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Original Article

Castration Had No Effect on Decreased Expression of the Neural Cell Adhesion Molecule in the Prefrontal Cortex of Rats Subjected to Chronic Mild Stress

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Abstract: The effect of chronic mild stress on protein levels of the neural cell adhesion molecule (NCAM) were evaluated in the prefrontal cortex and hippocampus of rat brain. Decreased NCAM protein expression in the prefrontal cortex was found in the rats subjected to stress, while the protein levels in sub-regions of hippocampus remained unchanged. The study also explored whether there was a testicular hormone influence on the behavioral response to stress and on the NCAM expression. We found chronic mild stress induced an anhedonia-like behavior in intact rats, but not in the castrated male rats. Furthermore, castration did not have influence on the stress induced reduction of NCAM expression in the prefrontal cortex. In conclusion, our findings indicate that NCAM mediated remodelling in the prefrontal cortex under chronic mild stress condition might be independent to the sex hormones during the adult period in male rat.

Key Words: Chronic mild stress (CMS), castration, neural cell adhesion molecule (NCAM), behaviour test, prefrontal cortex (PFC), hippocampus

Introduction

For a long time, depression is considered to be caused by a neuro-chemical imbalance in the brain, including abnormality of aminergic transmitter systems and hypothalamic peptidergic systems [1, 2]. Recently, besides this classical neuro-chemical hypothesis, more and more evidence revealed a network disorders that underlies depression, including reduced neurogenesis and neuronal structural remodeling [3].

Neural cell adhesion molecule (NCAM), which contains three main isoforms of different molecular weight: NCAM-120, NCAM-140 and NCAM-180 [4], plays an important role in structural remodelling of the nervous system [5]. Several lines of evidence indicated its involvement in depression. Vawter et al. found that differently spliced NCAM isoforms were increased in the hippocampus of patients with bipolar disorder [6]. Experiments on animal models also provide supportive evidence. Chronic restraint stress resulted in decreased NCAM mRNA levels as well as decreased NCAM-140 protein levels in hippocampus of rats, while NCAM-120 and NCAM-180 protein levels remained unchanged [7, 8]. Total NCAM expression in the prefrontal cortex (PFC) and in the hippocampus were significantly reduced by chronic corticosterone injections [9].

The role of environmental stress (so-called "life events") in triggering the early episodes of depression has long been appreciated. Chronic mild stress (CMS) which mimics a series of life events, has been proven to produce behavioural and neuroendocrine changes in rats similar to major depression in human [10] and this model is widely accepted as a model of depression for its validity, reliability and utility [10]. So to study the involvement of NCAM regulation in this animal model could have implications for the

understanding of neuronal structural remodeling in depression.

Sex hormones are involved in stress response and in the pathogenesis of depression. It has been demonstrated that long-term castration increases the sensitivity to "depression"-inducing conditions [11, 12]. Sex hormones also play a role in structural remodeling, exemplified by androgen induced synaptic remodelling of the spinal cord [13], and estrogen induced synaptic connectivity [14]. Therefore, in the present study, we carried out our experiments according to the following hypotheses: first, CMS may produce alteration of NCAM expression in the PFC and hippocampus in experimental animals; second, castration may influence the sensitivity to the CMS procedure; and third, castration may influence the CMS induced alteration of NCAM expression.

Materials and Methods

Animals

Male Sprague-Dawley rats aged at 2 month were used in this study. Animals were housed three to four to a cage in a room under controlled 12 h light/dark schedule (light on at 7:00 A.M.) and temperature (23±2°C) conditions with ad libitum access to food and water. Rats were divided into four groups: sham operation & control group (n=7), sham operation & stress group (n=8), castration & control group (n=8), castration & stress group (n=8). Castration or sham operation was carried out when rats were under anesthetization with chloral hydrate (7%, 1ml/100g body weight). Rats were used after one week recovery. All animal handling procedures were carried out in accordance with all relevant local guidelines and legislation to minimize suffering of the animals.

