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ORIGINAL ARTICLE

Repeated clomipramine treatment reversed the inhibition of cell proliferation in adult hippocampus induced by chronic unpredictable stress

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Adult hippocampal neurogenesis has been demonstrated in several species and is regulated by both environmental and pharmacological stimuli. Repeated exposure to stress is known to induce the reduction of neurogenesis in the dentate gyrus (DG) of hippocampus. The present study aimed at determining whether the clinically effective antidepressant clomipramine may influence hippocampal proliferation and neurogenesis in adult rats subjected to the chronic unpredictable stress (CUS) procedure, a model of depression with predictive validity. Repeated administration of clomipramine (5 mg kg^{-1}, intraperitoneal) for 3 weeks, starting 2 weeks after the beginning of the stress procedure, significantly reversed the reduction of behavior measured by open-field test and forced swimming test. Moreover, rats subjected to stress exhibited a 49.9% reduction of cell proliferation at the end of a 5-week stress period, an effect which was suppressed by clomipramine treatment. These results demonstrated that exposure to CUS, which results in a state of behavioral depression, decreases hippocampal cell proliferation and that these effects can be counteracted by chronic clomipramine treatment.
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Introduction

Depression and anxiety disorders, with 10–20% lifetime prevalence, are significant public health problems. Despite a number of preclinical and clinical investigations, little is known about the etiology of depression and the mechanisms responsible for the therapeutic effects of antidepressant drugs.[1] The hippocampus is an area of the brain that has been extensively studied with regard to the etiology of depression and antidepressant agents.[2,3] Clinical evidence is emerging that hippocampal volume is decreased in depressive disorders and antidepressants reverse this decrease.[4,5] Recently, imaging studies have reported that a reduction in hippocampal volume is correlated with the length of depressive illness, indicating that the change in hippocampal volume may be caused by depression.[6,7]

Within the hippocampal formation, dentate gyrus (DG) is one of the few brain regions where production of new neurons occurs even in the adult mammalian brain.[8–12] Neurogenesis is defined by the proliferation of progenitor cells, giving

rise to cells that migrate into the granule cell layers, differentiate into neurons[13,14] and ultimately make functional synaptic connections with the hippocampal circuitry.[15] A large number of variables regulate adult hippocampal neurogenesis such as age, strain, gender, hormones, environment, exercise and learning.[16] Among the regulatory factors of neurogenesis, stress, which has been identified as a potential inhibitor of dentate cell proliferation,[17,18] can precipitate or worsen depression[19,20] and is often used as a model in preclinical studies.[21] In contrast, antidepressant treatment increases adult cell proliferation and neurogenesis in the hippocampus of normal rats, and the time course for this effect (that is, chronic, but not acute treatment) is consistent with the time course for the therapeutic action of antidepressants.[22,23] Additionally, it has been reported that the hippocampal neurogenesis might play an important role in the behavioral effects of antidepressants.[24] All these demonstrated that impairments in hippocampal neurogenesis could lead to the development of the depressive state while the antidepressants could prevent the development of depressive state by improving neurogenesis.[16,23] Therefore, the present study was designed to investigate whether chronic administration of clomipramine, one of the tricyclic antidepressants, influences the behavioral activity and newborn cell proliferation in the DG of depression model rats.

On the basis of the clinical evidence that links stressful life events with depressive episodes,[25] several animal models of depression have been developed. The chronic unpredictable stress (CUS) procedure has been proposed as a model of depression,[26] in that it has been extremely useful in elaborating and detecting the effects of antidepressant drugs. Earlier investigation has shown that chronic stress affect cytogenesis in the DG.[27] Recently, application of CUS was shown to dramatically reduce synaptic long-time potential in the DG.[28] Since CUS causes the occurrence of physical abnormalities reminiscent of certain symptoms of human depression, we measured both open-field test and forced swimming test, two behavioral paradigms, which have frequently been used in studies over the entire stress and treatment period. Clomipramine is a well-established tricyclic antidepressant used in clinical practice and stressed animals.[29,30] In this study, the daily intraperitoneal (i.p.) application of vehicle and clomipramine started after the stress-induced behavioral alterations had been established. The action of clomipramine was followed across a clinically relevant time period of 3 weeks while the stress continued during the whole treatment period. In these experiments, cell proliferation was measured by incorporation of 5-bromo-2-deoxyuridine (BrdU), a thymidine analog that labels dividing cells in the S-phase.[31] Phenotypic development of newborn cells was determined using specific neuronal markers.

