

Effects of venlafaxine and chronic unpredictable stress on behavior and hippocampal neurogenesis of rat dams

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Abstract

OBJECTIVE: Epidemiological studies strongly support the theory that stressful life events play an important role in the etiology of depression. The mechanism of chronic stress induced depression involves a number of systems. Chronic stress represents a serious health issue especially during pregnancy and lactation. In this sensitive period, stress can lead to changes in emotion and cognitive behavior both of the mothers and the offspring. It is thus necessary to properly manage stress events during gestation. Venlafaxine belongs to the group of serotonin and noradrenaline re-uptake inhibitor drugs. It is used for the treatment of depression, anxiety disorders and other mood disorders. During pregnancy, however, the use of venlafaxine is questionable due to the lack of experimental and clinical studies. Therefore the aim of this study was to evaluate the effect of chronic unpredictable stress and/or venlafaxine treatment on maternal and open field behavior of dams. Moreover, hippocampal neurogenesis was investigated either.

METHODS: Female Wistar rats were subjected to 2-week chronic unpredictable stress induced by random stressors and treated with venlafaxine orally at a dose of 5 mg/kg twice a day. Maternal behavior was evaluated within 5-min observations twice a day. Mothers were also tested in the open field 8 weeks after chronic unpredictable stress procedure in a single 15-min session. Hippocampal neurogenesis was investigated by immunohistochemistry essay using DCX staining.

RESULTS: Results of the present study showed altered maternal and open field behavior of the dams. Stressed dams had lowered hippocampal neurogenesis, while venlafaxine treatment reversed this lowering.

CONCLUSIONS: These results suggest that stress and antidepressant therapy can have significant impact on behavior and hippocampal neurogenesis in rat dams.

INTRODUCTION

Depression is a serious mental illness that does not spare pregnant and breastfeeding women, rather the contrary. The prevalence of antenatal (AD) and postnatal depression (PD) is about 20% (Leung & Kaplan 2009; Rayen *et al.* 2013). The incidence of depression may actually be higher, due to the reluctance of many mothers to admit to depressive states at this time. Etiology of AD and PD is multifactorial and environmental factors are significantly involved in it (Sedlackova *et al.* 2015). The most important factors include excessive stress during pregnancy (death in the family, divorce, etc.), young maternal age, lack of family support, low family income, domestic, psychological and sexual violence as well as a negative attitude towards pregnancy (Hartley *et al.* 2011; Karmaliani *et al.* 2009; Mohammad *et al.* 2011). Both untreated and treated maternal depression may have serious consequences on the developing fetus and/or neonate (Csaszar *et al.* 2014). Gynecologists and obstetricians thus stand before the dilemma whether to treat or not to treat depression during this sensitive period. Thus depression is considered to be a major concern during pregnancy. Experimental and clinical studies are needed to address this issue.

Several experimental approaches have been designed to study effects of maternal depression and/or antidepressant treatment during gestation and lactation. Chronic unpredictable stress (CUS), which has been developed by Richard Katz, is the most used animal model of depression (Katz 1982; Katz *et al.* 1981). CUS involves exposure of animals to a series of mild and unpredictable stressors (periods of food and water deprivation, small temperature reductions, changes of cage mates, etc.) during at least 2 weeks (Willner 2005; Willner *et al.* 1992, 1987). In the rat, prenatal stress, and consequent changes in glucocorticoids levels, produces offspring that appear more anxious as adults (Wyrwoll & Holmes 2012). Higher placental expression of genes regulating fetoplacental glucocorticoid and serotonin exposure is characteristic for infants with more regulatory behavioral challenges (infants' crying, feeding, spitting, elimination such as bowel movements and sleeping) (Räikkönen *et al.* 2015) Untreated maternal depression during pregnancy increases risk of prematurity, incidence of low birth weight, and attention, emotional and behavioral problems later in life (Field 2011; Nulman *et al.* 2012).

