



Physical Exercise Counteracts Stress-induced Upregulation of Melanin-concentrating Hormone in the Brain and Stress-induced Persisting Anxiety-like Behaviors

Tae-Kyung Kim¹ and Pyung-Lim Han^{1,2*}

¹Department of Brain and Cognitive Sciences, and ²Chemistry and Nano Science, Ewha Womans University, Seoul 03760, Korea

Chronic stress induces anxiety disorders, whereas physical exercise is believed to help people with clinical anxiety. In the present study, we investigated the mechanisms underlying stress-induced anxiety and its counteraction by exercise using an established animal model of anxiety. Mice treated with restraint for 2 h daily for 14 days exhibited anxiety-like behaviors, including social and nonsocial behavioral symptoms, and these behavioral impairments lasted for more than 12 weeks after the stress treatment was removed. Despite these lasting behavioral changes, wheel-running exercise treatment for 1 h daily from post-stress days 1 - 21 counteracted anxiety-like behaviors, and these anxiolytic effects of exercise persisted for more than 2 months, suggesting that anxiolytic effects of exercise stably induced. Repeated restraint treatment up-regulated the expression of the neuropeptide, melanin-concentrating hormone (MCH), in the lateral hypothalamus, hippocampus, and basolateral amygdala, the brain regions important for emotional behaviors. In an *in vitro* study, treatment of HT22 hippocampal cells with glucocorticoid increased MCH expression, suggesting that MCH upregulation can be initially triggered by the stress hormone, corticosterone. In contrast, post-stress treatment with wheel-running exercise reduced the stress-induced increase in MCH expression to control levels in the lateral hypothalamus, hippocampus and basolateral amygdala. Administration of an MCH receptor antagonist (SNAP94847) to stress-treated mice was therapeutic against stress-induced anxiety-like behaviors. These results suggest that repeated stress produces long-lasting anxiety-like behaviors and upregulates MCH in the brain, while exercise counteracts stress-induced MCH expression and persisting anxiety-like behaviors.

Key words: Stress, Exercise, Anxiety, BLA, Hippocampus, MCH

INTRODUCTION

Chronic stress is a risk factor of anxiety disorders [1, 2]. Chronic or repeated stress in animal models induces anxiety-like behaviors in various behavioral tests [3-6]. Benzodiazepines were widely used to treat anxiety symptoms in the past, whereas in current years, antidepressants are considered to be the first

Received July 14, 2016, Revised August 10, 2016,
Accepted August 10, 2016

* To whom correspondence should be addressed.
TEL: 82-2-3277-4130, FAX: 82-2-3277-3419
e-mail: plhan@ewha.ac.kr

line of therapy for anxiety disorders [7]. Various antidepressants suppress stress-induced anxiety-like behaviors in different animal models [8, 9]. Studies in animal models have revealed that various neurotransmitters including GABA, monoamines, and cannabinoids, as well as neuropeptides, neurotrophins, and cytokines, regulate anxiety states [10-13]. These results suggest that multiple complex mechanisms are involved in the induction of anxiety disorders and inhibition of anxiety symptoms [7, 10].

Physical exercise helps to improve anxiety symptoms [14, 15], and thus has the opposite effects of stress. Resistance training might evoke certain stress responses, but at a low-to-moderate intensity it also has anxiolytic effects in humans [16]. In adolescents and young adults, physical activity reduces overall incidence of anxiety disorders as well as other co-morbid mental disorders [17]. In animal models, treadmill exercise reduces anxiety-like behaviors [18, 19], including those induced by stress [20]. Although the mechanisms by which exercise exerts anxiolytic effects are not clearly understood, exercise may induce physiological changes in the brain regions regulating emotional states, including the hippocampus [15, 21]. Exercise increases neurogenesis in the hippocampus [22] and prevents the stress-induced activation of granule neurons and stress-induced enhancing local inhibitory mechanisms of the dentate gyrus in the ventral hippocampus [23]. However, the detailed mechanisms by which exercise-induced activation of the hippocampus and other brain regions leads to anxiolytic effects need to be elaborated.

Melanin-concentrating hormone (MCH) is a 19 amino acid neuropeptide [24]. MCH is highly expressed in the lateral hypothalamus, and these MCH-containing neurons project throughout the brain, into regions including the cerebral cortex, amygdala, and nucleus accumbens [25, 26]. Several lines of evidence suggest that MCH regulates stress responses and anxiety [25, 27] among others. Intracerebroventricular injection of MCH increases corticosterone release and produces anxiety-like behaviors [28], while MCHR1 antagonists have anxiolytic effects [28-30]. Thus, available evidence supports a role of MCH in controlling anxiety states, but it is unknown whether MCH is a mediator of regulating stress-induced anxiety and exercise effects on anxiety.

In the present study, we demonstrate that repeated stress produces anxiety-like behaviors by up-regulating MCH in the hippocampus, amygdala and lateral hypothalamus, the areas important for emotional behaviors, whereas exercise downregulates the stress-induced increase in MCH expression and affords long-lasting anxiolytic effects.

MATERIALS AND METHODS

Animals

Male C57BL6 mice at 7 weeks of age were purchased from Daehan BioLink (Eumsung, Chungbuk, Republic of Korea). After their arrival, the mice were randomly divided into experimental groups as indicated and were housed in pairs in standard plastic cages in a temperature (23~24°C)- and humidity (50~60%)-controlled animal room. All animals were handled in accordance with the animal care guidelines of Ewha Womans University. All the proposed experimental procedures were approved by the Ewha Womans University IACUC (IACUC 16-018).

Repeated restraint

Scheduled repeated restraints were performed as described previously [31, 32]. In brief, mice were individually placed in a 50-ml conical tube with many holes for good ventilation and were restrained within this tube for 2 h daily beginning at 10 a.m. After each session of restraint, the mice were returned to their home cages and allowed to have free access to food and water. This procedure was repeated each day for the indicated number of days.

Drug administration

Imipramine was purchased from Sigma-Aldrich (St. Louis, MO, USA), and directly diluted in 0.9% saline. SNAP94847, an MCH receptor 1 antagonist, was purchased from Tocris Bioscience (#3347; Bristol, U.K), and was first dissolved in dimethylsulfoxide (DMSO), and then diluted in 0.9% saline. Imipramine (20 mg/kg/d) and SNAP94847 (30 mg/kg/d) were intraperitoneally (i.p) injected at a volume of 120 μ l per injection for the indicated periods.

Wheel-running exercise

Passive wheel-running exercise was performed as described previously [31, 32]. In brief, the running wheel consisted of a rotating drum with two circular ventilated plastic walls (20 cm, in inner diameter) with a rim (7 cm, in width) composed of evenly spaced aluminum bars (each 2 mm in width, spaced by 0.65 cm). Mice were placed on the running wheel for 20 min daily for 5 days so that they become familiar with the equipment and wheel running. This procedure was applied to animals prior to the start of the restraint session and was regarded as habituation to the running wheel. Each mouse was placed on a running wheel rotating at 9 m/min for 60 min daily, starting at 10:00 am, and this treatment was repeated for 21 days. All exercise performances for the 5 days of prehabituation and 21 days of scheduled exercise

were videotaped. Any mice that run on the running wheel for less than 80% of the training time for more than two consecutive days was regarded as having failed to perform the exercise properly and therefore was excluded from the final analysis.

