

Intranasal Administration of Melanin-Concentrating Hormone Reduces Stress-Induced Anxiety- and Depressive-Like Behaviors in Rodents

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Major depressive disorder is a complex neuropsychiatric disorder with few treatment options. Non-targeted antidepressants have low efficacy and can induce series of side effects. While a neuropeptide, melanin-concentrating hormone (MCH), is known to exhibit regulator of affective state, no study to date has assessed the anti-depressive effects of MCH in a stress-induced depression model. This study aimed to evaluate the pharmacological effects of intranasal administration of MCH on depression-related behavior in stressed rats and mice. Using a number of behavioral tests, we found that MCH treatment significantly decreased anxiety- and depressive-like behaviors induced by stress. Notably, the effects of MCH were equivalent to those of fluoxetine. MCH treatment also restored the activity of the mammalian target of rapamycin (mTOR) signaling pathway and normalized the levels of synaptic proteins, including postsynaptic density 95, glutamate receptor 1, and synapsin 1, which were all downregulated by stress. Interestingly, the protective effects of MCH were blocked by the mTOR inhibitor, rapamycin. These results suggest that MCH exhibits antidepressant properties by modulating the mTOR pathway. Altogether, this study provides an insight into the molecular mechanisms involved in the antidepressant-like effects of MCH, thereby paving the way for the future clinical application of MCH.

Key words: Depression, Mammalian target of rapamycin (mTOR), Melanin-concentrating hormone (MCH), Stress, Anxiety, Rapamycin

INTRODUCTION

Major depressive disorder (MDD) is a common but serious mood disorder that is diagnosed based on symptomatic criteria such as sadness or melancholia, irritability, feeling down, and a loss

of pleasure or interest in daily activities. It is often accompanied by other symptoms, including insomnia or hypersomnia, fatigue, and alterations in body weight, and is known to significantly increase the risk for suicide based on the Diagnostic and Statistical Manual of Mental Disorders (*DSM-V*) [1]. According to the World Health Organization [2], depression is the most common illness and the leading cause of disability worldwide. It has been estimated that 350 million people are affected by depression globally, with an increase of more than 18% between 2005 and 2015.

Depression results from the complex interaction between social, psychological, and biological factors. Among them, disrupted function in one or more steps of synaptic transmission may be a crucial mechanism underlying depression [3-7]. Numerous stud-

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ies have shown that the serotonergic system is involved in the pathology of depression, and the majority of serotonergic neurons are located within the dorsal raphe nucleus (DRN). They play an important role in the regulation of emotional states and several other functions, including motor activity and sleep-wakefulness [6, 7]. It has been shown that low levels of serotonin and/or its metabolites are present in the urine and cerebrospinal fluid of patients with depression [3] as well as in suicidal patients with depressive disorders [4, 5]. Among neuropeptides, melanin-concentrating hormone (MCH) has been reported to be associated with MDD [8]. Furthermore, MCH is a known regulator of affective state and has been shown to interact with the serotonergic system [8]. It was first discovered in teleost fish [9] and was subsequently found in humans, rodents, and other mammals [10-12]. MCH-expressing (MCHergic) neurons project throughout the central nervous system, including the somatosensory cortex, isocortex, caudate putamen, hippocampus, nucleus accumbens, and amygdala [13, 14]. These neurons are primarily located in the lateral hypothalamus and adjacent zona incerta, projecting extensively throughout the brain [13]. MCH has two types of receptors: MCH receptor 1 (MCHR1) and 2 (MCHR2). Only MCHR1 is found in rodents [15], and is distributed throughout various brain regions including the cerebral cortex, basal ganglia, hypothalamus, and brainstem [16], while both MCHR1 and MCHR2 are found in humans [17]. Central MCH displays a wide range of functions, including the regulation of sleep [18, 19], olfactory function [20], and depression [8], and has a major role in energy maintenance by decreasing energy expenditure and stimulating feeding behavior [21]. However, these findings, mostly based on preclinical models, need to be validated in humans [22].

In the previous study, intranasally administered MCH ameliorated pain-related symptoms, including depressive-like behavior [23]. These results suggest that MCH may have anti-depressive effects, however, to date, no study has revealed the antidepressant effects of MCH in a stress-induced depression model. Therefore, the objective of the present study was to evaluate the antidepressant-like effects of intranasal administration of MCH on stress-induced depressive-like behaviors in rats and mice.

MATERIALS AND METHODS

Animals

Eight-week-old adult male Sprague Dawley rats (Samtako Animal Co., Seoul, Korea) weighing 200~220 g were used in behavioral tests. The rats were maintained on a 12/12-h light/dark cycle (lights on at 8:00 am, lights off at 8:00 pm) under a controlled temperature of 22±2°C and relative humidity of 55±15%. All animals

were acclimated to this condition for 7 days after arrival.

For all experiments using mice, 6-week-old healthy male ICR mice (Orient Bio., Seoul, Korea) were allowed 1 week for quarantine and acclimatization. The mice were housed on a 12/12-h light/dark cycle (lights on at 7:00 am, lights off at 7:00 pm) under a controlled temperature of 22±1°C and a relative humidity of 55±1%. They were housed in polycarbonate cages and provided with tap water and commercial rodent chow (Samyang Feed, Daejeon, Korea) *ad libitum*.

The animal experiments in this study followed the 'Guide for Animal Experiments' provided by the Korean Academy of Medical Sciences, and the protocols were approved by the Institutional Animal Ethical Committee, Dongguk University, South Korea (Approval Number: IACUC-2016-034).

Single prolonged stress in rats

A single prolonged stress (SPS) model of post-traumatic stress disorder was used as an acute stress model. To examine the effects of different doses of MCH in the SPS model, the rats were divided into six groups (n=6/group): normal (Nor), SPS-induced stress (STR), STR+MCH 2 µg/30 µl, STR+MCH 8 µg/30 µl, STR+MCH 18 µg/30 µl, and STR+fluoxetine (Flu) as a positive control. To evaluate the mechanism(s) associated with the mTOR pathway, rapamycin, an inhibitor of mTOR, and four experimental groups were used (n=6/group): normal (Nor), SPS-induced stress (STR), STR+MCH 18 µg/30 µl (MCH), and STR+MCH+rapamycin (MCH+Rapa). For SPS, the limbs of rats were fixed with surgical tape on a metal board to restrict head motion. After immobilization for 2 h, the rats were immediately forced to swim for 20 min in a plexiglass cylinder (50 cm in height, 24 cm in diameter), which was filled with 24°C fresh water to two-thirds of its volume. Then, the animals were dried, allowed to recover for 15 min, and exposed to ether vapor until they became unconscious. After recovery from anesthesia, all animals were individually placed in a cage and left undisturbed for 7 days (Fig. 1A) [24]. The STR procedure was performed between 11 am and 4 pm.

Immobility stress in mice

To confirm the antidepressant-like effect of MCH in mice, mice were subjected to immobility-induced stress for 4 weeks. Stressed mice were treated with vehicle (STR), MCH (1, 5, or 25 µg/30 µl), or fluoxetine. In total, 36 male mice were blindly randomized into six different treatment groups (n=6/group): normal (Nor), immobility-stressed (STR), STR+MCH 1 µg/30 µl, STR+MCH 5 µg/30 µl, STR+MCH 25 µg/30 µl, and STR+fluoxetine (Flu) as a positive control. Animals in the stress group (STR) were individually placed into a flexible triangle-shaped vinyl screen with a nose-hole