Results

Exposure to CUS results in the decrease of behavioral activity and repeated clomipramine treatment reverses this effect

The behavioral tests were conducted as Figure 1. In the open-field test, stressed rats demonstrated a typical decrease in the number of crossings and rearings, whereas clomipramine treatment increased these behavioral activities (Figure 2). Before CUS, the number of crossings of all rats were not significantly different ($F_{(4, 35)} = 0.635$, $P > 0.05$). Animals in stress and stress + saline groups showed a significant decrease in the number of crossings in the second week, which continued to the fifth week when the experiment ended ($F_{(4, 35)} = 55.025$, $P < 0.05$). In stress with clomipramine group, the number of crossings decreased significantly 2 weeks after exposed to the stressors ($F_{(4, 35)} = 34.990$, $P < 0.05$). After clomipramine treatment for 3 weeks, the number of crossings had a significant increase compared to the saline treatment animals ($F_{(4, 35)} = 55.025$, $P < 0.05$) (Figure 2a). Similar changes were seen in the vertical activity (number of rearings) of all groups rats (Figure 2b).

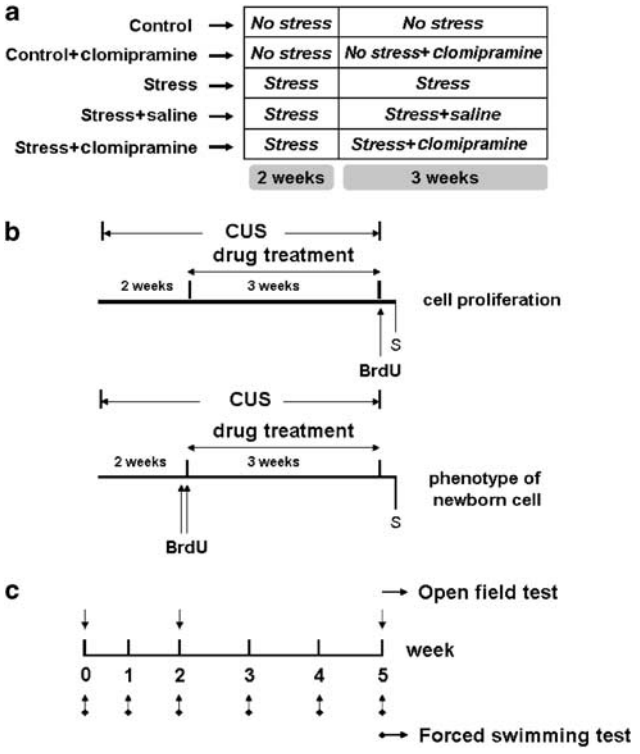

Figure 1 Animal groups (**a**), schematic representation of the experimental procedure (**b**) and behavioral test (**c**). Rats were randomly divided into five groups: control, control + clomipramine, stress, stress + saline, stress + clomipramine ($n = 8$ per group). Rats were subjected to a variety of chronic stressors (CUS) during 5 weeks, whereas animals of the normal group (Control) remained undisturbed. Two weeks after CUS initiated, rats were injected with clomipramine (5 mg per kg per day) or saline treatment once a day for 3 weeks. For analysis of cell proliferation, 50% of the rats received a single injection of BrdU (100 mg kg^{-1} i.p.) on the last day of 5 weeks period, and killed (S) 24 h after BrdU administration. For analysis of the cell survival and phenotype of newborn cells, another 50% of rats were administered with BrdU injection (100 mg per kg per day, i.p.) for 2 days before 3 weeks of drug treatment, and were killed 24 h following the final drug treatment. Parallel groups ($n = 8$ per group) of animals were prepared for behavioral tests. Open-field test was measured before stress, drug administration, and at the end of the experiment. Forced swimming test was measured, respectively, at the end of every week.