Chronic mild stress

Rats in the stress groups were exposed to a chronic unpredictable mild stress procedure for eight weeks. Stressors consisted of 24h social isolation (one rat per cage), paired housing in a small cage (narrow condition), intermittent overnight illumination (2h light-dark cycle), continuous illumination (overnight), wet cage, tilt cage, cage without padding, 24h food deprivation, 24h water deprivation, 20min swim at room temperature. The stressors were given twice a day, in a random manner. Control groups were housed undisturbed in a different room.

Open field test

The open field test was carried out 12h after ceasing the CMS procedure. The apparatus consisted of a rectangular area of 81 × 81cm surrounded by a 28cm high wall. The area was divided into 16 squares of 20 × 20cm by painted white lines. The rats were placed in the center of the open field and its activity was recorded in the following 5 minutes. Horizontal locomotion (number of times crossing of the white lines) and vertical activity (frequency of rearing) were observed.

Sucrose preference test

Animals were initially exposed to a 1% sucrose solution for 24h to acclimatize to the procedure. Then they were given free access to two bottles, one filled with water and the other with 1% sucrose solution for 24h. The weight of water and sucrose consumed were measured. The percentage of sucrose solution from the total liquid intake represented the parameter of hedonic behavior.

Tissue preparation

Twenty-four hours after the test procedure, the rats were anesthetized with chloral hydrate and decapitated. The brains were rapidly removed from the skull. The PFC was dissected out on ice and quickly frozen in liquid nitrogen and then stored at -80°C. Remained part of brains was fixed in 4% PFC at room temperature for 48h, and then dehydrated in 15% and 30% sucrose solution by turns.

Western-blotting

Rat PFC was homogenized in ice-cold homogenization buffer containing 50mM HEPES, 0.5mM $MgCl_2$, 50mM NaF, 100mM KCl, 0.1mM each for EDTA and EGTA, 5mM DTT, 10mM β-glycerophosphate, 1mM Na3VO4, 1% NP40 and protease inhibitor cocktail (Roche Diagnostics GmbH, Germany). Protein sample extract (30μg) was subjected 10% SDS-PAGE and then transferred to a PVDF membrane (Millipore, USA). The membrane was blocked with 5% skim milk for

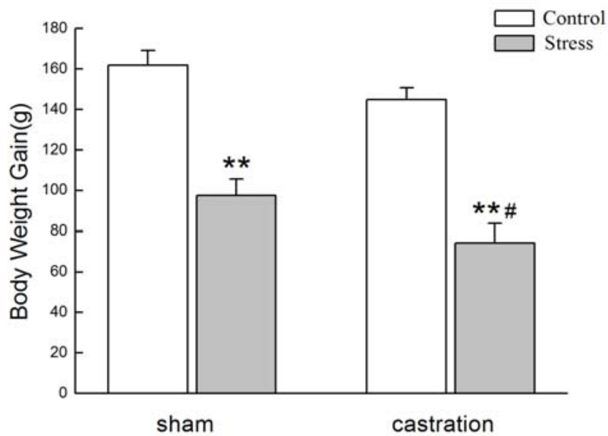

Figure 1. Body weight gain after the chronic mild stress procedure. Data presented as mean ± S.E.M. Open column: control groups; Closed column: CMS groups. **P<0.01 vs. control group; #P<0.05 vs. sham operated group.

1h at 37°C and then probed with the mouse monoclonal antibody against NCAM (1:400, Sigma, USA) for 2h at room temperature. α-tublin (1:200, Santa Cruz, USA) was probed as an internal control. Detection was performed using horseradish peroxidase (HRP) conjugated horse anti-mouse IgG (1:2000, DaKoCytomation, Denmark), and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA). The bands on the film were scanned and analyzed by Image J software.