Immunohistochemistry

Immunohistochemical analyses were carried out as previously described [31, 33]. Briefly, mice were anesthetized with a mixture (3.5:1) of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (23.3 mg/ml) at a dose of 2.5 µl/g body weight. They were then perfused with 4% paraformaldehyde by a trans-cardiac method, and brains were isolated. Brains were post-fixed further in the same solution overnight at 4°C. Brains were coronally cut into 40-µm-thick sections with a vibratome (Leica VT 1000S; Leica Instruments, Nussloch, Germany). These sections in a floating state were incubated with 4% bovine serum albumin in PBS with Tween-20 (PBST) for 1 h, then reacted with the primary antibody at 4°C overnight. The sections were washed with PBST, reacted with the secondary antibody, and visualized with an ABC Elite kit (PK-6200, Vector Laboratories, Burlingame, CA, USA) for DAB staining.

Anti-MCH was purchased from Phoenix Pharmaceuticals (#H-070-47; 1:500; Germany), while the secondary antibody, biotinylated anti-rabbit IgG, was purchased from Vector Laboratories (#BA-1000; 1:200, Burlingame, CA, USA). DAB-stained images were analyzed with an Olympus BX 51 microscope equipped with a DP71 camera and MetaMorph Microscopy Automation & Image Analysis software (Molecular Devices, Sunnyvale, CA, USA).

Real-time PCR analyses

Real-time PCR for the quantification of mRNA levels was carried out as described previously [31, 32]. Total RNA from tissue samples or cell cultures was purified with TRI reagent (15596-018, Invitrogen, CA, USA). Isolated RNA from each group was treated with DNase I to avoid genomic contamination. A reverse transcription system (Promega, MO, USA) was used to convert 1 µg of total RNA to cDNA. Real-time PCR was performed with 10 µl of 2X iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Foster City, CA, USA), 1 µl each of 5 pmol/µl primers, and 4 µl of the cDNA (1/8 dilution of the cDNA) in a volume of 20 µl using the CFX 96 Real-Time PCR System Detector (Bio-Rad Laboratories; Foster City, CA, USA). The primer sequences used were 5'-TGAACGATGATGACAATAAGAA-3' and 5'-TCAGAGCGAGGTAAGGTT-3' for MCH; 5'-AGAAGGTGGTGAAGCAGGCATC-3' and 5'-CGAAGGTGGAAGAGTGGGAGTTG-3' for GAPDH; and 5'-GCTGC

CATCTGTTTTACGG-3' and 5'-TGACTGGTGCCTGATGAAC-3' for L32. GAPDH and L32 were used as controls.

HT22 cells and corticosterone treatment

HT22 mouse hippocampal cells were cultured as described previously [34]. Briefly, HT22 cells were cultured in DMEM (Hyclone, Logan, UT, USA; SH30243.01) supplemented with 10% heat-inactivated FBS (CellNest, Charlotte, NC, USA; CNF303-0500), penicillin (20 U/ml), and streptomycin (20 mg/ml) at 37°C in a humidified incubator gassed with 95% air and 5% CO₂. For corticosterone treatment, HT22 cells at 40~50% confluence were incubated with corticosterone at 400 or 800 ng/ml in DMEM containing 1% FBS in 6-well plates. After 24 h, cells were washed in PBS and harvested.

Corticosterone measurement

Corticosterone levels in serum were measured as described previously [34]. Briefly, blood was collected from the hearts of sacrificed mice, centrifuged at 1,500 g for 15 min to obtain serum, and stored at -80°C until use. Sera were diluted 1: 50 in the EIA buffer provided in the EIA kit (Cayman Chemicals, MI, USA). Each diluted serum sample (50 µl) was mixed with an equal volume of a specific antibody to corticosterone (corticosterone EIA antiserum) and acetylcholinesterase-linked corticosterone (AChE Tracer) from the EIA kit on a 96-well plate. The reaction was incubated for 2 h at room temperature on an orbital shaker rotating at 150 rpm. Then, the reaction mixture in each well was discarded and rinsed with the washing buffer provided in the EIA kit. The Ellman's Develop Reagent in the kit was added at 200 µl/well, and the plate was incubated for 90 min at room temperature in the dark on an orbital shaker (150 rpm). Finally, the absorbance at 405 nm was measured using a spectrofluorometer (SpectraMax® M5; Molecular Devices, Sunnyvale, CA, USA).

Behavioral assessments

Behavioral tests were performed as described previously [31, 32, 35]. Mice were brought to the testing room 30 min prior to the start of each behavioral test. At all times non-specific background sounds were masked with 65-dB white noise. The behavior testing room was lit with indirect illumination of 20~30 lux for the social interaction test, elevated plus maze test, and novelty-suppressed feeding test and at 250 lux for the marble-burying test. Behavioral tests were carried out during the light cycle (9 A.M.~3 P.M.). The performance of each animal in the behavioral tests was recorded with a computerized video-tracking system (SMART; Panlab S.I., Barcelona, Spain) and/or webcam recording system (HD Webcam C210, Logitech, USA). All parts of the apparatus were cleaned with

70% ethanol between tests.

Social interaction test

The social interaction test was performed as described previously [32, 34]. In brief, a U-shaped field was prepared by partitioning an open field (45×45×40 cm) to the central point with a wall (20×40 cm, width by height), so that each field had one enclosed and one open square. On the test day, subject mice were individually placed in the empty U-shaped field for 5 min of exploration, during which the locomotive trajectory and the time spent in each field were recorded. This procedure was regarded as habituation to the test field. While the subject mice were returned to their home cages for 2~3 min, an active social target (10~12 weeks old male B6 mouse) was loaded in a circular grid cage (12 cm in diameter × 33 cm in height) and was positioned in the corner of the enclosed square (called the target zone), while an unanimated grid cage was placed on the opposite side (called the non-target zone). Then, each subject mouse was tested for its trajectory in the U-field for 10 min, during which the locomotive trajectory and the time spent in each zone were recorded.

Elevated plus maze test

The elevated plus maze test was performed as described previously [5, 35]. Briefly, the elevated plus maze apparatus consisted of four arms (30×7 cm each) made of gray FOAMEX (Expanded PVC; LG Ltd., Korea), which were elevated 50 cm above the floor and placed at right angles to each other. Two of the arms had 20-cm-high walls (enclosed arms), while the other two had no walls (open arms). The illumination at the open center was adjusted to 40 lux. For the test, subject mice were individually placed at the center of the two open arms and left to explore all arms for 5 min. The number of entries into the open and enclosed arms and the time spent in each arm were recorded. Entry into each arm was scored as an event if the animal crossed over the line of the cross square (7×7 cm) with all paws.

