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FLUOXETINE MODERATES AMPHETAMINE RESPONSE IN CHRONICALLY STRESSED RATS WITH HIGH AND LOW LEVELS OF POSITIVE AFFECTIVITY

Master's thesis

Running head: Effects of fluoxetine in chronically stressed rats with repeated exposure to amphetamine

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Fluoxetine moderates amphetamine response in chronically stressed rats with high and low levels of positive affectivity

ABSTRACT

High level of positive affectivity serves as a protective factor against adverse effects of stress and low positive affectivity increases vulnerability to mental disorders, i.e. mood disorders and drug abuse. In animal models, rat 50-kHz ultrasonic vocalizations index the level of positive affect, whereas there are stable, trait-like inter-individual differences in terms of vocalization activity. Previously, we have demonstrated that experience of chronic stress can suppress amphetamine-induced 50-kHz vocalizations, but only in animals with low levels of vocalization activity. In the present study it was tested, whether the chronic stress effect on USV activity is preventable with fluoxetine treatment. Male Wistar high (n=32) and low (n=30) 50-kHz vocalizing rats were subjected to 43-day chronic variable stress (CVS) regimen. On day 17 of the CVS, fluoxetine treatment was started, followed up by the 19-day amphetamine test with USV and locomotor data recording on the 1st, 10th and 19th day. Chronically stressed rats developed cross-sensitization between previous CVS regimen and repeated administration of amphetamine in both groups of HC, and also in fluoxetine-pretreated LC rats. Fluoxetine had a different effect in chronically stressed rats with high and low trait of positive affectivity. Fluoxetine pretreatment increased the frequency-modulated and trill calls in proportions in repeatedly amphetamine treated LC rats, but not in any other group. These findings suggest that fluoxetine treatment modulates the effect of chronic stress on the rewarding effects of amphetamine depending on inter-individual differences in positive affectivity.

Keywords: positive affectivity, 50-kHz vocalizations, chronic stress, amphetamine, behavioural sensitization

Fluoksetiin modereerib amfetamiini vastust kõrge ja madala positiivse afektiivsusega kroonilises stressis rottidel

KOKKUVÕTE

Madalat positiivset afektiivsust käsitletakse kui haavatavat faktorit patoloogiate, nagu meeleolu- ja sõltuvushäirete välja kujunemisel. Loomkatsemudelites mõõdetakse positiivset afektiivsust rottide 50-kHz ultrahelihäälitsustega ehk kudinatega. Kudisemisaktiivsuses esinevad seadumuslikud erinevused, mille alusel saab rotte jagada palju- (HC) ja vähekudisejateks (LC). Varasemalt oleme näidanud, et krooniline stress vähendab amfetamiini poolt esile kutsutud kudisemisaktiivsust vähem kudisevatel rottidel. Käesoleva väitekirja eesmärgiks on uurida, kas kroonilise stressi efekt kudisemisaktiivsusele on ennetatav fluoksetiiniga. Isased Wistar liini palju- ja vähekudisevad rotid läbisid 43-päevase kestusega kroonilise muutliku stressirežiimi (CVS), mille 17. päeval alustati fluoksetiini manustamist. Pärast stressirežiimi manustati pooltele HC ja LC rottidele amfetamiini (1 mg/kg; IP), koos kudisemis- ja liikumisaktiivsuse andmete salvestamisega 1., 10. ja 19. päeval. Kroonilises stressis rottidel kujunes amfetamiini korduvmanustamisel välja ristuv sensitisatsioon mõlemas HC grupis, ühtlasi ka fluoksetiini kuuri läbinud LC rottidel. Fluoksetiin moduleeris kroonilise stressi mõju amfetamiini poolt esile kutsutud häälitsustele HC ja LC gruppides erinevalt. Fluoksetiinikuuri läbinud kroonilises stressis rottidel kasvas amfetamiini korduvmanustamisel eksklusiivselt sagedusmuutlike ja trillielementidega häälitsuste protsentuaalne osakaal. Eelnevalt kirjeldatud tulemused näitavad, et fluoksetiin moduleerib kroonilise stressi mõju amfetamiini korduvamanustamisel kogetavatele tasustavatele efektidele HC ja LC rottidel erinevalt.

Märksõnad: positiivne afektiivsus, 50-kHz ultrahelihäälitsused, krooniline stress, amfetamiin, käitumuslik sensitisatsioon

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INTRODUCTION

Individual variability in vulnerability to stress related psychiatric disorders is usually understood in framework of diathesis-stress model, which sees vulnerability (the diathesis) and precipitation (the stress) as separable components in the process leading to pathological condition (Willner, Scheel-Krüger, and Belzung 2013). Vulnerability to stress is determined by diverse set of interacting factors of genetic, behavioural and environmental nature (Armario & Nadal 2013). The diathesis-stress concept has often been applied to depression studies, in which several risk factors for developing the pathology have been identified.

Large body of research has described neuroticism as one of the key risk factor for several psychopathologies, i.e. in affective disorders (Roelofs, Huibers, Peeters, Arntz 2008), and in schizophrenia (Blanchard, Mueser, Bellack 1998). Past research has however somewhat less emphasized the role of protective factors, which can convey resilience to stress and prevent the development of pathologies. Some recent findings have suggested that high level of positive affectivity serves as a protective factor (Werner-Seidler, Banks, Dunn & Moulds 2013; Nutt, Demyttenaere, Janka, Aarre, Bourin *et al* 2007), whereas low positive affectivity increases vulnerability (Mällo, Matrov, Kõiv, and Harro 2009; Clark & Watson 1991; Horan & Blanchard 2003).

Individual differences in rat 50-kHz ultrasonic vocalizations as an indicator of positive affectivity

Regarding behavioural readouts in potential animal models of resilience, 50-kHz ultrasonic vocalizations (USV-s; *often referred as* "chirps") most reliably index the level of positive affect in the rat (Knutson, Burgdorf & Panksepp 2002). Fifty-kHz vocalizations are naturally emitted in response to various positive stimuli; for example food (Brenes & Schwarting 2015), mating (Knutson *et al* 2002), natural or imitated rough-and-tumble play (Mällo *et al* 2007; Knutson, Burgdorf & Panksepp 1998), and in response to pharmacological activation of mesolimbic brain reward circuitry (Burgdorf, Knutson, Panksepp 2001; Browning, Browning, Maxwell, Dong, Jansen 2011; Simola & Morelli 2015). The presence of trait-like inter-individual differences in 50-kHz vocalization activity has been proposed on the grounds that rats can be selectively bred for high or low levels of USV activity (Burgdorf, Panksepp, Brudzynski, Kroes, Moskal 2005; Webber *et al* 2012). Such stable differences are also persistently expressed in response to experimentally imitated rough-and-tumble play (Mällo *et al* 2007) and in response to drug treatment (Taracha, Hamed, Krzaścik, Lehner, Skórzewska

2012). Furthermore, the rats with lower 50-kHz vocalization activity show elevated levels of anxiety in several tests (Burgdorf, Panksepp, Moskal 2011). Thus, low positive affectivity phenotype in rats appears to have potential in obtaining new information about development of psychopathologies.

It is now well-established that USV-s serve as social signals for rodents in emotionally valenced conditions. Vocalizations with the 50-kHz frequency component vary in their characteristics and are generally divided into two broader categories. Firstly, the ones being in near constant frequency, named flat calls, and secondly the calls, which include frequency modulations, often referred as "FM calls" (Brudzynski 2015). Both subtypes of USV-s have been associated with positive affective states; however, there is a difference in the context these are emitted. Flat calls have been proposed to have a social-coordinating function, for example in a situation of aggressive encounter between two male rodents (Wöhr, Houx, Schwarting, and Spruijt 2008). Calls with frequency modulation have been described in studies with rewards and psychostimulant-induced euphoria (Mahler *et al* 2013; Ahrens, Ma, Maier, Duvauchelle, and Schallert 2009). Moreover calls with frequency modulation can be divided by their acoustic parameters into 13 different categories (detailed description of the subtypes can be found in Wright, Gourdon, and Clarke 2010).

According to Wright *et al* (2010), the proportions of the calls seem to be affected by social context. The authors measured USV activity among rats in pairs and singly, resulting in significantly different call profiles between experimental conditions. Pair-tested rats had significantly higher proportion of trill calls and lower proportion of flat calls compared to their respective singly-tested group. These differences remained even when rats were administered amphetamine. Unfortunately no further work to my knowledge has done in studying the 50-kHz vocalization response, while considering proportions of different call subtypes. Larger body of research with USV-s focuses in measuring number of calls emitted, and testing the rats singly, not in pair, although the importance of the social context should be taken into account, especially in research on flat and frequency-modulated calls with trill elements.

The frequency-modulated calls with trill elements, or simply "trill calls", have gained more focus in pharmacological studies (Ahrens *et al* 2009; Simola & Morelli 2015). The widely acknowledged hypothesis suggests that trill calls have an association with psychostimulant-induced hedonic and euphoric effects. Inquiries about their neural substrates have established an association between trill calls and midbrain dopamine pathways, often referred as brain

reward system. Wright and colleagues (2013) have reported that blocking dopamine D_1 and D_2 receptors attenuates the number of trill calls elicited by rats, with the effect being strongest when both D_1 and D_2 receptors are blocked simultaneously. Furthermore, Ringel *et al* (2013) have shown that simultaneous blockage of D_1 and D_2 receptors leads to reduction in mean complexity and bandwidth in 50-kHz ultrasonic vocalizations, while also reducing the overall number of USV-s with frequency modulation component. Thus, the proportions of different subtypes of calls should be responsive to manipulation of midbrain dopamine levels, including amphetamine administration.

