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Item Type	Journal article
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Citation	Lv, Y., Chen, P., Kuang, L. et al. (2020) Role of corticotropin-releasing hormone in the impact of chronic stress during pregnancy on inducing depression in male offspring mice. Brain Research, 1747, 147029.
DOI	10.1016/j.brainres.2020.147029
Publisher	Elsevier
Journal	Brain Research
Download date	2026-04-17 02:50:48
License	https://creativecommons.org/licenses/by-nc-nd/4.0/
Link to Item	http://hdl.handle.net/2436/624619

Role of corticotropin-releasing hormone in the impact of chronic stress during pregnancy on inducing depression in male offspring mice

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Abstract

Although chronic stress during pregnancy (CSDP) caused male offspring to exhibit depression-like behavior, its mechanism has not been fully understood. The current study aimed to identify the mediation role of corticotropin-releasing hormone (CRH) in the impact of CSDP on inducing depression in male offspring mice and to explore its mechanism. Forced swimming test, tail suspension test and sucrose preference test were performed to evaluate the degree of depression. Neurons in the hippocampal CA3 region were stained by hematoxylin and eosin and observed under microscope. Mammalian target of rapamycin (mTOR) protein levels in the hippocampus were determined by Western blot analysis. The CRH concentrations in the hippocampus were measured by enzyme-linked immunosorbent assay (ELISA). The results showed that CSDP significantly increased depressive-like behavior and neuronal damage of hippocampal CA3 region, and elevated hippocampal CRH levels in male offspring. CRHR1 and N-methyl-D-aspartic acid receptor (NMDAR) antagonists (Antalarmin and MK-801, respectively) ameliorated the depressive behavior and pathological damage of the hippocampus in offspring. In addition, CRHR1 and NMDAR antagonists prevented reduced mTOR expression levels induced by CSDP in offspring. In vitro, CRH lowered the expression of mTOR in cultured hippocampal organotypic slices, whilst CRHR1 antagonist NBI30775 and NMDAR antagonist MK801 prevented decreased expression of mTOR induced by CRH. It is concluded that CRH may mediate the impact of CSDP on depression in male offspring. This is through activating NMDAR and decreasing mTOR protein expression in the hippocampus, which result in neuronal injury in the CA3 region of offspring hippocampus.

Keywords: Depression; corticotropin-releasing hormone; pregnancy; stress

1. Introduction

Depression is a common mood disorder that occurs in 7%–15% of men and 10%–30% of women during their lifetime. It has been predicted that by 2020 depression will be the second leading cause of disability in the world [1, 2]. While the etiology of depression is not fully understood, a large number of studies indicate that the incidence of depression is associated with abnormalities in the prefrontal cortex, hippocampus, amygdala and hypothalamus [3]. Brain tissue shrinkage, neuronal atrophy, and synaptic loss may be one of the causes of depression [4].

Stress is an emotional state caused by dangerous or unexpected changes in the external environment [4, 5]. Stress and stressful experiences have long been implicated in the etiology and pathophysiology of chronic physical and mental health conditions [6]. Stressors include natural disaster, threat from a predator, mugging, an accident, noise, pollution, hot or cold climate, toxic environment, interpersonal conflict, lack of nutrition, severe crowding, severe financial hardship, changes in life-style, critical illness or death of first-degree relatives [7-10]. A hallmark of the stress response system is the activation of the autonomic nervous system and hypothalamic-pituitary-adrenal (HPA). The “fight-or-flight” response is a classical way of envisioning the behavioral and physiological response to a threat from a dangerous situation [7]. Stressful experiences can be classified as “good”, “tolerable” or “toxic” depending on the extent to which an individual has control over a given stressor and access to coping systems [11]. Appropriate and acceptable levels of stressful experiences can result in resilience and well-being due to positive outcomes such as growth, adaptive learning. A recent study has shown that appropriate stress induced the formation of reactive oxygen species, up-regulated autophagy, and recycled nutrients, with a potential anti-aging effect and extension of lifespan [12]. By contrast, other stressful experiences can foster a proliferation of recursive neural, physiological, behavioral, cognitive, and emotional changes that increase vulnerability to ill health and premature death [13]. Previous studies suggested that compensatory enhancement in hippocampal neurogenesis was

related to the long-term individual differences in maladaptive responses to stress [14]. For some individuals, exposure to chronic stress leads to a pathologically enhanced allostatic response, which ultimately results in disease states such as major depressive disorder or posttraumatic stress disorder [7]. Animal studies have found that chronic stress during pregnancy (CSDP) can induce depression-like behavior in their offspring [15]. Furthermore, some studies have demonstrated that prenatal stress caused depression in male offspring rodents [16, 17]. However, the mechanism by which CSDP increases the risk of depression in their offspring remains to be clarified.

CSDP affects the HPA axis function in various animals [18]. Evidence from studies in animals such as rodents, sheep, and non-human primates has shown that exposure to CSDP led to significantly elevated levels of corticotropin-releasing hormone (CRH) and glucocorticoids (GCs) in the central nervous system and serum in offspring [19, 20].

