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EFFECTS OF POSITIVE EMOTIONALITY AND CHRONIC VARIABLE
STRESS ON BRAIN MONOAMINE LEVELS AND BEHAVIOUR IN RATS

Master's thesis

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Keywords: positive emotionality, chronic variable stress model, monoamines

Tartu 2013

Contents

Abstract

Background: Positive emotional states have been shown to play the role in modifying stress response both in animal models and in humans. Additionally positive emotional states have been related to higher resilience against many psychiatric disorders at population level. The main goal of current master's thesis was to experimentally analyze the effects of positive emotionality measured by 50 kHz ultrasonic vocalizations (USVs) in stress response both at behavioral level and also at the level of monoaminergic signaling by using chronically variable stress (CVS) animal model. *Method:* Male Wistar rats were tickled for 2 weeks and based on USVs elicited by tickling divided in low chirping (LC) and high chirping (HC) group that was followed by CVS condition. After the end of CVS half of the animals were subjected to open field and social preference tests, while others were sacrificed to assess the level of main monoamine neurotransmitters and their metabolites in amygdala and prefrontal cortex. *Results:* CVS decreased 5-HT turnover in frontal cortex of HC animals, while increasing it in LC animals. In open field test the HC animals displayed less anxious phenotype than LC animal at basal level but this difference was eliminated by CVS. In social preference test the HC differed from LC animals by having higher activity toward unanimated objects and spending larger proportion of time in active socialization out of total time spent near other rat. HC animals had higher sucrose preference 3 weeks after tickling, while this difference was not seen in following 3 tests. CVS affected the weight gain and increased the stress induced hyperthermia response.

Positiivse emotsionaalsuse ja kroonilise varieeruva stressi mõju rottide käitumisele ja aju monoamiinsete virgatsainete tasemele

Taust: Eelnevates uurimustes on näidatud, et nii loommudelites kui ka inimeste puhul muudavad positiivsed emotsioonid organismi stressile reageerimist ja selle mõjusid. Lisaks sellele on populatsioonipõhistes uuringutes näidatud, et kõrgem positiivsete emotsioonide kogemise tase on seotud madalama psühhiaatriliste häirete esinemise riskiga. Magistritöö eesmärgiks oli uurida loommudelit kasutades positiivse emotsionaalsuse mõju stressireaktsioonile nii käitumise kui ka monoamiinsete virgatsainete tasemel. *Meetod:* Isaseid Wistari rotte kõdistati kaks nädalat ning lähtuvalt nende piuksumisaktiivsustes jagati nad vähepiuksujate (LC) või paljupiuksujate (HC) rühma. Kõdistamisele järgnes kroonilise varieeruva stressi protseduur, misjärel pooled loomad läbisid avarvälja ja sotsiaalse eelistuse käitumiskatsed ja teine pool loomi ohverdati, et mõõta monoamiinsete virgatsainete ja nende peamiste metaboliitide taset mandeltuumast ja frontaalkoorest. *Tulemused:* Katsetest selgus,

et stress vähendab frontaalkoore serotoniini käivet HC loomadel, aga suurendab sama näitajat LC loomadel. Avarvälja katses olid HC loomad baastasemelt vähem äreva käitumisega kui LC loomad. Stressi mõjul aga LC ja HC loomade erinevused äreva käitumise tasemes kadusid. Sotsiaalse eelistuse testis iseloomustas HC loomi võrreldes LC loomadega suurem aktiivsus elutute objektide suhtes ning suurem aktiivset sotsiaalset uudistamise osakaal teise roti lähisuhtlusalas viidetud ajast. HC rottide suhkrueelistus oli 3 nädalat pärast kõdistamist kõrgem kui LC rottidel, erinevus aga kadus kolmes järgnevas mõõtmises. Kasutatud kroonilise stressi režiim tõi kaasa muutused kaalutõusus ja suurendas akuutsest stressist tingitud hüpertermilist reaktsiooni.

Introduction

Stress

Stress can be described as state where homeostasis is threated by external or internal factors that evokes the adaptive reaction of organism by activating complexes of physiological and behavioral adaptive responses (Chrousos, 2009). Chronic stress has varied effect on nervous, hormonal, and immune system (for review see McEwen, 2003). At the level of population higher levels of stress are related to higher levels of mental disorders (Rosen and Schulkin, 1998) and more specifically high stress levels have a direct positive causal effect on the neurochemical disturbances seen in depression (for review see Praag, 2004).

The main effectors of stress response at peripheral nervous system are glucocorticoids regulated by HPA-axis (hypothalamic–pituitary–adrenal axis) and norepinephrine and epinephrine (NA). At the level of central nervous system (CNS) the adaptive homeostasis involved in stress response includes the neural pathways related to arousal, vigilance and focused attention. Stress response in CNS is initiated by hypothalamic hormones arginine vasopressin (AVP) and corticotropin-releasing hormone signals (CRH) from paraventricular nuclei, pro-opiomelanocortin-derived peptides (α-melanocyte stimulating hormone and β-endorphin), norepinephrinergic signaling by *locus coeruleus* areas A1 and A2, autonomic norepinephrine centers in brainstem and ascending serotonergic pathway from the raphe nuclei. Activation of hypothalamic CHR and AVP centers stimulates the norepinephrine centers in brainstem, including *locus coeruleus* and *vice versa*, which constitutes the basis of stress response pathway at the level of CNS. (Chrousos, 2009). Activation of base stress response pathways (CRH and AVP-ergic nuclei in hypothalamus and NA-ergic neurons in *locus coeruleus*) also elevates the core temperature of organism (Chrousos, 1997), which constitutes the neural basis of stress induced hyperthermia seen in rats.

The activation of base stress response pathway affects monoaminergic pathways related to executive, cognitive, reward and fear functions mainly by interacting with mesocortical and mesolimbic dopamine system, amygdala and hippocampus. Amygdala is activated by ascending NA-egic neurons originating from brainstem or by inner emotional stressors generated in cortical association areas. Hippocampus has an important and mostly inhibitory effect both on amygdala and base stress response pathway that includes hypothalamus and *locus coeruleus*. (Chrousos, 1997). Higher order effects of chronic and

acute stress are at least partially mediated by interactions between different brain regions. For example the processing of emotional memories includes the interaction between amygdala and hippocampus and fear extinction requires interaction between frontal cortex and amygdala (McEwen, 2003).

In addition to changing the functioning of different brain regions stress response can also cause the morphological remodeling of those regions. It has been shown experimentally that chronic stress for 10 to 21 days is enough to cause morphological changes in prefrontal cortex (Cook et al., 2004), amygdala (Vyas et al., 2002) and hippocampus (Magarinos et al., 1997, Vyas et al., 2002) in rats.

Chronic variable stress model

The CVS regime is widely used in rodents to model depression in which rodents are exposed to array of unpredictable mild stressors with different durations over a sustained period of time (Hill et al., 2013). The main strength of using CVS animal model in study of depression is ecological validity of stressors used: the conditions included in stress regime are realistic simulation of real life stressors for rodents (Wu et al., 2010). CVS has been shown to cause array of neurobiological changes that mirror those seen in depressive disorders, further it has been suggested that CVS is suitable method to investigate novel biochemical disturbances in depression that could be used in future drug discoveries (for review see Hill et al., 2013). At the behavioral level the main outcome of CVS in respect to behavior of animals is anhedonia that constitutes one of the most central symptoms of depression (Willner, 1997). In addition other phenotypes seen in depression have been reported as an effect of CVS in rats: decreased weigh gain (Willner et al., 1996), increased corticosterone levels (Grippo et al., 2005), reduced self-care and grooming (Willner, 1995) and altered monoamine levels (Hill et al., 2013).

Behavioral changes caused by CVS are reversed by clinically effective antidepressants (Grippo et al., 2006) and other clinically effective depression interventions (Henningsen et al., 2013), while drugs without antidepressant properties have failed to reverse the behavioural effects caused by CVS (Wilner, 2005). The ability of clinically effective antidepressants to reverse behavioral changes caused by CVS supports the notion that CVS causes similar changes in neural substrate that also underline the pathology of depression. This makes CVS a suitable method to experimentally study the neurochemical basis of depression (for example the changes of monoamine neurotransmitters) and the

protective factor that modify behavioral and biochemical changes caused by stress (for example positive affect measured by chirping activity of rats). The chronic variable stress condition (CVS) [also called chronic mild stress (CMS)] used in this thesis has been described in detail in the corresponding section of method chapter of current thesis.