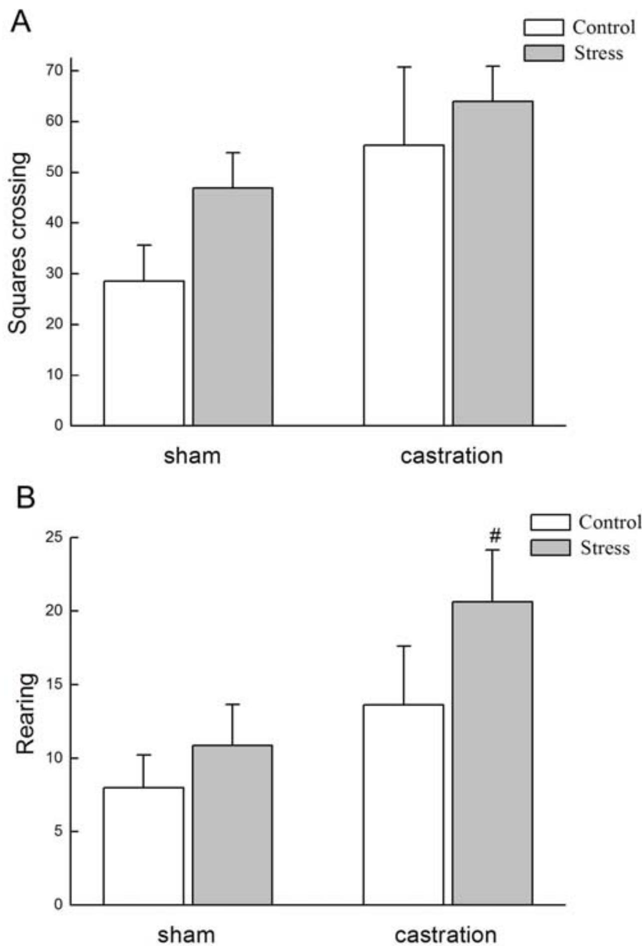

Figure 2. Locomotor activity in the open field test: square crossing (A) and rearing (B). Data presented as mean ± S.E.M. Open column: control groups; Closed column: CMS groups. #P<0.05 vs. sham operated group.

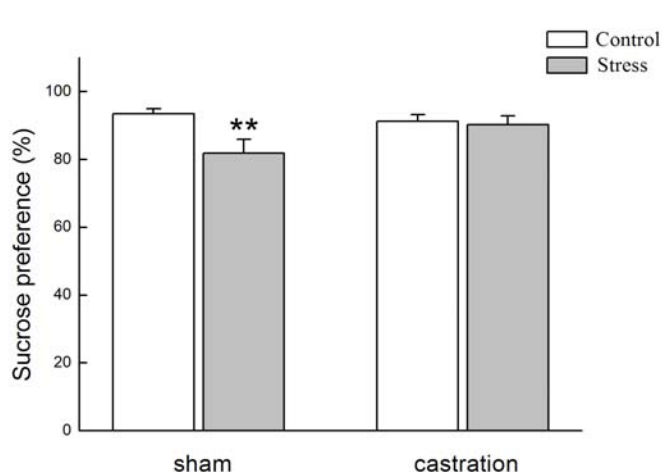

Figure 3. Sucrose preference test after the CMS procedure. Data presented as mean ± S.E.M. Open column: control groups; Closed column: CMS groups. **P<0.01 vs. control group.

Immunohistochemistry

After dehydration, coronal sections of the brain (15μm) were cut in a cryostat and mounted on 0.1% polylysine (Sigma, USA) coated slides. The hippocampal sections were washed in PBS, then incubated in PBS containing 0.3%H_2O_2 and 1% Triton X-100 for 30min at room temperature to quench endogenous peroxidase activity. After washing, sections were incubated with the mouse monoclonal antibody against NCAM (1:200) for 1h at 37°C. Detection was performed using biotinylated horse anti-mouse IgG (1:200, Vector Laboratories, USA) and visualized with DAB. After that, slides were dehydrated in ethanol, cleared in xylene and mounted in Entellan.