Light-dark box test

The light-dark box test was performed as described previously [11]. Briefly, the light box (36×25×18 cm) and dark box (12×25×18 cm) were made from a clear plastic rat cage partitioned with a separating wall with a shuttle door (5×5 cm) at the center of the two compartments at floor level. The light box was open at the top and illuminated to 700 lux at the bottom. The dark box was painted black and covered with a removable black lid. For the test, mice were individually placed into the light box with the shuttle door closed. After 10 sec, the shuttle door was opened, and the total number of transitions between the dark and light compartments,

latency to entry into the dark box, and time spent in the light/dark boxes were recorded for 5 min.

Open field test

The open field test was performed as described previously [11, 35]. Locomotor activity was measured in an open field (45 cm×45 cm×40 cm). The open field was lit with indirect illumination of 70 lux. Mice were individually placed at the center of the open field, and the locomotive trajectory, distance traveled, and times spent in the center and on the periphery were recorded for 30 min. The center area of the open field was defined as the inner rectangular area that constituted 30% of the open field.

Novelty-suppressed feeding test

The novelty-suppressed feeding test was performed as described previously [35]. In brief, mice were deprived of food, but not water, in their normal home cages for 24 h. On the test day, mice were individually transferred to a holding cage without food and water for 30 min. Then, each mouse was placed in a corner of the open field (45×45×40 cm), and a single food pellet (2×2×2.5 cm) placed on a circular piece of white filter paper (15 cm in diameter) in the center of the open field. The latency to the first bite of the food pellet was recorded. Immediately after the mouse began to eat the lab chow, the subject animal was removed and individually placed in its home cage with a weighed piece of chow. After 5 min, the amount of food consumed was determined based on the weight of the piece of chow.

Marble-burying test

The marble-burying test was carried out as described previously [35]. Empty normal plastic cages were filled with smooth bedding (JRS 3-4; J. Rettenmaier & Söhne, Rosenberg, Germany) up to 5 cm from the cage floor, and 12 marbles (glass balls with diameters of 1.5 cm) were placed evenly throughout the cage. Mice were individually allowed to freely explore the cage for 30 min, and the number of successfully buried marbles was counted. Marble “burying” was defined when less than 25% of a marble was visible.

Statistical analysis

Two-sample comparisons were carried out with Student's *t*-test, while multiple comparisons were conducted using one-way ANOVA followed by the Newman-Keuls multiple comparison test. Statistical significance analyses were performed with GraphPad PRISM 6 software (GraphPad Software, Inc, CA, USA). All data are presented as means±S.E.M. and statistical significance was established at the 5% level unless otherwise indicated.