The role of monoamines in stress response and depression

The noradrenergic and serotonergic systems originate from brainstem and are widely projected throughout the brain, exerting effects that are correspondingly widespread. Both noradrenaline (NA) and serotonin (5-HT) have widespread modulatory role at cellular level (Morilak and Frazer, 2004). Previous work has shown that noradrenergic and serotonergic systems regulate many of the same broader behavioral dimensions that are also affected by anxiety disorders and depression like motivation, arousal and positive/ negative affect. Especially the role of serotonergic system in pathology of depression has been examined because the serotonergic system has widespread innervate effect on most cortical and subcortical regions of brain through varied signaling ways and functional roles, which may explain the association of altered serotonergic activity with different symptoms of depression (Mann, 1999). Despite the global role of monoamine neurotransmitters in regulating many behavioral dimensions it is still possible to experimentally study specific roles of monoamine neurotransmitters by studding monoamine response in specific brain regions where they modulate key behavioral components that are dysregulated in depression. (Morilak and Frazer, 2004). The role of monoamines in depression is also supported by facts that several clinically effective antidepressants have been developed that are known to affect central adrenergic and serotonergic neurons (Frazer, 2001). One of the main effects of stress in central nervous system is the alteration of monoaminergic response (Ahmad et al., 2010). The repeatedly described effect of CVS in animal models is the decrease preference for sweet foods interpreted as the sign of anhedonia (Willner, 1997), which is caused by the alterations of serotonergic and dopaminergic monoamine systems (Gamaro et al., 2003).

Previous work has shown that CVS has varied brain region specific effect on the levels of monoamines and main monoamine metabolites that at whole parallel the neurobiological findings seen in human depression. Still somewhat inconsistent results between experiments can be accounted for by the fact that the effects of CVS on monoamine neurotransmitters seem to depend at least partially on the housing conditions, age and strain of rats used in experiments. Prefrontal cortex (PFC) and hippocampus seem to be the two

most studied brain areas in context of CVS effect on monoamine neurotransmitters. (For review see Hill et al., 2013. This article is also used as basis of following overview of brain area specific effects of CVS on monoamine neurotransmitters).

In Wistar strain rats the effect of CVS in 5-HT levels on PFC is either the decrease of 5-HT levels (Li et al., 2003, Yi et al., 2008, Xu et al., 2008, Li et al., 2009) or no effect (Berkis et al., 2005, Häidkind et al., 2003). The studies on 5-HT turnover (measured by 5-HT metabolite 5-HIAA to 5-HT ratio) for PFC in Wistar rats are sparse. Berkis with colleagues 2005 found that CVS does not alter the 5-HT level in PFC but decreases the 5-HT turnover in Sprague-Dawley rats but not in Wistar rats. CVS seems not to have an effect on NA levels in PFC in Wistar rats (Yi et al., 2008, Harro et al., 2001, Häidkind et al., 2003). Inconclusive results have been obtained about the effects of CVS in PFC for DA. CVS used on Wistar rats either increases DA levels (Yi et al., 2008), decreases DA levels (Berkis et al., 2005, Ahmad et al., 2010), decreases DA turnover (Dalla et al., 2008), increases DA turnover (Berkis et al., 2005) or has no effect (Harro et al., 2001, Häidkind et al., 2003).

In Wistar strain rats the effect of CVS on 5-HT levels in hippocampus is mainly the decrease of 5-HT levels (Li et al., 2003, Yi et al., 2008, Xu et al., 2008, Li et al., 2009). Gamaro et al., 2003 and Berkis et al., 2005 and Ahmad et al., 2010 have shown that CVS increases 5-HT turnover in hippocampus. As a notable exception, Dalla et al., 2005 have shown that CVS does not decrease 5-HT levels in hippocampus but decreases the 5-HT turnover in hippocampus of female but not in male rats. Hippocampal NA levels (Harro et al., 2001, Yi et al., 2008) and DA levels (Harro et al., 2001, Dalla et al., 2008) seem to be unchanged after CVS in Wistar rats

Ultrasonic vocalization in rats as marker of affective states

Positive mental states have been shown to have longitudinally widespread beneficial effects on varied social, work and health related outcomes (for review see Lyubomirsky, 2005). Higher levels of positive mental states are related to lower rates of depression and anxiety disorders. Contrary to popular belief that successful outcomes and desirable characteristics are primarily the causes rather than the consequences of happiness, a large amount of evidence now supports the notion that this relationship may be at minimum bidirectional. Positive emotional states are at least partially the prerequisite for future positive outcomes (*ibid.*).

At the level of neurobiology the positive affective states are related to ascending mesolimbic dopamine system. The positive affective state resulting of activation of mesolimbic dopamine system is different from mere pleasure of sensory affects. It also encompasses the elements of expectancy and seeking seen for example in drug craving. (Burgdorf et al., 2011).

One of the main markers of affective states in rodents is the ultrasonic vocalizations (USVs) they emit (Panksepp, 2007). UVSs emitted by rodents can be categorized in two: the 50 kHz short UVSs or chirps and longer 22 kHz UVSs. It has been proposed that 22 kHz UVSs have two related signaling roles in rodents. Firstly the 22 kHZ UVS are the part of defensive repertoire in rats and are directed at predatory animal prior to initiation of defensive attack. (Litvin et al., 2007). Secondly, the 22 kHz UVSs are seen as alarm cries in rats because they are emitted in situations of distress such as foot shock, social isolation, social defeat and cocaine withdrawal (Burgdorf and Panksepp, 2001). Fifty kHz chirps have been further classified as having two separate variations: 50 kHz chirps with frequency modulation (FM) component (also called trill chirps) and 50 kHz chirps without the FM component (Burgdorf et al., 2007). Fifty kHz chirps, especially the FM calls are related to positive affective states in rodents and are primarily elicited by stimuluses' with positive valence such as tickling and rough-and-tumble play (Burgdorf and Panksepp, 2001). FM 50 kHz USVs are elevated by hedonic stimuli and suppressed by adverse stimuli and the rate of FM 50 kHz USVs is positively correlated with the rewarding value of USVs eliciting stimuli (Burgdorf et al., 2011).

Panksepp (2007) goes even further by suggesting that 50 kHz vocalizations may represent something broader than previously mentioned simple behavioral response in rodents to stimulus with positive valence: chirping seen in rats in positive valence situations may be primordial evolutionary predecessor of human infantile laughter. He has also proposed that similarly to humans the rodent behavior is also at least partially guided by basic affective experiences. Evolutionary relationship between human laughter and 50 kHz chirps and similar role of affective states in regulating the behavior in rats and human opens up the possibility to study the role of affective factors in mental health (for example in depression) in controlled laboratory environment by using animal models.

Similarly to positive affective states in humans, the 50 kHz USVs are related to dopaminergic pathways (Brudzynski, 2013). Experiments show that disruption of mesolimbic

dopamine system by lesions or pharmacological blockade reduces the levels of FM 50 kHz USVs (Burgdorf et al., 2007). At the level of individual animal the 50 kHz and 22 kHz USVs seem to be uncorrelated (Burgdorf et al., 2008). However, selective breeding in rats for higher levels of 50 kHz USVs has shown that future generations of animals that show higher levels of 50 kHz chirps have also reduced rate of 22 kHz vocalizations and vice versa (*ibid.*), which may hint that at the genetic and epigenetic level the 22 kHz USVs and 50 kHz USVs are related and represent opposing ends of stable affective trait.

In adolescent rats the 50 kHz vocalizations are readily elicited during rough-and-tumble play that can be "reproduced" by human experimenter (Burgdorf and Panksepp, 2001). Tickling by experimenter consist of high activity playful whole-body stimulation and repeated pinning of tickled animal. Solid experimental results have been gathered that show that tickling of rats by human experimenter mimics the effects of natural rough-and-tumble play (*ibid.*): rats that have been tickled chirp significantly more than control group of lightly touched animals, also tickled rats approach the hand of experimenter more willingly, develop preference toward the tickling hand and tickling can be used as rewarding stimulus in operant bar pressing task, which all in all hints at the positive emotional valence of tickling. The parallels between tickling and the playfulness of rats in natural conditions is highlighted by the fact that socially deprived single housed animals that have been shown to be more playful than group housed animals did indeed chirp more in response to tickling than group housed rats (*ibid.*).

Differences between rats with high and low chirping activity

It should not go unnoticed that in addition to the effects of stable positive affective trait the tickling treatment used to elicit the chirping of rats can by itself have stress response modifying properties. Hori with colleagues (2013) has shown that tickled animals show lower level of freezing behavior in second retention test in auditory fear conditioning condition (96 hours after initial fear conditioning). Tickling also modified plasma catecholamine levels after 96 h fear conditioning retention test by lowering the adrenaline and nor-adrenaline levels in tickled group (*ibid.*). In humans major depression has been related to loss of hippocampal neurons due to reduced neurogenesis in this brain area (Sheline et al., 2003). Tickling applied on 4 week old rats for 5 minutes per day for 7 days has been shown to significantly increase the hippocampal neurogenesis in rats (Yamamuro et al., 2010).