For immunofluorescence, detection was performed using Cy3 conjugated horse anti mouse IgG (1:200, Chemicon, USA), and then the slides were mounted in 70% glycerol.

Quantitation of immunochemistry stain

Slides developed using DAB were used for quantitation. In the present study, an image analysis system including Metamorph image acquisition and processing software (Universal Imaging Corporation, USA), Spot cooled color digital camera (Diagnostic Instruments, Inc., USA), Nikon E800u microscope (Nikon Corporation, Japan) equipped with a Prior scanning stage (Prior Scientific Instruments Ltd., England) and HP computer, was used to evaluate the immunoreactivity of NCAM. The means of the optical density (OD) of each subregion were measured by Image-Pro software, and the values for each rat were calculated from an average of measurements in three to five matched sections after subtracting the background.

Statistical analysis

Two-way analyses of variance (ANOVA) were performed with castration and stress as independent factors. T-test was carried out for pairwise comparisons between groups when appropriate. The data were expressed as means ± SEM. P<0.05 was considered significant.

Results

Body weight gain

Two way ANOVA revealed a significant effect of both factor "CMS" and "castration" on body weight gain (P=0.000 and 0.017, respectively). Separate analysis of data showed a significant reduction of body weight gain in both sham operated and castrated animals exposed to CMS (P=0.000 for both).

When exposed to stress, castrated rats displayed decreased body weight gain compared with sham operated rats (P=0.044). Statistical analyses did not reveal a significant interaction between CMS and castration for body weight gain (**Figure 1**).

Open field behavior

In the open field test, the locomotor behaviors, square crossing and rearing, were not affected by CMS in either sham operated or castrated rats. When subjected to stress, castrated rats displayed increased rearing than sham