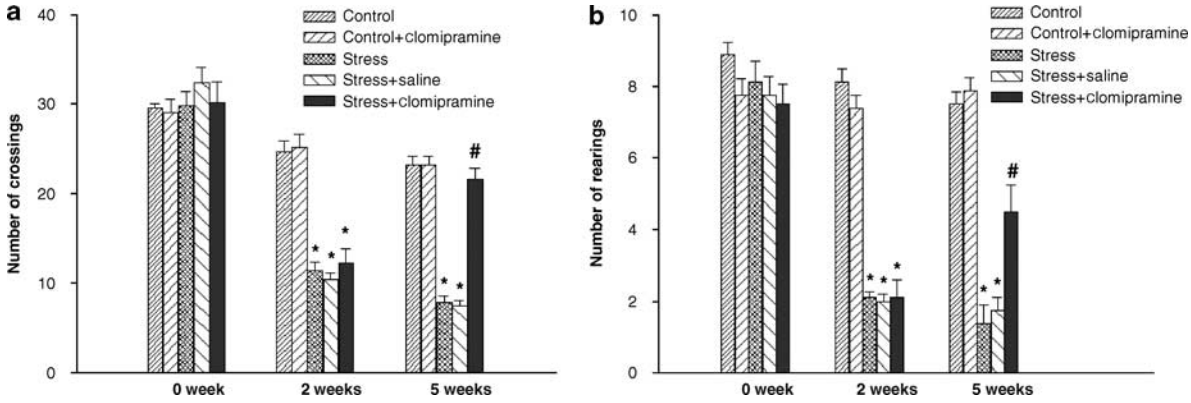

Figure 2 Open-field behaviour in rats after CUS procedure and clomipramine treatment. Rats were injected with either clomipramine (5 mg per kg per day) or saline 2 weeks after the stress procedure commenced. Animals performed the open-field test before CUS, 2 and 5 weeks after CUS was initiated. (**a**) Number of crossings during the 3-min session. (**b**) Number of rearings during the 3-min session. Results are given as mean ± s.e.m. ($n=8$ per group). *$P<0.05$, **$P<0.01$, stress groups compared to control; $^{\#}P<0.05$, stress + clomipramine group compared to stress + saline.

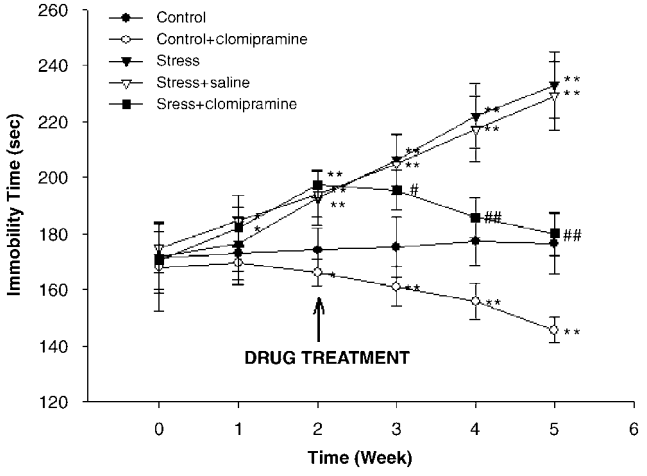

Figure 3 Immobility time in the forced swimming test during the experimental procedure. Rats were injected with either clomipramine (5 mg per kg per day) or saline 2 weeks after CUS was initiated for a further 3 weeks. Immobility time was measured during the first 5 min of forced swimming on the beginning of every week. Results are expressed as mean ± s.e.m. ($n=8$ per group). *$P<0.05$, **$P<0.01$, Stress groups compared to Control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, stress + clomipramine group compared to stress + saline.

In the forced swimming test, the immobility time was measured during the first 5 min of swimming. At the beginning of the experimental procedure, there were no significant differences among the groups exposed to forced swimming ($F_{(4,35)}=0.334$, $P>0.05$). After CUS for 2 weeks, stressed animals (stress, stress + saline and stress + clomipramine groups) showed a significant increase in immobility time ($F_{(4,35)}=28.207$, $P<0.01$). Stress + saline and stress + clomipramine group animals were injected with saline and clomipramine, respectively; 2 weeks after CUS was initiated for a further 3 weeks while the stress procedure were continued. At the end of the 5th week, the stress and stress + saline group animals had a significantly greater increase in immobility time ($F_{(4,35)}=70.163$, $P<0.01$). However, stress + clomipramine group animals revealed a significant decrease in immobility time compared with the saline-treated animals ($F_{(4,35)}=37.724$, $P<0.05$), which suggested that clomipramine blocked the stress-induced change of behavioral activity. In addition, the control + clomipramine group has a significant decrease in the immobility time during the experiment ($F_{(4,35)}=28.207$, $P<0.05$) (Figure 3).

Exposure to CUS results in a significant decrease in cell proliferation of hippocampus and clomipramine treatment suppresses the stress-induced effect

BrdU immunohistochemistry revealed dividing cells in the DG of five group rats. The majority of BrdU-positive cells were located in the subgranular zone and generally occurred singly or in small clusters of 3–5 cells (Figure 4a). CUS for 5 weeks resulted in a significant 49.9% ($F_{(4,15)}=14.091$, $P<0.01$) decrease in the number of BrdU-positive cells relative to control rats (Figures 4b and f). Clomipramine treatment of stressed rats showed a 102.3% ($P<0.01$) increase in the number of BrdU-positive cells in the DG compared with the stress + saline group (Figures 4e and f). There were no differences in the cell proliferation ($4650.26+556.01$ vs $4250.37+125.92$, $P=0.412$) between the control group and the control + clomipramine group (Figures 4c and f). The cell proliferation in the hippocampus was evaluated at the end of the experimental procedure (Figure 4f). Comparison of BrdU-labeled cells in saline treatment group with clomipramine treatment group showed that clomipramine remarkably increased the number of BrdU-positive cells in DG, which suggested that the clomipramine treatment suppressed the decrease of hippocampal cell proliferation induced by CUS.