CRH is produced predominantly by neurons in the paraventricular nucleus of the hypothalamus [21, 22]. Furthermore, CRH is also produced by neurons in extra-hypothalamic limbic structures like amygdala and hippocampus [23, 24]. CRH is synthesized and released by hypothalamus neurons, which play a major role in regulating activity of the HPA axis and triggers the classic endocrine stress response [25], whereas outside the HPA axis CRH acts as a modulator of synaptic transmission at pre- and postsynaptic sites within specific central neuronal circuits rather than as a hormone [26]. CRH evokes its effects by activating the G-protein coupled CRH receptor1 (CRHR1) and CRH receptor2 (CRHR2) [22, 27], which are critical for the development and integration of physiological responses to stressful stimuli. CRHR1 is highly expressed throughout the brain, particularly in regions associated with affective, stress and nociceptive circuits [28]. Previous studies showed that a high level of CRH was associated with the occurrence of depression [29, 30]. In vitro, exposure to CRH provoked spine loss and dendritic regression in hippocampal organotypic cultures, and CRHR1 antagonists significantly ameliorated this damage [31]. Since CSDP leads to

significantly elevated levels of CRH in the central system and serum of offspring, it can be hypothesized that CRH mediates the impact of CSDP on depression in offspring.

N-methyl-D-aspartic acid receptor (NMDAR) is one of Glutamate receptor, which plays an important role as a neurotransmitter during stress-induced structure and function changes in the hippocampus [32]. NMDAR is considered to be associated with depression [33]. Previous research suggested that dendritic spine damage caused by CRH was dependent on activation of NMDAR [34]. Therefore, we hypothesized that NMDAR also mediates the CSDP inducing the depression in offspring.

Mammalian target of rapamycin (mTOR) is a typical serine/threonine protein kinase. Animal studies have shown that mTOR signaling was closely associated with the risk of depression [35, 36]. Animal studies [37] also showed that the rapid antidepressant effect of ketamine was mediated by up-regulation of mTOR signaling, and this effect was blocked by inhibition of mTOR activation. Recent studies revealed a significant association between NMDAR and mTOR levels in that NMDAR activation down-regulates the expression of mTOR [38]. Therefore, we speculated that CSDP induces depression in offspring via the CRH-NMDAR-mTOR signal pathway.

In this study, we examined the depression related indices in male offspring mice born from the dams exposed to chronic stress, and assessed whether CRHR1 and NMDAR antagonist ameliorated depression-like symptom and pathological change in offspring mice induced by CSDP. We also explored whether CRHR1 and NMDAR antagonist could improve the expression of mTOR proteins. In vitro we further investigated whether inducing depression in male offspring by CSDP was through CRH-NADHR-mTOR signal pathway in hippocampus.

2. Experimental Procedures

2.1. Animals and experimental design

In this study, we used Kunming mice, which originated from Swiss mice and are commonly used as an animal model in China. Twenty five adult male and 83 female Kunming mice, aged approximately 8 weeks and weighing 30–40 grams, were obtained from the Experimental Animal Center of Anhui Medical University (China). Mice were housed for 7 days in pathogen-free environment under standard laboratory conditions (24±1°C room temperature, 50%±2% humidity, 12-h light cycles) and with free access to food and water. The purpose of this period of acclimatization was to allow the mice adapt to the new environment and reduce the stress caused by an unfamiliar environment before the start of the experiment.

All experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (<https://oacu.oir.nih.gov>) (NIH Publications No. 2010940400, revised 2010). All animal protocols were approved by the Animal Care of Anhui, China. Details of the timeline of the CSDP and experimental treatments in vivo are shown in Figure 1. Details of the timeline of organotypic hippocampal slice preparation and treatments in vitro are shown in Figure 2

2.1.1. Chronic stress during pregnancy (CSDP)

To obtain offspring mice, the female mice were allowed to mate with male mice when they were 10 weeks old. The day on which a seminal (copulation) plug was first observed was considered to be the gestational day 1 (G1). Subsequently, the pregnant females were housed individually with sawdust bedding. On gestational day 7 (G7), the pregnant females were randomly assigned to either the chronic stress during pregnancy (CPS) group or the control (CON) group. From G7 to G20, the pregnant females in the CPS group were subjected to a regime of chronic stress using a procedure (Table 1)

modified from that described by Kiryanova [39].

Table 1 Chronic unpredictable stress schedule used in pregnant mice (G7 to G20)

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
First week	Tail pinch (5 min)	Restraint (2 h)	Cold water swimming (5 min)	Warm water swimming (15 min)	Water deprivation (24 h)	Night illumination (12 h)	Tail pinch (5 min)
Second week	Cold water swimming (5 min)	Night illumination (12 h)	Restraint (2 h)	Tail pinch (5 min)	Cold water swimming (5 min)	Food deprivation (24 h)	Warm water swimming (15 min)
Third week	Tail pinch (5 min)	Water deprivation (24 h)	Warm water swimming (15 min)	Food deprivation (24 h)	Tail pinch (5 min)	Night illumination (12 h)	Cold water swimming (5 min)

Stressors consisted of tail pinches (2 cm apart from the end of the tail) for 5 min; restraint for 2 h; cold water swimming (4–8°C) for 5 min followed by towel-drying; warm water swimming (28–35°C) for 15 min followed by towel-drying; night illumination; water deprivation for 24 h; and food deprivation for 24 h. One stressor was applied per day. Consecutive selection of the same stressor was avoided. Mice in the control group were left undisturbed for the duration of their pregnancies. The chronic unpredictable stress procedure has been shown to produce neurobiological changes, including robust activation of the HPA axis in pregnant mice [39, 40].

