DISSERTATIONES PSYCHOLOGICAE UNIVERSITATIS TARTUENSIS
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TANEL MÄLLO

Exploratory behaviour and 50-kHz ultrasonic vocalizations in rats: behavioural and neurochemical profiles of persistent inter-individual differences

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"The whole business with the cheese and the squaking is just a front."
The old man paused, and with a sympathetic frown continued.
"They've been experimenting on you, I'm afraid."

Douglas Adams
The Hitchhiker's Guide to the Galaxy

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LIST OF ORIGINAL PUBLICATIONS

I Mällo T., Alttoa A., Kõiv K., Tõnissaar M., Eller M., Harro J. (2007). Rats with persistently low or high exploratory activity: behaviour in tests of anxiety and depression, and extracellular levels of dopamine. *Behavioural Brain Research*, 177: 269–81.

II Mällo T., Kõiv K., Koppel I., Raudkivi K., Uustare A., Rinken A., Timmusk T., Harro J. (2008). Regulation of extracellular serotonin levels and brain-derived neurotrophic factor in rats with high and low exploratory activity. *Brain Research*, 1194: 110–7.

III Mällo T., Berggård C., Eller M., Damberg M., Oreland L., Harro J. (2004). Effect of long-term blockade of CRF(1) receptors on exploratory behaviour, monoamines and transcription factor AP-2. *Pharmacology Biochemistry and Behavior*, 77: 855–65

IV Mällo T., Harro J. Effect of long-term blockade of CRF_1 receptors on rats with high or low spontaneous exploratory activity. (*submitted for publication)*

V Mällo T., Matrov D., Herm L., Kõiv K., Eller M., Rinken A., Harro J. (2007). Tickling-induced 50-kHz ultrasonic vocalization is individually stable and predicts behaviour in tests of anxiety and depression in rats. *Behavioural Brain Research*, 184: 57–71.

VI Mällo T., Matrov D., Kõiv K., Harro J. Effects of chronic variable stress on behaviour and cerebral oxidative metabolism in rats with high or low levels of 50-kHz ultrasonic vocalizations. *(submitted for publication)*

The author of the present dissertation contributed to all individual publications presented herein as the main author, creating experimental designs, carrying out most of the experimental work and preparing the publications.

ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
5-HTT	serotonin transporter
BDNF	brain-derived neurotrophic factor
COX	cytochrome c oxidase
CRF	corticotropin-releasing factor
CVS	chronic variable stress
DA	dopamine
DG	dentate gyrus
DOPAC	3,4-dihydroxyphenylacetic acid
DSP-4	[N(2-chloroethyl)-N-ethyl-2-bromobenzylamine]
HC	high chirping
HE	high exploratory activity
HPA	hypothalamic-pituitary-adrenal axis
HPLC	high performance liquid chromatography
HVA	homovanillic acid
i.p.	intraperitoneal
kHz	kilohertz
LC	low chirping
LE	low exploratory activity
FM	frequency modulated
NA	noradrenaline
NGF	nerve growth factor
PCA	parachloroamphetamine
PFC	prefrontal cortex
USV	ultrasonic vocalization

1. INTRODUCTION

1.1. Affective states and disorders

The concept of „affect“ labels the subjective experience of feeling or emotion and relates to the organism's reactions to and interactions with environmental stimuli. Affective states occur before (both evolutionally as well as in individuals) higher cognitive processes necessary for more elaborate emotions (Zajonc, 1980), although some basic perceptual and cognitive encoding is naturally necessary for the analysis of the stimuli. In the widest approach, it is possible to classify all affects according to two-dimensional Cartesian planes, with an approach-avoidance or good-bad valence dimension and an arousal dimension with "energized" and "enervated" at opposites (Burgdorf and Panksepp, 2006; Knutson *et al*., 2002; Russell, 2003). Affects influence perception, cognition and behaviour and are modified by internal and external causes, while the causal connections remain directly inaccessible. Hence the resulting state may be experienced as freefloating mood or an emotional episode which is attributable to some specific cause (Russell, 2003). In the World Health Organization International Statistical Classification of Diseases and Related Health Problems (ICD-10) the listed mood disorders are referred to as synonymical to „affective disorders".

Depression is characterized by lowered mood and energy, loss of interest or pleasure, feelings of guilt or worthlessness, disturbed sleep and/or appetite, poor concentration and suicidal ideations, while in anxiety, typical symptoms include worry, tension, sleep disorders, fatigue and irritability. Affective disorders are a major cause of disability in the world, with lifetime prevalence around 15–20% for depression alone (Kessler *et al*., 2005, Paykel *et al*., 2005). Significant comorbidity is reported between depression and anxiety disorders (Merikangas *et al*., 2003) and although the ICD-10 keeps these disorders in separate blocks, pathological anxiety is considered one of the key features of affective disorders (Hiller *et al*., 1989). Variation exists in the prevalence of different affective disorders between sexes, suggesting differences in the underlying mechanisms of vulnerability.

1.1.1. Neurobiology of affective states and disorders

Studies on the neurobiological basis of affective states and non-adaptive changes in these states were qualitatively changed in the middle of previous century by the serendipitous discovery of antidepressant action of monoamine oxidase inhibitors and monoamine reuptake blockers, and the diametrically opposite effect of reserpine that induces depletion of monoamine reserves.

These findings suggested that a decreased activity of noradrenergic (NA) and serotonergic (5-HT) systems leads to depression and sedated affective states, while drugs which increase or potentiate the activity of monoaminergic systems cause behavioural activation and antidepressant effects, leading to the catecholamine hypothesis of depression (Coppen, 1967; Prange, 1964; Schildkraut, 1965; Schildkraut and Ketty, 1967). Nevertheless, the decreased activity of monoaminergic systems has not always been proved sufficient for depressed affect neither in patients nor healthy subjects (Mendels and Frazer, 1974; Pare *et al*., 1969). It also has to be pointed out that some drugs that increase monoaminergic activity (i.e. psychostimulants) are not effective clinical antidepressants. Further studies have introduced more specificity to the initial hypothesis, with greater regard to receptor types involved and their function, as postmortem studies of depressed patients have revealed significant changes in β-adrenoceptors and 5-HT_2 receptors in particular (Mann *et al*., 1986). Currently, it is suggested that a complex pattern of dysregulations of monoaminergic systems, also involving influences of other types of transmitter molecules, constitutes the biological basis of depression (Bymaster *et al*., 2003; Harro and Oreland, 2001).

By now, various non-aminergic mechanisms have been implicated in the etiology of affective states and disorders (reviewed in Pacher and Kecskemeti, 2004). Among others, extensive research has been carried out on neuropeptide systems with greatest focus on corticotropin-releasing factor (CRF) (Grigoriadis, 2005; Holsboer, 1999) and neuropeptide Y (Husum *et al*., 2000). Significant findings have been made connecting affective disorders and their treatment to changes in molecules influencing transcriptional processes and neurotrophic factors (Duman, 2002; Duman and Monteggia, 2006; Schloss and Henn, 2004).

Stressful life events play a significant role in development of psychiatric disorders and depression in particular (Kendler *et al*., 1999; Paykel, 2001). The stress response is accompanied by an increased release of glucocorticoids via the activation of the hypothalamus–pituitary–adrenal (HPA) axis that is under CRF-ergic control (Koob, 1999), and changes in the balance of HPA axis are characteristic to depression (reviewed in Holsboer, 2000). CRF holds a central role in reactions to environmental stimulation. It has been found to mediate changes in neuroendocrinological functions and behaviour induced by external stimuli (Owens and Nemeroff, 1991). Intracerebroventricular administration of CRF to laboratory animals brings forth a wide range of physiological and behavioural changes comparable to those elicited by stressful stimuli, including an increase in heart rate, suppression of exploratory behaviour, reduction in food intake etc. (for a review, see Griebel, 1999). Of the two CRF receptor subtypes, the CRF_1 receptor has mainly been implicated in mediating the stress-related effects of the CRF-system (for a review, see Takahashi, 2001) and the anxiolytic effects of CRF system blockade (Gilligan *et al*., 2000). Reports of very high levels of CRF_1 -like immunoreactivity in noradrenergic brainstem

nucleus locus coeruleus neurons (Sauvage and Steckler, 2001) suggest that the CRF_1 receptor subtype could be an important mediator of CRF-ergic regulation of locus coeruleus function and hence, NA-ergic activity.

Molecules affecting transcriptional processes have been increasingly studied with focus on molecular mechanisms underlying mental disorders (Damberg *et al*., 2001a). The AP-2 family of transcription factors regulates gene expression in central nervous system monoaminergic neurons (Kim *et al*., 2001). The AP-2 levels in the brainstem correlate with monoamine levels in several brain regions (Damberg *et al*., 2001b) and have been found (as measured in the whole rat brain) to be influenced by chronic antidepressant treatment (Damberg *et al*., 2000). Also, AP-2β genotype has been associated with anxiety-related personality traits (Damberg *et al*., 2001a).

Neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4 are a family of secreted growth factors that promote the survival, differentiation and maintenance of specific neuronal populations and regulate activity-dependent synaptic plasticity (reviewed in Bibel and Barde, 2000) with perhaps the most prominent effect on 5-HT system (Mamounas *et al*., 1995). Decreases of BDNF levels are reported in and are believed to lead to several pathologies, including anxiety-related behaviours, depression, bipolar disorder and schizophrenia, as well as neurodegenerative disorders like Huntington's, Parkinson's and Alzheimer's diseases (reviewed in Binder and Scharfman, 2004; Angelucci *et al*., 2005; Duman and Monteggia, 2006) and BDNF signalling plays a significant role in mechanisms of antidepressant action (Castrén, 2004; Hashimoto *et al.,* 2004).

Regardless of the enormous number of studies dedicated to identifying the underlying neurobiological mechanisms, most recently including extensive candidate gene association studies, the biological basis of affective disorders as well as the relationship between the pharmacological profiles and therapeutic effects of antidepressant medications still lack a coherent theory (Hindmarch, 2002; Nestler *et al*., 2002; Sartorius *et al*., 2007). It is acknowledged that extensive changes develop all over the brain in depression (Harro and Oreland, 2001), and it is important to identify mechanisms central to these overall changes, and develop means to influence these mechanisms in order to restore the initial balance. A critical aspect in this context is the fact that the animal models extensively used in these studies have been developed to be sensitive to the already known antidepressant and anxiolytic drugs and may hence lack the features needed for identifying possible novel targets for depression treatment.

1.1.2. Animal models of affective states and disorders

Based on the assumption of evolutionary conservatism, the study of animal behaviour may give us powerful insights to human psychology. The study of animal affect and its use in the study of emotional systems in humans, and their disruption in mood disorders, is now based on thorough knowledge about neuroanatomical and neurochemical correspondence in these processes over many mammalian species. It is acknowledged that the observed emotional states derive from evolutionally old subcortical and limbic regions that are well conserved in most animals, including humans (Panksepp, 1998). The better understanding and experimental implementation of the homologies between emotional states in animals and humans allows us to better understand the underlying neural and biochemical mechanisms of human mood disorders and to develop strategies for treatment.

Animal models of mood disorders have historically been developed for two goals – to enable studying the disorder in a way that is not possible in humans and to provide a screening tool for new pharmacological and other treatments. To attain these goals, an ideal model would have to provide unambiguous symptoms that are similar to the disorder in humans and react to known treatments in a known and unambiguous way (Willner, 1984). Naturally, some symptoms in human mood disorders are impossible to create in animals, but it may also be pointed out that not all the symptoms listed in diagnostic manuals have to be present in order to diagnose a mood disorder in humans. Hence, many available animal models have focussed on one or a short list of behaviours or functions that reveal a human disorder-like features or changes after specific manipulations (Nestler *et al*., 2002). These behaviours may be naturally-occurring or induced by various treatments that have often capitalized on various stress reactions (Blanchard *et al*., 2001; Meaney, 2001; Willner, 1997).

1.1.3. Stress

Stressors are the physical and psychological events that threaten the organism's homeostatic state and trigger a multitude of behavioural and physiological responses in order to regain this state (for review, see Chrousos and Gold, 1992). Sustained exposure to stressors leads to changes in neuronal, hormonal and immune systems (McEwen, 2003) and greater susceptibility to psychiatric disorders (McEwen and Steller, 1993; Rosen and Schulkin, 1998). The main aim of the original chronic variable stress procedure developed by Katz and colleagues (1981) and its later modifications (for an example, Willner, 1997) is to produce a depressed state (mostly expressed as anhedonia) as the variation and unpredictiveness of a variety of stressors administered over a long period of

time adds up, while none of the stressors used is necessary or sufficient to have a significant effect on behaviour on its own (Muscat and Willner, 1992). The animals are subjected to the stressors usually over a period of several weeks and the treatment results in changes in activity levels in tests used in depression and anxiety studies, which are eliminated by antidepressant treatment (for review, see Willner, 2005). The most pervading effects of chronic stress regimes are decreases in reward-related behaviours – electrical brain stimulation (Moreau *et al*., 1992), place preference (Papp *et al*., 1991), and, most importantly, in sucrose intake and preference (Katz, 1982; Willner *et al*., 1987). Anhedonic tendencies have been considered among the core syptoms in depressive states in humans, which has justified the interpretation of decreased sucrose consumption and preference in chronically stressed rats as a measure of depressive-like states. Similarly to the fact that stressful life experience does not cause depression in all humans, inter-strain as well as inter-individual variability has been demonstrated in the effects of chronic variable stress on rats (Nielsen *et al*., 2000) and mice (Anisman and Zacharko, 1990).

1.1.4. Inter-individual differences in laboratory animals

It has been acknowledged that stable inter-individual differences in behaviour exist between animals used in biomedical research. If these traits are stable in their expression, they open up new possibilities in modelling of human psychology and psychopathology by studying relationships with other traits and the underlying neural mechanisms. Various standardized behavioural test procedures have been used to screen animals with regard to specific quantifiable criteria, and stable individual variations have been found in rearing and locomotor activity on a novel field, activity in the elevated plus-maze or more complex mazes, sucrose consumption, social behaviour, stress reactivity etc., and used in biomedical research (Brunelli and Hofer, 2007; Borta and Schwarting, 2005; Cools *et al*, 1993; Dellu *et al*., 1996; Desousa *et al*., 1998; Jama *et al*., 2008; Landgraf, 2003; Ramos *et al*., 2003; Stead *et al*., 2006; Taghzouti *et al*., 1999; Thiel *et al*., 1999; Tõnissaar *et al*., 2006 and 2008; White *et al*., 2007). In particular, these classifications have often been applied to study anxiety and vulnerability to stress and addictive conditions in animal models (Landgraf and Wigger, 2002; Piazza *et al*., 1989). When pre-selection of animals with regard to some specific behaviour is used, it is of great interest to evaluate, whether the differences in the test are specific to the test context or generalizable to a wider variety of tests, in order to assess the use of such "phenotype models" in the study of different human conditions (Hasler *et al*., 2004).

It is noteworthy that most of the research carried out on affective states and their dysfunction in both animals and humans has concentrated on negative affect. Given their effects on life quality, it seems reasonable that stimuli

eliciting psychological (and physical) distress have been focussed on more thoroughly. Nevertheless, positive affective reactions (and their dysfunction) may be considered equally important (Berridge, 2003; Burgdorf and Panksepp, 2006). The range of methods that can be used for studies of positive affect is lagging behind the multitude of those for the modelling and investigation of negative affect. The measurement of elicited behavioural or physiological affective reactions to the hedonic stimulation is one way to study the positive affect, and the parameters that enable such an approach are currently being specified (Panksepp, 2003; Berridge, 2003). The study of the instrumental performance and response reinforcement in rewarded tasks is another option with a somewhat restricted conceptual approach with regard to the interpretation of the results, while the most typically used approach in humans, subjective ratings, is of course not possible in animal studies.

1.2. Exploratory behaviour

Exploratory behaviour comprises behaviours triggered by and towards novel stimuli. It is essential for survival due to the possibilities it provides to find food, water, mating partner, shelter etc (Berlyne, 1955). It consists of behavioural acts and postures that permit the collection of information about new conspecifics, novel objects and unfamiliar parts of the environment (File and Hyde, 1978; Griebel *et al*., 1993; Harro, 1993). Exploratory behaviour is at any particular moment influenced by the conflicting motivations to get into contact with the potentially dangerous novel environment or to stay within the secure and familiar surroundings. Thus an animal's behaviour in a novel environment is always simultaneously influenced by curiosity or motivation to explore and by neophobia (Harro, 1993, Belzung, 1999). Behavioural tests in rodents which are based on exploration are often used for measurement of anxiety-related effects of psychoactive drugs, and blockade of the dopamine (DA)-based appetitive motivational or seeking system has been found to bring forth an apathetic depressive state in humans (Panksepp, 1998; Wise, 1982). It has been pointed out that a free choice for the animal to choose between novel and familiar environments plays a significant role in exploratory behaviour, forced access to novelty being more stressful than free access (Misslin and Cigrang, 1986).

1.2.1. Neurobiology of exploratory behaviour

Typical to a complex behaviour, various neural mechanisms have been found to influence exploratory behaviour. Monoaminergic mechanisms have been shown to play a significant role; in particular, intact NA-ergic projections ascending

from the locus coeruleus have been found to be important for normal exploration in rats, as increased neophobia has been observed after selective destruction of these projections (Harro *et al*., 1995). Disruption of DA-ergic neurons has also been found to affect exploration, as neurotoxic lesions of mesolimbicocortical DA terminals have been found to decrease this behaviour (Fink and Smith, 1980). 5-HT depletion has been associated with decreases in spontaneous exploratory activity (Lipska *et al*., 1992), and most drugs used to treat mood disorders via modulation of 5-HT system in humans have been found to modulate animal behaviour in tests used in anxiety and depression research dependently on the receptor subtype affected (reviewed in Millan, 2003). Also, antagonists of neuropeptides CCK, NPY and CRF have been shown to influence exploration (Hughes *et al*., 1990; Harro *et al*., 2001a,; Harro, 2006).

1.2.2. Inter-individual differences in exploratory behaviour

The approach of creating phenotype-based models of affective states has also been used with different aspects of exploratory behaviour as selection criterions. Rats that were selectively bred for higher anxiety in the elevated plus-maze test will consequently display similar behavioural patterns in other tests of anxiety, such as lower activity in the black-white box test (Henniger *et al*., 2000) and open field test, and use more passive coping strategies in the forced swimming test during the first but not the second test day (Liebsch *et al*., 1998). However, in acutely selected rats, Ho *et al*. (2002) reported no difference in forced swimming; neither did they find differences in rearing in open field between animals selected by plus-maze behaviour, suggestive that breeding for different behavioural patterns in the elevated plus-maze test yields somewhat different groups than acute testing. It is important to note, that in case of elevated plus-maze that has often been used for selection, repeated testing is impossible, due to markedly decreased activity on already second testing, accompanied by differences in pharmacological effects, i.e. decrease in sensitivity to benzodiazepines (File and Zangrossi, 1993), hence leading to the strategy of breeding animals with high vs. low anxiety in the plus-maze test (Landgraf and Wigger, 2002).

Differences in the expression of anxiety are accompanied by differences in neurochemistry of amino acids, monoamines and neuropeptides (Giorgi *et al*., 2003; Harro *et al*., 1990; Landgraf, 2005; Rägo *et al*., 1988; Singewald, 2007; Thiel *et al*., 1999; Umriukhin *et al*., 2002). Particular interest has been paid to the 5-HT-ergic function in medial prefrontal cortex (PFC) and hippocampus (Pollier *et al*., 2000; Giorgi *et al*., 2003; Keck *et al*., 2005), but the results have remained rather inconclusive, possibly due to large variation in behavioural paradigms and methods used and the single variables measured. Several neurochemical features are shown to be associated with inter-individual differences in

responding to novel stimuli — compared to low responders to novelty, high responders to novelty have been reported to have higher basal and stimulated DA release in the nucleus accumbens (Hooks *et al*., 1991; Rouge-Pont *et al*., 1998), higher basal firing rates and bursting activity of DA neurons in the ventral tegmental area (Marinelli and White, 2000) and lower 5-HT content in the medial prefrontal cortex (Thiel *et al*., 1999).

Inter-individual differences have also been seen in the reactivity to both typical and novel pharmacological treatments – i.e. diazepam exerting greater anxiolytic effects and CRF_1 receptor blockade being anxiolytic only in high-anxiety-related behaviour in animals selectively bred for their behaviour in the elevated plus-maze test (Keck *et al*., 2001; Liebsch *et al*., 1998). In the forced swimming test, desipramine was found to cause significant decrease in immobility in low response animals only, while fluoxetine reduced immobility in both low and high response groups (Jama *et al*., 2008). Also, Saigusa *et al*. (1999) found that the catecholamine synthesis inhibitor α-methyl-*p*-tyrosine decreased both novelty-induced behavioural activation and accumbal DA release in high responders to novelty, but did not affect that behaviour in low responders to novelty and conversely, increased novelty-induced DA release in the latter group. The authors hypothesized that this effect that lasted longer than behavioural activation elicited by novel environment helps animals to incorporate information about the new situation. Indeed, dopaminergic neurotransmission, particularly dopamine release in the nucleus accumbens, is increased in response to natural rewards and novelty (Rebec *et al*., 1997) and has been implicated in the mechanism of action of drugs with addictive properties.

1.3. Ultrasonic vocalizations

The understanding that the study of animal vocalizations may give insights into their nervous system functions, behaviour and affective states has gained widespread and growing interest over the past decade (for recent reviews, see Boissy *et al*., 2007; Burman *et al*., 2007; Cheng and Durand, 2004; Parr *et al*., 2005; Portfors, 2007). It has become an acceptable position that the vocalizations in animals carry a deep sociobiological significance, that, when studied at the level of the underlying neural mechanisms and their regulation allows us to better comprehend what the animals feel in experimental situations. This gives better means for constructing more complex and more adequate animal experiments that model the phenomena of interest in human beings. The study of ultrasonic vocalization (USV) in rodents has been lasting for more than three decades, mostly in the context of sexual and aggressive behaviours (McIntosh *et al*., 1978; Takahashi *et al*., 1983), maternal separation in rat pups (Brunelli *et al*., 1996; Winslow and Insel, 1991), anxiety and other negative affective states

in adult rats (Blanchard *et al*., 1991; Vivian and Miczek, 1991, for a recent review, see Litvin *et al*., 2007). Attention has also been paid to the concerns about the acoustic environment of laboratory animals (Sales *et al*., 1988). Over the last decade also other functional aspects of rat USVs have been started to be studied, with USVs associated with positive stimuli gaining significant attention (Panksepp, 2007; Portfors, 2007; Schwarting *et al*., 2007). By now it is clear that we can distinguish at least three different forms of USVs in common laboratory rats: 1) the 40-kHz distress vocalizations in rat pups that are routinely observed in maternal separation paradigms; 2) juvenile and adult USVs with long duration and frequency below 30 kHz (usually termed „22-kHz USVs“) that seem to be the adult counterpart of the 40-kHz calls in rat pups, and 3) short and high-frequency so-called 50-kHz USVs or “chirps” that have been found to range from 30 to 70 kHz (Brudzynski and Pniak, 2002; Kaltwasser, 1990; Knutson *et al*., 1998). The latter two seem to convey information on diametrically opposite affective states, namely aversion and anticipation in adult rats (Burgdorf *et al*., 2001b; Knutson *et al*., 1999) and have almost non-overlapping spectrographic parameters as well as different neurobiological substrates (Brudzynski, 2007).

It has been found that play is highly rewarding to young rats (Vanderschuren *et al*., 1997), and the USVs emitted around the frequency range of 50 kHz (the so-called „rat laughter“) were identified as a type of sociovocal communication that facilitated playfulness in studies of the sensory systems which control rough-and-tumble play in juvenile rats (Knutson *et al*., 1998; Panksepp and Burgdorf, 2003), for quantitative analysis see Brudzynski (2005). It was found that these chirps may easily be experimenter-induced by manipulating the animal in a way that mimics the rough-and-tumble play in juvenile rats, or literally by “tickling” the experimental animal (Panksepp and Burgdorf, 2003). After the initial findings, the chirping response itself and its relations with many other mostly reward-related behaviours have been thoroughly studied. The studies so far indicate that measurements of 50-kHz USVs can be used to investigate positive affective states such as playfulness and social joyfulness (Knutson *et al*., 1998), as well as pharmacologically conditioned reward-related behaviour (Knutson *et al*., 1999). Adult as well as adolescent rats emit these vocalizations in response to many rewarding stimuli such as play, sexual partners, and social contacts after a mild isolation, food, drugs of abuse, or electrical brain stimulation (Brudzynski and Pniak, 2002; Burgdorf *et al*., 2000; Burgdorf *et al*., 2001a; Knutson *et al*., 1999; Panksepp *et al*., 2002b). A procedure of experimenter-administered tickle-like stimulation is often exploited in order to mimic the natural rough-and-tumble play in juvenile rats (Burgdorf and Panksepp, 2001) and to simultaneously elicit very high levels of 50-kHz vocalization in rats (Burgdorf *et al*., 2005). The rate of 50-kHz vocalizing in response to tickling has been found to reflect the pleasantness and reinforcing value of stimuli (Burgdorf and Panksepp 2001).

Two subtypes of 50-kHz ultrasonic vocalizations have been focussed on: flat USVs with minimal bandwidth variation around 5–7 kHz and others that include a significant frequency modulated (FM), or so-called trill component (Burgdorf *et al*., 2007; Schwarting *et al*., 2007). The specific different roles of these two subgroups of USVs have not been clarified yet, although it has already been found that there are differences in the extent in which they are modulated by pharmacological manipulations (Burgdorf *et al*., 2007).

Stimuli with negative valence for the animals, such as bright light and predatory odours, reduce the 50-kHz USVs (Knutson *et al*., 1998), and increase the 22-kHz calls (Brudzynski, 2005; Burgdorf *et al*., 2001b). These vocalizations are considered to reflect a negative affective state in adult rats and are exhibited during social defeat, in response to danger, opiate withdrawal, and in anticipation of foot shock (Blanchard *et al*., 1991; Brudzynski, 2001; Portfors, 2007; Sánchez, 2003; Tonoue *et al*., 1986; Vivian and Miczek, 1991). It has previously been reported that these negatively valenced USVs correlate negatively with the 50-kHz USVs in certain conditions (Burgdorf *et al*., 2005), suggestive of an inverse relationship between the two types of vocalizations. Also, the 22-kHz USVs are decreased by anxiolytic substances similarly to separation distress calls (Miczek *et al*., 1995).

1.3.1. Neurobiology of ultrasonic vocalizations

In mediation of appetitive, reward-related behaviour, DA signal transmission is believed to play a major role (Panksepp *et al*., 2002b), whereas 5-HT system has been connected with anxiety (Morilak and Frazer, 2004), which, in turn, may control the active appetitive behaviours. Therefore the study of these neurotransmitter systems in particular may be considered essential in determining the neural basis of the expressed emotional states. The study of Fu and Brudzynski (1994) in which glutamate and carbachol were injected into anterior hypothalamic-preoptic area concluded that the 50-kHz and 22-kHz USVs have different underlying neurobiological mechanisms as the former were dose-dependently elicited by glutamate administration while the latter were elicited by carbachol injection. Later findings have specified that the 50-kHz USVs are induced by the activation of ascending DA-ergic system, while the 22-kHz USVs are induced by the ascending cholinergic system, with the regulation of the respective positive and negative affective states also controlled by these pathways (Brudzynski, 2007).

1.3.2. Inter-individual differences in ultrasonic vocalizations

By now, it has been found that stable individual variations exist in 50-kHz USVs (Panksepp *et al*., 2002a; Schwarting *et al*., 2007), suggesting that the extent of emitting these USVs refers to an individual disposition or trait. This trait is probably connected with genetic and physiological variables, since breeding studies have yielded distinguishable lines of high and low chirpers (Burgdorf *et al*., 2005). Animals selected for high levels of tickling-induced 50-kHz USVs have been found to exhibit more play behaviour with conspecifics (Panksepp and Burgdorf, 2000). When bred for high levels of 50-kHz chirps over five generations (Burgdorf *et al*., 2005), the animals exhibited significantly more 50-kHz USVs than animals in a randomly bred line. These rats also emitted fewer 22-kHz USVs, suggestive of lower levels of negative affect, while the animals bred for low levels of 50-kHz calls tended to emit more 22-kHz USVs and the same level of 50-kHz calls as a random group. The chirping response has been found not to habituate over repeated testing and remain stable irrespective of whether the animals are food-deprived or fed ad libitum (Schwarting *et al*., 2007).

2. AIMS OF THE PRESENT STUDIES

Inherent inter-individual differences in animals' behaviour and naturally occurring phenotypes may provide evolutionally valid models human psychology. The studies presented herein focussed on inter-individual differences in animals' responses to novel environment and experimenter-administered tickling-like stimulation.

The hypothesis was tested that stable inter-individual differences exist in animals' exploratory behaviour in novel environment and 50-kHz ultrasonic vocalizations in response to play-like stimulation, and one of the major aims of these studies was to develop adequate methods for measurement of these inter-individual variations. Also, it was hypothesized that these stable dispositions are reflected in behaviours in other tests used in the research of anxiolytic and antidepressant drugs.

DA-ergic neural circuits have been associated with motivational and reward systems, while NA-ergic, 5-HT-ergic and CRF systems are deeply interconnected in the regulation of organism's responses to stressful and aversive stimuli. Behavioural differences in exploratory activity or 50-kHz ultrasonic vocalizations between individual animals suggest underlying variations in the regulation of these behaviours by these neurobiological systems that were hence assessed via various methods. In order to further assess the functions of transcription factor AP-2 in the NA-ergic systems, it was measured after long-term CRF_1 receptor blockade. The possible involvement of BDNF in the regulation of differences in 5-HT-ergic system between high and low exploring rats was assessed.

In order to map brain regions associated with the high/low chirping phenotype, the long-term metabolic activity in various brain regions was studied. The effects of chronic variable stress on behaviour and brain metabolic activity were also studied in these animals with the hypothesis of possible differences in stress reactivity. Because males and females differ in prevalence of affective disorders and may have different underlying mechanisms of vulnerability, these experiments were carried out comparatively in male and female rats.

3. MATERIALS AND METHODS

3.1. Animals and housing conditions

The animals used in Papers I, II and IV originated from Scanbur BK AB, Sweden. The animals used for experiments in Paper III were acquired from Finnish Laboratory Animal Center (Kuopio, Finland) (Experiment 1) and from the National Public Health Institute (Kuopio, Finland) (Experiment 2). All animals arrived to our animal facilities approximately at the age of 3 weeks. The animals used for experiments in Papers V and VI were locally bred first generation offspring from males and females from Scanbur (five breeding pairs in Paper V and seven different breeding pairs in Paper VI) that were weaned at the age of 3 weeks. Wistar rats were used for all experiments, except for Experiment 1 in Paper I, in which both Sprague-Dawley and Wistar rats were used. Male rats were used in all studies, except for those reported in Papers V and VI, in which both male and female rat pups were used. The animals were single-housed (Paper V, Experiment 1), or housed in groups of three (Paper III, Experiment 1; Paper I, Experiment 1) or four (all the other Experiments) in standard transparent polypropylene cages under controlled unturned light cycle (lights on from 8 a.m. to 8 p.m. during all Experiments, except for studies reported in Paper V during which the light cycle was from 8.30 a.m. to 8.30 p.m.) and temperature (19–21°C), with free access to tap water and food pellets (diet R70, Lactamin, Sweden). All behavioural testing was carried out between 12 a.m. and 7 p.m. The lighting in the animal room was 320–400 lx, depending on the height of the shelf. In the microdialysis experiments, only data from the animals with correct probe location are presented. The experimental protocols were approved by the Ethics Committee of the University of Tartu.

3.2. Behavioural methods

3.2.1. Exploration box test and the procedure of division of animals into groups with high and low exploratory activity

The exploration box (Matto *et al*., 1996; Otter *et al*., 1997) was made of brushed metal and consisted of a 50×100 cm open area (height of side walls 40 cm) with a small (20×20×20 cm) compartment attached outside to one of the shorter sides of the open area. The open area was divided into eight squares of equal size (25×25 cm). In the open area, four objects were situated in certain places. Three of these objects were unfamiliar and one familiar (namely, a glass jar, a cardboard box, a wooden handle and a food pellet). The floor of the small compartment was covered with wood shavings, and the compartment was

directly linked to the open area through an opening (size 20×20 cm), allowing the animal free alternation between the two locations. The lighting conditions of the experiment room were dim, with approximately 3–7 lux in the open part of the apparatus. The observer was seated at the end of the apparatus opposite to the exit from the small compartment. The animals were moved from the housing room to the testing room in their home cages and allowed to habituate with the room for about 15 min with no experimentation carried out during this period. The exploration test was initiated by taking a random rat from its home cage and placing it into the small compartment of the apparatus, which was then covered with a lid. The following measures were taken by the 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) time spent exploring the open area. A single test session lasted 15 min, after which the animal was gently removed from the apparatus, weighted and returned to its home cage. The apparatus was cleaned with dampened laboratory tissue after each animal. When tested repeatedly in the exploration box test, the inter-day correlations for exploratory activity show an increasing trend with every successive day (reported in Paper I). The activity on the first testing session does not correlate highly with the following tests, but already the second testing, carried out 24 h after the first, gives a good prediction of activity levels on the consecutive test sessions. Therefore the rats were observed in the exploration box at least for 2 consecutive days for determination of inherent exploratory activity levels. 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. The rats were divided into low exploratory (LE) and high exploratory (HE) activity groups on the basis of the sum of exploratory activity during the second testing session.

3.2.2. Tickling-induced ultrasonic vocalizations and the procedure of division of animals into groups with high and low levels of 50-kHz USVs

Rat pups were weaned when 3 weeks old and single-housed immediately. Training sessions started the next day after single-housing. During the single-housing period, the rats were given daily sessions of experimenter-induced stimulation or “tickling” (Panksepp and Burgdorf, 2003). In the beginning of a tickling session, the animal was taken from the animal room to an adjacent room with similar lighting conditions, removed from its home cage and placed into an empty and smaller (32×14×13 cm) cage, located under a microphone about 20 cm from the floor of the cage for amplifying the ultrasonic vocalizations to an audible range to the human ear (Paper V) or recording (Paper VI) the

ultrasonic vocalizations. The animal was given 15 s to habituate with the new cage, followed by 15 s of handling by experimenter that mimicked natural rough-and-tumble play in juvenile rats. In short, the "tickling" session that each animal received consisted of stimulating the rat with one hand by the experimenter, that included rapid finger movements on the back of the neck, turning the animal on the back and letting it "wrestle" with the experimenter's hand with vigorous alternating finger movements administered on the animals' ventral surface, followed by release after 1–2 seconds of stimulation. Altogether, four 15 s sessions of stimulation were given over two minutes, after which the animal was again placed in its home cage, returned to the animal room and the test cage cleaned thoroughly. In studies presented in Paper V, the ultrasonic vocalizations were counted during tickling sessions, and in studies presented in Paper VI, the recorded audio files were later analyzed with the Avisoft SASLab Pro software, creating spectrograms from which the 22-kHz USVs, plain 50-kHz USVs and USVs containing a frequency modulated, or a "trill" component were manually counted. The animals were divided into groups with high and low levels of 50-kHz USVs by the median split of the average response on Days 12–14 of tickling, providing the 'high chirping' and 'low chirping' (HC and LC) groups.

3.2.3. Chronic variable stress

The chronic variable stress regimen (Harro *et al*., 2001b) lasted for four weeks and comprised of seven different stressors that were intermittently used once every week. The stressors, presented in the order of administration were: a) cold (4°C) water and wet bedding (initially, 400 ml of water was poured on the rats, and the sawdust bedding was kept wet for the following 22 h); b) imitation of a peritoneal injection with the rough and firm grasping of the animal using special glove and syringe without the needle, which was pressed to the animal's body for several seconds; c) stroboscopic light (for 14 h, 10 Hz, 2 lx); d) tail pinch with a clothes-pin placed 1 cm distal from the base of tail (5 min); e) cage tilt at 45° (for 24 h); f) movement restriction in a small cage (11×16×7 cm for 2 h); g) strong illumination (900 lx) during the predicted dark phase (for 12 h). Five sucrose preference tests were carried out during the stress regimen, with the first one on the night preceding the first stressor and the following ones at the end of every week. The stressors were administered during the light phase of the cycle (except for the ones that lasted overnight).

3.2.4. Other behavioural methods

Elevated zero-maze
The elevated zero-maze was designed in accordance with the original description (Shepherd *et al*., 1994) with a few modifications (as in Matto *et al*., 1997). The elevated zero-maze was an annular platform (width 10 cm) with a diameter of 105 cm, divided into two opposite open parts and two opposite closed parts (height of the side walls 40 cm). The open parts had borders (height 1 cm). All parts of the apparatus were made of non-transparent plastic, and the apparatus was elevated 50 cm above the floor. For the test, the animal was placed into one of the open parts facing the closed part of the apparatus and was observed for 5 min. Behavioural measures taken included a) number of open part entries; b) time spent in the open parts; c) number of head dips over the edge of the platform; d) number of stretched-attend postures. The experiments were carried out under bright lighting conditions.