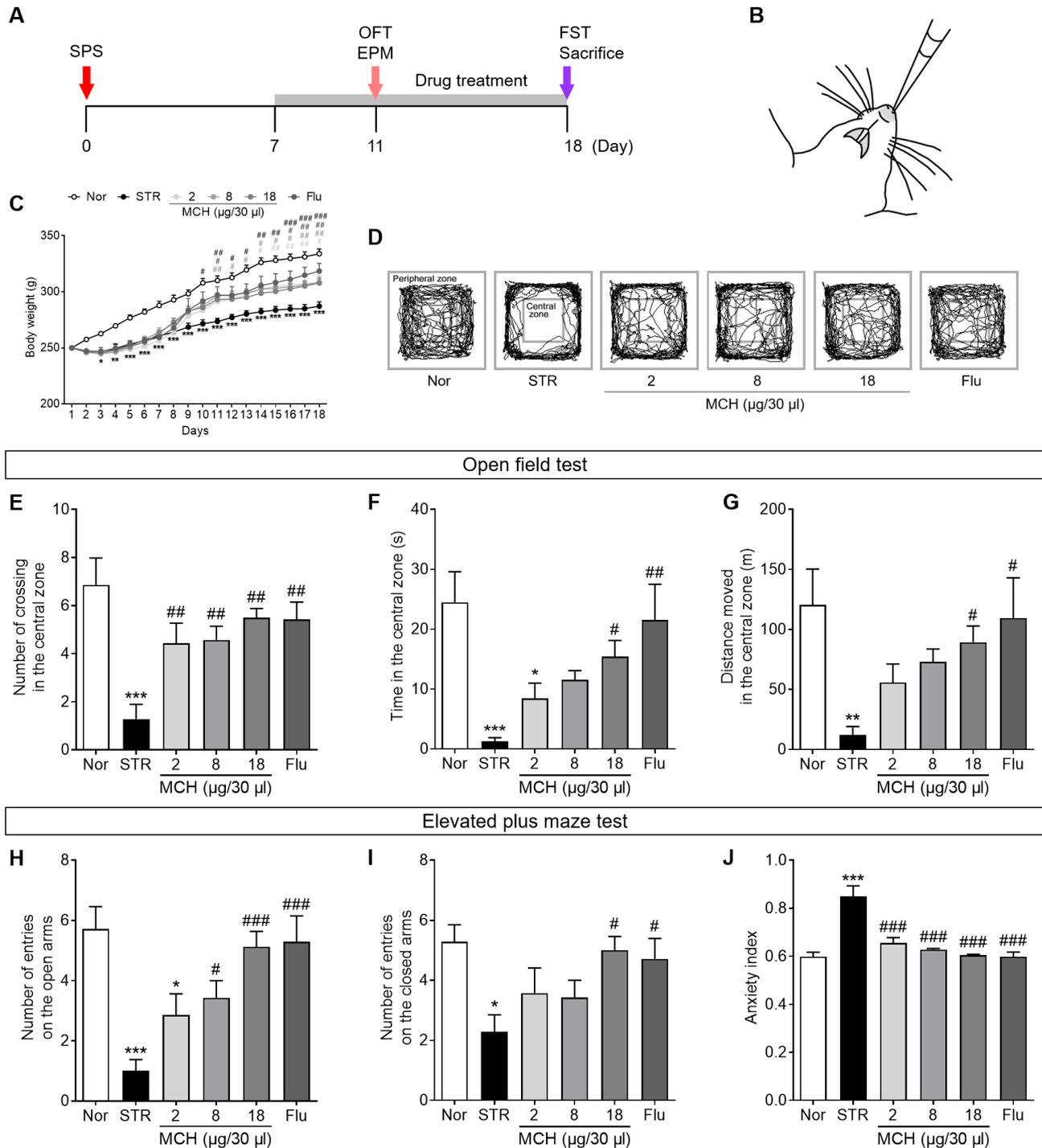


Fig. 1. Effects of MCH administration on depression- and anxiety-related behaviors in stressed rats. (A) Schematic representation of developing SPS and details of MCH administration. SPS was induced by immobilization (2 h), forced swim (20 min), rest (15 min), and ether anesthesia on day 1. Rats were left undisturbed after SPS. OFT and EPM tests were performed on day 11. An FST was conducted and animals were sacrificed on day 18. (B) Intranasal MCH treatment in rats. (C) Variations in body weight of rats over the course of 18 days. MCH and fluoxetine significantly induced weight loss in rats from day 10 compared with STR. Effects of MCH on depression- and anxiety-related behaviors in (D–G) OFT, (H–J) EPM test, and (K–M) FST in stressed rats. Data represent the means±SEM. ***p<0.001, **p<0.01, *p<0.05 vs. Nor group, ###p<0.001, ##p<0.01, #p<0.05 vs. STR group. SPS, single prolonged stress; OFT, open field test; EPM, elevated plus maze; FST, Forced swimming test; Nor, normal; STR, stress; Flu, fluoxetine.

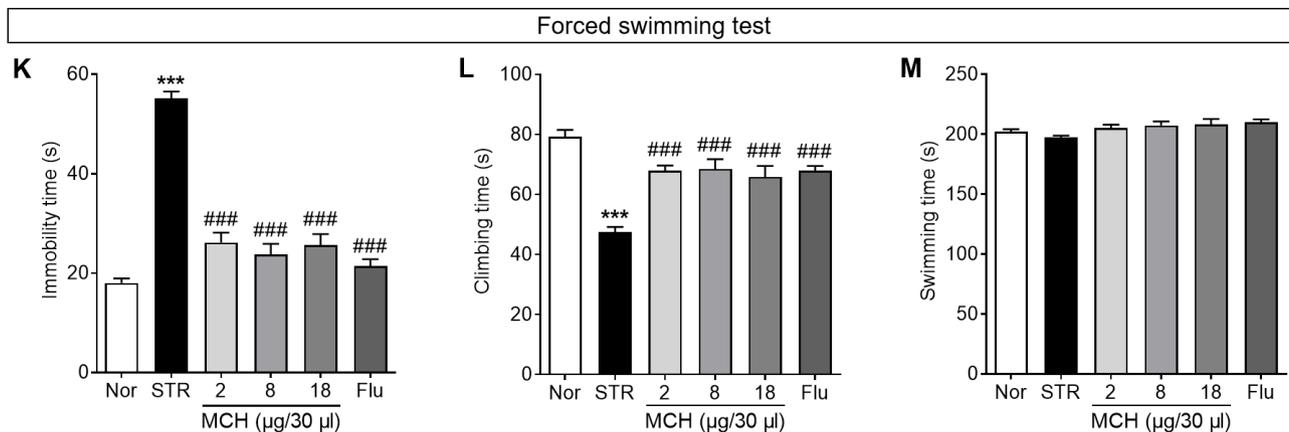


Fig. 1. Continued.

for ventilation and exposed to immobility stress for 4 h a day over 4 weeks. This model was used to assess the psychological consequences of chronic stress, weighed and compared between groups to investigate the nutritional status. In addition, to examine the effect of antidepressant-like effects of MCH in mice, we measured the energy homeostasis of each experimental group by calculating food efficiency ratio (FER) from food intake and total weight gain. At the end of the experimental period, all animals were decapitated, and the prefrontal cortices (PFC) were immediately collected and frozen at -80°C until further analysis. Blood samples were also collected from the heart via cardiac puncture prior to brain excision.

Pharmacological treatments

In the acute stress experiment in rats, MCH (2, 8, or 18 μg dissolved in 30 μl of saline; Tocris Biosciences, Bristol, UK; 3806) was administered intranasally to the rats for 12 consecutive days (Fig. 1A, B). Saline was used as a negative control. As a positive control, fluoxetine (10 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a sterile saline solution and applied intraperitoneally (i.p.). Fluoxetine was administered 2 h before behavioral tests to maintain fluoxetine effects in the central nervous system. Both MCH and fluoxetine were administered 7 days after exposure to stress. The mTOR inhibitor rapamycin (Rapa, 110 $\mu\text{g}/30 \mu\text{l}$, Cayman, Ann Arbor, MI, USA) was applied intranasally 30 min after administering MCH.

In the chronic immobility stress experiment in mice, MCH at 1, 5, or 25 μg in 30 μl was dropped into the nostrils under stress conditions for 4 weeks. As a positive control, fluoxetine hydrochloride was orally administered at 10 mg/ml for 4 weeks.

TC-MCH7c, an MCH receptor type 1 antagonist (10 mg/kg; catalog 4365, Tocris Bioscience), was dissolved in 80 μl of dimethyl

sulfoxide. It was then diluted to a total volume of 8 ml with saline and administered intraperitoneally 30 min before each MCH treatment.

Behavior tests

Open field test (OFT)

The potential effects of MCH on anxiety-related behaviors were assessed in the OFT in rats on day 11 (Fig. 1A). The test was conducted in a box contained in a dark room with dimensions of $60 \times 60 \times 30$ cm. The box was divided into 16 identical squares ("zones"). In each test, one rat was placed into the center of the arena and the frequency of crossing the center zone as well as the total distance traveled was recorded for 5 min. After each rat was tested, the box was thoroughly cleaned with 70% alcohol. The experimenter was blinded to the group information. The total distance traveled and the time spent in the zone during the test were analyzed automatically using an S-MART program (Pan Lab Co., Barcelona, Spain).

Elevated plus maze (EPM) test

The EPM test was performed on day 11, immediately following OFT, to analyze anxiety-related behaviors in rats (Fig. 1A). The plus maze consisted of four arms (50 \times 10 cm each) placed in a plus (+) shape, raised 50 cm above the floor. Two opposite arms were enclosed by 20 cm walls (closed arms), and the other two arms were not enclosed (open arms). Movements on the platforms were video-recorded for 5 min using the S-MART program located on the ceiling above the center of the maze. Arm entry was defined as entering an arm with all four paws. The following three measurements were taken: the number of open arm entries, the number of closed arm entries, and the percentage of open arm time.

Anxiety reduction, manifested by open arm exploration in the

EPM test, was defined as an increase in the number of entries into open arms compared with the total entries into either open or closed arms, or an increase in the fraction of time spent in open arms compared with total time spent on either arm. Total arm entries were used to monitor changes in the locomotor activity of rats.

To compare the overall effect on anxiety behavior, we defined anxiety index by incorporating the assessed values. The index was calculated as follows:

$$\text{Anxiety index} = 1 - \left[\frac{\left(\frac{\text{Time spent in the open arms}}{\text{Total time on the maze}} \right) + \left(\frac{\text{Number of entries to the open arms}}{\text{Total exploration on the maze}} \right)}{2} \right]$$

Anxiety index values range from 0 to 1, with an increase in the index indicating increased anxiety-like behavior [25].

