et al., 2003). This is at odds with our results, as these mice have increased forebrain serotonin levels whereas in our study the combination of decreased serotonin levels and chronic stress increased baseline body-temperature.

Stress-induced hyperthermia after chronic stress was best expressed in rats with intact serotonergic system, and the body-temperature remained elevated throughout the recovery period. This is in accord with the reports that rats subjected to repeated social stress displayed an increase in stress-induced hyperthermia induced by acute restraint and rats subjected to a week of chronic restraint prenatally displayed a steeper and more enduring body temperature elevation in response to acute social stress (Bhatnagar et al., 2006; Rimondini et al., 2003). Juvenile repeated stress of short duration did not cause changes in stress-induced hyperthermia in response to acute social defeat stress in adulthood, but when exposed to the acute stressor the second time, the juvenile stress group exhibited higher thermal reactivity probably because the animals had developed an anticipation to defeat (Yee et al., 2011). Interestingly, though chronic stress can cause anxious behaviour and potentiate stress-induced hyperthermia, several drugs with known anxiogenic effect in behavioural tests cannot increase stress-induced hyperthermia, probably because of the multi-factorial nature of anxiety (Houtepen et al., 2011).

Serotonin transporter knock-out rats have increased serotonin content and extracellular serotonin, anxious and depression-like behavioural phenotype, and in response to acute stress they display reduced stress-induced hyperthermia (Homberg et al., 2007; Olivier et al., 2008a; Olivier et al., 2008b). The latter finding is in conflict with the results presented here, showing that lower cerebral serotonin levels in rats with serotonergic lesions result in less pronounced hyperthermia – though only in chronically stressed animals. On the other hand, serotonin transporter knock-out rats have extremely high levels of serotonin, nine-fold increase compared to wild-types, and cannot probably be compared with “normal” rats with intact serotonin system (Homberg et al., 2007). Contrary to the presented results, it has been shown that serotonergic depletion by 5,7-dihydroxtryptamine renders the animals thermally more reactive to

repeated social defeat (Chung et al., 1999b). One major methodological difference between the latter and our study is the choice of acute stressor to induce stress-induced hyperthermia. In our study handling during temperature measurement induced hyperthermia, whereas Chung and colleagues measured temperature telemetrically during ten social defeat sessions (Chung et al., 1999b). Social defeat is presumably the most potent stressor in rodents, and this stressor was presented repeatedly, thus creating a strong anticipatory reaction (Chung et al., 1999b; Tornatzky and Miczek, 1994; Yee et al., 2011).

Conclusively, chronic variable stress and partial serotonergic denervation induce a depression-like state, as characterised by increased baseline body-temperature, and serotonergic lesion attenuates the stress-induced hyperthermia after chronic stress.

## 4.5. Chronic stress increases adrenal gland weight and endocrine stress response but does not affect baseline corticosterone levels

In these experiments (**Paper II and Study II**) two chronic stress paradigms were utilised and their impact on adrenal weight, plasma corticosterone levels and acute stress-induced corticosterone release was assessed. Furthermore, chronic social stress was applied to rats with high or low levels of hedonia, as measured by sucrose consumption, and chronic variable stress was used on animals with partial serotonergic lesions for detecting any eventual stress-diathesis effects.

The average weight of right and left adrenal glands increased in response to chronic social stress. This increase was derived from differences between low sucrose consuming animals, and it should be noted that control HS-rats tended to have higher adrenal weight than control LS-rats (**Figure 11A**). On the other

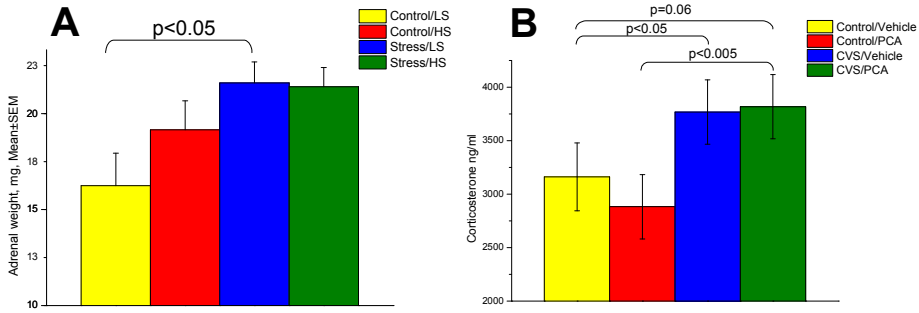


**Figure 11.** **A –** Adrenal weight (mg, mean±SEM) in response to chronic social defeat stress (CSS) in rats with high and low sucrose intake (HS and LS). **B** – Acute stress-induced plasma corticosterone levels (ng/ml) in rats with PCA induced serotonergic lesions submitted to chronic variable stress (CVS). For group-wise comparisons LSD post hoc test was used.