Social interaction
The test developed by File and Hyde (1978) was used as previously described (Tõnissaar *et al*., 2004). In this test, a pair of previously single-housed, weight-matched rats was placed into a novel, brightly lit chamber (30×30×60 cm) with floor covered with wood shavings. The total time spent in active social behaviour (allogrooming, sniffing the partner, crawling under and over, following) was recorded for 10 min. The behaviour of the animals was observed directly by two observers. Inter-rater reliability between observers was high (r = 0.92). The mean social interaction time was calculated for each rat. In each test, the partners were new to each other.

Sucrose preference
In Paper I, Experiment 1 and Paper V, Experiment 1, sucrose intake was measured in home cages of single-housed animals. In the rest of experiments, the animals were placed into single cages immediately before the sucrose preference testing. Sucrose preference test (as described in Tõnissaar *et al*., 2006) was carried out with two bottles, one filled with 1% sucrose solution and the other with water. For the animals that remained single-housed through the whole experiment, the water bottles were not changed, while for the animals that were single-housed only for sucrose preference testing, new bottles were provided for the single-housing period. Sucrose and water consumption was measured for the period of 1 h by weighing pre-weighed bottles at the end of the test. Sucrose preference was measured by calculating the proportion of sucrose consumption out of total consumption of water and sucrose solution. The test was carried out on two consecutive days in all Experiments except for Paper V, Experiment 2 and Paper VI. In Paper V, Experiment 2, an 18-h food and water deprivation was assigned after a 24-h resting period, and a third sucrose preference test carried out

immediately after that. In Paper VI, five sucrose preference tests were carried out with a weekly interval, 5–6 hours after the beginning of the dark phase, with the first test on the night before the stress regimen.

Forced swimming

The forced swimming test, first described by Porsolt *et al*. (1978) was carried out as described previously (Häidkind *et al*., 2004). Briefly, rats were placed into a vertical glass cylinder (diameter 22.5 cm and height 60 cm) containing about 35 cm water at 25ºC. On the first day of the experiment, the procedure lasted 15 min and the re-exposition 24 h later lasted 5 min. At the end of each session the rats were dried with laboratory tissues. The sessions were recorded with a video camera and the duration of immobility, swimming and struggling was later measured by two independent experimentators. The measurements were based on the behavioural categories described by Armario *et al*. (1988). In short, a rat was judged to be immobile when it remained floating in the water with all limbs motionless. 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 touching the walls of the tank. The time spent in swimming was recorded when the rat was making active swimming motions, more than necessary to merely maintain its head above the water, e.g. moving around in the cylinder. The average results of the two experimenters were used in the calculations. Inter-rater reliability between observers was high (r = 0.8–0.9).

Elevated plus-maze

The method first described by Handley and Mithani (1984) and modified for low baseline open arm activity in our laboratory (Harro *et al*., 1990) was used. In brief, the plus-maze consisted of two open arms (50×10 cm) without any walls, two enclosed arms of the same size with 40 cm high side walls and end wall, and the central arena (10×10 cm) interconnecting the arms. The arms of the same type were opposite to each other. Both open arms were divided into three parts of equal size by lines which also separated the central arena from all arms. At the beginning of the experiment the rat was placed into a closed arm. The central arena and the open arms formed the "open part" of the apparatus. An entry into open arms was counted when the rat crossed the line between the central arena and an open arm with all four paws. The rat was considered to explore the open part of the apparatus when it had clearly crossed the line between a closed arm and the central arena with its both forepaws. Behavioural measures taken during 4 minutes included: a) latency before entering the open part (i.e. the central arena); b) line crossing; c) time spent in the open arms of the apparatus; d) approaches towards the central arena which were not completed (nose crossed the line but not both of the forepaws); e) open arm entries; f) the total number of arm entries. From the two latter measures, the open/total arm entries ratio was calculated.

Fear conditioning

Fear conditioning was studied in a standard shuttle box following the procedure described by Wallace and Rosen (2001). In short, rat was placed in the chamber with metal grid floor for 3 min before the administration of a 1.5 mA, 1 s foot shock. Freezing, defined as a characteristic crouch position, was measured for 4 min immediately after the foot shock. Freezing was measured as a sample of freezing or not freezing every 10 s, for a total of 25 observations. A retention test of fear conditioning was conducted 24 h after the foot shock by placing the animal back into the same chamber and recording freezing for 4 min as described above.

Activity in a novel cage

The cage used in Paper I for the novel cage testing was identical to the home cages of the animals, except for the absence of cage mates, food pellets and water. The new cage was located in the animal room on the same shelf as the animal's home cage. The floor was covered with wood shavings and the cage was covered with standard metal grid ceiling. The animal was placed in the centre of the cage and observed for 10 minutes. In Experiment 4, the cage floor was divided into four squares and the following measures were counted: a) transitions from one square to another; b) rearing; c) digging in wood shavings; d) grooming; e) time of exploration. In Experiment 5, a novel object (a plastic tube of 1 cm in diameter and 7 cm in length) was placed in one corner of the cage and the episodes and time of exploration of the object were counted. Every animal was tested in a separate new cage.

Light-dark box

The light-dark box test was modified from Santucci *et al*. (1994). A metal box measuring 30×60×40 cm was divided into two compartments of equal size (30×30 cm). The bright compartment had no ceiling. Black lines divided the floor of the light compartment into four 15×15 cm squares. The dark compartment had a ceiling. A door of 10×10 cm in the partition wall allowed the rat to go from one compartment to the other (transition). The experiments were conducted at normal room light (approximately 190 lux at the centre of the light part). The following measures were counted: a) transitions between the light and dark compartments; b) latency (s) to enter the dark side after initial placement in the light side; c) latency (s) to re-enter the light side following the first cross into the dark side; d) number of transition attempts from the dark to the bright compartment (the animal places the head out of a dark side without crossing to the light side); e) total time spent in the light compartment; f) line crossing in open part; g) the number of stretched-attend postures made out from the dark compartment; h) rearing in open part.

3.3. Biochemical methods

3.3.1. High-performance liquid chromatography with tissue samples

Monoamines in brain tissue were measured by high performance liquid chromatography (HPLC) with electrochemical detection. The tissues were disrupted with an ultrasonic homogenizer (Bandelin, Germany) in ice cold solution of 0.1 M perchloric acid (10–20 µl/mg) containing 5 mM sodium bisulfite and 0.04 mM EDTA for avoiding oxidation. The homogenate was then centrifuged at 14000 × g for 20 min at 4˚C and 20 µl of the resulting supernatant was chromatographed on a Lichospher 100 RP-18 column (250×3 mm; 5 µm) (Paper III) or a Luna C18 column (150×2 mm; 5 µm) (Paper V). The separation was done in isocratic elution mode at column temperature 30˚C using the mobile phase containing (in Paper III and IV) 0.05 M citrate buffer at pH 3.6, 1 mM sodium octylsulfonate, 0.3 mM triethylamine, 0.02 mM EDTA, 1mM KCl and 6.25% acetonitril or (in Paper V) 0.05 M citrate buffer at pH 3.7, 1 mM sodium octylsulfonate, 0.02 mM EDTA, 1 mM KCl and 7.5% acetonitril.. The measurements were done at electrode potentials of a glassy carbon electrode +0.6V versus Ag/AgCl reference electrode with HP 1049 electrochemical detector (Hewlett Packard, Germany).

3.3.2. Extraction of nuclear proteins and enzyme-linked immunosorbent assay for AP-2 measurement

Nuclear proteins were extracted according to a modified protocol of Dignam and co-workers (1983). Tissue sections of the locus coeruleus area were homogenized in 3 ml of buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF). After incubation on ice for 15 min and addition of 0.25 ml 10% Nonidet P40 the homogenates were centrifuged for 1 min at 17 100 × g at 4ºC. The nuclear pellets were resuspended in 0.5 ml of ice cold buffer B (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF). Shaking for 15 min at 4ºC was followed by centrifugation at 17 100 × g for 5 minutes at 4ºC. The aliquots from the supernatants were frozen in liquid nitrogen and stored at –80ºC. Total protein concentration was determined for all nuclear extracts by the method of Lowry *et al*. (1951). Microtiterplates (96-well) were coated with 50 µl (0.06 µg/ml) nuclear extract diluted in 50 mM carbonate-biscarbonate buffer, pH 9.0. The plates were covered with parafilm and incubated overnight at 4ºC. Following the incubation the antigen solution was removed and 200 µl of blocking buffer (PBS and 1% BSA) was added to each well and the plates were incubated for two hours at room temperature. The blocking buffer was

removed and the plates were washed with PBS. Thereafter, the primary antibody (goat polyclonal AP-2α and AP-2β, 15 µl/ml respectively, Santa Cruz Biotechnology), diluted in blocking buffer, was added (50 µl per well) and the plates incubated overnight at 4ºC. After incubation the antibody was removed and the plates were washed three times with wash buffer I (PBS, 0.05% Tween-20). Thereafter the secondary antibody (donkey anti-goat IgG AP conjugated, SDS) diluted 1:350 in blocking buffer was added (50 µl to each well) and the plates were incubated for two hours at room temperature. After removal of the secondary antibody, the plates were washed three times with wash buffer I and once with wash buffer II (10mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.5). Therafter, 50 µl substrate (Phosphate substrate, Sigma, one 5 mg tablet diluted in 5 ml wash buffer II) was added to each well. The reaction continued for 20 minutes and was terminated by adding 50 µl of 0.1 M EDTA, pH 7.5. The plates were analysed in an ELISA reader at optical density 405/490. The optical density of the AP-2 isoforms for each rat was correlated to a value in a standard curve, where known concentrations of antibody are plotted against optical density. The value from the standard curve was then divided with the concentration of the total protein in the nuclear extracts. The quota was used as a relative amount of AP-2α and AP-2β. Samples from each rat were analysed twice for accuracy.

3.3.3. Microdialysis

The animals were anaesthetized with chloral hydrate (350 mg/kg, i.p.) and mounted in a Kopf stereotactic frame. A self-made concentric Y-shaped microdialysis probe with 7 mm shaft length and 3 mm active tip was implanted in the dorsal striatum according to the following coordinates: AP: +0.7; ML: +3.0; DV: –7.0; a probe with 7.5 mm shaft length and 2 mm active tip was implanted into nucleus accumbens to the following coordinates: AP +1.3; ML: +1.7; DV: –8.0; a probe with 5 mm shaft length and active membrane on the whole length was implanted into the PFC according to the following coordinates: AP: +3.3; ML: +0.8; DV: –5.0; and a probe with 4 mm shaft length and 1 mm active tip was implanted into dentate gyrus (DG) with the following coordinates: AP –4.3; ML: +2.2; DV: –3.8. All coordinates are according to Paxinos and Watson (1986). All probes were implanted to separate animals. The dialysis membrane used was polyacrylonitrile/sodium methalyl sulphonate copolymer (Filtral 12; inner diameter: 0.22 mm; outer diameter: 0.31 mm; AN 69, Hospal, Bologna, Italy). Two stainless steel screws and dental cement was used to fix the probe to the scull. After the surgery, rats were placed in 21×36×18 cm individual cages in which they remained throughout the experiment. Rats were given about 24 h for recovery and microdialysis procedure was conducted in awake freely moving animals. The microdialysis probe was connected to a syringe pump (World Precision Instruments, USA) and microfraction collector (CMA/142,

Sweden) and perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 0.2 mM NaH_2PO_4; pH 7.20–7.22) at a constant rate of 1.5 µl/min. Connections to the infusion pump and microfraction collector were made with flexible FEB-tubing (inner diameter 0.12 mm, AgnTho's AB, Sweden). After connecting the animal to the microdialysis system, the perfusate was discarded during the first 60 minutes to allow stabilization. Then 6 baseline samples were collected, followed by administration of D-amphetamine (0.5 mg/kg, i.p.; in animals with microdialysis probe in striatum or nucleus accumbens; Paper I) in the beginning of the collection of the 7th sample, after which another 9 samples were collected, or parachloroamphetamine (PCA) (2 mg/kg, i.p.; in animals with microdialysis probe in the PFC or DG; Paper II), after which another 18 samples were collected, or local administration of citalopram (1 µM; in animals with microdialysis probe in the PFC or DG; Paper II) by reverse dialysis for 2.5 h, after which another 14 samples were collected. The samples were collected in 15-min periods into vials prefilled with 7.5 µl of 0.02 M acetic acid (Paper I), or directly into a 50 µl loop of the electrically actuated injector (Cheminert C2V, Vici AG International, Switzerland) and injected automatically into the column in order to determine the quantity of 5-HT in the samples online by using HPLC with electrochemical detection (Paper II). In Paper I, the quantity of DA in the samples was determined by HPLC with electrochemical detection. The chromatography system consisted of Hewlett Packard series 1100 pump and autosampler, a Hypersil BDS C-18 column (250×2 mm, 5 µm), an ESA 5011 analytical cell (working electrode potential +250 mV) and an ESA Coulochem II controller unit. The column temperature was 30ºC. The mobile phase composition was 0.05 M sodium citrate buffer, pH 5.3, 0.02 mM EDTA, 3.1 mM sodium octylsuphonate, 9.5% acetonitrile. Upon completion of the experiment the animals were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) and decapitated; the brains were removed, immediately frozen in ice cold acetone and kept at –80ºC. The brains were sectioned on a cryostatic microtome (Microm GmbH, Germany) and probe placements were determined according to the atlas of Paxinos and Watson (1986). For statistical analysis, the data were expressed as a percentage of basal 5-HT values. Basal 5-HT values were calculated for every individual animal as the mean of the last three consecutive baseline samples before the start of drug administration.

3.3.4. Radioligand binding to serotonin transporter

In Paper V, the PFC and DG samples were collected from the right hemispheres of the animals that were decapitated and brains immediately dissected on ice. In Paper II, the PFC samples were collected from the right hemisphere (contralateral to the microdialysis site) of the frozen brains of the animals that had undergone

the microdialysis experiment immediately before the probe localization determination, while the DG samples were collected from the right hemisphere of naive animals that were decapitated and brains immediately dissected on ice. The cortical tissue were homogenized in 5 ml of homogenization buffer (50 mM Tris-HCl, pH=7.4) by Bandelin Sonopuls sonicator (three passes, 10 s each). The membrane fragments were collected by centrifugation at 30 000 × g for 20 min at 4˚C and washed by homogenization and centrifugation for two more times. The final pellet was resuspended in 100 vol (ww/v) of the incubation buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH=7.4). Binding of [N-methyl-^{3}H]Citalopram (75 Ci/mmol, Amersham Biosciences) was carried out by incubating membranes (1 mg of tissue per tube) in incubation buffer with different concentrations of radioligand (0.3–3.5 nM) for 60 min at 25˚C. Non-specific binding was determined in the presence of 1 μM nonradioactive fluoxetine. The reaction was stopped by rapid filtration through GF/B glass-fiber filters (Whatman Int. Ltd., presoaked with 0.3% polyethyleneimine before filtration) and the filters were washed three times with ice-cold incubation buffer. The filters were kept in 4 ml of scintillation cocktail (OptiPhase HiSafe3, Wallac Perkin Elmer Life Sciences, Cambridge, UK) overnight and counted using a RackBeta 1219 liquid scintillation counter (Wallac Inc., Gaithersburg, MD, USA). Raw data was analysed by means of a non-linear least squares fittings using GraphPad PRISMTM (GraphPad Software, San Diego, CA, USA) software.

3.3.5. D_2 receptor-stimulated [^{35}S]GTPγS binding

Membranes from nucleus accumbens and striatum were collected from animals that were decapitated and brains immediately dissected on ice. The final pellet of membranes were homogenized in 90 vol (ww/v, in the case of striatum) or 200 vol (nucleus accumbens) of the incubation buffer (20 mM K-HEPES, 7 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH=7.4). Binding of [^{35}S]-guanosine-5´-(γ-thio)-triphosphate ([^{35}SGTPγS]; Perkin Elmer Life Sciences, Boston, MA, USA) was carried out as described earlier (Rebec *et al*., 1997). In brief, the membranes (200 μg of accumbal and 500 μg of striatal tissue per tube) were incubated with 0.2 nM [^{35}S]GTPγS and different concentrations of GDP (3 mM – 1 μM) and 1 mM DA or 10 μM butaclamol (all from Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA) for 90 min at 30°C. The reaction was stopped by rapid filtration through GF/B glass-fiber filters and the filters were washed three times with 3 ml of ice-cold 20 mM phosphate buffer (pH=7.4) containing 100 mM NaCl. The radioactivity content of the filters was counted in 4 ml of scintillation cocktail with a RackBeta 1219 liquid scintillation counter. Raw data was analysed by means of a non-linear least squares fittings using GraphPad PRISMTM (GraphPad Software, San Diego, CA, USA) software.

3.3.6. RNA isolation, cDNA synthesis and quantitative real-time PCR

Six LE- and six HE-rats, not used in any experiments but for the behavioural selection, were decapitated and brains immediately dissected on ice. The dissected tissue samples were prepared for total RNA isolation and cDNA synthesis as described previously (Pruunsild *et al*., 2007). Levels of total BDNF and NGF mRNA were quantified with qPCR Core kit for SYBR® Green I No ROX (RT-SN10–05NR, Eurogentec, Belgium). All reactions were performed on LightCycler 2.0 thermocycler (Roche) using the following temperature cycling conditions: 10 min at 95°C (initial denaturation step), then 45 cycles of 5 s at 95°C, 10 s at 55°C and 10 s at 72°C. All PCR reactions were performed in triplicate and normalized to beta-actin (ACTB) mRNA levels. The following primers were used: BDNF_cod_s GGCCCAACGAAGAAAACCAT, BDNF_cod_as AGCATCACCCGGGAAGTGT, NGF_s TTGCCAAGGACG CAGCTTTCTA, NGF as CAACATGGACATTACGCTATGCA, ACTB_s ATGGAATCCTGTGGCATCCAT and ACTB_as CCACCAGACAGCACT GTGTTG. The Q-PCR data were expressed as BDNF or NGF mRNA levels relative to the reference ß-actin mRNA levels, with the expression level of 1.0 for a randomly selected sample.

3.3.7. Cytochrome c oxidase histochemistry and image analysis

The cytochrome c oxidase (COX) measurements were carried out as described previously (Kanarik *et al*., 2008). In short, the 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 was 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 with all the steps in 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 samples 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. 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. Eighty-nine regions of interest were detected from the stained images with the help of Paxinos and Watson (1986) rat brain atlas. Grayscale values were transformed to optical density values with the help of Kodak grayscale tablet with known grayscale and optical density values. The optical density of any given region was sampled and averaged from three consecutive slices of the same hemisphere in each brain but randomly from right or left hemisphere of different animals. The regions of interest were selected with a freehand selection tool covering the whole brain region, leaving out defected areas.

3.3.8. Preparation of blood samples and enzyme-linked immunosorbent assay for corticosterone measurement

Animals were decapitated and trunk blood was collected into pre-cooled tubes containing K3 EDTA. The blood samples were kept on ice and centrifuged after every 4 animals (4 000 × g for 10 min at room temperature). Plasma was pipetted into Eppendorf tubes and stored at –80°C until the assay. Plasma samples were thawed on ice and lightly vortexed and diluted 15 times. Plasma corticosterone was measured by ELISA (Correlate-EIA™, Assay Design, Inc; Ann Arbor, MI 48108, USA) according to manufacturer's directions. The sensitivity of this assay is 26.99 pg⁄mL. Upon completion of the assay, 96-well plates were read at 405 nm on a Labsystems Multiskan MCC/340 microplate reader.

3.4. Drugs and chemicals

In Experiment 1 of Paper III, each dose of neurotoxin DSP-4 (50 mg/kg, i.p.) was weighed separately, dissolved in distilled water and immediately injected one week before the behavioural experiments. In all experiments, CP-154,526 was suspended in distilled water by adding a few drops of Tween 85, and administered i.p. 30 min before behavioural observations, or at about the same time of day on days which did not include behavioural testing.

3.5. Statistics

In Paper I, data was analyzed with one (Experiments 2–6; *Exploration*) or two-factor ANOVA (Experiment 1; *Strain* × *Exploration*) with repeated measures, while data of elevated plus-maze and forced swimming tests obtained in Experiments 2 and 3 were merged for statistical analyses. In Paper II, microdialysis and 5-HT transporter (5-HTT) binding data were analysed with one-factor (*Exploration*) repeated measures ANOVA. Q-PCR data were analysed with a two-factor (*Exploration* × *Hemisphere*) ANOVA. In Paper III, data from the exploration box tests were analyzed with two-factor ANOVA (*Toxin* × *Treatment* in Experiment 1 and *Testing* × *Treatment* in Experiment 2) with repeated measures or one-factor (*Treatment*) repeated measures ANOVA in Experiment 2. Data from biochemical measurements were analyzed with two-factor ANOVA (*Toxin* × *Treatment* in Experiment 1 and *Testing* × *Treatment* in Experiment 2). In Paper IV, data for pre-selection measurements were analysed with unpaired *t*-test. The rest of the behavioural data of were analyzed with 2-factor ANOVA (*Exploration* × *Treatment*) with repeated testing. The monoamine data of Experiment 1 and corticosterone data of Experiment 2 were analysed with 2-factor ANOVA (*Exploration* × *Treatment*). In Paper V, the USV data was analyzed with one-factor (*Chirping*) ANOVA with repeated measures. The weight and behavioural data were analyzed with one-factor ANOVA (*Tickling* or *Chirping*) with repeated measures. The monoamine data were analyzed with two-factor ANOVA (*Tickling* × *Sex* or *Chirping* × *Sex*). In Paper VI, data were analyzed with a three-factor (*Sex* × *Stress* × *Chirping*) ANOVA for the elevated plus-maze behaviour and COX measurements, and with a three-factor (*Sex* × *Stress* × *Chirping*) ANOVA with repeated measures for the rest of data. In all Papers, when appropriate, the subsequent pair-wise comparisons were made with Fisher's LSD test. In correlative analyses Pearson correlation coefficients were used. Statistical significance was set at $P<0.05$ in all analyses. All statistics were made using StatView 5.0 software (SAS Institute Inc., Cary, NC, USA).

4. RESULTS AND DISCUSSION

4.1. Exploratory behaviour as a stable behavioural disposition

4.1.1. Stability of exploratory behaviour: high and low explorers

The exploration box test enables characterization of different alterations in exploratory behaviour elicited by anxiogenic, anxiolytic and antidepressant drugs and neurochemical manipulations (Harro *et al*., 1995; Matto *et al*., 1996; Otter *et al*., 1997). These alterations may be dependent on inter-individual differences in exploration (Alttoa *et al*., 2005). In the present studies, the persistency of the trait of novelty-related behaviour as expressed in exploratory activity was approached via repeated testing over longer periods of time in two different strains (Wistar and Sprague-Dawley) that are known to differ in their anxiety-related behavioural profiles as well as in sensitivity to antidepressant treatment (Tejani-Butt *et al*., 2003). It was found in both strains used that when repeatedly tested in the exploration box, the animals develop a characteristic level of activity in the apparatus (Figure 1). However, their activity during the first exploration box test was poorly predictive of subsequent exploratory behaviour. Repeated testing led to increasing correlation of the results with subsequently observed activities, the maximal accuracy reached by the third testing. However, already the second exposition to the test apparatus predicted the consequent behaviour of rats with acceptable accuracy. When animals were classified on this basis into HE- and LE-rats, the difference between groups was still observed in further experiments, including a second block of tests carried out one month later, and even as much as six months later (Experiment 4, Paper I). In the latter, the levels of activity decreased in HE group, which was probably due to age- and weight-related decrease in exploratory drive, while the already low levels of LE group show little if any change.

However, the difference between HE- and LE-rats was much more apparent in Wistar group, the patterns of exploratory activity over repeated testing being quite different in the two strains. In an entirely novel environment, Wistar rats were more neophobic and possibly anxious as compared to Sprague-Dawley rats. Sprague-Dawley animals initially seem to exhibit very high levels of motivation to explore, which is shortly followed by a decrease with repeated testing. It therefore suggests that assessing the behaviour in the exploration box using different rat strains enables studying different aspects of anxiety- and depression-related behaviours (like fear of novel environments and high or low motivation to explore novel areas).

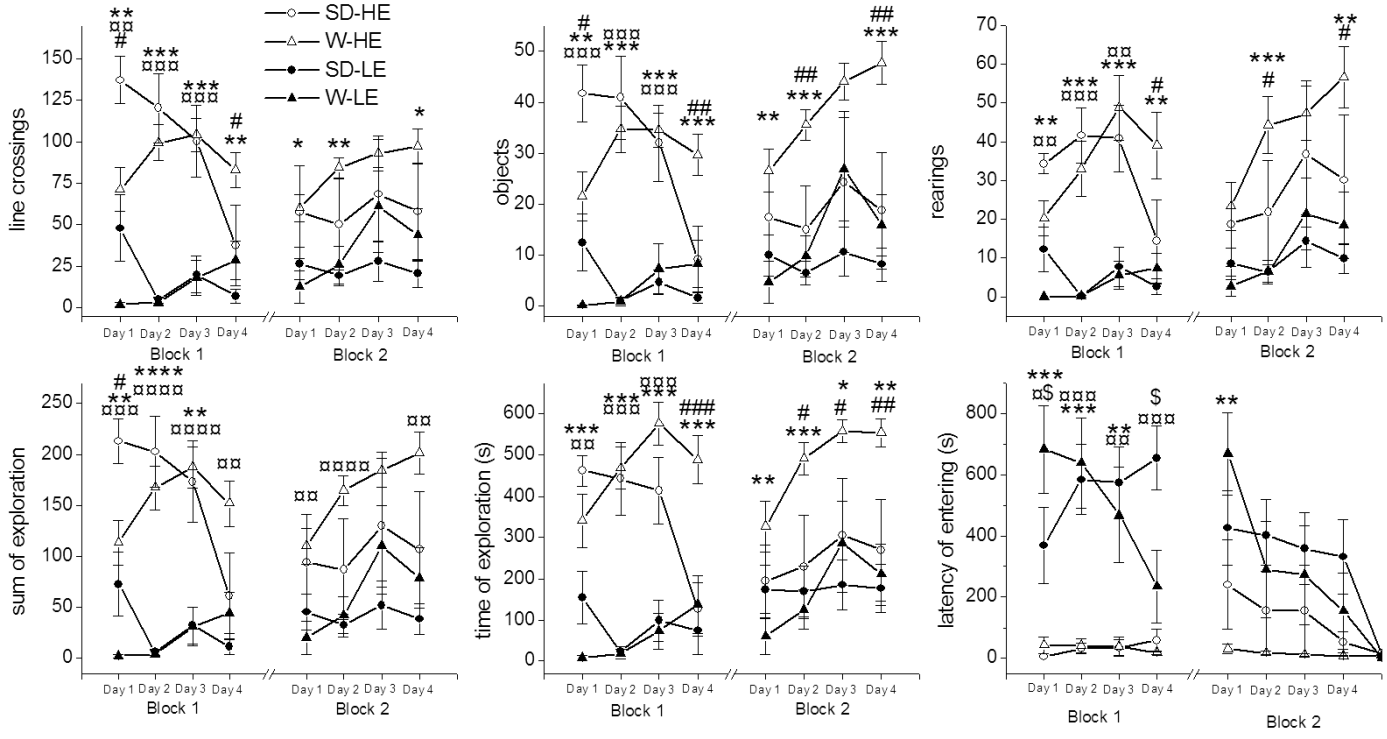

Figure 1. Exploratory behaviour during repeated testing in HE- and LE-rats of Wistar and Sprague-Dawley strains (Paper I). n(W-LE)=9; n(W-HE)=9; n(SD-LE)=9; n(SD-HE)=9. * – P<0.05, ** – P<0.01, *** – P<0.001 LE vs HE (Sprague-Dawley); ¤ – P<0.05, ¤¤ – P<0.01, ¤¤¤ – P<0.001 LE vs HE (Wistar); # – P<0.05, ## – P<0.01, ### – P<0.001 SD-HE vs W-HE; $ – P<0.05 SD-LE vs W-LE. SD – Sprague-Dawley; W – Wistar; HE – high exploratory behaviour rats; LE – low exploratory behaviour rats. Data are presented as mean ± SEM.

4.1.2. Behavioural differences in high and low explorers in tests used in anxiety and depression research

When the HE- and LE-rats were compared in various tests that have traditionally been used to measure different aspects of emotionality in studies on anxiolytic and antidepressant drugs, significantly different behavioural profiles were found.

When tested in a cage similar to home cage, no differences were found between HE and LE animals (Paper I), suggesting that the HE/LE differentiation is specific to the exploration box and not to more familiar environments. The important aspect of exploration box behaviour is probably the emergence of the animal from the small compartment attached to the open part of the apparatus.

The elevated plus-maze is one of the most widely used test paradigms to investigate anxiety-related behaviour (Carobrez *et al*., 2005). The present experiments show higher anxiety levels in elevated plus-maze in rats with low activity in exploration box test (Figure 2), and while the decrease in activity described by File (1993) on trial 2 is visible in both groups, the LE animals remain the less active group even then. Liebsch *et al*. (1998) have shown that in animals bred on the basis of their high or low anxiety-related behaviour in the plus-maze (HAB and LAB, respectively), the former were less active in the black-white box and open field test. It might therefore be concluded that the exploration box test and elevated plus-maze test measure partly the same construct.

No differences were observed in social activity between HE and LE animals (Paper I). Social interaction time has often been shown to be in inverse relation with anxiety levels (File and Seth, 2003), and Henniger *et al*. (2000) have found that rats, selectively bred for differences in anxiety-related behaviour in the elevated plus-maze, show consistent differences in social interaction test, with animals with high anxiety-related behaviour of both sexes being less active in both tests. In our previous studies we have found that sociability – measured with successive social interaction testing – is a stable trait, with an animals' social behaviour in each single test correlating highly with its mean social behaviour level (Tõnissaar *et al*., 2004). The present findings suggest that different mechanisms govern anxiety-related behaviours in those two different situations that are motivating and at the same time anxiety-provoking – namely a novel environment and a novel partner.

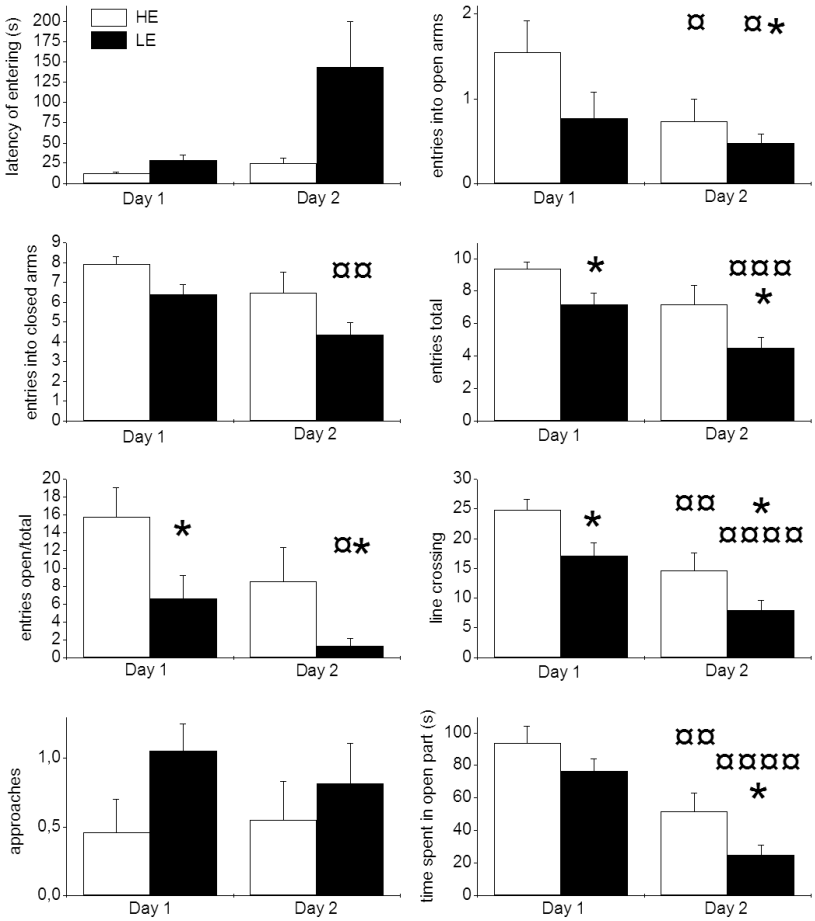

Figure 2. Plus-maze behaviour in HE- and LE-rats (Paper I). n(LE)=18; n(HE)=14. * – P<0.05 vs HE; ¤ – p<0.05, ¤¤ – p<0.01, ¤¤¤¤ – P<0.0001 vs Day 1. HE – high exploratory behaviour rats; LE – low exploratory behaviour rats. Bars represent mean ± SEM.

In the fear-conditioning paradigm, animals relate a formerly neutral environment or other cue to a stressful event and develop anxiety towards it. HE animals showed higher freezing levels on the first test day (Figure 3), suggesting higher proneness to develop anxiety in response to acute stressors in HE-rats. Nevertheless, the extinction rate was faster in this group as the decrease in freezing between the two test days was greater in the HE group, suggesting more active coping style. In contrast, Borta *et al*. (2005) have found

in previous experiments that rats selected for their low or high amount of time spent on the open arms of the elevated plus-maze also display differences in acute and conditioned responses in a fear conditioning paradigm – namely, rats with low levels of time spent in the open arms of the plus-maze showing more freezing and emitting more distress calls.

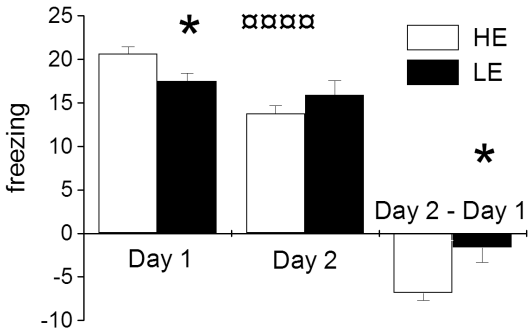

Figure 3. Freezing in fear conditioning test in HE- and LE-rats (Paper I). n(LE)=8; n(HE)=8. * – P<0.05 vs HE; ¤ – P<0.05 vs Day 1. HE – high exploratory behaviour rats; LE – low exploratory behaviour rats. Bars represent mean ± SEM.

Taghzouti *et al*. (1999) have shown a significant negative correlation (r=–0.61) between novelty-related behaviour in a novel circular corridor and immobility time on the second day of the forced swimming test, with high responders to novelty being less immobile and swimming more. Our present studies give a basically similar result, with HE animals showing less immobility and more swimming on Day 2 (Figure 4), indicative of more active coping strategies in the HE group.

The sucrose preference test has been used as a measure of anhedonic tendencies in stress and depression models. In the present experiments, the LE-rats consumed more sucrose solution (Paper I). In the light of previous findings (Harro *et al*., 2001b), which suggest that similarly to cases of atypical depression in clinical psychiatry in which both decrease and increase in body weight are considered as symptoms of depression, the higher levels of consumption of sweet solution in LE-rats might also be interpreted as a result of a negative affect. Nevertheless, in the second experiment on sucrose preference reported in Paper I, the differences waned off after 18 h of food and water deprivation, as the increase of sucrose intake after the fast was significantly greater in HE animals. This could be indicative of differences in reactivity to short-term stress like food deprivation between the HE- and LE-rats, or perhaps just of differences in attaining and maintaining individual energetic balance in these two groups.

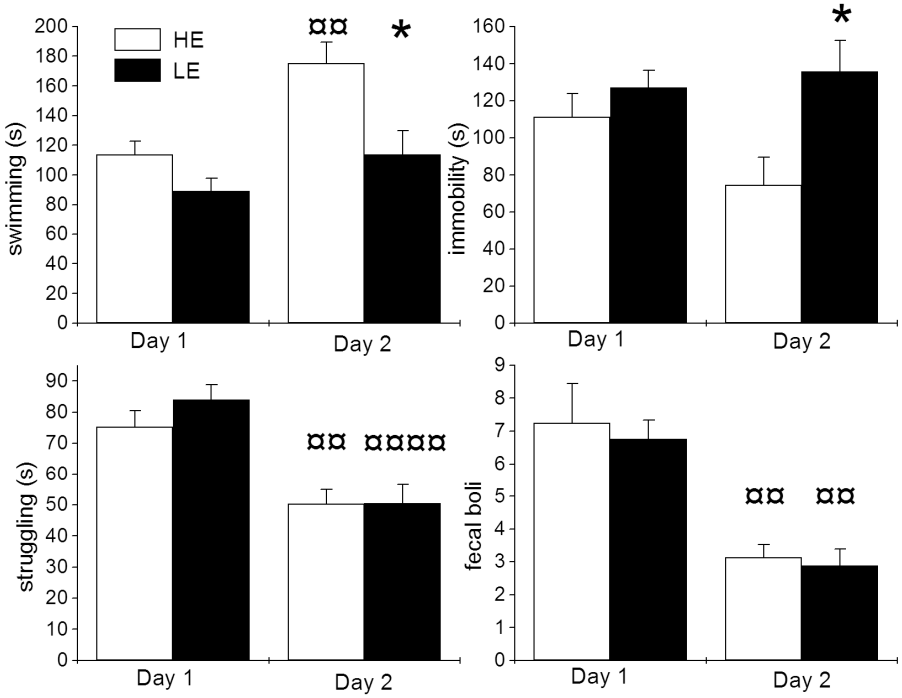

Figure 4. Forced swimming test in HE- and LE-rats (Paper I). n(LE)=18; n(HE)=14. * – P<0.05 vs HE; ¤ – P<0.05, ¤¤ – P<0.01, ¤¤¤¤ – P<0.0001 vs Day 1. HE – high exploratory behaviour rats; LE – low exploratory behaviour rats. Bars represent mean ± SEM.