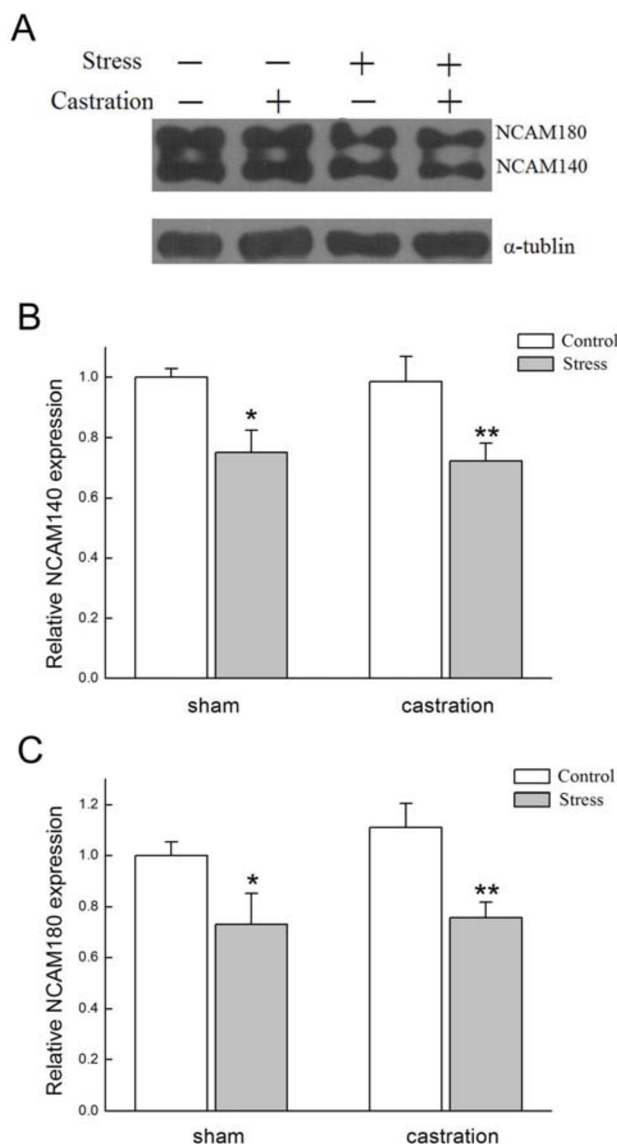

Figure 4. Decreased NCAM-140 (B) and NCAM-180 (C) expression in the PFC in rats subjected to CMS. Visualized bands from western-blotting are presented in (A). Data presented as mean ± S.E.M. Open column: control groups; Closed column: CMS groups. *P<0.05 vs. control group; **P<0.01 vs. control group.

operated rats (P=0.042). While separate analysis of data did not reveal significant differences in square crossing among the groups, two way ANOVA showed that factor "castration" significantly contribute to the increased crossing and rearing (P=0.036 and 0.027, respectively). No significant interaction between CMS and castration was detected for square crossing and rearing (**Figure 2**).

Sucrose preference

CMS resulted in a decrease in sucrose preference in sham operated rats (P=0.008) but not in castrated animals. Two way ANOVA test did not demonstrate a statistically significant interaction between CMS and castration or an effect of castration on sucrose preference. However, a significant effect of CMS was detected (P=0.035) that rats subjected to stress showed lower sucrose preference than control groups (**Figure 3**).

NCAM expression in PFC

In both sham operated and castrated rats, exposure to CMS resulted in significant decrease of NCAM180 expression in PFC (P=0.035 and 0.006, respectively). The CMS procedure also produced a decrease of NCAM140 in PFC in both sham operated and castrated animals (P=0.011 and 0.005, respectively). Two way ANOVA analyses failed to reveal an interaction between CMS and castration on NCAM expression in PFC (**Figure 4**).

NCAM expression in hippocampus

In the hippocampus, NCAM immunoreactivity was mainly detected in the hilus and inner molecular layer (IML) of the dentate gyrus (DG). The mossy fiber tract in the stratum lucidum of CA3 was also strongly stained. However, we did not observe any significant difference in these sub-regions among groups (**Figure 5** and **Figure 6**). Also, statistic analysis on DAB stained slides did not reveal any significant difference among those groups (data not shown).

Discussion

The present study showed that exposure to an 8-week chronic mild stress regime reduced the NCAM expression in the PFC, while the NCAM expression in hippocampus remains unchanged. Castration could significantly increase the locomotor activity of experimental animals, but it did not influence the stress induced anhedonia. The alteration

