REDUCED NEUROGENESIS AND PRE-SYNAPTIC DYSFUNCTION IN THE OLFACTORY BULB OF A RAT MODEL OF DEPRESSION

D. YANG,a,b,c1 Q. LI,a,b,c1 L. FANG,b,c K. CHENG,a,b,c
R. ZHANG,b,c P. ZHENG,a,b,c Q. ZHAN,a Z. QI,a,b,c
S. ZHONGa AND P. XIEa,b,c*

aDepartment of Neurology, The First Affiliated Hospital, Chongqing Medical University, Chongqing, China
bDepartment of Neurology, The Fifth People’s Hospital of Chongqing, China
cInstitute of Neuroscience, Chongqing Medical University, Chongqing, China

Abstract—A variety of evidence has a connection with hippocampal neurogenesis in the pathophysiology of depression. However, whether other neurogenic regions in the adult central nervous system would likewise be involved is a highly interesting question. The olfactory bulb (OB) is one of the post-developmental neurogenesis areas in the adult mammalian brain. Clinical studies have shown a decreased olfactory sensitivity in depressed patients, and a recent study disclosed cases of reduced OB volume in acute major depression, indicating the OB may be also affected. Here, animal models are superior to human studies, which may provide further insight into such complex processes. We therefore investigated OB neurogenesis using a chronic unpredictable mild stress (CUMS) rat model of depression. Considering the functional analysis of adult neurogenesis which has been carried out at the synaptic level as well as animal behavior level, we detected pre-synaptic and olfactory function in the OB of rats after 4 weeks of chronic stress. Immunohistochemistry and Western blot analysis showed a dramatic reduction of immature neurons marked by polysialylated neural cell adhesion molecule and doublecortin as well as mature neurons labeled by neuronal nuclei. Moreover, chronic stress down-regulated the expression of synaptophysin but up-regulated syntaxin in the OB, as demonstrated by Western blot, whereas a significant variation at the mRNA level was lacking. Notably, in the rat model of depression, both a decreased OB volume and olfactory dysfunction were present at the same time, which is consistent with clinical findings in depressed patients. In summary, reduced OB neurogenesis and pre-synaptic dysfunction were observed in the rat model, which may at least in part correspond to the reduced OB volume and olfactory malfunction in patients suffering from depression. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: depression, olfactory bulb, neurogenesis, synaptophysin, syntaxin.

Major depression is the most prominent and universal psychiatric disorders in modern society. About 16% of the population is estimated to be affected by major depression one or more times throughout life time (Kessler et al., 2005). In spite of high prevalence and socioeconomic impact, the etiology of depressive disorder is far from being understood. Strong evidence indicates that affected adult hippocampal neurogenesis may be both a candidate mechanism for the etiology of depression and a substrate for antidepressant action. In this context stress plays a major role due to data showing that psychosocial stress reduces hippocampal neurogenesis in rodents, whereas antidepressants leads to an increase thereby resisting the negative effects of stress (for review see: Dranovsky and Hen, 2006). We recently adopted a proteome approach to examine possible alterations of protein expression in the hippocampus of a chronic unpredictable mild stress (CUMS) rat model of depression and figured out several changed proteins, which at some level, may be responsible for neurogenesis (Mu et al., 2007). In humans, selective serotonin reuptake inhibitors and tricyclic antidepressants increase dividing and neural progenitor cells (NPCs) in the dentate gyrus (DG) of patients suffering from depressive disorder, compared with untreated depressed patients or controls (Boldrini et al., 2009).

