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Research report

Beneficial effects of benzodiazepine diazepam on chronic stress-induced impairment of hippocampal structural plasticity and depression-like behavior in mice

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ABSTRACT

Whether benzodiazepines (BZDs) have beneficial effects on the progress of chronic stress-induced impairment of hippocampal structural plasticity and major depression is uncertain. The present study designed four preclinical experiments to determine the effects of BZDs using chronic unpredictable stress model. In Experiment 1, several time course studies on behavior and hippocampus response to stress were conducted using the forced swim and tail suspension tests (FST and TST) as well as hippocampal structural plasticity markers. Chronic stress induced depression-like behavior in the FST and TST as well as decreased hippocampal structural plasticity that returned to normal within 3 wk. In Experiment 2, mice received p.o. administration of three diazepam dosages prior to each variate stress session for 4 wk. This treatment significantly antagonized the elevation of stress-induced corticosterone levels. Only low- (0.5 mg/kg) and medium-dose (1 mg/kg) diazepam blocked the detrimental effects of chronic stress. In Experiment 3, after 7 wk of stress sessions, daily p.o. diazepam administration during 1 wk recovery phase dose-dependently accelerated the recovery of stressed mice. In Experiment 4, 1 wk diazepam administration to control mice enhanced significantly hippocampal structural plasticity and induced an antidepressant-like behavioral effect, whereas 4 wk diazepam administration produced opposite effects. Hence, diazepam can slow the progress of chronic stress-induced detrimental consequences by normalizing glucocorticoid hormones. Considering the adverse effect of long-term diazepam administration on hippocampal plasticity, the preventive effects of diazepam may depend on the proper dose. Short-term diazepam treatment enhances hippocampal structural plasticity and is beneficial to recovery following chronic stress.

1. Introduction

Chronic mild or traumatic stress is detrimental to the central nervous system. In humans, stress is perceived to initiate or exacerbate a number of psychiatric disorders, such as posttraumatic stress syndrome and major depression [1–4]. In rodents, repeated restraint stress can induce depression-like behaviors [5–8].

Increasing number of data demonstrate that hippocampal structural plasticity (such as plastic changes in spine and dendrite morphology as well as adult neurogenesis) is disrupted in mood disorders and in animal models of stress [9–11]. Hippocampal atrophy has been repeatedly documented [12,13] and is possibly due to the irreversible loss of hippocampus pyramidal cells [14,15], atrophy of apical dendrites [16–18], and decreased cytoskeletal neurofilament levels [19,20]. Many different forms of chronic stress reduce adult neurogenesis in the hippocampus [21–24], and impaired neurogenesis has been hypothesized to represent a core pathophysiological feature of major depression [21].

Adrenal glucocorticoid hormones, principal effectors in the stress response, can be responsible for stress-induced impairment of hippocampal plasticity. The deleterious effects of chronically elevated glucocorticoids on hippocampal function, seen in Cushing's patients or as a result of steroid therapy, have long been recognized [25]. In rodents, chronic corticosterone injection caused a pronounced loss of synapses [18,26] and decreased brain-derived neurotrophic factor (BDNF) protein in hippocampal area CA3 [27]. The recent findings that blockade of glucocorticoid receptors or anti-glucocorticoid gene therapy reverse the impairing effects of chronic stress or elevated corticosterone further confirm the crucial role of glucocorticoid hormones in stress-induced impairment of hippocampal plasticity [28,29].

Benzodiazepines (BZDs) are among the most frequently prescribed drugs because of their anxiolytic, hypnotic, sedative, amnesic, antiepileptic, and muscle relaxant properties [30]. A significant increase in the use of BZDs for patients suffering from

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stress-induced anxiety, dysphoria, and insomnia has been noted for the last decade despite the known risks of these drugs [31–33]. The use of BZDs is especially controversial in China, where drug abuse is a serious challenge. However, whether BZDs have beneficial effects on stress-induced mental disorders remains unclear. Furthermore, whether BZDs are advantageous, the benefit is associated with the improved hippocampal structural plasticity and glucocorticoid hormones, or the benefit of BZDs depends on the correct duration and dosage remain to be elucidated.