It has been recently shown that individuals with genetic predisposition to depression are more vulnerable to the effects of chronic stress as the cause of depressive behavior (Murray *et al.* 2013). There are several systems involved in rise of depression in connection with chronic stress, such as monoaminergic system and mostly hypothalamic-pituitary-adrenal (HPA) axis, which has a key role in the regulation of stress responses (Massart *et al.* 2012). Stimulation of

HPA axis triggers changes in several neurotransmitter systems, including the serotonergic system. Corticotropin releasing hormone (CRH) acts through its own CRHR1 receptors and increases the sensitivity of signal pathways mediated through serotonin 5-HT_{2R} receptors. By this mechanism, anxiety and depressive disorder can appear as a response to stressors (Magalhaes *et al.* 2010). Glucocorticoids have an inhibitory effect on the expression of enzymes synthesizing noradrenaline and serotonin, which in turn may result in monoamine deficiency (Gass *et al.* 2001; Heydendael & Jacobson 2010). During CUS a decrease in the volume of the hippocampus occurs, however extent of this change depends on the length of stressor action. Xi *et al.* (2011) found no significant changes in the volume of the hippocampus after 6 weeks of CUS, however, the changes in the level N-acetylaspartate and cognitive functions were found. After 8 weeks, there were clearly significant changes in the volume of the hippocampus, though a significantly shorter time of stress exposure is sufficient for the appearance of behavioral changes (e.g. depression-like behavior), which means that individual structures and physiological systems may respond differentially to same stressful stimuli (Luo *et al.* 2014).

Venlafaxine (VENF) belongs to the category of serotonin and noradrenaline re-uptake inhibitors. In low doses, it is a potent inhibitor of serotonin and noradrenaline re-uptake, however with much higher affinity for the serotonin transporters. At higher doses, VENF is also a weak inhibitor of dopamine re-uptake (Plachá *et al.* 2016; Weikop *et al.* 2004). By these mechanisms, it increases cellular levels of monoamines in the prefrontal cortex (Umehara *et al.* 2013). VENF is used for treatment of depression, anxiety disorders and other mood disorders. FDA classifies VENF as the drug of category C, which means that there are no controlled or only limited studies available in women or animals. Possible advantages of VENF use, however, appear to outweigh the potential risks. Considering the number of affected neurotransmitter systems, VENF has a faster onset of action than SSRIs and is used in many cases when other drugs are not effective.

In the present study we evaluated effects of pre-mating CUS and/or prenatal and early postnatal VENF treatment on maternal and open-field behavior and hippocampal neurogenesis of lactating dams.

MATERIAL AND METHODS

Animals

Female primiparous Wistar rats (initial weight 180–200 g, age 3–4 months, n=60) were obtained from the Department of Toxicology and Laboratory Animal Breeding of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Dobra Voda, Slovak Republic (reg. No. SK CH 24 011). After 14 days of acclimatization, the animals were kept in a temperature and humidity controlled room (20–24°C,

relative humidity 50–60%) with 12/12 h light/dark cycle (6 a.m. – 6 p.m. light phase) and with *ad libitum* access to food and water. Females were mated with males in the ratio 1 male: 3 females and mating period took place for two weeks. The presence of spermatozoa in vaginal smears was considered day 0 of gestation and pregnant female was moved to other cage. Pregnant rats, 3 per cage, were housed in plastic cages with wooden shavings for bedding. On day 15 of gestation, the females were separated and housed individually. The experiments were conducted in compliance with the Principles of Laboratory Animal Care issued by the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, and the experimental design was approved by the State Veterinary and Food Administration of the Slovak Republic.

Chronic unpredictable stress procedure

After the acclimatization period, the rats were subjected to a 2-week chronic unpredictable stress procedure consisting of random stressors (food deprivation, water deprivation, cage decline, damp bedding, overcrowding and forced swim) (Table 1) (Gemmel *et al.* 2015) and then the females were mated.