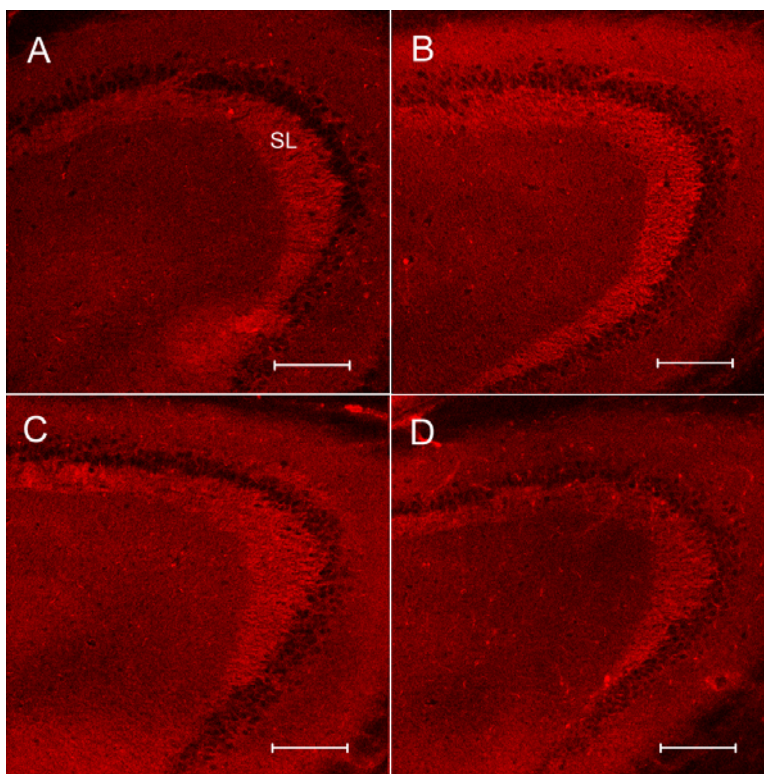

Figure 5. Expression of NCAM in the stratum lucidum of CA3 region of hippocampus in intact/control (A), intact/CMS (B), castrated/control (C) and castrated/CMS (D) animals. Bar: 100μm.

of NCAM expression resulted from CMS was also unaffected by castration.

Repeated restraint is a widely used model to study the structural plasticity in the PFC and hippocampus in stress response [15-18]. Previous studies reported altered expression of NCAM in this animal model [3]. In the present study, we use CMS model instead of the repeated restraint. Compared to repeated restraint stress, CMS has a similar effect on structural plasticity, for example, produced the same degree of apical dendritic atrophy in CA3 pyramidal neurons [19]. Moreover, the advantage of CMS is that the procedure simulates anhedonia, a loss of responsiveness to pleasant events, which is a core symptom of depression [10]. Also, decreased locomotor activity in an open field is observed in this experimental model [10]. In the present study, the CMS procedure failed to reduce the horizontal and vertical activity in the open field test while there is a tendency that the locomotor activity increased after the CMS procedure. This discrepancy could be due to different stress process. Correspondingly, there are data showing increased locomotor activity after CMS [20].

Gonadal hormones have been investigated as modulators in stress related behavior. The effect of ovariectomy on stress responses was widely studied. The duration of immobility in the tail suspension test was significantly increased by ovariectomy [11, 12]. Chronic mild stress induced anhedonia could not be observed in ovariectomized rats, in contrast to sham-operated females [20]. On the other hand, the effects of orchidectomy on behavioral responses to stress are less understood. Bernardi et al. reported that long-term castration significantly increase the immobile duration in the tail suspension test in male mice [11]. In the present study, we found that castration could increase the horizontal and vertical activity in the open field test, while no cross-work between castration and CMS was found. Also our results found an involvement of castration in chronic mild stress induced anhedonia. Reduced sucrose preference was observed in intact, but not castrated male rats. Our results seemed to be contrary with the hypothesis which is widely believed that castration could increased the sensitivity to stress and induce depression [11, 12, 21]. However, some other studies indicated that it is not a universal finding. For example, locomotor activity significantly increased after repeated exposure to psychological stress in sham-operated rats, but not in 2-week ovariectomized rats [22]. Compared with sham-operated control, increased locomotor activity in open field was observed in adult male rats that was castrated before sexual mature, but not observed in rats castrated after sexual mature [13]. Testosterone replacement had no effect on mood status in chemical castrated men at 60-75 year of age [23]. Thus it is possible that the effect of castration or hormone replacement