Studies with drug sensitization and repeated exposure to amphetamine

Previous work combining measurement of rodents' ultrasonic vocalizations has mostly been carried out in context of studying biological basis of drug addiction. In those studies the sensitization effect has been emphasized (Cador, Bjijou, Cailhol, Stinus 1999). Sensitization describes an effect, where repeated exposure to a certain stimulus enhances behavioural and physiological response to the same stimulus at subsequent time points (Steketee & Kalivas 2011). For example, if rats are repeatedly administered psychoactive drugs, it would result in significantly increased number of ultrasonic vocalization activity in contrast with the respective control group. Several studies have shown that repeatedly administering low doses of amphetamine induces behavioural sensitization in rodents, resulting in hyperlocomotion and increased numbers of USV-s emitted (Mu *et al* 2009; Vanderschuren *et al* 2001).

Previous research has revealed that some subtypes of USV-s are more susceptible to sensitization than others. Repeated amphetamine treatment seems to preferentially lead to sensitization of USV-s with frequency modulation component (Ahrens *et al* 2009; Wright *et al* 2013). Ahrens *et al* (2009) also showed concurrency of sensitization of calls with frequency modulation and locomotor activity. Also other dopaminergic drugs, such as cocaine seem to be capable of inducing sensitization of FM calls if administrated repeatedly (Maier, Abdalla, Ahrens, Schallert, and Duvauchelle 2012). It is important to note that at least one previous experiment hints at the possibility of presence of intra-individual differences in sensitization of 50-kHz USV-s in response to repeated amphetamine treatment. Namely, Taracha and colleagues (2012) found that sensitization of USV-s could only be described in some animals, whereas others showed no significant elevation in vocalization activity in response to repeated treatment with amphetamine. Thus, it is possible that the effect of repeated amphetamine treatment could be different in rats with high *vs.* low positive affectivity, if one rat phenotype would be more susceptible to sensitization than the other.

Previous work on chronic stress and antidepressants

Various chronic stress models have been developed to study the stress response and human depression in animals. Katz and colleagues (1982) introduced the chronic variable stress regimen, that later was further developed to chronic mild stress by Willner and colleagues (1987). There are methodological differences between these models, but the central idea is the same; animals are intermittedly presented with variety of uncontrollable stressors, which should result in subject's depression-like state, indicated by slower weight gain, decreased sugar preference and higher body temperature (Harro, Tõnissaar, Eller, Kask, Oreland 2001; Mällo *et al* 2009). These symptoms can be reversed with antidepressant treatment (Tõnissaar *et al* 2008).

Previous work has shown that rats show inhibited levels of USV activity in response to chronic variable stress regimen, while the effect is larger among male animals with lower levels of baseline vocalizing (Mällo *et al* 2009). Similar results were also described in Raudkivi *et al* (2012) study, and also found with the restraint stress model (Popik, Potasiewicz, Pluta, Zieniewicz 2012). Thus, animals with lower levels of USV activity seem to be more susceptible to stress regimen. Furthermore, in our most recent study we replicated that effect and found out that chronic stress can suppress amphetamine-induced fifty-kHz vocalizations, but that effect could only be described among animals with low levels of vocalization acitvity (Kõiv *et al* 2016). The attenuated number of calls was seen in all subtypes of calls we differentiated, that being trills and frequency-modulated calls, as well as flat and short calls. These results suggest, that chronic stress causes blunted hedonic response among rats with low levels of vocalizing.

Since the chronic stress effect can be reversed with antidepressants, it is possible that stress induced reduction of USV-s in repeated amphetamine treatment setting among rats with low level of positive affectivity is reversable by antidepressant treatment. To my knowledge, no previous work has been published, which studies the effect of antidepressant treatment on 50-kHz ultrasonic vocalizations in chronically stressed rats. In fact, there is only one study, published by Boulay and colleagues (2013), which describes the fluoxetine to have no effect on rat USV-s. The present experiment further studies the effect of fluoxetine on rat 50-kHz ultrasonic vocalizations, providing some new insights on one of the most used and well-known SSRI's effect on behaviour and emotion regulation in chronic stress setting.

Aims of this study

The aim of this thesis is to test the effect of fluoxetine on amphetamine-induced rat 50-kHz ultrasonic vocalizations in chronically stressed animals with high *vs.* low trait of positive affectivity. The present study addresses the following research questions:

- Does the antidepressant treatment affect the chronic stress effect on USV response to repeated amphetamine treatment?
- How the latter is reflected in different subtypes of USV-s?
- How does the USV response differentiate in chronically stressed rats with inter-individual differences in positive affectivity?

This study is part of a larger experiment and not all data will be reported here. The author of this thesis contributed to all assignments and parts of the experiment, mostly during chronic stress regimen, fluoxetine and amphetamine treatment and behavioural tests. Nevertheless this experiment was managed in full cooperation of all contributors and could not have been successful otherwise.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rat pups (n=62) were weaned at the age of three weeks. Parents were provided by Harlan Laboratories (the Netherlands) and the animals were bred on location. Juvenile rats were single-housed in standard transparent polypropylene cages with wood-chip bedding in a temperature controlled room (20–22°C) under 12:12 light/dark cycle, light-cycle starting at 08:00 hours. Rats had *ad libitum* access to tap water and food (diet R70, Lactamin AB, Sweden), except during testing. After the tickling sessions, animals were group-housed by four, each cage consisting of equivalently HC and LC animals. All behavioural experiments were performed during light-period. The experiments were in accordance with EU legislation (directive 2010/63/EU) and the experimental protocol was approved by the Animal Experimentation Committee at the Estonian Ministry of Agriculture.

General procedure

Table 1. Timetable of the experiment.

Procedure	Age of animals	Notes
Weaning from mother	Week 3	
Tickling by experimenter	Weeks 4-6 (period of 14 days)	
Intermediate group housing	Weeks 7-12	Time period for USV counting
Re-grouping the housing of animals	Week 13	
Pre-CVS behavioural testing	Week 14	Stress-induced hyperthermia; sucrose preference
Pre-fluoxetine CVS regimen	Weeks 14-17 (period of 16 days)	
Fluoxetine w/ CVS regimen	Week 17-20 (period of 27 days)	Fluoxetine at dose of 10 mg/kg
Behavioural testing	Week 21 (period of 5 days)	In following order: stress-induced hyperthermia; 0-maze; T-maze; re-tickling and NSF
Habituation with amphetamine experiment cage	End of week 21 (period of 4 days)	USV recording on day 2 of habituation (data not reported).
Amphetamine treatment	Weeks 22-23 (period of 10 days)	Amphetamine treatment at dose of 1 mg/kg for half of the animals. **USV and locomotor activity recording on 1st and 10th day.**
Sensitization testing/Amphetamine challenge	Week 24 (9 days after last chronic amphetamine dose)	19th day of amphetamine test. Amphetamine treatment at dose of 1 mg/kg for all animals. **USV and locomotor activity recording.**
Sacrifice of animals	Week 30 (5 days after challenge)	

Tickling sessions started one day after single-housing. Sessions lasted for 14 days, including one 2-min tickling session per day. Juvenile rats were divided between HC and LC groups by the median split of total chirping activity across three measurement days (days 12-14 of tickling procedure). At the age of four months, 32 animals were assigned to chronic variable stress regimen (CVS) with duration of 43 days, consisting of one short-lasting stressor during daytime and a whole night lasting stressor in the dark phase. Subsequently half of the CVS rats (n=16) were administered with fluoxetine for 27 days. Antidepressant treatment started on the 17th day of the CVS regimen and ended with CVS regimen (day 43 of CVS regimen). Immediately after CVS, behavioural testing was carried out during the next 5 days (data not reported).

The day after behavioural testing, animals were habituated with the experiment cage for four days for the amphetamine treatment procedure, which was carried out on the following 10 days. Nine days after the end of repeated amphetamine treatment all animals received a 1 mg/kg injection of amphetamine. Ultrasonic vocalizations and locomotor activity were recorded on day 1, 10 and 19. The rats were sacrificed by decapitation five days after amphetamine challenge.

Tickling

Tickling of juvenile animals carried out by an experimenter, imitates natural rough-and-tumble play, a characteristic behaviour in young rats. The animal was given 15 s to habituate with the new cage (30×15×13 cm), followed by 15 s of tickling by experimenter. The tickling sessions consisted of four manual stimulation sessions during 2 min. 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 s of stimulation. (Mällo *et al* 2007; Burgdorf and Panksepp 2001; Panksepp and Burgdorf 2000)

In the 2-min sessions chirping was recorded with an ultrasound microphone (Avisoft Ultra Sound Gate 116-200, Avisoft Bioacoustics, Berlin, Germany), located 20 cm from the cage floor. USV-s were recorded with a sampling rate of 300 kHz in 16 bit format and later manually scored with the Avisoft SASLab Pro (Avisoft Bioacoustics, Berlin, Germany) software by creating spectrograms using fast Fourier transformation (1024 FFT length, 75% frame, Hamming window, and 75% time window overlap). The average number of 50-kHz calls during the tickling periods in tickling days 12-14 was 227±3 in HC group and 135±8 in LC rats.