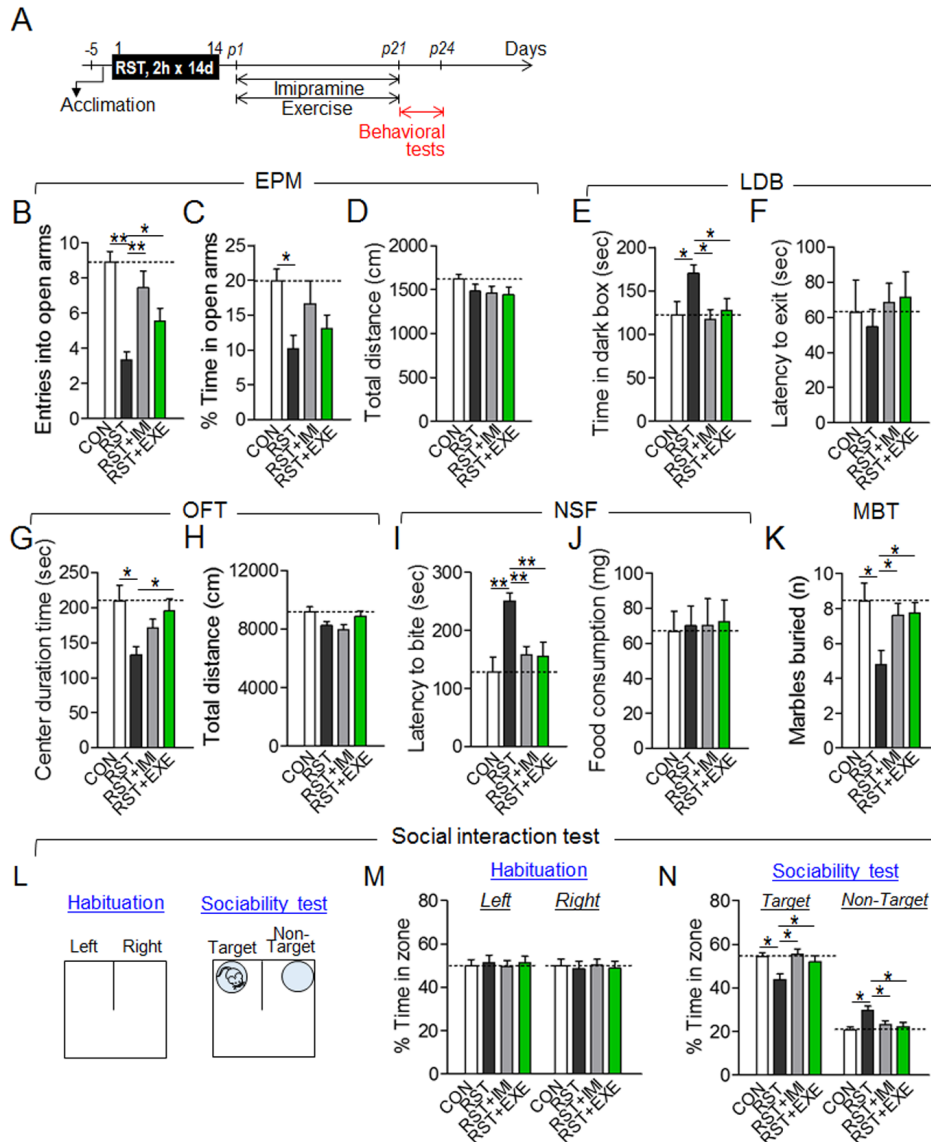


Fig. 1. Repeated restraint stress induced anxiety-like behaviors, whereas passive exercise counteracted stress-induced anxiety-like phenotypes. (A) Experimental design for treatment with 2 h×14 d RST (RST) and post-stress treatment with wheel-running exercise (RST+EXE) or imipramine (RST+IMI). Wheel-running exercise was treated for 1 h daily from post-stress days 1~21. Imipramine (20 mg/kg/day) was intraperitoneally (i.p) injected from post-stress days 1~21. Controls were aged-matched untreated naïve mice (CON). *p1*–*p24* represent post-stress days. (B–D) The number of entries and time spent in the open arms in the elevated plus maze test (EPM) among mice treated with 2 h×14 d RST (RST), mice treated with 2 h×14 d RST followed by exercise (RST+EXE), mice treated with 2 h×14 d RST followed by imipramine (RST+IMI), and their control mice (CON). (E, F) Times spent in the dark box and latency to first exit from the light box in the light-dark box test (LDB) among mice treated with 2 h×14 d RST (RST), mice treated with 2 h×14 d RST followed by exercise (RST+EXE), mice treated with 2 h×14 d RST followed by imipramine (RST+IMI), and their control mice (CON). (G, H) Time spent in the center area and the total distance traveled in the open field test (OFT) among mice treated with 2 h×14 d RST (RST), mice treated with 2 h×14 d RST followed by exercise (RST+EXE), mice treated with 2 h×14 d RST followed by imipramine (RST+IMI), and their control mice (CON). (I, J) Latency to eating a food pellet placed at the center of the open field and the amount of food consumed after the latency analysis in the novelty-suppressed feeding test (NSF) among mice treated with 2 h×14 d RST (RST), mice treated with 2 h×14 d RST followed by exercise (RST+EXE), mice treated with 2 h×14 d RST followed by imipramine (RST+IMI), and their control mice (CON). (K) The number of marbles buried in the marble-burying test (MBT) among mice treated with 2 h×14 d RST (RST), mice treated with 2 h×14 d RST followed by exercise (RST+EXE), mice treated with 2 h×14 d RST followed by imipramine (RST+IMI), and their control mice (CON). (L–N) The U-shaped field in the context without (for habituation test; left panel) or with (for sociability test; right panel) a social target (L). Times spent in the right and left sides of the empty U-shaped field (habituation test; M) and social interaction levels (N) in the sociability test among mice treated with 2 h×14 d RST (RST), mice treated with 2 h×14 d RST followed by exercise (RST+EXE), mice treated with 2 h×14 d RST followed by imipramine (RST+IMI), and their control mice (CON). Data are presented as mean±SEM (n=7~16). * and **, *p*<0.05 and *p*<0.01, respectively, for the differences between the control and indicated groups (one-way ANOVA and Newman-Keuls *post hoc* test).

RESULTS

Repeated restraint stress induced lasting anxiety-like behaviors, whereas passive exercise counteracted stress-induced anxiety-like phenotypes

Previously we reported that chronic restraints treatment for 2 h daily for 14 days (2 h×14 d RST) in mice produced anxiety-like behaviors [5]. In the present study, we investigated the mechanisms underlying stress-induced anxiety and its reversal by exercise using this stress-induced anxiety model. Mice treated with 2 h×14 d RST showed reduced dwelling times and entry numbers in the open arms in the elevated plus maze test, along with increased times in the dark box in the light-dark box test (Fig. 1A~F). Stress-treated mice also spent less time spent in the center area in the open field test, were more latent to eat a food pellet placed at the center of the open field in the novelty-suppressed feeding test, and buried fewer marbles in the marble-burying test (Fig. 1G~K). Stress-treated mice also exhibited decreased social interaction in the social interaction test (Fig. 1L~N). Thus, mice treated with repeated restraints displayed anxiety-like behaviors in various types of behavioral tests.

Toward understanding the mechanisms by which exercise produced anxiolytic effects, mice with stress-induced anxiety were treated with physical exercise for 1 h daily for 21 days (1 h×21 d EXE). Post-stress treatment with the 1 h×21 d EXE in mice with stress-induced anxiety reversed the reduced dwelling times in the open arms in the elevated plus maze test and suppressed the increased times in the dark box in the light-dark box test (Fig. 1A~F). Post-stress treatment with the 1 h×21 d EXE also increased the reduced times in the center in the open field test, reduced the increased latency to eating a food pellet placed at the center in the open field test, and increased the reduced marble burying in the marble-burying test (Fig. 1G~K). Post-stress treatment with the 1 h×21 d EXE increased the reduced social interaction in the social interaction test (Fig. 1L~N). Thus, post-stress treatment with exercise, although it was forcefully imposed, effectively blocked stress-induced anxiety-like behaviors in several types of behavioral tests. We included an imipramine group because this drug has shown to produce long-lasting effects in recovering impaired mood behaviors in the same stress-treated animal model [32, 34]. Post-stress treatment with imipramine also produced anxiolytic effects in this animal model (Fig. 1A~N).

Next, we examined whether the therapeutic effects of exercise on stress-induced anxiety were transient or long lasting. To address this, four groups of mice were prepared; untreated control mice (CON group), mice treated with the repeated stress (2 h×14 d restraints: RST group), mice subjected to the 2 h×14 d restraints,