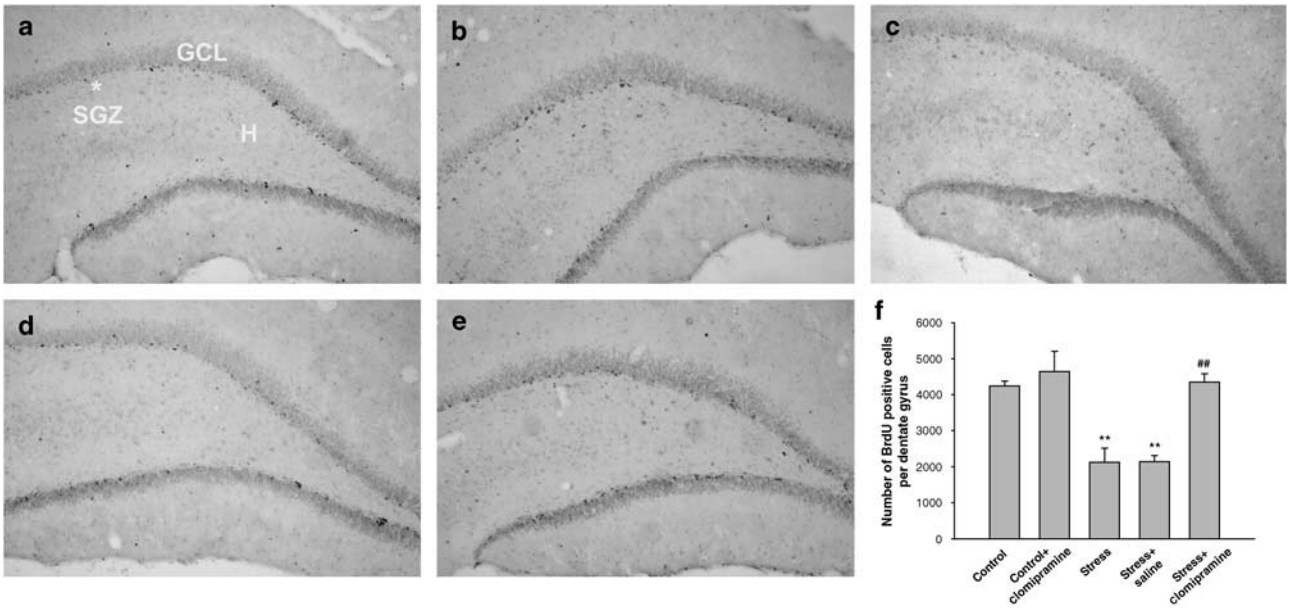

Figure 4 Exposure to CUS decreases the number of BrdU-positive cells in the adult hippocampus, whereas chronic clomipramine treatment suppressed the stress-induced effect. Rats received single injection of BrdU on the last day of 5 weeks stress period and were killed 24 h later. The majority of the BrdU-labeled cells are located in the subgranular zone (SGZ, indicated by asterisk in ((**a**)) of the hippocampus, the region between the granule cell layer (GCL) and hilus (H). The total number of BrdU-labeled cells were counted including GCL, SGZ and H. The representative photomicrographs ($\times 100$ magnification) of proliferating cells from (**a**) control, (**b**) stress (**c**) control + clomipramine, (**d**) stress + saline, (**e**) stress + clomipramine (**f**) are shown. The results are expressed as the estimated mean total number ($\pm$ s.e.m.) of BrdU-labeled cells per DG region ($n=4$ per group). $^{*}P<0.05$, $^{**}P<0.01$, stress groups compared to control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, stress + clomipramine group compared to stress + saline.

Exposure to CUS affects the cell survival and differentiation of DG and clomipramine counteracts the suppression of neurogenesis

To determine whether clomipramine treatment produces long-term effects on the incorporation of BrdU into dividing cells, animals were given BrdU before the clomipramine or saline treatment. Confocal microscopy analysis was carried out to verify the colocalization of fluorescent signals originated from the same cell. Colocalization studies of BrdU with neuronal markers revealed that the majority of the proliferating cells in the rats with clomipramime treatment were neurons (BrdU + PSA-NCAM: 68.2%, BrdU + TUJ-1: 63.3%). BrdU-positive cells coexpressed the marker for young neurons PSA-NCAM (Figures 5a–c) and differentiated into mature TUJ-1-positive granule neurons (Figures 5d–f).

The number and phenotype of DG BrdU-labeled cells was determined 3 weeks after the last BrdU administration, a time interval sufficient to allow newly generated cells to migrate and differentiate. At this time, BrdU-positive cells were mostly concentrated within the granule cell layer and their absolute number in nonstressed rats was lower than that evaluated 24 h after BrdU injection (number of BrdU-positive cells 24 h after BrdU: $4250.37 + 125.92$, $n=4$; 3 weeks after BrdU administration: $2499.18 + 85.38$, $n=4$). Analysis of BrdU labeling revealed a significant main effect for the number of BrdU-positive cells in the DG ($F_{(4, 15)} = 11.019$, $P<0.01$). CUS produced a 53% decrease in the number of BrdU immunoreactive cells ($P<0.01$) in saline-treated rats relative to the control animals (Figure 5g), while in clomipramine-treated animals, the number of BrdU immunoreactive cells was significantly increased when compared with saline-treated rats ($2340.23 + 375.47$ vs $1185.29 + 151.82$, $P<0.01$). There was no major difference in phenotypic expression patterns among five groups (Figure 5h).