Forced swimming test (FST)

To evaluate depression severity, a modified FST was performed as previously described [26]. Rats were considered immobile when they remained in the water without struggling, but only made the necessary movements to keep their head above the water. Immobility behavior was represented as the time interval in which rats did not show any escape response. Climbing behavior was defined as an upward-directed movement of the forepaws along the side of the swim chamber.

In the case of mice, each mouse was placed in a plexiglass cylinder (25 cm in height, 15 cm in internal diameter) containing water at a temperature of $25 \pm 1^\circ\text{C}$ with a depth of 10 cm, which prevented mice from escaping the cylinder or touching the bottom. The 5-min swim test was recorded for behavioral analysis of immobility, climbing, and swimming.

Tail suspension test (TST)

Mice were suspended individually by their tails 40 cm above the floor with adhesive tape placed approximately 1 cm proximal from the tip of their tails, while isolated both acoustically and visually. After a few minutes of vigorous activity, the mice became passive and motionless. The total immobility period was scored throughout a 6-min test. Mice were considered immobile when there was no limb or body movement, except for those caused by respiration. A decrease in the duration of immobility indicated an antidepressant-like effect.

Corticosterone levels

Serum was isolated by centrifugation at 3,000 rpm for 15 min at 4°C immediately after trunk blood collection and kept at -80°C . Serum corticosterone levels were measured in duplicate using an enzyme-linked immunoassay (ELISA, Enzo Life, USA) according

to the manufacturer's instructions.

Western blotting

Brain samples were homogenized in 200 μl of lysis buffer (CY-QUANT; Invitrogen, Eugene, OR, USA) supplemented with phosphatase inhibitor cocktail and protease inhibitor cocktail tablets (Thermo Scientific, Waltham, MA, USA). The samples were centrifuged at 12,000 rpm for 15 min at 4°C , and the supernatants were collected. Protein levels were determined using a bicinchoninic acid assay (Thermo Scientific). Equal amounts of protein (10 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). For immunodetection, the blots were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature and incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: p-Akt (1:1,000, #4058), total Akt (1:1,000, #4691), p-ERK1/2 (1:3,000, #4370), total ERK1/2 (1:3,000, #9102), p-mTOR (1:500, #2971), total mTOR (1:500, #2983), p-p70S6K (1:500, #9204), p-PSD95 (1:1,000, #45737), total PSD95 (1:1,000, #3409), p-GluR1 (1:1,000, #8084), total GluR1 (1:500, #13185), p-Synapsin1 (p-Syn1, 1:1,000, #88246), and total Syn1 (1:1,000, #9297) from Cell Signaling Technology (Danvers, MA, USA) and β -actin (1:30,000, #A1978) from Sigma-Aldrich. After washing with TBS-T three times (10 min per wash) at room temperature, the membrane was incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit (Pierce, Rockford, IL, USA) or anti-mouse (Thermo Scientific; 31430) antibodies. The signal was visualized using a chemiluminescence kit (Super Signal West Pico; Pierce). Signal intensity was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Distribution of FITC-labeled MCH after intranasal administration

Thirty minutes after the intranasal administration of either MCH-FITC (10 $\mu\text{g}/30 \mu\text{l}$, GL Biochem; Shanghai, China) or saline (30 μl), the slightly anesthetized mice were transcardially perfused with PBS followed by ice-cold 10% formalin. The brains were immediately removed and stored at -80°C for 1 h. Next, coronal sections (40- μm thick) of the brain were cut to encompass the entire brain. Coverslips were mounted using a mounting medium containing DAPI (H-1200, Vector Laboratories Inc., Burlingame, CA, USA), and all images were collected using an Olympus FV-1000 laser confocal scanning microscope (Olympus Corporation, Shinjuku, Tokyo, Japan).

Intracerebroventricular injection of MCH

The mice were anesthetized with an i.p. injection of a mixture of Zoletil:Rompun (3:1) and fixed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). An injection cannula (CMA/7; CMA Microdialysis, Sweden) was inserted into the DRN at coordinates 4.5 mm posterior to bregma, 1.5 mm lateral to the midline, 2.8 mm ventral to the dura, and at a 30° angle from the vertical plane to avoid the sagittal sinus. The guide cannula and three small stainless steel screws serving as anchors were cemented to the skull with dental cement. MCH (0.2 µl) was infused over a period of 5 min at a constant flow using a microinjection pump (KDS 310 plus Nano Legacy syringe pump, Kd Scientific, Holliston, MA, USA), and the injector was left in place for 5 min after injection to allow diffusion. After surgery, the mice were singly housed.

Statistical analysis

GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analyses. Data are expressed as the mean ± standard error of the mean (SEM). Group comparisons were performed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls *post hoc* test. Analyses for body weight were performed using a two-way ANOVA with repeated measures and the Bonferroni *post hoc* test for pairwise multiple comparisons. In all analyses, differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of MCH on the body weight of stressed rats

Stress efficacy was monitored by measuring body weight. The rats were weighed over the course of STR induction and MCH intranasal administration for 18 days (Fig. 1A, B). Two-way ANOVA showed a significant difference among the groups in weight change [$F_{5,648}=105.3$, $p < 0.0001$] and in the timing of the effect (days) [$F_{17,648}=184.5$, $p < 0.0001$]. *Post hoc* tests revealed that the STR group showed a significant decrease in body weight compared with the normal (Nor) group from day 3 of STR induction ($p < 0.05$). However, after day 10 of STR induction, rats in the MCH 18 µg/30 µl group gained a significant amount of body weight compared with the STR group ($p < 0.05$, Fig. 1C).

Effect of MCH on the anxiogenic behavior of stressed rats

OFT was performed to measure changes in anxiety and exploratory behavior after treatment with MCH. Rats were exposed to a novel environment in an open field apparatus, and their activities were analyzed. A one-way ANOVA of the OFT data revealed a significant effect of MCH on anxiety behavior in the numbers of

crossings in the central zone [$F_{5,42}=6.469$, $p=0.0002$], time in the central zone [$F_{5,42}=5.572$, $p=0.0006$], and distance moved in the central zone [$F_{5,42}=3.586$, $p=0.0096$]. *Post hoc* analyses revealed that the total number of line crossings in the center zone was significantly reduced in the STR group (vs. Nor, $p < 0.001$, Fig. 1D~G). However, this decrease was restored in the MCH group ($p < 0.01$, Fig. 1D~G), indicating an improvement in anxiety-related behavior.

To further investigate the effects of MCH on anxiety behaviors, rats were assessed during open arm exploration in the EPM test. A one-way ANOVA revealed significant group effects in the number of entries into the open arms [$F_{5,42}=7.815$, $p < 0.0001$] and closed arms [$F_{5,42}=3.425$, $p=0.0121$]. *Post hoc* comparisons showed that the STR group displayed a decrease in the number of entries into the open arms ($p < 0.001$) and closed arms ($p < 0.05$) compared with the Nor group (Fig. 1H~I). A significant restoration in the number of entries into the open arms was observed in the MCH (18 µg/30 µl) group compared with that in the STR group ($p < 0.001$). Overall, the anxiety index revealed a significant group effect [$F_{5,42}=17.95$, $p < 0.0001$]. The anxiety index calculated based on the number of visits and the time spent in the open and closed arms was lower in the MCH group than in the STR group ($p < 0.001$, Fig. 1J).

Effect of MCH on depressive-like behavior of stressed rats

To determine the effects of MCH on depressive-like behaviors, rats were exposed to FST. A one-way ANOVA of performance data in FST indicated significant group effects on immobility [$F_{5,37}=49.64$, $p < 0.0001$] and climbing time [$F_{5,37}=17.64$, $p < 0.0001$] but not swimming time [$F_{5,37}=2.480$, $p=0.0524$]. *Post hoc* analyses revealed a significant increase in depressive phenotype (duration of immobility) in the STR group compared with that in the Nor group ($p < 0.001$, Fig. 1K). In contrast, the MCH group exhibited a marked ($p < 0.001$) decrease in the duration of immobility compared with the STR group, indicating that MCH alleviated depressive-like behaviors. Similarly, an analysis of climbing time, another key indicator of depressive-like behavior, revealed a significant decrease in the STR group (vs. Nor, $p < 0.001$), which was restored by treatment with MCH (vs. STR, $p < 0.001$, Fig. 1L). The stressed groups displayed only slight variations in swimming time, suggesting that the results observed in this study were caused by increased depressive-like behavior and not by motor function deficits (Fig. 1M).

