Increased apoptosis and different regulation of pro-apoptosis protein bax and anti-apoptosis protein bcl-2 in the olfactory bulb of a rat model of depression

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\textbf{ABSTRACT}

Reduced olfactory bulb (OB) volume and olfactory sensitivity have been observed in depressed patients, the exact mechanisms underlying, however, are still unknown. Our previous study found that decreased neurogenesis and pre-synaptic dysfunction in the OB of a rat model of depression may be responsible for the phenomena. Nevertheless, whether the apoptosis would also play a certain role in this process is not clear. In this study, we investigated the apoptosis in the OB of a chronic unpredictable mild stress (CUMS) rat model of depression using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Simultaneously, the pro-apoptosis protein bax and anti-apoptosis protein bcl-2 were detected by Western blot. The results showed that the number of TUNEL-positive cells increased dramatically in the glomerular layer of the OB of the CUMS rats, accompanied with up-regulated expression of bax protein and down-regulated expression of bcl-2 protein. The findings indicate that increased apoptosis may be attributed to explain at some level for the reduced OB volume and olfactory dysfunction in depressed patients. Moreover, the mitochondria–death pathway might be involved in apoptosis in the OB of the CUMS rats.

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Major depression is regarded as the most universal psychiatric disorder, yet the pathogenesis of this mental disorder is poorly understood. Altered hippocampal neurogenesis has been wildly investigated as an imperative element in the pathophysiology of depression [3]. Our previous study reported several changed proteins in the hippocampus of a chronic unpredictable mild stress (CUMS) rat model of depression, to some extent, may be accounted for the decreased neurogenesis by means of a proteomic approach [15]. Moreover, recent evidence suggests that implicated neurogenesis may exist not only in the hippocampus but also in the subventricular zone (SVZ) of depression [6,14]. Furthermore, the olfactory bulbectomised rat has been validated as a model of depression over the past 30 years [26], suggesting a close relationship between olfactory bulb (OB) and depression. Our latest study observed that reduced neurogenesis occurred in the OB of the CUMS rat model of depression [29], which is the most universal utilized model of depression [16]. Notably, in the rat model both a decreased OB volume and olfactory dysfunction were present at the same time [29], what is consistent with clinical profiles in depressed patients [9,18,21,24].

Albeit neurogenesis is affected by depression, the heterogeneity of depression implies that manifold neural substrates and mechanisms that underlie its etiology [4,12,19]. Apoptosis, which is well known as a cell death process that normally occurs in tissue development as well as pathological disorders [1,7], has been profoundly investigated in depression [13]. In one such study, low level of cell death (measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining) was found in the entorhinal cortex, subiculum and the dentate gyrus, CA1 and CA4 regions of the hippocampus of 11 out of 15 depressed patients [10]. Furthermore, increased cell death and expression of pro-apoptosis protein bax as well as decreased expression of anti-apoptotic protein bcl-2 were observed in the hippocampus in the animal models of depression [11,28]. Unlike the hippocampus, little is known about the OB region following chronic stress exposure. Hereby, we aim to investigate whether increased apoptosis would exist in the OB of the CUMS rat model,

\textbf{Abbreviations:} CON, control; CUMS, chronic unpredictable mild stress; GCL, granule cell layer; GL, glomerular layer; HE, hematoxylin and eosin; OB, olfactory bulb; PBS, phosphate buffer saline; RMS, rostral migratory stream; SEZ, subependymal zone; SVZ, subventricular zone; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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and the expression of bax and bcl-2 would be changed in the rat model.

22 healthy adult male Sprague-Dawley rats (weights: 230–280 g; age: 3–4 months) were 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 acclimatized to the laboratory conditions (7 days prior to the start of the experiments), all animals were randomly divided into two main groups: chronic stress (n = 11) or no stress (n = 11). The time schedule is illustrated in Fig. 1. This 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.

The stress procedure followed our previously described method [29]. 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 continuously in 2 days so that animals could not predict the occurrence of stimulation. The stress procedure lasted for four weeks prior to behavioral testing. Control animals were group housed by 3–4 per cage and given ordinary daily care.