2.1.2. Offspring

Pups were weaned at postnatal day 21 (PND21). Male and female mice were housed separately, while mice of the same sex were housed two per cage. To identify the role of CRH in the induction of depression in offspring following CSDP, only male offspring were used in this study. On PND41, the behavioral experiments were conducted to measure the degree of depression in offspring. Offspring from the dams exposed to chronic stress were assigned to the CPS group, and offspring from the dams without any treatment were assigned to CON group.

2.1.3. Treatment with CRHR1 antagonist or NMDAR antagonist

To identify the mediation role of CRH in impact of CSDP on inducing depression

in offspring, male offspring were divided into three groups (20 animals/group) On PND 21: (1) CON group, the prenatal control; (2) CPS group, the prenatal stress-offspring vehicle group, offspring animals received daily intraperitoneal (ip) injection of vehicle (warm saline with 20 % Tween-80) starting from PND21 to PND41; (3) CPS+CRHR1 antagonist group, the offspring mice received daily injection of Antalarmin (suspended in warm saline with 20% Tween-80, 20 mg/kg, ip, Sigma-Aldrich, St. Louis, USA) from PND21 to PND41. The selection of an effective dose of Antalarmin was based on previous reports [41]. No inflammatory reactions were observed on the abdominal skin after multiple injections of the vehicle, and there were no significant differences in body weight and food consumption among different groups. To further study the effect of NMDAR on offspring depression induced by CSDP, NMDAR antagonist MK-801 (0.1 mg/kg, ip, Sigma-Aldrich, St. Louis, USA) was administered once daily from PND21 to PND41. The design of this experiment was similar to the design of the experiment mentioned above.

2.2. Forced swim test (FST)

On PND42–43, the forced swim test was used to measure the degree of depression in offspring. As previously described [42], mice were placed in a 2 liter (L) beaker containing 1.3 L of water (18–20°C). Each mouse (n = 10 per group) was randomly placed in a beaker, in which the level of water prevented mice from escaping or reaching the bottom of the container. During a 6 minutes trial, mice were continuously monitored for immobility behavior from 1 to 6 minutes. Immobility was defined as the lack of all motion except respiration and that required to keep the mouse afloat. At the end of the trial, the animal was removed from water, towel-dried, and returned to its cage.

2.3. Tail suspension test (TST)

The TST is another well-established model for screening depression [43, 44]. Briefly, mice were suspended by the bands and hung from a hook mounted 50 cm above the floor for a 6-min trial. On PND43–44, each mouse (n = 10 per group) was randomly

suspended and assessed. The time that the mice were immobile during the 6-min testing period was recorded. Immobility was considered as lack of all movement except for whisker movement and respiration.

2.4. Sucrose preference test (SPT)

SPT was carried out as described by Willner et al [45]. On PND44-46, each mouse (n = 10 per group) was measured. Briefly, 72 h before the test, mice were adapted to 1% sucrose solution (w/v): two bottles of 1% sucrose solution were placed in each cage, and 24 h later 1% sucrose in one bottle was replaced with tap water and kept for further 24 h. After this, mice were deprived of water and food for 24 h. The SPT was conducted at 9:00 a.m. on mice which were housed in individual cages and had free access to two bottles containing 100 mL of sucrose solution (1%, w/v) and 100 mL of water. After 1 h, the volumes of sucrose solution and water consumed were recorded and the sucrose preference was calculated as the sucrose preference (%) = sucrose consumption / (sucrose consumption + water consumption).

2.5. Measurement of corticotropin-releasing hormone

Concentrations of CRH in the hippocampus were measured 2 h after the last behavior test (our preliminary study showed that the concentrations of CRH increased slightly in 1 h after behavioral experiments and returned to a stable state in 2 h after behavioral experiments). CRH levels in hippocampus of the offspring were determined using a commercially available enzyme-linked immune sorbent assay (ELISA) kit (Cusabio, #CSB-E08038r, Wuhan, China) according to the manufacturer's instructions. Optical densities were read at 450 nm using the Epoch microplate reader (BioTek, USA). In addition, protein concentrations were determined using the Lowry protein assay. CRH levels were recorded as ng/mg protein.

2.6. Morphological observations

Tissue damage was evaluated using hematoxylin and eosin (H&E) staining. Four

mice were randomly selected from each group and the brain tissues were harvested after perfusion with 4% paraformaldehyde and placed into 4% paraformaldehyde, paraffinized. Serial coronal tissue sections (10- μ m thick) were prepared. Sections from the same location in the hippocampus of different groups were then stained with H&E. Two sections from each mouse (with one from each level, separated by approximately 60 μ m) were then observed under an optical microscope (Carl Zeiss, Axiovert. A1, Germany). The images were subsequently digitized and quantified using Image J. Finally, the number of neurons in each field of vision (400 \times magnification) was counted.