Effects of MCH on the mTOR signaling pathway in the PFC following stress

The mTOR pathway in the PFC is demonstrated to be closely involved in depression [27, 28]. To investigate whether the anxio-

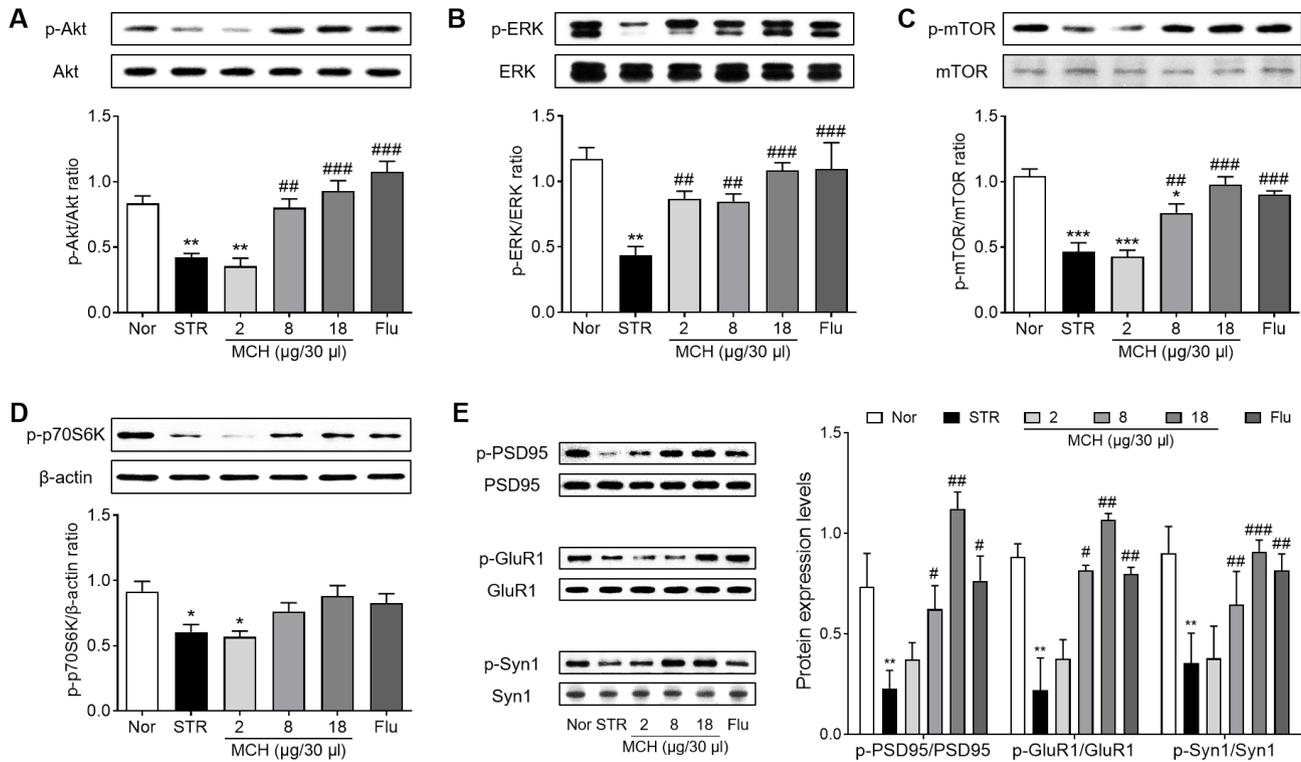


Fig. 2. Activation of the mTOR signaling pathway and synaptic proteins after MCH administration. Western blot analysis of protein expression levels of (A) Akt, (B) ERK, (C) mTOR, (D) p70S6K, and (E) synaptic proteins in stressed rats. The amount of phosphorylated proteins was normalized to that of total proteins and presented in bar graphs except p70S6K. The amount of phosphorylated p70S6K was normalized to that of β -actin. Data represent the means \pm SEM. *** p <0.001, ** p <0.01, * p <0.05 vs. Nor group, ### p <0.001, ## p <0.01, # p <0.05 vs. STR group. Nor, normal; STR, stress; Flu, fluoxetine.

lytic and antidepressant-like effects of MCH are associated with the mTOR signaling pathway, the expression of major proteins in the mTOR signaling pathway were analyzed after MCH administration in stressed rats. Statistical analyses showed significant phosphorylation levels of several proteins in the PFC: p-Akt/Akt [$F_{5,17}=19.36, p<0.0001$], p-ERK/ERK [$F_{5,17}=11.67, p=0.0003$], p-mTOR/mTOR [$F_{5,17}=21.40, p<0.0001$], and p-p70S6K/ β -actin [$F_{5,17}=4.526, p=0.0150$].

Post hoc analyses revealed that the levels of p-Akt ($p<0.01$, Fig. 2A), p-ERK ($p<0.01$, Fig. 2B), p-mTOR ($p<0.001$, Fig. 2C), and p-p70S6K ($p<0.05$, Fig. 2D) in the STR group were significantly lower than those in the Nor group. However, the expression levels of p-Akt ($p<0.01$, Fig. 2A), p-ERK ($p<0.01$, Fig. 2B), and p-mTOR ($p<0.01$, Fig. 2C) were restored following the administration of MCH (8 $\mu\text{g}/30 \mu\text{l}$). Moreover, the levels of p-Akt ($p<0.001$, Fig. 2A), p-ERK ($p<0.001$, Fig. 2B), and p-mTOR ($p<0.001$, Fig. 2C) were significantly increased in the MCH (18 $\mu\text{g}/30 \mu\text{l}$) group compared with those in the STR group.

Effects of MCH on synaptic proteins in the PFC following stress

Synaptic plasticity has been shown to be involved in the pathogenesis of depression. Therefore, we examined the phosphorylation of major synaptic proteins, including postsynaptic density 95 (PSD95), glutamate receptor 1 (GluR1), and synapsin 1 (Syn1), which are known to be associated with antidepressant-like effects and synaptic plasticity.

Statistical analyses indicated a significant effect of MCH on p-PSD95/PSD95 [$F_{5,17}=20.70, p<0.0001$], p-GluR1/GluR1 [$F_{5,17}=10.06, p=0.0006$], and p-Syn1/Syn1 [$F_{5,17}=9.136, p=0.0009$] in the PFC. A *post hoc* test revealed that p-PSD95 ($p<0.01$), p-GluR1 ($p<0.01$), and p-Syn1 ($p<0.01$) were downregulated in the STR group compared with the Nor group (Fig. 2E). However, the levels of p-PSD95 ($p<0.01$), p-GluR1 ($p<0.01$), and p-Syn1 ($p<0.001$) were restored in the MCH (8, 18 $\mu\text{g}/30 \mu\text{l}$) group (Fig. 2E).

Pretreatment with the mTOR inhibitor rapamycin blocked the effect of MCH

To confirm the involvement of the mTOR signaling pathway in the effects of MCH, we intranasally injected rapamycin, an inhibi-