Recording apparatus and the amphetamine administration

The experiment was carried out in the colony room, with the experiment cage (floor - 1815 cm^2) isolated from the home cages with a screen in the most far corner of the room. The experiment cage was identical to their home cage with wood-chip bedding and wire-mesh lid. The ultrasound microphone was located approximately 30 cm above the cage floor.

Rats were weighed every day before amphetamine administration. After four days of habituation with the experiment cage, half of the animals (n=31) were administered amphetamine 1 mg/kg, while the other half (n=31) were administered with equivalent amount of saline IP. Subjects were instantaneously placed in the recording cage for 15 min, and the ultrasonic vocalizations with locomotor activity data were collected on day 1, 10 and 19 (otherwise the procedure was identical except data was not recorded). On the day 19, all animals were administered a single dose of amphetamine IP (1 mg/kg).

Analysis of USVs and locomotor activity

Seven time-bins were chosen out of the 15-min recorded data, based on their correlation to overall chirping scores during 15 min. Hence the minutes 1; 3; 5; 7; 9; 11 and 13 from audio recordings were scored. The vocalizations were manually scored from spectrogram by experimenter blind to experimental conditions. Categorization was made according to the method of Wright et al. (2010), and further grouped together into four categories: 1) trill calls (which included trill, flat-trill combination, trill with jumps); 2) frequency-modulated calls (consisting of complex, upward ramp, downward ramp, split, step up, step down, multi-step, inverted U, composite type of calls); 3) flat calls; 4) short calls.

Locomotor activity was measured by counting rearings (rat on two hind paws, at least at an angle of 45-degrees) and line crossings (all four paws were over the line), scored from 15-min digital video recordings, and scored in three 5-min bins. In order to estimate the line crossings, experimental cage was divided into six equal-sized squares.

Chronic variable stress

The chronic variable stress regimen lasted for 6 weeks, consisting of various stressors interchangeably used during the week. The CVS regimen included one short stressor during daytime and one long lasting stressor during nighttime. Control rats were left undisturbed in the colony room; all the stressors were presented in separate rooms. The short stressors included: 1) rats were exposed to cold temperature for 1 h; 2) placing the animal under strong illumination (900 lx) on a round 10 cm diameter platform, which was located 75 cm above ground for 30 min; 3) tail-pinch with a clothespin near base of the tail for 5 min; 4) restricted mobility in an tight space (25x9 cm) lasting 2 h; 5) grabbing the rat with a thick glove, immobilizing it for 5 min. Night-time long stressors were: 1) stroboscopic light (10-50 Hz; 12 h); 2) lights on during night-time, lasting 12 h; 3) tilted cage at 45-degree angle for 12 h, 4) loud white noise in 10-minute time-bins, followed by 1 h of silence in 6 repeated cycles; 5) removing the home cage bedding and replacing it with 1 cm cold water for 12 h.

Measurement of weight gain

Weight gain was used as an indicator to test the effectiveness of chronic variable stress regimen. It was calculated for 5 successive time points by subtracting the pre-CVS bodyweight from bodyweight of respective measurement. The first measurement corresponds to the 16-day period of CVS before starting fluoxetine administration. Measurements 2-4 correspond to simultaneous CVS and fluoxetine administration (in total 27 days). Data used

for analysis were collected on the 16th (Measurement 1), 23rd (Measurement 2); 30th (Measurement 3), 37th (Measurement 4), 43rd (Measurement 5) day of CVS regimen.

Drugs

Fluoxetine hydrochloride (Tokyo Chemical Industries, Japan) was administered in sterile saline solution 10 mg/kg IP after daily CVS procedures in home cage. Fluoxetine treatment began on the first day of week 3 of CVS regimen and lasted for 27 days (ended simultaneously with CVS regimen).

***D*-Amphetamine** sulphate (Tocris Bioscience, Bristol, UK) was dissolved in sterile 0.9% saline and administered in the dose of 1 mg/kg (as for salt) in a volume of 1 ml/kg IP before placing the animal to the experiment cage.

Statistical analysis

Statview (SAS Institute Inc., USA) and Statistica (StatSoft Inc., Tulsa, USA) were used for factorial and repeated-measures analysis of variance, with independent factors being *HC vs LC, Stress* (*vs* control), *Fluoxetine* (*vs* vehicle), *Amphetamine* (*vs* saline) and the dependent factors being total amount of USV-s per recording and separately all the subcategories (trill, flat, frequency-modulated and short calls) of USV-s. Locomotor activity on day 19 was analyzed with four-factor ANOVA with the same categorical variables as for analysis of USV-s. Two-tailed Pearson correlation coefficient was calculated in order to describe the associations between USV and locomotor activity. Recording day was used as a repeated measures factor where appropriate. Fisher's PLSD was used for post-hoc testing.

RESULTS

Weight gain

The measure of weight gain was used to assess the effectiveness of CVS regimen and fluoxetine treatment. Weight gain was calculated for 5 successive time points by subtracting the pre-CVS bodyweight from bodyweight of respective measurement. Figure 1 depicts the cumulative weight across 5 measurements between groups of stressed, fluoxetine-treated, and their respective control animals. In detail, animals gained weight over the 43 day period [$F(4,216)=45.97$; $p<.0001$], whereas CVS and fluoxetine decreased the weight gain [*Measurement* × *Stress* - $F(4,216)=24.82$; $p<.0001$ and *Measurement* × *Fluoxetine* – $F(4,216)=130.28$; $p<.0001$, respectively]. In terms of HC/LC affiliation, there were no significant differences, although the slowest weight gain in both groups resulted from interaction of CVS and fluoxetine administration [*Measurement* × *Stress* × *Fluoxetine* –

F(4,216)=2,59; p<.05]. Repeated measures ANOVA displayed within measurement main effects with stress [F(1,54)=61.41; p<.0001] and fluoxetine [F(1,54)=78.77; p<.0001].

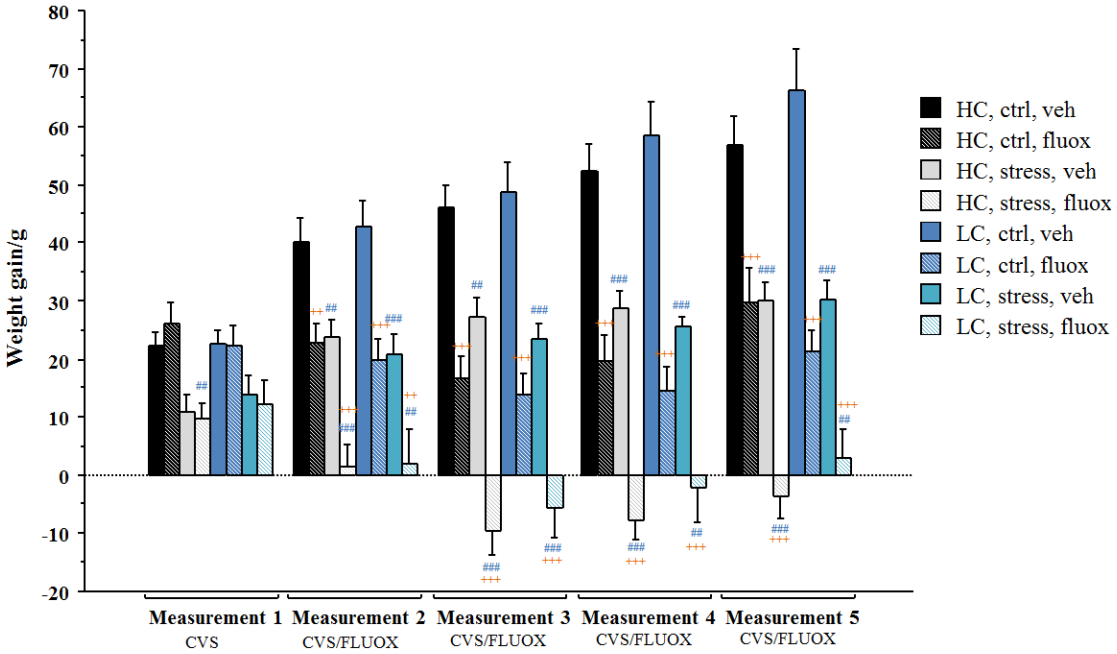

Figure 1. Weight gain during chronic variable stress regimen and fluoxetine administration. Data reported as mean scores + SEM. #, ##, ### - p<.05, .01 vs control, respectively; +, ++, +++ - p<.05, .01, .001 vs vehicle, respectively. Measurements 2-4 correspond to simultaneous CVS and fluoxetine administration (in total 27 days). Data used for analysis were collected on the 16th (Measurement 1), 23rd (Measurement 2); 30th (Measurement 3), 37th (Measurement 4), 43rd (Measurement 5) day of CVS regimen.

Overall number of ultrasonic vocalizations during amphetamine administration

Four-factor repeated measures ANOVA showed that the overall number of 50-kHz ultrasonic vocalizations was significantly different on testing days 1, 10 and 19 [F(2,92)=117.3; p<.0001], depending on CVS regimen and amphetamine treatment [*Day* × *Stress* - F(2,92)=3.6; p<.05 and *Day* × *Amphetamine* - F(2,92)=3.8; p<.05, respectively]. Moreover these variables presented an interaction effect [*Day* × *Stress* × *Amphetamine* - F(2,92)=5.5; p<.005], which tended to have differential effect on vocalization activity across testing days. As seen on Figure 2, the amphetamine treatment increased vocalization activity on day 1, whereas CVS regimen had no significant effect on number of USV-s. However on the 10th and 19th day, stress potentiated the effect of repeated amphetamine on overall number of USV-s. Across three testing times ANOVA revealed main effects on HC/LC affiliation [F(1,46)=8.7; p<.01] and amphetamine treatment [F(1,46)=17.5; p<.0001]. Generally HC groups emitted higher number of vocalizations in response to amphetamine than LC rats

[*HC/LC* × *Amphetamine* - F(1,46)=4.9; p<.05], and chronic stress potentiated the amphetamine response [*Stress* × *Amphetamine* - F(1,46)=8.1; p<.01].