2.7. Western blot analysis

The hippocampal tissues were dissected quickly on ice and stored at -80°C for later use. Total protein extraction and Western blotting analysis were performed according to previously described methods [46]. Proteins (35 mg per lane) were separated by 8% SDS-PAGE and subsequently transferred to nitrocellulose membranes. The blots were blocked with 5% skimmed milk powder in 0.1% Tris-buffered saline/Tween-20 at room temperature for 2 h, then incubated overnight at 4°C with primary antibodies for the detection of mTOR (1:2,000, Abcam, USA); anti- β -actin antibody (1:1,000, Elabscience, Wuhan, China) was used as a reference. After three washes with Tris-buffered saline/Tween-20, the membrane was incubated with the horseradish peroxidase-conjugated secondary antibodies (1:200, Zhongshanjinqiao, Beijing, China) for 1 h at room temperature. Immunoreactive proteins were detected and visualized using the enhanced chemiluminescence Western blot detection system (Tanon 5200 Imager, China). To control for sampling errors, the band intensities were normalized against β -actin to quantify the relative protein expression levels.

2.8. Preparation of organotypic hippocampal slice cultures

The inhibitory effects of CSDP on mTOR activation might occur via two mechanisms: through CRH-mediated activation of CRHR1 and NMDAR in the hippocampus, or alternatively through glucocorticoid-mediated activation of the

glucocorticoid receptor. On further examining whether CSDP inhibits mTOR activation by directly activating CRHR1 and NMDAR in hippocampus, organotypic hippocampal slice cultures were prepared. In fact, using organotypic hippocampal slice from mice is better suited to complement the study performed in juvenile mice. Since it is very difficult to dissect out the hippocampus and prepare 200- μ m thick slices in 10-day-old mice, 10-day-old Sprague–Dawley rats were used as an alternative [47, 48]. After sacrifice by cervical dislocation, the rats were placed in a beaker containing 75% alcohol for sterilization and then quickly transferred to culture dishes containing ice. The whole brain was removed and placed in Hank's balanced salts (HBSS) solution at 4°C. The hippocampus was dissected out, washed in HBSS solution, and 200- μ m thick coronal slices were prepared using a vibratome (HA752 Vibroslice Tissue Slicer, Campden instruments, Loughborough, UK). Hippocampus slices (3–4) were then placed on Millicell-CM microporous membrane (0.4 μ m pore size; Millipore, Marlborough, MA, USA) inserts that had been pre-incubated in 1 ml of culture medium at 37°C and placed in 35 mm 6-well culture plates. The medium was composed of 36 mM glucose, 50% Minimum Essential Media (MEM, Thermo Fisher, Gibco, USA), 25% heat-inactivated horse serum (Thermo Fisher, Gibco), 24% HBSS, and 1% 100 UL/ml penicillin/streptomycin. Plates were incubated at 37°C under 5% CO₂ for one week and the medium was changed every other day. The hippocampus slices were cultured in a medium with sufficient nutrient medium and gas-liquid interface.

2.9. Medicine treatment *in vitro*

NBI 30775 (2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylamino-pyrazolo[1, 5-a]pyrimidine) is a potent small-molecule CRHR1 antagonist with high affinity for the CRHR1. It is easily dissolved in dimethyl sulfoxide (DMSO). NBI 30775 has been shown to reduce both anxiety-like behavior and depressive behavior in rodents [49]. Moreover, a small open-label clinical study suggested that NBI 30775 reduced measures of both anxiety and depression in depressive patients [50]. MK-801 [(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo [a,d]cyclohepten-5, 10-imine

hydrogen maleate, Dizocilpine hydrogen maleate] is an NMDAR antagonist, which exhibited antidepressant activity in patients and in a range of preclinical screening procedures [51]. To further investigate the ability of CSDP to inhibit mTOR activation by directly activating CRHR1 and NMDAR in the hippocampus, synthetic rat/human CRH (Tocris, Bio-Techne Corporation) and the CRHR1 antagonist NBI 30775 (Sigma–Aldrich, St. Louis, USA) or the NMDAR antagonist MK-801 (Sigma–Aldrich, St. Louis, USA) were applied to the organotypic hippocampal slice cultures. From day 2 to day 7, the slices were transferred into fresh culture medium containing 100 nM CRH and various concentrations of the CRHR1 antagonist NBI 30775 (0, 0.1, 1, and 10 μ M) or the NMDAR antagonist MK-801 (0, 1, 5, and 50 μ M). The ambient levels of CRH in extracellular space of control and offspring mice from CSDP are difficult to estimate, and available studies showed that the level of CRH released within the hippocampus was about 0.16 pg/mg wet tissue/1 hour [52]. Therefore, the dose of CRH used in this study was set according to previous reports [31, 53]. Dose setting for NBI 30775 and MK-801 was also based on previous reports [31, 54, 55]. On day 8, mTOR expression was measured by Western blot analysis.

2.10. Statistical analysis

Differences in FST, TST and SPT results, CA3 neuronal counts and hippocampal CRH levels between the CPS and CON groups were analyzed using Student's *t*-test. One-way ANOVAs followed by Tukey post hoc comparisons were used to examine changes in mTOR expression, FST and SPT behavioral responses and CA3 neuronal counts among CON group, CPS group and CPS+CRHR1 antagonist group (or NMDAR antagonist). Differences in mTOR expression among the Control, CRH, NBI 30775 (or MK-801) groups were analyzed in one-way ANOVA with Tukey's post-hoc tests. Results were expressed as the mean \pm standard deviation (SD) of at least three independent experiments. The level of significance was $P \leq 0.05$.