hand, basal corticosterone levels measured in the same animals did not reveal any impact of stress. Basal corticosterone levels were also unresponsive to chronic variable stress and partial serotonergic lesioning. Serotonergic lesions and chronic variable stress exaggerated the endocrine stress response, though: The increase in corticosterone levels caused by acute stress (15 minute-stay on an elevated platform) was larger in rats with previous chronic stress exposure. This effect was more clearly expressed in animals with serotonergic lesions (acute stress main effect $F(1, 85)=232.0$; $p<0.0001$; chronic stress main effect $F(1, 85)=7.21$; $p<0.01$; acute stress x chronic stress $F(1, 85)=4.22$; $p<0.05$ **Figure 11B**).

Elevated glucocorticoid levels and enlarged adrenals are, in both humans and animal models, considered a hallmark of depression and a mechanism by which stress can exert its harm to the organism (Gao and Bao, 2011; McEwen and Wingfield, 2003; Ulrich-Lai et al., 2006). In the present study we found that chronic stress regimens that induced a large decrease in body weight gain and caused anxious/depression-like behaviour did not increase baseline corticosterone levels. Furthermore, we could dissociate the impact of chronic stress on different measures of the condition of the endocrine system. In case of chronic social defeat we observed a dissociation between adrenal enlargement and corticosterone levels. Nevertheless, in the study of the effects of acute stress and chronic variable stress we found that baseline levels of corticosterone were not predictive of acute stress response as only the latter was increased after chronic stress. Hyperplasia and hypertrophy of adrenal glands induced by HPA axis hyperactivity generally leads to higher adrenal corticosterone secretion (Ulrich-Lai et al., 2006). Thus it could be that in the developmental phase of the model the HPA axis was more active and induced adrenal weight gain, but by the time of measurement, HPA hyperactivity had ceased. Because social stress is not subject to habituation this scenario is not very likely (Koolhaas et al., 2011). It is also known that corticosteroid levels may be increased phasically while the tonic levels are stable. During stress adrenals are more active and possibly fifteen one-hour sessions had been sufficient to produce a gain in adrenal weight. Chronic stress causes a dysregulation in adrenal activity (e.g., lack of feedback inhibition), thus acute stress leads to enhanced and prolonged glucocorticoid secretion. Though glucocorticoid levels eventually decrease to baseline levels, the prolonged and enhanced acute endocrine activation could lead to increased adrenal growth (Koolhaas et al., 2011; McEwen, 2007). Since the HPA hyper-reactivity after chronic variable stress was present, but baseline levels were not increased, it can be hypothesised that dysregulation and more frequent activation of HPA axis is among the mechanisms that lead to adrenal growth.

Working with methods that measure cerebral long-term activity we have mapped regional activation in several depression models and in none of them have we ever registered a persistent increase in oxidative metabolism in paraventricular hypothalamus, the main regulatory region of endocrine stress response (Paper I; Paper II; Mällo et al., 2009). It must be noted, though, that

cerebral energy mapping of congenital helplessness did reveal increased paraventricular activity as do most studies measuring short-term activation after acute stress (Shumake et al., 2001).

Thus, in chronically stressed animals baseline endocrine activity remained unchanged, but adrenal weight gain suggested that adrenal function had at some point been elevated, and acute endocrine stress reaction was more potent.