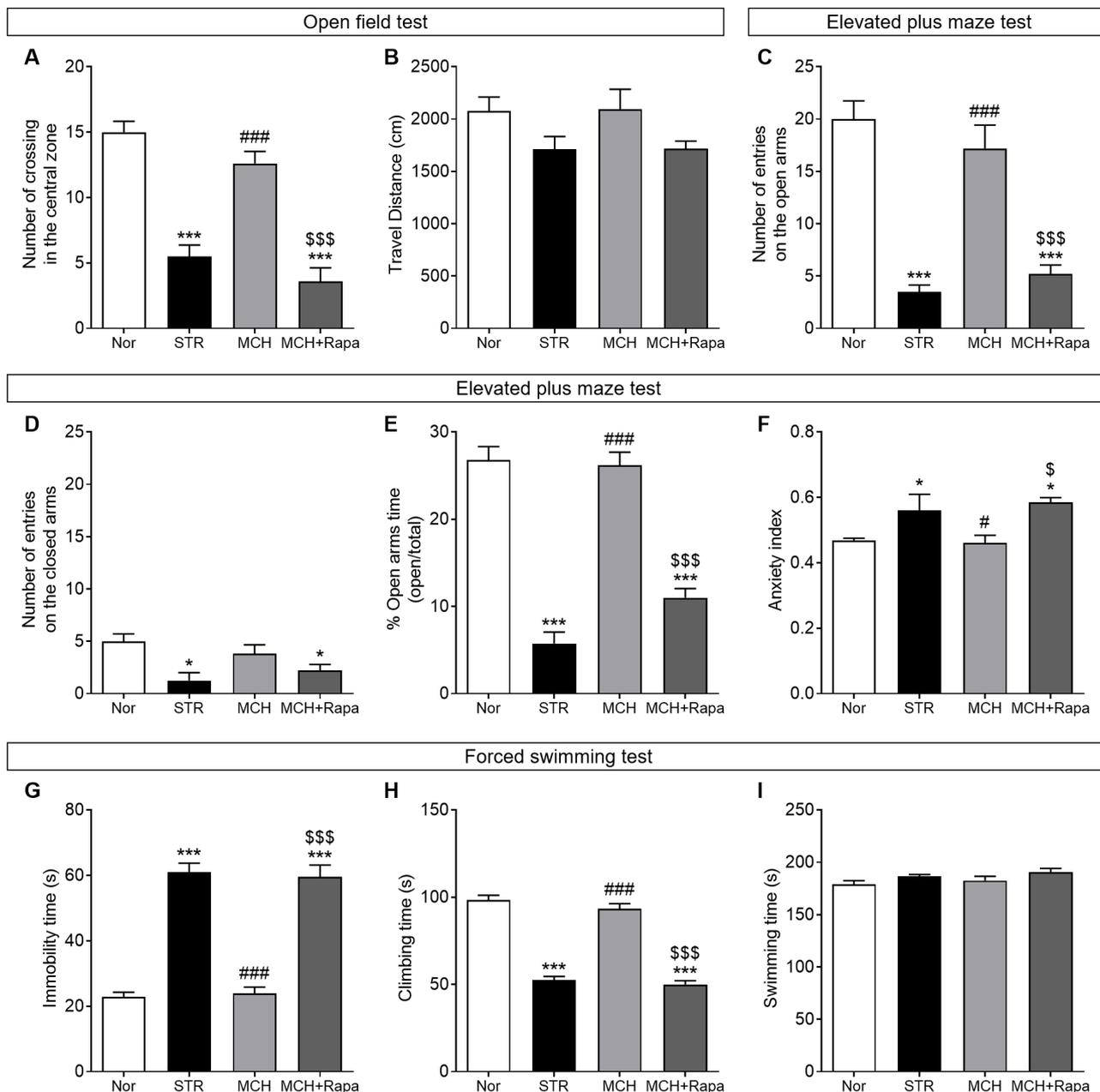


Fig. 3. Rapamycin blocks the antidepressant-like and anxiolytic-like effects of MCH. Effects of MCH (18 µg/30 µl) administration on depression and anxiety behaviors in (A, B) OFT, (C–F) EPM test, and (G–I) FST in stressed rats. Pretreatment with the mTOR inhibitor rapamycin (110 µg/30 µl, intranasal) blocked the antidepressant and anxiolytic effects of MCH. Data represent the means±SEM. ***p<0.001, *p<0.05 vs. Nor group, ###p<0.001 vs. STR group, \$\$\$p<0.001, \$p<0.05 vs. MCH group. Nor, normal; STR, stress; Rapa, rapamycin; OFT, open field test; EPM, elevated plus maze; FST, forced swim test.

tor of the mTOR pathway, at a dose of 110 µg/30 µl and then performed behavioral tests.

First, the rats were subjected to OFT. A one-way ANOVA of the OFT data indicated that the anxiolytic effect of MCH was significantly blocked in the MCH+Rapa group [$F_{3,18}=35.59, p<0.0001$]. *Post hoc* analyses showed that the MCH+Rapa group exhibited

a substantially decreased number of lines crossing the central zone compared with the MCH group ($p<0.001$, Fig. 3A). However, the travel distance did not differ among groups [$F_{3,18}=2.397, p=0.1088$], indicating that their motor function was not affected (Fig. 3B).

A one-way ANOVA of the EPM test data revealed significant ef-

fects of rapamycin treatment on the number of entries into open [$F_{3,18}=27.30, p<0.0001$] and closed arms [$F_{3,18}=4.977, p=0.0136$] of the maze. According to *post hoc* analyses, the MCH+Rapa group showed fewer entries into the open arms ($p<0.001$, Fig. 3C), but not into the closed arms, compared with the MCH group (Fig. 3D), supporting that rapamycin suppressed the anxiolytic effects of MCH. Statistical analyses of EPM data showed that the percentage of time in open arms [$F_{3,18}=58.38, p<0.0001$] differed significantly among the four groups. *Post hoc* analyses revealed that rats in the STR group spent significantly less time in the open arms of the maze compared with the Nor group ($p<0.001$), which was restored by MCH treatment ($p<0.001$, Fig. 3E). However, this improvement was abolished in the MCH+Rapa group, with significantly less time spent in the open arms compared with that in the MCH group ($p<0.001$, Fig. 3E). Overall, the anxiety index revealed a significant group effect [$F_{3,18}=55.70, p=0.0049$]. The anxiety index calculated based on the number of visits to and time spent in the open and closed arms was lower in the MCH group than in the MCH+Rapa group ($p<0.05$, Fig. 3F).

Lastly, the FST was conducted to test the role of the mTOR signaling pathway in the antidepressant-like effects of MCH. A one-way ANOVA of the FST data showed that immobility time [$F_{3,18}=69.59, p<0.0001$] was significantly increased, and climbing time [$F_{3,18}=102.0, p<0.0001$] was significantly decreased in the MCH+Rapa group, and that swimming time was not different between groups [$F_{3,18}=1.996, p=0.1579$]. *Post hoc* analyses showed that the MCH+Rapa group exhibited a pronounced depression-like phenotype (increased immobility) compared with the MCH group ($p<0.001$, Fig. 3G). In addition, the MCH+Rapa group exhibited a decrease in climbing time compared with the MCH group ($p<0.001$, Fig. 3H). However, the groups showed no significant difference in swimming time (Fig. 3I). Taken together, our findings from the behavioral tests suggest that pretreatment with the mTOR inhibitor rapamycin suppresses the effects of MCH on anxiety and depressive-like behaviors.

Effects of MCH on depressive-like behavior in immobility-induced mice

To confirm the antidepressant-like effects of MCH in mice, we examined the energy homeostasis of each experimental group by calculating FER from food intake and total weight gain (Fig. 4A~D). A one-way ANOVA revealed differences in total weight gain [$F_{5,23}=24.09, p<0.0001$], total food intake [$F_{5,23}=5.440, p=0.0032$], and total FER [$F_{5,18}=1.079, p=0.405$]. Compared with the STR group, the Nor group showed increased food intake and weight gain. However, there was no significant difference in food intake or weight gain between the MCH and STR groups, sug-

gesting that administration of MCH did not affect body energy metabolism.

Next, we examined the effect of MCH on depression-like behavior using the TST [$F_{5,23}=8.549, p=0.0003$]. The STR group showed increased immobility time compared with the Nor group ($p<0.01$), whereas MCH (1 or 5 $\mu\text{g}/30\ \mu\text{l}$) and fluoxetine eliminated the stress-induced increase (MCH 1 μg or fluoxetine, $p<0.001$; MCH 5 μg , $p<0.01$; Fig. 4E). We then assessed the effect of MCH in the FST. A one-way ANOVA revealed differences in immobility [$F_{5,23}=4.523, p=0.0076$], climbing [$F_{5,23}=15.67, p<0.0001$], and swimming time [$F_{5,23}=3.529, p=0.0213$]. *Post hoc* analyses showed no significant difference in immobility and climbing time between the Nor and STR groups, while the MCH (1 $\mu\text{g}/30\ \mu\text{l}$) group exhibited a marked difference (immobility, $p<0.05$; climbing, $p<0.001$) compared with the STR group, indicating that MCH alleviates depression-like behaviors (Fig. 4F, G). Similarly, the MCH (1 $\mu\text{g}/30\ \mu\text{l}$) and fluoxetine groups showed a significantly longer swimming time compared with the STR group ($p<0.05$, Fig. 4H). However, a higher dose of MCH (25 $\mu\text{g}/30\ \mu\text{l}$) did not result in improvements in depression-like behaviors (Fig. 4E~H).

Effects of MCH on serum corticosterone levels following stress

Serum corticosterone was examined as an induction of hypothalamic-pituitary-adrenal (HPA) axis activity. Analysis of serum corticosterone levels revealed significant differences among the groups [$F_{5,24}=40.94, p<0.0001$]. *Post hoc* comparisons showed a significantly increased level of serum corticosterone in the STR group compared with that in the Nor group ($p<0.001$). In contrast, the MCH 1, 5 $\mu\text{g}/30\ \mu\text{l}$ groups showed a significant decrease in the concentration of corticosterone after fluoxetine compared with that in the STR group ($p<0.001$). These data suggest that MCH inhibits HPA axis hyperactivity in the STR (Fig. 4I).