3. Results

3.1. CSDP induced depression in male offspring mice

Compared with the CON group, male offspring mice in the CPS group exhibited more immobility time in the FST (Fig. 3A; $t_{(v=18)} = 6.057, P < 0.01$) and TST (Fig. 3B; $t_{(v=18)} = 9.455, P < 0.01$). Moreover, compared with the CON group, mice in the CPS groups exhibited a significant decreased sugar preference rate in SPT (Fig.3C, $t_{(v=18)} = 3.574, P < 0.01$).

3.2. CSDP enhanced CRH levels and aggravated neuropathological damage in the hippocampus of male offspring mice

To examine if the depression in male offspring was caused by the damage in hippocampus, the mice hippocampal CA3 region was observed by HE staining. The data showed that in CON groups most pyramidal neurons were clearly stained, neatly arranged, round spheres, round nucleus, and obvious nucleoli in CA3 region of the hippocampus (Fig.4A). Compared with the CON group, the number of neurons in CPS groups declined ($t_{(v=6)} = 3.278, P < 0.01$), and some pyramidal neurons were disordered, exhibiting loose arrangement and dark-staining, with visible nuclear shrinkage (Fig.4B).

As shown in Figure 4C, hippocampal CRH levels were significantly higher in the CPS group compared with those in the CON group ($t_{(v=6)} = 3.133, P < 0.01$).

3.3. CRHR1 antagonist ameliorated depressive-behavior and pathological damage of hippocampus in male offspring mice

To determine whether CSDP inducing depression in male offspring is mediated by CRH, mice were injected intraperitoneally with CRHR1 antagonist Antalarmin (ANT) (20 μ g/g mice/d, PND21–PND41). Behavioral performance analysis showed that compared with the CON group, the offspring mice in the CPS group exhibited more immobility time in FST (Fig. 5A, $F_{(2,27)} = 137.71, P < 0.01$) and decreased sugar preference rates in SPT ($F_{(2,27)} = 7.18, P < 0.01$) (Fig. 5B,). The neuropathological

changes were characterized by soma condensation, loosened neurons, decreased number of neurons, and nuclear pyknosis in the CA3 region of offspring hippocampus (Fig. 5D). Compared with the CPS group, the offspring mice in the CPS+ANT group had longer motion time in FST (Fig. 5A, $F_{(2,27)} = 137.71$, $P < 0.01$) and increased sugar preference rate in SPT ($F_{(2,27)} = 7.18$, $P < 0.05$) (Fig. 5B). The neuropathological changes were characterized by reduced neuronal damage and increased neuron number in the CA3 region of the hippocampus in the CPS+ANT group compared with that in the CPS group (40.57 ± 8.65 vs. 63.45 ± 5.56 , $F_{(2,9)} = 12.12$, $P < 0.05$) (Fig. 5E).

3.4. NMDAR antagonist treatment ameliorated depressive-behavior and pathological damage of hippocampus in male offspring mice

To determine whether CSDP inducing depression in male offspring is mediated by the NMDAR too, mice were injected intraperitoneally with NMDAR antagonist MK801 (0.1mg/kg mice/d, PND21-PND41). The behavior performance analysis showed that compared with the CON group, the offspring mice in the CPS group had less motion time in FST ($F_{(2,27)} = 145.578$, $P < 0.01$) (Fig. 6A) and decreased sugar preference rates in SPT ($F_{(2,27)} = 7.626$, $P < 0.01$) (Fig. 6B). Compared with the CPS group, the offspring mice in CPS+MK-801 group had longer motion time in FST (Fig. 6A, $F_{(2,27)} = 145.578$, $P < 0.01$) and an increased sugar preference rates in SPT (Fig. 6B, $F_{(2,27)} = 7.626$, $P < 0.05$). H&E staining revealed that some neurons in the CPS group were loosely arranged and disordered, with pyknosis observed in some cells (Fig. 6D). Compared with the CPS group (45.67 ± 16.56), the total number of neurons in the CPS+MK-801 group (77.33 ± 6.69) was increased ($F_{(2,9)} = 12.12$, $P < 0.05$) and the damaged neurons were reduced (Fig. 6E).

3.5. CRHR1 or NMDAR antagonist prevented the decreased expression of mTOR induced by CSDP in the hippocampus of male offspring mice.

On examining the level of mTOR expression in the hippocampus of the offspring mice, we found that CPS group had significantly decreased expression of mTOR in

hippocampus tissues when compared with CON group ($F_{(2,9)}=95.360$, $P < 0.01$). Compared with the CPS group, the protein levels of mTOR in hippocampus in CPS+ANT was increased ($F_{(2,9)}=95.360$, $P < 0.01$) (Fig.7A), suggesting that CRHR1 receptor antagonist prevents the decreased mTOR caused by CSDP. In addition, compared with CPS group, the protein levels of mTOR in CPS+MK801 group were also significantly elevated ($F_{(2,9)}=5.301$, $P < 0.05$) (Fig. 7B), indicating that NMDAR antagonist MK801 treatment improves mTOR expression in offspring.