Irrespective of stress and depression affecting hippocampal neurogenesis, the heterogeneity of depression implies that manifold neural substrates and mechanisms contribute to its etiology (Manji et al., 2001; Duman, 2002; Nestler et al., 2002). In a recent study using mice exposed to CUMS, a substantial decrease of neurogenesis was not only observed in the hippocampus but also in the subventricular zone (SVZ), which is another brain region with continuous neurogenesis throughout adulthood (Mineur et al., 2007). In addition, chronic forced-swim stress could also reduce neural stem cells (NSCs) in the SVZ (Hitoshi et al., 2007). With respect to depression these data point to the significance of disturbed neurogenesis in brain areas other than the hippocampus. The olfactory bulb (OB) is one of the prominent post-developmental neurogenesis areas in the adult mammalian brain (Lledo et al., 2006).
Neuronal precursors generated in the SVZ migrate along the rostral migratory stream (RMS) into the OB, where they differentiate into granular and periglomerular neurons, and integrate into established neuronal networks and respond to odor stimulations (Lledo and Saghatelzhan, 2005). Whether depression would also correlate with the OB neurogenesis is still unknown. It should, however, be noted that a significant reduction of the OB volume was observed in patients with acute major depression, and a significant negative correlation between the olfactory bulb volume and depression scores has been shown lately (Negoias et al., 2010). Moreover, series of clinical studies found a decreased olfactory sensitivity especially in patients with acute major depression (for review see: Atanasova et al., 2008). Furthermore, in rodents bilateral olfactory bulbectomy induces changes in behavior, simulating alterations in the endocrine, immune and neurotransmitter systems, that parallels to many of those seen in patients with major depression (Song and Leonard, 2005). These data suggested that part of the abnormalities seen in depression could be related to changes of the OB. Based on all these evidences, we addressed the question whether reduced neurogenesis in the OB could be measured by the CUMS rat model of depression. Given that the functional analysis of adult neurogenesis has been manipulated at three different levels: individual synapses and cells, neuronal circuits, and whole animals (Ming and Song, 2005), we investigated whether preturbed pre-synaptic function in the OB and altered olfactory behavior would occur simultaneously in the CUMS rat model of depression.

Our approach was to firstly investigate the number of immature neurons (marked by polysialylated neural cell adhesion molecule (PSA-NCAM) and doublecortin (DCX)) and mature neurons (labeled by Neuronal Nuclei (NeuN)) in the OB of the CUMS rats by immunohistochemistry and Western blot. Secondly, we examined the effect of chronic stress on the expression of two pre-synaptic proteins, synaptophysin (SYN) and syntaxin (STX), in the OB by Western blot and RT-PCR. SYN is highly expressed in pre-synaptic vesicles, performs like a chaperone to synaptobrevin (Edelmann et al., 1995) and plays an important role in regulating activity-dependent synapse formation (Tarsa and Goda, 2002). Additionally, STX performs imperative impact on regulating vesicle docking and fusion which is essential for neurotransmitter release (Hu et al., 2003). Taken together, SYN and STX are well-established markers of pre-synaptic function and could be used to study variations of pre-synaptic function under different circumstances (Gray et al., 2010). Finally, we evaluated the general olfactory function of the rats exposed to chronic stress by buried food pellet test.

**EXPERIMENTAL PROCEDURES**

**Animals**

This study was carried out using 32 healthy adult male Sprague–Dawley rats (weights: 230–280 g; age: 3–4 months), purchased from the animal facility of the Chongqing Medical University (Chongqing, China). The rats were kept under standard conditions (12 h light/dark cycle; lights on at 7:00 AM; 22 ± 1 °C ambient temperature; 52 ± 2% relative humidity; food and water ad libitum), unless otherwise stated. After adaptation to the laboratory conditions (7 days prior to the start of the experiments), the animals were randomly divided into two groups, those kept under chronic stress conditions (n = 16) and controls without stress (n = 16). The time schedule is given in Fig. 1. The study was approved by the Ethics Committee of Chongqing Medical University, and all procedures were in accordance with the National Institutes of Health Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals). Special care was taken to minimize number of and suffering of animals.

**CUMS procedure**

The stress procedure followed our previously described strategy (Mu et al., 2007), with minor modifications. In brief, the chronic stress group was kept in isolation, each rat in a single cage, subjected to a variety of mild stressors: cage tilting for 24 h, swimming in 4 °C cold water for 5 min, swimming in 45 °C hot water for 5 min, fasting for 48 h, water deprivation for 24 h, shaking for 10 min, nip tail for 1 min, wet bedding for 24 h, and inversion of the light/dark cycle. Rats received one of these stressors per day, but the same stressor was not applied in two consecutive days. The stress procedure lasted for 4 weeks prior to behavioral testing, and was finished by food deprivation for 24 h as final stressor. Control animals were kept in groups (four rats per cage) and given ordinary daily care.