Discussion

The CUS paradigm in rats is a valid model of depression, as it satisfies the criteria of correlation, isomorphism and homology.[32,33] Here, we confirmed that chronically stressed rats exhibited a marked degradation of behavior, an effect which lasted until the end of the CUS. Because the etiology of depression is very complicated, the estimates of depression animal models include many behavioral tests, such as open-field test, forced swimming test, elevated plus maze test and so on. It is very necessary to integrate those tests to prove the depression model or the efficiency of antidepressants. In line with the idea that stress-induced behavioral change may represent a valid measure of depression in rats are the present findings that animals exposed to the CUSs have been depressed. Furthermore, clomipramine treatment improved the CUS-induced depressive state. This result was in line with previous researches on behavioral change of antidepressants. It has been reported that repeated admin-

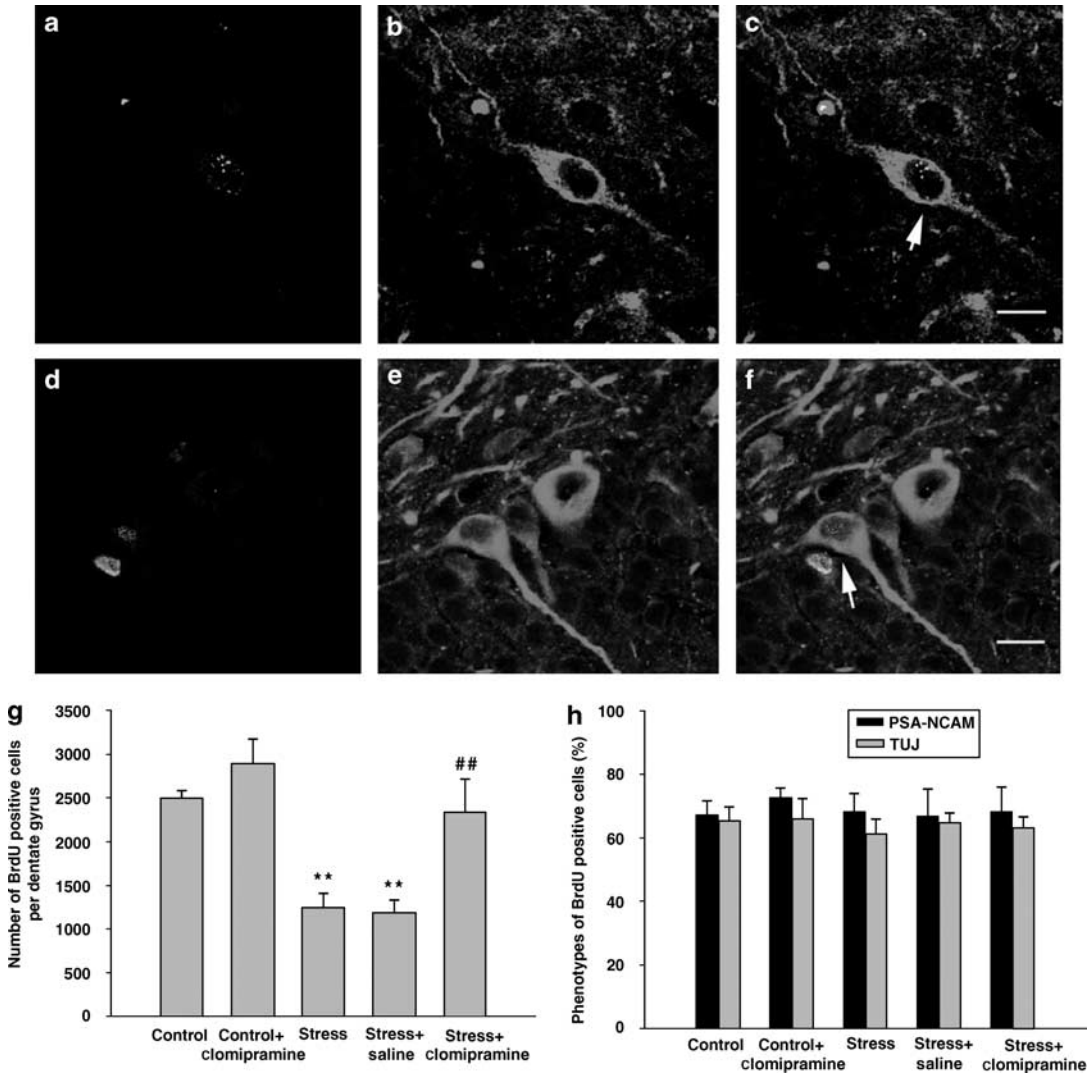

Figure 5 Phenotype of proliferating cells in the DG of hippocampus and clomipramine counteracted the CUS-induced suppression of neurogenesis. Confocal scanning laser images of sections were double-labeled to show BrdU (**a** and **d**) and a marker for post-mitotic young neurons PSA-NCAM (**b**), or a marker for mature neurons TUJ-1 (**e**). These images are merged in the right panels (**c** and **f**). Scale bar: 25 μm. Double-labeled cells are indicated by arrows. Rats were subjected to a 5-week stress period and were treated with clomipramine during the last 3 weeks. BrdU was administrated for 2 days before 3 weeks of drug treatment. The number of surviving BrdU-labeled cells in the DG was significantly decreased in stressed as compared to nonstressed controls, whereas chronic clomipramine treatment counteracted the stress-induced effect (**g**). The data was expressed as the estimated mean total number (± s.e.m.) of BrdU-labeled cells per DG region ($n = 4$ per group). *$P < 0.05$, **$P < 0.01$, Stress groups compared to the control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, stress + clomipramine group compared to the stress + saline. In nonstressed and stressed rats, the majority (67%) of surviving BrdU immunoreactive cells matured into neurons, and the phenotypic expression patterns remained unchanged in drug-treated rats (**h**).

istration of fluoxetine, a serotonin selective reuptake inhibitor and one of the most prescribed antidepressants, improved the degradation of the physical state of the coat and the loss of coping behavior produced by stress.[34] Recent study also showed that agmatine could reverse the chronic stress-induced decrease of open-field behavior.[35] Our research provided further evidence for the efficacy of clomipramine in depression models, notably in the CUS procedure.

The newborn cell proliferation was observed after stressed for 5 weeks. The results showed that CUS remarkably reduced the rate of newborn cell proliferation, as evidenced by the marked decrease (49.9%) in the number of BrdU-positive cells in the DG, whereas clomipramine treatment of stressed rats showed a 102.3% increase in the number of BrdU-positive cells in the DG compared with the saline treatment group. These demonstrated that the decreased cell proliferation induced by CUS in rats was suppressed by repeated treatment with clomipramine. Previous reports showed the deleterious effect of stressful events on hippocampal newborn cell proliferation in various animal species, which have been reported following predator odor exposure