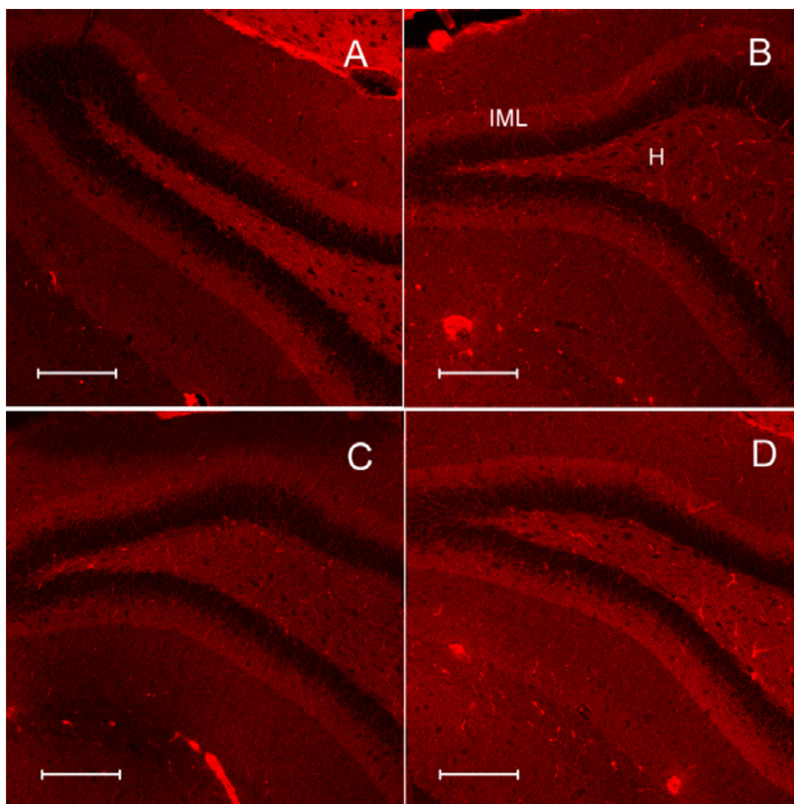

Figure 6. Expression of NCAM in the hilus and inner molecular layer of the DG region of hippocampus in intact/control (A), intact/CMS (B), castrated/control (C) and castrated/CMS (D) animals. Bar: 100μm.

on mood related behavior is a much more complicated process, which may be related to age, basal hormone level or stress process.

The three major isoforms NCAM-120, NCAM-140 and NCAM-180 are generated by alternative splicing of the transcript from a single NCAM gene. They share similar extracellular parts, but differ in their length of their cytoplasmic domain and their attachment to the cell membrane [4]. The antibody we used in this study could recognize the NCAM-140 and NCAM-180 isoforms. By western-blotting, we found a significantly reduced expression of these two isoforms of NCAM in the PFC of stressed animals. NCAM-140 has been shown to be critically involved in neurite outgrowth through different transduction pathways [24, 25]. While NCAM-180 has been shown to play an important role in synaptic remodelling [26]. Our results first find that expression of these two NCAM isoforms altered in the PFC in CMS animal model, and this may provide hint to understand the structural plasticity in depression.

To investigate NCAM expression in different sub-region of the hippocampus, we used immunochemistry assays. However, our results did not show any significant effect of CMS on NCAM expression in hippocampus. This is may be because of the limitation of the method. It has been shown that chronic restraint stress resulted in decreased NCAM-140 protein levels in hippocampus of rats, while NCAM-120 and NCAM-180 protein levels remained unchanged [7]. So it might be that the CMS procedure decreased NCAM-140 expression but this effect was diluted by the NCAM-180 expression in the hippocampus.

Circulating steroid hormones have been shown to be involved in neurochemical response to stress. Castrated male rats showed significantly more Fos-ir cells in the paraventricular nucleus when subjected to stress [27]. A reduction of NCAM-140 gene transcription in hippocampus was reported in adult male but not female mice subjected to chronic restraint stress [28]. In the present study, we tested the possibility that androgen may influence the CMS induced alteration of NCAM expression in prefrontal cortex and hippocampus. However, we found no cross-work between castration and CMS on NCAM expression. One possible explanation for this is that the animals were subjected to CMS only one week after castration, although it is reported that plasma testosterone levels is reduced by more than 90% 7 days after surgery [29], it is still possible that circulating testosterone is not completely depleted and it may influence our result. On the other hand, these results may suggest that NCAM mediated remodelling in the prefrontal cortex under chronic mild stress condition might be independent to androgen during the adult period in male rat.

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