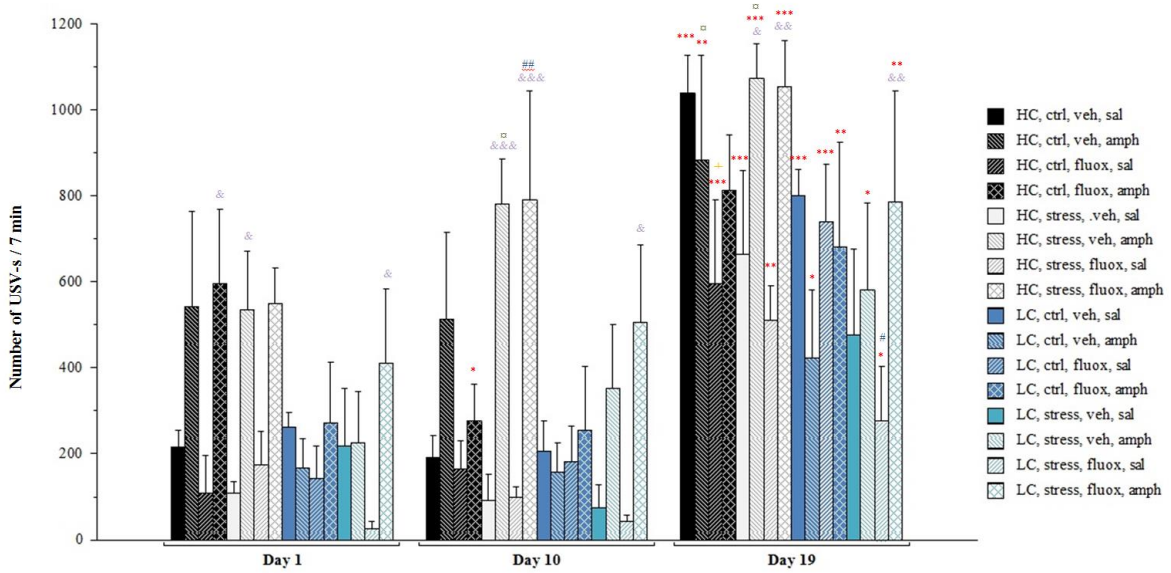

Figure 2. Mean scores of 50-kHz ultrasonic vocalizations scored from 7 selected time-bins on day 1, 10 and 19 (data expressed as mean + SEM). On day 19 all animals (n=62) were injected with amphetamine (1 mg/kg, IP) to examine the sensitization effect *, **, *** - p<.05, .01, .001 vs day 1, respectively; #, ##, ### - p<.05, .01 stress vs control, respectively; +, ++, +++ - p<.05, .01, .001 vs vehicle, respectively; &, &&, &&& - p<.05, .01, .001 vs saline, respectively; ¤, ¤¤, ¤¤¤ - p<.05, .01, .001 vs LC, respectively.

On day 1, amphetamine increased USV numbers among all HC animals, although this effect was significant only among non-stressed fluoxetine-treated HC rats and stressed vehicle-treated HC group. Interestingly, there were no differences between LC rats' vocalization activity, except among stressed animals, which had been treated with fluoxetine. There was a significant amphetamine effect in non-stressed fluoxetine HC animals on day 1 (p<.05), but not on day 10 and 19. In fact, there seemed to be reduction in number of USV-s on day 10, compared with testing day 1 (p<.05), with significantly smaller number of USV-s emitted than their respective stress group (p<.01). On day 10, amphetamine had a USV-increasing effect among HC-stress groups (p<.001), and in fluoxetine-treated LC-stress group (p<.05).

On day 19, a single dose of amphetamine elicited a significant increase in number of USV-s in comparison to day 1 across all groups (p<.05). Exceptions were non-stressed fluoxetine HC animals, which had been previously repeatedly treated with amphetamine, and LC stressed fluoxetine group, which were previously saline-treated. CVS regimen had an USV-inhibiting effect in LC-stress fluoxetine group (p<.05), despite the significant increase in USV numbers due to a single dose of amphetamine (p<.05).

Repeated treatment with amphetamine increased 50-kHz vocalization response among stressed HC animals (p<.05) and in fluoxetine-treated LC-stress group (p<.05). On day 19

these groups emitted significantly higher number of ultrasonic vocalizations, in contrast to their scores on day 1 and their respective saline-treated groups. Thus, emergence of sensitization effect can be concluded in aforementioned groups.

Trill calls during amphetamine administration

Number of trill calls differed across testing days [$F(2,92)=39,7$; $p<.0001$], with only significant main effect being amphetamine treatment [$F(1,46)=4.8$; $p<.05$]. Interestingly, a single dose of amphetamine increased the number of trill calls on day 19 across several groups, while there were no significant differences between groups on days 1 and 10. (Figure 3)

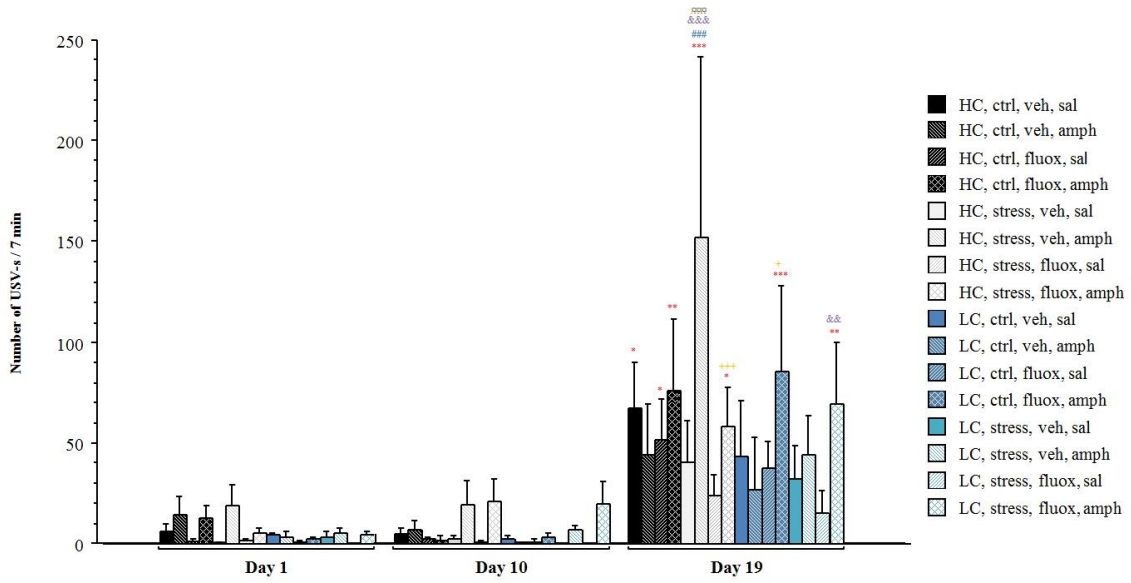

Figure 3. Mean scores of trill vocalizations scored from 7 selected time-bins on day 1, 10 and 19 (data expressed as mean + SEM). On day 19 all animals (n=62) were injected with amphetamine (1 mg/kg, IP) to examine the sensitization effect. *, **, *** - p<.05, .01, .001 vs day 1, respectively; #, ##, ### - p<.05, .01 vs control, respectively; +, ++, +++ - p<.05, .01, .001 vs vehicle, respectively; &, &&, &&& - p<.05, .01, .001 vs saline, respectively; ¤, ¤¤, ¤¤¤ - p<.05, .01, .001 vs LC, respectively.

Interestingly, fluoxetine elicited increase in numbers of trill calls on day 19 among LC non-stressed animals, who had previously received amphetamine treatment ($p<.05$). In addition there was a prevalent sensitization effect among vehicle-treated stressed HC animals, compared to their number of trill calls on day 1 and their respective saline-treatment group (both - $p<.001$). However the latter effect emerged due to small group size, since one animal made 416 trill calls on day 19, while the others in this group had a mean number of 63 calls. Excluding this rat from analysis, there was no significant increase in mean number of trill calls in this group, contrasting to their number of trill calls emitted on day 1 and respective saline-treated group on day 19 (data not reported on Figure 3).

Repeated treatment with amphetamine exclusively evoked sensitization among fluoxetine-treated stressed LC group, which showed increased 50-kHz vocalization activity on day 19, in contrast to their USV activity on day 1 and their respective saline-treated group (both - p<.01)

The proportion of trill calls in number of overall USV-s differed across testing days [F(2,92)=54; p<.0001], depending on interaction between CVS regimen and fluoxetine treatment (*Day* × *Stress* × *Fluoxetine*) [F(2,92)=4.6; p<.01]. (Table 2)

Table 2. Trill call percentage from the overall number of USV-s elicited on day 1, 10 and 19. *, **, *** - p<.05, .01, .001 vs day 1, respectively; #, ##, ### - p<.05, .01 vs control, respectively; +, ++, +++ - p<.05, .01, .001 vs vehicle, respectively; &, &&, &&& - p<.05, .01, .001 vs saline, respectively.