Venlafaxine treatment

Venlafaxine hydrochloride (VENF) with molecular weight of 313.87 (Chemoz, Czech Republic, purity, 98.5%) was diluted in distilled water and administered orally in cookies to pregnant rats from day 15 of gestation till the end of experiment at a dose of 5 mg/kg twice a day (Figure 1). The animals from the control groups received vehicle. Pregnant animals were divided into 4 groups: non-stress + vehicle, non-stress + VENF, stress + vehicle, stress + VENF.

Evaluation of maternal behavior

Maternal behavior was evaluated in home cages within 5-min observations twice a day (at about 9 a.m. and 3 p.m.) from day 2 to day 6 *post partum*. Time spent with all kind of nursing (arched back-dam displays an obvious arch in her back while nursing; blanket-dam engages in nursing postures with no obvious arch in her back; and passive nursing-dam is lying on her side or back while nursing her pups), nest building (dam is reorganizing bedding and creating a nest), off nest (no maternal contact with offspring), licking-nursing (licking of pups with nursing) and licking (licking of pups without nursing) were observed and recorded (expressed in seconds). Observations were performed by two investigators in real time.

Open-field test

The mothers were tested 8 weeks after chronic unpredictable stress procedure in the plastic open field box sized 60×60 cm in the light phase (illumination 60 lx). They were placed in the central zone of the box for a

single 15 min session. The intensity of motor activity (distance traveled) in the peripheral and central zones was recorded and analyzed by computer software ANY-MAZE™ (Stoelting Europe, Ireland). Habituation of motor activity was determined by linear regression. All tests took place between 8 a.m. – 12 a.m. The apparatus was cleaned with 70% ethanol and dried between individual testing.

Immunohistochemistry assay

The females were deeply anesthetized (between 8:00 a.m. and noon) with an injection of Zoletil/xylazine. Brains were post-fixed in 4% paraformaldehyde for 24 h, cryoprotected in 30% sucrose/phosphate-buffered saline solution for up to 1 week, frozen and kept at – 80 °C. Brain tissue was sliced in 40 µm sections on a cryostat (Leica). Tissue sections were stored in anti-freeze solution and maintained at –15 °C. The dentate gyrus of the hippocampus was assessed for the number of immature neurons, by using the endogenous marker doublecortin (DCX, Santa Cruz) Sections throughout

Tab. 1. Schedule of stressors in chronic unpredictable stress procedure.

day	Stressor	duration	time
1	cage decline	7 h	7 am
2	damp bedding	8 h	8 am
3	food deprivation	12 h	9 pm
4	forced swim	5 min	1 pm
5	damp bedding	7 h	8 am
6	food deprivation	12 h	8 pm
7	overcrowding	24 h	8 am
8	forced swim	5 min	2 pm
9	water deprivation	5 h	10 am
10	cage decline	8 h	8 pm
11	overcrowding	24 h	8 am
12	damp bedding	8 h	8 am
13	food deprivation	12 h	8 pm
14	overcrowding	24 h	8 am

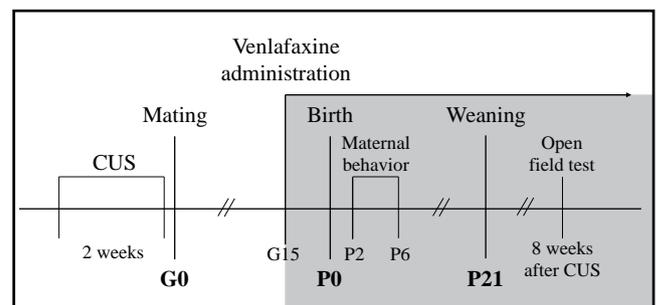


Fig. 1. Time schedule of procedures applied during this study.