3.6. CRHR1 and NMDAR antagonist increased mTOR expression in organotypic hippocampal slices in vitro

To investigate how the CRH signal contributed to mTOR expression, hippocampal organotypic slice were treated with the CRHR1 antagonist NBI30775. Western blot analysis showed significantly reduced mTOR expression in the hippocampal slices of the CRH group compared with those in the CON group ($F_{(4,20)} = 128.117$, $P < 0.01$). Compared with the CRH group, the expression of mTOR in the groups treated with three different doses of NBI 30775 were significantly increased ($F_{(4,20)} = 128.117$, $P < 0.01$), with the most marked increase observed in the middle dose group (Fig. 8A), indicating that the CRHR1 antagonists prevented the CRH-induced decrease in mTOR levels in vitro. To further verify that CRH inhibited mTOR activation by activating NMDAR, hippocampal organotypic slices were treated with CRH in the presence of the NMDAR antagonist MK-801. As shown in Figure 8B, CRH effectively reduced the expression of mTOR proteins ($F_{(4,20)} = 15.268$, $P < 0.01$). Compared with the CRH group, mTOR expression was significantly increased following MK-801 treatment at all three doses ($F_{(4,20)} = 15.268$, $P < 0.05$), with the most marked increase observed in the middle dose group (Fig. 8B). These suggested that CRH inhibits mTOR expression by activating NMDAR.

4. Discussion

In this study, we examined the role of CRH in the impact of CSDP on the induction of depression in male offspring mice. We verified that CSDP induced depression in male offspring mice and was associated with neuronal damage in the hippocampal CA3 region as well as elevated hippocampal CRH levels. CRHR1 and NMDAR antagonists ameliorated the CSDP-induced depressive behavior and pathological damage of the hippocampus in male offspring mice. In addition, CRHR1 and NMDAR antagonists prevented the decreased levels of mTOR induced by CSDP in male offspring. In vitro, CRHR1 and NMDAR antagonists inhibited the decreased expression of mTOR induced by CRH.

Some studies have shown that prenatal stress mainly caused depression in male offspring rodents [16, 17, 56, 57]. Our preliminary studies also showed that prenatal stress caused depression in male but not in female Kunming offspring mice. In this study we focused on the impact of CSDP on depression in male offspring mice. The FST and the TST have been widely used for measuring the pharmacological effects of antidepressant. The immobility and escape responses were interpreted not only as “depression” or “depression-like”, but also as coping with stressors in order to survive [58, 59]. Our results showed that compared with control group, male offspring mice in CPS group exhibited more immobility time in the FST and TST, and a lower sugar preference rate in the SPT. These findings suggest that CSDP could induce depressive symptoms in male offspring.

In previous studies of depression in mice, the hippocampus was found to be closely related to emotions as well as being rich in various neurotransmitters and receptors. It was also the main tissue that was likely to be damaged by stress [60]. In this study, the pathological results showed that the number of neurons in the hippocampal CA3 region of the male offspring in the CPS group was lower than that of the Control group. We also found that the neurons were loosely arranged and disordered, indicating that CSDP

induced the neuronal damage in the hippocampal CA3 region of male offspring. Previous studies showed that there are lots of CRHR1 in hippocampal CA3 region [24], and exposure to CRH provoked spine loss and dendritic regression in hippocampal organotypic cultures [31]. Thus, it can be speculated that increased CRH is involved in the mechanism by which CSDP induces damage in hippocampal CA3 neurons. Furthermore, some studies have reported a close relationship between CA3 dysfunction and depression [61, 62]. It is thus possible that CA3 neuronal injury induced by CSDP may accentuate depressive-like behaviors in the CPS group. Several studies have shown that the dendrites of CA3 neurons appear to be the more sensitive to chronic stress or glucocorticoids than the dendrites from the CA1 region [63]. Therefore, we checked the pathological changes in CA3 region of the hippocampus. Although CA1 region of the hippocampus might be sensitive to chronic stress too [64], unfortunately, this study did not examine the pathological changes in CA1 region. We will observe the pathological changes in CA1 region of the hippocampus in the future study.

As mentioned above, CRH is an important hormone contributing to stress, and may be associated with the occurrence of depression. In the current study, the levels of hippocampal CRH in the male offspring in the CPS group were significantly higher (by 28%) than those detected in the CON group, suggesting that the mechanism by which CSDP induces depressive symptoms in male offspring may be associated with elevated CRH levels. In addition, our study showed that treatment with the CRHR1 antagonist improved the pathological changes induced by CSDP in neurons in the CA3 region of male offspring. This suggests that the hippocampal neuronal damage induced by CSDP in male offspring is mediated by CRH. Because CRHR1 is highly expressed throughout the brain, particularly in regions associated with affective, stress and nociceptive circuits, the current use of a single drug at single doses represents just a starting point for more adequate experimentation. The effects of different doses of CRHR1 antagonist on the pathological changes induced by CSDP in neurons in the CA3 region of male offspring will be investigated in future studies.

At present, glutamate and its NMDAR are widely associated with various neurological conditions and depression. NMDAR blockers and related drugs have been shown to have anti-depressant effects [65]. A previous study by Carozzi et al. [32] suggested that stress caused a significant increase in rat hippocampal glutamate levels, and excessive activation of NMDAR resulted in excitatory neurotoxic effects, which are associated with a variety of neurological diseases. Our data also indicated that CSDP induced marked depression-like behavior, and that the NMDAR antagonist MK-801 alleviated depressive symptoms. In addition, our data showed that NMDAR antagonists alleviated the pathological damage of neurons in the CA3 region of offspring mice induced by CSDP, suggesting that CSDP inducing depression and hippocampal neuronal damage in male offspring is also mediated by NMDAR.