HC/LC	CVS regimen	FLUOX/SAL	AMPH/SAL	GROUP SIZE	DAY 1	DAY 10	DAY 19
HC	ctrl	veh	sal	4	2.6%±1.1%	2.2%±1.3%	6%±1.5%
HC	ctrl	veh	amph	4	1.9%±1.2%	0.8%±0.5%	3.7%±2.1%
HC	ctrl	fluox	sal	4	0.3%±0.3%	1.2%±0.5%	6.4%±2.3%***
HC	ctrl	fluox	amph	4	2.9%±1.3%	0.7%±0.7%	10.6%±5.7%***++
HC	stress	veh	sal	4	0.4%±0.4%	0.7%±0.7%	6.9%±3.6%**
HC	stress	veh	amph	4	2.8%±1.1%	2.1%±1.1%	12.7%±6.6%***&###
HC	stress	fluox	sal	4	0.7%±0.4%	0.5%±0.5%	4.4%±1.5%
HC	stress	fluox	amph	4	0.8%±0.3%	1.8%±0.9%	5.3%±1.5%++
LC	ctrl	veh	sal	4	1.6%±0.3%	0.9%±0.3%	5.3%±3.2%
LC	ctrl	veh	amph	4	1%±0.8%	0.3%±0.3%	3.4%±3.4%
LC	ctrl	fluox	sal	4	0.4%±0.2%	0.3%±0.3%	4.3%±1.5%+
LC	ctrl	fluox	amph	4	2.2%±1.8%	0.5%±0.3%	10%±2.9%***&+
LC	stress	veh	sal	3	0.6%±0.6%	0%±0%	5.6%±2.7%
LC	stress	veh	amph	3	2.7%±1.1%	2%±0.3%	7%±0.7%
LC	stress	fluox	sal	4	0.4%±0.4%	0.7%±0.7%	3.4%±1.7%
LC	stress	fluox	amph	4	1.1%±0.3%	2.5%±1.5%	8%±2%**

Fluoxetine treatment increased trill call proportions in HC and LC non-stressed rats, who received repeated treatment with amphetamine, in contrast to their respective vehicle-treated groups (p<.05). On the other hand, the antidepressant caused lower trill call rate in stressed HC group, who had been repeatedly administered amphetamine, compared to the respective vehicle-treated group on day 19 (p<.05).

On day 19, there were no sensitization in trill call proportions among repeatedly amphetamine-treated groups, but the former had a tendency to increase the trill call percentage in several HC groups (p<.01). Significant increase in trill call proportions was also apparent in fluoxetine-treated LC groups, which had been repeatedly administered amphetamine (p<.05).

Frequency-modulated calls during amphetamine administration

Number of frequency-modulated calls differed across testing days [F(2,92)=83; p<.0001], depending on CVS regimen [*Day* × *Stress* - F(2,92)=6.7; p<.01], amphetamine treatment [*Day* × *Amphetamine* - F(2,92)=4.3; p<.01] and their interaction effect [*Day* × *Stress* × *Amphetamine* - F(2,92)=6.1; p<.01] (Figure 4). Amphetamine treatment [F(1,46)=6.5; p<.01], as well as an interaction with CVS regimen [*Stress* × *Amphetamine* - F(1,46)=11.3; p<.001] had a main effect on number of FM-calls emitted. The amphetamine and chronic stress effect on 50-kHz vocalization activity resulted in similar differences in FM-call numbers as they did with overall number of vocalizations.

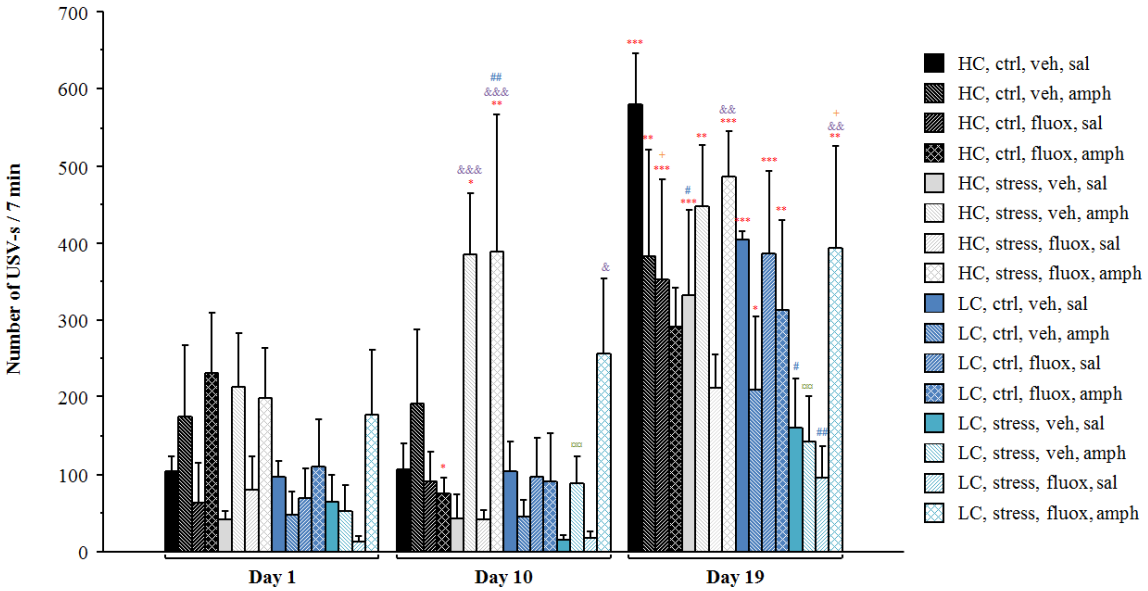

Figure 4. Mean scores of frequency-modulated vocalizations scored from 7 selected time-bins on day 1, 10 and 19 (data expressed as mean + SEM). On day 19 all animals (n=62) were injected with amphetamine (1 mg/kg, IP) to examine the sensitization effect. *, **, *** - p<.05, .01, .001 vs day 1, respectively; #, ##, ### - p<.05, .01 vs control, respectively; +, ++, +++ - p<.05, .01, .001 vs vehicle, respectively; &, &&, &&& - p<.05, .01, .001 vs saline, respectively; ¤, ¤¤, ¤¤¤ - p<.05, .01, .001 vs LC, respectively.

According to Fisher's LSD, there were no between-group differences on day 1. However there was a non-significant tendency for increased number of vocalizations among all HC rats, and fluoxetine-treated LC-stress group. On day 10, stressed HC and fluoxetine-treated LC-stress animals showed an increased vocalization activity in response to repeated amphetamine treatment, in contrast to their respective saline-treated groups (p<.05). Furthermore, the CVS regimen potentiated amphetamine response among fluoxetine-treated HC-stress rats on day 10, since their number of USV-s emitted was significantly higher than respective non-stress group. In fact, there was a significant reduction in vocalization activity

in fluoxetine-treated HC non-stressed group, compared to their mean score of USV-s on day 1 ($p<.05$).

On day 19, CVS regimen reduced the number of FM calls in HC-stress vehicle group ($p<.05$) and among fluoxetine-treated LC-stress animals ($p<.01$), who had been previously treated with saline. It seemed that fluoxetine-treated LC-stress group had a slightly lower FM call count than their respective vehicle group, but that effect did not reach statistical significance.

Repeated treatment with amphetamine elicited sensitization of frequency-modulated calls in fluoxetine-treated HC- and LC-stress groups, since the number of FM calls had increased compared to testing day 1 ($p<.001$ and $p<.01$ respectively), while their respective saline-treated groups had significantly lower FM call count on day 19 (both – $p<.01$). Furthermore, fluoxetine treatment increased the number of FM calls in LC group, in contrast with vehicle-treated animals on day 19 ($p<.05$). Interestingly, the FM call rate in fluoxetine-treated HC-stress group was significantly higher than their respective non-stress group on day 10 ($p<.01$), since the non-stress animals had a reduced number of FM calls compared to day 1 ($p<.05$).

The proportion of FM calls differed across testing days [$F(2,92)=14.5$; $p<.0001$], depending on the interaction of CVS regimen, fluoxetine treatment and amphetamine administration (*Day* × *Stress* × *Fluoxetine* × *Amphetamine* – $F(2,92)=3.3$; $p<.05$) (Table 3). Amphetamine treatment had a main effect on the proportion of FM calls [$F(1,46)=4.7$; $p<.05$] and interactions with CVS regimen and fluoxetine [*Amphetamine* × *Stress* - $F(1,46)=3.9$ and *Amphetamine* × *Fluoxetine* - $F(1,46)=5.1$, respectively; both – $p<.05$).

Table 3. Frequency-modulated call percentage from the overall number of USV-s elicited on day 1, 10 and 19. *, **, *** - p<.05, .01, .001 vs day 1, respectively; #, ##, ### - p<.05, .01 vs control, respectively; +, ++, +++ - p<.05, .01, .001 vs vehicle, respectively; &, &&, &&& - p<.05, .01, .001 vs saline, respectively; ¤, ¤¤, ¤¤¤ - p<.05, .01, .001 vs LC, respectively.