**Behavioral tests**

Rats were placed in the testing room 30 min before the behavioral tests started. All tests took place in a soundproof room between 8:00 AM and 1:00 PM, unless otherwise stated. After each test, rats were returned to their home cages and then to the holding room once every animal was tested.

*Buried food pellet test.* To measure general olfactory function, a set of buried food pellet tests were applied, following the method of Nathan et al. (2004). The baseline olfactory function test was the same as the first buried food pellet test after the CUMS period as following: in the first place, all the rats underwent food deprivation for 24 h but with access to water ad libitum prior to the very first testing after chronic stress. In each trial a single rat was placed at random in a test cage (42 × 35 × 40 cm³) to search for a 0.5 g food pellet which was buried approximately 2 cm below the surface of a 3 cm deep layer of the bedding material. The pellet was always buried at the same site, and the latency to find the food pellet was defined as the time between placing the rat in the cage and detection of the food pellet and grasping it in its forepaws and/or teeth. Animals were allowed to consume the pellet they found and were then returned to their home cages. If an animal couldn’t locate the food pellet within 5 min, it was removed.
returned to its home cage and given a score of 5 min. The bedding in the test chamber was changed between each trial. All testing periods were recorded using a Sony DCR-SR45E camera. After finishing the tests, each rat was given a 0.5 g food pellet to eat. After 4 h, a second buried food pellet test was performed, in which we increased the food pellet weight to 15 g. Similarly like above, each rat received a 0.5 g food pellet after the test was completed. In the morning of the next day, a third buried food pellet test was carried out, in which we diminished the volume of the test cage to 28×19×40 cm$^3$ and buried a 15 g food pellet under the bedding material.

Open field test. The open field test was performed to measure spatial exploration behavior in rodents. Briefly, the apparatus consisting of a black square cage with 100×100×40 cm$^3$ was divided into 25×25 cm$^2$ equal squares on the floor of the arena. A single rat was placed in the center of the cage and after 30 s of adaptation, the total behaviors including the number of locomotion (with the four paws) and the number of rears (posture sustained with hindpaws on the floor) were recorded for 5 min using a Sony DCR-SR45E camera located 190–200 cm above the arena. The cage was thoroughly cleaned after each trial. Following this, open field activity was scored manually from a computer screen by a researcher and scored for each trial. The Open field behavior was scored for a total of six sections from the sagittal series of the left OB. The volume of the left OB was measured using a method described previously (Mirich et al., 2002). Every 30th section was selected from each rat for a total of six sections from the sagittal series of the left OB, stained with HE, and then used to evaluate the volume of the left OB. Under light microscopy, a 4× objective was used to reconstruct images of each section. The area of the OB of each section was measured using Nikon imaging software (NIS-Elements BR 64-bit version 3.2, Laboratory Imaging). The end of the olfactory bulb or beginning of the olfactory tract could clearly be monitored as a change in diameter. The volume could then be calculated by determining the mean of every two successive areas multiplied by the interval between them, and summing up each of these volumes.

**Immunohistochemistry**

After conventional dewaxing with xylene and rehydration, microwave antigen retrieval was performed at 95 °C for 15 min by using a citrate buffer (pH 6.0) (NeuN labeled) or a Tris-EDTA buffer (pH 9.0) (PSA-NCAM and DCX labeled). Then the sections were incubated with a 3% hydrogen peroxide solution for 10 min to block the endogenous peroxidase at room temperature (RT), and a blocking solution containing 10% goat serum was used to inhibit nonspecific antibodies binding at RT for 20 min. After that, mouse polyclonal anti-NeuN (1:100, Chemicon/Millipore), mouse monoclonal anti-PSA-NCAM (1:100, Chemicon/Millipore), or rabbit polyclonal anti-DCX (1:200, Cell Signaling) was used as the primary antibodies. After incubation overnight at 4 °C and several washes with PBS (PH 7.4), biotinylated anti-mouse IgG or anti-rabbit IgG (secondary antibody) was added, and the mixture was incubated at 37 °C for 30 min; then horseradish peroxidase-labeled streptavidin was added after several washes with PBS, followed by 30 min incubation time at 37 °C. After additional washes with PBS, the immune reaction was visualized by the diaminobenzidine reaction. After washes with distilled water, the sections were counter-stained with hematoxylin and eosin (HE) staining or immunohistochemistry as given below.

