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Low molecular mass chondroitin sulfate suppresses chronic unpredictable mild stress-induced depression-like behavior in mice

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The present study is aimed at testing the antidepressant-like effects and probable mechanisms of action of low molecular mass chondroitin sulfate (LMMCS) on depression induced by chronic unpredictable mild stress (CUMS) in mice. Four weeks of CUMS exposure resulted in depressive-like behavior, expressed by a significant decrease in the locomotor activity and sucrose consumption and increased immobility time in the forced swim test. Further, there was a significant reduction of 5-HT level in the hippocampus region of depressed mice. Treatment of mice for four weeks with LMMCS ameliorated significantly both the behavioral and biochemical changes induced by CUMS. These novel results suggest that LMMCS produces an antidepressant-like effect in mice subjected to CUMS, which might be related, at least in part, to the increase of 5-HT concentration in the hippocampus.

Keywords: low molecular mass chondroitin sulfate, chronic unpredictable mild stress, depression, mice, hippocampus, 5-HT

As a common mood disorder, depression is often characterized by several key symptoms such as anhedonia, despair, hopelessness and memory impairment. In clinics, many antidepressants have been used to treat depression. Among them, selective serotonin reuptake inhibitors (SSRIs), like fluoxetine, remain the first-line antidepressants for major depressions (1). However, most of these drugs are limited by certain negative features, such as delayed efficacy (4–8 weeks) and noticeable side effects (1, 2). Moreover, studies have reported that only 22–40 % of patients benefit from drug treatment (3). It is therefore necessary to seek more effective drugs with fewer side effects and better tolerability.

Chondroitin sulfate (CS), a natural glycosaminoglycan (GAG), exists in the extracellular matrix (ECM) or on the surface of animal cells. In the central nervous system (CNS), CS has been suggested to be contributing to the pathological processes of diseases such as

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epilepsy, stroke and Alzheimer's disease (4), but it might also be involved in some biological activities such as neuroprotection, since it exerts protective effects on Aβ-induced damage in mouse hippocampus (5). Previous studies have demonstrated that low molecular mass GAGs, including low molecular mass chondroitin sulfate (LMMCS, molecular mass is about 3500–5300 Da), could cross the blood-brain barrier (6).

In the present study, we investigated the influence of chronic treatment with LMMCS on behavioral and neurochemical changes induced by chronic unpredictable mild stress (CUMS) protocol in mice, as an animal model of depressive-like behaviors.

EXPERIMENTAL

Animals

Seventy-two male Kunming (Swiss) mice (18–24 g, 4 weeks old) were purchased from the Laboratory Animal Centre, Qingdao Food and Drug Inspection Institute (Qingdao, China). They were kept in standard plastic cages (six mice per cage) and maintained in a 12-h light/dark cycle at room temperature 23–25 ° C and humidity 55 ± 5 %. Normative laboratory food and water were freely available, except during experimental procedures. Animals were acclimatized to the laboratory conditions for at least one week prior to use. They were then randomly allocated to one of six groups (n = 12/group) for behavioral tests (see below).

All procedures involving animals were carried out in compliance with the National Institutes of Health and institutional guidelines for the humane care of animals and were approved by the Animal Care Committee of Qingdao University (Qingdao, China).

Drugs and treatment

LMMCS was obtained from Qingdao Belt Biological Technology Co., Ltd. (China). Fluoxetine hydrochloride was purchased from Sigma (USA).

After adaptation to the cages for seven days, the mice were subjected to the baseline behavioral testing [open field test (OFT), sucrose preference test (SPT) and forced swimming test (FST)]. They were then divided into six groups based on their baseline FST results (to make the average immobility time equal for each group): control group (C) and CUMS model group (CUMS) were given vehicle (deionized distilled water), LMMCS groups (CS) were administered 30, 100, and 300 mg kg^{-1} bm of LMMCS, resp., and fluoxetine group (Flu 20) received 20 mg kg^{-1} bm of fluoxetine.