Effects of MCH on the mTOR signaling pathway in the PFC following stress

To substantiate the molecular mechanisms underlying the antidepressant-like effects of MCH, we examined the phosphorylation of mTOR in the PFC of immobility-induced depressed-like mice. A one-way ANOVA revealed significant differences in p-mTOR/mTOR [$F_{5,23}=44.42, p<0.0001$]. The phosphorylation level of mTOR was significantly decreased in the PFC of stressed mice ($p<0.001$) (Fig. 4J), which was restored by MCH (1 $\mu\text{g}/30\ \mu\text{l}$) or fluoxetine treatment (MCH 1 μg , $p<0.001$; fluoxetine, $p<0.01$) (Fig. 4J). Next, the phosphorylation levels of synaptic proteins were examined. Western blotting confirmed that the phosphorylation levels of p-PSD95/PSD95 [$F_{5,11}=57.70, p<0.0001$], p-GluR1/GluR1

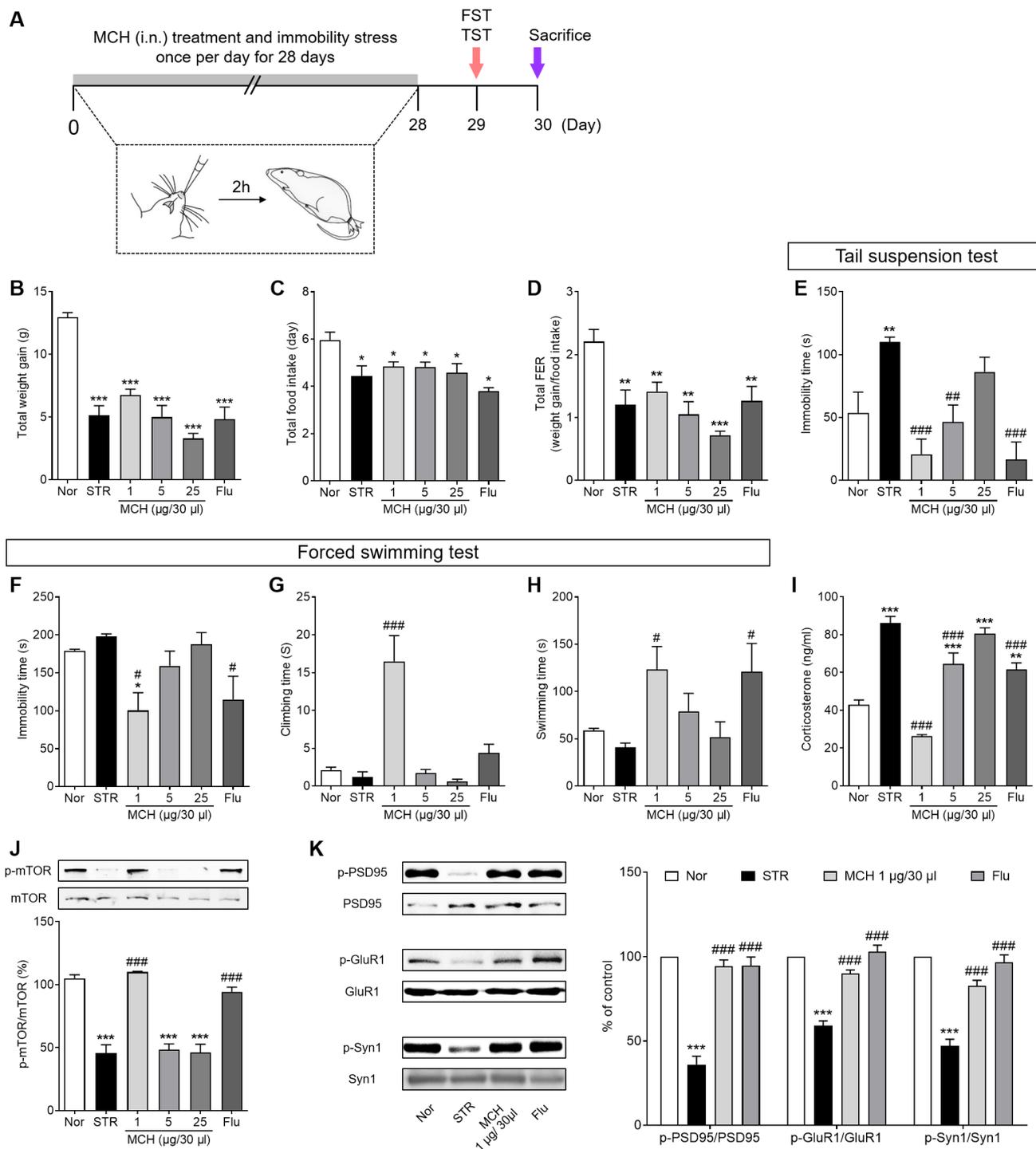


Fig. 4. Antidepressant-like effects of MCH in stressed mice. (A) Schematic representation of the STR paradigm. (B) Total weight gain was divided by (C) total food intake to calculate (D) total FER. (E) TST was administered during the 6-min test period and immobility time was recorded. (F) Immobility, (G) climbing, and (H) swimming behaviors in mice were observed during the last 4 min test period and climbing or swimming time was recorded. (I) Concentration of corticosterone was measured using an ELISA kit. The phosphorylation levels of (J) mTOR, (K) PSD95, GluR1 and Syn1 in the PFC were measured by immunoblotting. The amount of phosphorylated proteins were normalized to that of total proteins and presented as bar graphs (right panel). Data represent the means±SEM. ****p*<0.001, ***p*<0.01, **p*<0.05 vs. Nor group, ###*p*<0.001, ##*p*<0.01, #*p*<0.05 vs. STR group. Nor, normal; STR, stress; Flu, fluoxetine; FST, forced swim test; TST, tail suspension test.

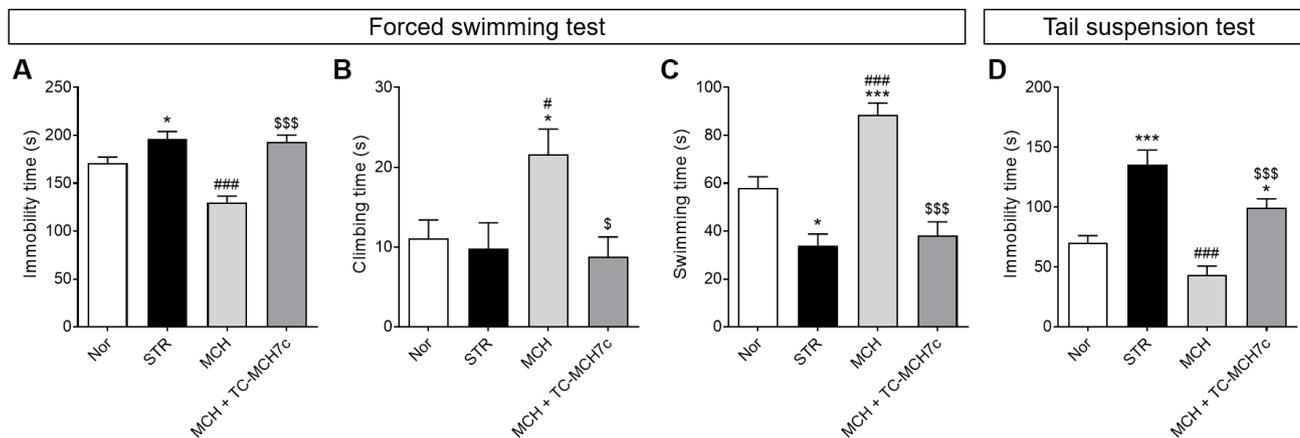


Fig. 5. Antidepressant-like effect of MCH is mediated by MCHR1. The FST was examined during the last 4 min of the test period and (A) immobility, (B) climbing, and (C) swimming behaviors were recorded in stressed mice. In the TST, (D) immobility behavior in mice were observed during the 6 min test period. $n=5/\text{group}$. Data represent the means \pm SEM. *** $p<0.001$, * $p<0.01$ vs. Nor group, ### $p<0.001$, # $p<0.05$ vs. STR group, \$\$\$ $p<0.001$, \$ $p<0.05$ vs. MCH group. Nor, normal; STR, stress; MCH, melanin-concentrating hormone.

[$F_{3,11}=59.95$, $p<0.0001$], and p-Syn1/Syn1 [$F_{3,11}=51.25$, $p<0.0001$] were significantly altered in the PFC between groups. Immobility stress reduced the phosphorylation levels ($p<0.001$), while MCH had a reverse effect ($p<0.001$) (Fig. 4K).

Pretreatment with the MCH antagonist TC-MCH7c blocked the effect of MCH

To determine the involvement of MCHR1 in the regulation of depression-like behavior, we injected mice with TC-MCH7c, an MCH antagonist, at a dose of 10 mg/kg, prior to an injection of MCH (1 $\mu\text{g}/30 \mu\text{l}$) and then proceeded to behavioral changes assessment. A one-way ANOVA of the FST data showed a significant difference in immobility [$F_{3,19}=19.39$, $p<0.0001$], climbing [$F_{3,19}=4.376$, $p=0.0198$], and swimming time [$F_{3,19}=23.58$, $p<0.0001$]. *Post hoc* analyses showed that the MCH+TC-MCH7c group exhibited a pronounced depression-like phenotype (increased immobility) compared with the MCH group ($p<0.001$, Fig. 5A). The MCH+TC-MCH7c group exhibited a decrease in climbing time compared with the MCH group ($p<0.05$, Fig. 5B). In addition, the MCH+TC-MCH7c group showed a significant decrease in swimming time ($p<0.001$, Fig. 5C). Next, we performed a TST [$F_{3,19}=21.46$, $p<0.0001$], with the MCH group showing decreased immobility time compared with the STR group ($p<0.001$), but not the MCH+TC-MCH7c group (Fig. 5D).

Dual effects of MCH according to the administration pathway

Our results show that MCH intranasal administration improves stress-induced depression- and anxiety-like effects by regulating

the mTOR signaling pathway and synaptic plasticity (Fig. 6A). However, in previous studies, microinjection of MCH has been shown to cause depression (Fig. 6B). Therefore, we analyzed TST after administering MCH in two different ways in naïve unstressed mice. As a result, depression-like behavior was not observed after intranasal administration of MCH at 0, 1, 10, or 20 μg (Fig. 6C), but was observed following a microinjection into the DRN at 0, 50, or 100 ng (Fig. 6D). Therefore, MCH has a dual effect depending on the route of administration.