**OB volume**

The OB volume was measured using a method described previously (Mirich et al., 2002). Every 30th section was selected from each rat for a total of six sections from the sagittal series of the left OB/frONTAL cortex, stained with HE, and then used to evaluate the volume of the olfactory bulb. Under light microscopy, a 4× objective was used to reconstruct images of each section. The area of the OB of each section was measured using Nikon imaging software (NIS-Elements BR 64-bit version 3.2, Laboratory Imaging). The end of the olfactory bulb or beginning of the olfactory tract could clearly be monitored as a change in diameter. The volume could then be calculated by determining the mean of every two successive areas multiplied by the interval between them, and summing up each of these volumes.

**Sucrose preference test and body weight**

According to the method in a previous study (Luo et al., 2008), rats were trained to adapt to 1% (w/v) sucrose solution 72 h before testing; two bottles of 1% sucrose solution were placed in each cage, and 24 h later 1% sucrose in one bottle was replaced with tap water for 24 h. After adaptation, rats were deprived of water and food for 24 h, followed by the sucrose preference test, in which all rats housed in individual cages had free access to two bottles, one containing 1% sucrose and the other tap water. The position of the two bottles (left/right sides of the cages) was varied randomly. All fluid consumption was recorded by weighing the two bottles before testing and after 24 h, and the sucrose preference was calculated as the sucrose preference (%) = sucrose consumption (sucrose consumption + water consumption). Body weights of all animals were measured before beginning the CUMS procedure, and then weekly throughout the CUMS period under similar conditions.

**Fixation and tissue preparation**

Following anesthesia administered by an overdose of chloral hydrate, rats (CUMS group $n=5$, CON group $n=5$) were quickly perfused intracardially, each with chilled phosphate buffer saline (PBS) (0.01 M, pH 7.4) followed by chilled 4% paraformaldehyde for about 10 min (about 300 ml of each solution per animal). Through the rostral SVZ, the brain (including RMS and OBs) was removed from the skull and then post-fixed for 24 h in the 4% paraformaldehyde at 4 °C, followed by embedding in paraffin according to standard methods. Sagittal sections (4 μm thick) through the left OB/frONTAL cortex were performed using a rotary microtome (Leica RM 2135, Meyer Instruments, Houston, TX, USA) for hematoxylin and eosin (HE) staining or immunohistochemistry as given below.

**Immunohistochemistry**

After conventional dewaxing with xylene and rehydration, microwave antigen retrieval was performed at 95 °C for 15 min by using a citrate buffer (pH 6.0) (NeuN labeled) or a Tris-EDTA buffer (PH 9.0) (PSA-NCAM and DCX labeled). Then the sections were incubated with a 3% hydrogen peroxide solution for 10 min to block the endogenous peroxidase at room temperature (RT), and a blocking solution containing 10% goat serum was used to inhibit nonspecific antibodies binding at RT for 20 min. After that, mouse polyclonal anti-NeuN (1:100, Chemicon/Millipore), mouse monoclonal anti-PSA-NCAM (1:100, Chemicon/Millipore), or rabbit polyclonal anti-DCX (1:200, Cell Signaling) was used as the primary antibodies. After incubation overnight at 4 °C and several washes with PBS (PH 7.4), biotinylated anti-mouse IgG or anti-rabbit IgG (secondary antibody) was added, and the mixture was incubated at 37 °C for 30 min; then horseradish peroxidase-labeled streptavidin was added after several washes with PBS, followed by 30 min incubation time at 37 °C. After additional washes with PBS, the immune reaction was visualized by the diaminobenzidine reaction. After washes with distilled water, the sections were counter-stained with hematoxylin and eosin (HE) staining or immunohistochemistry as given below.