Raudkivi et al., 2012 have shown that LC (low chirping) male Wistar rats are more sensitive to CVS compared to their HC counterparts: the effect of CVS on the weight gain and corticosterone levels measured from trunk blood was higher in LC rats. Also the post-CVS increase of extracellular 5-HT level induced by citalopram was greater in group of LC rats. One of the main behavioral effects of CVS in rats is the anhedonia measured by sucrose preference test (Willner, 1997). Mällo et al., 2007 have shown that in non-stressed Wistar rats the chirping activity by itself influences the results sucrose preference test toward higher levels of sucrose preference in LC rats. The same experiment also found the effect of chirping on general level of exploratory activity in female rats as the LC-rats had higher activity levels. Both of those results are interpreted by the authors hinting toward the lower level of motivated behavior in HC-rats.

The modifying effects of positive affectivity in stress response are shown by Mällo et al., 2009: 4 week CVS regime increased the long-term oxidative metabolic activity in amygdala, hippocampus and anterior thalamus in male LC-rats only. The behavioral effects of CVS were also more pronounced in male LC-rats, who had lower sucrose preference, higher reduction of body weight gain during the CVS and increased immobility in forced swimming test (*ibid.*). Above-mentioned results are supported by Burgdorf et al., 2009, who show that rats breed for high level of chirping display higher level of preference for sucrose, are less aggressive than control animals and show lower level of anxiety in an open field test by exhibiting more crosses into the center of open field apparatus.

Aims of master's thesis

The main goal of current master's thesis is to experimentally analyze the effects of positive emotionality in stress response both at the level of behavioral and also at the level of monoaminergic signaling. Because of the relations between altered stress response and pathology of depression, the wider aim of the thesis is the analysis of the role of positive emotionality in depressive pathology.

Materials and method

Animals

In total 80 male Wistar rats were used during the study. The animals were bred at location from parent Wistar rats provided by Harlan Laboratories, Netherlands. All animals were single housed in polypropylene cages with wood chips bedding throughout the tickling

sessions, recordings and analysis of USVs was carried out. After that the rats were housed in group housing of 4 animals per polypropylene cage with wood chips bedding. Both in single and group housing the experimental animals were granted ad libitum access to food (Lactamin R70, Sweden) and tap water and were housed in constant environmental conditions with controlled light cycle (lights on from 8:30 to 20:30) and temperature (temperature form 19-21 degrees Celsius). All the behavioral experiments were carried out between 12:00 and 20:00. Animals were balanced between cages by their chirping activity: each cage housed 2 LC and 2 HC rats. Similarly the housing groups of 4 were balanced in terms of weight of the animals in order to have similar average weight and weight variability between different cages.

All the experiments were in accordance of European Union Directive "Directive 2010/63/EU on the protection of animals used for scientific purposes". All efforts were made to minimize the suffering and number of animals used in this study.

General procedure

The rat pups were weaned from mother at the age of 3 weeks and housed in separate cages. A day later the tickling sessions were started that lasted for 14 days. USVs from tickling at days 12, 13 and 14 were used to divide animals between groups of HC and LC animals. At the age of 15 weeks rats were assigned into group housing. At the age of 16 weeks the pre-CVS sucrose preference test was performed. 4 days later the pre-CVS body temperature measurements were recorded followed by the beginning of CVS on next day for 25 days. During the 10th and 11th days of CVS the mid-CVS sucrose preference test was performed. At the end of CVS the post-CVS sucrose preference test was performed followed by post-CSV body temperature measurement a day later. Out of 80 animals 40 were sacrificed by decapitation for biochemical measurements. Remaining 40 animals were assigned to behavioral tests performed on sequential days in following order: open field test, social preference test, memory test, and elevated zero maze.

Recording and analysis of tickling induced ultrasonic vocalization

The tickling procedure of juvenile rats was carried out as previously described by Mällo et al., 2009. Single housed animals were individually removed from home cage and placed in a different smaller cage. Animals were given 15s for habituation followed by 15s tickling session. In total 4 tickling sessions were given during 2 minute time period, after which animal was returned to home cage and the test cage was cleaned. The tickling sessions

of juvenile rats lasted for 14 days as it has been shown by Mällo et al., 2007 that 14 days of tickling is enough for animals to develop stable level of 50 kHz ultrasonic vocalizations that allows animals to be categorized between high-chirping (HC) and low-chirping groups (LC). The USVs were recorded by microphone located 20cm above the test cage during the tickling sessions in days 12, 13 and 14. Recordings were analyzed by creating a spectrogram in Avisoft SASLab Pro (Avisoft Bioacoustics, Germany). Spectral data of sound recordings were used to manually count the number of 50 kHz USVs with and without trill component.

Out of 100 animals the 40 animals that showed the lowest median number of 50 kHz USVs across 3 recordings were assigned in LC group and the 40 animals that showed the highest median number of 50 kHz USVs across 3 recordings were assigned in HC group. The middle 20 rats sized group of animals in terms of the 50 kHz USVs were left out in order to maximize the intergroup variability between HC and LC groups. The remaining 80 animals were divided in groups of 4 for group housing.

Measurement of body weight and sucrose preference

During the experiment the weights of animals were registered on six different time points including pre-CVS, during CVS and post-CVS. The sucrose preference test was carried out on 4 occasions including measurements pre-CVS, mid-CVS and post-CVS. For sucrose preference test the animals were placed from group housing into single cages 1h before the lights-off period. At the beginning of lights-off period the animals were given a choice between two equal bottles, one filled with 1% sucrose solution and the other with tap water. The 1h sucrose and water consumption was measured by weighting previously weighted sucrose and water bottles one hour after the beginning of sucrose preference test. The 12h sucrose and water consumption was measured by weighting previously weighted sucrose and water bottles 12 hours after the beginning of sucrose preference test. To minimize the unnecessary handling and stress to animals the weighting of animals was carried out at the end of the 12h sucrose preference test if possible.

In each of four tests of sucrose preference the amount of sugar and water consumed by each animal in 1h and 12h time window was recorded and based on this data the sucrose preference ratio (%) was calculated by dividing the amount of sugar consumed with the sum of sugar and water consumed. The calculated index was interpreted as proxy to describe the sucrose preference level of animals.

Measurements of body temperature

The measurement of rectal body temperature was carried out as described by Kõiv and Harro, 2010. Rectal temperature of animals was measured with accuracy of 0.1 °C by flexible temperature probe (YSI, 400 series) by inserting a lubricated thermo sensing probe 50 mm into the rectum of rat and the readings from the temperature control unit (HB 101, Panlab, Harvard Apparatus) were taken after 1 min. The rats were lightly manually handled from the base of the tail during the measurement of body temperature. The measurements were carried out by two experimenters in a separate room.

The stress induced hyperthermia (SIH) response of animals was measured two times - first shortly before the beginning of CVS regime (pre-CVS measurement) and second time immediately after the end of CVS regime (post-CVS measurement). In both times the body temperature of animals was registered twice – as baseline (T0) and 15 minutes after the first measurement (T15). In total 8 variables were analyzed: pre-CVS body temperature at T0, pre-CVS body temperature at T15, post-CVS body temperature at T0, post-CVS body temperature at T15, difference of body temperatures at time point T0 post- and pre-CVS difference of body temperatures at time point T15 post- and pre-CVS, difference of body temperatures pre-CVS between T0 and T15 and difference of body temperatures post-CVS between T0 and T15.

Chronic variable stress (CVS) regime

The CVS model used was a modified version of CVS procedure used in previous experiments (Harro et al., 2001, Tõnissaar et al., 2008). Different stressors with different duration were applied one by one every day. Each stressor was used no more than 3 times in total. The stressors presented included: 1) movement restriction in cage made out of 2 l plastic bottle for 2h, 2) tilted cage at 45 degree angle for 24h, 3) tail pinch with a clothes-pinch placed at the base of the tail for 5min 4) cold (4 degrees) for 2h, 5) stroboscopic lights for 12h at varying frequency of 10-50Hz, 6) strong illumination (900 lx) during the predicted dark phase of day for 12h, 7) imitation of injection for 5min, 8) cage without wood chips bedding and 5mm deep water level for 12h during dark phase of the day 9) round 10cm diameter platform lifted 75cm from the ground with strong illumination (900 lx) for 1h and 10) loud white noise for 1h during the dark phase of the day, 11) crowded cage (8 rats from different cages instead of 4) for 12h). All stressors were carried out in separate room while control rats stayed undisturbed in their home cages in the animal room.