the hippocampus were stained as previously described (Gemmel *et al.* 2015; Pawluski *et al.* 2012; Pawluski *et al.* 2011; Rayen *et al.* 2011). DCX staining sections were rinsed between steps in Tris-buffered saline (TBS). Tissue was first incubated in 0.6% H₂O₂ for 30 minutes at room temperature, followed by application of goat antiodoublecortin (1:200; Santa Cruz Biotech). Sections were then incubated for 2 h in biotinylated rabbit anti-goat (1:500 Vector) secondary antibody at room temperature. Brain sections were further processed by using the avidinebiotine complex (ABC Elite kit; 1:1000; Vector laboratories) and DAB kit (Vector labo-

ratories). Sections stained for DCX were mounted on Starfrost Advanced Adhesive for IHC (Bamed) dried, dehydrated and coverslipped with Permount (Fisher Scientific) (Gemmel *et al.* 2015; Rayen *et al.* 2015). Cells were observed by light microscope (Leica DM4000M) and counted manually by single observer.

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate differences in the individual variables studied (STATISTICA 10). *Post-hoc* comparisons utilized the Fisher LSD test ($p \leq 0.05$).

We evaluated habituation of motor activity (rate of this activity) by using exponential function $Y_{(t)} = Y_0 \cdot e^{-kt}$ (Y = intensity of motor activity in individual 1 minute intervals, k = individual rate of habituation, t = time of observation) (Dubovicky & Jezova 2004).

RESULTS

Maternal behavior

There was no significant main effect of stress on maternal behavior. However, there was a significant main effect of treatment ($F(5,26) = 3.4419$, $p = 0.01612$). *Post-hoc* analysis revealed a significant decrease in licking-nursing ($p = 0.0032$) and a marginal significance increase in nursing behavior ($p = 0.0542$) in animals treated with VENN compared to vehicle controls (Figure 2).

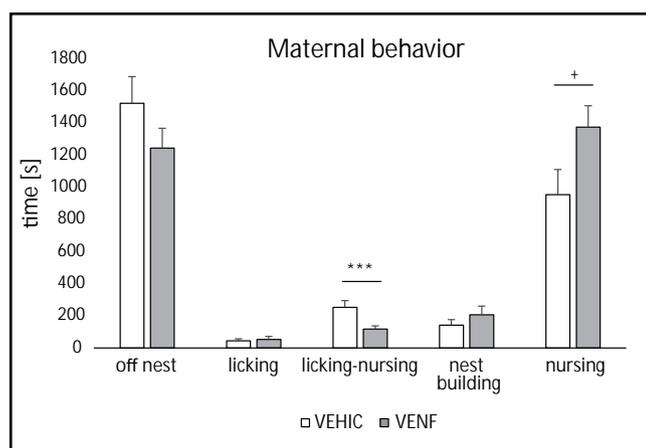


Fig. 2. Distribution of individual variables in maternal behavior taking together stress- and non-stress animals. VENN – venlafaxine, VEHIC – vehicle, *** $p < 0.005$, + $p = 0.054$ – marginal significance. $n = 15-19$.

Open field test

There was no main significant effect of VENN and stress on the habituation of motor activity (Figure 3). We found main effect of stress in combination with treatment on the total distance traveled ($F(1,28) = 4.456$, $p = 0.043$) and distance traveled in the central zone ($F(1,28) = 8.124$, $p = 0.008$) in the open-field test. There was marginally significant decrease in the total distance traveled in the non-stress VENN treated animals compared to non-stress vehicle controls ($p = 0.056$). Concerning the distance traveled in the central zone, there was statistically significant decrease in the non-stress VENN treated animals ($p = 0.024$) as well as stress-vehicle ($p = 0.023$) compared to non-stress vehicle controls (Figure 4).

Immunohistochemistry

There was main effect of stress ($F(1,30) = 14.07$, $p = 0.0007$) as well as combination of stress and treatment ($F(1,30) = 14.717$, $p = 0.0006$) on the number of new neurons in *gyrus dentatus*. CUS significantly ($p = 0.00001$) decreased proliferation of neurons in the vehicle group compared to vehicle non-stress animals. In the VENN groups, there were no differences between stress and non-stress animals. In the stress groups, the mean number of new neurons in the VENN treated animals was significantly increased compared to vehicle controls ($p = 0.00007$) (Figure 5).