Both LMMCS and fluoxetine were dissolved in deionized distilled water. In all experiments, LMMCS, fluoxetine or vehicle were administered intragastrically (*i.g.*) 1 h prior to stress exposure. The mice were weighed every day and the doses were adjusted to their weight gain.

The procedural sequence is given in Fig. 1: (*i*) adaptation: one week, (*ii*) baseline behavioral tests (pre-tests for OFT, SPT, and FST, resp.): four days (every test takes one or two days), (*iii*) CUMS and LMMCS treatment: four weeks, (*iv*) post behavioral tests (OFT, SPT, and FST, resp.): four days, (*v*) brain tissue isolation.

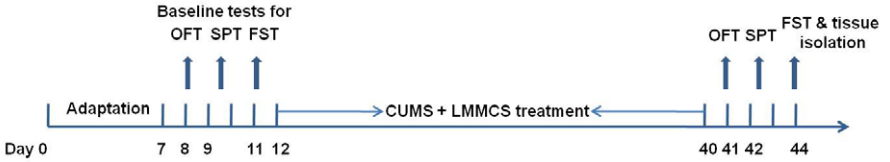

Fig. 1. Schematic representation of the experimental design. OFT – open field test, SPT – sucrose preference test, FST – forced swimming test, CUMS - chronic unpredictable mild stress, LMMCS – low molecular mass chondroitin sulfate.

Chronic unpredictable mild stress procedure

The CUMS procedure was performed as described previously, with a slight modification (7). All groups of animals, except for the control group, were exposed to CUMS. In brief, the CUMS protocol consisted of sequential application of a variety of mild stressors: fasting for 24 h, water deprivation for 24 h, cage tilting for 7 h, swimming (cold water 4 °C, 5 min), behavior restriction for 2 h, light-dark-cycle reversal (12 h/12 h), finally, 1 h with an empty bottle; damp bedding for 24 h, foreign object for 24 h. To prevent habituation and to ensure unpredictability of the stressors, all stressors were randomly scheduled over a one week period and were continued for the duration of the experiment (Table I). Mice were subjected to one or two of the stressors (in random order) on one day and without repetitive stressors on the two consecutive days. Control animals were isolated in a separate room and had no contact with the stressed animals.

Open field test

The open field test (OFT) is a measure of spatial exploration behavior of mice (total distance and entries in the center zone: horizontal movement scores reflect range of motion, rearing number: vertical movement scores reflect exploratory behaviors), as previously described (8). The open-field apparatus, a four-sided organic plastic square chamber (40 × 40 × 40 cm) with a dark floor, was divided into 16 equal squares by white lines. Each mouse was gently put in the center square. The first 60 seconds was set for adaptation. The OFT (the number of locomotion and rearing frequency) were observed for 5 min (Sony SSC-G218 camera located 190–200 cm above the arena, Sony, Japan) and were automatically calculated by the ANY-maze video tracking system (SMART, Panlab SL, Spain). The cage was entirely cleaned with alcohol and dried to exclude potential interaction of the dejections in the trail. The value for each group was calculated as a percentage of the post-test value relative to the mean pre-test value.

Sucrose preference test

Briefly, the mice were trained to adapt to 1 % (*m*/*V*) sucrose solution 24 h before testing (7). In the test phase, mice were able to access both tap water and a 1 % sucrose solution for 24 h. The position of the two bottles (left/right sides of the cage) was changed randomly in order to avoid place preference. The sucrose preference value was calculated as a percentage of the volume of the consumed 1 % sucrose solution relative to the total volume of liquid intake.