The open field test was performed to measure spatial exploration behavior in rodents. Following the procedure, rats were placed in the testing room 30 min before the test started, which took place in a soundproof room between 8:00 AM and 1:00 PM. The apparatus consisting of a black square cage with 100 cm × 100 cm × 40 cm was divided into 25 cm × 25 cm 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, all the 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 technical person, who was blinded to whether the animals were in the control (CON) or CUMS group. The scores were computed for further statistical analysis.

The sucrose preference test, used to define anhedonia, was performed using a two-bottle choice procedure according to our previously described method [29]. Body weight of all animals was measured before beginning the CUMS procedure, and then conducted weekly throughout the CUMS period under similar conditions.

After behavioral tests, rats (n = 5 for each group, selected at random) anesthetized by a chloral hydrate overdose were quickly perfused intracardially with chilled phosphate buffer saline (PBS) (0.01 M, pH 7.4) followed by chilled 4% paraformaldehyde for about 10 min for each rat (about 300 ml of each solution per animal). Through the rostral SVZ, the brain (including frontal cortex 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 TUNEL histochemistry as given below.

For visualization of the DNA fragmentation in the OB, TUNEL staining was performed using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol. To quantify the number of TUNEL-labeled cells, sections were examined microscopically by a Nikon E100 Eclipse microscope, and their images were 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 anatomically between animals. Five predetermined areas (40× objective) (Fig. 2) in the glomerular layer (GL) for TUNEL-labeled cells were analyzed on each section. The number of positive cells in each GL area was averaged to obtain a mean value for each animal.

To detect the expression of the bax and bcl-2 proteins, rats (n = 6 for each group) were sacrificed and the OBs were removed by dissection, immediately frozen in liquid nitrogen and stored at −80 °C. Left OBs were homogenised in a standard lysis buffer, and then sonicated to promote lysis. After that, the samples were 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 25 μg protein were boiled at 95 °C in SDS sample buffer for 5 min, electrophoresed on 15% SDS PAGE gels, and transferred to polyvinylidene difluoride membranes. The membranes were blocked, incubated with the primary antibodies (monoclonal mouse anti-bax 1:1000, Santa Cruz; polyclonal rabbit anti-bcl-2 1:1000, Bioworld). The specific bands were detected using an ECL reagents and the chemiluminescence signal was imaged using a ChemiDoc XRS (Bio-Rad, Hercules, CA). Immunoblots were quantified using Quantity One software (Bio-Rad, Hercules, CA).

![Fig. 1. Time schedule in days for the different procedures used in the study. Open field tests were conducted on days 7 and 38. Sucrose preference test was completed at day 41.](image)

![Fig. 2. Anatomical overview of the olfactory bulb (OB) in a HE stained sagittal section. The boxed areas in the OB are the regions from which counts of TUNEL-positive cells were made. Scale bar = 100 μm. RMS, rostral migratory stream; SEZ, subependymal zone; GCL, granule cell layer; GL, glomerular layer.](image)
All results are given as mean ± SD values. The statistical analyses were carried out using SPSS software. Body weight was analyzed by the 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 for the analysis of significant difference between two groups. All tests were two-tailed. Significance level was set at P < 0.05.

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. With respect to body weight (Fig. 3A), an ANOVA yield a main effect of time \(F_{(4,48)} = 208.27, P < 0.001\), a main effect of group \(F_{(1,24)} = 31.63, P < 0.001\) and significant group by time interaction \(F_{(4,96)} = 29.07, P < 0.001\). No significant differences were 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 three weeks of the CUMS period \(t = 5.08, P < 0.001; t = 10.70, P < 0.001; t = 6.95, P < 0.001\). For the scores of the open field test, no differences were detected at baseline, but animals subjected to CUMS for 28 days showed a significant decrease in the number of locomotion and rears \(t = 7.35, P < 0.001; t = 10.30, P < 0.001\), respectively (Fig. 3B and C). Regarding preferences of sucrose, the CUMS rats showed a dramatic reduction in relative sucrose intake compared with the CON rats \(t = 8.43, P < 0.001\) (Fig. 3D).