in rats,[36] social stress in marmosets[17] and tree shrews,[37] and prenatal or repeated restraint stress in rats.[18,38] So far, antidepressant drugs such as clomipramine were shown to stimulate newborn cell proliferation in stressed tree shrews,[39] a finding confirmed here in rats. Moreover, we examined the effect of clomipramine on normal rats. The results showed that clomipramine treatment displayed no effect on the cell proliferation of DG, while improved the CUS-induced depressive behavior. Interestingly, previous research showed that antidepressants treatment, such as fluxetine, tranylcypromine, olanzapine and so on, could promote the cell proliferation in normal animals.[22] The possibility induced the difference between former researches and present research might be due to the distinct mechanism of different antidepressants.

In the present study, BrdU was injected before the clomipramine treatment, which was followed by exposure to unpredictable stressors for 2 weeks, and this enabled us to detect the cell survival and the phenotype of newborn cells. Heine *et al*.[40] investigated whether suppressed proliferation changes in the rat DG after chronic stress could be recovered after 3 weeks. However, stress exposure for longer periods may have different effects and the detection of behavior is deficient. We found that hippocampal neurogenesis could be correlated with the behavioral changes of rats. The present study showed that clomipramine treatment not only improved the stress-induced depressive behavior but also suppressed the decreased hippocampal neurogenesis in stress rats. Previous investigations have demonstrated that antidepressant treatment can block the effects of stress on neurogenesis in the adult brain. The influence of maternal separation stress on neurogenesis in young rats (14–21 days) is reversed by chronic fluoxetine administration.[41] Chronic administration of an atypical antidepressant, tianeptine, blocks the effects of subordination stress on neurogenesis in the hippocampus of adult tree shrews.[42] A recent study also found that chronic administration of either a corticotrophin releasing factor receptor-1 or arginine vasopressin receptor-1b antagonist blocks the downregulation of neurogenesis caused by chronic mild stress.[43] Another study reported that fluoxetine treatment also counteracted the suppressed cytogenesis in the medial prefrontal cortex and neurogenesis induced by chronic stress.[44] In line with these researches, the normalization of cell proliferation by 3 weeks of clomipramine treatment in present study could reflect an action of antidepressant treatment (increase) and CUS (decrease) on cell proliferation of DG. The present results suggested that the effect of clomipramine on the depression model rats was a specific response to normalize the CUS-induced behavioral deficits and the change of hippocampal neurogenesis.