HC/LC	CVS regimen	FLUOX/SAL	AMPH/SAL	GROUP SIZE	DAY 1	DAY 10	DAY 19
HC	ctrl	veh	sal	4	48.8%±3.6%	52.9%±6.3%	55.5%±2.6%*
HC	ctrl	veh	amph	4	22.5%±8.5%&&	28.2%±7.7%&	35.7%±10.2%
HC	ctrl	fluox	sal	4	25.6%±15.2%+	41.6%±14%*	56.2%±5.2%***
HC	ctrl	fluox	amph	4	39.2%±4.9%	27.5%±5.5%&	35.6%±2.1%
HC	stress	veh	sal	4	34.3%±4.1%	49.9%±3.1%	49%±4.3%
HC	stress	veh	amph	4	36.7%±5.4%	47.7%±6.7%¤	42.6%±8.8%
HC	stress	fluox	sal	4	45.3%±8.9%	38.7%±5.7%	41%±3.2%
HC	stress	fluox	amph	4	34.6%±5.9%	39.8%±9.8%	46.2%±3.2%
LC	ctrl	veh	sal	4	35.9%±3.7%	51.5%±4%*	51.2%±3.2%*
LC	ctrl	veh	amph	4	22%±5.9%	27.5%±6.8%&	42.9%±6.1%**
LC	ctrl	fluox	sal	4	28%±13.2%	46.1%±6.4%*	49.5%±7.6%**
LC	ctrl	fluox	amph	4	39.9%±11.1%	31%±3.5%	48%±3.1%

LC	stress	veh	sal	3	32.5%±4.2%	34.8%±15.1%	31.9%±7.9%
LC	stress	veh	amph	3	20.7%±3.3%	25.4%±0.8%	23.3%±1.6%
LC	stress	fluox	sal	4	28.8%±11.7%	39.2%±5.4%	42.6%±9.2%
LC	stress	fluox	amph	4	43.2%±5%$^{+}$	45.9%±5%	55.3%6.4%$^{++}$

Fluoxetine significantly increased FM call percentage in LC-stress amphetamine group on days 1 ($p<.05$) and 19 ($p<.01$), while the effect was not apparent on day 10. Interestingly, repeated treatment with amphetamine decreased FM call proportions in vehicle-treated HC non-stressed rats on days 1 and 10 ($p<.05$). The same effect was apparent among fluoxetine-treated HC non-stressed group and in vehicle-treated LC non-stressed rats on day 10 ($p<.05$). However, a single dose of amphetamine increased frequency-modulated call proportions in HC and LC non-stressed rats, who had been previously treated with saline, on day 19 ($p<.01$). No sensitization effect can be concluded amid any group, since there were no significant differences in response to amphetamine in terms of FM call proportions.

Flat calls during amphetamine administration

Repeated measures ANOVA showed differences in number of flat calls across testing days [$F(2,92)=60$; $p<.0001$], whereas amphetamine significantly increased the flat call rate [$F(1,46)=29.7$; $p<.0001$]. Generally HC animals tended to emit higher number of flat calls than LC rats on all testing days [*HC/LC x Amphetamine treatment* - $F(1,46)=8.5$; $p<.01$]. (Figure 5)

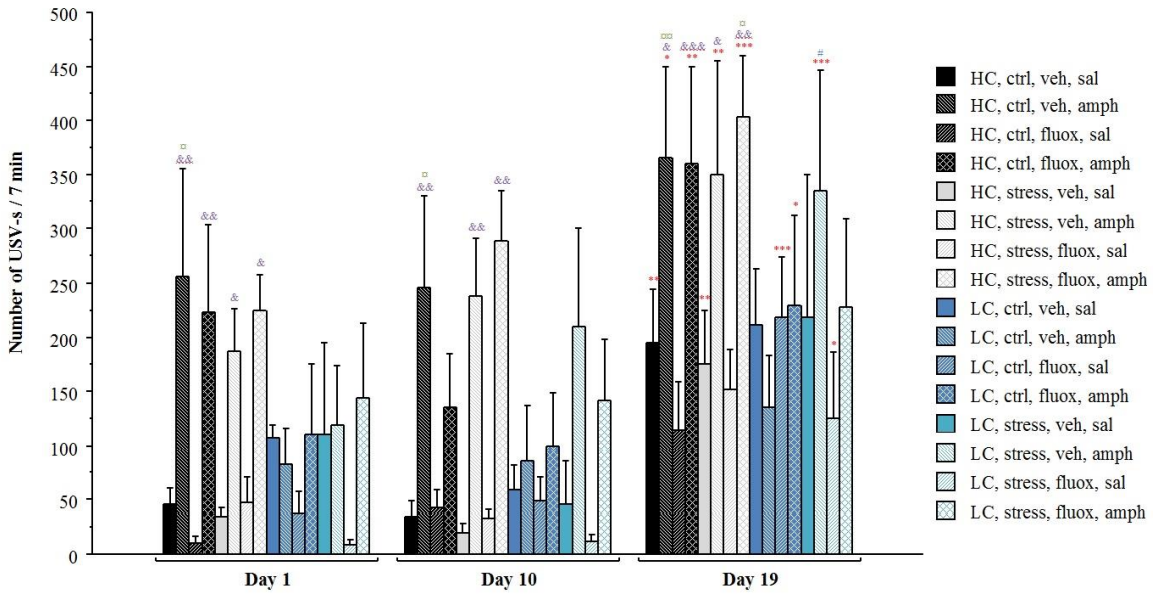

Figure 5. Mean scores of flat vocalizations scored from 7 selected time-bins on day 1, 10 and 19 (data expressed as mean + SEM). On day 19 all animals (n=62) were injected with amphetamine (1 mg/kg, IP) to examine the sensitization effect. *, **, *** - p<.05, .01, .001 vs day 1, respectively; &, &&, &&& - p<.05, .01, .001 vs saline, respectively; ¤, ¤¤, ¤¤¤ - p<.05, .01, .001 vs LC, respectively.

According to post-hoc tests, all HC groups showed increased flat call rate in response to amphetamine across all testing days ($p<.05$), although this effect was not significant among HC-stress fluoxetine-treated rats on day 10. Of note, there was a slight non-significant tendency for this effect among LC rats as well on day 10, but not on day 1 or 19.

Repeated treatment with amphetamine provoked sensitization in flat calls across all HC groups, since the call rate on day 19 was significantly higher than the number of flat calls elicited on testing day 1 ($p<.05$), while remaining higher than the number of flat calls in their respective saline-groups on day 19 ($p<.05$). Interestingly, the effect could not be described among LC groups. Several LC groups had higher flat call rate than day 1, but a single dose of amphetamine on day 19 did not provoke statistically significant differences between previously saline and amphetamine treated rats.

According to repeated measures ANOVA, the proportion of flat calls remained statistically the same across testing days, while the proportion depended on fluoxetine [interaction *Day* × *Fluoxetine* - $F(2,92)=3.9$; $p<.05$] and the interaction between HC/LC affiliation and CVS regimen [*Day* × *HC/LC* × *Stress* – $F(2,92)=3.4$; $p<.05$]. Proportion of flat calls within day depended on amphetamine treatment [$F(1,46)=25.3$; $p<.0001$] and fluoxetine [$F(1,46)=4.8$; $p<.05$], the latter having an interaction effect between [*HC/LC* × *Stress* × *Fluoxetine* – $F(1,46)=6.1$; $p<.05$]. Interestingly repeated treatment with amphetamine seemed to increase flat call proportions, while fluoxetine attenuated this effect among LC rats. Moreover HC rats tended to have higher flat call rate than LC rats in these conditions. On the other hand, vehicle-treated stressed LC rats showed higher flat call percentage than respective HC animals.

Table 4. Flat call percentage from the overall number of USV-s elicited on day 1, 10 and 19. *, **, *** - p<.05, .01, .001 vs day 1, respectively; &, &&, &&& - p<.05, .01, .001 vs saline, respectively; ¤, ¤¤, ¤¤¤ - p<.05, .01, .001 vs LC, respectively.

HC/LC	CVS regimen	FLUOX/SAL	AMPH/SAL	GROUP SIZE	DAY 1	DAY 10	DAY 19
HC	ctrl	veh	sal	4	20.3%±5.4%¤	19.5%±6.2%	18.6%±4.7%
HC	ctrl	veh	amph	4	55.7%±9.1%&&&	55%±6%&&&	50%±11%&&
HC	ctrl	fluox	sal	4	6.6%±4.5%	19.8%±6.7%	20.8%±4.5%
HC	ctrl	fluox	amph	4	35.8%±5.7%&&	48.8%±6.3%&&	43.3%±7.5%&
HC	stress	veh	sal	4	32.5%±3.2%	27.5%±4.7%	26.8%±1.7%
HC	stress	veh	amph	4	39.1%±6.8%	32%±8.8%&#¤	32.5%±10.2%¤
HC	stress	fluox	sal	4	24.2%±7.9%	32.2%±6.3%	29.3%±4.1%
HC	stress	fluox	amph	4	41.9%±5.6%	45.9%±10.1%	37.9%±1.6%
LC	ctrl	veh	sal	4	42.4%±5%	26.7%±3.2%*	25.4%±5.4%*
LC	ctrl	veh	amph	4	49.3%±4.9%	51.7%±8.1%	36.1%±8.2%
LC	ctrl	fluox	sal	4	26.6%±12.5%	37.4%±8.3%	32.5%±8%
LC	ctrl	fluox	amph	4	42.5%±14.8%	48.2%±7.8%	33.5%±4%
LC	stress	veh	sal	3	37.6%±10.8%	41.2%±16.5%	43.1%±10.6%
LC	stress	veh	amph	3	52.7%±6.5%	59.6%±3.2%	57.9%±3.1%
LC	stress	fluox	sal	4	16.8%±9.8%	26.2%±7.9%	35.7%±12.1%*
LC	stress	fluox	amph	4	31.1%±7.3%	30.1%±5.7%++	26.1%±5%++

Amphetamine treatment seemed to increase flat call rate in proportion of overall USV activity in HC-control rats (p<.05). Effect was also apparent on testing day 10 among HC-stress vehicle animals (p<.05). Fluoxetine treatment decreased flat calls in proportion within LC-stress amphetamine group on days 10 and 19 (p<.01). On days 10 and 19 HC-stress vehicle rats who had been repeatedly treated with amphetamine shower a lower proportion of flat call in contrast with their respective LC group (p<.05). The same effect appears to be the baseline difference between HC and LC subjects on day 1 (p<.05), since the HC non-stressed vehicle saline-treated animals had a lower flat call rate in proportions on day 1 than their respective LC group. The effect was not significant on days 10 and 19.