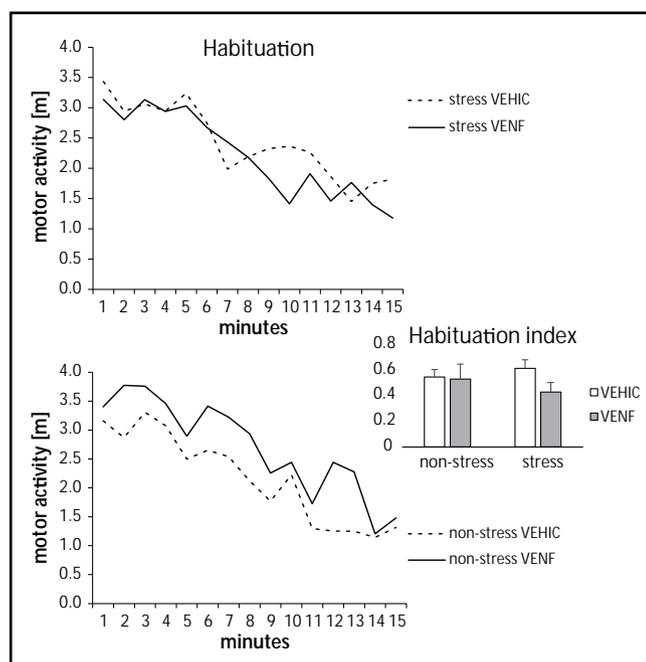


Fig. 3. Effect of venlafaxine and chronic unpredictable stress on habituation in open-field test. VEHIC – vehicle, VENN – venlafaxine. $n = 6-10$.

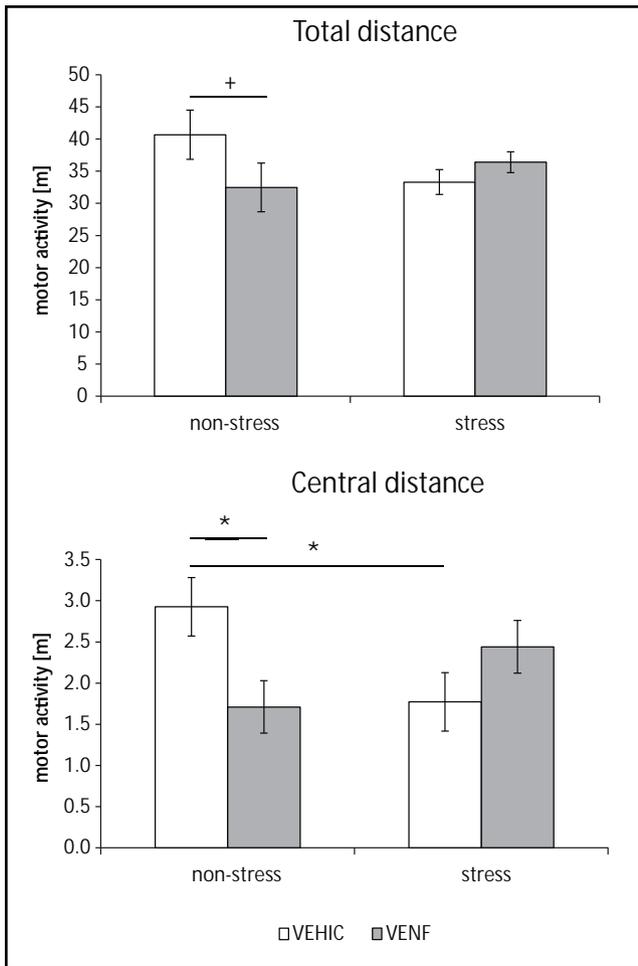


Fig. 4. Effect of venlafaxine and CUS on motor activity of mothers in central zone and total in open-field test. VEHIC – vehicle, VENF – venlafaxine. $n=6-10$, $*p<0.05$; $+p=0.056$ – marginal significance.