Four preclinical experiments were performed in male C57BL/6N mice in the current paper. First, the effects of stress on the hippocampal structural plasticity and depression-like behavior were dynamically observed to obtain the proper time point of BZD administration and index observation in the following experiments using behavioral tests and structural plasticity markers (BDNF, a marker of neurogenesis; SYP, synaptophysin, a synapse marker; and NF-L, neurofilament light chain, a dendrite marker) (Experiment 1). Second, whether diazepam (one of the most widely used BZDs) administered during stress may retard the development of depression-like behaviors in mice under chronic stress was determined (Experiment 2). Third, whether diazepam may promote the stressed mice to recover following stressor withdrawal (Experiment 3) was also examined. Finally, the impact of short- and long-term dosages of diazepam administration on depression-like behavior and hippocampal structural plasticity in the control mice was investigated to discuss the suitable administration duration and dosage (Experiment 4).

2. Materials and methods

2.1. Experimental animals

Male C57BL/6N mice (18–20 g, 2 months old) obtained from the Laboratory Animal Center of Nanjing Medical University, Nanjing, China were housed in groups of five in home cages made of Plexiglas (25 cm × 15 cm × 10 cm) with sawdust bedding. The animals were maintained under a standard dark–light cycle (lights on between 7:00 and 19:00) at room temperature of 22 ± 2 °C. The mice had free access to food and water, except for the stressed group, during the period when the stressor applied required no food or water. Prior to the experiments, mice were habituated to daily handling during the week after delivery. All animal treatments were in accordance with the Guidelines of Accommodation and Care for Animals formulated by the Chinese Convention for the protection of vertebrate animals used for experimental and other scientific purposes. All efforts were made to minimize animal suffering and reduce the number of animals used for experiments.

2.2. Chronic unpredictable stress model

The chronic unpredictable stress protocol was modified from other models [34,35]. The animals were divided into two groups, namely, control and stressed. Controls were kept undisturbed in their home cages during the treatment. A variate-stressor paradigm was used for the animals in the stressed group. Individual stressors and length of time applied each day during the week are listed in Table 1. The following stressors were used: (a) 24 h food deprivation, (b) 24 h water deprivation, (c) 6 h restraint, as described below, (d) flashing lights for 120–210 min, as described below, (e) 20 min of random intermittent foot shock, as described below, and (f) 24 h wet sawdust bedding. Stress application started at different times to minimize predictability. For restraint stress, the animal was placed in a 3 cm × 10 cm plastic tube enclosed at one end with plaster tape to immobilize the animal. There was a 1 cm hole at the other end for breathing. For flashing light stress, animals were placed in a 25 cm high, 20 cm × 30 cm open field made of brown plywood with a frontal glass wall. A 40 W lamp flashed at 60 flashes/min. Foot shocks were administered through the grid floor of shock boxes (NatureGene Corp., Beijing, China). The 15 cm wide and 18 cm long boxes were partitioned so that two mice could be shocked in one box. A random shock generator was used to deliver approximately 20 shocks of 0.2 mA for a duration of 1 s within a 20 min period.

Table 1
One week representative schedule[a] of stressor agents used during the treatment.

Day of treatment	Stressor used	Duration
1	Water deprivation	24 h
2	No stressor applied	–
3	Flashing light	3 h
4	Food deprivation	24 h
5	Restraint	6 h
6	Wet sawdust	24 h
7	Foot-shock	20 min

[a] Schedule for each week was randomly generated by the use of above seven stressor agents.

2.3. Experimental treatment groups

2.3.1. Experiment 1: time course of behavior and hippocampus response to chronic unpredictable stress

Mice were randomly assigned to 14 experimental groups (n = 10/group). There were seven stressed groups and seven control groups. After 7 wk of stress treatment, the stressed groups were allowed to recover for 3 wk. The depression-like behavior of the mice was observed in the forced swimming test (FST) and the tail suspension test (TST) at weeks 1, 3, 5, 7, 8, 9, and 10 (Fig. 1a). Behavioral assessments were conducted during the light period of the light:dark cycle. A minimum of 1 h after behavioral tests, the mice (10 controls and 10 stressed) were decapitated to obtain venous blood. Serum was separated by centrifugation at 3000 rpm and stored at −20 °C until assayed for corticosterone concentrations. Brain tissues were removed rapidly from the skulls, and hippocampal tissues were dissected on an ice-cold plate. Five hippocampal tissue samples per group were randomly chosen and stored at −80 °C until Western blot analysis. When the stressor applied was food or water deprivation, no tests were performed on the stress day or on the days immediately following the stress.