Short calls during amphetamine administration

Four-factor repeated measures ANOVA displayed differences in number of short calls across testing days [F(2,92)=27.6; p<.0001], depending on the subjects' response to amphetamine [*Day* × *Amphetamine* - F(2,92)=20.3; p<.0001] (Figure 6). On days 1 and 10, amphetamine [F(1,46)=6.6; p<.01] tended to increase the number of short calls emitted among HC rats, but not on day 19. Furthermore, HC groups showed a higher number of short calls in response to amphetamine, than LC rats on these days [*HC/LC* - F(1,46)=13.5; p<.001], while this effect did not occur on day 19.

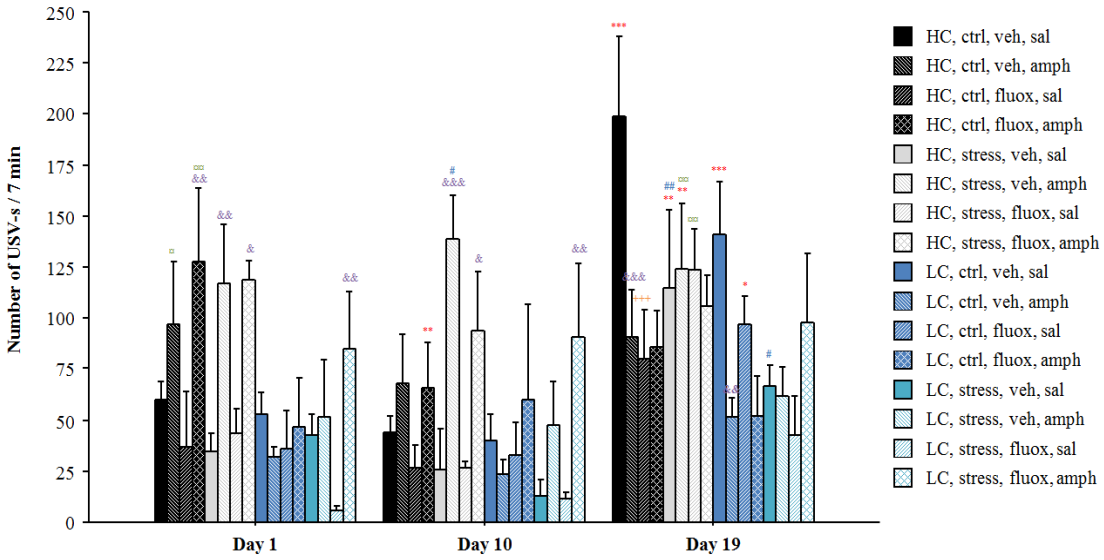

Figure 6. Mean scores of short vocalizations scored from 7 selected time-bins on day 1, 10 and 19 (data expressed as mean + SEM). On day 19 all animals (n=62) were injected with amphetamine (1 mg/kg, IP) to examine the sensitization effect. *, **, *** - p<.05, .01, .001 vs day 1, respectively; #, ##, ### - p<.05, .01 vs control, respectively; +, ++, +++ - p<.05, .01, .001 vs vehicle, respectively; &, &&, &&& - p<.05, .01, .001 vs saline, respectively; ¤, ¤¤, ¤¤¤ - p<.05, .01, .001 vs LC, respectively.

There was an increase in short call numbers across all HC groups (p<.05), but not among LC animals on day 1. The only exception was fluoxetine-treated stressed rats (p<.01). Furthermore, there were baseline differences between HC and LC rats in response to acute amphetamine administration on day 1, since the HC non-stressed vehicle-treated group had higher number of short calls than contrasting LC rats (p<.05). However on day 10, all groups showed a tendency for increased short call numbers in response to repeated amphetamine administration, although this effect was significant only among HC-stressed rats (p<.05) and LC-stress fluoxetine treated group (p<.01).

On day 19, when all animals received a single dose of amphetamine, vehicle-treated HC and non-stressed LC animals, showed a significantly higher number of short calls than their respective amphetamine-treated animals (p<.05). On the other hand, their contrasting stress-groups had no such effect, provoking a significant stress effect (p<.05). No groups showed indications of sensitization on day 19, since the single dose of amphetamine did not provoke any differences in respective amphetamine and saline groups.

Proportion of short calls differed across testing days [F(2,92)=19.1; p<.0001], depending on interaction between HC/LC affiliation, fluoxetine and amphetamine treatment [*Day* × *HC/LC* × *Fluoxetine* × *Amphetamine* – F(2,92)=4; p<.05]. Generally amphetamine-treated rats showed lower percentage of short calls, while the effect was most noticeable among LC

animals. Moreover fluoxetine significantly increased saline-treated LC rats' short call rate on day 1, but not on day 10 and 19. Amphetamine treatment had a main effect on proportion of short calls within testing day [F(1,46)=13.7; p<.001].

Table 5. Short call percentage from the overall number of USV-s elicited on day 1, 10 and 19. *, **, *** - p<.05, .01, .001 vs day 1, respectively; #, ##, ### - p<.05, .01 vs control, respectively; +, ++, +++ - p<.05, .01, .001 vs vehicle, respectively; &, &&, &&& - p<.05, .01, .001 vs saline, respectively; ¤, ¤¤, ¤¤¤ - p<.05, .01, .001 vs LC, respectively.

HC/LC	CVS regimen	FLUOX/SAL	AMPH/SAL	GROUP SIZE	DAY 1	DAY 10	DAY 19
HC	ctrl	veh	sal	4	28.3%±3.5%	25.4%±2.6%	19.9%±4.8%
HC	ctrl	veh	amph	4	19.8%±3.4%	16%±2.5%	10.6%±0.9%
HC	ctrl	fluox	sal	4	17.5%±10.2%¤¤¤	12.4%±4.1%	16.7%±3.6%
HC	ctrl	fluox	amph	4	22.1%±0.9%	22.9%±3.1%	10.6%±1.1%
HC	stress	veh	sal	4	32.8%±2.9%	21.8%±4.2%	17.3%±2.6%*
HC	stress	veh	amph	4	21.5%±1.8%	18.2%±3.2%	12.1%±3.3%
HC	stress	fluox	sal	4	29.7%±4.2%¤¤	28.7%±4.3%	25.3%±4.1%
HC	stress	fluox	amph	4	22.6%±2.5%	12.4%±1.5%	10.5%±2.1%
LC	ctrl	veh	sal	4	20.2%±2.2%	21%±2.0%	18.1%±3.7%
LC	ctrl	veh	amph	4	27.6%±9.7%	20.6%±7.8%	17.5%±6.4%
LC	ctrl	fluox	sal	4	45%±18.4%++	16.2%±2.5%***	13.7%±1.1%***
LC	ctrl	fluox	amph	4	15.4%±3.2%&&&	20.3%±4.3%	8.5%±1.3%
LC	stress	veh	sal	3	29.2%±8.2%	24.1%±5.2%	19.4%±6.5%
LC	stress	veh	amph	3	23.9%±5.3%	13%±2.3%	11.7%±2.6%
LC	stress	fluox	sal	4	53.9%±21.2%++	33.9%±8.2%**#	18.3%±3.7%***
LC	stress	fluox	amph	4	24.6%±3.6%&&&	21.6%±4.5%	10.7%±1.9%

On day 1, amphetamine decreased short call proportions among fluoxetine-treated LC rats, compared to their respective saline-treated groups (p<.001). Moreover, fluoxetine increased short call percentage among saline-treated LC animals (p<.01), while remaining significantly higher when compared to their respective HC groups (p<.001). Interestingly, these effects did not occur on day 10, or day 19 on a single dose of amphetamine. In fact, fluoxetine-treated LC groups showed significantly lower proportions of short calls on day 19, compared to their scores on day 1 (p<.001).

Locomotor activity on day 19

Four-factor ANOVA did not display any statistically significant factors on line crossings or rearings on day 19. There was a strong correlation between line crossings and rearings (r=.641; p<.001). In addition these variables correlated to USV activity differently; line crossings had a correlation of r=.332 (p<.01) and rearings r=.421 (p<.001). Furthermore, if summed into single variable, it presented a moderate correlation with overall number of USV-s (r=.41; p<.001). On the other hand USV subtypes appeared to correlate differently with line crossings and rearings. Trill calls correlated with line crossing and rearings (r=.340 and .346

respectively, both – $p<.01$). FM-s correlated with line crossings and rearings ($r=.307$ and .341 respectively, both – $p<.01$). Flat calls correlated only with rearings ($r=.278$; $p<.05$). Short calls had a significant correlation only with rearings ($r=.330$; $p<.01$).