Behavioral tests

Open field test

The behavior in open field test has been related to the level of non-pathological anxiety in animals (Prut and Belzung, 2003). In open field test animals were placed at the center of a square shaped box with dimensions 78 cm by 78 cm with 34 cm high side walls. The area was virtually divided into 16 (4 by 4) squares with side length of 19.5 cm. The 4 squares in the center of 4 by 4 field represent the center of open field. Four squares in outer corners of the 4 by 4 field represented the corners of open field and the remaining 8 squares next to sidewalls represented the thigmotaxis area. The duration of open field test was 600 s.

During the open field experiment 10 behavioral variables were recorded and included in later statistical analysis. Those variables were: number of entries into the center of open field, time spent in center of open field, number of entries into corners, duration of time spent in corners, latency of first entrance into the corner, latency of first entrance into the center of open field, latency of first entrance of thigmotaxis area of open field, number of rearings by animal, number of entries into thigmotaxis area and time spent in thigmotaxis area. The number of rearings was registered manually by experimenter. For the scoring of other parameters the video recording of experiment and automated scoring by EthoVision XT8 software (Noldus Information technology, Netherlands) was used.

Social preference test

Used social preference test was based on the work of Berton and colleagues (2006). The social preference test field consisted of a square shaped black colored floor area with dimensions of 98 cm by 98 cm surrounded by 40cm high black colored side walls. The social preference test area was virtually divided into four equal quarters. In two diagonally opposite corners of social preference test area two animal cages were placed. The virtual quarters that contained cages were defines as socialization quarters. In each of the two socialization quarters an additional virtual area was defined as closer socialization area, which constituted the closer area neighboring the cages placed in corners. Social preference test was carried out in two days. During the firsts day animals were given 600 s to explore the social preference test area without social stimulus (both cages being empty). During the second day the social preference measurements were carried out by placing the animals one after another in center area of the social preference test arena with social stimulus (other rat in one of the cages).

The activity of animal in social preference arena with social stimulus was recorded for time period of 600 s.

During the social preference test in total 19 variables were measured and included in statistical analysis. Those variables were: number of entrances and time stayed in corners without cages; number of entrance, latency time of first entrance and total time stayed in socialization quarter without social stimulus (empty cage); number of entrance, latency time of first entrance and total time stayed in closer socialization area without social stimulus (empty cage); number of entrance, latency time of first entrance and total time stayed in socialization quarter with social stimulus (cage with other rat); number of entrance, latency time of first entrance and total time stayed in closer socialization area with social stimulus; distance moved during social preference test; number of entrances, latency of first entrance and total time stayed in center of social preference field; total time of active socialization and ratio of active socialization to total time stayed in closer socialization area of cage with other rat. The time spent in active socialization by rat was registered manually by experimenter during the social preference test. For the scoring of other parameters the video recording of experiment and automated scoring by EthoVision XT8 software (Noldus Information technology, Netherlands) was used.

High performance liquid chromatography (HPLC)

Biochemical measurements were carried out in amygdala and frontal cortex brain regions. The analysis of amygdala was based on 40 samples of 40 animals while the analysis of frontal cortex was based on 27 animals out of total 40. The remainder of collected samples of frontal cortex region was used to analyze the neuronal sprouting differences between HC and LC group animals (data not reported in current master's thesis). The animals were sacrificed by decapitation and the brains were dissected on ice and stored at -80 degree Celsius until measurements with high-performance liquid chromatography (HPLC).

The monoamine levels and their metabolites were measured from tissue with high-performance liquid chromatography with electrochemical detection as described by Raudkivi, 2012. In short, rat brain tissues were homogenized with an ultrasonic homogenizer in ice-cold solution of 0.1 M perchloric acid (30 or 50 μl/mg) containing 5 mM of sodium bisulphite and 0.4 mM EDTA to avoid oxidation. The homogenate was then centrifuged at 14.000 rpm for 10 min at 4°C. Aliquots (10 μl) of the obtained supernatant were chromatographed on a Luna C18(2) column (150 x 2 mm, 5 μm). The separation was done in isocratic elution mode at

column temperature of 30 °C using the mobile phase containing 0.05 M sodium citrate buffer at pH 3.7, 0.02 mM EDTA, 1 mM KCl, 1 mM sodium octanesulphonate and 7.5% acetonitrile. The chromatography system consisted of an isocratic pump (Hewlett Packard HP1100), a temperature-regulated autosampler, a temperature-regulated column compartment and an HP 1049 electrochemical detector (Agilent, Waldbronn, Germany) with glassy carbon electrode. The measurements were done at an electrode potential of +0.7 V versus the Ag/AgCl reference electrode. The limits of detection at signal-to-noise ratio (S/N)=3 were as follows: 0.08 pmol/mg tissue for DA; 0.10 pmol/mg tissue for HVA; 0.05 pmol/mg tissue for DOPAC; 0.08 pmol/mg tissue for 5-HT; 0.04 pmol/mg tissue for 5-HIAA. (Protocol described by Raudkivi, 2012).

In total 7 biochemical parameters were measured from samples of amygdala and frontal cortex. The tissue levels of three monoamine neurotransmitters serotonin (5-HT), noradrenaline (NA) and dopamine (DA) were assessed. The main metabolites of previously named monoamines were assessed: the metabolite of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), the metabolite of norepinephrine, normetanephrine (NMN), the metabolite of dopamine, 3-methoxytyramine (3-MT) and the metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA). In addition the turnover of 5-HT, DA and NA (ratios between the neurotransmitter metabolites and the corresponding neurotransmitter, NMN/NA, DOPAC/DA, 3-MT/DA and 5-HIAA/5-HT) were calculated and included in statistical analysis.

Statistical Analysis

Two-way ANOVA models were constructed and analyzed by using STATA 12.0 SE (StatCorp, USA) with stress group and HC/LC group as factors for continuous outcome measures from behavioral tests and biochemical measurements. Because the balanced design of experiments (i.e. the number of animals is equal in groups in comparison) Tukey HSD test was used for post hoc testing. To analyze repeatedly measured weight and sucrose preference data repeated measures ANOVA models with stress group and HC/LC factors were used in STATISTICA 12.0 software package (StatSoft, USA). Software packages STATA (StatCorp, USA) and IBM SPSS 20.0 (IBM, USA) were used for graphing purposes.

Results

Weight change and sucrose preference test

CVS by measurement time interaction ($F(5,380) = 9.6$, $p < .01$) was confirmed by repeated measures ANOVA. Figure 1.0 depicts the weights of animals in CVS and control group. Based on difference of weight gain trend between CVS and control animal groups the used chronically variable stress (CVS) regime has been effective. The weights of animals over 6 measurements did not show HC/LC group by measurement time interactive effects ($F(5,380) = 0.26$, $p > .10$) nor chirping activity and CVS by measurement time ($F(5,380) = 0.9$, $p > .10$) interactive effects.