2.3.2. Experiment 2: effects of diazepam on the development of stress-induced behavioral and hippocampal changes

As depicted in Fig. 1b, animals were divided into five experimental groups, as follows: (1) *Control group* (n = 10). Mice remained in their cages except for daily oral (p.o.) administration of saline; (2) *Chronically stressed group* (n = 10). For 5 wk, mice received daily p.o. administration of saline immediately prior to variate stress sessions; (3) *Chronic high dosage of diazepam-treated stressed group* (HDS, n = 10). After 1 wk of stress sessions, these mice received p.o. administration of 2 mg/kg diazepam in saline immediately prior to each variate stress session for the next 4 wk; (4) *Chronic medium dosage of diazepam-treated stressed group* (MDS, n = 10). Mice were treated as described above, but with 1 mg/kg diazepam. (5) *Chronic low dosage of diazepam-treated stressed group* (LDS, n = 10). Mice were treated as described above, but with 0.5 mg/kg diazepam. The three doses of diazepam were based on the recommended adult daily dosage (2.5–10 mg) for treatment of anxiety [30]. After the last stress session and behavioral testing, the mice were sacrificed to obtain serum and hippocampal tissues for corticosterone and Western blot analysis.

2.3.3. Experiment 3: therapeutic effects of diazepam on stress-induced behavioral and hippocampal changes

As depicted in Fig. 1c, animals were divided into five experimental groups, as follows: (1) *Control group* (n = 10). Mice remained in their cages except for the daily p.o. administration of saline during the last week; (2) *Chronically stressed group* (n = 10). After 7 wk of stress sessions, mice were allowed to recover for 1 wk. Saline was administered daily p.o. to the mice during the 1 wk recovery phase; (3) *Chronic high dosage diazepam-treated stressed group* (HDS, n = 10). Diazepam (2 mg/kg) was administered daily p.o. to the stressed mice during the 1 wk recovery phase; (4) *Chronic medium dosage diazepam-treated stressed group* (MDS, n = 10). Mice were treated as described above, but using 1 mg/kg diazepam; (5) *Chronic low dosage diazepam-treated stressed group* (LDS, n = 10). Mice were treated as described above, but with 0.5 mg/kg diazepam. After 1 wk daily diazepam administration, mice were subjected to behavioral testing. Then, the animals were sacrificed to obtain serum and hippocampal tissues for corticosterone as well as Western blot analysis.

2.3.4. Experiment 4: short- and long-term effects of diazepam treatment on animal behavior and hippocampal neurons

Mice were randomly assigned to eight experimental groups (n = 10/group). Two groups comprised the control groups, whereas the other six groups were the diazepam-treated groups (two groups for each dosage). After daily p.o. administration for 1 wk or 4 wk, the mice (10 controls, 10 high dosage treated, 10 medium dosage-treated, and 10 low dosage-treated) were subjected to behavior testing and then decapitated to obtain hippocampal tissues for Western blot analysis (Fig. 1d).

2.4. Behavioral tests

Behavioral tests were performed in JLBehv-FSG-4 sound insulation boxes controlled by the DigBehav animal behavior video analysis system (Shanghai Jiliang Software Technology Co. Ltd., Shanghai, China) that can automatically record and analyze animal movements to provide total immobility times in the FST and TST. Depression-like behavior was inferred from increases in time spent immobile during these tests. The FST was similar to that described by Porsolt et al. [36]. Mice were placed individually in 10 cm of ambient temperature water (25 ± 1 °C) in 2000 ml glass beakers and were allowed to swim for 5 min. The durations of immobility were recorded during the last 4 min of the test.