Table I. Schedule for the chronic unpredictable mild stress procedure

Week	Day	Stress protocol	Duration
	Sun	Foreign object	24 h
	Mon	Fasting and water deprivation	24 h
	Tue	Cage tilting	7 h
1	Wed	Overnight illumination / empty bottle	12 h/1 h
	Thu	Cold water swimming	5 min
	Fri	Behavior restriction	2 h
	Sat	Damp bedding	24 h
	Sun	Overnight illumination / empty bottle	12 h/1 h
	Mon	Cage tilting	7 h
	Tue	Cold water swimming	5 min
2	Wed	Fasting and water deprivation	24 h
	Thu	Behavior restriction	2 h
	Fri	Damp bedding	24 h
	Sat	Foreign object	24 h
	Sun	Cold water swimming	5 min
	Mon	Overnight illumination / empty bottle	12 h/1 h
	Tue	Fasting and water deprivation	24 h
3	Wed	Behavior restriction	2 h
	Thu	Foreign object	24 h
	Fri	Cage tilting	7 h
	Sat	Damp bedding	24 h
	Sun	Fasting and water deprivation	24 h
	Mon	Cage tilting	7 h
	Tue	Overnight illumination / empty bottle	12 h/1 h
4	Wed	Foreign object	24 h
	Thu	Damp bedding	24 h
	Fri	Cold water swimming	5 min
	Sat	Fasting and water deprivation	24 h

Mice were exposed to the stressors according to this schedule for four weeks. At the same time, animals were treated with LMMCS (30, 100 and 300 mg kg^{-1} bm) and fluoxetine (20 mg kg^{-1} bm) or vehicle intragastrically.

Forced swimming test

The forced swimming test (FST) was carried out following the protocol of Porsolt *et al.* (9). The mice were placed in clear glass cylinders (40 cm tall × 18 cm diameter) filled with water (25 °C), approximately 23 cm deep, to prevent their tails from touching the bottom. Immobility was recorded during the total 4 min testing period. Mice were considered to be immobile when they made only small movements necessary to float and keep their

heads above the water surface. Test sessions were recorded with a video camera and the duration of immobility was scored using a video-computerized tracking system (SMART, Panlab SL).

Brain tissue sample preparation

The mice were sacrificed by decapitation after completion of the behavioral tests. The hippocampus was quickly isolated from the mice brain on ice, weighed and frozen in liquid nitrogen and transported to –80 °C until assays were performed.

ELISA assay for 5-HT

Hippocampus of one mouse from each group was homogenized in ice-cold buffer. The homogenates were centrifuged at 10000×*g* for 10 min at 4 °C and the supernatants were collected for further analysis. The supernatants were assayed using a serotonin ELISA kit (Abcam, UK) according to the manufacturer's protocol and quantified with a microplate reader (450 nm). The results were shown as nanograms per gram of tissue.

Western blot for 5-HT$_{1A}$ receptor

Hippocampus of two mice from each group was homogenized in an ice-cold RIPA buffer (Solarbio, China), and centrifuged at 12000×*g* for 10 min at 4 °C. The supernatants were collected. Protein concentration was determined by a BCA (bicinchoninic acid) protein assay using bovine serum albumin as a standard. The proteins were separated with the aid of sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking in 3 % bovine serum albumin/Tris buffered saline with Tween 20 (TBST) at room temperature for 2 h, the membranes were incubated with the primary antibody at 4 °C overnight (anti-5HT$_{1A}$ 1:1000, Abcam, UK). After washing with TBST three times, the membranes were incubated with HRP conjugated anti-rabbit or anti-mouse lgG secondary antibodies (1:10000, Proteintech, USA) for 1 h at room temperature. The blots were washed again three times with TBST buffer, and the immunoreactive bands were detected using a chemiluminescence system (Bio-Rad ChemiDox XRS, USA). Protein band densities were quantified using the Image J software (Bio-Rad ChemiDoc XRS) while GAPDH (1:5000, Proteintech) was used as a loading control.

Statistical analysis

All data were normally distributed and are presented as mean ± standard error of the mean (SEM). The data were analyzed by One-way ANOVA, followed by Dunett's (in the case of SPT and 5-HT tests) or Newman-Keuls (in the case of OFT and FST tests) multiple comparison *post hoc* test. A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

LMMCS improved range of motion and exploratory aspiration

In the OFT, horizontal scores reflect the range of motion including two parameters: total traveled distance and entries in the center zone; vertical scores reflect exploratory