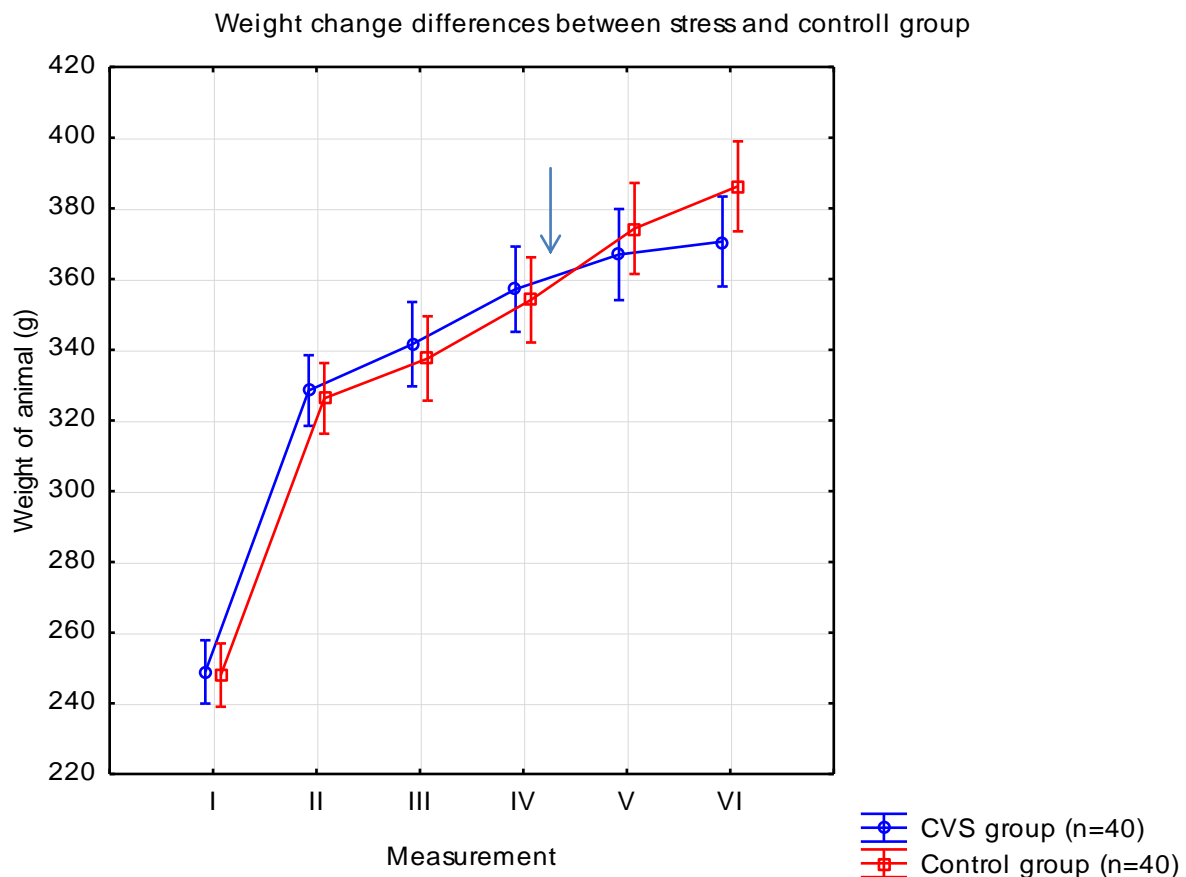Weight change differences between stress and controll group

Figure 1.0 Weight of animals in CVS and control group. (Note: whiskers denote the 95% confidence interval of measure; arrow denotes the beginning of CVS).

The sucrose preference was affected by the chirping activity by time interaction ($F(3,228) = 4.3$, $p < .01$). Figure 2.0 shows the sucrose preference relative to water over 12h sucrose preference tests between HC and LC rats. Only at post tickling the sucrose preference differed between HC and LC animals ($F(1,76) = 5.2$, $p < .05$). CVS regime by time interaction did not reach statistical significance ($F(3,228) = 1.0$, $p > .10$). Similarly the CVS

and chirping activity by measurement time together did not have effect on the 12h sucrose preference ($F(3,228) = 0.6$, $p > .10$).
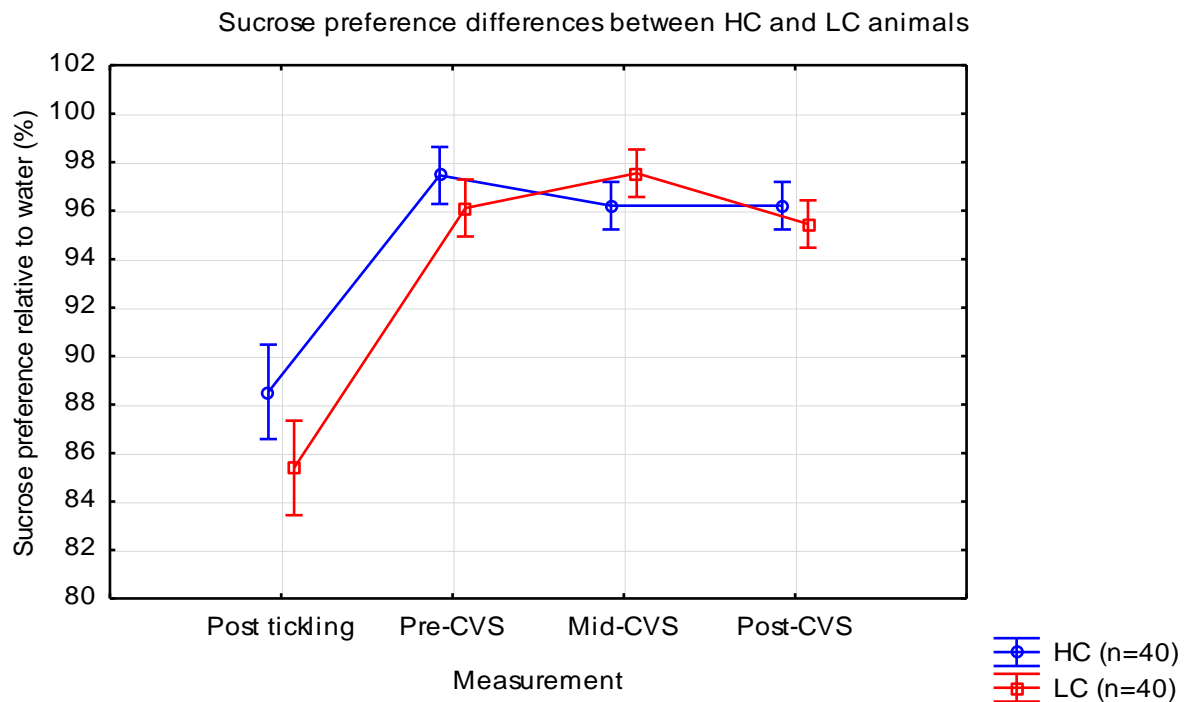

Figure 2.0 Sucrose preference of animals in HC and LC group. (Note: The 95% percent confidence intervals are denoted on every single measurement).

Stress induced hyperthermia

The post-CVS body temperature was similar between CVS and control group at T0. However the CVS had effect on body temperature measured at T15 post-CVS ($F(1,79) = 11.0$, $p < .01$). Animals from CVS group had on average 0.29 degrees Celsius higher body temperature compared to control group. Figure 3.0 (a) depicts the difference of body temperature difference between control and CVS group at T15 post-CVS regime. Chronic stress also had effect on the change of temperature between time points T0 post-CVS and T0 pre-CVS ($F(1,79) = 4.2$, $p < .05$), post hoc test showing the differences between CSV and control group ($p < .05$). CVS group animals had 0.055 degrees Celsius increase and control group animals 0.185 degree Celsius decrease in body temperature between T0 post-CVS and T0 pre-CVS. CVS also had effect on the change of temperature between time points T15 post-CVS and T15 pre-CVS ($F(1,79) = 4.2$, $p < .05$). In CVS group the body temperature was 0.047 degrees Celsius lower and in the control group 0.285 degrees Celsius lower at T15 post-CVS compared to pre-CVS body temperature at T15. Body temperature changes

between pre-CVS regime and post-CVS regime at time points T0 and T15 are also depicted on figure 3.0 (b).
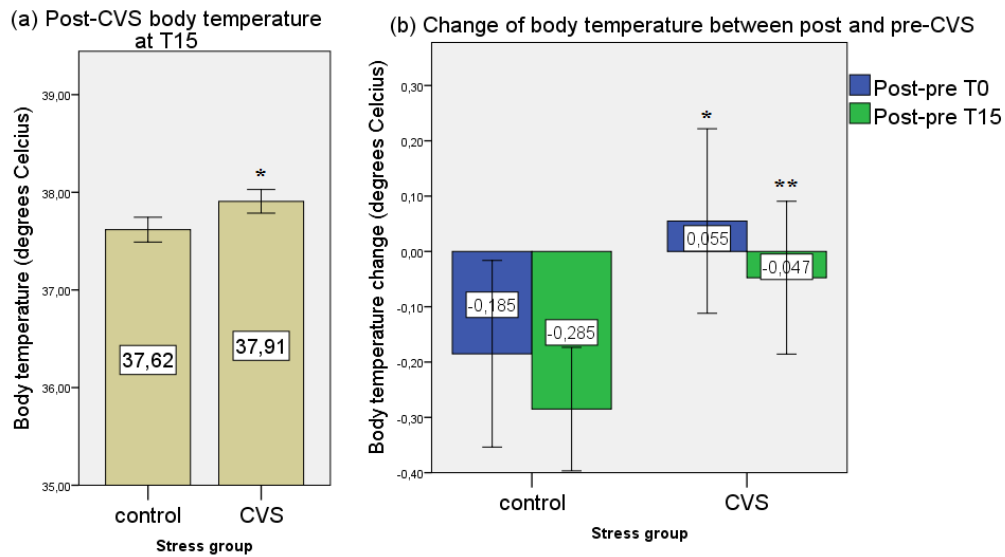