**Quantitative analyses**

To quantify the number of labeled cells, sections were examined microscopically by a Nikon E100 Eclipse microscope, and their images analyzed utilizing Nikon imaging software (NIS-element BR 64-bit version 3.2, Laboratory Imaging). Two sections per animal were examined, and all were carefully matched anatomi-
cally between animals. One predetermined area (40× objective) in the subependymal zone (SEZ) for PSA-NCAM and DCX positive cells, and five predetermined areas (40× objective) in the glomerular layer (GL) and another five predetermined areas (40× objective) in the granule cell layer (GCL) of the OB for NeuN labeled cells were analyzed in each section (Fig. 2). The number of positive cells in each GL area was averaged to obtain a mean value for each animal. In the GCL and SEZ, the density of positive cells required electronic densitometry to measure the NeuN, PSA-NCAM, and DCX signal intensities. The intensity values of each chosen areas were then normalized against background staining of the section and a mean (average) value for each rat was calculated.

Western blot

Rats (CUMS group n=6, CON group n=6) were sacrificed and the OBs were removed by dissection, immediately frozen in liquid nitrogen and stored at −80 °C. Left OBs were homogenized in a standard lysis buffer, and then sonicated to promote lysis. The samples were then centrifuged at 13,000 rpm at 4 °C for 60 min. The supernatants were collected, and protein amounts were quantified by Bradford method. Lysates containing equal amounts of protein was boiled at 95 °C in SDS sample buffer for 5 min, electrophoresed on SDS PAGE gels, and transferred to polyvinylidifluoridine membranes. Subsequently membranes blocked in 5% (w/v) skimmed milk solution for 1 h at RT and incubated overnight at 4 °C with either primary antibody (mouse polyclonal anti-NeuN 1:500, Chemicon/Millipore; mouse monoclonal anti-PSA-NCAM antibody 1:1000, Chemicon/Millipore; rabbit polyclonal anti-DCX 1:1000, Cell Signaling; mouse monoclonal anti-synaptophysin 1:500, Chemicon/Millipore; mouse monoclonal anti-syntaxin 1:1500, Invitrogen). After three washings for 30 min in TTBS buffer, membranes were incubated at 37 °C for 60 min with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG, and washed three times for 30 min with TTBS buffer. Then membranes were developed with ECL reagents and the chemiluminescence signal was imaged using a ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). To normalize for protein content, blots were stripped in stripping buffer containing 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCL (PH 6.7) and then probed with anti-β-tub antibody (1:1000, bioworld). Immunoblots were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

RT-PCR

The semi-quantitative RT-PCR was used to detect mRNA corresponding to pre-synaptic proteins. Total RNA was isolated from the left OBs (CUMS group n=5, CON group n=5) by the acid...
guanidinium thiocyanate method and quantified by spectrophotometry. First-strand cDNA synthesis was performed with the Reverse Transcription System from Promega. PCR reactions were conducted using rat-specific primers for SYN (NM_012664.2) forward (5′-CAGTGGGTCTTTGCCATCTT-3′) and reverse (5′-TTCAGCCGACGAGTAGTATG-3′); syntaxin-1A (STX1A) (NM_053788.2) forward (5′-GGCTTTATTGACAAGATGTGC-3′) and reverse (5′-TCGTTAGTCGAGTAGTGTC-3′). To normalize the SYN and STX1A data for semi-quantitative analysis, we applied the same cDNA samples to a RT-PCR using /H9252-actin (NM_031144.2) primers forward (5′-GACGTTGACATCCGTAAAGACC-3′) and reverse (5′-TAGGAGCCAGGGCAGTAATCT-3′). The PCR cycles were optimized to amplify each primer-specific RT-PCR product within a linear range of amplifications as follows: denaturation 94 °C 30 s; annealing 57 °C 30 s; elongation 72 °C 40 s. Thirty-five cycles were performed. Each pair of primers produced a single amplicon of an expected size: SYN, 222 bp, STX1A, 304 bp, /H9252-actin, 112 bp. The PCR products were then analyzed by electrophoresis in 2% agarose gels. Digital images were captured and quantified by Quantity One analysis software on the ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA).