Regulation of neurogenesis occurs on several levels, such as cell proliferation, differentiation, migration and survival. Over time, progenitor cells in the DG give rise to cells that migrate into the granule cell layers and ultimately differentiate into mature neurons or astroglia.[12,22,45,46] To identify the phenotype of the newborn cells in the present study, rats were injected with BrdU before clomipramine treatment and killed after 3 weeks of drug treatment. Immunohistochemical results showed that the majority of the proliferating cells were neurons. Previous researches also demonstrated that 3 weeks after BrdU injection, about 65% of BrdU-labeled cells expressed the neuronal marker neuron-specific enolase, and were incorporated into the granule cell layer.[22,24] In addition, a recent study suggested that the decrease in BrdU-labeled cell number reflects diminished adult neurogenesis, as double-labeled with BrdU and TUJ-1 revealed no differences in the percentage of new cells that expressed this marker among our groups.[39] The similar result was observed in our research. All these results indicated that incorporation of new neurons into the DG was severely decreased by chronic stress, whereas clomipramine normalized the decrease in neurogenesis induced by CUS in hippocampus.

In summary, the present researches suggested that clomipramine could improve stress-induced depressive behavior and suppressed neurogenesis in the DG of hippocampus. Clomipramine might play an antidepressant role through regulating neurogenesis in hippocampus. The underlying mechanisms how neurogenesis contributed to the external stress-induced depression should be further investigated.

Materials and methods

Animals

Experiments were performed on adult male Sprague–Dawley rats (Experimental Animal Center, Shanghai Medical College of Fudan University, China) weighing 250–270 g. All rats were allowed to acclimatize for 1 week before experimental manipulation and were maintained on a 12:12 h light/dark cycle with free access to food and water. These rats were randomly divided into five groups: control, control + -clomipramine, stress, stress + saline, stress + clomipramine ($n = 16$ per group) and housed in separate cages. Each stress procedure and behavioral test was carried out in a separate quiet room. All the experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications no. 8023, revised 1978) and the number of experimental rats was kept to a minimum.

Experimental procedure and drug treatment

The experiment was conducted as Figure 1. Two weeks after the beginning of the CUS, rats were received clomipramine (5 mg kg^{-1}, i.p.) or saline treatment once a day for 3 weeks. Parallel groups of animals were prepared for behavioral tests. Open-field test was measured before stress, drug administration and at the end of the experiment. Forced swimming test was measured, respectively, at the end of every week.

CUS procedure

Stress-group rats were subjected to the CUS procedure.[47] Various stressors were changed randomly each day. The stressors applied included (frequency every 3 weeks in parentheses) water deprivation for 40 h (3), swimming in

4 °C water for 5 min (3), food deprivation for 40 h (3), flatly shaking at high speed for 30 min ((120 r.p.m.) (3)), reversal of the light/dark cycle (2), and heating in a 45 °C chamber for 5 min (3). All efforts were made to minimize animal suffering. The control rats remained undisturbed in their cage for the procedure.

Open-field test

The open-field test was performed as described previously,[48] and was carried out before stress (0 week), 2 weeks after stress (2 weeks) and 5 weeks after stress (5 weeks). The open-field apparatus consisted of a $100 \times 100 \times 40$ cm wooden cuboids, which was covered inside with foil to increase the reflectivity of the walls. The floor of the cube was divided into 16 squares. A 60-W light bulb was positioned 90 cm above the base of the apparatus, and was the only source of illumination in the room. Each animal was placed in the center of the apparatus and allowed to explore freely for 3 min. During the test time the number of crossings (defined as at least three paws in a quadrant) and the number of rearings (defined as the animal standing upright on its hind legs) were measured. After exploring each animal, the test apparatus was cleaned with a 10% ethanol solution and water to remove any olfactory cues.

Forced swimming test

The design of the forced swimming test was adapted from previous description.[49] Briefly, rats were forced to swim individually in a cylindrical glass container (40 cm height, 18 cm diameter), which contained tap water (25 ± 1 °C) to a depth adjusted for the weight of the individual animal, so that its hind paws could just touch the bottom of the container. At first, rats were placed in the water for 15 min, and retested for another 5 min after 24 h. After the rest, rats were dried with a towel and returned to their home cages. After 1 week, the animals were re-exposed to the forced swimming for a further 5 min. The test sessions were recorded and scored by an observer who was blind to the groups of animals. The behavioral test were subsequently scored according to the criteria of previous investigation.[50] The duration of immobility was measured at the beginning of every week during the experimental procedure, judging the rat to be immobile whenever it remained floating passively in the water in a slightly hunched but upright position, with its head just above the surface.

BrdU injection

To examine the proliferation of precursor cells, rats were killed 24 h after a single injection of BrdU (200 mg kg^{-1}, i.p.). To study the effects of clomipramine treatment on the phenotype of proliferating cells, rats received BrdU (100 mg per kg per day, i.p.) injection for 2 days before the drug and saline treatment, and were killed 24 h following the final drug treatment.