The mTOR signaling is involved in neuronal differentiation, and increases the synaptic signaling protein and the length and number of synaptic ridges. This in turn mediates the occurrence of depression [66]. In this study, we found that CSDP significantly lowered mTOR protein expression in the hippocampus of male offspring mice, while the CRHR1 antagonist enhanced mTOR expression. These findings suggest that the decreased expression of mTOR induced by CSDP in the hippocampus of male offspring mice is mediated by CRH. In addition, the expression of mTOR in hippocampus of CPS group was significantly decreased compared with that in the CON group. Whereas after male offspring were treated with the NMDAR antagonist MK-801, the expression of mTOR was increased too, indicating that CSDP inhibits mTOR expression via the NMDAR signaling pathway.

In vivo, the inhibitory effects of CSDP on mTOR activation might occur via two mechanisms: through CRH-mediated activation of CRHR1 and NMDAR in the hippocampus, or alternatively through glucocorticoid-mediated activation of the glucocorticoid receptor. CSDP has been reported to increase plasma glucocorticoid levels in rodent offspring [20]. The increased glucocorticoids may penetrate the blood brain barrier and act on receptors found in hippocampal neurons, resulting in decreased

expression of mTOR and dendritic atrophy [67]. Involvement of glucocorticoids in the mechanisms by which CSDP causes decreased levels of mTOR and hippocampal defects could not be excluded by systemic administration of a CRHR1 antagonist, because the antagonist blocked both pituitary and central CRH receptors [68, 69]. Blocking pituitary CRHR1 would attenuate adrenocorticotrophic hormone and glucocorticoid release and interaction with hippocampal neurons. Therefore, it is conceivable that systemic administration of the CRHR1 antagonist ameliorates the effects of CSDP by reducing glucocorticoid. On further examining whether CSDP inhibits mTOR activation by directly activating CRHR1 in hippocampus, CRH, CRHR1 antagonist NBI 30775, were applied to organotypic hippocampal slice cultured in medium in vitro. We found that CRH could significantly reduce the expression of mTOR, and CRHR1 antagonist NBI 30775 prevented the CRH-induced decreased mTOR levels in vitro. On further examining whether CRH inhibits mTOR activation by activating NMDAR in hippocampus, we observed that CRH intervention effectively reduced the mTOR expression, while mTOR expression was significantly increased in the CRH+MK-801 groups. These results suggest the CSDP inhibits mTOR activation by CRH through activation of CRHR1 and NMDAR in the hippocampus.

Krishnan et al found that compensatory enhancement in hippocampal neurogenesis was related to the long-term individual differences in maladaptive responses to stress [14]. In this study, we randomly selected four mice from each group to observe the pathological morphology and mTOR levels in the hippocampus. Unfortunately, we did not record the behavioral changes and corresponding hippocampal pathological morphology and mTOR levels of each mouse. Therefore, we were unable to analyze individual differences in behaviorally coping with stressors related to the cellular and molecular changes in the hippocampus. This is one limitation of our study that we will address in future research. In addition, the immobility and escape responses in FST and TST were interpreted not only as “depression” or “depression-like”, but also as coping with stressors in order to survive. The chief

consistent false positive in the FST are psychomotor stimulants, which reduce immobility and are probably not clinically effective as antidepressants [58, 59]. Therefore, an alternative method that provides a more accurate measure of the antidepressant effect of CRHR1 and NMDAR antagonist should be employed in future studies.

In conclusion, CSDP induced depressive symptoms in male offspring accompanied by neuronal damage in the CA3 region and elevated CRH levels in the hippocampus. CRHR1 and NMDAR antagonists ameliorated the depressive-behavior and pathological damage of hippocampus in male offspring mice. CRHR1 and NMDAR antagonists prevented decreased levels of mTOR induced by CSDP in male offspring. In vitro, CRHR1 and NMDAR antagonist ameliorated the decreased expression of mTOR induced by CRH. These findings suggest that CRH mediating the impact of CSDP on depression in male offspring was through CRH-NADHR-mTOR signaling pathway in hippocampus.

Conflicts of interest

There are no conflicts of interest concerning any of the authors.

Funding

This work was supported by the National Natural Science Foundation of China (grant no. 81773452).

Authors contribution statement

Yili Lv: conducted experiments, and drafted manuscript; Peng Chen: conducted experiments; Liang Kuang: conducted experiments; Zhenmin Han: conducted experiments; Bhawna Solanki: provided critical feedback on manuscript; Weiju Zhou: provided critical feedback on manuscript; Fangbiao Tao: designed experiments; Ruoling Chen: supervised manuscript writing and provided critical feedback on manuscript; Yuyou Yao: designed experiments, conducted experiments and wrote manuscript. All interpreted data and reviewed and revised the manuscript.

Acknowledgment

The authors express heartfelt thanks to Wei Tang and Qunang Wang for their excellent technical assistance.

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Figure Legends

Fig. 1 Timeline of chronic stress during pregnancy (CSDP) and experimental treatments CSDP: Chronic stress during pregnancy, FST: Forced swim test, TST: Tail suspension test, SPT: Sucrose preference test, ELISA: Enzyme-linked immune sorbent assay.