4.1.3. Neurobiological differences in high and low explorers

4.1.3.1. Serotonin transporter

The LE-rats were found to have higher levels of 5-HTT in the prefrontal cortex, but not in hippocampus (Figure 5). Giorgi *et al*. (2003) have previously reported higher levels of [^{3}H]-citalopram binding to 5-HT reuptake sites and greater increase in 5-HT availability in the frontoparietal cortex elicited by local application of chlorimipramine and fluoxetine in Roman high-avoidance rats, that also have been found to be more anxious in the elevated plus-maze and light/dark compartment test (Chaouloff *et al*., 1994), while the release of 5-HT was similar at baseline conditions with the low-avoidance rats. Parenthetically, it should be noted that the above mentioned high-avoidance rats are also more susceptible to drugs of abuse than the low-avoidance rats (Giorgi *et al*., 2007),

and this may also be the case for our LE-rats which develop sensitization to a low dose of amphetamine that did not elicit this behavioural change in HE-rats (Alttoa *et al*., 2007). It is noteworthy that similarly, in studies on patients with bipolar disorder, 5-HTT levels have been found to be increased in medial prefrontal cortex, but not in hippocampus (Cannon *et al*., 2006). Petty *et al*. (1994) have shown that after exposure of rats to tail-shock stress, 5-HT release in the medial frontal cortex correlates significantly with subsequent helpless behaviour, although the basal 5-HT levels did not. It therefore seems that the increased reuptake of 5-HT in prefrontal cortical areas may develop in anxious subjects to reduce behavioural consequences of aversive stimuli. On the basis of these results, microdialysis studies were carried out to assess the 5-HT system in vivo.

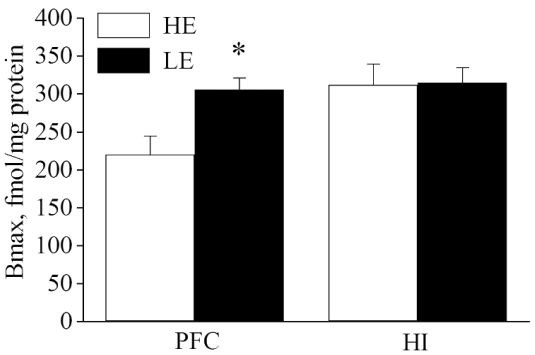

Figure 5. 5-HTT levels measured with [^{3}H]citalopram binding in membranes of the medial prefrontal cortex (PFC) and hippocampus (HI) of HE- and LE-rats (Paper II). * – $P<0.05$ compared to the HE group. HE – high exploratory activity rats (open bars, n=6 and 7 respectively in PFC and HI); LE – low exploratory activity rats (filled bars, n=6 in both regions).

4.1.3.2. Extracellular serotonin and dopamine

Differences in the expression of anxiety in animals bred or pre-selected on the basis of anxiety-related behaviour are accompanied by differences in neurochemistry of amino acids, monoamines and neuropeptides (Harro *et al*., 1990; Landgraf, 2005; Rägo *et al*., 1988; Singewald, 2007). Particular interest has been paid to the 5-HT-ergic function in PFC and hippocampus (Giorgi *et al*., 2003; Keck *et al*., 2005; Pollier *et al*., 2000). The 5-HT-ergic system has been proven to play a role in anxiety and emotional reactivity in animals and in corresponding animal models of human disorders (for a review, see Griebel, 1995). 5-HT-ergic pathways innervating such brain regions as the frontal cortex, amygdala, hypothalamus and hippocampus have been found to be

activated by anxiogenic stimuli, including psychosocial stress, conditioned fear and conflict procedures (for a review see Millan, 2003). The relationship between 5-HT-ergic activity and the affective state of the animal is not always straightforward, as it has also been shown that an increase in 5-HT-ergic activity in different forebrain regions may be linked simply to general behavioural activity (Rueter and Jacobs, 1996). Both reduced as well as increased 5-HT-ergic neurotransmission have been associated with negative emotionality (see Tõnissaar *et al*., 2004 and references therein). The prefrontal cortex has widespread influences on multiple components of forebrain circuits regulating anxiety states and anxiety-related behaviour. The hippocampal 5-HT-ergic system has been acknowledged to mediate an anxiogenic response (for an example, see File *et al*., 2000), and dysfunction in hippocampal region, especially in DG has been ascribed an important role in stress reactions (Gould et al., 1997). With regard to animals pre-selected in the exploration box test, different effects on spontaneous, amphetamine-stimulated (Alttoa *et al*., 2005) and amphetamine-sensitized (Alttoa *et al*., 2007) behaviour have been reported in high- and low-exploring rats with selective denervation of locus coeruleus NA-ergic projections. DSP-4 treatment also decreased the ex vivo 5-hydroxyindoleacetic acid (5-HIAA) levels in the nucleus accumbens and striatum in LE-rats only. We have found higher levels of metabolic activity in dorsal raphe in LE animals (Matrov *et al*., 2007) that are probably connected with their passive coping style.

The baseline levels of 5-HT release in PFC and DG were similar in HE- and LE-rats, and no differences were detected in PCA-induced depolarization-independent 5-HT release in these regions (Paper II). After local infusion with citalopram, the increase in extracellular 5-HT levels in LE group was greater in the PFC (Figure 6). It is possible that the firing rate of the 5-HT-ergic projections to the PFC is higher in LE-rats, resulting in higher extracellular 5-HT levels after blockade of 5-HTT, while at baseline conditions the greater release of 5-HT is balanced by the increased re-uptake in the LE-rats that we found to have higher levels of 5-HTT in this region.

Extracellular DA levels in dorsal striatum in baseline conditions as well as in response to amphetamine administration were significantly higher in HE animals than in LE animals (Figure 7), while no difference was detected in the nucleus accumbens. We have previously shown that acute administration of a low dose of amphetamine induces a similar proportional increase in locomotor activity in HE- and LE-rats with the activity levels of the HE-rats remaining significantly higher as compared to the LE animals (Alttoa *et al*., 2005). Also, higher basal and evoked DA release has been reported in animals with higher locomotor activity levels (Hooks *et al*., 1992). It is therefore possible that absolute differences in the activity of midbrain DA-ergic circuits are at least partly responsible for the differences in novelty-related behaviours in those two groups.

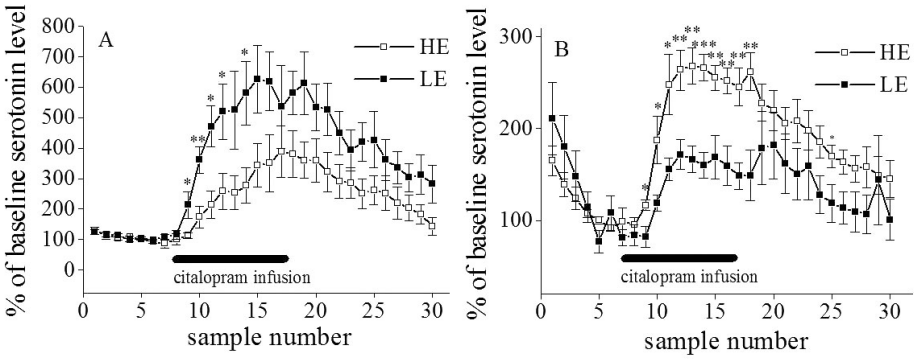

Figure 6. Extracellular serotonin levels in the medial prefrontal cortex (A) and dentate gyrus (B) of HE- and LE-rats, after local infusion of citalopram (Paper II). Samples were collected every 15 min and are presented as percentage of baseline levels (mean of samples 4.–6.). Infusion with 1 μM solution of citalopram was made during the collection of samples 7.–16. * – $P<0.05$; ** – $P<0.01$; *** – $P<0.0001$ difference between HE and LE groups. HE – high exploratory activity rats (open squares, n=6 and 7, respectively in PFC and HI); LE – low exploratory activity rats (filled squares, n=6 in both regions). Data are presented as mean ± SEM.

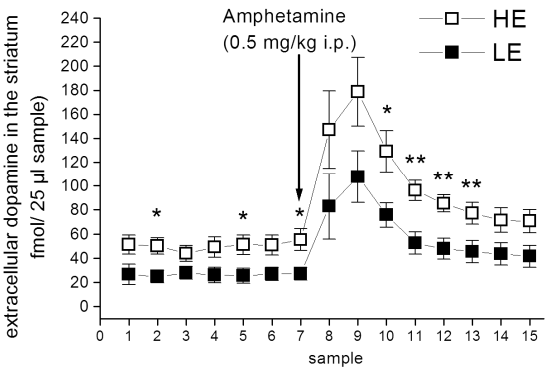

Figure 7. Dopamine release in the dorsal striatum in response to amphetamine administration in HE- and LE-rats (Paper I). n(LE)=6; n(HE)=6. * – P<0.05, ** – P<0.01 vs LE). HE – high exploratory behaviour rats; LE – low exploratory behaviour rats; i.p. – intraperitoneal. Data are presented as mean ± SEM.

4.1.3.3. Brain-derived neurotrophic factor

Previously it has been proposed that 5-HTT-mediated increases in 5-HT-ergic neurotransmission upregulate BDNF expression via increases in cAMP response element binding protein phosphorylation (Duman *et al*., 1997 and 1999; Nibuya *et al*., 1996). In PFC of LE-rats, the levels of BDNF mRNA were found to be higher as compared to HE-rats, suggesting that BDNF gene expression is increased on the level of transcription or mRNA stability (Figure 8). NGF mRNA levels were found not to be different in these two groups. This, combined with the higher levels of extracellular 5-HT in LE-rats after citalopram infusion, fits with the findings of BDNF going hand in hand with increased 5-HT availability (Altar, 1999), possibly through a 5-HTT-controlled process. Nevertheless, it should be acknowledged that our results are not indicative of any direct association between BDNF and 5-HTT expression.

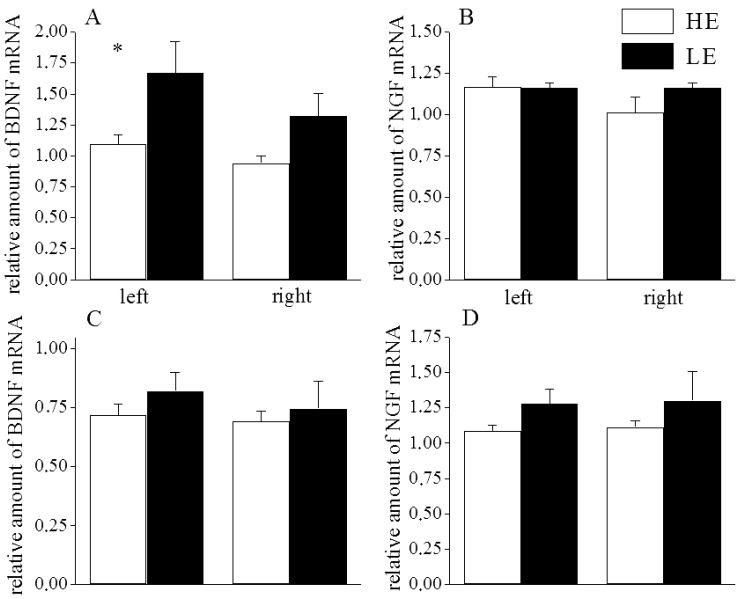

Figure 8. Relative levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) mRNA in prefrontal cortex (PFC) and hippocampus (HI) of HE- and LE-rats separately in left and right hemisphere (Paper II). A) BDNF in PFC; B) NGF in PFC; C) BDNF in HI; D) NGF in HI. BDNF and NGF mRNA were normalized to ß-actin. * – difference versus respective HE or LE group. HE – high exploratory activity rats (open bars, n=5–6); LE – low exploratory activity rats (filled bars, n=5–6). Data are presented as mean ± SEM.

The results contradict with the common BDNF hypothesis of depression that predicts decreased levels of BDNF mRNA in depression-related brain areas (Licinio and Wong, 2002). However, this view is already questioned, as bulbectomized mice that have depressed-like phenotype have recently been found to have significantly increased levels of BDNF protein in hippocampus and frontal cortex (Hellweg *et al*., 2007). Also, in two rat models of depression-like states, no differences as compared to controls or even an upregulation of BDNF has been found in several brain areas (Angelucci *et al*., 2004; Vollmayr *et al*., 2001). These results suggest that the role of this neurotrophic factor in mood disorders is more complex than originally thought. For example, the possibility has to be considered that the change in BDNF levels in rodent models of anxiety and depression might indicate an attempt towards neurochemical adaptation that remains unsuccessful in behavioural regulation.

4.1.4. CRF_1 receptors and exploratory behaviour: drug-environment interactions and inter-individual differences

CRF_1 receptor knock-out mice have been found to display a reduced anxiogenic response (Contarino *et al*. 1999; Timpl *et al*. 1998). Acutely administered selective nonpeptide CRF_1 antagonists have been found to display only limited or no potency in exploration-based anxiety models (Griebel *et al*., 1998; Lundkvist *et al*., 1996; Okuyama *et al*., 1999). Nevertheless, CRF_1 antagonists produce a downright anxiolytic-like activity in animal models involving inescapable stress, conflict procedures, social defeat-induced anxiety and the mouse defense test battery (Griebel *et al*., 2002; Mansbach *et al*., 1997). CRF_1 antagonists have also been found to block the anxiogenic effects of CRF in the elevated plus-maze, without affecting anxiety-like behaviour in a vehicle-pretreated group (Okuyama *et al*., 1999; Zorrilla *et al*. 2002) when administered acutely. Chronic treatment with CRF_1 receptor antagonists also has significant anxiolytic and antidepressant effects, with decreases in defensive withdrawal behaviour (Arborelius *et al*., 2000) and inhibition of olfactory bulbectomy-induced hyperemotionality (Okuyama *et al*., 1999).

4.1.4.1. Effect of previous denervation of locus coeruleus *projections and CRF_1 receptor blockade with contingent behavioural testing on exploratory behaviour*

In the present study, a clear anxiolytic effect of repeated administration of the CRF_1 antagonist (CP-154,526) was found on exploratory behaviour when the drug was daily administered for five consecutive days of behavioural testing (Figure 9). CRF_1 receptor blockade did not have an acute anxiolytic effect on

exploratory behaviour, which is similar to what has been found with another selective antagonist, CRA1000 (Harro *et al*., 2001a). Selective destruction of NA-ergic projections with neurotoxin DSP-4 significantly reduced all measures of exploratory activity and repeated administration of CP-154,526 attenuated this effect, although the drug effects were less pronounced than in control group. Stress, as well as intra-ventricular and intra-coerular injections of CRF have been found to increase the discharge rates of locus coeruleus neurons in rats (Valentino *et al*., 1983; Curtis *et al*., 1997; Lejeune and Millan, 2003) and increase NA release in PFC (Curtis *et al*., 1997). CRF_1 receptor antagonists have been found to counteract the activation of locus coeruleus neurones by intracerebroventricular administration of CRF (Schulz *et al*., 1996; Okuyama *et al*., 1999; Lejeune and Millan, 2003) or stress (Kawahara *et al*., 2000; Griebel *et al*., 2002). The anxiolytic effects of CRF_1 blockade have also been found to depend on the inherent anxiety levels in rats (Keck *et al*., 2001). The present results suggest that the NA-ergic projections are involved in the anxiolytic-like effect of CRF_1 receptor blockade.

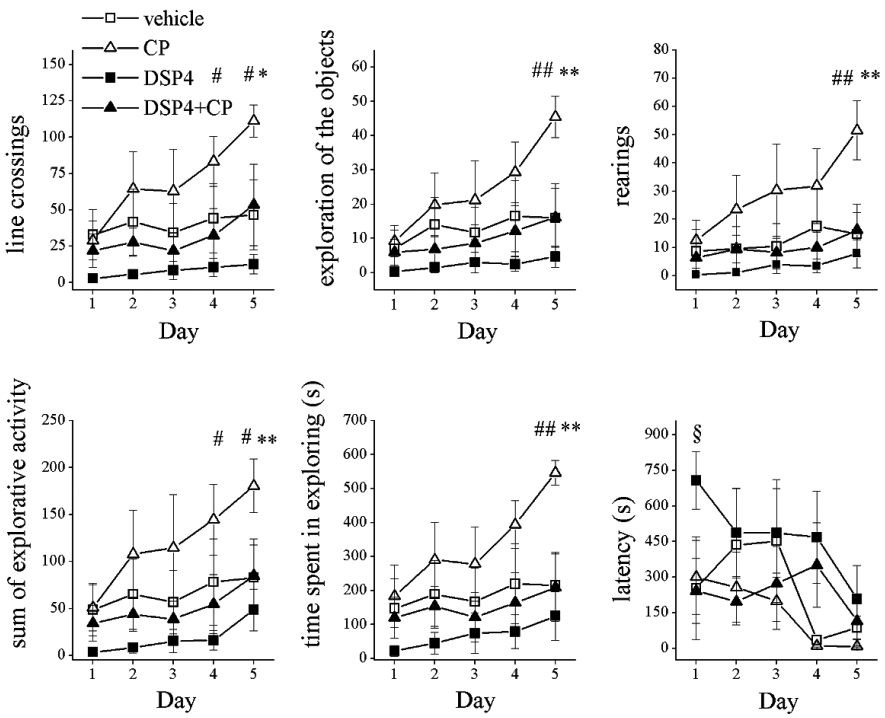

Figure 9. The effect of DSP-4 pretreatment and CP-154,526 (2.5 mg/kg) on behaviour in exploration box on five consecutive days (Paper III). * – $p<0.05$, ** – $p<0.01$ vehicle vs. CP-154,526; # – $p<0.05$, ## – $p<0.01$ CP-154,526 vs. CP-154,526 + DSP-4; § – $p < 0.05$ – DSP-4 vs. DSP-4 + CP-154,526. Data are presented as mean ± SEM.

4.1.4.2. Effect of CRF_1 receptor blockade with non-contingent behavioural testing

Six days of administration of the CRF_1 receptor antagonist had a clear anxiolytic-like effect in the elevated zero-maze on the risk assessment measure "stretched-attend postures" (Figure 10) which has been considered to be a sensitive criterion in measurement of the anxiogenic or anxiolytic action of a drug (Dawson and Tricklebank, 1995). This suggests that repeated administration of CP-154,526 in the dose of 2.5 mg/kg had an anxiolytic effect also in this experiment. On the other hand, no anxiolytic effects of CP-154,526 administration were revealed in the exploration box test, when the daily drug administration was followed by behavioural testing on days 1, 5 and 12 only (Figure 11). On the contrary, chronic administration of CP-154,526 blocked the increase in exploration with time that was present in the vehicle group. In rats that were tested in the exploration box test only on the 12th day of drug administration, no difference was found between drug and vehicle groups and neither with repeatedly tested vehicle group (Paper III). The increase in exploration in the vehicle group and similar levels of activity in the groups that were tested only on day 12 were in the former group probably due to habituation with the test apparatus and in all groups due to daily handling, which has previously been shown to increase exploration in rats (Reboucas and Schmidek, 1997; Harro *et al*., 2001a). This suggests that drug-environment interaction is important in the manifestation of anxiolytic effects of CRF_1 receptor blockade and repeated testing under the influence of CRF_1 receptor blockade prevents habituation-induced increase in exploration.

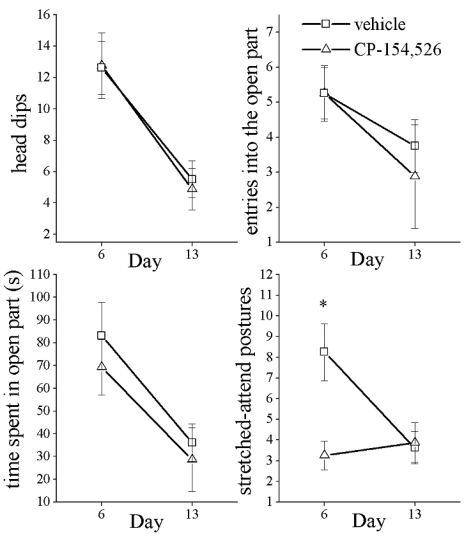

Figure 10. The effect of CP-154,526 (2,5 mg/kg) on behaviour in zero-maze (Paper III). * – $p<0.05$ vs. vehicle. Data are presented as mean ± SEM.

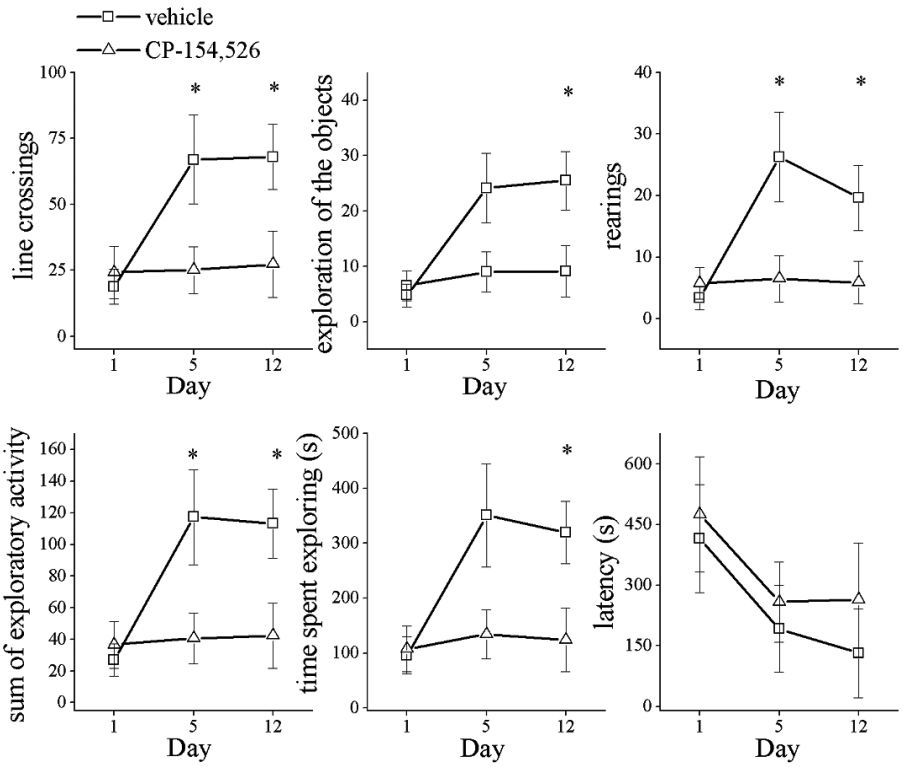

Figure 11. The effect of chronic administration of CP-154,526 (2.5 mg/kg) on changes in behaviour in the exploration box (Paper III). * – $p<0.05$ vs vehicle. Data are presented as mean ± SEM.

4.1.4.3. The influence of previous experience with the test apparatus and inter-individual differences in exploratory activity on CRF_1 receptor blockade

In HE animals that had been tested for pre-selection twice in the exploration box test before the start of drug administration, CP-154,526 decreased exploratory behaviour on first administration but after 12-days administration with non-contingent behavioural testing there was no difference between drug- and vehicle-treated groups due to an increase in activity in the former group (Figure 12). No drug effect was evident in LE animals. In rats that had been tested for pre-selection in a version of the light-dark box test before the start of drug administration and hence had no previous experience of the exploration box apparatus, CRF_1 blockade decreased exploratory behaviour on first administration in both HE and LE animals (Figure 13). In drug-treated HE-rats

activity levels increased to higher levels than in vehicle-treated animals after 5 days of administration and remained so also after 10 days. In LE-rats the drug still had an activity-decreasing effect after 5 days of non-contingent administration and no effect after 10 days.

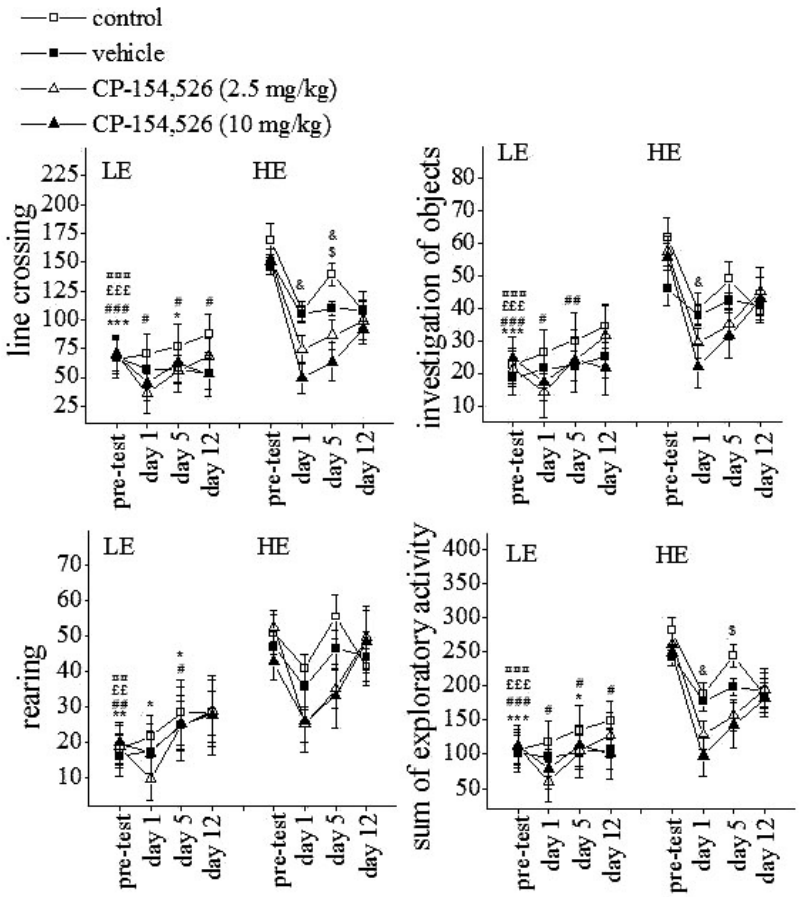

Figure 12. The effect of CP-154,526 on exploratory behaviour in animals pre-tested in the exploration box test (Paper IV). * – p<0.05, ** – p<0.01; *** – p<0.001, as compared to the respective HE control; # – p<0.05; ## – p<0.01; ### – p<0.001, as compared to the respective HE vehicle; ££ – p<0.01; £££ – p<0.001, as compared to the respective HE CP-2.5 mg/kg; ¤¤ – p<0.01; ¤¤¤ – p<0.001, as compared to the respective HE CP-10 mg/kg; $ – p<0.05, veh vs control group; & – p<0.05, CP-10 mg/kg as compared to respective vehicle group. CP 2.5 – CP-154,526 2.5 mg/kg group; CP10 – CP-154,526 10 mg/kg group; HE – high exploratory behaviour rats; LE – low exploratory behaviour rats; pre – second baseline exploratory activity measurement. Data are presented as mean ± SEM.

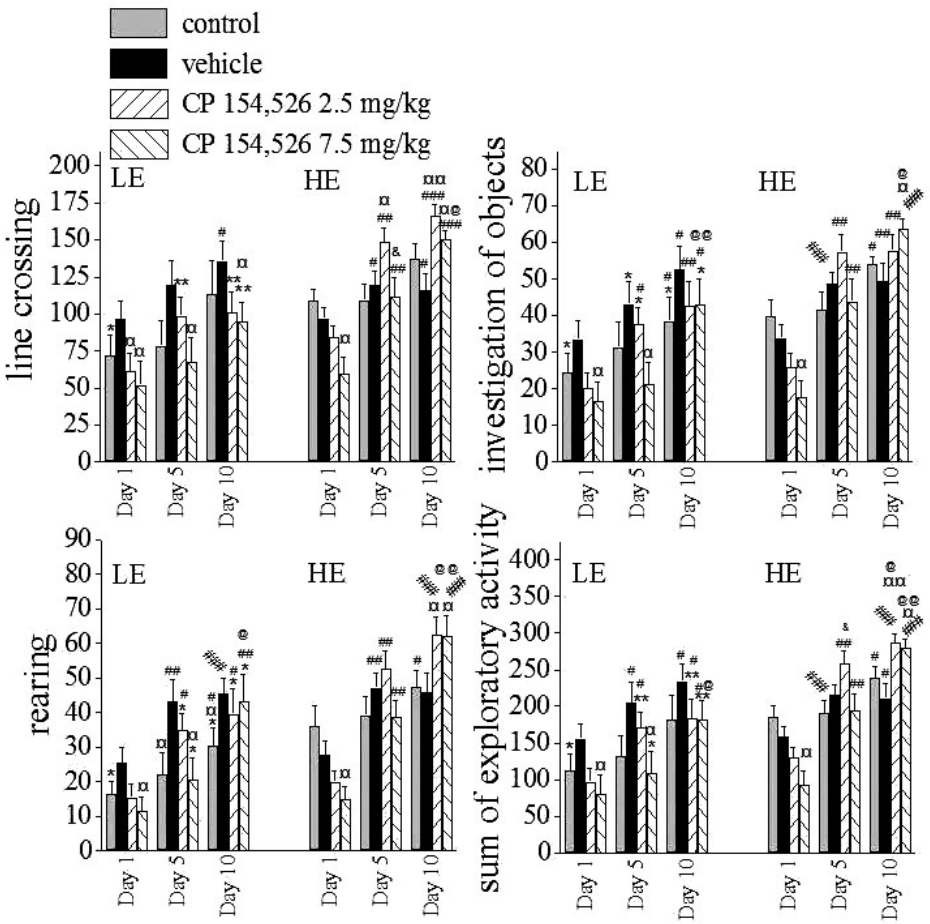

Figure 13. The effect of CP-154,526 on exploratory behaviour in animals pre-tested in the light-dark test (Paper IV). * – $p<0.05$, ** – $p<0.01$, as compared to respective HE group; $ – $p<0.05$, $$ – $p<0.01$ as compared to respective control group; ¤ – $p<0.05$, ¤¤ – $p<0.01$ as compared to respective vehicle group; & – $p<0.05$ as compared to the respective CP-154,526 7.5 mg/kg group; # – $p<0.05$, ## – $p<0.01$, ### – $p<0.001$ as compared to Day 1; @ – $p<0.05$, @@ – $p<0.01$ as compared to Day 5. HE – high exploratory behaviour rats; LE – low exploratory behaviour rats. Data are presented as mean ± SEM.

Blockade of CRF_1 receptors has been found to have an anxiolytic effect in many studies (reviewed in Seymour *et al*., 2003), but the effects are most clearly expressed in previously stressed animals, while the results are more variable in animals with no pretreatment. The stress elicited by injection procedure in the present studies probably caused the decrease in activity levels in surroundings

not associated with such aversive stimuli in vehicle-treated rats, as vehicle treatment has been found to act as a significant stressor in rats (Kondashevskaya and Nikolskaya, 2004). CRF_1 blockade aggravated this effect. It may be that in the context where the animals were desensitized to handling and testing during the pre-testing session via repeated contacts with the experimenter and test conditions (which may in part be mediated by adaptive changes in the CRF-ergic systems), the disruption in the acquired balance by CRF_1 blockade results in an effect diametrically opposite to the reported anxiolytic effects of handling (Rebouças and Schmidek, 1997). Probably the HE animals are more sensitive to such manipulations. White *et al*. (2007) have reported that animals with high motor response to novelty show less anxiety in the elevated plus maze and defensive withdrawal tests, but more anxiety in the acoustic startle-induced vocalization test, suggesting higher reactivity to stressful stimuli in this group. It has also been reported that previous testing experience affects consequent behaviour in other tests of anxiety differently in animals with different inherent levels of novelty seeking (Ballaz *et al*., 2007). This is in good accordance with the finding that in LE animals with previous experience with the test apparatus, acute CRF_1 blockade did not affect exploratory activity, while in LE-rats with no such experience the drug effects were similar to the HE group. These results suggest disparities in adaptation to the environment in subgroups of animals that depend on both the inherent level on anxiety as well as on specific features of the testing procedure that may have either anxiolytic, anxiogenic or no effect on subsequent behaviours. The aggravation of the injection stress effect in the HE animals after acute CRF_1 receptor blockade suggests that an intact CRF-system is needed particularly in HE-rats for adequate coping with acute stressors. The similar gradual patterns of increasing activity in drug groups that were similar to the anxiolytic effects of repeated administration of CP-154,526 in unselected animals suggest that on repeated administration the CRF_1 receptor antagonist had an anxiolytic effect in HE animals, while the previous drug-free experience of the test apparatus delayed this effect. This relates quite well to the fact that while CRF increases behavioural activity in a familiar environment, low doses of CRF may also increase locomotor activity in a novel environment (Sutton *et al*., 1982), suggesting that an intact CRF system is needed for coping in a familiar surrounding in HE animals in particular. The long-term CRF_1 blockade did not have an anxiolytic effect in the LE-rats, suggesting that the neuro-chemical basis of higher anxiety in this group is less affected by CRF-ergic activity.

4.1.5. Effect of CRF_1 receptor blockade on neurobiological measures

4.1.5.1. Ex vivo monoamines

The administration of CP-154,526 (2.5 mg/kg) for five days did not have any significant effect on monoamines in the PFC (Paper I), which is in concordance with a similar finding with CRA1000 (Harro *et al*., 2001a). Also, acute i.p. and intra-coerulear administration of CP-154,526 has previously been found not to influence monoamine levels in freely moving rats (Millan *et al*., 2001, Kawahara *et al*., 2000). Nevertheless, there was a tendency of an increase in 5-HT turnover in the prefrontal cortex in vehicle but not in DSP-4-pretreated rats after CP-154,526 in the present study.

In the experiment with selected animals and 12-days administration of CP-154,526, ex vivo DA levels were found to be lower in PFC and striatum in the HE control group as compared to all other groups, and 12-days treatment with CP-154,526 decreased DA turnover in PFC and DA levels in striatum in this group (Paper I). The higher levels of striatal DA are in good accordance with the higher extracellular DA levels in this region in HE animals. Previously, acute blockade of D_1 receptors has been found to reduce CRF-induced increases in startle amplitude (Meloni *et al*., 2006) suggesting that DA-ergic systems are at least partly responsible for the mediation of CRF-related responses to stressors. Acute administration of CP-154,526 has been found not to affect extracellular DA levels in PFC (Isogawa *et al*., 2000), but a 10-days CRF_1 blockade with antalarmin has been reported to reverse the isolation rearing-increased D_2 receptor density in the central amygdala and nucleus accumbens (Djouma *et al*., 2006). Together with the results presented herein, an interplay of mutual influences between the CRF and DA systems may be suggested that has greater role in the regulation of exploratory activity in HE-rats.

The 12-days administration of the drug also increased 5-HIAA and 5-HT turnover in PFC and decreased these measures in hippocampus in HE animals. Dorsal raphe 5-HT-ergic neurons are innervated by CRF-immunoreactive fibers and the administration of CRF has inhibitory effects on dorsal raphe discharge (Kirby *et al*., 2000). It has also been found that the electric footshock stress-induced increases in 5-HT turnover in several brain regions are further elevated by the administration of the nonselective CRF antagonist alpha-helical $CRF_{9–41}$ (Li *et al*., 1998). It might hence be that in HE-rats CRF has a stronger inhibitory effect on 5-HT neurons. Conversely, in the hippocampus that is mainly innervated by 5-HT-ergic projections from the median raphe, CP-154,526 dose-dependently reduced 5-HIAA and 5-HT in HE-rats only. Although straight-forward associations have been drawn between the activity of 5-HT-ergic systems and anxiety-related behavioural states (Griebel, 1995), it nevertheless needs to be pointed out that while CRF_1 receptor blockade eliminated the

differences between HE and LE animals in 5-HT measures in the present study, it did not attenuate behavioural differences. This suggests that the differences in 5-HT-ergic system do not directly form the basis of the baseline behavioural differences between HE and LE animals, but rather differently modify the patterns of habituation in an increasingly familiar environment.

4.1.5.2. Transcription factor AP-2

In the present studies, neither DSP-4 nor CP-154,526 had any significant effect on the levels of transcription factor AP-2 isoforms in the locus coeruleus area in the unselected animals (Paper I). A positive correlation between AP-2 levels in the locus coeruleus area and PFC NA levels was found in vehicle-treated animals, and a similar trend was found in the hippocampus (Figure 14). Yet, there was a negative correlation between hippocampal NA content and AP-2 levels in naïve animals, and in animals with chronic CRF_1 receptor blockade, and similar trends with regard to PFC NA. Thus, it seems that in naïve rats there is a negative association between AP-2 and NA, and that CRF_1 receptor blockade reverses the handling stress-induced change in the associations between locus coeruleus AP-2 levels and brain monoamine content that has also been previously reported in animals that had been subjected to various manipulations (Damberg *et al*., 2001b). The present finding agrees with the notion that CRF_1 receptor antagonists are able to counteract the changes in the brain monoaminergic systems caused by different stressors (Seymour *et al*., 2003).