Statistic analysis

All results are given as mean±SD values. The statistical analyses were carried out using SPSS software. Body weight was analyzed by means of a repeated measurement ANOVA (CUMS or CON group as independent factor and time as repeated measure). A Student’s t-test was used to analyze significant differences between the two groups. All tests were two-tailed. Significance level was set at P<0.05.

RESULTS

The quality of the depressed rat model

To evaluate the quality of the rat depression model, three parameters were used, changes of body weight, scores of open field test, and preferences for sucrose. Our data revealed significant differences between the two groups (CUMS group and CON group) for each of these indexes, what was, notably, consistent with our previous study (Mu et al., 2007).

With respect to body weight (Fig. 3A), an ANOVA yield a main effect of time [F(4,128)=294.64, P<0.001], a main effect of group [F(1,30)=68.00, P<0.001] and significant group by time interaction [F(4,120)=52.30, P<0.001]. No significant difference was found in the baseline body weight of the two groups, but the body weight of the CUMS group was significantly lower than that of the CON group during last 3 weeks of the CUMS period (t=7.66, P<0.001; t=14.76, P<0.001; t=9.37, P<0.001). For the scores of open field test, no differences were detected at baseline,
but animals subjected to chronic unpredictable stress for 28 days showed a significant decrease in the number of locomotion and rears ($t=5.53, P<0.001; t=8.50, P<0.001$, respectively) (Fig. 3B, C). Regarding preferences of sucrose, the CUMS rats showed a dramatic reduction in relative sucrose intake compared with the CON rats ($t=4.29, P<0.01$) (Fig. 3D).

Neurogenesis

The neurogenesis in the OB was evaluated by analyzing immature (PSA-NCAM and DCX labeled) and mature neurons (NeuN labeled). In the OB of the CUMS rats the number of immature neurons, based on the density of PSA-NCAM and DCX positive cells in the SEZ, was significantly less than that in the CON rats ($t=3.85, P<0.01; t=11.22, P<0.001$, respectively) (Figs. 4A, B and 5A, B). Western blot analysis was consistent with these outcomes showing that the expression of PSA-NCAM and DCX proteins in the OB was considerably down-regulated in the CUMS rats as compared with the CON rats ($t=3.65, P<0.05; t=5.72, P<0.001$, respectively) (Figs. 4C, D and 5C, D). Likewise, a significant reduction in NeuN positive cells was observed in the GL of the CUMS rats ($t=3.19, P<0.05$) (Fig. 6A, B), while the density of NeuN immunoreactivity in GCL did not change significantly ($t=1.30\times10^5\pm0.46\times10^5$ for CUMS vs. $1.61\times10^5\pm0.37\times10^5$ for CON; $t=1.19, P=0.27$). This could be confirmed by Western blot analysis of NeuN protein, which showed a significant decreased level of NeuN protein in the CUMS rats compared with the CON rats ($t=3.05, P<0.05$) (Fig. 6C, D).

The expression of pre-synaptic proteins

We measured the alterations of both protein and mRNA expression for SYN in the OB of the CUMS rats. As illustrated in Fig. 7, chronic stress for 28 days significantly reduced the expression of SYN protein in the OB ($t=3.03, P<0.05$), while the SYN mRNA level did not reach statistical significance ($t=1.65, P=0.14$). Syntaxins comprise a large family of membrane-associated proteins. Syntaxin 1A (STX1A) resides in the nerve terminals of sensory neurons (Aguado et al., 1999) and plays an important role in the secretion of neurotransmitters by neuronal cells (Nakayama et al., 1998). Therefore, not only the STX
protein expression was evaluated but also the STX1A mRNA level in the OB. The results showed that chronic stress up-regulated the expression of STX protein in the OB (t=11005 2.75, P=11021 0.05), notwithstanding the STX1A mRNA expression which remained unchanged in the CUMS rats (t=11005 1.42, P=11005 0.19) (Fig. 7).

The OB volume

It was of particular interest whether the reduction of the different functional stages of neurons in the OB would be in parallel with changes of the OB volume. The data showed that this was, indeed, the case. Chronic stress reduced the OB volume of the CUMS rats to 78.7% of that of the CON rats (t=2.54, P<0.05) (Fig. 8).