Distribution in the brain region after intranasal administration of MCH

We administered MCH-FITC to assess the extent of brain infiltration. Starting from the olfactory bulb (Fig. 7A), MCH was detected in depression-related regions, including the PFC (Fig. 7B), thalamus (Fig. 7C), hippocampus (Fig. 7D), and amygdala (Fig. 7E).

DISCUSSION

In this study, we investigated the antidepressant-like effects of MCH using stress models in rats and mice (Figs. 1, 4). We found that these effects were mediated by the activation of mTOR signaling molecules (Fig. 2) and were inhibited by an mTOR inhibitor (Fig. 3) and MCHR1 antagonist (Fig. 5). As far as we know, this is the first study to show the therapeutic potential of MCH in the stress-induced depression model.

In our study of stressed rats, we showed that MCH exhibited antidepressant-like effects at all 2, 8, and 18 $\mu\text{g}/30 \mu\text{l}$ doses (Fig. 1).

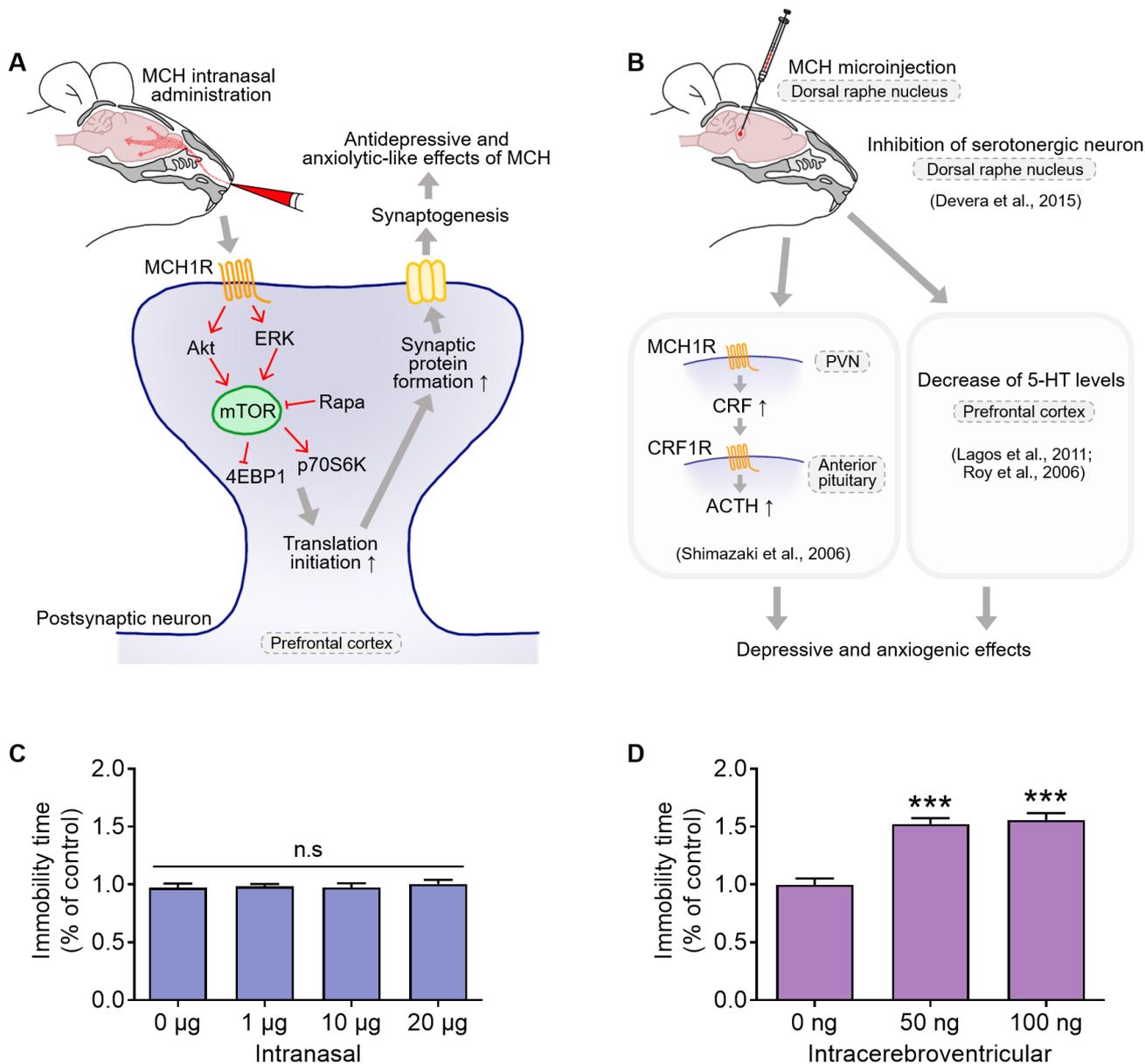


Fig. 6. The effect of MCH on depression- or anxiety-like behaviors is based on the route of administration. (A) In this study, intranasal administration of MCH produced antidepressant- and anxiolytic-like behaviors in both rodent models. MCH treatment intranasally increases the activation of Akt and ERK, and these induced the phosphorylation of mTOR. Phosphorylation of mTOR hyperphosphorylates 4EBP1 and p70S6K, thus promoting an increase in synaptic protein formation in the PFC. However, pretreatment with the mTOR inhibitor, rapamycin prevented these changes. (B) In contrast, a microinjection of MCH into the DRN induced depression- and anxiogenic-like behaviors. Previous studies have suggested that a microinjection of MCH induces depression-like and anxiogenic effects possibly via the inhibition of serotonergic neurons in the DRN, increasing the secretion of CRF, which promotes the release of ACTH from the anterior pituitary, and a decrease in 5-HT levels in the PFC. (C) Intranasal administration of MCH produced no changes in immobility time in the TST, whereas (D) microinjection of MCH into the DRN induced depression-like behaviors in the TST. Data represent the means ± SEM. ***p < 0.001 vs. 0 µg or 0 ng.

However, in our study of stressed mice, only a low dose of MCH (1 µg/30 µl) elicited antidepressant-like effects, whereas a high dose of MCH (25 µg/30 µl) did not (Fig. 4). When the doses were normalized by body weight, each dose was equivalent to 10, 40, and 90 µg/kg in rats and 50, 250, and 1,250 µg/kg in mice. In addition, the

mice received twice the dose (20, 80, and 180 µg/kg) administered to rats, when scaled to rat size by surface area [29]. These results suggest that efficacious doses of MCH are actually similar between rats and mice.