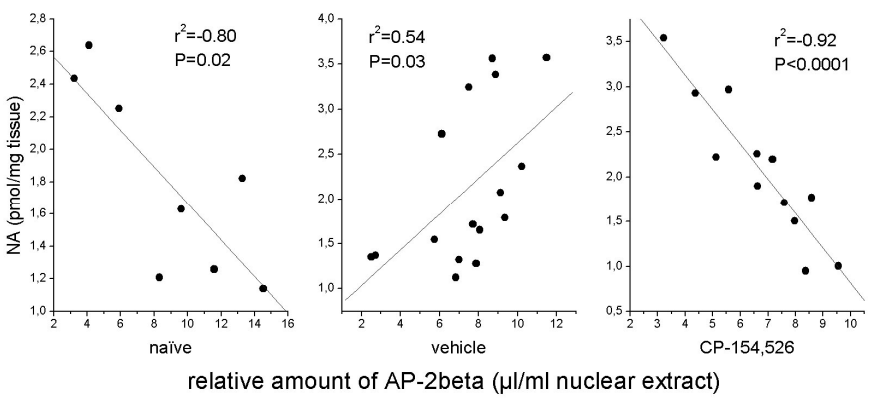

Figure 14. Effect of chronic administration of vehicle and CP-154,526 (2.5mg/kg) on the association of AP-2ß levels in the locus coeruelus area and noradrenaline levels in the hippocampus (Paper I).

The changes in transcriptional processes mediated by AP-2 may be a significant mechanism of adaptation with stress in the locus coeruleus region, although the exact mechanism by which these dynamic associations between AP-2 levels and NA-ergic systems are executed remains to be thoroughly discussed. AP-2 has been identified co-localizing with tyrosine hydroxylase in NA-ergic neurons and co-regulating the promoter activities of the tyrosine hydroxylase and DA β-hydroxylase genes (Kim *et al*., 2001) and hence suggested as an important regulator of gene expression in CNS monoaminergic systems. However it has recently been shown that AP-2 is a transcription factor with dual functions, acting as an activator or repressor depending on conditions yet to be specified (Ren and Liao, 2001). One way of explaining the negative correlations between AP-2 levels and brain NA content in naïve animals would be that AP-2, directly or indirectly, exerts a repressor function for genes involved in the formation of NA. If so, manipulations with animals have the potential to switch the role AP-2 plays in regulating NA-ergic neurons, and this switching is mediated by release of CRF acting via CRF_1 receptors.

4.1.5.3. Plasma corticosterone

Corticosterone levels were lower in naïve HE animals, and behavioural testing decreased corticosterone to a similar level in both HE and LE group (Figure 15), which associates well with the decreased anxiety usually accompanying repeated behavioural testing. Behavioural testing with vehicle injections increased corticosterone levels in HE-rats only, while after CP-154,526 treatment corticosterone levels were decreased in HE-rats and increased in LE-rats as compared to the vehicle groups. Thus, chronic CRF_1 receptor blockade eliminated the increasing effect of daily injection stress on corticosterone levels that appeared in the HE-rats, while in LE-rats it prevented the habituation effect that decreased corticosterone levels in controls. As a net effect, after repeated CP-154,526 treatment differences in corticosterone levels in HE- and LE-rats were as in naïve animals. Thus far, different CRF_1 receptor antagonists have been found to reduce corticosterone levels when administered both acutely and chronically, and to prevent stressor-induced elevation of corticosterone levels when administered acutely (Bornstein *et al*., 1998; Gutman *et al*., 2003; Heinrich *et al*., 2002; Lelas *et al*., 2004). Also, when administered chronically to obese and lean Zucker rats (Doyon *et al*., 2007), the CRF_1 receptor antagonist SSR125543 was shown to reduce plasma corticosterone levels in lean rats only, partly supporting the present finding that the results of long-term CRF_1 blockade may have different effects on HPA axis activity measures in selected subpopulations of rats. It is noteworthy that there is an interesting similarity of these results with the effects of long-term CRF_1 blockade on the association between AP-2 and NA levels. Together these results suggest that chronic CRF_1

blockade is able to reverse the effects of handling- and injection procedure stress on biological measures of HPA stress axis and locus coeruleus-related stress system activity. The diametrically opposite effects of CRF_1 receptor blockade on corticosterone levels in HE and LE animals suggest differences in the stress-related neurochemical systems in these groups.

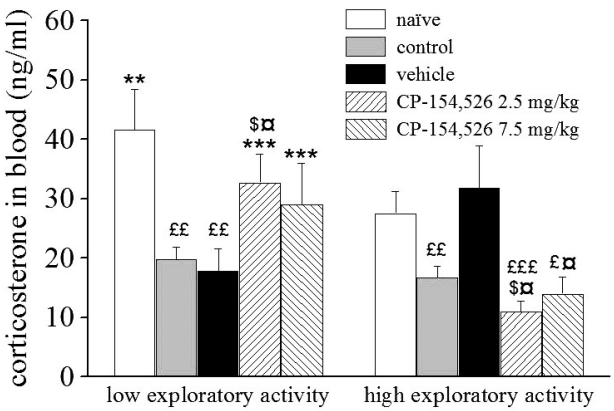

Figure 15. The effect of long-term administration of CP-154,526 on plasma corticosterone levels (Paper IV). ** – $p<0.01$, *** – $p<0.001$ as compared to respective HE group; £ – $p<0.05$, ££ – $p<0.01$, £££ – $p<0.01$ as compared to respective naïve group; $ – $p<0.05$ as compared to respective control group; ¤ – $p<0.05$ as compared to respective vehicle group. Data are presented as mean ± SEM.

The behavioural profiles of HE control and vehicle-treated animals did not differ significantly in this experiment, and neither did the activity levels of control and drug-treated LE animals differ in spite of differences in corticosterone levels. It has been previously demonstrated that the behavioural effects of another CRF_1 antagonist, R121919 are independent of the drug-induced changes in adrenocorticotropic hormone secretion, as the drug increased activity only in animals selectively bred for high anxiety in the elevated plus-maze, while its effects on the adrenocorticotropic hormone response to plus-maze exposure were similar in both high and low anxiety groups (Keck *et al*., 2001). The differential effects of CRF_1 blockade on corticosterone levels in HE and LE animals suggest that its effects on exploratory activity in these subgroups are at least partly independent of the HPA axis.

4.2. Tickling-induced ultrasonic vocalizations as a stable behavioural disposition

4.2.1. Stability of 50-kHz ultrasonic response: male and female high and low chirpers

In all experiments, the gradual rise in the number of 50-kHz chirps over repeated tickling sessions stabilized on an individually specific level by the second week of manipulation (Figures 16 and 17 and Paper V). The average 50-kHz USV response to daily tickling on days 12–14 proved to be a good predictor of the subsequent USV response. Rats divided into groups of high and low chirpers (HC and LC, respectively) by the median split of this measure revealed respectively high and low levels of 50-kHz USVs both over prolonged periods of continued tickling as well as after a pause in tickling sessions and regardless of housing conditions (single- or group housing) and sex. Also, a significant between-days correlation of the 50-kHz responses on different test days was found in the first week already, but this response did not predict the animal's chirping levels on the subsequent weeks. Similar pattern of change over repeated tickling sessions was visible in both sexes in the FM 50-kHz USVs (Figure 17), suggesting that at least in the context of daily experimenter-induced stimulation in juvenile rats there is no qualitative difference between the flat and FM USVs as a response to this stimulation. The levels of FM calls were somewhat lower in both sexes as compared to the flat USVs at baseline levels, while after social housing this difference was visible in male rats only. Previously, Burgdorf and Panksepp (2006) have reported more trill-type USVs than flat USVs in response to tickling in adult females and that with regard to the flat-type USVs the high-chirping rats do not differ from the low-chirping group. It seems reasonable to assume that differences in experimental design stand behind these variations, suggesting that if the tickling procedure is started when the animals have reached adult age, the resulting USV profile may differ to a certain extent from the conditions where tickling sessions have been started at weaning already. Age-related decreases in USV response to tickling have been reported (Panksepp and Burgdorf, 2003), and in the light of the above-mentioned results it may be suggested that the flat-type 50-kHz USVs show a greater and earlier decrease over aging process that may be related to different relevance and „meaning" of the two types of USVs at different age points.

The animals were always single-housed during the tickling sessions over the first two weeks since the 50-kHz response to tickling has been shown to be greater in such conditions (Panksepp and Burgdorf, 2000). The number of tickling-induced 50-kHz USVs decreased over periods of social housing that has been found to reduce tickling-induced USVs already in 48 h after moving isolate-housed animals to group housing (Burgdorf and Panksepp, 2001), but

the differences between HC and LC animals were retained. It is noteworthy that the levels of FM chirps did not decrease in females over the period of social housing, and the decrease in flat USVs was not significant in female LC animals. This suggests that at least in LC females social housing has a different effect on USVs than in other groups (and more so on FM chirps), that seems a reasonable assumption in the context that females have been found to have different social behaviour profiles than males (Douglas *et al*., 2004; Pellis *et al*., 1997), and remain playful longer after puberty as compared to males (Panksepp *et al*., 1984).

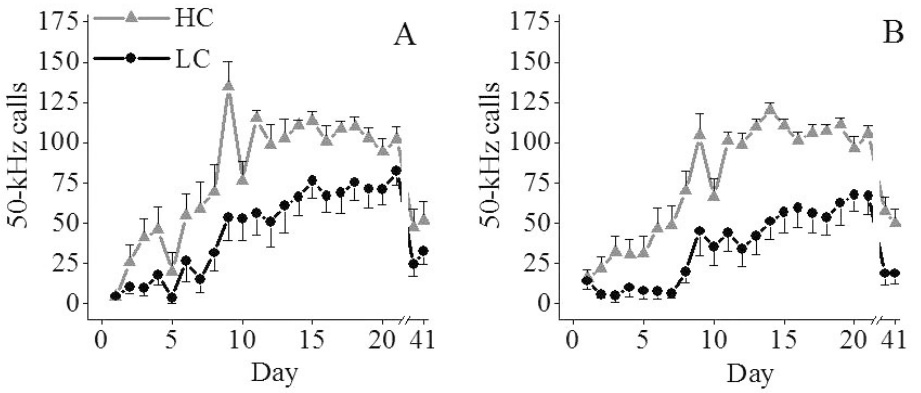

Figure 16. The levels of responding to tickling (four 15 s sessions of tickling intermittently with 15 s pauses) with 50-kHz chirping in male (A) and female (B) rats over 41 days of treatment, with a pause in tickling between Days 21–40 (Paper V). The animals were single-housed over the first 21 days of the Experiment, and group-housed by 4 after that. n = 6, 9, 10, 10(male HC and LC; female HC and LC, respectively). The animals were divided into groups with high and low levels of 50-kHz USVs by median split of the average result of Days 12–14 of tickling. Significant differences between the male groups: Days 3, 7, 12, 15–18 $p<0.05$; Days 9, 11, 14 $p<0.01$. Significant differences between the female groups: Days 3, 6, 20, 41 $p<0.05$; Days 7, 8, 9, 16, 17, 19, 21, 40 $p<0.01$; Days 11, 12, 15, 18 $p<0.001$; Days 13, 14 $p<0.0001$. HC – high chirping rats; LC – low chirping rats; Day – day of tickling (zero equals to the day of weaning). Data are presented as mean ± SEM.

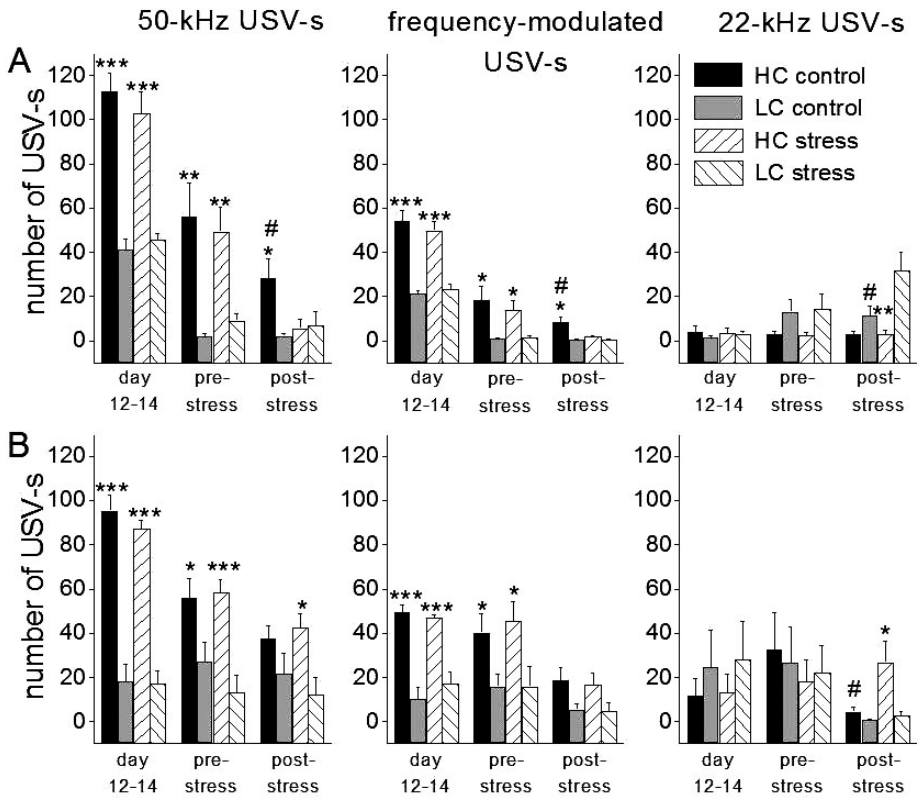

Figure 17. Ultrasonic vocalizations in male (A) and female (B) rats before and after chronic stress (Paper VI). * – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$ vs respective LC; # – $p<0.05$ vs respective stress. The animals were divided into groups with high and low levels of 50-kHz USVs by median split of the average result of Days 12–14 of tickling. HC – high chirping rats; LC – low chirping rats. Data are presented as mean ± SEM.

We also studied the emission of low-frequency USVs in the range of 20 kHz, that have long been associated with negative events and stimuli (Vivian and Miczek, 1991). In order to learn whether the 22- and 50-kHz USVs and hence the emotional messages they carry are mutually exclusive or not, the associations between the two types of USVs were assessed, and no correlations were found in untreated rats, although some female HC-rats tended to emit increasing levels of 22-kHz USVs over repeated testing (Paper V). Previously, Burgdorf *et al*. (2005) have found a negative correlation between the two types of USVs in rats that were bred for high levels of 50-kHz chirps. It is possible that there are differences in 22-kHz USV levels between animals from this breeding experiment and from the present first generation studies that play a

significant role in this regard. It has been suggested that the vocalizations and movements made by the tickled subject carry signals of both pleasure and submission, warning that, when ignored by the tickler, the normally pleasant stimulation may become unpleasant (Brudzynski and Ociepa, 1992). This implies that the 22- and 50-kHz USVs should be emitted simultaneously in specific situations, which was true in the present experiments, in which the two types of USVs were detected in identical experimental conditions. Thus, the 22- and 50-kHz USVs are not mutually exclusive, but permit the expression of different aspects of affective states in the animal quite simultaneously.

4.2.2. Behavioural differences in male and female high and low chirpers

Tickling on its own induced many changes in animals' behaviour in standard tests used in anxiety and depression research, and sometimes the essence of these changes depended on the animal's level of emitted 50-kHz USVs. It is important to note that the level of 50-kHz USVs on the first week, although stable, did not associate with the subsequent behaviour in other tests, while the level of 50-kHz USVs emitted by the end of the second week did.

Tickling was found to increase activity levels in the elevated plus-maze and HC-rats of both sexes tended to be more active as compared to the respective LC group in this test (Figure 18). In the exploration box test, tickling had almost no effect on HC-rats of both sexes, while the activity levels of LC-rats were consistently higher as compared to both control and HC-rats in both sexes (Figure 19). It seems that the lower activity of HC-rats in the exploration box test is rather due to lower motivation to explore than heightened levels of anxiety, as the exploration box as an „unforced exploration" test allows the animal to remain inactive more easily than the elevated plus-maze. Exploring new areas has been found to be rewarding for rats (Klebaur and Bardo, 1999), and the HC-rats do not seem have deficiencies in relating to the rewarding aspects of tickling, suggesting inertness towards a situation that demands active involvement. The context of the elevated plus-maze seems to have a more anxiogenic effect on the LC-rats, while lower levels of fear-provoking stimulation from the environment (i.e. as in the exploration box test) bring forth higher levels of exploratory activity in male LC-rats.

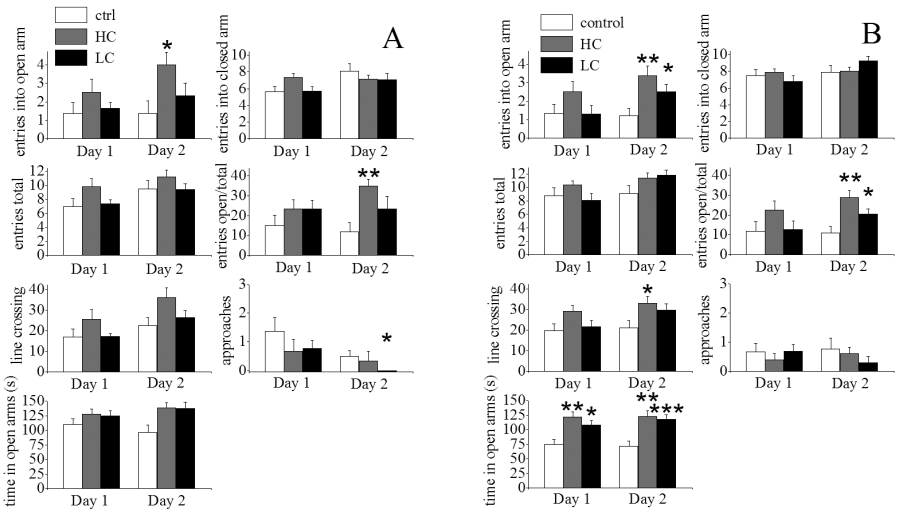

Figure 18. Behaviour of male (A) and female (B) HC- and LC-rats in the elevated plus-maze test (Paper V). n = 9, 10, 10 (female control, HC and LC, respectively). * – $p<0.05$ vs. respective control; ** – $p<0.01$ vs. respective control; *** – $p<0.001$ vs. respective control. HC – high chirping rats; LC – low chirping rats; Day – consecutive days of testing. Data are presented as mean ± SEM.

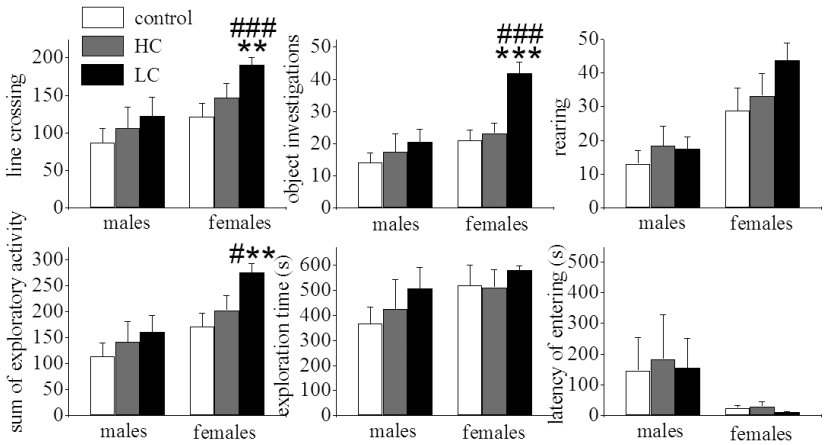

Figure 19. Exploration box test (second exposure) in male and female HC- and LC-rats (Paper V). n = 8, 6, 9, 9, 10, 10 (male control, HC and LC; female control, HC and LC, respectively). ** – $p<0.01$ vs. respective control; *** – $p<0.01$ vs. respective control; # – $p<0.05$ vs. respective HC; ### – $p<0.001$ vs. respective HC. HC – high chirping rats; LC – low chirping rats. Data are presented as mean ± SEM.

Tickling had no effect on sucrose consumption and preference in female rats, but tended to decrease these measures in male rats, and more systematically in the HC group (Paper V). The lower levels of taking in sweet solution in HC-rats are somewhat similar to the propensity of these animals to be less interested to explore novel areas in the exploration box test, suggesting deficiencies in motivational systems.

In the forced swimming test, tickling eliminated the increase in immobility visible on the second exposure to the forced swimming situation in male rats (Paper V). No significant differences were found between HC- and LC-rats, but the HC-rats tended to have higher levels of immobility, suggestive of greater stress response in this group. Also, no significant differences were found between HC and LC animals in foot-shock induced freezing, but while the freezing brought about by acute foot-shock was relieved similarly in both groups by history of having been tickled, the effect was somewhat greater in HC-rats. It is possible that the behavioural profile of HC-rats in this test reflects not so much a lower stress reactivity, but a difference in learning mechanisms, i.e. that the LC-rats learn better an association with negative reinforcers than HC-rats.

Although the HC animals show a propensity for a greater 50-kHz USV response to tickling, indicative of greater level of subjectively felt pleasure, these animals display an inhibited state in some behavioural tests, expressed in lower motivation to explore novel areas and to consume sweet solution, tend to adopt more passive coping strategies in the forced swimming test, and acquire associations with negative stimuli less efficiently. It is possible that the daily tickling procedure may condition the animals to receive certain stimulation on a daily basis that decreases in the HC animals the interest towards other rewarding stimuli (i.e. novel areas, sweet solution). The LC-rats seem to be less susceptible to such manipulations and have increased levels of activity in some behavioural tests which are similar to commonly reported effects of handling (Roy and Chapillon, 2004).

4.2.3. Oxidative metabolism and chronic variable stress in male and female high and low chirpers

The activity of cytochrome c oxidase determines the amount of ATP available in a neuron and, therefore, it can serve as a reliable metabolic marker for neuronal activity (Wong-Riley *et al*., 1998). There is now ample evidence that cytochrome c oxidase activity corresponds under normal conditions to the functional long-term changes in the neuronal metabolism of various nuclear groups (Sakata *et al*., 2005). Therefore, this method is most appropriate to relate differences in metabolic activity to stably expressed differences in a behavioural phenotype.

Inter-individual variations in reactivity to stress have been found in animals with high sociability that developed lower levels of sucrose intake after three weeks of chronic variable stress (Tõnissaar *et al*., 2008), elaborating on the previous finding that greater vulnerability to stress may occur in subgroups of unselected rats (Nielsen *et al*., 2000). Tickling on its own was found to have an anxiolytic effect on behavioural measures in the first studies (Paper V), and the behavioural differences between HC and LC rats suggested that variations in vulnerability to chronic stress may also develop between these groups. Also, the behavioural differences that were found between male and female rats (Paper V) regardless of similar chirping levels elicited by tickling suggested studying both sexes in order to specify possible differences in the mechanisms governing these processes.

It is noteworthy that significant differences were found in neuronal metabolic activity in various brain regions between control male and female rats (Paper VI). Lower metabolic activity was detected in females in raphe regions that provide input to corticolimbic structures involved in the control of anxious states (Millan, 2003). Higher metabolic activity was found in females in amygdala and related limbic regions that have been appointed a significant role in regulation of anxiety and fear responses (Davis and Shi, 1999) and mood disorders (Drevets, 2003). In humans, it has been observed that the lateralization of amygdala participation in emotionally influenced memory is sex-dependent (Cahill *et al*., 2001). Differences in anxiety and stress reactivity between male and female rats have been reported both in behavioural and neurobiological measures (Bowman *et al*., 2004; Luine, 2002; Palanza, 2001; Toufexis, 2007) and it seems reasonable that these variations may at least partly stem from differences in neuronal activity in baseline conditions.

Significant stress-induced changes in COX activity have been reported in various brain regions in male rats (Kanarik *et al*., 2008), and it is noteworthy that in some regions in which stress had no apparent effect in unselected animals in that study (namely, anteroventral thalamic nucleus and CA3), stress significantly increased COX levels in LC animals only (Paper VI). Chronic variable stress also increased COX levels in male LC-rats in medial amygdala, hippocampal zones CA1-CA2, perirhinal cortex, ventrolateral nucleus of thalamus, nucleus of the diagonal band (horizontal) and inferior colliculi (central nucleus and external cortex), resulting in significantly higher levels of metabolic activity in stressed LC-rats as compared to the stressed HC animals in these regions. The nucleus of the diagonal band has been demonstrated to provide cholinergic innervation to the hippocampus (Gaykema *et al*., 1990), influencing learning and memory processes, while amygdala and thalamic regions are incorporated in the mediation of stress effects with inputs from hippocampal structures, perirhinal cortex and subiculum (in which also a tendency of a similar stress effect on metabolic activity was visible) (Pitkänen *et al*., 2000). Together, these changes in metabolic activity in male LC-rats

support the notion of greater sensitivity to stress in this group with regard to learning and memory processes.

4.2.4. Behaviour and chronic variable stress in male and female high and low chirpers

Four weeks of chronic variable stress decreased the levels of flat and FM 50-kHz USVs in male HC animals (Figure 17), while no effect was visible on either measure in LC animals in which the number of chirps was very low already after social housing. The decrease in 50-kHz USVs in male HC animals after stress to the level of LC-rats suits with the previous findings of decreasing effects of aversive stimuli on 50-kHz vocalizations (Panksepp and Burgdorf, 1999). Due to very low levels of 50-kHz USVs in LC-rats it cannot of course be concluded that stress affected this measure in HC animals selectively. In females, stress had no effect on either type of 50-kHz chirps.

No difference was found between HC and LC animals in 22-kHz USVs at baseline and pre-stress testing (Figure 17). In male rats, chronic variable stress increased 22-kHz USVs in the LC group to a significantly higher level as compared to the HC group. This fits in with the previous findings that aversive events that decrease the 50-kHz USVs simultaneously increase the 22-kHz vocalizations in unselected rats (Panksepp and Burgdorf, 1999). Apparently, the male LC animals are more vulnerable and/or reactive in this regard. In females, the levels of 22-kHz USVs tended to decrease in all groups except for the HC stress group over the period of stress regimen, resulting in a significant difference between the HC and LC stress groups that was opposite to that of the males' groups. This effect partly associates with the increasing number of 22-kHz USVs over daily tickling sessions in female HC-rats in the previous experiment, suggesting greater reactivity to stimulation in female HC-rats. Hence, the low-chirping males and high-chirping female rats are similar with regard to higher reactivity to stressful experience in both groups.

Chronic variable stress regimen significantly affected weight gain in male rats (Figure 20), but no difference was found between the HC and LC animals in this regard, although the differences with control groups tended to develop earlier and remained more stable in LC animals as compared to the HC group. Weight gain suppression has been considered an important marker of stress regimen effectiveness (for an example, see Harro *et al*, 1999), and the more expressed effect in the LC group is in concordance with the result of increased 22-kHz USVs in the male LC group after the stress regimen, suggesting that this group was more affected by the stress procedure than HC animals. In females, no differences were found between any groups in weight gain, suggesting that the stress procedure had only minor effects (detectable in some behavioural parameters) on these animals in the present experiment. It has been previously

found that females are less sensitive to stress (Bowman *et al*., 2001) and that social housing can improve stress coping in female rats, while in male rats it was reported to increase the negative effects of chronic stress (Westenbroek *et al*., 2003). It is hence likely that in the present experiment, group housing prevented most of the negative effects of stress regimen in female rats.

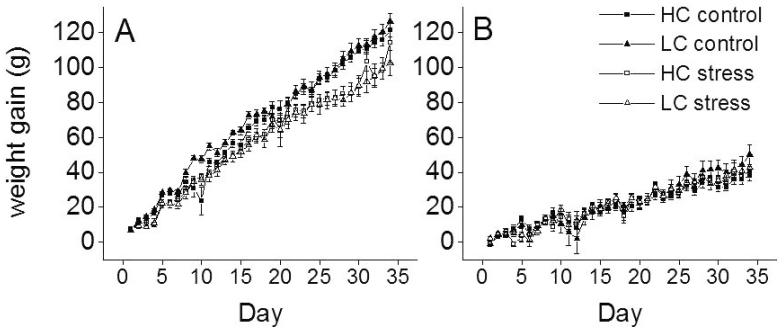

Figure 20. The effect of chronic stress on cumulative weight gain (g) in male (A) and female (B) rats (Paper VI). Significant differences between groups: male control LC vs male control HC: day 9 and day 10 ($p<0.01$); day 11 ($p<0.05$); male control LC vs male stress LC: day 3 ($p<0.001$); day 4 ($p<0.01$); day 5 ($p<0.05$); day 6–9 ($p<0.01$); day 11 ($p<0.001$); day 12 ($p<0.05$); day 13 ($p<0.01$); day 14 ($p<0.001$); day 15 ($p<0.05$); day 16 ($p<0.001$); day 17 ($p<0.05$); day 18 ($p<0.01$); day 22 and 23 ($p<0.05$); day 25–27 ($p<0.05$); day 28–30 ($p<0.001$); day 32 and 33 ($p<0.01$); male control HC vs male stress HC: day 3 ($p<0.05$); day 4 ($p<0.01$); day 5, 6, 10, 14, 17, 23, 25, 26 ($p<0.05$); day 27–30 ($p<0.01$); day 32 ($p<0.01$); day 33 ($p<0.05$). The animals were divided into groups with high and low levels of 50-kHz USVs by median split of the average result of Days 12–14 of tickling. HC – high chirping rats; LC – low chirping rats. Data are presented as mean ± SEM.

In the exploration box test, control LC animals had higher activity in male rats, and stress increased the difference on the first day of testing (Figure 21). In females, HC animals of both control and stress groups tended to be more active on some measures. Possibly, male LC-rats have higher motivation to explore novel environments that is suppressed by higher anxiety on the first encounter with the test apparatus as the differences between HC and LC animals became significant on the second testing. Although stress did not affect exploratory behaviour in female rats, it is of interest that in both sexes the groups with higher activity in the exploration box test were the same in which stress increased 22-kHz USVs. Hence, the higher exploration levels in these groups may rather reflect higher reactivity to various environmental stimuli than lower levels of anxiety.

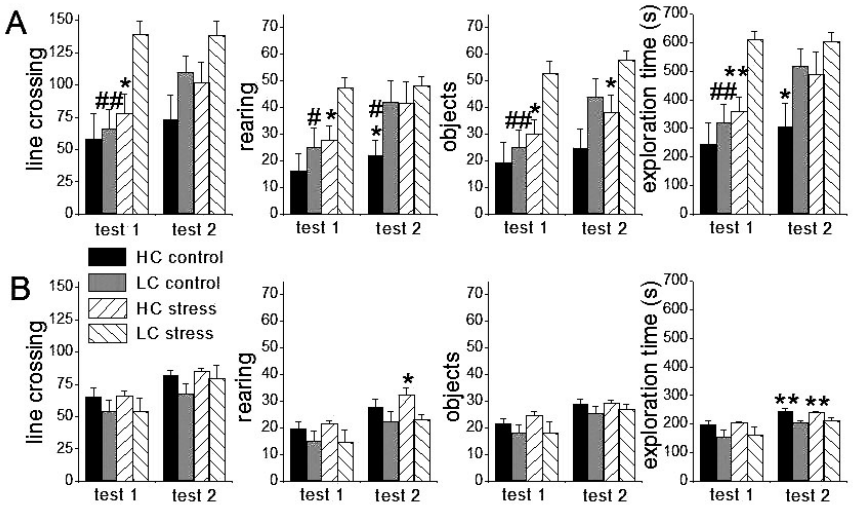

Figure 21. The effect of chronic stress on exploration box behaviour in male (A) and female (B) rats (Paper VI). * – $p<0.05$; ** – $p<0.01$ vs respective LC group; # – $p<0.05$; ## – $p<0.01$ vs respective stress group. The animals were divided into groups with high and low levels of 50-kHz USVs by median split of the average result of Days 12–14 of tickling. HC – high chirping rats; LC – low chirping rats. Data are presented as mean ± SEM.

The lack of differences in sucrose preference between HC and LC animals at baseline conditions (Figure 22) was similar to that of the previous experiment in which the animals had previously been group-housed, again confirming that the differences in sucrose consumption are only manifested in specific conditions. In general, these results suggest that the measures of hedonic states like sucrose preference and the level of tickling-induced 50-kHz USVs are dependent on the previous social stimulation level. Indeed, it has been reported that social housing reduces tickle-induced vocalizations and approach speed to the rewarding stimuli (in the specific case, the tickling hand) compared to isolate housing (Burgdorf and Panksepp, 2001). Over the repeated testing during the stress regimen, sucrose consumption increased in control groups of both sexes. No systematic differences were found between control and stressed HC animals, but sucrose consumption and preference were significantly lower in LC stress groups as compared to the respective controls, and sucrose preference also as compared to the HC stress group. Hence, stress significantly blocked the increase in sucrose preference in LC animals selectively, suggestive of an anhedonic state caused by higher sensitivity to chronic stress in this group.

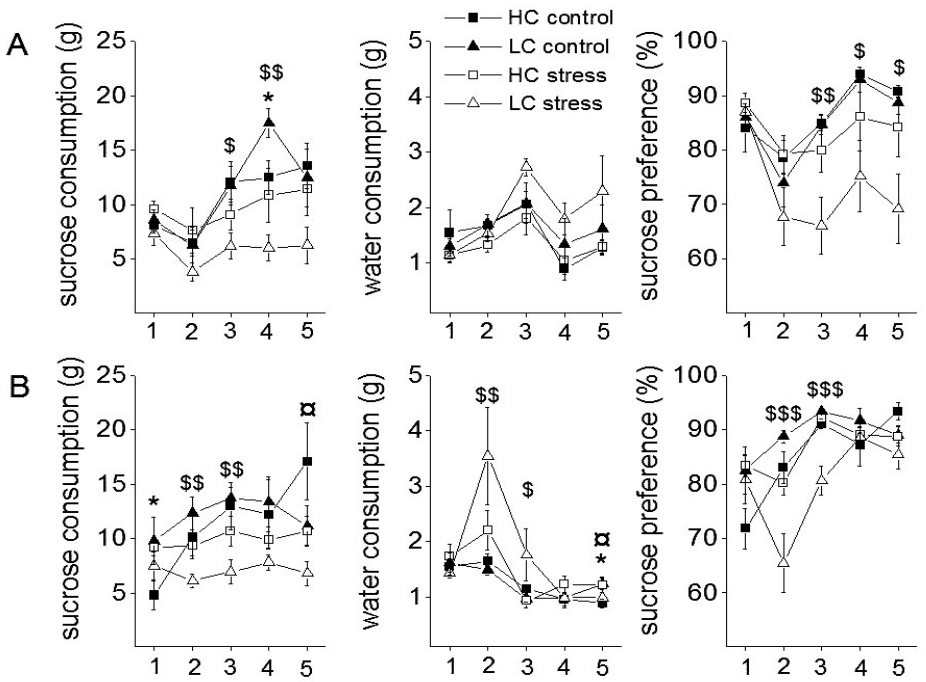

Figure 22. The effect of chronic stress on sucrose consumption, water consumption and sucrose preference in male (A) and female (B) rats (Paper VI). * – $p<0.05$ control HC vs control LC; ¤ – $p<0.05$ HC control vs HC stress; $ – $p<0.05$; $$ – $p<0.01$; $$$ – $p<0.001$ LC control vs LC stress. The animals were divided into groups with high and low levels of 50-kHz USVs by median split of the average result of Days 12–14 of tickling. The numbers 2–4 on *y*-axis represent sucrose test numbers (test no. 1 was carried out before the start of stress regimen). HC – high chirping rats; LC – low chirping rats. Data are presented as mean ± SEM.

In the elevated plus-maze, HC-rats of both sexes showed higher levels of activity on some measures of activity like in previous experiments, while no effect of stress was detected (Paper VI). The lack of stress effects on elevated plus-maze behaviour suggests that the tickling procedure that was found to significantly increase activity in the elevated plus-maze (Paper V) may also be effective in eliminating the stress effect in this test. In a partly similar experimental paradigm, postnatal handling has been demonstrated to yield attenuation of stress reactions in various tests in adult rats (Núñez *et al*., 1996).

Similarly to the previous experiments, no differences were detected between control female HC and LC animals in forced swimming, and chronic variable stress also had no effect on females in this test (Figure 23). In males, the control LC group had higher levels of immobility on the re-exposure as compared to

HC controls, and stress increased immobility in the re-exposure session to a greater extent in LC animals. This suggests greater susceptibility to both acute stress (i.e. the forced swimming stress in the control group) and chronic stress (in the stress group) in male LC animals. Interestingly, in the male HC control group swimming decreased the most by the second testing, but struggling levels increased slightly to a significantly higher level as compared to all other groups, while stress withheld both of these changes. Together with the lowest immobility levels in male HC rats, these results suggest lower acute stress reactivity in this group, which is nevertheless impaired by chronic stress procedure, resulting in similar behavioural profile with the LC group.