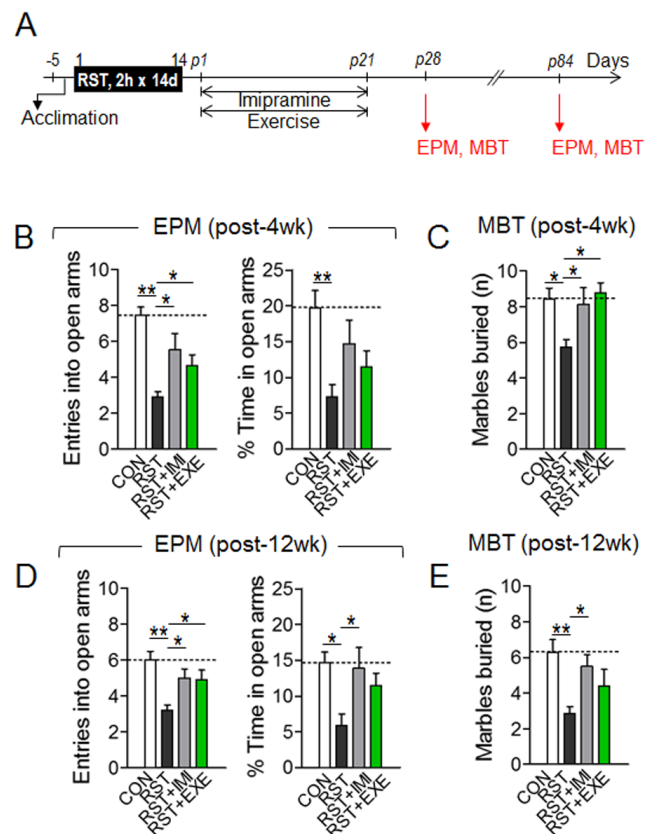


Fig. 2. Scheduled physical exercise stably counteracted long-lasting stress-induced anxiety. (A) Experimental design for treatment with 2 h×14 d RST (RST), post-stress treatment with wheel-running exercise (RST+EXE) and imipramine (RST+IMI), and subsequent behavioral assessments. Behavioral analyses were carried out on post-stress days 28 (4 weeks after treatment with 2 h×14 d RST) and 84 (12 weeks after treatment with 2 h×14 d RST). *p1*–*p84* represent post-stress days. (B) The number of entries and time spent in the open arms in the elevated plus maze test on post-stress day 28 (4 weeks after RST; post-4wk) among mice treated with 2 h×14 d RST (RST), mice treated with 2 h×14 d RST followed by exercise (RST+EXE), mice treated with 2 h×14 d RST followed by imipramine (RST+IMI), and their control mice (CON). (C) The number of marbles buried in the marble-burying test on post-stress day 28 (4 weeks after RST; post-4wk) among mice treated with 2 h×14 d RST (RST), mice treated with 2 h×14 d RST followed by exercise (RST+EXE), mice treated with 2 h×14 d RST followed by imipramine (RST+IMI), and their control mice (CON). (D) The number of entries and time spent in the open arms in the elevated plus maze test on post-stress day 84 (12 weeks after RST; post-12wk) among mice treated with 2 h×14 d RST (RST), mice treated with 2 h×14 d RST followed by exercise (RST+EXE), mice treated with 2 h×14 d RST followed by imipramine (RST+IMI), and their control mice (CON). (E) The number of marbles buried in the marble-burying test on post-stress day 84 (12 weeks after RST; post-12wk) among mice treated with 2 h×14 d RST (RST), mice treated with 2 h×14 d RST followed by exercise (RST+EXE), mice treated with 2 h×14 d RST followed by imipramine (RST+IMI), and their control mice (CON). Data are presented as mean±SEM (8~10). * and **, $p < 0.05$ and $p < 0.01$, respectively, for the differences between the control and indicated groups (one-way ANOVA and Newman-Keuls *post hoc* test).

followed by exercise or imipramine treatments (RST+EXE and RST+IMI groups). Post-stress treatments with exercise or imipramine were terminated on post-stress day 21 (Fig. 2A). When examined 7 days after the last exercise or imipramine treatment (i.e., post-stress day 28), mice treated with repeated stress (RST group) displayed reduced entries and visiting times in the open arms in the elevated plus maze test, and reduced marble burying in the marble-burying test (Fig. 2A~C). In contrast, mice subjected

to repeated stress followed by exercise (RST+EXE group) or imipramine (RST+IMI group) exhibited significantly or partially increased entries into the open arms of the elevated plus maze and control levels of marble burying in the marble-burying test (Fig. 2A~C). When the same mouse groups were subjected to the elevated plus maze test and marble-burying test on post-stress day 84 (that is, 12 weeks after the 2 h×14 d RST), the anxiolytic effects of exercise were sustained (Fig. 2D and E). These results suggest that repeated stress caused by the 2 h×14 d RST produces long-lasting anxiety-like behaviors and the therapeutic effects of exercise on stress-induced anxiety are also persisting.

Repeated restraint stress upregulated MCH expression in the brain, whereas exercise reversed the enhanced expression of MCH

Previously, we demonstrated that the neuropeptide MCH was upregulated in the amygdala in mice treated with 2 h~14 d RST [31]. Consistent with this report, real-time PCR analysis indicated that the expression of MCH increased in both the amygdala and the hippocampus in mice treated with 2 h~14 d RST (Fig. 3A and B).

Mice exposed to restraint for 2 h had markedly higher serum corticosterone levels than controls (Fig. 3C and D). We wondered whether the stress-dependent increase of MCH in the brain was induced by this stress hormone. To address this question, we performed an *in vitro* experiment using HT22 cells, a murine hippocampal cell line. When HT22 cells were treated with corticosterone at 400 ng/ml or 800 ng/ml for 24 h, MCH expression increased significantly (Fig. 3E and F). This result supports the possibility that the stress hormone corticosterone triggers the up-regulation of MCH in the brain.

Next, we examined whether stress-induced up-regulation of MCH in the brain was reversed by post-stress treatment with scheduled exercise. Immunohistochemical analysis of mice treated with repeated restraint indicated that MCH expression was upregulated in the hippocampus, basolateral amygdala (BLA), and lateral hypothalamus. The increased expression of MCH in each of these brain regions was reversed in animals that were treated with the scheduled exercise (Fig. 4A~E).

Pharmacological inhibition of the MCH receptor system had anxiolytic effects in mice with stress-induced anxiety-like behaviors

We examined whether pharmacological inhibition of MCH receptors in the brain in the post-stress period blocked stress-induced anxiety-like behaviors. Mice treated with repeated restraints (2 h×14 d RST) exhibited reduced social interaction

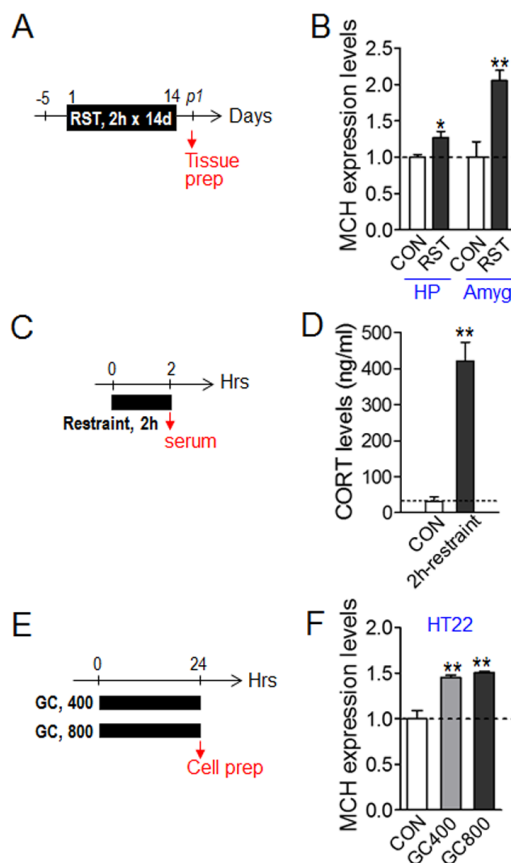


Fig. 3. Repeated restraint stress upregulated the expression of MCH in the brain and corticosterone increased the expression of MCH in HT22 cells. (A) Experimental design for treatment with 2 h×14 d RST, and the time point for brain tissue preparation (arrow). (B) Real-time PCR showing increased expression of MCH in the hippocampus (HP) and amygdala (Amyg) after treatment with repeated restraint stress (n=6 each). (C) Experimental design for treatment with a single 2-h restraint and the time point for blood preparation (arrow). (D) Corticosterone levels in the blood of mice treated with 2 h restraint (restraint) and their control (CON) (n=7 each). (E) Experimental design for treatment with corticosterone (final concentration: 400 or 800 µg/ml) and the time point for cell harvesting (arrow). (F) Real-time PCR showing increased expression of MCH in HT22 cells after treatment with corticosterone (n=5 animals each; three repeats of duplicated PCR data). Data are presented as mean ± SEM. * and **, p<0.05 and p<0.01, respectively for the differences between the control and indicated groups (one-way ANOVA and Newman-Keuls *post hoc* test).