DISCUSSION

The present study aimed to examine the effect of antidepressant treatment on amphetamine-induced 50-kHz ultrasonic vocalizations in chronically stressed HC *vs* LC rats. The chronic variable stress regimen decreased weight gain, although the stress-induced attenuation of weight gain did not differ between HC and LC animals. These results are in accordance with previous works of our group (Mällo *et al* 2009; Raudkivi *et al* 2012). Somewhat unexpectedly fluoxetine treatment attenuated weight gain, compared to vehicle-treated rats. The effect of fluoxetine on weight gain may be related to group housing used during concordant CVS regimen and fluoxetine treatment. At observational level an increase of in-cage fighting was noted in animals after daily fluoxetine treatment, especially so in first weeks of fluoxetine treatment. Those results are corroborated by findings presented in meta-analysis by Carrillo and colleagues (2009), which indicate that increased serotonergic activity in Wistar rats, especially in context of stress-induced aggression, and treatment periods of less than 3 weeks has paradoxical aggression enhancing effect. The increase in aggression towards conspecifics might have decreased food intake, as it has been previously displayed with social defeat stress model (Meerlo, Overkamp, Daan, van den Hoofdakker, Koolhaas 1996).

Previous chronic stress exposure facilitated the development of sensitization of USV response to subsequent repeated amphetamine treatment, as sensitization appeared only in previously chronically stressed animals. Both stressed and amphetamine treated groups of HC rats developed USV sensitization, whereas stressed LC rats showed USV sensitization only with fluoxetine pretreatment. These results resemble the phenomenon of cross-sensitization, where prior acute or chronic stress exposure increases the subsequent responsiveness to the effects of psychostimulant drugs (Cruz, Marin, Leão, Planeta 2012, Marinelli & Piazza 2002). Brain regions responsible for initiation and development of cross-sensitization are A9 dopaminergic neurons located in ventral tegmental area, projecting to nucleus accumbens (NAcc) (Kalivas & Stewart 1991). These structures are affected directly and/or indirectly by exposure to stress and amphetamine administration (Saal, Dong, Bonci, and Malenka 2003), and thus could explain the prevalence of cross-sensitization in the present study as well, although further

work is necessary, to model the cross-sensitization effect on rat USV-s. Of note, to my knowledge, this study is the first to describe cross-sensitization in the context of drug induced USV activity as all previous relevant studies have used measures of locomotor activity.

These results also suggest that fluoxetine pretreatment modulates the effect of chronic stress on amphetamine-induced USV-s. This was observed in stressed LC rats that did not develop sensitization unless having previously received fluoxetine. This effect of fluoxetine treatment could be associated with the functional level of the mesolimbic dopamine system, since it has been demonstrated that dopamine D_1 and D_2 receptors in nucleus accumbens shell are the key neural substrate in production of USV-s of all subtypes (Thompson, Leonard, and Brudzynski 2006), while antidepressants of all classes increase the responsiveness of D_2 dopamine receptors in NAcc (Willner *et al* 2013). Studies in humans have found that administration of dopamine receptor antagonists during treatment with SSRI-s causes reinstatement of depressed mood (Willner, Hale, and Argyropoulos 2005). In animal studies, it has been demonstrated that exposure to chronic stress induces several neurochemical changes in mesolimbic brain (Chaudhury *et al* 2013), and we have recently reported that some of these changes are different in HC *vs.* LC rats (Kõiv *et al* 2016). Therefore fluoxetine treatment could modulate the chronic stress effect on mesolimbic brain differently, depending on inter-individual differences in positive affectivity. However, it should be noted that the present study does not incorporate any neurochemical measurements. Thus, this hypothesis is highly speculative, and should be tested in future studies.

We have recently reported that prior chronic stress exposure affects the USV response to subsequent repeated amphetamine treatment dependently on the trait of positive affectivity (Kõiv *et al* 2016). More specifically, CVS regimen attenuated the USV response to amphetamine treatment only in animals characterized by low level of positive affectivity. Results from the present study thus differ from this finding as no CVS-induced attenuation of USV response to repeated amphetamine treatment occurred. This may be related to differences in chronic stress regimen used – i.e. two phase chronic stress regimen used previously vs. overall longer stress regimen without intermittent stopping of stress regimen used in the present experiment. Exposure to chronic stress provokes allostatic changes in the brain, which are partially differentiated by the ontogeny, i.e. what stressors are presented and how often, and duration of stress (Herman 2013). This effect is mainly prevalent in hypothalamo-pituitary-adrenocortical (HPA) axis, and is regulated mainly by glucocorticoids (Harvey, Brand, Jeeva, and Stein 2006), although mesencephalic dopamine regulation is also

affected (Imperato, Angelucci, Casolini, Zocchi, and Puglisi-Allegra 1992). In terms of chronic stress effect on USV activity, the changes in mesolimbic dopamine pathway's synaptic long-term potentiation is of most importance, since the effects of dopaminergic psychostimulants like *d*-amphetamine are directly affected by the latter mechanism (Saal *et al* 2003). It is possible that this mechanism is differently altered by the CVS regimen in our two studies, which induces distinct changes in dopaminergic neurotransmission, and hence could modulate the USV response to amphetamine.

Previous studies have often linked 50-kHz vocalizations to rewards, and these calls are thought to express approach behaviour in some conditions, i.e. social incentive stimuli, feeding, and anticipation of pharmacological rewards (Knutson *et al* 2002; Wöhr & Schwarting 2007; Browning *et al* 2011). It has been previously postulated that 50-kHz USV subcategories reflect different positive emotional and motivational states, indicated by the contextual differences between them (Brudzynski 2015). More importantly, sensitization in frequency-modulated and trill calls, but not in flat and short calls, has previously been observed across some studies with repeated amphetamine administration (Ahrens *et al* 2009; Rippberger, van Gaalen, Schwarting, Wöhr 2015). On the other hand, there have been reports, which describe decrease or no change in FM and trill calls (for review, see Simola 2015). These differences may occur due to data recording during different stages of reward anticipation and acquisition. Opiol and colleagues (2015) have demonstrated that FM calls are exhibited mostly during anticipation stages of food reward, while flat calls seem to be noticeably associated with acquisition of food and social signaling of the latter. These findings could apply to pharmacological rewards as well, although further research is required for more specific conclusions.

In the present study, the sensitization of trill and FM calls was noticeable in call proportions in fluoxetine-treated stressed LC animals, which received repeated treatment with amphetamine, but not in any other group: fluoxetine pretreatment increased the frequency-modulated and trill calls in proportions, and decreased the percentage of flat calls during amphetamine administration. Previous work has suggested that frequency-modulated calls, especially vocalizations with trill-elements, reflect highly rewarding and motivated affective states, and are mostly emitted in response to psychostimulant treatment (Brudzynski 2015; Burgdorf, Panksepp, Moskal 2011). The findings in the present study support this hypothesis to some extent; however, it is important to note that these results were reflected in proportions, not in overall number of call subtypes.

The increase in the mean number of frequency-modulated calls emitted after repeated administration of amphetamine was noticeable in stressed HC rats, while significant only in the fluoxetine-pretreated group at the sensitization testing. Interestingly there were no changes in proportions of FM calls across testing days in this group, while there was a tendency for increased proportion of flat calls. In fact there was a substantial increase of flat call rate among all HC groups in response to repeated treatment with amphetamine. It has been previously postulated that flat calls serve as contact signals and are not necessarily emitted in rewarding situations (Wöhr *et al* 2008; Burgdorf *et al* 2008). The present findings suggest that flat calls do reflect some kind of positive affective state in response to repeated psychostimulant treatment, although it may not be similar to the state which is expressed with FM and trill calls.

Repeated treatment with amphetamine did not significantly increase locomotor activity in any group at the sensitization testing. These results suggest that sensitization in USV-s is not necessarily accompanied with increase in locomotor activity, which is in accordance with previous findings, indicating that fifty-kHz USV-s are independent from locomotor activity (Knutson, Burgdorf, Panksepp 2002). Nonetheless this study presents a significant correlation between USV and locomotor activity, while demonstrating some interesting differences between subtypes of 50-kHz vocalizations and indicators of locomotor activity. These correlations were moderate, but unlikely to be random, since we demonstrated similar results in our previous experiment (Kõiv *et al* 2016). The more detailed understanding of associations between locomotor activity and ultrasonic vocalizations should be addressed in future studies.

CONCLUSIONS

This study provides new insights into antidepressant action on positive affect regulation and how chronic stress modulates the development of drug abuse. The main conclusions in the current study were:

- fluoxetine, while in interaction with chronic stress, has a different effect on amphetamine-induced ultrasonic vocalization activity among animals with high *vs.* low baseline level of positive affectivity, since chronically stressed LC rats did not develop sensitization unless having previously received fluoxetine, whereas sensitization occurred in both stressed HC groups repeatedly treated with amphetamine;
- rats with lower baseline levels of positive affectivity showed increased proportions of frequency-modulated and trill calls in response to repeated treatment with amphetamine, while respective HC rats did not, which suggests that HC *vs.* LC rats might have experienced different positive affective state in response to amphetamine;
- rats with higher positive affectivity showed increased flat call rate in response to amphetamine treatment, which suggests that flat calls do have an association with positive affective stimuli.

The present experiment had a group size of 3-4 rats, which is a clear limitation in interpretation of aforementioned results due to large individual variability in vocalization activity. In addition the animals used in experiments were group-housed, and any environmental differences with single-housing cannot be excluded. Hence it would be wise to draw conclusions with some precaution.

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