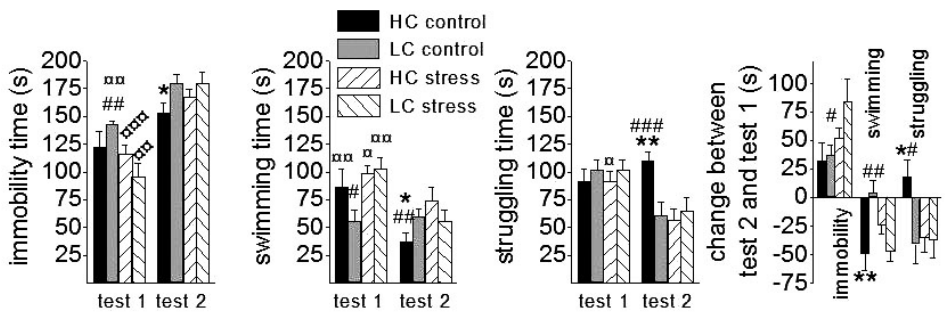

Figure 23. The effect of chronic stress on forced swimming in male rats (Paper VI). * – p<0.05; ** – p<0.01 vs respective LC; ¤ – p<0.05; # – p<0.05; ## – p<0.01; ### – p<0.001 vs respective stress; ¤¤ – p<0.01 ¤¤¤ – p<0.001 vs respective test 2. The animals were divided into groups with high and low levels of 50-kHz USVs by median split of the average result of Days 12–14 of tickling. HC – high chirping rats; LC – low chirping rats. Data are presented as mean ± SEM.

4.2.5. Oxidative metabolism and behaviour in male and female high and low chirpers

The stress-induced increase in 22-kHz USVs appeared in male LC and female HC groups. Also, the stress-induced changes in brain metabolic activity appeared almost exclusively in these two goups, while the pattern of these changes was distinct. In the brain regions that were affected by chronic variable stress, in male LC-rats metabolic activity increased (except for septum), and in female HC-rats it was decreased (except for locus coeruleus). As compared to the HC-rats, greater stress effects were also visible in male LC-rats in more stable suppression of weight gain and sucrose intake and greater increase in immobility time on re-exposure to the forced swimming situation. Also, significantly higher levels of activity were found in stressed groups of male LC

and female HC animals in the exploration box test. In females, no significant stress effects on behaviour were visible, which is in line with previous findings of lower sensitivity to stress in females (Bowman *et al*., 2001). Nevertheless, the stress-induced changes in metabolic activity in HC females rather suggest that the behavioural manifestation of stress effects were attenuated by some other factors, most likely social housing.

Stress increased activity in the exploration box test in male LC-rats, and it is noteworthy that in this group, stress also increased metabolic activity in hippocampus that has been associated with exploratory activity (Lever *et al*., 2006) with NA injections into hippocampus increasing behavioural activity in the open field (Płaźnik *et al*., 1983). Stress decreased metabolic activity in locus coeruleus in HC-rats, possibly leading to lower NA-ergic input to hippocampus in this group which may be partly responsible for the lower activity of the HC animals in the exploration box test. In the median raphe, chronic variable stress increased COX in male HC-rats. The 5-HT-ergic pathways innervating higher brain regions are mainly activated by anxiogenic stimuli (Millan, 2003), suggesting that the stress effects on male rats were to a greater extent mediated by changes in the 5-HT-ergic system in the HC group. Greater stress effects in male LC-rats were also evident in the increased metabolic activity in this group in the medial amygdala that has a central role in emotional processes (Phelps and LeDoux, 2005). Female rats had lower levels of COX activity as compared to males in both dorsal and medial raphe that were not affected by stress that is in line with the relative lack of stress effects on behaviour in females. In the red nucleus, stress also increased COX in male HC-rats and female LC-rats, while it decreased metabolic activity in this region in HC- female rats. The chages in metabolic activity in the red nucleus suggest possible changes in motor functions (Houk, 1991). It is noteworthy that in females, stress changed red nucleus metabolic activity in both HC and LC animals, and the tendencies of both higher activity in the exploration box test in HC control animals and difference in metabolic activity in this region became significant in stress groups only, suggesting that red nucleus activity may be one of the key factors to determining individual behavioural activity levels.

5. GENERAL SUMMARY

All together, the present studies confirmed that the division of rats by their levels of activity in the exploration box test and by the 50-kHz ultrasonic vocalizations elicited by tickling yields groups with significant differences in anxiety- and depression-like behaviours. Methods were developed for measurement of the differences in exploration and emitting of USVs.

Animals with high exploratory behaviour appear less anxious in other behavioural tests and also use more active coping strategies. The midbrain DA and 5-HT neurotransmission in PFC and hippocampus (with possible influences from BDNF and brainstem AP-2) contribute to the inter-individual variations in these dispositions. CRF_1 receptor blockade modulates exploratory behaviour, whereas this modulation is highly dependent on drug-environment interactions, animals' former experience with the test apparatus and inter-individual differences in exploratory behaviour. Apparently, intact CRF system is necessary for normal exploratory behaviour. Long-term CRF_1 blockade decreased corticosterone levels in HE animals and increased in LE animals, suggesting differences in the stress-related neuroendocrine systems in these groups.

The differences in animals' disposition to respond to tickling with high or low levels of 50-kHz USVs relate to the animal's more general tendency for emotional reactivity over different situations, and the HC-rats are more sensitive to stimulation. Tickling on its own induces changes in behaviour that refer to decreased anxiety levels and are paralleled by different changes in monoaminergic systems in HC- and LC-rats. In female rats, the effects of chronic variable stress in the present study were mostly not observed, being possibly attenuated by social housing, but the increased 22-kHz USVs and changes in the brain metabolic activity in HC animals suggest greater sensitivity to stress in this group. The male rats with low 50-kHz USV response to tickling present a phenotype behaviourally more vulnerable to stress, that also corresponds to stronger stress effects on brain metabolic activity in this group, especially in increases in metabolic activity in limbic regions.

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SUMMARY IN ESTONIAN

Uudistav käitumine ning 50-kHz ultrahelihäälitsused rottidel: indiviididevaheliste erinevuste käitumuslikud ja neurokeemilised profiilid

"Afektide" all mõistetakse subjektiivset emotsionaalset kogemust ning sellel on oluline osa organismi reaktsioonides keskkondlikele stiimulitele. Häired emotsionaalsetes protsessides, sealhulgas depressioon ning ärevushäired, on ühed olulisimad inimkonda vaevavad patoloogilised probleemid, mille raviks on küll olemas teatud osa patsientide jaoks efektiivsed farmakoteraapiad, kuid mille tekke ning kasutusel olevate ravimite toimemehhanismide kohta puudub ammendav teooria. Mõjukaim teooria depressiooni neurobioloogiliste aluste kohta on eelmise sajandi 60-ndatel aastatel loodud katehhoolamiinergiliste transmittersüsteemide vähenenud aktiivsusele tuginev hüpotees, mida on hilisemalt täpsustatud nii mõjustatud retseptorite alatüüpide ning nende funktsionaalsuse kui ka mitmete mitte-amiinergiliste mehhanismide kaasamise kaudu. Viimastest ühed olulisimad on endokriinse hüpotalamuse-ajuripatsi-neerupealiste (HPA) stressitelje aktivatsiooni kontrolliv peptiidne transmitter kortikotropiini vabastusfaktor (CRF) ning erinevad geenitranskriptsiooni ning neuronaalset arengut mõjutavad molekulid nagu monoamiinergilise aktiivsusega seostuv transkriptsioonifaktor AP-2 ning serotonergiliste neuronite diferentseerumist ja funktsioneerimist reguleeriv neurotroofne faktor BDNF.

Ajalooliselt on enimkasutatavateks depressiooni ning ärevuse loommudeliteks kujunenud niisugused, mis töötati algselt välja monoamiinergiliste transmittersüsteemide mõjutamise kaudu toimivate ravimite efektiivsuse skriinimiseks. Nende sisuks on reeglina teatud käitumistele või funktsioonidele fokusseerumine, mis on loomulikult (nt katseloomade vaheliste individuaalsete erinevuste tasandil) või peale spetsiifilisi manipulatsioone (nt stressiga mõjustamine) analoogilised inimestel esinevate haigusnähtudega.

Uudistav käitumine hõlmab uudsete stiimulite poolt vallandatud ning neile suunatud käitumisi, mille eesmärk on organismi efektiivne toimimine keskkonnas, sh toidu, varjupaiga, seksuaalpartneri jms leidmine. Uudistavat käitumist mõjutavad samaaegselt nii hirm kui ka uudishimu võõrate objektide suhtes, mistõttu sellel põhineb suur hulk ärevuse mõõtmisega seotud käitumisteste. Uudistava käitumise neurobioloogilises regulatsioonis on leitud olulist rolli mängivat nii monoamiinergilised kui ka neuropeptiidsed transmittersüsteemid, samuti on sellega seonduvates käitumistes täheldatud olulisi indiviididevahelisi variatsioone katseloomadel.

Katseloomade poolt esile toodud häälitsustele ning spetsiifilisemalt rottide ultrahelihäälitsustele kui võimalikele afektiivseid seisundeid vahendavale käitumisele on viimastel aastatel pööratud üha suurenevat tähelepanu. Peale väga

levinud negatiivsete afektiivsete seisundite uurimise on leitud, et rottide poolt 50-kHz sagedusel esitatud ultrahelihäälitsuste hulk seondub positiivse valentsiga seisunditega. Efektiivseks stiimuliks, mis rottidel 50-kHz ultrahelihäälitsusi esile kutsub, on leitud olevat eksperimentaatori-poolne manuaalne stimulatsioon, mis imiteerib noorloomade omavahelist "müramist". Negatiivse afektiga seonduvad 22-kHz sagedusel esitatavad ultrahelihäälitsused. 50-kHz ultrahelihäälitsusi kutsub esile ülenevate dopamiinergiliste närviteede aktivatsioon, samas kui 22-kHz ultrahelihäälitsuste hulk seondub ülenevate koliinergiliste juhteteede aktiivsusega. 50-kHz ultrahelihäälitsustes on leitud ilmnevat indiviididevahelised erinevused, mis on püsivad ning päritavad.

Käesolevas töös kirjeldatud uuringute eesmärk oli uudistavas käitumises ning 50-kHz ultrahelihäälitsustes ilmnevate katseloomade vaheliste indiviididevaheliste erinevuste stabiilsuse selgitamine ning meetodite väljatöötamine nende objektiivseks mõõtmiseks eesmärgiga kasutada nimetatud käitumuslikke fenotüüpe afektiivsete seisundite loommudelite loomiseks. Testiti hüpoteesi, et stabiilselt esinevad variatsioonid uudistavas käitumises ning 50-kHz ultrahelihäälitsustes peegelduvad ka erinevates käitumisprofiilides teistes ärevuse ning depressiooniuuringute kontekstis kasutatavates loomkatsetes.

Eelnevate uuringute põhjal võis eeldada monoamiinergiliste transmittersüsteemide ning nende aktiivsuse ja arenguga seotud transkriptsioonifaktori AP-2 ning neurotroofse faktori BDNF olulist rolli afektiivsete käitumiste aluseks olevates neurobioloogilistes mehhanismides. Käesolevate uuringute käigus läheneti neile süsteemidele erinevate meetoditega uurimaks nii närvikoes paiknevate transmitterainete varude, neuronitevahelises ruumis asuva transmitteraine ning selle muutuste ja retseptorite ja teiste sünaptiliste mehhanismide funktsionaalsust, testimaks hüpoteesi nende olulisuse kohta uuritavates käitumistes ilmnevates indiviididevahelistes erinevustes. Tuginedes leidudele CRF-süsteemi suurenenud aktiivsuse kohta stressirohketes tingimustes, testiti hüpoteesi CRF 1-alatüüpi retseptorite blokeerimise võimaliku ärevustleevendava toime kohta, mis avalduks ka monoamiinergilistes süsteemides ning neuroendokriinse HPA telje aktiivsust demonstreeriva vereplasma kortikosterooni tasemete muutustes.

Uuriti metaboolse aktiivsuse erinevusi erinevates ajupiirkondades emastel ning isastel rottidel, kes toovad eksperimentaatori-poolse stimulatsiooni peale esile vähe või palju 50-kHz ultrahelihäälitsusi. Samuti uuriti kroonilise stressi mõju aju metaboolsele aktiivsusele ja käitumisele nimetatud loomadel, eesmärgiga kaardistada positiivse afektiivsusega seonduvaid ajupiirkondi ning erinevusi stressitundlikkuses.

Uuringutes tuvastati, et katseloomade jagamine uudistava käitumise tasemete ning eksperimentaatori-poolse stimulatsiooniga esilekutsutud 50-kHz ultrahelihäälitsuste alusel annab stabiilsete omadustega rühmad, mis erinevad omavahel ka ärevuse ja depressioonitestides mõõdetavate käitumiste poolest.

Kõrge uudistamisaktiivsusega loomad on teistes käitumiskatsetes vähem ärevad ning kasutavad rohkem aktiivseid toimetulekustrateegiaid. Madala ning kõrge uudistamisaktiivsusega loomade käitumuslikes erinevustes mängivad olulist rolli erinevused keskaju dopamiinergilistes ning prefrontaalkoore serotoniinergilistes süsteemides, mida mõjutavad rühmadevahelised variatsioonid BDNF tasemetes. CRF 1-alatüüpi retseptorite blokeerimine moduleerib uudistavat käitumist, samas sõltub see väga palju ravimi-keskkonna interaktsioonidest, katseloomade eelnevast kogemusest katseaparaadis ning indiviididevahelistest erinevustest. Pikaajaline CRF süsteemi blokaad langetas kortikosterooni tasemeid kõrge uudistamisaktiivsusega loomadel ning suurendas madala uudistamisaktiivsusega loomadel, viidates erinevustele neuroendokriinsetes stressisüsteemides.

Loomade erinevused eksperimentaatori-poolsele stimulatsioonile 50-kHz ultrahelihäälitsustega reageerimises on seotud nende üldisema emotsionaalse reaktiivsusega, ning palju 50-kHz ultrahelihäälitsusi tegevad loomad on stimulatsioonile tundlikumad. Eksperimentaatori-poolne stimulatsioon põhjustas muutusi käitumises mis viitavad vähenenud ärevusele, ning mis on vähe ning palju 50-kHz ultrahelihäälitsusi tegevatel loomadel vahendatud erinevate muutuste kaudu dopamiini- ning serotoniinisüsteemides. Isased vähe ultrahelihäälitsusi tegevad loomad on tundlikumad negatiivsetele stiimulitele, mis avaldub ka muutustes aju metaboolses aktiivsuses reageeringuna kroonilisele stressile. Emaste loomade hulgas on stressile tundlikumad palju 50-kHz ultrahelihäälitsusi tegevad loomad.

PUBLICATIONS

I

Mällo T., Alttoa A., Kõiv K., Tõnissaar M., Eller M., Harro J. (2007). Rats with persistently low or high exploratory activity: behaviour in tests of anxiety and depression, and extracellular levels of dopamine. *Behavioural Brain Research*, 177: 269–81.

II

Mällo T., Kõiv K., Koppel I., Raudkivi K.,
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Regulation of extracellular serotonin levels and brain-derived neurotrophic factor in rats with high and low exploratory activity.
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III

Mällo T., Berggård C., Eller M., Damberg M., Oreland L., Harro J. (2004).
Effect of long-term blockade of CRF(1) receptors on
exploratory behaviour, monoamines and transcription factor AP-2.
Pharmacology Biochemistry and Behavior, 77: 855–65

IV

Mällo T., Harro J.
Effect of long-term blockade of CRF_1 receptors on rats with high or low spontaneous exploratory activity. (*submitted for publication)*

EFFECT OF LONG-TERM BLOCKADE OF CRF_1 RECEPTORS ON RATS WITH HIGH OR LOW SPONTANEOUS EXPLORATORY ACTIVITY

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ABSTRACT

While increased activity of corticotropin-releasing factor (CRF) is anxiogenic, CRF has been found to increase behavioural activity in familiar environments. Individual differences in anxiety-related behaviours are based on differences in monoaminergic systems that interact with the CRF-ergic neurotransmission. We have studied the effects of CRF_1 receptor blockade in rats with low (LE) or high (HE) levels of exploratory behaviour. In HE-rats acute CRF_1 blockade with the selective antagonist CP-154,526 decreased exploratory activity, but increased it after repeated administration in animals with no previous experience with the test apparatus, while in animals with previous experience no effect was found after 12-days drug treatment. In LE-rats CP-154,526 had no effect on behaviour in animals with previous experience with the test apparatus. In LE-rats without previous experience with the equipment the drug decreased exploration both acutely and after 5 days of treatment, but when drug administration was continued this effect disappeared. In HE animals selectively, CRF_1 blockade decreased dopamine turnover and increased serotonin turnover in frontal cortex, and decreased the serotonin and 5-HIAA levels in hippocampus. Corticosterone levels were lower in naïve HE-rats, and behavioural testing decreased corticosterone in both groups. Vehicle treatment increased corticosterone levels in HE-rats only, while after CRF_1 blockade corticosterone levels were decreased in HE-rats and increased in LE-rats as compared to vehicle groups. Conclusively, the effects of CRF_1 blockade depend on individual differences in anxiety-related behaviours, possibly because of differential changes in the dopamine and serotonin systems and the hypothalamus-pituitary-adrenal stress axis activity.

INTRODUCTION

Corticotropin-releasing factor (CRF) holds a central role in reactions to various environmental stimuli. It has been found to mediate changes in neuroendocrinological functions and behaviour induced by external stimuli (Owens and Nemeroff, 1991). Of the two CRF receptor subtypes, the CRF_1 receptor has mainly been implicated in mediating the stress-related effects of the CRF-system (for a review, see Takahashi, 2001). Blockade of the CRF_1 receptor has mainly been found to have anxiolytic effects after both acute (Schulz *et al*., 1996; Mansbach *et al*., 1997) and chronic administration (Griebel *et al*., 2002; Arborelius *et al*., 2000). With regard to exploratory behaviour we have found that chronic CRF_1 receptor blockade may either increase or decrease exploratory behaviour in rats depending on the experimental design (Harro et al., 2001a; Mällo *et al*., 2004), suggesting inhibitory effects of CRF_1 blockade on adaptive behaviours.

CRF – likely via CRF_1 receptors – has been found to influence dopamine (DA)-ergic (Djouma *et al*., 2006; Meloni *et al*., 2006) and serotonin (5-HT)-ergic (Price *et al*., 1998; Isogawa *et al*., 2000) systems in the regulation of stress- and motivation-related states, and its effects on DA-system have also been found to depend on the test animals' locomotor response to novelty (Rougé-Pont *et al*., 1998). The anxiolytic effects of CRF_1 blockade have also been found to depend on the inherent anxiety levels in rats (Keck *et al*., 2001). We have previously reported variations in DA and 5-HT systems possibly underlying the stable individually different levels of exploratory activity (Alttoa *et al.,* 2005 and 2007; Mällo *et al*., 2007 and 2008). The results of these studies, as well as variations in the behavioural effects of CRF_1 blockade reported by other laboratories (reviewed in Seymour *et al*., 2003; Nielsen, 2006) motivated us to examine whether the inter-individual differences in exploratory behaviour influence the effects of CRF_1 receptor antagonists. Hence, the aims of the present experiments were to study the effects of acute and long-term CRF_1 receptor blockade (using a selective CRF_1 antagonist, CP-154,526) on exploratory behaviour in rats preselected on the basis of low or high levels of inherent exploratory activity (LE and HE animals, respectively) (Mällo *et al*., 2007). Also, the effects of the treatment on brain tissue monoamine levels and blood plasma corticosterone concentrations were measured.

METHODS

Animals

Male Wistar (Experiments 1 (n=64) and 2 (n=80)) rats were housed in groups of 4 in transparent polypropylene cages under controlled light cycle (lights on from 08:00 h to 20.00 h) and temperature (19–21°C), with free access to tap water and food pellets (diet R70, Lactamin, Sweden). Experiments were carried

out 12:00–19:00. The experimental protocol was approved by the Animal Experimentation Committee at the Estonian Ministry of Agriculture.

General procedure

Experiment 1: Rats were pre-tested in the exploration box on two consecutive days for determination of baseline activity levels (Mällo *et al*., 2007). The rats were then divided into animals with low exploratory activity (LE) and high exploratory activity (HE) on the basis of the median value of the sum of exploratory activity during the second testing, a measure which we have previously found to predict future behaviour better than the activity during the first testing. The average scores of exploratory activity varied between 0–24 and 110–257 activity units for LE and HE animals, respectively. Both LE- and HE-rats were divided into four groups (n=7–9): control (no treatment), vehicle, 2.5 mg/kg of CP-154,526 and 10 mg/kg of CP-154,526. The drug treatment started 4 days after the preselection testing, and daily treatment was carried out on 14 consecutive days, during which behavioural testing in the exploration box was carried out on days 1, 5 and 12. Animals were decapitated on day 14, and tissue samples dissected and immediately frozen on dry ice until the measurement of the levels of monoamines.

Experiment 2: Ninety-six animals were tested in a light-dark box. We have found in our previous experiments that the animals' behaviour in the used version of the light-dark box predicts well the behaviour in the exploration box. Best correlations were found between the sum of exploratory activity in the exploration box test and the sum of line crossing and rearing in the light compartment of the light-dark box test (r=0.65–0.70) (unpublished data), which enabled us in Experiment 2 to use these measures in order to make the division of animals into high and low explorers without subjecting them to the exploration box before the start of drug administration. The average scores for light-dark box behaviour varied between 2–22 and 54–129 activity units for LE and HE animals, respectively. Sixteen animals with medium activity levels were excluded from the further studies and the rest were assigned for the following treatments: naïve (no treatment), control (behavioural testing only), vehicle (behavioural testing and daily i.p. injection of vehicle) and drug groups of 2.5 and 7.5 mg/kg CP-154,526 (behavioural testing and daily i.p. injection of the respective dose of the CRF_1 receptor antagonist) (n=8 in each group). As compared to Experiment 1, the larger dose was reduced from 10 to 7.5 mg/kg and the period of drug administrations shortened from 14 to 10 days in Experiment 2 in order to optimize the experimental design in the context of low available amount of the substance. Based on the previous results, we had reasons to believe that these modifications would not significantly affect the results. The animals were re-housed so that rats receiving different treatments would not share cages, and given 7–10 days for habituation before drug treatment and exploration box testing started. The naïve group received no

treatment or handling until sacrifice. The exploration box test was conducted on days 1, 5 and 10 of drug administration. Two to three hours after the end of exploration box test on day 10 of drug administration the animals were sacrificed by decapitation. Trunk blood was collected and immediately centrifuged for the extraction of plasma samples. The samples were collected into Eppendorf vials and immediately frozen on dry ice and stored at –80°C until the corticosterone assay.

Drug administration
CP-154,526 was suspended in distilled water by adding a few drops of Tween 85. Other animals received vehicle or no treatment. CP-154,526 was administered intraperitoneally (i.p.) 30 min before behavioural observations, or at about the same time of day on the days that did not include behavioural testing.

Exploration box test
The exploration box was carried out as previously described (Otter *et al.*, 1997; Mällo *et al*., 2007). In short, the apparatus was made of metal and consisted of an open area 0.5 x 1 m (side walls 40 cm) with a small 20 x 20 x 20 cm compartment 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 all experiments). The small compartment, which had its floor covered with wood shavings, was directly linked to the open area through an opening (size 20 x 20 cm). The apparatus was cleaned with dampened cloth 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: 1) latency (s) of entering the open area with all four paws on it; 2) entries into the open area; 3) line crossing; 4) rearing; 5) exploration of the objects in the open area; 6) time spent in exploring on 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 in the first experiment. A single behavioural test session lasted 15 min.

Light-dark box test
The light-dark box test was modified from Santucci *et al*. (1994). A metal box measuring 30 x 60 x 40 cm was divided into two compartments of equal size (30 x 30 cm). The bright compartment had no ceiling. Black lines divided the floor of the light compartment into four 15 x 15 cm squares. The dark compartment had a ceiling. A door of 10 x 10 cm in the partition wall allowed the rat to go from one compartment to the other (transition). The experiments were conducted at normal room light (approximately 190 lux at the centre of the

light part). The following measures were counted: 1) transitions between the light and dark compartments; 2) latency (s) to enter the dark side after initial placement in the light side; 3) latency (s) to re-enter the light side following the first cross into the dark side; 4) number of transition attempts from the dark to the bright compartment (the animal places the head out of a dark side without crossing to the light side); 5) total time spent in the light compartment; 6) distance travelled – line crossing in open part; 7) the number of stretched-attend postures made out from the dark compartment; 8) rearing in open part.

High performance liquid chromatography
Monoamines were measured by high performance liquid chromatography with electrochemical detection as described in Mällo *et al*. (2004).

Corticosterone assay
Animals were decapitated and trunk blood was collected into pre-cooled tubes containing K3 EDTA. The blood samples were kept on ice and centrifuged after every 4 animals (4,000 rpm for 10 min at room temperature). Plasma was pipetted into Eppendorf tubes and stored at –80°C until the assay. Plasma samples were thawed on ice and lightly vortexed and diluted 15 times. Plasma corticosterone was measured by ELISA (Correlate-EIA™, Assay Design, Inc; Ann Arbor, MI 48108, USA) according to manufacture's directions. The sensitivity of this assay is 26.99 pg⁄mL. Upon completion of the assay, 96-well plates were read at 405 nm on a Labsystems Multiskan MCC/340 microplate reader.

Statistics
The data for preselection measurements in both Experiments were analysed with unpaired *t*-test. The rest of the behavioural data of both Experiments were analyzed with 2-factor ANOVA (Exploration x Treatment) with repeated testing. The monoamine data of Experiment 1 and corticosterone data of Experiment 2 were analysed with 2-factor ANOVA (Exploration x Treatment). Subsequent pairwise comparisons were made with Fisher's LSD test.

RESULTS

Experiment 1

The effect of CP-154,526 (2.5 and 10 mg/kg, i.p.) on exploratory behaviour in animals pre-tested in the exploration box test

The groups of low and high exploring animals differed significantly on the second day of testing on latency (t=–2.57; $p<0.05$), number of entries, line crossing, object investigations, rearing, sum of exploratory events and time of exploration (t=5.15, 9.29, 9.24, 8.58, 7.46, 10.1, respectively; all $p<0.001$), while the treatment groups did not differ in any measures from each other

among LE and HE animals (Fig. 1). A significant Exploration effect was found on latency [$F(1, 56)=8.79$; $p<0.01$], entering, line crossing, investigation of objects, rearing, time of exploration and sum of exploratory activity [$F(1, 56)=$ 19.6, 30.2, 27.6, 25.6, 24.9, 30.5, respectively; all $p<0.001$]. A Repeated testing effect was found on latency [$F(3, 168)=2.82$; $p<0.05$], entering, line crossing, investigation of objects, rearing, time of exploration and sum of exploratory activity [$F(3, 168)=46.8$, 30.8, 12.5, 9.84, 9.63, 19.2, respectively; all $p<0.001$]. An Exploration x Repeated testing interaction was revealed on enterings, line crossing, investigation of objects, sum of exploratory activity [$F(3, 168)=6.66$, 15.3, 11.7, 12.2 respectively; all $p<0.001$], rearing and time of exploration [$F(3, 168)=3.66$ and 3.69; both $p<0.05$]. Also, a significant Treatment x Repeated testing interaction was found on line crossing, investigation of objects and sum of exploratory activity [$F(9, 168)=1.98$, 1.82, 1.80, respectively; all $p<0.05$]. Post-hoc tests revealed that in HE groups the decrease in activity levels on first behavioural testing after the pre-selection procedure was greatest in the drug groups and remained significantly lower as compared to the pre-testing activity levels as long as through the second test. Over the 12 days of drug administration, behavioural activity increased the most in drug groups and reached the pre-testing level in most groups on most measures by the third test on the 12^{th} day of drug administration. In LE animals, no significant changes were detected on acute drug administration, while over repeated testing activity levels tended to increase in all groups on most measures, but not significantly.

The effect of long-term administration of CP-154,526 (2.5 and 10 mg/kg, i.p.) on tissue monoamine levels

In the prefrontal cortex (PFC), a two-factor ANOVA (Treatment x Exploration) revealed a significant Exploration effect on DA [$F(1, 56)=4.06$; $p<0.05$]. HE animals had lower levels of DA in control group, as compared to the corresponding LE animals (Table 1). No effect was found on 3,4-dihydroxyphenylacetic acid (DOPAC) levels, but a significant Treatment effect was found on DA turnover (calculated as DOPAC/DA) [$F(3, 56)=3.76$; $p<0.05$]. HE animals in both 2.5 and 10 mg/kg CP-154,526 groups had significantly lower DA turnover as compared to the corresponding control animals. The 10 mg/kg CP-154,526 LE group had significantly higher DA turnover as compared to the corresponding vehicle and 2.5 mg/kg CP-154,526 groups. A significant Treatment effect was revealed on 5-HT [$F(3, 56)=2.86$; $p<0.05$]; post-hoc tests detected significantly lower levels of 5-HT in 2.5 mg/kg CP-154,526 LE group, as compared to the corresponding vehicle group. A significant Treatment effect, as well as an Exploration effect were found on 5-hydroxyindoleacetic acid (5-HIAA) [$F(3, 56)=3.37$; $p<0.05$ and $F(1, 56)=9.87$; $p<0.01$, respectively]. HE animals had significantly lower levels of 5-HIAA than the respective LE animals in vehicle and 2.5 mg/kg CP-154,526 groups. HE animals in the 10 mg/kg CP-154,526 group had significantly higher levels

of 5-HIAA as compared to all other three HE groups. A significant Treatment effect, as well as an Exploration effect were revealed on 5-HT turnover (calculated as 5-HIAA/5-HT) [$F(3, 56)=3.27$; $p<0.05$ and $F(1, 56)=10.4$; $p<0.01$, respectively]. HE animals had significantly lower 5-HT turnover in control and vehicle groups, as compared to the LE animals. Also, HE animals in the 10 mg/kg CP-154,526 group had significantly higher 5-HT turnover as compared to all other three HE groups.

In the hippocampus, a two-factor ANOVA (Treatment x Exploration) revealed a significant Exploration effect on NA levels [$F(1, 56)=7.62$; $p<0.01$]; HE animals had significantly higher levels of NA as compared to the LE animals in control and vehicle groups (Table 1). A significant Exploration effect was found on DA levels [$F(1, 56)=4.50$; $p<0.05$]; HE vehicle group had significantly higher levels of DA as compared to the corresponding LE group. A significant Treatment effect, as well as an Exploration effect were revealed on 5-HT [$F(3, 56)=6.67$; $p<0.001$ and $F(1, 56)=8.42$; $p<0.01$, respectively]. HE animals had higher levels of 5-HT in all groups except for the 10 mg/kg CP-154,526 group. Also, the 10 mg/kg CP-154,526 HE group had significantly lower levels of 5-HT as compared to all other three HE groups, and both LE and HE 2.5 mg/kg CP-154,526 groups had significantly lower levels of 5-HT as compared to the corresponding control groups. A significant Treatment effect, as well as an Exploration effect were found on 5-HIAA [$F(3, 56)=4.85$; $p<0.01$ and $F(1, 56)=11.9$, $p<0.01$, respectively]. A significant Treatment x Exploration interaction effect was also revealed on 5-HIAA [$F(3, 56)=3.53$; $p<0.05$]. Post-hoc tests showed that HE animals had significantly higher levels of 5-HIAA in control and vehicle groups, as compared to the corresponding LE animals; also, as compared to the control HE group, all other three HE groups displayed significantly lower levels of 5-HIAA, while the 10 mg/kg CP-154,526 HE group also displayed significantly lower levels of 5-HIAA as compared to the vehicle HE group. The Treatment effect on 5-HT turnover remained just above the level of statistical significance [$F(3, 56)=2.65$; $p=0.057$]; there was a tendency for lower 5-HT turnover in the control LE group as compared to all other three LE groups. Also, the 10 mg/kg CP-154,526 HE group displayed higher 5-HT turnover as compared to the corresponding vehicle and 2.5 mg/kg CP-154,526 groups.

In the striatum, a significant Exploration effect was revealed on DA levels [$F(1, 56)=5.45$; $p<0.05$], with a tendency in control and vehicle HE animals for higher levels of DA as compared to the respective LE groups (Table 1). Also, a significant Exploration effect was found on 5-HIAA levels [$F(1, 56)=4.82$; $p<0.05$], with HE animals displaying a tendency for higher levels of 5-HIAA in all groups except for vehicle-treated group.

In the septum, a two-factor ANOVA (Treatment x Exploration) revealed a significant Treatment x Exploration interaction effect on DA levels [$F(3, 56)=3.43$; $p<0.05$]. The 10 mg/kg CP-154,526 HE group had lower levels of

DA as compared to the corresponding LE group, while the same difference was reversed, remaining just above the level of statistical significance in the 2.5 mg/kg CP-154,526 group (Table 1). Also, the 10 mg/kg CP-154,526 LE group had significantly higher levels of DA as compared to the corresponding 2.5 mg/kg CP-154,526 group. A significant Exploration effect on DA turnover was found [$F(1, 56)=5.63$; $p<0.05$]. HE animals in the 10 mg/kg CP-154,526 group had significantly higher DA turnover, while the same effect remained just above the level of statistical significance in the vehicle group. A significant Exploration effect was revealed on 5-HIAA levels [$F(1, 56)=9.32$; $p<0.01$], as HE animals had significantly higher levels of 5-HIAA in both control and vehicle groups.

Experiment 2

The effect of CP-154,526 (2.5 and 7.5 mg/kg, i.p.) on exploratory behaviour in animals pre-tested in the light-dark test

A significant Exploration effect was found on latency [$F(1, 56)=4.64$; $p<0.05$], line crossing, investigation of objects, rearing, sum of exploratory activity [$F(1, 56)=15.0$, 14.6, 20.2, 18.0, respectively; all $p<0.001$] and time of exploration [$F(1, 56)=8.72$; $p<0.01$] as HE animals tended to have higher levels of activity on most measures in most treatment groups (Fig. 2). A significant Exploration x Treatment interaction was revealed on line crossing [$F(3, 56)=2.81$; $p<0.05$]. A significant Repeated testing effect was found on latency, [$F(2, 112)=5.33$; $p<0.01$], line crossing, investigation of objects, rearing, sum of exploratory activity and time of exploration [$F(2, 112)=43.1$, 67.1, 74.5, 65.3, 55.8, respectively; all $p<0.001$] as the levels of activity increased in most groups over repeated testing. Also, significant Treatment x Repeated testing interactions were found on line crossing [$F(6, 112)=2.97$; $p<0.01$], investigation of objects, rearing, sum of exploratory activity [$F(6, 112)=4.55$, 6.12, 4.45, respectively; all $p<0.001$] and time of exploration [$F(6, 112)=3.20$; $p<0.01$]. Post-hoc comparisons revealed that in the higher dose drug group, acute drug administration decreased activity levels in both LE and HE animals. Repeated drug administration increased activity levels significantly in the HE drug groups, becoming similar or even higher as compared to the respective vehicle-treated animals already by the second testing, and remaining higher also on the third testing on the 10^{th} day of drug administration. In the LE animals treated with CP-154.526, the increase in activity was less significant as the activity levels remained lower as compared to the vehicle-treated animals on the second testing and became similar to the vehicle group on most measures by the 10^{th} day of drug administration.

The effect of long-term administration of CP-154,526 (2.5 and 7.5 mg/kg, i.p.) on plasma corticosterone levels

A significant Exploration effect was found on corticosterone levels in blood [$F(1, 65)=7.59$; $p<0.01$] since LE animals had higher levels of corticosterone in the naïve and both drug-treated groups (Fig. 3). A significant Treatment effect and an Exploration x Treatment interaction [$F(4, 65)=5.00$; $p<0.01$] were found on corticosterone levels [$F(4, 65)=3.11$; $p<0.05$], as behavioural testing decreased corticosterone levels to a similar level in both control groups, while vehicle treatment eliminated this effect in HE animals. Treatment with CP-154,526 increased corticosterone levels as compared to the vehicle group in LE animals and decreased it in HE-rats.