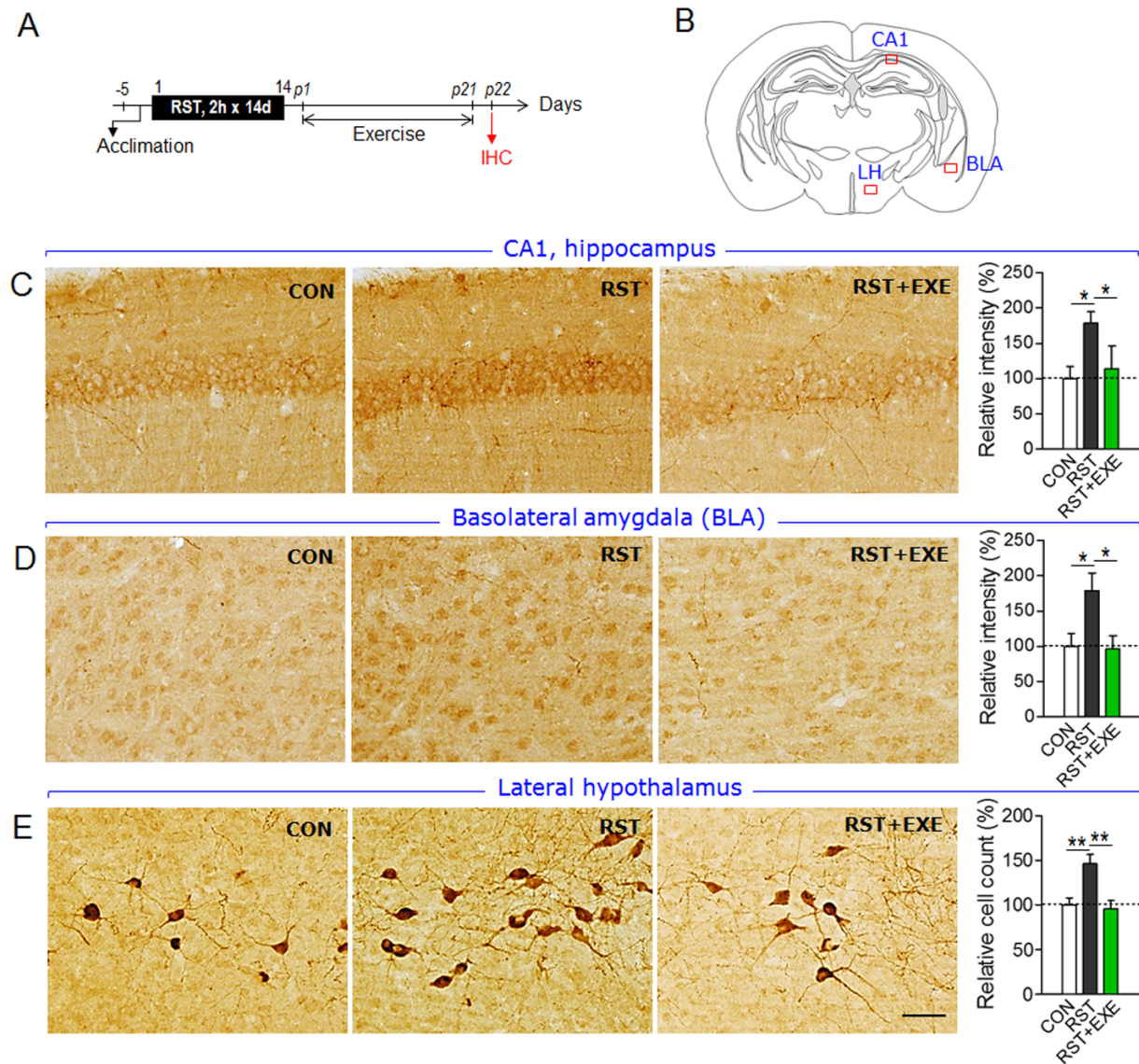


Fig. 4. Repeated restraint stress upregulated the expression of MCH in the brain, while exercise reversed the increased expression of MCH. (A) Experimental design for treatment with 2 h×14 d RST and post-stress treatment with wheel-running exercise, and time point for tissue preparation (arrow). Wheel-running exercise was treated for 1 h daily from post-stress days 1~21. (B) Brain regions marked by rectangles were used for analysis of MCH expression and for the high magnifications in Fig. 4C~E. CA1, CA1 of the hippocampus; BLA, basolateral amygdala; LH, lateral hypothalamus. (C~E) Photomicrographs showing the expression of MCH in the CA1 of the hippocampus (C), basolateral amygdala (BLA) (D), and lateral hypothalamus (LH) (E) of control animals (CON), mice treated with 2 h×14 d RST (RST), and mice treated with 2 h×14 d RST followed by exercise (RST+EXE). The quantified levels of MCH in the CA1 (C), BLA (D), and LH (E) are represented with bar graphs to the right of the photomicrographs. Scale bar; 200 μ m. Data are presented as mean \pm SEM ($n=10\sim24$). * and **, $p<0.05$ and $p<0.01$, respectively, for the differences between the control and indicated groups (one-way ANOVA and Newman-Keuls *post hoc* test).

in the social interaction test (Fig. 5A and B) and reduced marble burying in the marble-burying test (Fig. 5C). On the contrary, intraperitoneal administration of SNAP-94847 (an antagonist for MCH receptor 1) to mice subjected to the 2 h×14 d RST reversed the reduced social interaction (Fig. 5B) and increased the reduced marble burying (Fig. 5C) in the behavioral tests.

DISCUSSION

In the present study, we demonstrated that repeated stress produced long-lasting anxiety-like behaviors and up-regulated MCH in brain regions important for emotional behaviors, whereas physical exercise counteracted stress-induced MCH expression and persisting anxiety-like behaviors. Our results support the