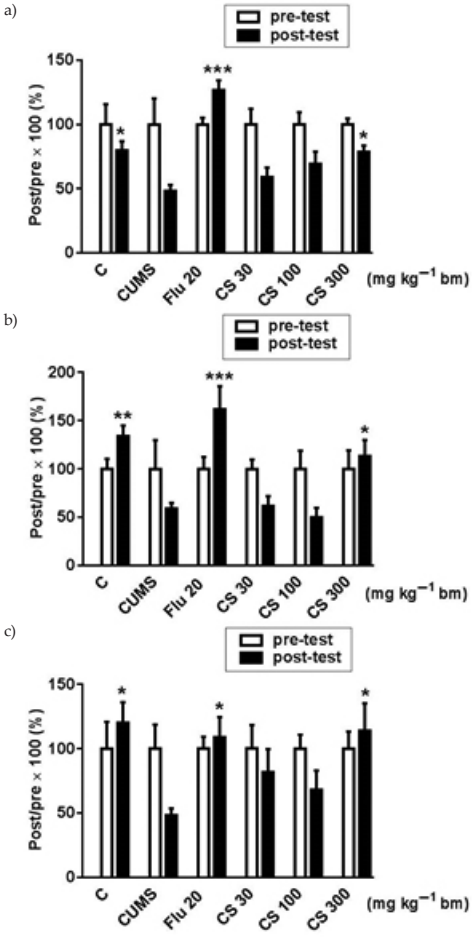

Fig. 2. Effects of LMMCS administration on OFT: a) total traveled distance in the chamber, b) entries in the center zone, c) total rearing numbers in the chamber. Data are represented as mean ± SEM, n = 8–10 mice per group. Statistically significant difference *vs*. CUMS group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. C – control, CS – LMMCS, CUMS – chronic unpredictable mild stress, Flu – fluoxetine.

aspiration (expressed as the total rearing number). In line with the decrease in the locomotor activity induced by CUMS reported in previous studies (10), the horizontal and vertical scores of mice in the CUMS group (total traveled distance: 48.3 ± 4.6 %, $p < 0.05$, entries in center zone: 59.1 ± 5.8 %, $p < 0.05$, total rearing number: 48.4 ± 5.2 %, $p < 0.01$, Fig. 2) were reduced significantly compared to the control group (total traveled distance: 79.8 ± 7.0 %, entries in center zone: 134.2 ± 10.7 %, total rearing number: 120.3 ± 15.9 %, Fig. 2).

Repeated LMMCS 300 mg kg^{-1} bm treatments reversed significantly these reduced scores (total traveled distance: 78.6 ± 4.9 %, $p < 0.05$, entries in center zone: 113.3 ± 16.6 %,

$p < 0.05$, total rearing number: 114.2 ± 21.1 %, $p < 0.05$, Fig. 2). However, neither 30 nor 100 mg kg^{-1} bm showed a significant difference compared to the CUMS group for any parameter (Fig. 2). Fluoxetine at 20 mg kg^{-1} bm reversed all measured parameters (total traveled distance: 126.7 ± 7.5 %, $p < 0.05$, entries in center zone: 162.0 ± 23.9 %, $p < 0.001$, total rearing number: 109.1 ± 15.6 % $p < 0.001$, Fig. 2) compared to the CUMS group (Fig. 2). Taken together, these results suggest that repeated treatment with LMMCS could improve the range of motion and exploratory activity in mice subjected to the CUMS protocol. Interestingly, our OFT results also revealed that the number of entries in the center zone showed significant differences between LMMCS treated and untreated groups, indicating that LMMCS could also improve the symptom of anxiety accompanied with depression (11).

LMMCS improved anhedonia

To evaluate the effect of LMMCS on improving anhedonia, SPT was carried out. As shown in Fig. 3, the *post hoc* test analysis showed that a four-week CUMS exposure decreased significantly the percentage of sucrose consumption in the CUMS mice (53.4 ± 3.8 %, $p < 0.01$) compared to the control group (76.3 ± 5.4 %). Furthermore, the four-week treatment with LMMCS (100 mg kg^{-1} bm) increased the sucrose preference compared to the CUMS mice (71.1 ± 5.4 %, $p < 0.05$), and similarly to the fluoxetine-treated group (67.4 ± 5.1 %, $p < 0.05$). However, the other two doses did not change sucrose consumption compared to the CUMS group. These results suggest that LMMCS treatment could improve anhedonia-like status in the CUMS mice.