DISCUSSION

In these experiments we have studied the effects of CRF_1 receptor blockade with selective antagonist CP-154,526 on exploratory behaviour, plasma corticosterone and brain ex vivo monoamine levels in rats with low or high inherent exploratory activity and with or without previous drug-free experience of the test apparatus. In HE animals with previous experience of the test apparatus, CP-154,526 decreased exploratory behaviour on first administration but after 12-days administration with non-contingent behavioural testing there was no difference between drug- and vehicle-treated groups due to an increase in activity in the former. No drug effect was evident in LE animals. In animals with no previous experience of the test apparatus, CRF_1 blockade decreased exploratory behaviour on first administration in both LE and HE animals, but in the latter drug group activity levels increased to higher levels than in vehicle-treated animals after 5 days of administration and remained so also after 10 days. In LE-rats the drug still had an activity-decreasing effect after 5-days non-contingent administration and no effect after 10 days. Long-term treatment with CP-154,526 decreased DA turnover in PFC and DA levels in striatum in HE animals. It also increased 5-HIAA and 5-HT turnover in PFC and decreased these measures in hippocampus selectively in HE animals. Corticosterone levels were lower in naïve HE animals, and behavioural testing decreased corticosterone in both LE and HE groups. Behavioural testing with vehicle injections increased corticosterone levels in HE-rats only, while after CP-154,526 treatment corticosterone levels were decreased in HE-rats and increased in LE-rats as compared to the vehicle groups.

Blockade of CRF_1 receptors has been found to have an anxiolytic effect in many studies (reviewed in Seymour *et al*., 2003), but the effects are most clearly expressed in previously stressed animals, while the results are more variable in animals with no pretreatment. We have also reported anxiolytic effects of long-term CRF_1 antagonists, as a 5-days administration of either

CRA1000 (1.25 mg/kg) (Harro *et al*., 2001a) or CP-154,526 (2.5 mg/kg) (Mällo *et al*., 2004) with contingent behavioural testing increased exploratory behaviour in an anxiolytic-like manner. We have also pointed out that modifications in contingency of the CRF_1 antagonist administration and behavioural testing (Mällo *et al*., 2004) as well as drug dose (Harro *et al*., 2001a) may result in blocking the habituation-related increase in behavioural activity. These results suggest that CRF_1 receptor blockade could disturb adaptive behaviour in an increasingly familiar environment. Also, we have distinguished significant variations in the level of exploratory behaviour between individual animals, which, due to the underlying differences in monoaminergic neurotransmitter systems (Alttoa *et al*., 2005 and 2007; Mällo *et al*., 2007 and 2008) that are affected among others by the CRF-system, may have partly contributed to the variations in those results.

In the present experiments we found that CRF_1 blockade with CP-154,526 affected more the animals with high levels of exploratory activity, as the acute administration of the drug dose-dependently decreased activity levels in these animals. In the experiment in which the animals had been previously tested twice in the exploration box test and hence received treatment on their third encounter with the test apparatus, the activity levels were decreased in all HE groups as compared to pre-selection testing, and CRF_1 blockade aggravated this effect. As the activity levels in control and vehicle-treated HE groups on the first day of drug administration were similar in both experiments, the possibility can not be ruled out that a similar effect also occurred in the study in which animals were pre-tested in the light-dark box test. The stress elicited by injection procedure probably caused the decrease in activity levels in surroundings not associated with such aversive stimuli in vehicle-treated rats, as vehicle treatment has been found to act as a significant stressor in rats (Kondashevskaya and Nikolskaya, 2004). As the animals were submitted to the behavioural testing in a random order and hence shared the space during injections, the experiments may have also caused psychological stress in the control animals and been similarly anxiogenic for this group as the injection itself for the vehicle- and drug-treated animals. The animal's previous experience in both experiments may also be interpreted in the light of the effects of handling that have been found to increase activity in some tests of anxiety (Rebouças & Schmidek, 1997). It may be that in the context where the animal has been desensitized to an acute but mild stressor (i.e. handling and testing during the pre-testing session) which may in part be mediated by adaptive changes in the CRF-ergic systems, the disruption in the acquired balance in these systems by CRF_1 blockade results in an diametrically opposite effect. Probably the HE animals are more sensitive to such manipulations. It is noteworthy that in another study (White *et al*., 2007), animals with high motor response to novelty showed less anxiety in the elevated plus maze and defensive withdrawal tests, but more anxiety in the acoustic startle-induced vocalization test, suggesting

higher reactivity to stressful stimuli in this group. It has also been reported that previous testing experience affects consequent behaviour in other tests of anxiety differently in animals with different inherent levels of novelty seeking (Ballaz *et al*., 2007). This is in good accordance with the present finding that in LE animals with previous experience with the test apparatus, acute CRF_1 blockade did not affect exploratory activity, while in LE-rats with no such experience the drug effects were similar to the HE group. These results suggest disparities in adaptation to the environment in subgroups of animals that depend on both the inherent level on anxiety and specific features of the testing procedure that may have either anxiolytic, anxiogenic or no effect on subsequent behaviours. The aggravation of the injection stress effect in the HE animals after acute CRF_1 receptor blockade suggests that an intact CRF-system is needed particularly in HE-rats for adequate coping with acute stressors.

The differences in the test animals' former experience with the test apparatus did not affect the drug effects in HE animals on acute administration, but over repeated drug administration with non-contingent behavioural testing, a greater increase in behavioural activity was apparent in these HE-rats that had not been subjected to the test apparatus in drug-free conditions. The similar gradual patterns of increasing activity in both experiments, as well as our previous results of anxiolytic effects of repeated administration of CRF_1 antagonists (Harro *et al*., 2001a; Mällo *et al*., 2004) suggest that if the drug administration had been continued in the present Experiment 1, it would have increased activity levels in HE-rats higher as compared to the respective vehicle group. Hence, the previous drug-free experience of the test apparatus interacts with the effects of CRF_1 blockade on the behavioural activity levels on subsequent tests in HE-rats, while a significant anxiolytic effect of the drug appears in animals with no such experience already at the second encounter with the test apparatus. This relates quite well to the fact that while CRF increases behavioural activity in a familiar environment, low doses of CRF may also increase locomotor activity in a novel environment (Sutton *et al*., 1982), suggesting that an intact CRF system is needed for coping in a familiar surrounding. Apparently the blockade of CRF_1 receptors disrupts the mechanisms of habituation in HE animals in a way that gives the previous experience with the test apparatus an anxiogenic denotation for the individual animals that may last well into subsequent testing and becomes less significant over another round of gradual desensitization to the anxiogenic properties of the environment. In LE-rats, the long-term CRF_1 blockade apparently has less effect on exploratory behaviour as the activity levels in drug groups were similar to the vehicle-treated animals in both experiments after repeated administration of CP-154,526. Most importantly, it did not have an anxiolytic effect in the LE-rats, suggesting that the neurochemical basis of higher anxiety in this group is not directly controlled by CRF-ergic activity. Altogether, these results suggest a more specific role in the

regulation of novelty-related actions by the CRF_1 receptor in animals with high levels of exploratory activity.

In the prefrontal cortex, which is a brain region critical in novelty-related behaviour, ex vivo DA levels were found to be lower in the HE control group in Experiment 1 as compared to all other groups, and this group had a significantly higher DA turnover (calculated as DOPAC/DA) compared with both CP-154,526 treated HE groups. Also, in the striatum, HE animals had higher levels of tissue DA that is in good accordance with our previous results of higher extracellular DA levels in this region both at baseline conditions and in response to amphetamine administration (Mällo *et al*., 2007). CRF_1 blockade also dose-dependently decreased DA levels in the striatum in HE animals only. Previously, acute blockade of D_1 receptors has been found to reduce CRF-induced increases in startle amplitude (Meloni *et al*., 2006) suggesting that DA-ergic systems are at least partly responsible for the mediation of CRF-related responses to stressors. Acute administration of CP-154,526 has been found not to affect extracellular DA levels in PFC (Isogawa *et al*., 2000), but a 10-days CRF_1 blockade with antalarmin has been reported to reverse the isolation rearing-increased D_2 receptor density in the central amygdala and nucleus accumbens (Djouma *et al*., 2006). Together with the current results an interplay of mutual influences between the CRF and DA systems may be suggested that has greater role in the regulation of exploratory activity in HE-rats.

Dorsal raphe 5-HT-ergic neurons are innervated by CRF-immunoreactive fibers and the administration of CRF has inhibitory effects on dorsal raphe discharge (Kirby *et al*., 2000). It has also been found that the electric footshock stress-induced increases in 5-HT turnover in several brain regions are further elevated by the administration of the nonselective CRF antagonist alpha-helical CRF_{9-41} (Li *et al*., 1998). LE-rats had higher ex vivo 5-HIAA levels and 5-HT turnover in the PFC as compared to the respective HE groups, while treatment with CP-154,526 (10 mg/kg) eliminated this difference. In HE-rats there was a dose-dependent increase of 5-HIAA levels and 5-HT turnover in PFC after treatment with CP-154,526, and the higher dose group had significantly higher 5-HIAA levels and 5-HT turnover as compared to all other HE groups, while the drug had no effect on LE animals. We have previously reported a statistically nonsignificant trend in the same direction in the PFC in two experiments, using 2.5 mg/kg of CP-154,526 and animals not preselected (Mällo *et al*., 2004). It might be that in HE-rats CRF has a stronger inhibitory effect on 5-HT neurons. Conversely, in the hippocampus that is mainly innervated by 5-HT-ergic projections from the median raphe, CP-154,526 dose-dependently reduced 5-HIAA and 5-HT in HE-rats only. Based on the current contrasting effects of CRF_1 blockade on 5-HT-ergic activity in PFC and hippocampus in HE-rats, we have studied and reported that acute administration of citalopram via reverse microdialysis results in smaller increase in the levels of extracellular 5-HT in the HE animals in PFC and in higher increase in

dentate gyrus in this group (Mällo *et al*., 2008), while at baseline conditions no difference was found between the LE and HE groups. Together these results suggest that in HE animals, the effects of manipulations affecting the 5-HT system are more variable than in LE animals that may in part be responsible for the greater variation in their behavioural profiles as compared to the LE-rats. Although straightforward associations have been drawn between the activity of 5-HT-ergic systems and anxiety-related behavioural states (Griebel, 1995), it nevertheless needs to be pointed out that while CRF_1 receptor blockade eliminated the differences between LE and HE animals in 5-HT measures in the present study, it did not attenuate behavioural differences. This suggests that the differences in 5-HT-ergic system do not directly form the basis of the baseline behavioural differences between le and HE animals, but rather differently modify the patterns of habituation in an increasingly familiar environment.

Trunk blood samples were collected on the 10^{th} day of CP-154,526 administration, 2–3 h after the last exploration box test. Previous studies have shown that behavioural testing has an effect on blood corticosterone levels, but the results are controversial regarding as to when the corticosterone levels normalize after testing. For an example, it has been reported that in two hours after a situation as anxiogenic as an elevated platform (for 60 min) the corticosterone levels have normalized at baseline levels (Degroot *et al*., 2004), while in a similar experiment by another group, the corticosterone levels remained fairly high 125 min after 5 min on the open arm of an elevated plus-maze (Kinzig *et al*., 2003). So, regarding the current results, it has to be taken into account that the corticosterone levels may in part reflect the effect of the last behavioural testing (except for the naïve group). Comparing the naïve groups, LE animals had significantly higher baseline levels of corticosterone than HE animals. Repeated testing without injections significantly decreased corticosterone to a similar level in both LE- and HE-rats, which associates well with the decreased anxiety accompanying repeated behavioural testing. The corticosterone levels in the vehicle-treated group were similar to behaviourally tested controls in case of LE-rats, but similar to experimentally naïve animals in the HE-rats, indicating that injection stress had greater effect on corticosterone in the latter group. Ten days of administration of CP-154,526 reduced corticosterone levels in HE-rats, but siginificantly increased the stress hormone levels in LE-rats as compared to vehicle. Thus, chronic CRF_1 receptor blockade eliminated the increasing effect of daily injection stress on corticosterone levels that appeared in the HE-rats, while in LE-rats it prevented the habituation effect that decreased corticosterone levels in controls. As a net effect, after repeated CP-154,526 treatment differences in corticosterone levels in LE- and HE-rats were as in naïve animals. Thus far, different CRF_1 receptor antagonists have been found to reduce corticosterone levels or prevent stressor-induced elevation of corticosterone levels when administered acutely (Heinrich *et al*., 2002; Gutman *et al*., 2003; Lelas *et al*., 2004). When administered chronically, another

CRF_1 antagonist, antalarmin, has been found to significantly decrease plasma corticosterone levels as compared to vehicle-treated rats (Bornstein *et al*., 1998). Also, when administered chronically to obese and lean Zucker rats (Doyon *et al*., 2007), the CRF_1 receptor antagonist SSR125543 was shown to reduce plasma corticosterone levels in lean rats only, partly supporting the present finding that the results of long-term CRF_1 blockade may have different effects on HPA axis activity measures in selected subpopulations of rats. Interestingly, we have previously found a highly similar pattern of correlations between the transcription factor AP-2 levels in locus coeruleus and noradrenaline levels in PFC (Mällo *et al*., 2004). Namely, the correlation between AP-2 in the locus coeruleus and NA levels in the hippocampus were negatively correlated in naïve animals and in animals chronically treated with CP-154,526, while in vehicle-treated group the correlation was positive. Together these results suggest that chronic CRF_1 blockade is able to reverse the effects of handling- and injection procedure stress on biological measures of hypothalamic-pituitary-adrenal (HPA) stress axis and locus coeruleus-related stress system activity.

The increase in behavioural activity in the drug-treated HE groups in Experiment 2, as compared to vehicle group, suggests an anxiolytic effect of CP-154,526. The behavioural profiles of HE control and vehicle-treated animals did not differ significantly, although the latter had higher corticosterone levels. Also, in LE animals the behavioural activity on the third testing did not differ between treatment groups, although the drug groups had higher levels of corticosterone as compared to controls and vehicle-treated animals. It has been previously demonstrated that the behavioural effects of another CRF_1 antagonist, R121919 may be mediated independently of the drug-induced changes in adrenocorticotropic hormone (ACTH) secretion, as the drug increased activity only in animals selectively bred for high anxiety in the elevated plus maze, while its effects on the ACTH response to plus maze exposure were similar in both high and low anxiety groups (Keck *et al*., 2001). Together, the differential effects of CRF_1 blockade on corticosterone levels in LE and HE animals suggest that its effects on exploratory activity in these subgroups are at least partly independent of the HPA axis.

The present findings suggest that the behavioural effects of chronic blockade of the CRF_1 receptor are dependent on the test animals' former experience with the test apparatus. In animals that have previously been tested in exploration tests, the acute CRF_1 blockade decreases activity in the exploration box test. The extent of this effect depends on the inherent levels of exploratory activity in the test animals and on whether the test environment in which the drug is administrated is the same or different as in previous drug-free conditions. It is important to point out that possible similar effects of former testing experience should be paid attention to in the studies of other antidepressant and anxiolytic manipulations. Chronic CRF_1 blockade with CP-154,526 decreased corticosterone levels in HE animals and increased in LE animals, as compared to vehicle

and control groups probably by eliminating the effect of daily injection stress on corticosterone levels in the HE group and by preventing the handling effect in LE animals. The diametrically opposite effects of CRF_1 receptor blockade on corticosterone levels in LE and HE animals suggest differences in the stress-related neurochemical systems in these groups.

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ABBREVIATIONS

5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HE, high exploratory activity; HPA, hypothalamic-pituitary-adrenal axis; HVA, homovanillic acid; i.p., intraperitoneal; LE, low exploratory activity; NA, noradrenaline; PFC, prefrontal cortex.

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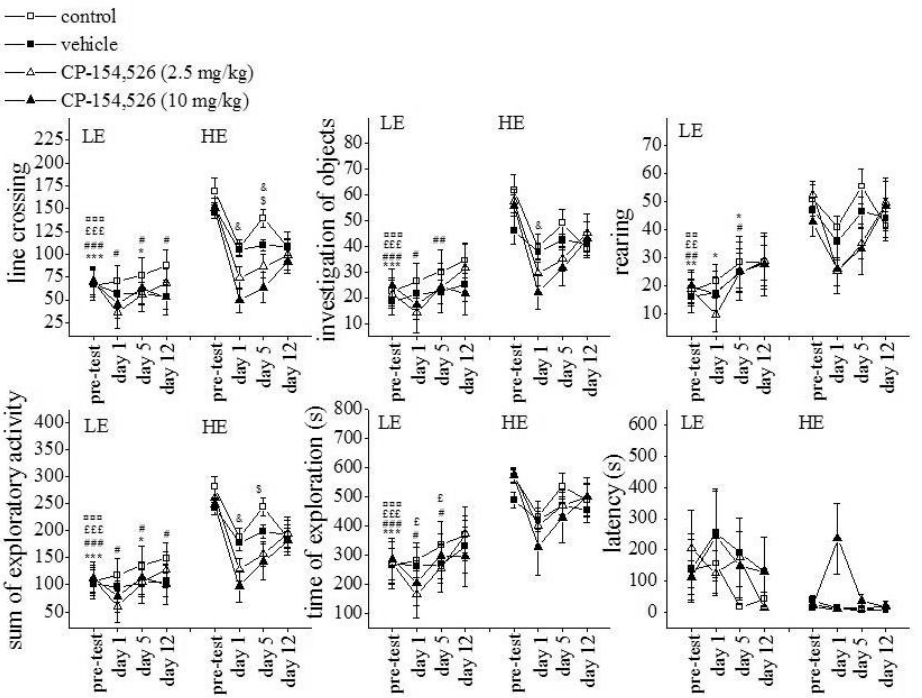

Figure 1. The effect of CP-154,526 on exploratory behaviour in animals pre-tested in the exploration box test (Paper IV). Data are given as mean ± SEM. * – $p<0.05$, ** – $p<0.01$; *** – $p<0.001$, as compared to the respective HE control; # – $p<0.05$; ## – $p<0.01$; ### – $p<0.001$, as compared to the respective HE vehicle; £ – $p<0.05$; ££ – $p<0.01$; £££ – $p<0.001$, as compared to the respective HE CP-2.5 mg/kg; ¤¤ – $p<0.01$; ¤¤¤ – $p<0.001$, as compared to the respective HE CP-10 mg/kg; $ – $p<0.05$, veh vs control group; & – $p<0.05$, CP-10 mg/kg as compared to respective vehicle group. The treatment groups between which significant differences exist are given in parenthesis on the graph. Data on entries is not shown. CP 2.5 – CP-154,526 2.5 mg/kg group; CP10 – CP-154,526 10 mg/kg group; HE – high exploratory behaviour rats; LE – low exploratory behaviour rats; pre – second baseline exploratory activity measurement.

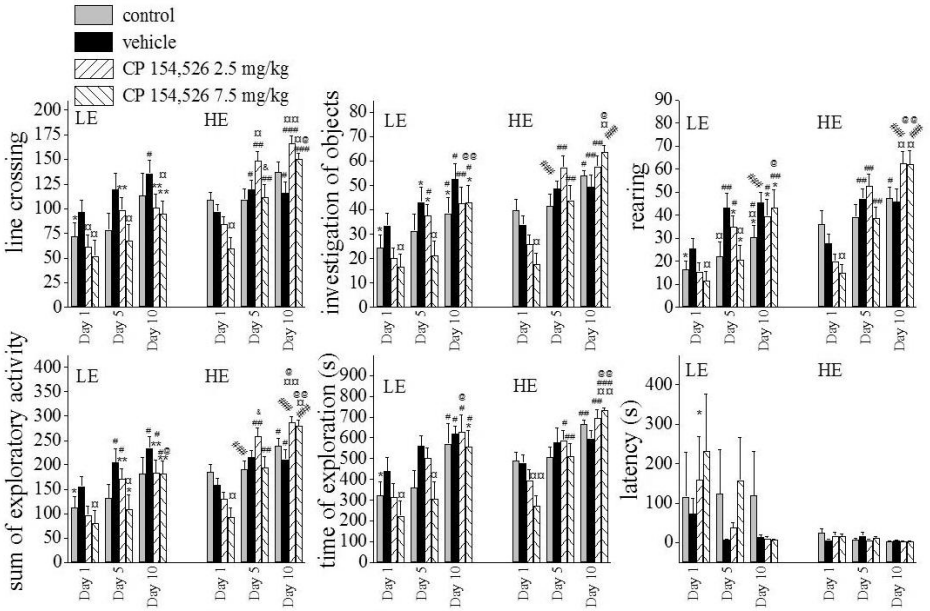

Figure 2. The effect of CP-154,526 on exploratory behaviour in animals pre-tested in the light-dark test. Data are given as mean ± SEM. * – $p<0.05$, ** – $p<0.01$, as compared to respective HE group; $ – $p<0.05$, $$ – $p<0.01$ as compared to respective control group; ¤ – $p<0.05$, ¤¤ – $p<0.01$ as compared to respective vehicle group; & – $p<0.05$ as compared to the respective CP-154,526 7.5 mg/kg group; # – $p<0.05$, ## – $p<0.01$, ### – $p<0.001$ as compared to Day 1; @ – $p<0.05$, @@ – $p<0.01$ as compared to Day 5. LE – low exploring animals; HE – high exploring animals.

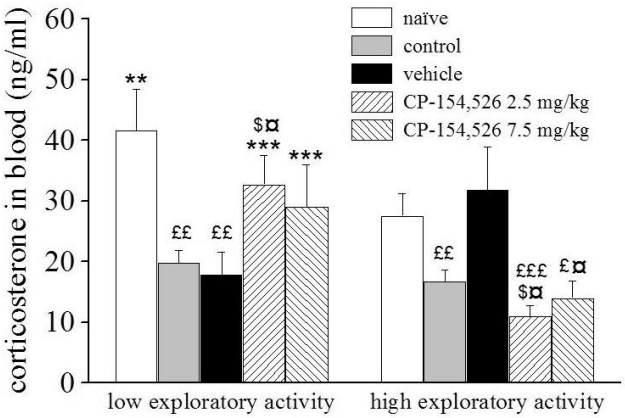

Figure 3. The effect of long-term administration of CP-154,526 on plasma corticosterone levels. Data are given as mean ± SEM. ** – $p<0.01$, *** – $p<0.001$ as compared to respective HE group; £ – $p<0.05$, ££ – $p<0.01$, £££ – $p<0.01$ as compared to respective naïve group; $ – $p<0.05$ as compared to respective control group; ¤ – $p<0.05$ as compared to respective vehicle group.

Table 1. The effect long-term treatment with CP-154,526 on monoamine concentrations in the frontal cortex, hippocampus, striatum and septum (n=7–9).

		control		vehicle		CP-154,526 2.5 mg/kg		CP 154,526 10 mg/kg	
		LE	HE	LE	HE	LE	HE	LE	HE
frontal cortex	NA	1.88±0.05	1.71±0.06	2.02±0.15	1.94±0.13	1.94±0.09	2.01±0.10	1.90±0.05	1.94±0.08
	DA	0.13±0.01	0.09±0.01**	0.13±0.01	0.12±0.01	0.13±0.01	0.13±0.01	0.12±0.01	0.12±0.00
	DOPAC	0.33±0.01	0.29±0.02	0.31±0.02	0.32±0.01	0.29±0.02	0.31±0.01	0.35±0.03	0.32±0.03
	DA turnover	2.68±0.18	3.16±0.23	2.39±0.18	2.79±0.18	2.28±0.09$^{\#\#}$	2.45±0.12	2.91±0.16§	2.57±0.20$^{\#\# \$}$
	5-HT	3.48±0.11	3.48±0.22	3.20±0.09	3.03±0.22	3.66±0.17§	3.29±0.12	3.64±0.14	3.36±0.16
	5-HIAA	1.65±0.18	1.17±0.33	1.55±0.28	0.73±0.27*	1.84±0.06	1.16±0.28*	1.81±0.09	1.85±0.06$^{\# \S\S\S \$}$
	5-HT turnover	0.48±0.06	0.31±0.08*	0.48±0.08	0.21±0.06**	0.51±0.02	0.34±0.08	0.50±0.01	0.56±0.03$^{\#\# \S\S\S \$}$
hippo-campus	NA	3.74±0.28	5.22±0.45*	3.80±0.43	5.26±0.44*	4.10±0.45	4.27±0.47	4.21±0.40	4.39±0.41
	DA	0.16±0.02	0.19±0.03	0.13±0.01	0.25±0.09*	0.14±0.02	0.18±0.03	0.14±0.01	0.17±0.02
	5-HT	3.68±0.28	4.50±0.28*	3.05±0.33	3.92±0.29*	2.86±0.16$^{\#}$	3.66±0.27$^{\# *}$	3.13±0.22	2.78±0.19$^{\#\#\# \S\S \$}$
	5-HIAA	3.07±0.21	4.07±0.29***	2.85±0.18	3.48±0.22$^{\# *}$	2.79±0.08	3.17±0.15$^{\#\#}$	3.01±0.15	2.82±0.15$^{\#\#\# \S}$
	5-HT turnover	0.84±0.03	0.91±0.05	0.97±0.06	0.90±0.04	0.99±0.05	0.89±0.03	0.98±0.04	1.04±0.07§
striatum	NA	0.59±0.04	0.59±0.04	0.68±0.05	0.61±0.02	0.64±0.05	0.72±0.05	0.53±0.03	0.62±0.05
	DA	64.94±3.40	73.66±3.95	64.59±0.88	71.68±4.47	59.25±2.74	65.90±4.24	64.80±1.85	64.53±3.20
	DOPAC	10.14±0.38	10.22±0.54	10.86±0.51	9.93±0.24	9.65±0.43	10.06±0.39	9.61±0.22	10.10±0.24
	HVA	4.04±0.24	3.71±0.19	4.11±0.20	3.72±0.12	3.58±0.14	3.80±0.24	3.79±0.10	4.01±0.12
	DA turnover	0.22±0.01	0.19±0.00	0.23±0.01	0.19±0.01	0.23±0.01	0.22±0.02	0.21±0.01	0.22±0.01
	5-HT	2.86±0.12	3.04±0.13	2.74±0.09	2.73±0.06	2.76±0.08	2.95±0.13	2.73±0.06	2.91±0.09
	5-HIAA	2.68±0.08	2.89±0.07	2.63±0.13	2.60±0.07	2.59±0.11	2.77±0.11	2.61±0.05	2.84±0.09
	5-HT turnover	0.94±0.03	0.96±0.02	0.96±0.03	0.95±0.02	0.94±0.02	0.94±0.02	0.96±0.02	0.98±0.04

		control		vehicle		CP-154,526 2.5 mg/kg		CP 154,526 10 mg/kg	
		LE	HE	LE	HE	LE	HE	LE	HE
septum	NA	5.8±0.39	6.26±0.34	5.85±0.56	6.46±0.27	6.01±0.42	5.74±0.42	6.15±0.19	5.82±0.34
	DA	5.97±0.94	5.46±0.57	4.86±0.54	5.84±0.87	4.31±0.42	6.34±0.74	6.82±1.00$^{\$}$	4.42±0.37*
	DOPAC	1.51±0.38	1.44±0.20	1.18±0.17	1.64±0.27	1.03±0.10	1.64±0.21	1.54±0.19	1.21±0.09
	HVA	0.63±0.09	0.61±0.07	0.60±0.04	0.67±0.05	0.63±0.05	0.63±0.06	0.67±0.06	0.66±0.07
	DA turnover	0.33±0.04	0.37±0.02	0.33±0.04	0.41±0.03	0.40±0.03	0.36±0.02	0.33±0.03	0.43±0.04*
	5-HT	3.14±0.16	3.65±0.27	2.88±0.25	3.50±0.24	2.94±0.12	3.22±0.19	3.46±0.17	3.10±0.17
	5-HIAA	2.40±0.14	2.78±0.16*	2.24±0.13	2.71±0.07*	2.42±0.12	2.51±0.10	2.41±0.07	2.57±0.17
	5-HT turnover	0.77±0.04	0.77±0.04	0.79±0.03	0.79±0.05	0.82±0.02	0.79±0.04	0.71±0.03	0.83±0.05

* – significantly different as compared to the corresponding LE group; § – significantly different as compared to the corresponding vehicle group; # – significantly different as compared to the corresponding control group. $ – significantly different as compared to the corresponding CP 2.5 mg/kg group. Data are given as mean ± SEM. one symbol – $p<0.05$, two symbols – $p<0.01$, three symbols – $p<0.001$; Fisher's PLSD after significant ANOVA. LE – low exploring animals; HE – high exploring animals.

V

Mällo T., Matrov D., Herm L., Kõiv K., Eller M., Rinken A., Harro J. (2007).
Tickling-induced 50-kHz ultrasonic vocalization is individually
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Behavioural Brain Research, 184: 57–71.

VI

Mällo T., Matrov D., Kõiv K., Harro J.
Effects of chronic variable stress on behaviour and cerebral oxidative metabolism in rats with high or low levels of 50-kHz ultrasonic vocalizations.
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EFFECTS OF CHRONIC VARIABLE STRESS ON BEHAVIOUR AND CEREBRAL OXIDATIVE METABOLISM IN RATS WITH HIGH OR LOW LEVELS OF 50-KHZ ULTRASONIC VOCALIZATIONS

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ABSTRACT

The 50-kHz ultrasonic vocalizations (USVs) in rats have been associated with positive and rewarding experience. We have previously reported that stable inter-individual differences are expressed in the level of these USVs that are associated with differences in anxiety-related behaviours. Brain metabolic activity and behaviour in male and female high- and low-chirping untreated rats and after four weeks of chronic variable stress were studied. Significant differences in brain metabolic activity were found between male and female rats with lower metabolic activity in females in brainstem regions and higher metabolic activity in amygdala and related limbic regions. Stress almost exclusively affected male LC-rats and female HC-rats, increasing metabolic activity in all regions affected in the first group and decreasing it in the latter, suggesting greater vulnerability to chronic stress in these groups. In females, vulnerability to stress seems to be generally lower as compared to males in group housing conditions. Nevertheless, the females emitting high levels of 50-kHz chirping when tickled, revealed more changes in brain metabolic activity, suggesting that at least on cellular level these animals were more sensitive to the stress procedure. In males, stress increased metabolic activity in limbic brain regions in LC animals that was accompanied by increased levels of 22-kHz USVs, earlier and more sustained appearance of the decreasing effect of stress on weight gain, the inhibitory effect of stress on increase in sucrose preference, and higher levels of immobility in both control (i.e. acutely stressed) and stressed animals in the forced swimming test, indicative of greater vulnerability to stress in low-chirping animals.

Key words: ultrasonic vocalization; 50-kHz calls; 22-kHz calls; emotionality; anxiety and depression tests; cytochrome c oxidase, rat.

INTRODUCTION

The study of ultrasonic vocalizations (USVs) in laboratory rats has approached vocalizations associated with both negative and positive connotation. Beside the USVs elicited in the contexts of maternal separation in rat pups (Brunelli and Hofer, 2007) and anxiety and other negative affective states in adult rats (for a recent review, see Litvin *et al*., 2007), rat ultrasonic vocalizations associated with various positive stimuli are gaining increasing attention (Panksepp, 2007; Portfors, 2007; Schwarting *et al*., 2007). It is possible to distinguish at least three different forms of USVs in common laboratory rats: 1) the 40-kHz distress vocalizations in rat pups that are routinely observed in maternal separation paradigms; 2) juvenile and adult USVs with long duration and frequency below 30 kHz (usually termed „22-kHz USVs") that seem to be the adult counterpart of the 40-kHz calls, and 3) short and high-frequency so-called 50-kHz USVs that have been found to range from 30 to 70 kHz (Kaltwasser, 1990; Knutson *et al*., 1998; Brudzynski and Pniak, 2002). The latter two seem to convey information on diametrically opposite affective states, namely aversion and anticipation in adult rats (Knutson *et al*., 1999; Burgdorf *et al*., 2001) and have been found to have almost non-overlapping spectrographic parameters as well as different neurobiological substrates (Brudzynski, 2007).

It has been found that play is highly rewarding to young rats (Vanderschuren *et al*., 1997), and the USVs emitted around the frequency range of 50 kHz or so-called „rat laughter" was first discovered in the context of play behaviours in juvenile rats (Knutson *et al*., 1998). By now this type of USVs has been related to many phenomena that can be associated to positive affective states (for reviews, see Knutson *et al*., 2002; Brudzynski and Pniak, 2002; Panksepp *et al*., 2002b). A procedure of experimenter-administered tickle-like stimulation is often exploited in order to mimic the natural rough-and-tumble play in juvenile rats (Burgdorf and Panksepp, 2001) and to simultaneously elicit very high levels of 50-kHz vocalization in rats (Burgdorf *et al*., 2005). Also, many other rewarding stimuli from drugs of abuse to electrical brain stimulation have also been found to evoke these USVs (Knutson *et al*., 1999; Burgdorf *et al*., 2000). The rate of 50-kHz vocalizing has been found to predict the pleasantness and reinforcing value of stimuli (Burgdorf and Panksepp 2001). Two types of these calls have been focussed on: flat USVs with minimal bandwidth variation around 5–7 kHz and others that include a significant frequency modulation (FM), or so-called trill component (Schwarting *et al*., 2007; Burgdorf *et al*., 2007). The specific different roles of these two subgroups of USVs have not been clarified yet, although it has already been found that there are differences in the extent in which they are modulated by pharmacological manipulations (Burgdorf *et al*., 2007).

The type of ultrasound calls at about the range of 20–30 kHz that adolescent and adult rats elicit in aversive situations like foot shock and drug withdrawal

(Brudzynski, 2001; Portfors, 2007), are decreased by anxiolytic substances similarly to separation distress calls (Miczek *et al*., 1995). Also, there seems to be a negative correlation between the 22-kHz and 50-kHz USVs in certain conditions (Burgdorf *et al*., 2005) and the aversive stimuli that increase the 22-kHz USVs have been found to simultaneously decrease the 50-kHz calls (Burgdorf *et al*., 2001).

By now, it has been found that stable individual variations exist in 50-kHz USVs (or more colloquially, „chirps“) (Mällo *et al*., 2007; Panksepp *et al*., 2002a; Schwarting *et al*., 2007), suggesting that the extent of emitting these USVs refers to an individual disposition or trait. This trait is probably connected with genetic and physiological variables, since breeding studies have yielded distinguishable lines of high and low chirpers (Burgdorf *et al*., 2005). We have previously reported that juvenile rats of both sexes, when given daily tickling sessions, develop an individually characteristic level of 50-kHz USV response, that remains stable over long periods of time, and enables the classification of animals into high and low chirpers (HC and LC animals, respectively) that emit high and low levels of 50-kHz USVs in response to tickling (Mällo *et al*., 2007). We have also found inter-individual variations in reactivity to stress in animals with high sociability that developed lower levels of sucrose intake after three weeks of chronic variable stress (Tõnissaar et al., 2008), elaborating on the previous finding that greater vulnerability to stress may occur in subgroups of unselected rats (Nielsen et al., 2000). While tickling on its own was found to have an anxiolytic effect on behavioural measures, the high-chirping rats tended to be more affected by different environmental and experimental stimuli like strong stressors or incentives, suggesting that variations in vulnerability to chronic stress may also develop between the HC and LC animals. Behavioural differences were found between male and female rats, as tickling had less effects in the latter group in our previous studies (Mällo et al., 2007), although the chirping levels elicited by tickling of respective HC and LC groups were similar between sexes.

The study of Fu and Brudzynski (1994) in which glutamate and carbachol were injected into anterior hypothalamic-preoptic area, concluded that the 50-kHz and 22-kHz USVs have different underlying neurobiological mechanisms as the former were dose-dependently elicited by glutamate administration, while the latter were elicited by carbachol injection. Later findings have specified that the 50-kHz USVs are induced by the activation of ascending dopamine(DA)ergic system, while the 22-kHz USVs are induced by the ascending cholinergic system, with the regulation of the respective positive and negative affective states also controlled by these pathways (Brudzynski, 2007). Our own previous results suggested that tickling induces changes in brain serotonergic and DA-ergic systems that are to some extent dependent on the animals' USV response to tickling procedure (Mällo *et al*., 2007).

In the current experiments, we have studied the effects of chronic stress regimen on male and female HC- and LC-rats, in order to further elucidate the distinction between these two groups. We have also studied metabolic activity in brain in order to map long-term changes in brain in response to tickling and stress for identifying brain regions underlying the behavioural variations between sexes and HC and LC subgroups.

METHODS

Animals

Seven breeding pairs of male and female Wistar rats (Scanbur BK AB, Sweden) were used to acquire rat pups. Male (n=33) and female (n=29) pups were weaned when 3 weeks old and single-housed in standard transparent polypropylene cages under controlled light cycle (lights on from 08:30 h to 20:30 h) and temperature (19°–21°C), with free access to tap water and food pellets (diet R70, Lactamin, Sweden). Tickling sessions started the next day after single-housing. The animals were group-housed (by four) after the end of the tickling sessions two weeks later, and remained so until the end of the experiment.