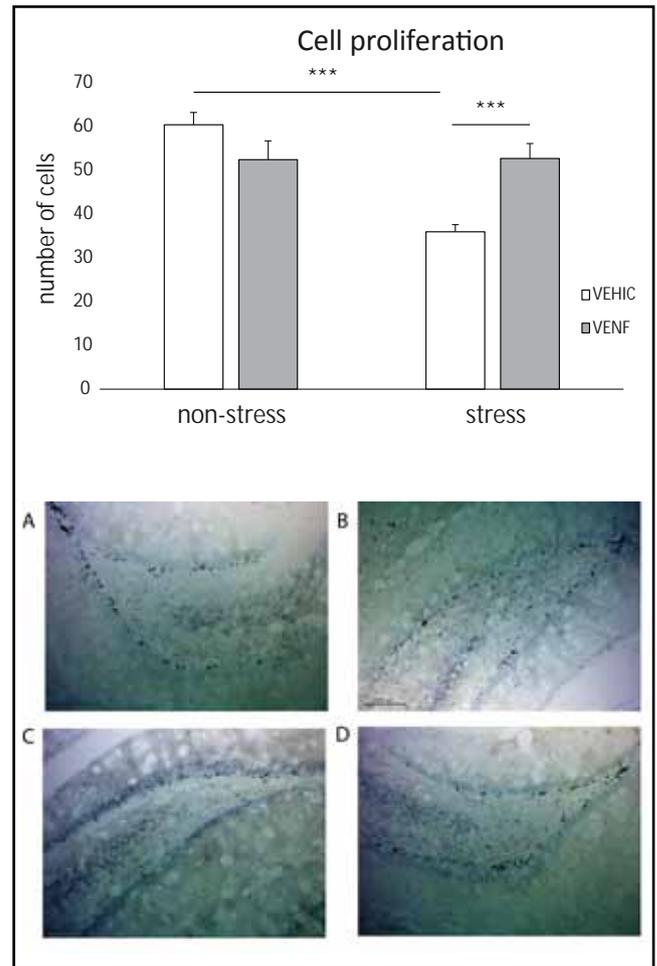


Fig. 5. Effect of venlafaxine and CUS on number of new neurons in *gyrus dentatus*. Picture A non-stress+VEHIC, B non-stress+VENF, C stress+VEHIC, D stress+VENF, scale bar: 200 μ m. VEHIC – vehicle, VENF – venlafaxine. $n=5-12$, $***p<0.001$

DISCUSSION

Results of the present study showed that stress and administration of VENF during gestation and lactation resulted in subtle alterations in maternal behavior of the dams. The females exposed to an antidepressant therapy showed increase in nursing behavior and decrease in licking-nursing behavior. In turn, dams not exposed to VENF spent more time out of the nest. Dams exposed to stress showed increased anxiety-like behavior compared to non-stress group after weaning. We found significantly lower level of hippocampal neurogenesis of stressed+vehicle group, while VENF reversed this lowering.

Dams exposed to VENF in the dose of 5 mg/kg spent more time nursing their pups, compared to vehicle groups, while females not exposed to VENF spent slightly but not significantly more time out of the nest. We moreover found that VENF decreased licking-nursing behavior of the dams. Similar results were obtained