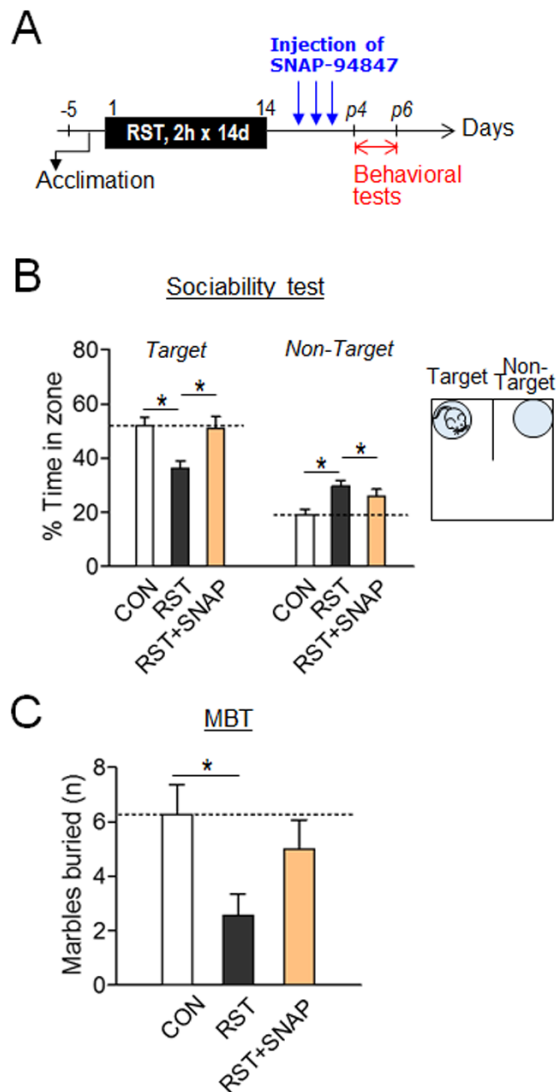


Fig. 5. Pharmacological inhibition of the MCH receptor system conferred anxiolytic effects. (A) Experimental design for treatment with the 2 h×14 d restraint and post-stress treatment with the MCH receptor 1 antagonist (SNAP-94847). SNAP-94847 was intraperitoneally administered at a dose of 30 mg/kg/day on post-stress days 1~3. Behavioral assessments were carried out on post-stress days 4~6. (B) Stress-induced reduced social interaction in the sociability test was reversed by post-stress treatment with SNAP-94847. The U-field with a social target are shown (right panel). (C) Stress-induced reduction in the number of marbles buried in the marble-burying test was partially reversed by SNAP-94847. Data are presented as mean±SEM (n=7~8). *, p<0.05 for the differences between the control and indicated groups (one-way ANOVA and Newman-Keuls *post hoc* test).

notion that MCH is an important player mediating, and/or a molecular biomarker representing, stress- and/or exercise-related behavioral changes. First, MCH expression was up-regulated by stress in the lateral hypothalamus, hippocampus, and basolateral amygdala, the brain regions important for emotional behaviors,

whereas it was downregulated in these areas following exercise (Fig. 3 and 4). These changes in MCH expression are consistent with the notion that exercise and stress have opposing effects on anxiety-like behaviors. Second, corticosterone treatment in HT22 cells induced MCH mRNA expression (Fig. 3F). This result supported the possibility that the up-regulation of MCH expression was initially triggered by corticosterone. Recently, we demonstrated that MCH expression could also be regulated by G9a [32]. Regarding this, it will be interesting to test whether stress- and exercise-dependent behavioral changes are mediated at least in part by a G9a-dependent mechanism. Regarding that repeated stress markedly increased p-CaMKII and p-ERK levels in the BLA [31, 33], it will be interesting to examine whether MCH expression is affected by p-CaMKII and/or p-ERK pathways. Third, intraperitoneal injection of the MCH receptor antagonist, SNAP-94847, in mice subjected to repeated stress (2 h×14 d RST) ameliorated their anxiogenic social interactions and partially restored the reduced level of marble burying in the marble-burying test (Fig. 5). In fact, it was reported that SNAP94847 has anti-anxiety effects [36]. These results, together with the findings that stress increased MCH expression while exercise reversed this effect, support the notion that repeated restraint stress produces anxiety-like behaviors by up-regulating MCH in the brain, while exercise counteracts stress-induced MCH expression and stress-induced anxiety-like behaviors.

The finding that daily 2-h restraint treatment for 14 days (2 h×14 d RST) induced anxiety-like behaviors in several behavioral tests (Fig. 1), while these behavioral changes persisted for 3 months (Fig. 2) raises the question of whether the repeated stress had produced long-term physiological and/or genomic changes in the brain. We speculate that these persisting behavioral changes are likely related to neural changes, such as stress-induced structural changes in neurons in the hippocampus, amygdala, and prefrontal cortex [37] or stress-induced gene expression alterations in the hippocampus and amygdala [5, 32]. Despite the finding that the stress-induced behavioral changes were long-lasting in our stress model, physical exercise treatment for 1 h daily from post-stress days 1~21 significantly reversed the anxiety-like behaviors to control levels in several behavioral tests (Fig. 1). These anxiolytic effects of exercise persisted for more than 2 months (Fig. 2). Considering that the stress-induced long-lasting behavioral changes were stably reversed by exercise, physical exercise likely reverses the stress-induced changes in the brain or exercise builds up a new change that blocks stress-induced impairments. Our recent microarray analysis of brains from stress- and exercise-treated mice revealed that exercise indeed reversed a number of stress-induced up- or down-regulated genes to their control-like states, while a number

of genes remained uncorrected and others changed to an exercise-specific pattern [32]. It was also reported that stress and exercise have opposing effects on the histone methyltransferase G9a in the BLA, the brain region wherein G9a-mediated epigenetic changes in gene expression of neuropeptides produce mood-related behavioral changes [32, 34]. Therefore, it will be interesting to investigate whether stimulus-dependent changes in G9a expression in the BLA are responsible for stress- and exercise-induced anxiety-related behaviors.

Wheel-running exercise was reported to activate the HPA axis, resulting in increased corticosterone release in the blood [38], and this HPA axis activating effect of exercise is similar to that of restraint stress [11, 39]. Nonetheless, as demonstrated in the present study, exercise produced anxiolytic effects, opposing to the effects of repeated restraint stress (Fig. 1 and 2). In relation to the activation of the HPA axis, the questions of how stress and exercise produce such complex and opposing effects remain unsolved. It was reported that wheel-running exercise produced a more rapid decay of the corticosterone surge following restraint stress [39]. However, the rapid regulation of the HPA axis might not be sufficient to explain the therapeutic effects of exercise that was treated during the post-stress period. Considering that the complex genomic responses occur in the brain after stress and exercise treatment, as revealed by microarray analysis [32], it will be interesting to explore brain regions that are specifically or commonly activated by stress and exercise.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education of the Republic of Korea and the National Research Foundation of Korea (NRF-2015S1A5B5A01015944).