In the present study, we demonstrated that stress reduced the

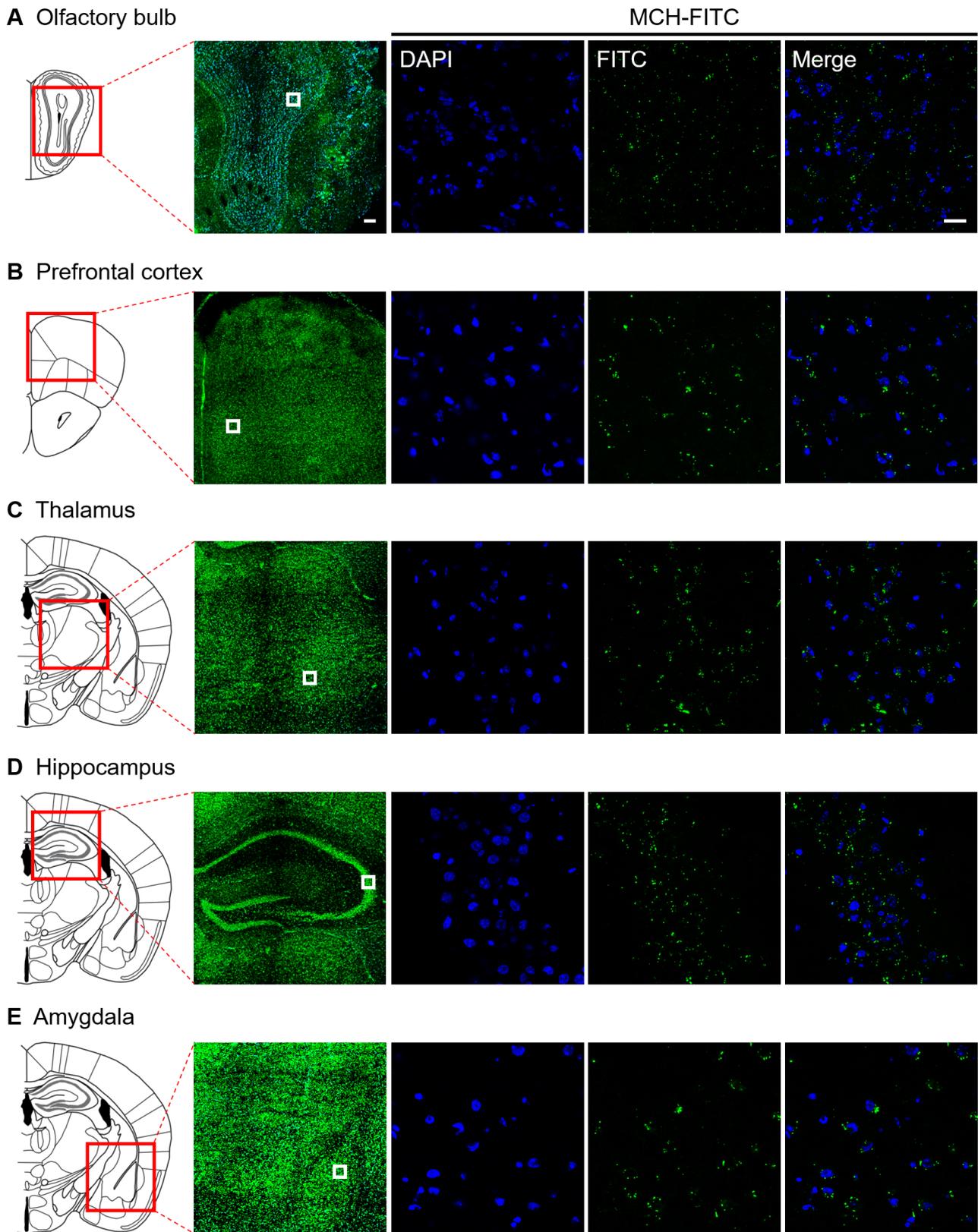


Fig. 7. Distribution of MCH in the brain after intranasal administration. Representative figure showing the distribution of MCH after 30 min of intranasal administration of MCH-FITC (10 $\mu\text{g}/30 \mu\text{l}$) in the (A) olfactory bulb, (B) PFC, (C) thalamus, (D) hippocampus, and (E) amygdala of mouse brain. Scale bar: 400 and 30 μm .

phosphorylation of mTOR, Akt, and ERK in the PFC, whereas MCH reversed this stress-induced dephosphorylation (Fig. 6A). Activation of the mTOR signaling pathway is associated with many physiological processes of the nervous system, including neurogenesis, axonal sprouting, dendritic spine growth, and psychiatric disorders such as MDD [28, 30]. The upstream activators of mTOR signaling are Akt and ERK, which can inhibit tuberous sclerosis complexes (TSC1 and TSC2), known inhibitors of mTOR [31]. MCH interacts with its receptor MCHR1 which leads to activation of the intracellular signaling pathway. MCHR1 signaling increases intracellular calcium mobilization via G-coupled pathways and activates ERK phosphorylation in recombinant cell lines and hippocampal brain slices [32, 33]. Moreover, MCH-induced neurite outgrowth is required for ERK activation in SH-SY5Y cells [34], suggesting that MCH-induced ERK activation may be involved in mTOR phosphorylation in the brain. The downstream targets of mTOR are ribosomal protein S6 kinases (S6Ks) and eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BP), which regulate protein biosynthesis [31]. We also showed that MCH reverses S6K activation in the PFC in stressed rats (Fig. 3). Previous studies using animal models of depression have reported decreased mTOR activity and reductions in the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway in the PFC, hippocampus, and amygdala of stressed rats [28, 30]. Congruent with PFC, the hippocampal mTOR protein levels in STR was significantly decreased compared to NOR, while it was restored after intranasal administration of 18 μ g/30 μ l MCH (data not shown). In addition, our previous study found that MCH increased hippocampal LTP [35]. These results suggest that MCH exerts its antidepressant-like effect through activation of the Akt/mTOR/S6K signaling pathway. Additionally, to produce the antidepressant-like effects of MCH, other brain regions, such as the hippocampus, contribute to the activity of these pathways [36, 37].

The ability of synapses to undergo dynamic rearrangement is important for brain function, and the number or function of synapses can be altered by stress [38, 39]. Among synaptic proteins, Syn1 and PSD95 play important roles in synaptic plasticity [40, 41] and synapse maturation [40]. Syn1 is widely distributed in presynaptic vesicle membranes, and PSD95 is the main protein in excitatory postsynaptic density material. Both of these proteins are activated by phosphorylation of the serine residues for synaptic function, Ser-295 of PSD95 [42], and Ser-603 of Syn1 [43]. In the present study, Syn1 and PSD95 expression was induced by MCH treatment, suggesting that MCH may increase synaptic strength through the activation of these proteins. Moreover, GluR1 is regulated by phosphorylation and dephosphorylation. The phosphorylation of Ser-845 of GluR1 enhances native and recombinant

AMPA receptor currents [42, 44], whereas dephosphorylation induces long-term depression [45]. Furthermore, MCHR1 activation increases intracellular calcium mobilization via G-coupled pathways [32], and postsynaptic Ca^{2+} influx is critical for LTP induction [46]. Consistent with these findings, our data suggest stress induced dephosphorylation of Syn1, PSD95, and GluR1 in the PFC, whereas MCH restored the phosphorylation of these proteins (Figs. 2, 4).

Several studies have related MCH to depression, although not consistently [8, 47]. For example, MCH has been shown to evoke depression-like behavior after a microinjection into the DRN [47] or locus coeruleus [48] in the brainstem, as demonstrated by a significant increase in immobility time in the FST, a decrease in climbing behavior, or a decrease in sucrose preference [47, 48]. In fact, when 50 ng of MCH is administered into the DRN, rats display depression-like behavior [49]. In contrast, an injection of a lower dose of MCH (25 ng) has been shown to produce a significant decrease in immobility time [49]. Moreover, a microinjection of MCH is sufficient to induce depression-like behavior 30 min after the injection, but the effect is lost after 60 min. These findings suggest that the effect of MCH on depression-like behaviors is transient and varies depending on its dose. Several studies have produced results that contrast with our findings, particularly those involving MCHR antagonists or transgenic mice. Borowsky et al. [50] first suggested that the small-molecule MCHR1 antagonist SNAP-7941 may be useful in the management of obesity and for the treatment of depression and anxiety. MCHR1 antagonists reverse depression-like behavior in both acute and chronic models of depression [51-53]. The genetic inactivation of MCHR1 in female mice also produces antidepressant-like effects in the FST [54]. However, genetic deletion of MCHR1 in male mice does not produce an antidepressant-like effect [54], and some MCHR1 antagonists have failed to show antidepressant-like effects in other models [55], adding to the uncertainty regarding the roles of MCH in depression-related behaviors.

One possible explanation for these discrepancies is the differences among sites of action. As reported in this study, intranasal delivery of MCH allows for distribution to various brain regions within 30 min, including the olfactory bulb, PFC, thalamus, hippocampus, and amygdala (Fig. 7). Although intranasally administered MCH diffuses to reach various brain regions [23], the anterior or middle parts of the brain, including the PFC and hippocampus, are more likely to play important roles in inducing therapeutic effects. Our recent findings also support this hypothesis. Intranasal administration of MCH improved pain-induced depression-like behaviors as well as pain, and these effects were correlated with the expression of cannabinoid receptor 1 in the PFC [23]. Intranasal MCH has

also been shown to attenuate cognitive impairment in Alzheimer's disease models; these effects are mediated by enhancing long-term potentiation and increasing levels of phosphorylated CREB and GSK3 β in the hippocampus [56]. In contrast, previous studies have mainly focused on regions of the hindbrain, such as the raphe nucleus [47] or locus coeruleus [48]. To verify the occurrence of region-specific effects of MCH, we compared the immobility time of two differentially administered MCH groups using the TST, and found that the intranasal group did not display an increase in immobility time, whereas the DRN microinjection group did (Fig. 6C, D). Our speculations about the differential effects and mechanisms of these MCH administration pathways are shown in Fig. 6A, B.

We administered MCH intranasally, which allows for rapid entry of peptides into the brain, bypassing the blood-brain barrier [57]. Distributed to various brain regions within 30 min, including the olfactory bulb, PFC, thalamus, hippocampus, and amygdala (Fig. 7), intranasally administered MCH may exert its antidepressant-like effect more promptly. Recently, intranasal administration of MCH has been shown to have neuroprotective effects through the enhancement of neuronal synapses and long-term potentiation (LTP) in the hippocampus of 5XFAD AD mice [56]. However, future studies are needed to elucidate the differential expression of MCH-FITC in rats and mice.

MCH overexpression may enhance food intake, reduce glucose tolerance, and provoke insulin resistance, resulting in obesity in mice [21]. However, we did not observe weight gain or a FER difference between the control and MCH-treated groups, suggesting that MCH has no adverse effects on energy homeostasis after intranasal administration of the doses used in this study, even when compared with fluoxetine at the same doses.

In conclusion, we demonstrated that the antidepressant-like effects of intranasally administered MCH in stressed rats and mice is mediated via the mTOR signaling pathway. Our findings provide novel insight into the molecular mechanism(s) underlying the protective effects of MCH in depression and promote MCH as a potential therapeutic agent for depression.

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