LMMCS reduced the immobility time in FST

The four-week exposure to CUMS increased the immobility time significantly when mice were subjected to the forced swimming test (131.08 ± 7.97 s, $p < 0.05$, compared to control mice, Fig. 4). Chronic LMMCS (30 and 100 mg kg^{-1} bm) or fluoxetine treatment reduced significantly the immobility time as a measure of helplessness and despair compared to CUMS mice (LMMCS 30 mg kg^{-1}: 83.07 ± 10.61 s, $p < 0.05$; LMMCS 100 mg kg^{-1}:79.62 ± 11.71 s, $p < 0.05$; fluoxetine 20 mg kg^{-1}: 73.02 ± 15.54 s, $p < 0.05$; Fig. 4). However, the LMMCS 300 mg kg^{-1} treatment showed no significant change compared to the CUMS group. These

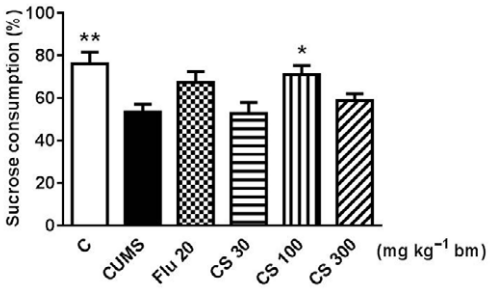

Fig. 3. Effects of LMMCS administration on the sucrose preference in the SPT. Data are represented as mean ± SEM from 9-12 mice per group. Statistically significant difference *vs.* CUMS group: * $p < 0.05$, ** $p < 0.01$. C – control, CS – LMMCS, CUMS – chronic unpredictable mild stress, Flu – fluoxetine.

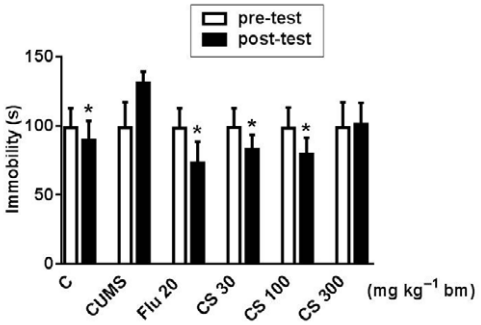

Fig. 4. Effects of LMMCS administration on the immobility time in the FST. Data are represented as mean ± SEM from 9–12 mice per group. Statistically significant difference *vs*. CUMS group: * $p < 0.05$. C – control, CS – LMMCS, CUMS – chronic unpredictable mild stress, Flu – fluoxetine.

results are in line with the results obtained in OFT and SPT, suggesting an antidepressant-like effect of LMMCS.

LMMCS reversed 5-HT concentration in the hippocampus region of mice

5-HT levels in the hippocampus correlate closely with depression (12). Our ELISA assay results revealed that 5-HT concentration decreased significantly in the hippocampus region of mice exposed to four weeks of CUMS compared to the control group (76.62 ± 5.42 ng g^{-1}, $p < 0.01$, Fig. 5). The four-week treatment with LMMCS (100 and 300 mg kg^{-1} bm) or fluoxetine increased significantly the 5-HT concentration in the hippocampus region compared to the CUMS group (LMMCS 100 mg kg^{-1}: 112.03 ± 11.18 ng g^{-1}, $p < 0.05$; LMMCS 300 mg kg^{-1}: 125.11 ± 6.57 ng g^{-1}, $p < 0.01$, fluoxetine 20 mg kg^{-1}: 126.68 ± 4.82 ng g^{-1}, $p < 0.001$), except for the lowest dose of 30 mg kg^{-1} bm, suggesting that LMMCS could increase the 5-HT level in the hippocampus of depressed mice.