General procedure

Single-housed rats were given daily sessions of experimenter-induced stimulation ("tickling", Burgdorf and Panksepp, 2001) for 14 days as we have previously shown that by this period the animals develop a stable level of USV response that remains similar for a long period (Mällo *et al*., 2007). In the beginning of a tickling session, the animal was taken to an adjacent room from the animal room with similar lighting conditions, removed from its home cage and placed into an empty and smaller (32 x 14 x 13 cm) cage, located under a microphone about 20 cm from the floor of the cage. The animal was given 15 s to habituate with the new cage, followed by 15 s of handling by experimenter that mimicked natural rough-and-tumble play in juvenile rats. In short, the "tickling" session that each animal received consisted of stimulating the rat with one hand by the experimenter, that included rapid finger movements on the back of the neck, turning the animal on the back and letting it "wrestle" with the experimenter's hand with vigorous alternating finger movements administered on the animals' ventral surface, followed by release after 1-2 seconds of stimulation. Altogether, four 15 s sessions of stimulation were given over two minutes, after which the animal was again placed in its home cage and the test cage was cleaned thoroughly. The recorded audio files were later analyzed with the Avisoft SASLab Pro software, creating spectrograms from which the 22-kHz USVs, plain 50-kHz USVs and those USVs that contained a frequency modulated, or a "trill" component 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, providing the HC and LC groups. At the age of two months, a chronic variable stress procedure was started, that lasted for 4 weeks. During the stress period, weekly sucrose preference testing were carried out, first on the night before the stress regimen. The animals were weighed daily during the stress regimen. After the cessation of the stress regimen, several behavioural tests were carried out as follows: elevated plus-maze, exploration box test on two consecutive days, and a forced swimming test on two consecutive days. On the day after the last behavioural test, the animals were sacrificed by decapitation and whole brains immediately frozen on dry ice.

Chronic variable stress

The chronic variable stress regimen lasted for four weeks and comprised of seven different stressors that were intermittently used once every week. The stressors, presented in the order of administration were: 1) cold (4°C) water and wet bedding (initially, 400 ml of water was poured on the rats, and the sawdust bedding was kept wet for the following 22 h); 2) imitation of a peritoneal injection with the rough and firm grasping of the animal using special glove and syringe without the needle, which was pressed to the animal's body for several seconds; 3) stroboscopic light (for 14 h, 10 Hz, 2 lx); 4) tail pinch with a clothes-pin placed 1 cm distal from the base of tail (5 min); 5) cage tilt at 45° (for 24 h); 6) movement restriction in a small cage (11×16×7 cm for 2 h); 7) strong illumination (900 lx) during the predicted dark phase (for 12 h). Five sucrose preference tests were carried out during the stress regimen, with the first one on the night preceding the first stressor and the following ones at the end of every week. The stressors were administered during the light phase of the cycle (except for the ones that lasted overnight).

Sucrose preference test

The sucrose preference tests were carried out 5–6 hours after the beginning of the dark phase. Sucrose preference test was carried out with two bottles, one filled with 1% sucrose solution and the other with water. The animals were placed into single cages immediately before the sucrose preference testing and group-housed again after that. Sucrose and water consumption were measured for the period of 1 h by weighing the bottles both at the beginning and at the end of the test. Sucrose preference was measured by calculating the proportion of sucrose solution consumption out of total consumption of water and sucrose solution.

Elevated plus-maze test

The method first described by Handley and Mithani (1984) and modified for low baseline open arm activity in our laboratory (Harro *et al*., 1990) was used. In brief, the plus-maze consisted of two open arms (50 x 10 cm) without any

walls, two enclosed arms of the same size with 40 cm high side walls and end wall, and the central arena (10 x 10 cm) interconnecting the arms. The arms of the same type were opposite to each other. Both open arms were divided into three parts of equal size by lines which also separated the central arena from all arms. At the beginning of the experiment the rat was placed into a closed arm. The central arena and the open arms formed the 'open part' of the apparatus. An entry into open arms was counted when the rat crossed the line between the central arena and an open arm with all four paws. The rat was considered to explore the open part of the apparatus when it had clearly crossed the line between a closed arm and the central arena with its both forepaws. Behavioural measures taken during 4 minutes included a) the latency period before entering the open part (i.e. the central arena); b) the number of line crossings; c) time spent in the open arms of the apparatus; d) the number of approaches towards the central arena which were not completed (nose crossed the line but not both of the forepaws); e) the number of open arm entries, and f) the total number of arm entries. From the two latter measures, the open/total arm entries ratio was calculated.

Exploration box test

The exploration box test has been previously described in more detail in Otter *et al* (1997) and Mällo *et al*., (2007). In short, the apparatus was made of metal and consisted of an open area 0.5 m x 1 m (height of side walls 40 cm) with a small compartment 20 cm x 20 cm x 20 cm attached to one of the shorter sides of the open area. The open area was divided into eight squares of equal size, with four objects, three unfamiliar and one familiar (a glass jar, a cardboard box, a wooden handle and a food pellet) situated in certain places. The small compartment, which had its floor covered with wood shavings, was directly linked to the open area through an opening (size 20 cm x 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).

Forced swimming test

The forced swimming test, first described by Porsolt (1978) was carried out as described previously (Häidkind *et al*., 2004). Briefly, rats were placed into a vertical glass cylinder (diameter 22.5 cm and height 60 cm) containing about

35 cm water at 25°C. On the first day of the experiment, the procedure lasted 15 min and the re-exposure 24 h later lasted 5 min. At the end of each session the rats were dried with laboratory tissues. The sessions were recorded with a video camera and the durations of immobility, swimming and struggling were measured afterwards. The measurements were based on the behavioural categories described by Armario (1988). A rat was judged to be immobile when it remained floating in the water with all limbs motionless. 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 touching the walls of the tank. The time spent in swimming was recorded when the rat was making active swimming motions, more than necessary to merely maintain its head above the water, e.g., moving around in the cylinder.

Cytochrome c oxidase histochemistry and image analysis

The measurements were carried out as described previously (Kanarik *et al*., 2008). In short, the 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 with all the steps in 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. 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. Eighty-nine regions of interest were detected from the stained

images with the help of Paxinos and Watson (1986) rat brain atlas. Grayscale values were transformed to optical density values with the help of Kodak gray-scale tablet with known grayscale and optical density values. Optical density of any given region was sampled and averaged from three consecutive slices of the same hemisphere in each brain but 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.

Data analysis

The data were analyzed with a three-factor (Sex x Stress x Chirping) ANOVA for the elevated plus-maze behaviour and COX measurements, and with a three-factor (Sex x Stress x Chirping) ANOVA with repeated measures for the rest of data. When appropriate, post-hoc comparisons were made with Fisher's PLSD test. Statistical significance was set at $p<0.05$.

RESULTS

Ultrasonic vocalizations

In both sexes, chirping levels were significantly higher in HC groups as compared to LC animals *[Chirping, $F_{1, 54}=93.2$; $p<0.001$]*, except for the stress group on post-stress testing in males and in control group on post-stress testing in females (Fig. 1). In males, chirping levels were higher at baseline testing as compared to the pre-stress and post-stress testing in both HC and LC animals *[Repeated testing, $F_{2, 108}=123.6$; $p<0.001$; Sex x Repeated testing, $F_{2, 108}=24.0$; $p<0.001$; Chirping x Repeated testing, $F_{2, 108}=37.5$; $p<0.001$]*. In female animals, only the HC groups had significantly lower levels of chirping on both pre- and post-stress tests as compared to the baseline levels. As compared to the pre-stress levels, the number of 50-kHz chirps was significantly lower after stress in HC animals only, while no such changes were visible in the male LC group and in either female group.

The FM USV levels were significantly higher in HC groups of both sexes as compared to the respective LC animals in baseline and pre-stress testing *[Chirping, $F_{1, 54}=59.0$; $p<0.001$; Repeated testing, $F_{2, 108}=111.1$; $p<0.001$; Chirping x Repeated testing, $F_{2, 108}=21.2$; $p<0.001$]*. After stress, the HC-LC difference was maintained only in male controls. In male rats, FM USV levels were lower as compared to the baseline on both pre-stress and post-stress testing in both HC and LC animals, while in females only HC-rats in post-stress test had significantly lower levels as compared to the baseline *[Sex, $F_{1, 54}=8.33$; $p<0.001$; Sex x x Repeated testing, $F_{2, 108}=28.1$; $p<0.001$]*. In male HC-stress rats, FM USV levels were lower in post-stress test as compared to the pre-stress test, while in females similar decrease was significant in both control and stress HC groups.

In male rats, LC stress group had significantly higher levels of 22-kHz USVs in the post-stress test, while in females the HC stress group had higher levels of 22-kHz USVs in the post-stress test *[Sex, $F(1, 53)=4.38$; $p<0.05$; Sex x Repeated testing, $F(2, 106)=7.24$; $p<0.01$; Stress x Repeated testing, $F(2, 106)=3.43$; $p<0.05$; Sex x Chirping x Repeated testing, $F(2, 106)=7.80$; $p<0.001$]*. In male rats, LC stress group had significantly higher levels of 22-kHz USVs in the post-stress test as compared to both baseline and pre-stress tests. In females, the same was true for the HC stress group. In males, stress significantly increased 22-kHz USVs in the LC group, while in females stress increased 22-kHz USVs in HC animals.

Weight gain during chronic stress

Stress significantly decreased weight gain in male rats already by the third day of stress regimen in both HC and LC animals and this difference remained significant until the end of the experiment in LC-rats, while in HC-rats the difference became consistent during the fourth week of stress regimen (Fig. 2A) *[Sex, $F_{1, 54}=541.2$; $p<0.001$; Stress, $F_{1, 54}=8.66$; $p<0.01$; Sex x Stress, $F_{1, 54}=8.28$; $p<0.01$; Repeated testing, $F_{33, 1782}=631.7$; $p<0.001$; Sex x Repeated testing, $F_{33, 1782}=127.6$; $p<0.001$; Stress x Repeated testing, $F_{33, 1782}=4.05$; $p<0.001$; Sex x Stress x Repeated testing, $F_{33, 1782}=3.47$; $p<0.001$; Sex x Chirping x Repeated testing, $F_{33, 1782}=1.81$; $p<0.01$; Sex x Stress x Chirping x Repeated testing, $F_{33, 1782}=1.71$, $p<0.01$]*. Neither stress nor chirping levels affected weight gain in female rats (Fig. 2B).

Sucrose preference

In either sex, no difference was found between HC and LC animals in sucrose or water consumption per kg, and on sucrose preference before the start of the chronic stress regimen. Sex, stress and chirping levels affected the pattern of changes in these measures. In the male HC group, no difference was found in sucrose consumption between stress and control animals, while in LC-rats stress prevented the increase in sucrose consumption that occurred with repeated testing (Fig. 3A) *[Sex, $F_{1, 54}=27.1$; $p<0.001$; Stress, $F_{1, 54}=14.4$; $p<0.001$; Repeated Testing, $F_{4, 216}=4.63$; $p<0.01$; Sex x Repeated testing, $F_{4, 216}=2.53$; $p<0.05$; Stress x Repeated testing $F_{4, 216}=4.16$; $p<0.01$ and $p<0.05$; Chirping x Repeated testing, $F_{4, 216}=2.90$; $p<0.05$]*. In females, sucrose consumption was lower in both stress groups as compared to the respective controls on some test days (Fig. 3B). Stress initially increased water consumption in LC groups of both sexes, but at the end of the stress regimen there was no difference between male groups, while in females HC control animals had lower levels of water consumption as compared to both respective LC-rats and stress group *[Sex, $F_{1, 54}= 20.5$; $p<0.001$; Stress, $F_{1, 54}= 5.04$; $p<0.05$; Chirping, $F_{1, 54}= 4.81$; $p<0.05$; Repeated Testing, $F_{4, 216}=14.6$; $p<0.001$; Sex x RT, $F_{4, 212}=11.8$; $p<0.01$; Stress x Repeated testing, $F_{4, 212}= 2.67$; $p<0.05$; Sex x Stress x Repeated testing,*

$F_{4,\ 212}$=4.57; p<0.01; Stress x Chirping x Repeated testing, $F_{4,\ 212}$=2.53; p<0.05; Sex x Stress x Chirping x Repeated testing, $F_{4,\ 212}$=2.43; p<0.05]. Stress significantly decreased sucrose preference in the male LC group as compared to the respective controls as well as the respective HC group *[Sex, $F_{1,\ 51}$=4.02; p<0.05; Stress, $F_{1,\ 51}$=12.1; p<0.01; Chirping, $F_{1,\ 51}$=5.08; p<0.05; Repeated Testing, $F_{4,\ 204}$=15.2; p<0.001; Sex x Repeated testing, $F_{4,\ 204}$=8.97; p<0.001; Stress x Repeated testing, $F_{4,\ 204}$=6.33; p<0.001; Chirping x Repeated testing, $F_{4,\ 204}$=2.97; p<0.05; Sex x Stress x Repeated testing, $F_{4,\ 204}$=4.15; p<0.01]*. No difference in sucrose preference was found between control and stress groups in HC animals. In female rats, significantly lower levels of sucrose preference were found in stress animals of the LC group on the second and third testing as compared to both respective controls as well as the HC stress group.

Elevated plus-maze

Female rats appeared to be more active in the plus-maze with lower in latency and higher number of entries into open part, entries into closed part, total number of entries and line crossing *[Sex, $F_{1,\ 54}$=5.17, 11.06, 41.2, 37.1, 33.8, respectively; p<0.05, p<0.01, p<0.001, p<0.001, p<0.001, respectively]*. HC groups of both sexes made a higher number of entries into closed part and total entries as compared to LC animals (data not shown) *[Chirping, $F_{1,\ 54}$=10.87, 7.13, respectively; p<0.01, p<0.05, respectively]*. In both sexes the difference between HC and LC rats became significant in stress animals. In both sexes, LC animals had a strong tendency for higher levels of stretched-attend postures in the elevated plus-maze, which became significant in female controls and male stress group (data not shown) *[Chirping, $F_{1,\ 54}$= 6.23, p<0.05]*.

Exploration box test

Female rats had higher latency and lower levels of entering *[Sex, $F_{1,\ 54}$=6.74 and 5.78; both p<0.05]* and lower levels of line crossing, sum of exploratory activity, investigation of objects, rearing and time of exploration on first test in the LC-stress group and on the second test in all groups except HC control *[Sex, $F_{1,\ 54}$=9.55, 11.8; 13.6, 12.44, 45.7, respectively; p<0.05, p<0.05, p<0.01, p<0.01, p<0.01, respectively]*. In male rats, line crossing, investigation of objects, rearing, sum of exploratory activity and time of exploration were higher and increased more on second testing in the LC control group as compared to HC-rats *[Sex x Chirping, $F_{1,\ 54}$=7.28, 9.77, 9.30, 8.82, 8.97, respectively; all p<0.01; Repeated Testing, $F_{1,\ 54}$=36.5, 49.1, 33.9, 47.9, 45.8, respectively; all p<0.001]*. Stress significantly increased the activity levels in the male LC group on both test days (Fig. 4A, data on latency, entering and sum of exploratory activity not shown) on line crossing *[Stress, $F_{1,\ 54}$=9.55; p<0.01; Sex x Stress Chirping x Repeated testing, $F_{1,\ 54}$=5.53; p<0.05]*, sum of exploratory activity *[Stress, $F_{1,\ 54}$= 11.8; p<0.01; Sex x Stress Chirping x Repeated testing, $F_{1,\ 54}$=6.05; p<0.05]*, investigation of objects *[Stress, $F_{1,\ 54}$=13.6; p<0.001; Sex x*

Stress Chirping x Repeated testing, $F_{1, 54}=4.81$; $p<0.05$], rearing *[Stress, $F_{1, 54}=12.4$; $p<0.001$; Stress x Chirping x Repeated testing, $F_{1, 54}=4.34$; $p<0.05$]* and time of exploration *[Stress, $F_{1, 54}=6.65$; $p<0.05$; Stress x Chirping x Repeated testing, $F_{1, 54}=9.54$; $p<0.01$]*. Both control and stress female HC animals had higher levels of activity as compared to the respective LC group (Fig. 4B, data on latency, entering and sum of exploratory activity not shown), while stress had no effect on females.

Forced swimming

Immobility time increased in all male groups in the second test except for the HC control group (Fig. 5) *[Repeated Testing, $F_{1, 54}=131.1$; $p<0.001$]*. On the initial test, stress decreased immobility time in male LC animals that was lower as compared to both the respective control groups, but this effect was not apparent on the second test. The increase in immobility time between the two tests was significant in the LC stress group in males, as compared to the respective controls, while no difference was found between the male HC groups *[Stress, $F_{1, 54}=6.06$; $p<0.05$]*. Female rats tended to have higher immobility *[Sex x Repeated Testing, $F_{1, 54}=6.37$; $p<0.05$]* as compared to males on the second test. Swimming time decreased by the second testing in all male groups except for LC controls *[Repeated Testing, $F_{1, 54}=61.8$; $p<0.001$]*. Male LC stress rats swam more on the first day, but this effect was lost with the decrease in swimming time in this group by the second test. Male HC stress group swam more on the second day, as compared to the respective controls, while there was no difference on the first day *[Stress x Chirping x Repeated Testing, $F_{1, 54}=5.17$; $p<0.05$]*. In the first test, male HC controls tended to swim more as compared to the respective LC animals, but the formers' swimming time became significantly lower by the second testing as it made through the largest decrease. A slight increase in swimming time in the LC control group caused a significant difference in the level of change as compared to the HC controls. The same pattern of change was visible when LC controls were compared to the LC stress group. Struggling levels decreased by the second test in all male groups except for HC controls, in which there was a small rise *[Repeated Testing, $F_{1, 54}=30.1$; $p<0.001$]*. There was no difference in struggling levels on the first day, but the change between the test days as well as the struggling levels on the second day in HC controls differed significantly from all the other groups *[Sex x Stress and Sex x Stress x Chirping, $F_{1, 54}=11.4, 6.26$; $p<0.001$, $p<0.05$, respectively]*. Female rats tended to have lower struggling levels *[Sex, $F_{1, 54}=4.10$; $p<0.05$]* as compared to males on the second test. In female rats, immobility increased while swimming and struggling decreased significantly by the second test day in most groups (data not shown). No differences were found in the extent of changes from the first test to the second between any groups in any measures. Stress significantly increased struggling in female HC animals on the first test day, while the difference disappeared on the second day.

COX histochemistry

Males had higher levels of COX in all groups in centromedial nucleus of thalamus *[Sex, $F_{1, 54}$=4.50; p<0.05]*, anterior olfactory nuclei (lateral and medial), agranular insular frontal cortex, dorsomedial frontal cortex, laterofrontal frontal cortex, lateral and medial orbital frontal cortex, prelimbic frontal cortex, temporal cortex area 1, piriform cortex, anterior cingulate cortex, ventral and dorsolateral striatum, nucleus accumbens core and shell regions, nucleus of the diagonal band (horizontal and vertical), medial preoptic area of hypothalamus, supraoptic and suprachiasmatic nucleii of hypothalamus, anterior, lateral and lateral anterior hypothalamic area, anterior amygdaloid area (dorsal), reticular thalamic nucleus, medial septum, globus pallidus, ventral pallidum, dorsal and median raphe, infralimbic cortex, paratenial nucleus and pontine nucleus *[Sex, $F_{1, 54}$=29.1, 23.9, 103.0, 83.1, 106.2, 88.2, 95.4, 81.3, 30.9, 74.0, 75.6, 92.4, 58.7, 84.8, 98.0, 96.6, 55.7, 127.5, 82.5, 33.7, 75.5, 107.7, 59.9, 86.6, 75.5, 52.7, 75.3, 67.3, 349.4, 414.0, 57.9, 70.9, 504.8; all p<0.001]*. Females had higher levels of COX in parietal cortex areas 1 and 2, perirhinal cortex, dorsal hypothalamic area, ventromedial thalamic nucleus, basolateral, medial and central amygdala, substantia nigra pars compacta and substantia nigra pars reticulata, central nucleus and external cortex of inferior colliculi, interpeduncular nucleus, paraventricular thalamic nucleus (posterior), presubiculum and subiculum *[Sex, $F_{1, 54}$=137.0, 104.5, 90.0, 90.8, 68.6, 122.9, 17.0, 122.7, 126.5, 138.5, 42.6, 85.0, 231.4, 85.8, 113.1, 130.0; all p<0.001]*.

Significant Stress effects were found in lateral septum, locus coeruleus and median raphe *[$F_{1, 54}$=4.53, 4.09, 5.18; all p<0.05]*. A Chirping effect was found in nucleus of the diagonal band (horizontal), temporal cortex areas 1 and 3 and ventromedial nucleus of hypothalamus *[$F_{1, 54}$=5.84, 5.19, 4.43, 6.21; all p<0.05]*. A significant Sex x Stress interaction was revealed in anterior cingulate cortex, anterior olfactory nuclei (ventral), CA regions 1–3, central and medial amygdala, central nucleus of inferior colliculi, median raphe, nucleus accumbens core and shell regions, perirhinal cortex, subiculum and ventrolateral nucleus of thalamus *[$F_{1, 54}$=5.16, 6.01, 7.32, 10.15, 4.00, 6.95, 5.96, 5.41, 9.54, 4.53, 5.85, 4.83, 6.41, 4.13; all p<0.05]*. A Sex x Chirping interaction was found in dorsomedial and ventrolateral anteroventral thalamic nucleii, CA regions 1 and 3, occipital cortex areas 1M and 2L and dorsolateral periaqueductal grey *[$F_{1, 54}$=5.96, 4.26, 5.31, 5.49, 4.78, 4.92, 4.55; all p<0.05]*. A Stress x Chirping interaction was revealed in dorsomedial and ventrolateral anteroventral thalamic nuclei, CA regions 1 and 2, external cortex of the inferior colliculi, temporal cortex area 1 and ventral tegmentum *[$F_{1, 54}$=5.83, 7.32, 4.03, 3.25, 4.29, 4.29, 4.40; all p<0.05]* and a Sex x Stress x Chirping interaction in red nucleus and ventrolateral nucleus of thalamus *[$F_{1, 54}$=10.43, 5.45; p<0.01, p<0.05]*. In anteromedial thalamic nucleus, both control and stressed female HC rats had higher levels of COX as compared to males, but there was no difference between sexes in LC animals. In anterior olfactory nuclei (ventral and

medial) female rats had lower COX levels and stress increased the difference between sexes. In the bed nucleus of stria terminalis and medial preoptic nucleus of hypothalamus, no difference was found between sexes in control animals, but stress decreased metabolic activity in female rats. In medial amygdala, hippocampal zones CA1-CA3, temporal cortex (areas 1 and 3), ventrolateral nucleus of thalamus, anteroventral thalamic nucleus, perirhinal cortex, nucleus of the diagonal band (horizontal) and inferior colliculi (central nucleus and external cortex) stress increased COX levels in male LC-rats, resulting in a difference in metabolic activity between stressed HC and LC rats in these regions. In the median raphe, red nucleus and ventral anterior olfactory nuclei, stress increased COX in male HC-rats, while in locus coeruleus stress decreased COX in all male rats. In the anteroventral thalamic nucleus female HC-rats had higher COX levels as compared to LC-rats. No difference was found between HC and LC animals in stress groups as stress increased metabolic activity in the LC group and decreased it in the HC group. In occipital cortex female LC rats had higher COX as compared to HC-rats, and stress eliminated the difference in area 1M. In perirhinal cortex, female LC-rats had higher COX in the stress group as compared to the controls. In central and medial amygdala, ventral tegmental area and subiculum stress decreased COX levels in female HC animals. In nucleus accumbens stress decreased COX in female LC animals only. In the red nucleus, stress decreased COX levels in female HC rats and increased it in female LC rats, while the difference between HC and LC animals was significant in both control and stressed animals, but reversed after stress.

DISCUSSION

In these experiments, we have studied the brain metabolic activity and behaviour in male and female high- and low-chirping untreated rats and after four weeks of chronic variable stress. Stress decreased 50-kHz USVs in male HC animals and increased 22-kHz USVs in male LC group. In females, stress had no effect on 50-kHz USVs, but significantly increased 22-kHz USVs in the HC group. Stress decreased weight gain in male rats only, and the effect was more stable in LC animals. No differences were found in sucrose intake and preference in either sexes, but stress significantly decreased both measures in LC animals of both male and female groups. HC animals of both sexes made a higher number of closed part entries in the elevated plus-maze, but the difference between HC and LC groups were significant in stressed animals only. Stress significantly increased activity in the exploration box test in mal LC-rats on the first test, while the difference with the respective HC and control groups were not significant on the second test. In female rats, both control and stressed HC-rats tended to be more active in the exploration box test. Male LC-rats were

more immobile and swam less in the forced swimming test, and stress eliminated these differences, while no differences were found between female HC and LC animals and stress also had no effect in these groups. Significant differences in brain metabolic activity were found between male and female rats with lower metabolic activity in females in brainstem regions and higher metabolic activity in amygdala and related limbic regions. Stress almost exclusively affected male LC-rats and female HC-rats, increasing metabolic activity in all regions affected in the first group and decreasing it in the latter.

By now numerous studies have confirmed that the tickling-induced 50-kHz ultrasonic vocalizations present a test animals reaction that most directly relates to the subjectively experienced positive affective state that is mediated by brain reward systems in a way similar to various inherently reinforcing stimuli (for review see Panksepp *et al*., 2002b). Provided with such a tool for studying positive affect in laboratory animals, it is possible to approach and better comprehend the affective states and their impairment in humans. It is important to analyze this phenomen in association with other emotionality-related behaviours in the experimental animals in order to put the new knowledge of positive emotions into the context of existing animal models of affect that has so far been focussing largely on negatively-valenced experience. There has been a considerable amount of findings over the previous decade about individual differences in laboratory animals in many different behavioural parameters, suggesting inborn tendencies for specific behaviours in subgroups of test animals. Hence, it came as no surprise to find that even without selective breeding over numerous generations, the animals steadily differ in their levels of tickling-induced chirping (Mällo *et al*., 2007) and that these subgroups show substantial differences in behaviour in different tests used in anxiety and depression models in animals. In the present experiments, we studied the effects of chronic variable stress on male and female rats that emitted high or low levels of chirping in response to experimenter-administered tickling, in order to investigate further our previous findings that suggested possible differences in reactivity to environmental stimulation between the HC and LC animals.

In recent studies, the 50-kHz USVs have been divided in two subgroups – namely, flat and frequency-modulated (Burgdorf *et al*., 2007; Schwarting *et al*., 2007). The former show very narrow bandwidth (5-7 kHz) and are hence visualized on the spectrogram as relatively flat, while the FM USVs may include a variation in the frequency in the range between 30-80 kHz, or so-called trill component. These two types have been found to have some different characteristics relating to pharmacological manipulations (Burgdorf *et al*., 2007), suggesting that their neurobiological substrate may differ to a certain extent and hence the functional properties of these two types of USVs may be somewhat different. The recording method used in the current experiment also allowed us to differentiate between flat and FM USVs. It also has to be noted that several other different types of ultrasonic vocalizations were detected by

manual checking of the spectrograms, but due to extremely low levels and/or high inter- and intra-individual variation of these USVs, the present paper only concentrates on data on the two types of 50-kHz and one type of 22-kHz USVs. It seems that in the methodological context used in the current study, the 50-kHz and 22-kHz vocalizations heavily dominate over others, which, nevertheless, may carry an important message about the emitters' affective or motivational state in other experimental or natural contexts and hopefully will be approached in further studies.

In both male and female rats, the change in the levels of flat 50-kHz USVs over the two-weeks period of daily tickling sessions was similar to our previous findings, i.e. the gradual rise in the number of chirps stabilized on an individually specific level by the second week of manipulation. Similar pattern of change was visible in both sexes in the FM 50-kHz USVs, suggesting that at least in the context of daily experimenter-induced stimulation in juvenile rats there is no qualitative difference between these two types of USVs as a response to this stimulation. The levels of FM calls were somewhat lower in both sexes as compared to the flat USVs at baseline levels, while after social housing this difference was visible in male rats only. Previously, Burgdorf and Panksepp (2006) have reported more trill-type USVs than flat USVs in response to tickling in adult females and that with regard to the flat-type USVs the high-chirping rats do not differ from the low-chirping group. It seems reasonable to assume that differences in experimental design stand behind these variations, suggesting that if the tickling procedure is started when the test animals have reached adult age, the resulting USV profile may differ to a certain extent from the conditions where tickling sessions have been started at weaning already. Age-related decreases in USV response to tickling have been reported (Panksepp and Burgdorf, 2003), and in the light of the abovementioned results it may be suggested that the flat-type 50-kHz USVs show a greater and earlier decrease over aging process that may be related to different relevance and „meaning of the two types of USVs at different age points.

After the cessation of initial tickling sessions the rats were group-housed for several weeks that probably accounted for the decrease in both kinds of chirps at the pre-stress testing in all male groups and in flat USVs in HC females, as social housing has been shown to have such an effect already in 48 h when isolate-housed animals were moved to group housing (Panksepp and Burgdorf, 2000). Nevertheless, the differences between LC and HC groups remained significant after social housing. It is noteworthy that the levels of FM chirps did not decrease in females over the period of social housing, and the decrease in flat USVs was also significant in HC animals only. This suggests that at least in LC females social housing has a different effect on ultrasonic vocalizations (and more so on FM chirps), that seems a reasonable assumption in the context that females have been found to have different social behaviour profiles than males (Douglas *et al*., 2004; Pellis *et al*., 1997). It also relates well with the notion that

females remain playful longer after puberty (Panksepp *et al*., 1984), while in males, probably greater competitiveness and hence, aggressiveness develops as animals enter adulthood.

After four weeks of chronic variable stress period, the levels of flat 50-kHz USVs had decreased in both male HC groups as compared to the pre-stress testing, but to a larger extent in the stress group. The frequency-modulated 50-kHz USVs decreased only in the stressed HC animals and became significantly lower as compared to the controls, while no effect was visible on either measure in LC animals in which the number of chirps was very low already after social housing. The decrease in 50-kHz USVs in male HC animals after stress to the level of LC-rats suits with the previous findings of decreasing effects of aversive stimuli on 50-kHz vocalizations (Panksepp and Burgdorf, 1999). Due to very low levels of 50-kHz USVs in LC-rats it cannot of course be concluded that stress affected this measure in HC animals selectively. In females, stress had no effect on 50-kHz chirping in HC animals as chirping levels decreased similarly in all groups that may be ascribed to the continued effect of social housing. The FM USVs decreased significantly in female HC groups, and a similar tendency was visible in the LC groups. Since this decrease was similar in both control and stress HC groups, it seems likely that stress had no effect on these measures in female rats.

No difference was found between HC and LC animals in 22-kHz USVs at baseline and pre-stress testing. In male rats, chronic variable stress increased 22-kHz USVs in the LC group to a significantly higher level as compared to the HC group. This is in part comparable to the previous findings that aversive events that decrease the 50-kHz USVs, simultaneously increase the 22-kHz vocalizations (Panksepp and Burgdorf, 1999). Apparently, the male LC animals are more vulnerable and/or reactive in this regard. In females, the levels of 22-kHz USVs tended to decrease in all groups except for the HC stress group over the period of stress regimen, resulting in a significant difference between the HC and LC stress groups that was opposite to that of the males' groups. In our previous studies, we have found that the number of 22-kHz USVs increased over three weeks of daily tickling sessions in female HC-rats (Mällo *et al*., 2007b), although there was no simultaneous decrease in 50-kHz USVs in this group. Together with the present results, it suggests greater reactivity to stimulation in female HC-rats. Hence, the low-chirping males and high-chirping female rats are similar with regard to higher reactivity to stressful experience in both groups. It is noteworthy, that stress-induced changes in brain metabolic activity appeared almost exclusively in these two groups in the present study, while the pattern of these changes was distinct. Out of the brain regions affected by chronic variable stress, it increased metabolic activity in all regions (except for septum) in male LC-rats, and decreased metabolic activity in all regions (except for locus coeruleus) in female HC-rats.

It is noteworthy that significant differences were found in neuronal metabolic activity in various brain regions between control male and female rats. Lower metabolic activity was detected in females in raphe regions that provide input to corticolimbic structures involved in the control of anxious states (Millan, 2003). Higher metabolic activity was found in females in amygdala and related limbic regions that have been appointed a significant role in regulation of anxiety and fear responses (Davis and Shi, 1999) and mood disorders (Drevets, 2003). In humans, it has been observed that the lateralization of amygdala participation in emotionally influenced memory is sex-dependent (Cahill *et al*., 2001). Differences in anxiety and stress reactivity between male and female rats have been reported both in behavioural and neurobiological measures (Bowman *et al*., 2004; Luine, 2002; Palanza, 2001; Toufexis, 2007) and it seems reasonable that these variations may at least partly stem from differences in neuronal activity in baseline conditions.

We have previously reported significant stress-induced changes in COX activity in various brain regions in male rats (Kanarik *et al*., 2008), and it is noteworthy that in some regions in which stress had no apparent effect in unselected animals in that study (namely, anteroventral thalamic nucleus and CA3), we currently report significantly increased COX levels after stress in LC animals only. In addition to these regions, the chronic variable stress also increased COX levels in male LC-rats selectively in medial amygdala, hippocampal zones CA1-CA2, perirhinal cortex, ventrolateral nucleus of thalamus, nucleus of the diagonal band (horizontal) and inferior colliculi (central nucleus and external cortex), resulting in significantly higher levels of metabolic activity in stressed LC-rats as compared to the HC animals in these regions. The nucleus of the diagonal band has been demonstrated to provide cholinergic innervation to the hippocampus (Gaykema *et al*., 1990), influencing learning and memory processes, while amygdala and thalamic regions are incorporated in the mediation of stress effects with inputs from hippocampal structures, perirhinal cortex and subiculum (in which also a tendency of a similar stress effect on metabolic activity was visible) (Pitkänen *et al*., 2000). Together, these changes in metabolic activity in male LC-rats suggest a greater sensitivity to stress in this group with regard to learning and memory processes.

In the exploration box test, we have previously reported higher levels of exploratory activity in LC animals of both sexes (Mällo *et al*., 2007b). In the present experiment, a similar difference was visible on most measures between HC and LC control animals in male groups, while in female rats, HC animals of both control and stress groups were more active on some measures. Obviously, male LC-rats have higher motivation to explore novel environments that is suppressed by higher anxiety on the first encounter with the test apparatus as the differences between HC and LC animals became significant on the second testing. Chronic variable stress increased activity levels in male LC animals and had no effect in females. It is noteworthy that in male LC-rats, stress also

increased metabolic activity in hippocampus that has been most directly associated with exploratory activity (Plaznik *et al*., 1983; Lever *et al*., 2006). Injections of noradrenaline into hippocampus have been reported to increase behavioural activity in the open field (Plaznik *et al*., 1983). Stress decreased metabolic activity in locus coeruleus in HC-rats, possibly leading to lower noradrenergic input to hippocampus in this group which may be partly responsible for the lower activity of the HC animals in the exploration box test. Although stress did not affect exploratory behaviour in female rats, it is of interest that in both sexes the groups with higher activity in the exploration box test were the same in which stress increased 22-kHz USVs and elicited the bulk of changes in metabolic activity in various brain regions. Hence, the higher exploration levels in these groups may rather reflect higher reactivity to various environmental stimuli than lower levels of anxiety.

In the median raphe, chronic variable stress increased COX in male HC-rats. The 5-HT-ergic pathways innervating higher brain regions are mainly activated by anxiogenic stimuli (Millan, 2003), suggesting that the stress effects on male rats were to a greater extent mediated by changes in the serotonergic system in the HC group. Female rats had lower levels of COX activity as compared to males in both dorsal and medial raphe that were not affected by stress that is in line with the relative lack of stress effects on behaviour in females. In the red nucleus, stress also increased COX in male HC-rats and female LC-rats, while it decreased metabolic activity in this region in HC- female rats. The chages in metabolic activity in the red nucleus suggest possible changes in motor functions (Houk, 1991). It is noteworthy that in females, stress changed red nucleus metabolic activity in both HC and LC animals, and the tendencies of both higher activity in the exploration box test in HC control animals and difference in metabolic activity in this region became significant in stress groups only.

Chronic variable stress regimen significantly affected weight gain in male rats, but no difference was found between the HC and LC animals in this regard, although the differences with control groups tended to develop earlier and remained more stable in LC animals as compared to the HC group. Weight gain suppression has been considered an important marker of stress regimen effectiveness (for an example, see Harro *et al*., 1999), and the more expressed effect in the LC group is in some concordance with the result of increased 22-kHz USVs in the male LC group after the stress regimen, suggesting that this group was more affected by the stress procedure than HC animals. In females, no differences were found between any groups in weight gain, suggesting that the stress procedure had only minor effects (detectable in some behavioural parameters) on these animals in the present experiment. It has been previously found that social housing can improve stress coping in female rats, while in male rats it was reported to increase the negative effects of chronic stress (Westenbroek *et al*., 2003). It is hence very likely that in the present

experiment, group housing prevented most of the negative effects of stress regimen in female rats.