Figure 3.0 (a) the body temperature at T15 of post CVS regime between stress and control group. Part (b) the change of body temperature between pre-CVS regime and post-CVS regime at time points T0 and T15. (Note: * denotes statistically significant difference between control and stress group at level $p < .05$; ** denotes statistically significant difference between control and stress group at level $p < .01$; whiskers denote the 95% confidence interval of measure)

Behavioral tests

Social preference test

The main differences in social preference test emerge between groups of HC and LC animals. Chirping activity affected total time spent in socialization quarter without stimulus ($F(1,39) = 4.2$, $p < .05$). HC animals spent 42.5s more in social stimulus quarter with empty cage compared to LC animals. Chirping activity also affected the number of entries into closer socialization area ($F(1,39) = 5.0$, $p < .01$). On average HC group animals had 2.8 more entries compared to LC group respective value of 10.5. HC animals had tendency for higher number of entries into the corners without cages ($F(1,39) = 3.6$, $p < .07$) and for longer duration of time spent in closer socialization area near empty cage ($F(1,39) = 3.9$, $p < .07$).
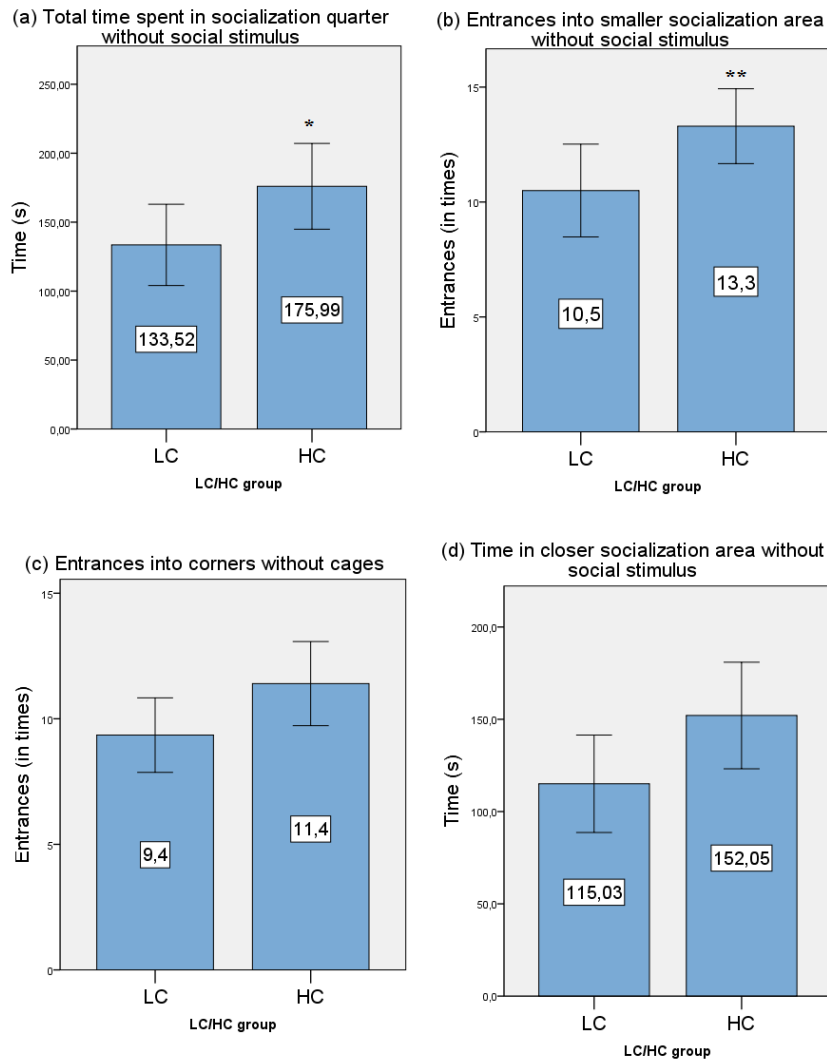
Figure 4.0. (a) time spent in socialization quarter without rat. (b) the number of entries into smaller socialization area without social stimulus (c) the entries into the corners without cages. (d) the time spent in socialization area. (Note: * denotes statistically significant difference between HC and LC group at level $p < .05$; ** denotes statistically significant difference between HC and LC group at level $p < .01$; whiskers denote the 95% confidence interval of measure)

Chirping activity affected the time spent in quarter with social stimulus ($F(1,39) = 4.6, p < .05$). LC animals stayed on average 50.69s longer in socialization quarter with other rat compared to HC animals. Similarly chirping activity affected the time spent in closer socialization area with social stimulus ($F(1,39) = 4.2, p < .05$). LC animals spent on average 47.91s longer in closer socialization area compared to HC animals. The results discussed previously are also depicted on figure 5.0 (a) and (b).
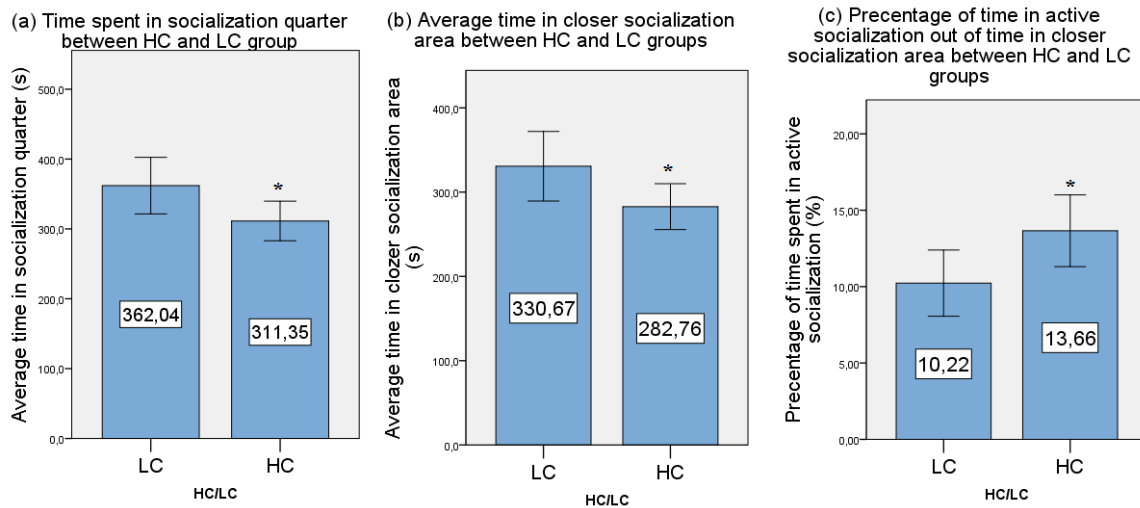
Figure 5.0 (a) time spent in socialization quarter with rat. (b) average time in closer socialization area. (c) percentage of active socialization out of the total time spent in closer socialization area with social stimulus. (Note: * denotes statistically significant difference between HC and LC group at level $p < .05$; whiskers denote the 95% confidence interval of measure)

Chirping activity also affected the percentage of time spent in active socialization out of total time stayed in closer socialization area ($F(1,39) = 4.8$, $p < .05$). Contrary to time spent in quarter with social stimulus and time spent in closer socialization area HC animals were 3.43 percent points more active in socialization compared to LC animal's respective value of 10.22 percent points.

At the level of overall movement activity in social preference test a tendency of difference emerged toward higher distance covered by LC animals group ($F(1,39) = 2.9$, $p < .10$) compared to HC animals. Based on this tendency it was tested if the higher activity of LC animals in respect to quarter with social stimulus in two above-named time measures may be related to the higher average movement activity of those animals. One-way ANCOVA models were constructed to test this with total distance moved as covariate in both cases with time spent in quarter with social stimulus and time spent in closer socialization area with social stimulus as dependent variables. In both cases the inclusion of covariate was enough to reduce previously significant statistical inference value to non-significant level – for time spent in quarter with social stimulus ($F(2,39) = 2.7$, $p > .08$) and time spent in closer socialization area with social stimulus ($F(2,39) = 2.9$, $p > 0,06$).

Open field

CVS condition had effect on time stayed in corners of open field ($F(1,39) = 6.1$, $p <$ .05). CVS group animals stayed on average 45.24s longer in the corners compared to the control group's corresponding value of 208.62s. The CSV regime also affected the latency time of first entry into the corner ($F(1,39) = 8.2$, $p < .01$). On average it took 6.5s for CVS group animals until the first entry into the corners was made, which is 4.97 (s) lower latency time than the corresponding value of control group animals. The results discussed previously are also depicted on figure 6.0 (a) and (b).