# 5. General summary

This doctoral dissertation focuses on the functional neuroanatomy and behaviour in animal models of affective disorders. Depression is caused by stressful life events, by inherent individual vulnerability, and most potently by a combination of these two factors. Thus modelling depression in a laboratory should follow the same aetiopathological principle. Vulnerability to depression can be modelled in rats by selection in behavioural tests for a trait related to low affect or by experimentally producing a putative neurobiological state that serves as the substrate of depression e.g., a weak serotonergic system. In this dissertation four vulnerability phenotypes were studied: partial serotonergic denervation mimics the deficits of the serotonin system found in depressed patients; neonatal maternal separation mimics the impact of adverse developmental conditions; low expression of the sociability trait focuses on the inhibited personal relations often seen in depression; low sucrose consumption trait represents a low hedonic trait of the animal, as anhedonia is one of the core symptoms of depression and most commonly measured in animal models by measuring sucrose intake. In order to induce environmental stress two paradigms were utilised. The depressogenic effect of chronic variable stress was attained by repeated administration of several mildly noxious stimuli to the animal. The chronic social stress method is based on the resident-intruder paradigm – one rat is placed repeatedly in the home cage of a bigger and more aggressive animal that will protect its territory and defeat the intruder rat. In order to construct a stress-diathesis model chronic stress was applied on rats with a vulnerability phenotype. Rats with higher baseline sucrose intake, i.e. with higher levels of the hedonic trait, were more susceptible to chronic social stress, as indicated by more passive coping in the forced swimming test. Behavioural responses in other models had been revealed in previous studies. To detect regional cerebral long-term neural activation the function of mitochondrial electron transport chain was assessed via cytochrome c oxidase histochemistry. Cytochrome c oxidase is the rate-limiting enzyme of the mitochondrial respiratory chain and the long-term energy demand of neurons determines the expression levels of cytochrome oxidase. All the vulnerability phenotypes and both chronic stress regimens caused a change in long-term neuronal activity on their own in specific brain regions, but there was minimal overlap between the regional activity patterns in different models. When chronic stressors were applied in combination with vulnerability factors, commonalities between different models in regional brain activity arose. Chronic variable stress with partial serotonergic denervation and chronic social stress in rats with high and low levels of hedonic trait, caused a change in metabolic activity in anterior thalamus, hippocampal CA3 area and medial amygdala, areas crucially involved in stress-response, fear and learning. When cytochrome oxidase activity data from all models was collapsed and analysed jointly, several regions emerged, where vulnerability and stress caused an activation common to all models. Vulnerability increased energy metabolism in

two inter-connected brain regions, retrosplenial cortex and reticular thalamus, and stress reversed this activation. Energy metabolism in habenula decreased due to chronic stress and in median raphe cytochrome oxidase activity was increased by the combination of stress and vulnerability. Since brain regions can be involved in multiple functions via different circuits, functional connectivity analysis was conducted to reveal the possible neural networks underlying depression. Vulnerability to depression was foremost associated with a loss of connectivity between olfactory bulbi and central amygdala from the rest of the brain regions. Chronic stress caused a decrease in connectivity between bed nucleus of stria terminalis from other nuclei. Chronic stress, when applied to rats with a vulnerability phenotype, resulted in attenuated connectivity between prefrontal and both anterior and posterior cingulate cortices from other brain regions. Thus this work has indicated several new anatomical targets for more detailed study, for example the highly interconnected nuclei of anterior thalamus, retrosplenial cortex, anterior cingulate, hippocampus and reticular thalamus.

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# SUMMARY IN ESTONIAN

## Indiviididevahelised erinevused depressioonisoodumuses: aju regionaalne energiametabolism loomkatsemudelites, serotoniinisüsteemi talitlus ja käitumine