Perfusion and tissue storage

Rats were given an overdose of urethane (1.5 g kg^{-1}, i.p.) and perfused through the ascending aorta with 200 ml of normal saline followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed from the skulls, postfixed for 4 h in the identical fixative solution at 4 °C, and immersed in 30% sucrose in phosphate buffer for 24–48 h at 4 °C for cryoprotectant. Serial sections of brain were cut (35 μm sections) through the entire DG on a freezing microtome (Leica CM1900, Germany) and stored in cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05 M phosphate buffer (pH 7.4)) at −20 °C until ready for use.

BrdU immunohistochemistry

Free-floating tissue sections were processed for BrdU immunohistochemistry. DNA denaturation was conducted by incubation for 2 h in 50% formamide/2 × SSC at 65 °C, followed by several phosphate-buffered saline rinses. Sections were incubated for 30 min in 2 N HCl at 37 °C and then 10 min in boric acid. After washing in phosphate-buffered saline, sections were incubated for 10 min in 0.3% H_2O_2 to eliminate endogenous peroxidases. After blocking with 3% normal horse serum in 0.01% Triton X-100, sections were incubated with sheep anti-BrdU (1:200; Biodesign, Saco, ME, USA) overnight at 4 °C. Sections were then incubated for 1 h with a secondary antibody (1:200; biotinylated donkey anti-sheep, Jackson Immunoresearch, West Grave, PA, USA) followed by amplification with an avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA), and were visualized by catalysis of 3,3-diaminobenzidine by horseradish peroxidase in the presence of 0.03% H_2O_2. Finally, the sections were dehydrated and mounted. To test the specificity of the primary antibody, controls were performed including the substitution of normal horse serum for the primary antibody and omission of the primary antibody. None of these controls showed any sign of immunohistochemical reaction.

Immunofluorescence labeling

To determine the phenotypes of proliferating cells in clomipramine treatment rats, sections were double-labeled for BrdU, PSA-NCAM (polysialic acid-neural cell adhesion molecule, a marker of immature neurons) or TUJ-1 (class III-tubulin, a marker of mature neurons). Sections were first pretreated by incubation in 50% formamide/2 × SSC for 2 h at 65 °C, rinsed in phosphate-buffered saline, incubated in 2 N HCl for 30 min at 37 °C and rinsed in borate buffer (0.1 M, pH 8.5) for 10 min. After blocking in 0.1% Triton X-100, 3% normal horse serum, phosphate-buffered saline, sections were incubated for 2 days at 4 °C in sheep anti-BrdU (1:200; Biodesign) and one of the following: mouse anti-PSA-NCAM monoclonal immunoglobulin G (1:200; Millipore, Billerica, MA, USA), and mouse anti-TUJ-1 monoclonal immunoglobulin G (1:100; R&D system Inc., Minneapolis, MN, USA). Secondary antibodies were fluorescein isothiocyanate-conjugated donkey anti-sheep immunoglobulin G (1:200; Sigma, St Louis, MO, USA) and Cy3-conjugated donkey anti-mouse F(ab)2 fragment (1:100; Jackson Immunoresearch), and were then applied for 1 h. The sections were rinsed in phosphate-buffered saline, mounted in with MOWIOL reagent (Calbiochem, San Diego, California, USA)

and visualized with confocal Z-plane sectioning by a Leica TCS SP2 (Leica, Germany).

At least 50 BrdU-positive cells per animal were analyzed using Z-plane sectioning (1 μm steps) to confirm the colocalization of BrdU and the marker TUJ-1 or PSA-NCAM. The percentage of BrdU-labeled cells of neuron phenotype was determined.

Quantitation of BrdU labeling

A modified unbiased stereology protocol was used that has been reported to successfully quantify BrdU labeling. Every eighth section throughout the hippocampus of each rat (Bregma −2.8 to −4.8) was processed for BrdU immunohistochemistry. All BrdU-labeled cells in the DG (subgranular zone, granule cell layer and hilus) were counted in each section by an experimenter blinded to the study code. To distinguish single cells within clusters, all counts were performed at $\times 400$ and $\times 1000$ under a light microscope (Leica, Germany), omitting cells in the outermost focal plane. The total number of BrdU-labeled cells per slice was determined and multiplied by 8 to obtain the total number of cells per DG. To confirm that the BrdU was labeling newborn cells and not cells undergoing DNA repair, mitotic figures were observed in each hippocampal slice.

Statistical analysis

Data are presented as mean ± s.e.m. and analyzed by SPSS 11.0. Repeated measures analysis of variance followed by Student–Newman–Keul test was used for *post hoc* analysis for differences between groups. $P<0.05$ was considered statistically significant.

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Duality of interest

We declare that we have no duality or conflict of interest.

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