General olfactory function

Through a variety of olfactory function tests, we monitored the latency to locate a buried food pellet. Two important parameters were considered, food odor intensity and space of the test chamber. No differences were detected at baseline in case of a 0.5 g food pellet, but chronic stress elongated the time to locate the food significantly (CUMS vs. CON rats; t=10.66, P<0.001). Elevated odor intensity by increased food weight led to similar differences between CUMS and CON rats (t=14.89, P<0.001). Finally, reduced space of the test chamber, in which the food pellet was buried almost under the rat’s body, revealed the same prolonged latency of the CUMS rats to find the food pellet as compared to unstressed controls (t=12.33, P<0.001) (Fig. 9).

DISCUSSION

This is the first study about the impact of chronic stress on the neurogenesis in the OB using the CUMS rat model of depression. The results clearly displayed a reduction in the number of immature as well as mature neurons, after chronic stress had been applied to these rats. Moreover, evidence for an altered neurogenesis in the OB, simultaneously accompanied with pre-synaptic dysfunction and olfactory deficit, could be demonstrated after chronic stress.

The present study aimed to clarify to which extent impaired neurogenesis may contribute to the pathophysiology of depression. Using the CUMS rat model of depression, we addressed the question whether neurogenic regions other than hippocampal, namely OB, would be promptly impaired in the early phase after chronic unpredictable mild stress. It is known that OB neurogenesis covers differentiation, maturation, integration and survival of neuronal cells. To analyze OB neurogenesis in the initial phase of the CUMS model, the proliferation marker bromodeoxyuridine (BrdU) could not be used. Instead, PSA-
NCAM and DCX were chosen, widely used to mark a stage of immature neurons in adult central nervous system (Bédard and Parent, 2004; Jin et al., 2004; Kempermann et al., 2004; Rao and Shetty, 2004; Couillard-Despres et al., 2005; Ming and Song, 2005; Curtis et al., 2007). In agreement with our anticipation, a significant reduction of PSA-NCAM and DCX labeled cells was observed in the OB of the rats applied to chronic stress. Simultaneously, diminished amounts of PSA-NCAM and DCX labeled cells accompanied with a significant reduction of mature neurons (NeuN labeled), indicated a considerable decrease of newborn cells reaching the mature state. Our results are corresponding well with other studies showing lower levels of hippocampal neurogenesis in animal models of depression (for review see: Dranovsky and Hen, 2006), suggesting that impaired neurogenesis may exist more widely than expected in the adult brain during depression. The finding

![Fig. 7. Effect of chronic stress on the SYN and STX expression in the OB.](image)

**(A)** Western blot analysis of the expression level of SYN and STX proteins in the OB following chronic stress. **(B)** RT-PCR analysis of the expression level of SYN and STX1A mRNA in the OB following chronic stress. **(C)** Results of Western blot analysis showing a drastic reduction of SYN and increasing of STX in the OB after chronic stress treatment. The data are presented as mean±SD (n=6 animals per group). **(D)** Results of RT-PCR analysis lacking significant changes in the level of SYN and STX1A mRNA after chronic stress treatment. The data are presented as mean±SD (n=5 animals per group). *P<0.05.

![Fig. 8. Quantification of the left OB volume. The data are presented as mean±SD (n=5 animals per group). *P<0.05.](image)

![Fig. 9. Latency to uncover the food pellet at baseline and after chronic stress. Baseline and the first buried food pellet test with 0.5 g food under the bedding material in a 42×35×40 cm³ test cage. The second buried food pellet test with increase amount of food (weighting 15 g) beneath the bedding material in the same cage as the first test. The third buried food pellet test with 15 g food under the bedding material in a test cage reduced in size (28×19×40 cm³). The data are presented as mean±SD (n=16 animals per group). ***P<0.001.](image)
of reduced numbers of different stages of neurons may be one of the reasons for the decreased OB volume in the rat model used in this study. Likewise, decreased OB volume was also observed in patients suffering from acute major depression (Negoias et al., 2010).