by Pawluski *et al.* (2012) where fluoxetine treatment increased maternal arched-back nursing, while treatment with haloperidol (dopamine receptor antagonist) inhibited licking of the pups (Stern & Taylor 1991). According to Stern & Taylor (1991) motorically-active and -inactive components of maternal behavior in rats could be differentially affected by dopamine. We suppose that VENF (serotonin and noradrenaline agonist) could have a different effect also on the quality of motorically-active (licking-nursing) and -inactive (nursing) components of maternal behavior. Interestingly, in the present study stress failed to affect maternal behavior. These results are in accordance with previous studies where some other stressors such as REM sleep restriction or restraint stress did not influence maternal behavior (Pires *et al.* 2015). However, data from other authors support that early life stress impairments of nursing behavior associated with postpartum depression and anxiety (Adewuya *et al.* 2008; Murgatroyd and Nephew, 2013; Pippins *et al.* 2002). The discrepancies

between the present findings and those of other authors may be due to the different stress models used as well as to the different time periods of the stress exposure.

In the present study, dams previously subjected to 2 weeks of chronic unpredictable stress showed decreased intensity of motor activity in the central zone of the open field test, which is an indicator of increased anxiety-like behavior of the animals. In addition, we found that females from the non-stress group treated with VENF had lower motor activity compared to the non-stress+vehicle group. Decrease in motor activity of animals in open field test and hence increased anxiety-like behavior after 3 weeks lasting chronic unpredictable stress was described already in 1981 by the founder of this method (Katz *et al.* 1981). This decrease can be observed from day 1 after CUS procedure and lasts for at least 6 weeks, according to our study and to many other authors (Boyko *et al.* 2015; Cai *et al.* 2015; Luo *et al.* 2013; Xu *et al.* 2015). Changes in motor activity of unstressed animals treated with antidepressant are controversial and this behavior may be increased as well as decreased by antidepressant treatment (Homberg *et al.* 2011; Koprdová *et al.* 2016; Sass and Wörtwein, 2012). This result also indicates the importance of using the animal model of depression, otherwise one could get false results from intact animals.

Moreover, in the stress group there was a tendency of VENF to ameliorate the effect of stress by slightly increasing motor activity. A similar effect of VENF on stressed animals was also observed in another study (Xing *et al.* 2013). We did not observe any effect of VENF or CUS on habituation of animals, which is a form of learning in which an organism decreases or ceases responses to a stimulus after repeated presentations.

In the present study, stress significantly decreased neurogenesis in the *gyrus dentatus*, while VENF had a neuroprotective effect and protected neurons from the harmful action of chronic stress. Increase in neuronal proliferation during adult neurogenesis in the rodent hippocampus by antidepressants is well known (D'Sa and Duman, 2002; Malberg *et al.* 2000). This phenomenon was lately proved by various different staining techniques, focusing especially on fluoxetine as the most widely used antidepressant in the US (Keith *et al.* 2007; Kodama *et al.* 2004; Lyons *et al.* 2011; Marcussen *et al.* 2008). A few studies have tested the effect of VENF on neurogenesis yet without the stress/depression factor (Asokan *et al.* 2014; Xu *et al.* 2003). In fact, studies investigating the consequences of stress and fluoxetine therapy showed increased number of proliferating cells in the hippocampus of the pregnant female, significantly shorter apical dendrites and fewer apical branch points in CA3 pyramidal cells in late pregnant females (J L Pawluski *et al.* 2012). Moreover, repeated stress during the early postpartum period interfered with the rate of hippocampal cell proliferation and increased hippocampal volume in the maternal brain during lactation (Hillner *et al.* 2014), while repeated stress during gesta-

tion had minimal effects on hippocampal neurogenesis in the dam shortly after weaning (Pawluski *et al.* 2012; Pawluski *et al.* 2015). However VENF as a representant of SNRI drugs has not been sufficiently studied from this point of view and to our best knowledge our study is the first to evaluate the effect of VENF in combination with CUS on hippocampal neurogenesis.

In conclusion, our study showed effect of VENF on maternal behavior. Chronic unpredictable stress as well as VENF during gestation and lactation increased anxiety-like behavior in the open field test. In the stressed dams there was a lower hippocampal neurogenesis, while venlafaxine treatment ameliorated this lowering.

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