In the sucrose preference test, we have previously reported no difference between HC and LC-rats of either sexes that had been group-housed after the end of initial tickling sessions, while in male rats that were single-housed and received tickling during the period that included sucrose preference testing, HC animals consumed less sucrose and had lower sucrose preference levels as compared to LC-rats (Mällo *et al*., 2007b). In the current experiment, we found no difference in sucrose consumption or preference between HC and LC animals of either sex on the initial test session that was carried out before the start of the chronic variable stress procedure. Although in the previous experiments the testing was carried out during the light phase and in the current experiment during the dark phase that we have found to provide more stable measures of sucrose preference (Tõnissaar *et al*., 2006), the findings are converging to suggest that differences between HC and LC animals in sucrose preference only show in specific conditions. It is also noteworthy that while we have found a significant positive correlation between sucrose intake and dopamine D2 receptor dependent [35S]GTPγS binding in striatum (Tõnissaar et al., 2006), no differences were found between HC and LC rats in our previous study (Mällo et al., 2007), suggesting that the hedonic properties of tickling and sucrose consumption are to some extent mediated by different neurobiological systems. Altogether, these results suggest that sucrose preference level, as well as the level of tickling-induced 50-kHz ultrasonic vocalizations, both theoretically measures of hedonic states, are dependent on the previous social stimulation level. Indeed, it has been reported that social housing reduces tickle-induced vocalizations and approach speed to the rewarding stimuli (in the specific case, the tickling hand) compared to isolate housing (Burgdorf and Panksepp, 2001). Although our previous results suggest a contrary effect at least in case of the HC-rats in which sucrose consumption was decreased in the experiment that included isolate housing, it seems plausible that social housing is an important factor in eliminating differences in this regard between HC- and LC-rats.

Over the repeated testing during the stress regimen, sucrose consumption increased in control groups of both sexes. No systematic differences were found between control and stressed HC animals, but sucrose consumption and preference were significantly lower in LC stress groups as compared to the respective controls and in sucrose preference also as compared to the HC stress group. Hence, stress significantly blocked the increase in sucrose preference in LC animals selectively, suggestive of an anhedonic state caused by higher sensitivity to chronic stress in this group.

In the elevated plus-maze, HC-rats of both sexes showed higher levels of activity on some measures of activity, while no effect of stress was detected. The higher levels of activity of HC animals in this test are in accordance with

our previous results (Mällo *et al*., 2007). The lack of stress effects on elevated plus-maze behaviour suggests that the tickling procedure that we have previously reported to significantly increase activity in the elevated plus-maze may also be effective in eliminating the stress effect in this test. In a partly similar experimental paradigm, postnatal handling has been demonstrated to yield anxiolytic effects in various tests in adult rats (Núñez *et al*., 1996). Previously, (Schwarting *et al*., 2007) have found a only a positive correlation between 50-kHz USVs emitted during tickling and risk assessment in the elevated plus-maze test, while in the present study, the number of stretched-attend postures tended to be higher in the LC animals of both sexes and general activity levels higher in the HC groups. We have previously (Mällo *et al*., 2007) compared the experimental procedure of Schwarting and colleagues to ours and suggested that the USV response that animals emit when tickling procedure is started in adult age and not done repeatedly over longer period, is qualitatively different from the response given in our procedure, and the present results suggest the same. Indeed, it has been reported by Panksepp (2007) that adult rats tend to emit very low levels of chirps unless they have been tickled during adolescence, but it also remains negotiable whether these chirps carry a similar affective or socioemotional connotation as in rats that have been tickled since juvenile age.

In our previous experiments, we have not detected significant differences between HC and LC animals in the forced swimming test, although the HC animals tended to have longer immobility periods on the second exposure as compared to LC animals (Mällo *et al*., 2007). In the present study, no differences were detected between female HC and LC animals in forced swimming, and chronic variable stress also had no effect on females in this test. In males, the control LC group had higher levels of immobility on the re-exposure as compared to HC controls, and stress increased immobility in the re-exposure session to a greater extent in LC animals. This suggests greater susceptibility to both acute stress (i.e. the forced swimming stress in the control group) and chronic stress (in the stress group) in male LC animals. Interestingly, in the HC control group swimming decreased the most by the second testing, but struggling levels increased slightly to a significantly higher level as compared to all other groups, while stress withheld both of these changes. Together with the lowest immobility levels in this group, these results suggest lower acute stress reactivity in HC-rats, which is nevertheless impaired by chronic stress procedure, resulting in similar behavioural profile with the LC group.

In the anteroventral thalamic nucleus, chronic variable stress increased metabolic activity in the LC group and decreased it in the HC group. The anteroventral thalamic nucleus is innervated by noradrenergic terminals exclusively originating in the locus coeruleus, but there was no change in metabolic activity in this region after stress in females. This suggests that the stress-induced change in metabolic activity in anteroventral thalamic nucleus may have been

mediated by input from other structures. Female stressed LC-rats had higher COX as compared to the controls in the perirhinal cortex, and both antero-ventral thalamic nucleus and perirhinal cortex have been shown to have robust direct and indirect connections with the limbic system (Aggleton & Sahgal, 1993; Lavenex and Amaral, 2000). As stress decreased COX levels in amygdala in females, it may hence be that stress-induced changes in this region or the limbic system in general affect metabolic activity levels in other brain regions differently in female HC and LC animals.

In anterior olfactory nuclei female rats had lower COX levels and chronic variable stress increased the difference between sexes as it increased COX in male HC-rats. As stress was also found to decrease COX in the locus coeruleus region male HC-rats and locus coeruleus lesions have been found to decrease noradrenaline content in the anterior olfactory nuclei (Fallon and Moore, 1978), it is possible that stress affected the metabolic changes in the latter via the former region.

It has been found that the more the animals chirp in response to tickling, the more they seem to enjoy the tickling experience (Burgdorf and Panksepp, 2001) and it seems reasonable that the individual tendency for such a response may be generalized to a more extensive affective disposition towards other stimuli of significant value to the test animal, whether they be aversive or rewarding. Nevertheless, it has to be noted that the complex design of such studies is prone to leaving important factors uncontrolled, as the current results suggest that it is very likely that the group housing and previous tickling experience prevented most of the negative effects of chronic variable stress regimen in female rats. Besides that, it is quite clear that the male rats with low 50-kHz USV response to tickling are more vulnerable to chronic stress that is best expressed in stressed-induced increases in metabolic activity in limbic brain regions in this group. This susceptibility was expressed as increased levels of 22-kHz USVs, earlier and more sustained appearance of the decreasing effect of stress on weight gain, the inhibitory effect of stress on increase in sucrose preference, and higher levels of immobility in both control (i.e. acutely stressed) and stressed animals in the forced swimming test. In females, vulnerability to stress seems to be generally lower as compared to males in group housing conditions. Nevertheless, the females emitting high levels of 50-kHz chirping when tickled, revealed more changes in brain metabolic activity, suggesting that at least on cellular level these animals were more sensitive to the stress procedure, although these effects were not reflected in behavioural measures.

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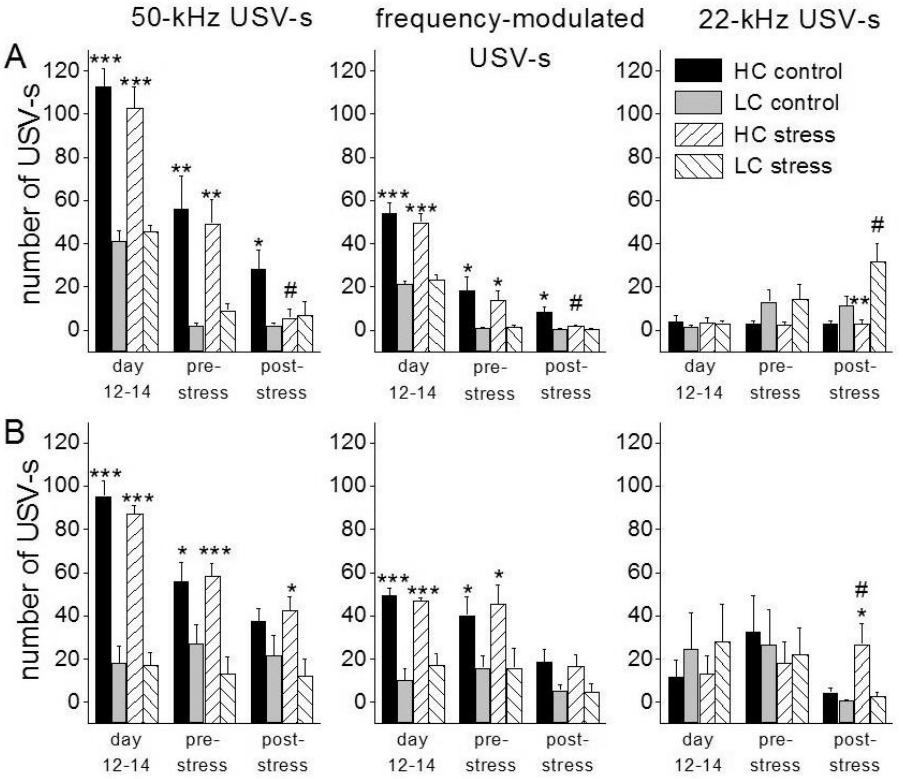

Figure 1. Ultrasonic vocalizations in male (A) and female (B) rats before and after chronic stress. * – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$ vs respective LC; # – $p<0.05$ vs respective control. Data expressed as means ± S.E.M. The animals were divided into groups with high and low levels of 50-kHz USV-s by median split of the average result of Days 12-14 of tickling. HC – high chirping rats; LC – low chirping rats.

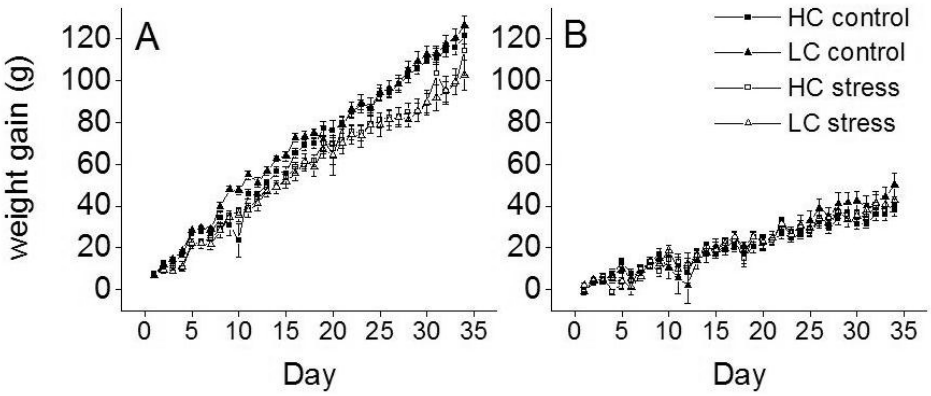

Figure 2. The effect of chronic stress on cumulative weight gain (g) in male (A) and female (B) rats. Significant differences between groups: male control LC vs male control HC: day 9 and day 10 ($p<0.01$); day 11 ($p<0.05$); male control LC vs male stress LC: day 3 ($p<0.001$); day 4 ($p<0.01$); day 5 ($p<0.05$); day 6–9 ($p<0.01$); day 11 ($p<0.001$); day 12 ($p<0.05$); day 13 ($p<0.01$); day 14 ($p<0.001$); day 15 ($p<0.05$); day 16 ($p<0.001$); day 17 ($p<0.05$); day 18 ($p<0.01$); day 22 and 23 ($p<0.05$); day 25–27 ($p<0.05$); day 28–30 ($p<0.001$); day 32 and 33 ($p<0.01$); male control HC vs male stress HC: day 3 ($p<0.05$); day 4 ($p<0.01$); day 5, 6, 10, 14, 17, 23, 25, 26 ($p<0.05$); day 27–30 ($p<0.01$); day 32 ($p<0.01$); day 33 ($p<0.05$). Data expressed as means ± S.E.M. The animals were divided into groups with high and low levels of 50-kHz USV-s by median split of the average result of Days 12–14 of tickling.

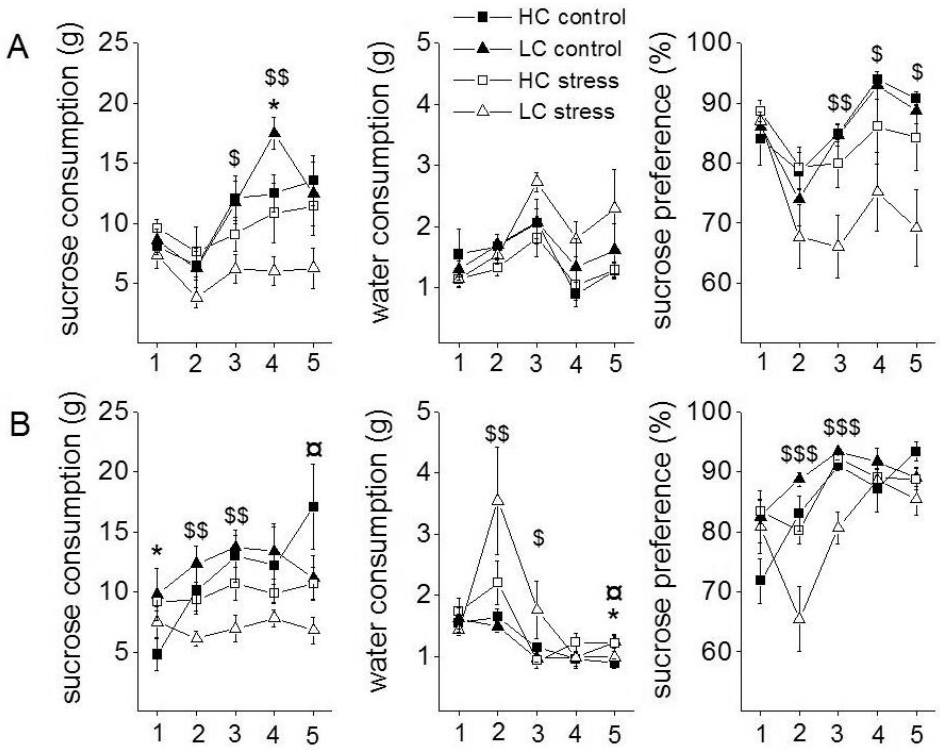

Figure 3. The effect of chronic stress on sucrose consumption, water consumption and sucrose preference in male (A) and female (B) rats. * – $p<0.05$ control HC vs control LC; ¤ – $p<0.05$ HC control vs HC stress; $ – $p<0.05$; $$ – $p<0.01$; $$$ – $p<0.001$ LC control vs LC stress. Data expressed as means ± S.E.M. The animals were divided into groups with high and low levels of 50-kHz USV-s by median split of the average result of Days 12–14 of tickling. The numbers 2–4 on *y*-axis represent sucrose test numbers (test no. 1 was carried out before the start of stress regimen). HC – high chirping rats; LC – low chirping rats.

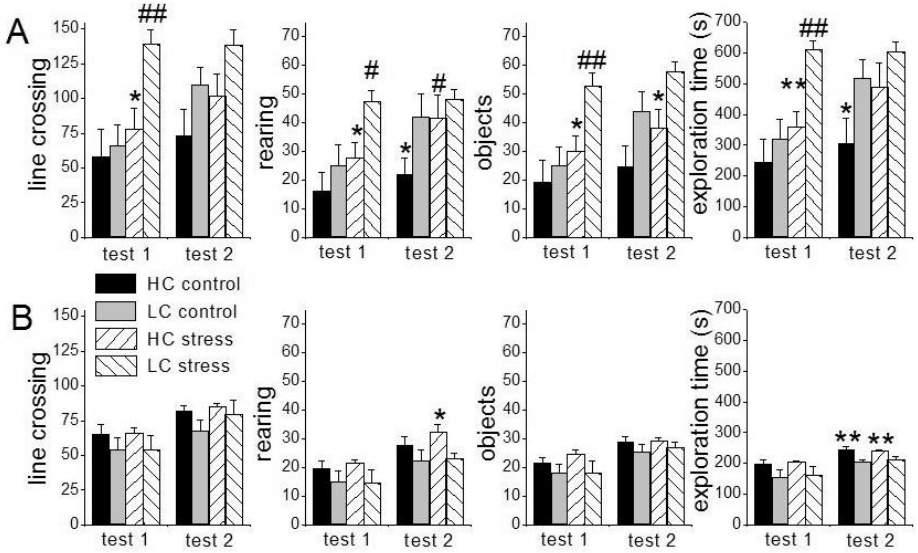

Figure 4. The effect of chronic stress on exploration box behaviour in male (A) and female (B) rats. * – $p<0.05$; ** – $p<0.01$ vs respective LC group; # – $p<0.05$; ## – $p<0.01$ vs respective control group. Data expressed as means ± S.E.M. The animals were divided into groups with high and low levels of 50-kHz USV-s by median split of the average result of Days 12-14 of tickling. HC – high chirping rats; LC – low chirping rats.

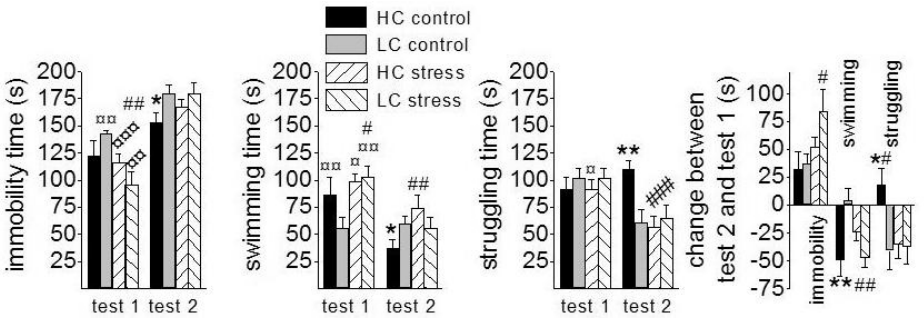

Figure 5. The effect of chronic stress on forced swimming in male rats. * – p<0.05; ** – p<0.01 vs respective LC; ¤ – p<0.05; # – p<0.05; ## – p<0.01; ### – p<0.001 vs respective control; ¤¤ – p<0.01 ¤¤¤ – p<0.001 vs respective test 2. Data expressed as means ± S.E.M. The animals were divided into groups with high and low levels of 50-kHz USV-s by median split of the average result of Days 12–14 of tickling. HC – high chirping rats; LC – low chirping rats.

Table 1. The effect of chronic stress on cytochrome c oxidase activity in male and female HC and LC rats.

sex	male				female			
stress	control		stress		control		stress	
HC/LC	HC	LC	HC	LC	HC	LC	HC	LC
Agranular insular frontal cortex	0.76±0.03	0.74±0.02	0.76±0.03	0.76±0.04	**0.57±0.02$$$**	**0.56±0.01$$$**	**0.54±0.02$$$**	**0.57±0.03$$**
Anterior amygdaloid area, dorsal	0.71±0.03	0.71±0.03	0.72±0.02	0.72±0.02	**0.57±0.02$$**	**0.57±0.02$$**	**0.54±0.02$$$**	**0.56±0.02$$$**
terior cingulate cortex	0.84±0.02	0.79±0.02	0.83±0.02	0.83±0.03	**0.70±0.02$$$**	**0.73±0.02$**	**0.66±0.02$$$**	**0.66±0.02$$$ #**
Anterior hypothalamic area	0.80±0.03	0.77±0.03	0.78±0.03	0.79±0.05	**0.61±0.01$$$**	**0.63±0.02$$**	**0.59±0.02$$$**	**0.63±0.01$$**
Anterior olfactory nuclei, dorsal	0.57±0.02	0.59±0.03	0.58±0.03	0.62±0.03	0.57±0.02	0.57±0.02	0.53±0.01	0.57±0.02
Anterior olfactory nuclei, lateral	0.65±0.02	0.67±0.03	0.66±0.02	0.69±0.03	**0.60±0.02$**	0.59±0.03	**0.55±0.02$$**	**0.58±0.01$$**
Anterior olfactory nuclei, medial	0.66±0.04	0.63±0.02	0.69±0.03	0.71±0.06	**0.55±0.03$**	0.59±0.02	**0.52±0.03$$$**	**0.57±0.02$**
Anterior olfactory nuclei, ventral	0.61±0.02	0.62±0.04	**0.70±0.04#**	0.69±0.05	0.55±0.02	0.54±0.03	**0.51±0.02$$$**	**0.53±0.01$**
Anterodorsal thalamic nucleus	1.00±0.04	1.00±0.04	0.92±0.04	1.11±0.08	0.95±0.02	0.94±0.03	0.93±0.03	0.92±0.02
Anteromedial thalamic nucleus	0.77±0.02	0.79±0.04	0.76±0.02	0.82±0.03	**0.88±0.03$$**	0.87±0.04	**0.82±0.02$**	0.83±0.02
Anteroventral thalamic nucleus, dorsomedial	0.83±0.04	0.86±0.03	0.78±0.03	**0.99±0.07*** #**	0.84±0.02	**0.78±0.01$***	0.79±0.02	0.82±0.02
Anteroventral thalamic nucleus, ventrolateral	0.71±0.03	0.68±0.03	0.68±0.03	**0.87±0.08*** #**	0.77±0.02	0.72±0.02	**0.70±0.02#**	0.75±0.02
Basolateral amygdala	0.71±0.02	0.70±0.04	0.66±0.02	0.75±0.03	**0.97±0.02$$$**	**0.98±0.03$$$**	**0.93±0.05$$$**	**0.95±0.05$$**
Bed nucleus of stria terminalis, lateral	0.69±0.02	0.73±0.03	0.71±0.02	0.74±0.03	0.64±0.01	0.67±0.03	**0.62±0.03$**	**0.62±0.02$$**
Bed nucleus of stria terminalis, medioanterior	0.67±0.04	0.69±0.02	0.74±0.03	0.71±0.04	0.63±0.01	0.65±0.02	**0.60±0.02$$**	**0.61±0.02$$**
Bed nucleus of stria terminalis, ventral	0.64±0.03	0.67±0.03	0.68±0.02	0.71±0.02	0.57±0.02	0.59±0.03	**0.54±0.02$$$**	**0.56±0.01$$$**
CA1	0.64±0.02	0.63±0.03	0.63±0.01	**0.70±0.01*** #**	0.69±0.02	0.65±0.02	0.64±0.01	0.62±0.02
CA2	0.67±0.01	0.66±0.02	0.67±0.01	**0.74±0.02** #**	0.73±0.02	0.71±0.02	0.69±0.01	0.67±0.02
CA3	0.69±0.02	0.68±0.03	0.67±0.01	**0.74±0.01****	0.73±0.02	0.70±0.02	0.70±0.01	0.67±0.02
Central amygdala	0.69±0.01	0.67±0.03	0.66±0.02	0.73±0.02	**0.90±0.02$$$**	**0.90±0.02$$$**	**0.82±0.03$$$ #**	**0.85±0.03$$**
Centromedial nucleus of thalamus	0.81±0.02	0.79±0.02	0.79±0.02	0.83±0.03	0.85±0.02	0.87±0.05	0.80±0.03	0.85±0.02

sex	male				female			
stress	control		stress		control		stress	
HC/LC	HC	LC	HC	LC	HC	LC	HC	LC
Cortical amygdala	0.65±0.02	0.66±0.02	0.65±0.02	0.71±0.03	**0.72±0.01$$**	0.72±0.02	0.67±0.03	0.70±0.03
Dentate gyrus	0.70±0.03	0.70±0.03	0.70±0.02	0.77±0.02	0.75±0.03	0.73±0.02	0.73±0.02	0.70±0.03
Dorsal hypothalamic area	0.73±0.02	0.71±0.03	0.73±0.02	0.77±0.04	**0.97±0.02$$$**	**0.94±0.04$$$**	**0.94±0.05$$$**	**0.95±0.05$$**
Dorsal raphe	0.82±0.03	0.82±0.02	0.80±0.02	0.80±0.05	**0.46±0.02$$$**	**0.47±0.01$$$**	**0.45±0.02$$$**	**0.45±0.01$$$**
Dorsomedial frontal cortex	0.84±0.03	0.83±0.02	0.85±0.03	0.84±0.05	**0.65±0.02$$$**	**0.65±0.02$$$**	**0.64±0.02$$$**	**0.65±0.03$$**
Entorhinal cortex	0.59±0.02	0.55±0.02	0.56±0.02	0.60±0.02	**0.50±0.01$$$**	0.51±0.02	**0.48±0.01$$**	**0.50±0.01$$$**
Globus pallidus	0.51±0.02	0.49±0.02	0.52±0.01	0.53±0.03	**0.44±0.01$$**	**0.41±0.01$$**	**0.40±0.01$$$**	**0.43±0.01$$**
Habenula	0.70±0.02	0.69±0.04	0.70±0.03	0.74±0.03	0.70±0.04	0.65±0.02	0.67±0.03	0.65±0.03
Inferior colliculi, central nucleus	0.85±0.03	0.81±0.05	0.78±0.10	**0.97±0.03#**	**1.11±0.02$$$**	**1.20±0.05$$$**	**1.09±0.04$**	1.10±0.08
Inferior colliculi, external cortex	0.79±0.03	0.73±0.03	0.79±0.02	**0.83±0.03#**	**0.98±0.03$$$**	**0.98±0.02$$$**	**0.92±0.04$$**	**1.00±0.03$$$**
Infralimbic cortex	0.89±0.03	0.84±0.04	0.87±0.04	0.85±0.04	**0.69±0.01$$$**	**0.70±0.04$**	**0.67±0.02$$$**	**0.68±0.02$$**
Interpeduncular nucleus	0.69±0.04	0.72±0.03	0.69±0.03	0.79±0.08	**1.20±0.04$$$**	**1.20±0.03$$$**	**1.13±0.03$$$**	**1.25±0.06$$$**
Lateral anterior hypothalamic area	0.77±0.03	0.78±0.03	0.77±0.03	0.80±0.05	**0.61±0.03$$**	**0.61±0.03$$**	**0.56±0.03$$$**	**0.63±0.02$$**
Lateral hypothalamic area	0.74±0.04	0.70±0.03	0.72±0.02	0.75±0.04	**0.54±0.02$$$**	**0.54±0.01$$$**	**0.52±0.01$$$**	**0.56±0.01$$$**
Lateral orbital frontal cortex	0.84±0.02	0.83±0.02	0.84±0.03	0.83±0.05	**0.66±0.03$$$**	**0.66±0.01$$$**	**0.62±0.01$$$**	**0.68±0.04$**
Lateral septum	0.85±0.03	0.87±0.04	0.82±0.03	0.85±0.01	0.81±0.02	0.78±0.03	0.77±0.02	**0.73±0.02$$$**
Laterofrontal frontal cortex	0.82±0.03	0.82±0.02	0.83±0.03	0.83±0.04	**0.63±0.02$$$**	**0.62±0.01$$$**	**0.61±0.02$$$**	**0.64±0.03$$**
Locus coeruleus	0.91±0.02	0.85±0.02	**0.76±0.10#**	0.80±0.05	0.74±0.04	0.84±0.05	0.71±0.03	0.75±0.04
Medial amygdala	0.66±0.02	0.65±0.02	0.65±0.02	**0.72±0.02#**	**0.77±0.01$$$**	**0.75±0.02$**	**0.70±0.03#**	0.72±0.03
Medial orbital frontal cortex	0.82±0.02	0.78±0.03	0.84±0.03	0.80±0.04	**0.62±0.02$$$**	**0.61±0.02$$$**	**0.59±0.02$$$**	**0.63±0.04$**
Medial preoptic area of hypothalamus	0.74±0.02	0.72±0.04	0.72±0.02	0.74±0.03	**0.56±0.02$$$**	**0.54±0.02$$**	**0.53±0.02$$$**	**0.54±0.02$$$**
Medial septum	0.77±0.03	0.76±0.03	0.75±0.03	0.81±0.05	**0.66±0.02$$**	**0.64±0.03$**	**0.58±0.01$$$**	**0.59±0.02$$**
Median preoptic nucleus of hypothalamus	0.74±0.02	0.71±0.05	0.74±0.02	0.73±0.02	0.67±0.03	0.62±0.03	**0.65±0.03$**	**0.65±0.03$**
Median raphe	0.53±0.02	0.57±0.01	**0.61±0.02##**	**0.63±0.03#**	**0.33±0.01$$$**	**0.31±0.02$$$**	**0.30±0.02$$$**	**0.32±0.01$$$**
Mediodorsal thalamic nucleus	0.86±0.02	0.82±0.02	0.82±0.02	0.86±0.03	0.84±0.02	0.88±0.04	0.80±0.02	0.84±0.02
Nucleus accumbens core	0.88±0.02	0.87±0.02	0.87±0.04	0.92±0.03	**0.73±0.02$$$**	**0.75±0.02$$**	**0.69±0.02$$$**	**0.68±0.04$$$**

sex	male				female			
stress	control		stress		control		stress	
HC/LC	HC	LC	HC	LC	HC	LC	HC	LC
Nucleus accumbens shell	0.98±0.02	0.98±0.03	0.97±0.03	1.03±0.04	**0.81±0.02$$$**	**0.85±0.02$$**	**0.77±0.03$$$**	**0.74±0.03$$$ #**
Nucleus of the diagonal band, horizontal	0.66±0.03	0.68±0.02	0.64±0.02	**0.72±0.02***	**0.55±0.01$$**	**0.55±0.02$$**	**0.51±0.02$$$**	**0.55±0.01$$$**
Nucleus of the diagonal band, vertical	0.70±0.03	0.73±0.02	0.67±0.01	0.73±0.02	**0.62±0.01$**	**0.61±0.02$$**	**0.58±0.02$$**	**0.60±0.02$$$**
Occipital cortex area 1B	0.83±0.01	0.83±0.04	0.82±0.02	0.80±0.02	0.80±0.02	0.85±0.02	0.81±0.02	0.85±0.03
Occipital cortex area 1M	0.86±0.01	0.84±0.04	0.86±0.02	0.82±0.02	**0.79±0.02$$**	**0.85±0.02***	**0.80±0.02$**	0.82+0.03
Occipital cortex area 2L	0.79±0.02	0.80±0.04	0.79±0.02	0.76±0.01	0.81±0.01	**0.86±0.01***	0.81±0.02	**0.88±0.04$$**
Paratenial nucleus	1.01±0.03	0.99±0.04	0.94±0.04	0.95±0.05	**0.80±0.02$$$**	**0.80±0.02$$**	**0.77±0.01$$$**	**0.77±0.02$$**
Paraventricular thalamic nucleus, anterior	0.73±0.03	0.73±0.04	0.70±0.04	0.83±0.07	0.77±0.02	0.71±0.03	0.71±0.02	0.71±0.02
Paraventricular thalamic nucleus, posterior	0.85±0.02	0.79±0.03	0.87±0.03	0.87±0.02	**1.12±0.04$$$**	**1.11±0.06$$$**	**1.15±0.05$$$**	**1.07±0.06$$**
Parietal cortex area 1	0.83±0.02	0.78±0.03	0.79±0.02	0.83±0.01	**0.97±0.02$$$**	**1.00±0.02$$$**	**0.95±0.03$$$**	**0.98±0.01$$$**
Parietal cortex area 2	0.77±0.02	0.77±0.03	0.74±0.02	0.78±0.02	**0.95±0.03$$$**	**0.97±0.02$$$**	**0.90±0.04$$**	**0.92±0.02$$$**
Periaqueductal grey, dorsal	0.90±0.02	0.89±0.03	0.95±0.02	0.89±0.03	0.97±0.04	0.92±0.04	0.88±0.04	0.97±0.05
Periaqueductal grey, dorsolateral	0.92±0.02	0.90±0.02	0.94±0.02	0.88±0.03	0.94±0.03	0.95±0.05	0.88±0.03	0.97±0.03
Periaqueductal grey, ventrolatral	0.91±0.02	0.90±0.02	0.93±0.02	0.92±0.04	0.93±0.03	0.90±0.02	0.91±0.03	0.99±0.03
Perirhinal cortex	0.70±0.02	0.68±0.03	0.69±0.01	**0.75±0.02***	**0.86±0.03$$$**	**0.90±0.04$$$**	**0.83±0.03$$$**	**0.84±0.01$$**
Piriform cortex	0.76±0.02	0.73±0.02	0.74±0.03	0.76±0.04	**0.58±0.02$$$**	**0.57±0.01$$$**	**0.55±0.02$$$**	**0.59±0.04$**
Pontine nucleus	0.69±0.04	0.71±0.01	0.69±0.02	0.72±0.04	**0.30±0.01$$$**	**0.31±0.01$$$**	**0.31±0.01$$$**	**0.35±0.02$$$**
Prelimbic frontal cortex	0.83±0.02	0.79±0.02	0.82±0.03	0.82±0.05	**0.64±0.02$$$**	**0.64±0.02$$$**	**0.62±0.01$$$**	**0.66±0.04$**
Presubiculum	0.54±0.04	0.53±0.02	0.53±0.01	0.58±0.04	**0.80±0.03$$$**	**0.75±0.02$$$**	**0.72±0.03$$$**	**0.76±0.03$$**
Red nucleus	0.74±0.03	0.78±0.02	**0.82±0.02#**	0.79±0.02	0.82±0.04	0.73±0.04	**0.71±0.04$$ #**	**0.83±0.04#****
Reticular thalamic nucleus	0.82±0.04	0.83±0.03	0.83±0.01	0.84±0.05	**0.66±0.02$$**	**0.66±0.02$**	**0.64±0.02$$$**	**0.65±0.02$$**
Retrosplenial cingulate cortex, agranular	0.83±0.02	0.73±0.05	0.79±0.02	0.80±0.04	**0.63±0.03$$$**	0.66±0.02	**0.65±0.03$$**	**0.63±0.03$$**
Retrosplenial cingulate cortex, granular	0.85±0.02	0.79±0.05	0.81±0.01	0.85±0.03	**0.74±0.02$$$**	0.73±0.02	**0.70±0.03$$**	**0.70±0.02$$**

sex	male				female			
stress	control		stress		control		stress	
HC/LC	HC	LC	HC	LC	HC	LC	HC	LC
Striatum, dorsolateral part	0.71±0.01	0.76±0.01	0.70±0.02	0.74±0.02	**0.64±0.01$$**	**0.64±0.03$$$**	**0.64±0.02$**	**0.64±0.01$$**
Striatum, ventral part	0.77±0.02	0.78±0.02	0.75±0.02	0.79±0.02	**0.65±0.01$$$**	**0.66±0.03$$**	**0.62±0.02$$$**	**0.63±0.01$$$**
Subiculum	0.57±0.03	0.55±0.02	0.56±0.01	0.62±0.03	**0.84±0.03$$$**	**0.83±0.03$$$**	**0.74±0.03$$$ #**	**0.79±0.03$$**
Substantia nigra pars compacta	0.47±0.03	0.48±0.02	0.46±0.01	0.52±0.04	**0.70±0.02$$$**	**0.68±0.02$$$**	**0.63±0.02$$$**	**0.69±0.02$$**
Substantia nigra pars reticulata	0.51±0.03	0.49±0.01	0.48±0.01	0.54±0.03	**0.73±0.03$$$**	**0.72±0.02$$$**	**0.68±0.02$$$**	**0.69±0.03$$**
Suprachiasmatic nucleus of hypothalamus	0.69±0.04	0.73±0.03	0.70±0.03	0.77±0.06	0.59±0.03	**0.57±0.02$$**	**0.54±0.03$$**	**0.59±0.03$**
Supraoptic nucleus of hypothalamus	0.74±0.04	0.76±0.03	0.78±0.02	0.81±0.06	**0.56±0.03$$**	**0.55±0.02$$$**	**0.54±0.03$$$**	**0.59±0.02$$**
Temporal cortex, area 1	0.78±0.02	0.79±0.03	0.74±0.02	**0.84±0.03****	**0.69±0.02$$**	**0.69±0.01$**	0.69±0.02	**0.73±0.03$$**
Temporal cortex, area 3	0.69±0.02	0.72±0.03	0.66±0.02	**0.74±0.02****	0.63±0.02	**0.63±0.02$**	0.63±0.02	**0.64±0.03$$**
Ventral pallidum	0.63±0.02	0.64±0.02	0.61±0.02	0.67±0.01	**0.55±0.01$$**	**0.54±0.02$$**	**0.50±0.02$$**	**0.52±0.02$$$**
Ventral tegmentum	0.58±0.01	0.55±0.04	0.56±0.02	0.61±0.02	0.58±0.02	0.54±0.03	**0.51±0.02 #**	**0.52±0.01$$**
Ventrolateral nucleus of thalamus	0.78±0.02	0.75±0.03	0.75±0.03	**0.84±0.02***	0.74±0.02	0.76±0.03	0.72±0.01	**0.72±0.01$$$**
Ventromedial nucleus of hypothalamus	0.69±0.03	0.71±0.03	0.70±0.02	0.78±0.02	0.64±0.02	0.65±0.02	0.63±0.04	0.71±0.03
Ventromedial thalamic nucleus	0.71±0.01	0.68±0.03	0.69±0.01	0.74±0.02	**0.86±0.03$$$**	**0.87±0.02$$$**	**0.83±0.03$$$**	**0.89±0.05$**
Vermis	0.98±0.03	0.92±0.03	0.88±0.01	0.97±0.04	0.91±0.03	0.95±0.02	0.81±0.03	0.88±0.03

Significant differences: $ – $p<0.05$, $$ – $p<0.01$, $$$ – $p<0.001$ vs respective male; # – $p<0.05$, ## – $p<0.01$ vs respective control; ** – $p<0.01$, *** – $p<0.001$ vs respective HC. Data expressed as means ± S.E.M. The animals were divided into groups with high and low levels of 50-kHz USV-s by median split of the average result of Days 12-14 of tickling. HC – high chirping rats; LC – low chirping rats.

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