When comparing the neurogenesis data with the pre-synaptic function, it did appear that reduced OB neurogenesis might impact on the pre-synaptic function. Albeit a significant down-regulated expression of SYN protein and up-regulated expression of STX protein were measured in the OB of the CUMS rats at the pre-synaptic protein level, no clear variations were found at the mRNA level. This consequence suggests that translation is altered under these circumstances due to immediate and selective changes of protein levels (Holcik and Sonenberg, 2005). Our results comply with a previous study showing that chronic stress differentially regulates pre-synaptic proteins SYN and synaptotagmin expression in the hippocampus (Thome et al., 2001). The information gathered on olfactory dysfunction in our chronic stress model using buried food pellet tests, correlates well with the OB representing the most important relay station in odor processing. The results presented here are in agreement with clinical data showing that patients in an acute episode of major depression were suffering from deficit in olfactory function (Pause et al., 2001; Negoias et al., 2010). It can be assumed that the mechanism underlying these olfactory dysfunction could be associated with reduced OB neurogenesis and perturbed pre-synaptic function. For instance, enriched olfactory environments increase the survival of neurons generated in adult brain, which is accompanied with improved performance in olfactory memory tasks (Rochefort et al., 2002). Conversely, mice with an impaired production of new interneurons, namely shown in neural cell adhesion molecule-deficient mice (Gheusi et al., 2000) and aged mice (Enwere et al., 2004), displayed deficits in olfactory discrimination. It is therefore conceivable that diminished neurogenesis in the OB, especially the decrement of periglomerular cells observed in this study, may be at least partially responsible for olfactory deficit. Moreover, the differential regulation of the expression of pre-synaptic proteins may represent a molecular correlation of olfactory behavioral alterations.

Although the mechanism(s) underlying the reduced OB neurogenesis remains to be determined, it should be noted that the deficiency in proliferation of stem cells in the SVZ of a CUMS mouse model of depression (Mineur et al., 2007) and a forced-swim model of stress in the mouse (Hitoshi et al., 2007), may provide additional explanations. A previous study showed that a significant increase in the number of proliferating cells in the subventricular zone coincided with a pronounced enlargement of newborn cells in the OB 2 weeks after ischemia in rats (Zhang et al., 2001). Contrarily, down-regulation of cell proliferation in the SVZ led to a decreased number of newborn cells in the OB 3 weeks after a short term treatment with estradiol in adult female C57B16/J mice (Brock et al., 2010). On the other hand, alterations inside the OB may regulate the neurogenesis in SVZ. This would go along with a previous study showing that cell proliferation was significantly reduced in the SVZ of adult rats which underwent bilateral olfactory bulbectomy (Keilhoff et al., 2006). Taken together, the relationship between OB neurogenesis and SVZ neurogenesis in the CUMS rat model of depression requests further research. Which role the reduced OB neurogenesis plays in the mechanism of depression will raise compelling questions to pursue in future investigations, especially whether changes in OB neurogenesis are modulated by antidepressants.

**CONCLUSION**

In summation, our study provides evidence that reduced OB neurogenesis occurs in the CUMS rat model of depression. Simultaneously, impaired neurogenesis was accompanied by down-regulated SYN and up-regulated STX expression in OB. These results may explain why reduced OB volume and olfactory sensitivity occurred in both depressed patients and the CUMS rat model of depression. Furthermore, our results indicate that impaired neurogenesis may be a general phenomenon in all brain stem cells in the CUMS rat model of depression. Further studies are needed to clarify whether antidepressant treatment would reverse the stress-induced responses in OB and how dysfunctions at the molecular and cellular level in the OB can be correlated to the mechanism of depression.

Acknowledgments—We thank associated professor Dan Li from the department of pathology of Chongqing Medical University for image analysis. We thank professor Hans Ludwig from Institute of Virology of Free University of Berlin and Dr. Liv Bode from Robert Koch Institute for valuable comments and critical revision of the manuscript. This work was supported by the National Basic Research Program of China (973 Program) (Grant No. 2009CB18300), National Natural Science Foundation of China (Grant No. 30900456) and Natural Science Foundation Project of Chongqing (CSTC, 2008BBB238 and 2010BB5393).

**REFERENCES**