Käesolev doktoriväitekiri keskendub depressiooni funktsionaalse neuroanatoomia ja depressiivse käitumise uurimisele kasutades meeleoluhäirete loomkatsemudeleid. Depressiooni põhjustavad nii korduvad stressirikkad elusündmused kui ka kaasasündinud individuaalne soodumus ja eelkõige nende kahe teguri koosmõju. Depressiooni mudeldamiseks laboritingimustes peaks lähtuma nendest samadest etiopatogeneetilistest teguritest. Depressioonisoodumus saab mudeldada selekteerides rotte afektiivse käitumise testide alusel või pikaajaliselt mõjustades nende ajus depressiooni tõenäolisi neuraalseid alusmehhanisme, näiteks kahjustades serotoniinisüsteemi. Käesolevas doktoriväitekirjas käsitletakse nelja depressioonisoodumuse mudelit, mis kajastavad erinevaid depressiooniga seonduvaid väärtalitlusi ajukeemias, mis omakorda ilmnevad käitumises. Need mudelid on: i) osaline serotonergiline närvikahjustus, mis mudeldab serotoniinsüsteemi puudulikkust; ii) vastsündinueas emahoolest ilmajätmine, mille läbi mudeldatakse ebasoodsa arengukeskkonna mõju; iii) vähene püsisotsiaalsus, mis iseloomustab depressiooniga kaasnevat vähenenud suhtlust; iv) püsi-magusatarbimine, mis mudeldab anhedooniat, ühte depressiooni keskset sümptomit. Keskkonnast tuleneva stressi mudeldamiseks kasutati käesolevas töös kaht kroonilise stressi paradigmat. Kroonilise muutliku stressi depressiooni-põhjustav mõju rajaneb mitmete mõõdukalt ebameeldivate stiimulite korduval esitamisel. Kroonilise sotsiaalse stressi meetod rajaneb peremeessissetungija paradigmal – rott asetatakse korduvalt suurema ja agressiivsema liigikaaslase kodupuuri, misjärel viimane hakkab oma eluala kaitsma ja alistab sissetungija. Stressi-diateesi mudeli saamiseks rakendati kroonilist stressi depressioonisoodumusega loomadele. Ajupiirkondade pikaajalise aktivatsiooni määramiseks hinnati mitokondriaalse elektronide transpordi-ahela talitlust tsütokroom c oksüdaasi histokeemilise mõõtmise kaudu. Tsütokroom c oksüdaas on mitokondriaalse hingamisahela reaktsioonkiirust määrav ensüüm, selle ensüümi aktiivsuse määrab närviraku pikaajaline aktivatsioonitase. Kõik depressioonisoodumuse ja ka kroonilise stressi mudelid eraldiseisvana põhjustasid mõnedes ajupiirkondades muutuse pikaajalises närviaktiivsuses, kuid erinevate mudelite närviaktiivsuse piirkondlikud aktivatsioonimustrid ei kattunud kuigi hästi. Kui kroonilist stressi rakendati haavatavuse-fenotüübiga loomadele, ilmnesid mudelitevahelised kokkulangevused aju piirkondlikus aktivatsioonis. Krooniline muutlik stress koos osalise serotonergilise närvikahjustusega ja krooniline sotsiaalne stress kutsusid esile muutuse energiametabolismis eesmises taalamuses, hippokampuse CA3 alas ja mediaalses mandelkehas, s.o. piirkondades, mis on keskel kohal organismi stressivastuse, õppimise ja hirmuga seotud käitumiste kontrollis. Kui tsütokroom c oksüdaasi aktiivsuse andmed erinevatest katsetest kokku koguti ja koos analüüsiti, ilmnes mitmeid piirkondi, kus depressioonisoodumus ja krooniline stress põhjustasid muutuse

kõiki mudeleid arvestades. Depresioonisoodumusega rottidel oli närvitegevus aktiivsem kahes tugeva vastastikuse innervatsiooniga piirkonnas – retrospleniaalses ajukoores ja retikulaarses taalamuses, krooniline stress aga taandas selle aktiivsuse kontroll-loomadega samale tasemele. Krooniline stress vähendas pikaajalist närviaktiivsust habenulas ning kroonilise stressi ja haavatavuse kombinatsioon viis energia-metabolismi tõusule mediaanses raphe-tuumas. Kuna ajupiirkonnad võivad osaleda erinevates funktsioonides eri närviringete kaudu, viidi läbi piirkondadevahelistel korrelatsioonidel rajanev funktsionaalse ühenduvuse analüüs, et leida võimalikke depressiooni vahendavaid närviringeid. Depressioonisoodumus seostus eelkõige ühenduste kaoga haistesibulate ja tsentraalse mandelkeha ning ülejäänud ajupiirkondade vahel. Krooniline stress põhjustas ühenduvuse langust terminaaljuti sängituumade ja ülejäänud ajupiirkondade vahel. Depressioonisoodumuse taustal vähendas krooniline stress ühenduvust prefrontaalkoore, eesmise ja tagumise vöökääru ja ülejäänud ajupiirkondade vahel. Käesolev töö tõi välja mitmeid ajupiirkondi, mida tasuks täpsemalt edasi uurida, nagu näiteks võrgustik, mis hõlmab eesmist taalamust, retrospleniaalset ajukoort, eesmist vöökääru, hippokampust ja retikulaarset taalamust.