Chirping activity and CVS regime together had effect on the number of entries into the center of open field ($F(1,39) = 5.2$, $p < .05$). In LC animals the CVS increases the number of entries into the center, while in group of HC animals CVS reduces the number of entries into the center of open field. In addition the HC and LC animals of control group differed in number of entries into the center of open field ($F(1,19) = 5.5$, $p < .05$). The results discussed previously are also depicted on figure 6.0 (c).
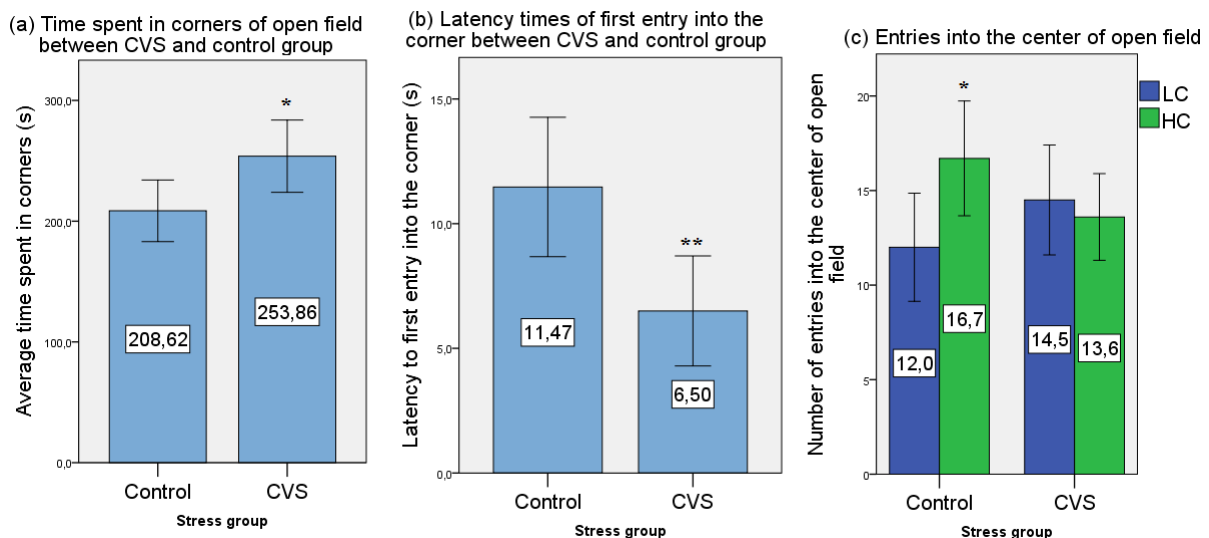

Figure 6.0. (a) time spent in corners of open field. (b) latency time of first entry into the corner between stress and control group. (Note: * denotes statistically significant difference between control and stress group at level $p < .05$; ** denotes statistically significant difference between control and stress group at level $p < .01$; whiskers denote the 95% confidence interval of measure).

Levels of monoamine neurotransmitters

CVS and chirping activity together had effect on the serotonin turnover (ratio of 5-HIAA and 5-HT) in frontal cortex ($F(1,26) = 7.3$, $p < .05$). In HC animals CVS regime

decreased the serotonin turnover contrary to LC animals where stress increased serotonin turnover. At the level of tendency CVS and chirping activity together also had effect on tissue 5-HT level ($F(1,26) = 3.2$, $p < .10$) in frontal cortex. In HC group the CVS seemed to increase the amount of 5-HT contrary to LC group where CVS seemed to decrease the level of extracellular 5-HT. Also at the level of tendency CVS had effect on 3-MT level in frontal cortex ($F(1,18) = 3.5$, $p < .10$) toward the higher level of 3-MT in frontal cortexes of control group animals. It is worth noting that the statistical hypothesis testing in two previously described tendencies (5-HT levels and 3-MT levels in frontal cortex) is somewhat hindered by lack of sample size (n=19 for 3-MT and n=27 for 5-HT). The CVS and chirping activity alone nor together affected the monoamine neurotransmitters in amygdala.
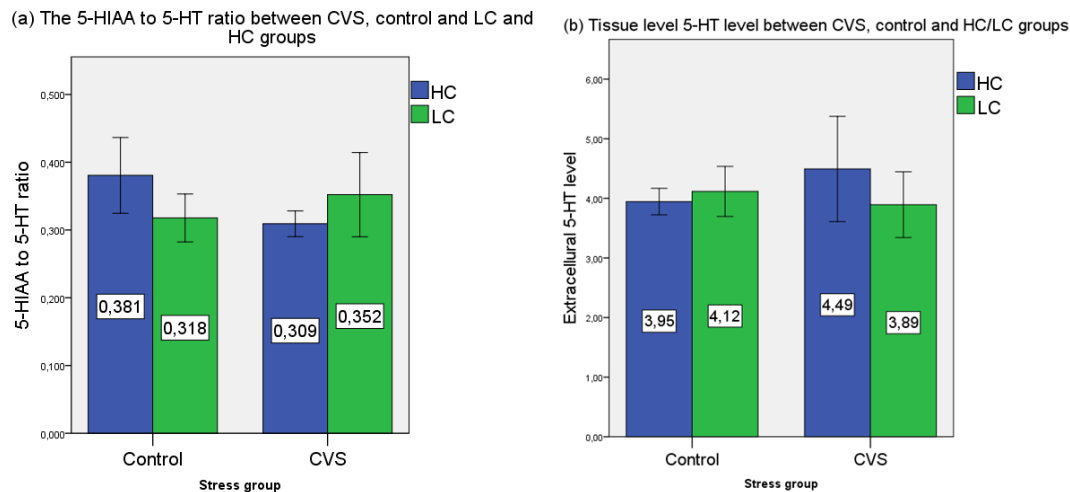

Figure 7.0. (a) 5-HT turnover in frontal cortex in HC and LC rats after CVS. (b) 5-HT levels in frontal cortex in HC and LC rats after CVS. (Note: whiskers denote the 95% confidence interval of measure).

Discussion

Body weight change and sucrose preference

CVS regime had statistically significant effect on the body weight of animals, decreasing the body weight increase over time. This result is on line with numerous previous experiments that show the effect of CVS on body weight (Willner et al., 1996) and indicate the effectiveness of the used CVS condition. Somewhat differently compared to some previous studies (Willner, 1997) we could not show the effect of CVS on sucrose preference test, which may hint that used CVS did not cause the behavior similar to anhedonia in our rat model of depression and thus can be seen as one of the limitations of current work. Still it is

important to consider the effects of tickling that was received by both the CVS and control group animals during 14 days. Previous work has shown that tickling by itself can increase the resilience of animals toward stress (Mällo et al., 2009) and change the behavior in hedonic situations (Mällo et al., 2007), which also could had effect on behavior of rats in sucrose preference test in this experiment.

Interestingly the baseline values in sucrose preferences measured 3 weeks after the tickling differed between HC and LC groups toward the higher sucrose preference in HC animals. The pre-CVS sucrose preference test 1 month later showed the equalizing of sucrose preference between HC and LC animals that was also seen in mid-CVS measurement and post-CVS-measurement. Previous work has shown that tickling decreases sucrose preference in HC animals if the sucrose preference test is closely followed by tickling (Mällo et al., 2007), which has been interpreted as showing lower motivation of HC animals in sucrose preference test. Above mentioned finding may be explained by the fact that tickling is more hedonic for HC animals than for LC animals and in closely following sucrose preference test the previously obtained hedonic stratification from tickling causes HC animals to have lower motivation toward the sweet food. During the current experiment the gap between the end of tickling and first sucrose preference test was 3 weeks that may have been long enough to cancel out the carry–over hedonic stratification effects from tickling to sucrose preference test. The possibility of explaining the apparent equalization of sucrose preference between HC and LC animals between post-tickling and pre-CVS measurements may relate to the effect of stress caused by the change from single housing to the group housing. Introduction of group housing causes the in-cage fighting between males to settle the power hierarchy, which is itself a stressor to animals. Additionally the effect of stress from in-cage fighting might have been emphasized in effect in HC animals that had previously shown higher appraisal to tickling stimuli that mimics the natural positively valenced interaction of rough-and-tumble play between rats. Additionally the sucrose preference did not differ in following mid-CVS and post-CVS tests adding to the interpretation that stress might had been the contributing factor eliminating the differences between HC and LC animals in sucrose preference test.