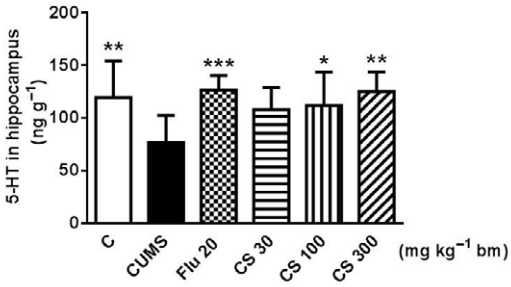

Fig. 5. Effects of LMMCS administration on the 5-HT level in the hippocampus region of mice. Data are represented as mean ± SEM (n = 8–10). Statistically significant difference *vs*. CUMS group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. C – control, CS – LMMCS, CUMS – chronic unpredictable mild stress, Flu – fluoxetine.

LMMCS did not change the 5-HT$_{1A}$ protein expression in the hippocampus region of mice

Brain 5-HT$_{1A}$ autoreceptor is important to maintain the 5-HT level in synapse, controlling 5-HT release by a negative feedback mechanism (12). To elucidate the possible primary mechanism of 5-HT increase in the hippocampus, the Western blot was conducted for the 5-HT$_{1A}$ receptor expression. As shown in Fig. 6, the CUMS group did not show any change in the 5-HT$_{1A}$ receptor expression compared to the control group. Although the 5-HT$_{1A}$ receptor expression slightly increased in LMMCS (100 and 300 mg kg^{-1} bm) or fluoxetine-treated groups compared to the CUMS group, these were not significant, thus indicating that the 5-HT$_{1A}$ receptor might not be involved in the antidepressant-like effect of LMMCS. In contrast, a recent report mentioned that the 5-HT$_{1A}$ receptor expression in the hippocampus of CUMS mice was reduced compared to the control group (13). Consequently, we suppose that two possibilities could explain the above result. First, the CUMS procedure in our study may not be strong enough to change the expression of the t5-HT$_{1A}$ receptor in the hippocampus of tested mice. Second, the 5-HT level decrease in the hippocampus may be a consequence of other receptors' interplay or signaling pathways like the brain derived neurotrophic factor (BDNF), since several studies suggested that 5-HT can stimulate the BDNF gene or protein expression in depressive patients (14) and rodents (15). Further studies are therefore necessary to elucidate this issue.

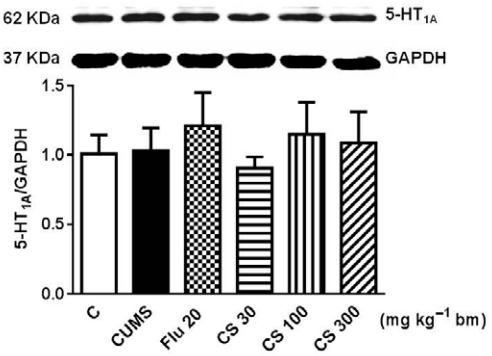

Fig. 6. Effects of LMMCS administration on the 5-HT$_{1A}$ receptor expression in the hippocampus region of mice. Data are represented as mean ± SEM (n = 3). C – control, CS – LMMCS, CUMS – chronic unpredictable mild stress, Flu–fluoxetine.

CONCLUSIONS

In the present study, a four-week LMMCS treatment improved significantly the depressive-like behaviors of CUMS mice, and increased 5-HT levels in the hippocampus. These novel findings suggest possible benefits of LMMCS application in depression treatment. However, further experiments that would provide more precise information about the potential antidepressant effect of LMMCS are warranted.

Abbreviations, acronyms, symbols. – BDNF – brain-derived neurotrophic factor, ELISA – enzyme-linked immune sorbent assay, Flu 20 – fluoxetine group, CS – chondroitin sulfate, CUMS – chronic unpredictable mild stress, ECM – extracellular matrix, FST – forced swimming test, GAG – glycosaminoglycan, HRP – horseradish peroxidase, 5-HT – serotonin, LMMCS – low molecular mass chondroitin sulfate, OFT – open field test, SPT – sucrose preference test, SSRIs – selective serotonin reuptake inhibitors, TBST – Tris buffered saline with Tween 20.

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