# PUBLICATIONS

# CURRICULUM VITAE

## Margus Kanarik

Date and place of birth: 3. april, 1976. a., Tartu
Citizenship: Estonian
Contact: Ravila 14a, 50411, Tartu, Estonia (Department of Psychology, University of Tartu, psychophysiology laboratory)
Tel.: 737 6660
*e-mail*: Margus.Kanarik@ut.ee

### Current position

Research fellow, University of Tartu, Faculty of Social Sciences and Education, Department of Psychology

### Education

| | |
|---|---|
| 1983–1986 | Tallinn, Secondary school No. 44 |
| 1986–1994 | Tallinn, Secondary school No. 7 |
| 1999–2000 | University of Tartu |
| 2000–2005 | University of Tartu, BSc in psychology |
| 2005–2007 | University of Tartu, MSc in psychology |
| Since 2007 | University of Tartu, PhD student in psychology |

### Employment

| | |
|---|---|
| 1994–1995 | Expodesign, artist/artisan |
| 1995–1996 | Journal „Kroonika”, lay-out editor |
| 1996–1997 | Grafix, artist/artisan |
| 1998–2000 | theatre „Old Town Studio”, stagehand, props manager, stage manager |
| 2000 summer | Wismari hospital, attendant |
| 2002 | Psychiatric hospital, attendant |
| 2004–2005 | Tartu Descartes Lyceum, psychology teacher |
| 2005–2007 | University of Tartu, Faculty of Social Sciences and Education, Department of Psychology, laboratory technician |
| Since 2007 | University of Tartu, Faculty of Social Sciences and Education, Department of Psychology, research fellow |

| | |
|---|---|
| Since 2011 | Associate member of European College of Neuropsychopharmacology (ECNP) |

# ELULOOKIRJELDUS

## Margus Kanarik

Sünniaeg ja koht: 3. aprill, 1976. a., Tartu
Kodakondsus: Eesti
Kontakt: Ravila 14a, 50411, Tartu, Eesti (psühholoogia instituut, psühhofüsioloogia osakond)
Tel.: 737 6660
*e-mail*: Margus.Kanarik@ut.ee

### Praegune töökoht

Teadur, Tartu Ülikool, Sotsiaal- ja haridusteaduskond, psühholoogia istituut, psühhofüsioloogia osakond

### Haridus

| | |
|---|---|
| 1983–1986 | Tallinna 44. Keskkool |
| 1986–1994 | Tallinna 7. Keskkool |
| 1999–2000 | Tartu Ülikool, AÜ, psühholoogia eriala bakalaureuseõpe |
| 2000–2005 | Tartu Ülikool, psühholoogia eriala bakalaureuseõpe |
| 2005–2007 | Tartu Ülikool, psühholoogia eriala teadusmagistriõpe |
| Alates 2007 | Tartu Ülikool, psühholoogia eriala doktoriõpe |

### Teenistuskäik

| | |
|---|---|
| 1994–1995 | Expodesign, kunstnik-teostaja |
| 1995–1996 | ajakiri „Kroonika”, küljendaja |
| 1996–1997 | Grafix, kunstnik-teostaja |
| 1998–2000 | teater „Vanalinnastuudio”, lavameister, rekvisiitor, inspitsient |
| 2000 suvi | Wismari haigla, hooldaja |
| 2002 | TÜK, Psühhiaatriakliinik, lasteosakond, hooldaja |
| 2004–2005 | Tartu Descartes’i Lütseum, psühholoogiaõpetaja |
| 2005–2007 | Tartu Ülikool, Sotsiaal- ja haridusteaduskond, psühholoogia instituut, laborant |
| Alates 2007 | Tartu Ülikool, Sotsiaal- ja haridusteaduskond, psühholoogia instituut, teadur |
| Alates 2011 | Euroopa Neuropsühhofarmakoloogia Kolledži (European College of Neuropsychopharmacology, ECNP) vaatlejaliige |

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