Stress induced hyperthermia

The effect of repeated stress regimes on body temperature has been shown previously experiments (Kõiv and Harro, 2010). The increased body temperature as a result of CVS regime observed in this experiment has been related to heightened activity of sympathetic

autonomic nervous system (Chrousos, 1997). Increased sympathetic autonomic nervous system activity is also seen in major depression (Veith et al., 1994) and the stimulation of vagus nerve over a longer period has been shown to have antidepressive effects in humans (Schlaepfer, 2008). Based on this we could show the effect of used CVS stress regime on the sympathetic autonomic nervous system activity of rats, which confirms the effectiveness and validity of used CVS condition. At the same time we could not show the effect of chirping activity or any interactive effects between CVS and chirping activity on any of the body temperature measurements.

In detail we could not find any difference in body temperatures at T0 post-CVS between CVS and control group animals. However the body temperatures differed between CVS and control group at T15 post-CVS regime. The body temperature measurement procedure is itself an acute stressor to animals. So the emergence of post-CVS body temperature differences between CVS and control group at T15 but not at T0 may indicate impaired adaptive responses in CVS group animals in acute stress situation like the procedure of body temperature measurement by experimenters.

This hypothesis is also supported by comparison of body temperature change between post-CVS and pre-CVS. In both measurements (T0 and T15) the control group showed bigger decrease in body temperature compared to CVS group, when corresponding post-CVS and pre-CVS measurements were compared. Further, higher post-CVS body temperature was observed at T0 compared to corresponding pre-CVS value. The post-CVS body temperature measurement elicited at least as strong body temperature change as the first pre-CVS measurement in the stressed animals, which suggests the loss of adaption toward repeatedly presented acute stressor after exposure to chronic stress. In contrast in control group the post-CVS body temperature measurement procedure elicited much milder response compared to pre-CVS body temperature measurement, which reveals adaption toward repeatedly presented acute stressor in this group. Those results support the impairing effect of CVS on adaption of repeatedly presented acute stressor such as the SIH procedure.

Social preference test

The behavior in social preference test was differed between LC and HC animals, while CVS by itself did not change the behavior of animals in this test. We found the effect of chirping on the time spent in socialization quarter without stimulus, where HC rats spent more time than LC group counterparts. Similarly the HC animals made more entrances into

the socialization area without social stimulus and at the level of tendency had more entrances into the corners without cages and spent more time in closer socialization area without social stimulus. In general terms, HC animals displayed higher activity toward the unanimated objects in social preference test. This is also supported by the fact that at the level of tendency the LC had higher total movement activity during the social preference test, which implies that the differences in activity between HC and LC rats toward the unanimated objects cannot be accounted on the chance resulting from higher total movement activity of HC animals. Mällo and colleagues (2007b) have found that HC rats display higher activity in exploration test compared to LC animals. In addition both the chirping activity (Burgdorf et al., 2011) and stable trait of explorative activity (Mällo et al., 2007b) are related to the functioning of dopaminergic system. Therefore a possible explanation for higher activity of HC animals toward unanimated objects may be related to the higher level of exploration in HC animals.

At the same time the LC animals displayed higher activity toward the areas related to social stimulus by spending longer time in socialization area and closer socialization area near the cage with another rat. However an interesting disparity emerged, when we compared the percentage of time spent in active socialization out of total time spent in closer socialization area between HC and LC rats. While LC rats stayed in this area longer than the HC animals, the HC rats engaged larger percentage of time in active socialization with other rat. Therefore we propose that at least in part the higher score of LC animals in time spent in the quarter with social stimulus and time spent in closer socialization area with social stimulus is rather than being related to social stimulus the result of higher overall movement activity of LC animals.

Open field test

The level of activity toward the center of open field has been described as showing the level of anxiety in rodents, where higher activity toward the center of open field suggests lesser level of anxiety. The higher number of entries into the center of open field by HC animals in control group is in accordance with previous findings that show higher number of center entries in animals bred for higher level of 50 kHz USVs (Burgdorf et al., 2009). The foregoing results have been interpreted as showing less anxious and more stress resilient phenotype for HC rats. Contrary to last interpretation we found CVS to have higher impact for HC animals as compared to LC animals. We showed that the chirping had interactive effect with the CVS regime in affecting the number of entrances into the center part of open field. In LC animals the stress increased the number of entries into the center of open field,

while in HC animals it decreased the corresponding value, which resulted in equalization of open field center entries between HC and LC animals in CVS group. Thus the lower initial anxiety of HC animals seems to be reversed by CVS in current experiment, which does not support the notion of higher stress resilience of HC animals. Nevertheless, open field test is a notoriously unreliable method to measure anxiety unless pharmacologically validated, and conditions in this particular setting (relatively small area and extensive previous handling) prevent any firm conclusions in term of anxiety. The higher activity of rodents toward the corners of open field has been interpreted as showing higher levels of anxiety. We found that the CVS increased the level of anxiety related behaviors by increasing the time spent in corners of open field and also decreasing the time for first entry into one of the corners.

Levels of monoamine neurotransmitters

Serotonin turnover in frontal cortex (FC) was interactively affected by CVS and chirping activity. CVS decreased the 5-HT turnover (ratio of 5-HIAA to 5-HT) in FC in HC rats, while 5-HT turnover was increased by CVS in LC rats. We also found the tendency for CVS to increase the 5-HT at tissue level in HC animals, while it was decreased in LC animals as a result of CVS. Further we could not show the sole effect of CVS on the 5-HT turnover or the level of 5-HT in FC, which is in accord of previous findings by Berkis et al., 2005 and Haidkind et al., 2003. At the same time current results are contrary to findings of Li et al., 2003, Yi et al., 2008, Xu et al., 2008 and Li et al., 2009, who have shown the effect of CVS to be the decrease of 5-HT levels in FC. The interactive effects of CVS and chirping activity on the serotonergic activity in FC seen in this study may help to explain the contrary results seen in previous works in respect to serotonergic alterations caused by CVS condition. The possible confounding effect of positive emotionality measured by chirping activity was not controlled in any of the above-mentioned works, which may have caused the confounding effects, which in turn may help to explain conflicting results seen in previous studies.

Postmortem studies in humans have shown that cerebrospinal fluid (CSF) 5-HIAA level is correlated with 5-HIAA level in prefrontal cortex in humans, which may suggest that CSF 5-HIAA levels are reasonable index of prefrontal cortex 5-HT turnover. This is relevant because some, but not all, of the previous work has shown that major depression is associated with reduced 5-HIAA level in CSF (Mann, 1999). Hou et al., 2006 have shown that drug free patients of major depression do not show alterations of 5-HT or 5-HIAA levels in CSF, but in the group of severe major depression the females had higher 5-HT turnover compared to males, which might be associated with higher risk of major depression seen in women at

population level. Based on the prior explanation the increased 5-HT turnover seen in LC rats as a response to CVS regime may hint at the lower stress resilience of those animals.

Limitations of current work and considerations for future studies

One of the main limitations of current work is the large variability of results between individual animals in behavioral and biochemical measurements. It was often seen that removing one or two possible outlier (data points greater than 1.5 times of upper quartile or data points less than 1.5 times of lower quartile) observations was enough to change the results seen in statistical analysis. By using the behavioral variables to predict HC or LC and CVS or control group membership of animal it was possible to construct logistic models that had hit ratio of 70% for HC/LC group membership and hit ratio of 82.5% for CVS and control group membership (detailed data not presented in current thesis). In both cases the percentage of correct classifications was significantly greater compared to naive hit ratio of 50% that would had been expected from balanced experimental design as used in the thesis. Those results support the validity of experimental design used by showing that both the chirping activity and CVS had systematic (nonrandom) effect on the behavior of animals across different behavioral experiments. As a side note an interesting pattern emerged by which most of the misclassifications of logistic models happened in animals that had shown "outlier like" behavior in some of the behavioral tests, which may hint that the behavior of outlier animals is somehow systematically different compared to rest of the rodents, which should be considered in future studies.

Conclusion

The used CVS regime was effective by decreasing the weight gain of rodents and by increasing the SIH response. CVS decreased 5-HT turnover in frontal cortex of HC animals, while increasing it in LC animals, which may suggest lower stress resilience of LC animals at serotonergic level. In open field test the HC animals displayed less anxious phenotype than LC animal at basal level but this difference was eliminated by CVS showing that chronic stress may had greater impact on HC animals compared to LC animals at behavioral level. In social preference test the HC differed from LC animals by having higher activity toward unanimated objects while having tendency toward lower overall locomotive activity. Above-mentioned results may be related to higher explorative activity of HC animals. HC animals also spent larger proportion of time in active socialization out of total time spent near other, which suggest higher activity of HC animals toward social stimulus. HC animals had higher sucrose preference 3 weeks after tickling, while this difference was not seen in following 3 tests. The equalization of sucrose preference after first test between HC and LC animals may be caused by stress of group housing and CVS condition.

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