To detect the apoptosis in the OB, tissues were analyzed for TUNEL-positive cells. TUNEL-positive cells were wildly distributed in the GL but very few were observed in the GCL (one or two per section) of the OB in the two groups. The number of TUNEL-positive cells in the GL of the CUMS rats was significantly more than that of the CON rats \(t = 3.97, P < 0.01\) (Fig. 4).

To investigate the possibility that the mitochondrion-dependent death pathway might be involved in causing cell death in the OB, Western blot analysis was utilized to measure the levels of two proteins from the bcl-2 family of death-related proteins. Significant increase in the expression of bax but decrease in the expression of bcl-2 was observed in the OB of the CUMS rats \(t = 3.91, P < 0.01; t = 3.57, P < 0.01\), respectively (Fig. 5).

The present data showed that increased TUNEL-positive cells in the OB of the CUMS rats, accompanied with up-regulated expression of bax and down-regulated expression of bcl-2 in the OB. To our knowledge, this is the first study showing increased apoptosis in the OB of the CUMS rat model of depression.

The OB is one of the few areas in the adult mammalian central nervous system that receives a continuous supply of newly generated neurons in the SVZ. In another way, neurogenesis is accompanied by an amount of apoptotic cells in the granule cell layer (GCL) and the glomerular layer (GL) where the newly generated cells are incorporated [5,17] and the volume of the OB does not increase in adult mice [22]. Accumulating evidence suggests that modifications to adult neurogenesis change olfactory processing in various ways, including olfactory perception, odor memory, and fear responses [8]. Here, increased apoptosis was observed in GL of the OB of the CUMS rats, in which input from olfactory receptor neurons is first organized and processed. In terms of olfactory glomeruli act as functional units in coding olfactory information and involve a complex network of synaptic
connections [27], the increased apoptosis in the GL may play a role in the olfactory dysfunction and decreased OB volume in the depressed patients [9,18,21,24]. Moreover, the results further support our previous findings showing the number of mature neurons decreased in the GL of the OB of the CUMS rats [29].

It is well known that the mitochondria plays a crucial role in apoptosis, and bcl-2-related proteins are key factors in the mitochondrial-dependent pathway in the apoptotic cell death process [13]. It has been verified that bcl-2 can block cell death induced by the diverse stimuli [23]. Whereas, a bcl-2-related protein, bax, forms heterodimers with bcl-2 and when overwhelming expressed would antagonize the protective effects of bcl-2 [2,20,23,30,31]. Therefore, the ratio of bax/bcl-2 in cells may play an important role in regulating the sensitivity of the tissue to apoptotic cell death [20,31]. In our present study, significant up-regulated expression of bax and down-regulated expression of bcl-2 were observed in the OB of the rat treated with chronic stress, suggesting an imbalance between bax and bcl-2 in the OB of the CUMS rats. Our results are consistent with previous studies showing different regulation of bax and bcl-2 in the hippocampus of the animal models of depression [11,28].

Although we observed increased apoptosis in the OB of the CUMS rats in the present study and decreased neurogenesis in our previous study [29], it calls for further research to investigate whether patients with depression would also exist the same phenomena. It should be aware of that in the rat olfaction is a vital sensory modality, while the sensory neocortex is less well developed than that in primates [25], suggesting there might at least be some neuroanatomical differences between rats and humans at the OB level.

In summary, the present study provides evidence that increased apoptosis does exist in the OB of the CUMS rats. Furthermore, up-regulated expression of bax and down-regulated expression of bcl-2 might play a key role in the increased apoptosis in the OB of the CUMS rats. Further studies are needed to investigate the molecular and cellular mechanisms underlying the differential expression of the bax and bcl-2 proteins and whether antidepressants would coordinate the expression of anti- and pro-apoptotic proteins as well as counteract the increased apoptosis in the OB of the CUMS rats.

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