Fig. 2 Timeline of preparation of organotypic hippocampal slice and Medicine treatments

Fig. 3 The effects of chronic stress during pregnancy on behavioral experiments in male offspring mice. The offspring from the dams exposed to chronic stress were used as a CPS group, and the offspring from the dams not subjected to any treatment were used as a CON group. On the postnatal day (PND) 41 days, behavioral experiments were conducted to measure the degree of depression in male offspring mice. (A) Immobility time in FST. (B) Immobility time in TST. (C) Percentages of sucrose consumption in SPT. The values represent the mean \pm SD, $n = 10$, $**P < 0.01$, compared with CON group.

Fig. 4 The effects of chronic stress during pregnancy on neuropathological changes in the CA3 region of hippocampus and the level of CRH in hippocampus in male offspring mice. 2 hours after the behavioral experiment, 4 mice from each group were subjected to quick decapitation, their hippocampus was immediately dissected on ice, and the supernatant of hippocampus was centrifuged for ELISA experiment. For HE staining, brains were removed after perfusion with 4% paraformaldehyde and placed into 4% paraformaldehyde followed by conventional paraffin embedding. The sections were observed under an optical microscope. (A) HE staining in hippocampal CA3 region in CON group in male offspring mice. (B) HE staining in hippocampal CA3 region in CPS group in male offspring mice. $n = 4$, Bar=20 μ m (C) Levels of hippocampal CRH in male offspring mice. The values represent the mean \pm SD, $n = 4$, $**P < 0.01$, compared with CON group.

Fig. 5 The effects of CRHR1 antagonist on behavioral experiments and neuropathological changes in the CA3 region of hippocampus in male offspring mice. Offspring animals in CON, CPS or CPS+ANT groups received a daily intraperitoneal injection (ip) of vehicle or CRHR1 antagonist Antalarmin (ANT) (20mg/kg mice/d) from PND21 to PND41. 24 hours after last injection of vehicle or ANT, FST and SPT was used to detect the degree of depression in mice. After the behavioral experiment, for HE staining, brains were removed after perfusion with 4% paraformaldehyde and placed into 4% paraformaldehyde followed by conventional paraffin embedding. The sections were observed under an optical microscope. (A) Immobility time in forced swimming test. (B) Percentages of sucrose consumption in SPT. The values represent the mean \pm SD, n = 10, The columns with different symbol (*, #) are significantly different. **P<0.01, compared with CON group. # P<0.05, ##P<0.01, compared with CPS group. (C) HE staining in hippocampal CA3 region in CON group. (D) HE staining in hippocampal CA3 region in CPS group. (E) HE staining in hippocampal CA3 region in CPS+ANT group. n = 4, Bar=20 μ m

Fig. 6 The effects of MK801 treatment on behavioral experiments and neuropathological changes in the CA3 field of hippocampus in male offspring mice. Offspring mice in CON, CPS or CPS+MK801 groups received daily intraperitoneal injection (ip) of vehicle or NMDAR antagonist MK801 (0.1mg/kg mice/d) from PND21 to PND41. 24hours after last injection of vehicle or MK801, FST and SPT were used to detect degree of depression in mice. After the behavioral experiment, for HE staining, brains were removed after perfusion with 4% paraformaldehyde and placed into 4% paraformaldehyde followed by conventional paraffin embedding. The sections were then observed under an optical microscope. (A) Immobility time in forced swimming test. (B) Percentages of sucrose consumption in SPT. The values represent the mean \pm SD, n = 10, The columns with different symbol (*, #) are significantly different. **P<0.01, compared with CON group. # P<0.05, ##P<0.01, compared with CPS group. (C) HE staining in hippocampal CA3 region in CON group. (D) HE staining

in hippocampal CA3 region in CPS group. (E) HE staining in hippocampal CA3 region in CPS+MK801 group. n = 4, Bar=20 μ m

Fig. 7 CRH or NMDA receptor antagonist increased the expression of mTOR in the hippocampus of male offspring mice. After the behavioral experiment, 4 mice in each group were subjected to quickly decapitation and each hippocampus was immediately dissected on ice. The mTOR expression was measured by western blotting. Bar graphs showing the quantification of the protein band density of mTOR after normalization with β -actin. (A) Representative immunoblots showing the expression of mTOR in the hippocampus of offspring which received CRHR1 antagonist treatment. (B) Representative immunoblots showing the expression of mTOR in the hippocampus of offspring which received MK801 treatment. Data presented are the mean \pm SD, n = 4. The columns with different symbol (*, #) are significantly different. *P<0.05, **P<0.01, compared with CON group, # P<0.05, ##P<0.01, compared with CPS group.

Fig. 8 Effects of CRHR1 antagonist NBI 30775 and NMDAR antagonist MK801 on mTOR expression in organotypic hippocampal slice exposed to CRH in vitro. From day 2 to day7, cultures were transferred into fresh culture medium containing 100 nM CRH and various concentrations (0-10 μ M) of CRHR1 antagonist (NBI 30775) or various concentrations (0-50 μ M) of NMDAR antagonist MK-801. On day 8, the mTOR expression was measured by western blotting. Bar graphs showing the quantification of the protein band density of mTOR after normalization with β -actin. (A) Representative immunoblots showing the expression of mTOR in the hippocampal slices treated with various concentrations (0-10 μ M) of NBI 30775. (B) Representative immunoblots showing the expression of mTOR in the hippocampal slices treated with various concentrations (0-50 μ M) of MK-801. Data presented are the mean \pm SD, n = 5. *P<0.05, **P<0.01, compared with CRH group.