REFERENCES

- Hoge EA, Ivkovic A, Fricchione GL (2012) Generalized anxiety disorder: diagnosis and treatment. *BMJ* 345:e7500.
- Sadock E, Auerbach SM, Rybarczyk B, Aggarwal A, Lanoye A (2014) The relationship of life stressors, mood disorder, and health care utilization in primary care patients referred for integrated behavioral health services. *J Nerv Ment Dis* 202:763-766.
- Vyas A, Chattarji S (2004) Modulation of different states of anxiety-like behavior by chronic stress. *Behav Neurosci* 118:1450-1454.
- Strekalova T, Spanagel R, Dolgov O, Bartsch D (2005) Stress-induced hyperlocomotion as a confounding factor in anxiety and depression models in mice. *Behav Pharmacol* 16:171-180.
- Kim KS, Han PL (2006) Optimization of chronic stress paradigms using anxiety- and depression-like behavioral parameters. *J Neurosci Res* 83:497-507.
- Yang L, Shi LJ, Yu J, Zhang YQ (2016) Activation of protein kinase A in the amygdala modulates anxiety-like behaviors in social defeat exposed mice. *Mol Brain* 9:3.
- Bystritsky A, Khalsa SS, Cameron ME, Schiffman J (2013) Current diagnosis and treatment of anxiety disorders. *Pharmacy Therpeu* 38:30-57.
- Lecci A, Borsini F, Volterra G, Meli A (1990) Pharmacological validation of a novel animal model of anticipatory anxiety in mice. *Psychopharmacology (Berl)* 101:255-261.
- Kim KS, Kwon HJ, Baek IS, Han PL (2012) Repeated short-term (2h×14d) emotional stress induces lasting depression-like behavior in mice. *Exp Neurobiol* 21:16-22.
- Millan MJ (2003) The neurobiology and control of anxious states. *Prog Neurobiol* 70:83-244.
- Kim KS, Lee KW, Baek IS, Lim CM, Krishnan V, Lee JK, Nestler EJ, Han PL (2008) Adenylyl cyclase-5 activity in the nucleus accumbens regulates anxiety-related behavior. *J Neurochem* 107:105-115.
- Pardon MC (2010) Role of neurotrophic factors in behavioral processes: implications for the treatment of psychiatric and neurodegenerative disorders. *Vitam Horm* 82:185-200.
- Bowers ME, Choi DC, Ressler KJ (2012) Neuropeptide regulation of fear and anxiety: Implications of cholecystokinin, endogenous opioids, and neuropeptide Y. *Physiol Behav* 107:699-710.
- Herring MP, O'Connor PJ, Dishman RK (2010) The effect of exercise training on anxiety symptoms among patients: a systematic review. *Arch Intern Med* 170:321-331.
- Anderson E, Shivakumar G (2013) Effects of exercise and physical activity on anxiety. *Front Psychiatry* 4:27.
- Strickland JC, Smith MA (2014) The anxiolytic effects of resistance exercise. *Front Psychol* 5:753.
- Ströhle A, Höfler M, Pfister H, Müller AG, Hoyer J, Wittchen HU, Lieb R (2007) Physical activity and prevalence and incidence of mental disorders in adolescents and young adults. *Psychol Med* 37:1657-1666.
- Fulk LJ, Stock HS, Lynn A, Marshall J, Wilson MA, Hand GA (2004) Chronic physical exercise reduces anxiety-like behavior in rats. *Int J Sports Med* 25:78-82.
- Patki G, Li L, Allam F, Solanki N, Dao AT, Alkadhi K, Salim S (2014) Moderate treadmill exercise rescues anxiety and depression-like behavior as well as memory impairment in

- a rat model of posttraumatic stress disorder. *Physiol Behav* 130:47-53.
20. Vollert C, Zagaar M, Hovatta I, Taneja M, Vu A, Dao A, Levine A, Alkadhi K, Salim S (2011) Exercise prevents sleep deprivation-associated anxiety-like behavior in rats: potential role of oxidative stress mechanisms. *Behav Brain Res* 224:233-240.
 21. Leuner B, Gould E (2010) Structural plasticity and hippocampal function. *Annu Rev Psychol* 61:111-140, C1-3.
 22. Van Praag H (2008). Neurogenesis and exercise: past and future directions. *Neuromolecular Med* 10:128-140.
 23. Schoenfeld TJ, Rada P, Pieruzzini PR, Hsueh B, Gould E (2013) Physical exercise prevents stress-induced activation of granule neurons and enhances local inhibitory mechanisms in the dentate gyrus. *J Neurosci* 33:7770-7777.
 24. Saito Y, Nagasaki H (2008) The melanin-concentrating hormone system and its physiological functions. *Results Probl Cell Differ* 46:159-179.
 25. Hervieu G (2003) Melanin-concentrating hormone functions in the nervous system: food intake and stress. *Expert Opin Ther Targets* 7:495-511.
 26. Pissios P, Bradley RL, Maratos-Flier E (2006) Expanding the scales: The multiple roles of MCH in regulating energy balance and other biological functions. *Endocr Rev* 27:606-620.
 27. Shimazaki T, Yoshimizu T, Chaki S (2006) Melanin-concentrating hormone MCH1 receptor antagonists: a potential new approach to the treatment of depression and anxiety disorders. *CNS Drugs* 20:801-811.
 28. Smith DG, Davis RJ, Rorick-Kehn L, Morin M, Witkin JM, McKinzie DL, Nomikos GG, Gehlert DR (2006) Melanin-concentrating hormone-1 receptor modulates neuroendocrine, behavioral, and corticolimbic neurochemical stress responses in mice. *Neuropsychopharmacology* 31:1135-1145.
 29. Borowsky B, Durkin MM, Ogozalek K, Marzabadi MR, DeLeon J, Lagu B, Heurich R, Lichtblau H, Shaposhnik Z, Daniewska I, Blackburn TP, Branchek TA, Gerald C, Vaysse PJ, Forray C (2002) Antidepressant, anxiolytic and anorectic effects of a melanin-concentrating hormone-1 receptor antagonist. *Nat Med* 8:825-830.
 30. Chaki S, Funakoshi T, Hirota-Okuno S, Nishiguchi M, Shimazaki T, Iijima M, Grottick AJ, Kanuma K, Omodera K, Sekiguchi Y, Okuyama S, Tran TA, Semple G, Thomsen W (2005) Anxiolytic- and antidepressant-like profile of ATC0065 and ATC0175: nonpeptidic and orally active melanin-concentrating hormone receptor 1 antagonists. *J Pharmacol Exp Ther* 313:831-839.
 31. Kim TK, Kim JE, Park JY, Lee JE, Choi J, Kim H, Lee EH, Kim SW, Lee JK, Kang HS, Han PL (2015a) Antidepressant effects of exercise are produced via suppression of hypocretin/orexin and melanin-concentrating hormone in the basolateral amygdala. *Neurobiol Dis* 79:59-69.
 32. Kim TK, Lee JE, Kim JE, Park JY, Choi J, Kim H, Lee EH, Han PL (2015b) G9a-mediated regulation of OXT and AVP expression in the basolateral amygdala mediates stress-induced lasting behavioral depression and its reversal by exercise. *Mol Neurobiol* 53:2843-2856.
 33. Choi J, Kim JE, Kim TK, Park JY, Lee JE, Kim H, Lee EH, Han PL (2015) TRH and TRH receptor system in the basolateral amygdala mediate stress-induced depression-like behaviors. *Neuropharmacology* 97:346-356.
 34. Seo JS, Park JY, Choi J, Kim TK, Shin JH, Lee JK, Han PL (2012) NADPH oxidase mediates depressive behavior induced by chronic stress in mice. *J Neurosci* 32:9690-9699.
 35. Baek IS, Park JY, Han PL (2016) Chronic antidepressant treatment in normal mice induces anxiety and impairs stress-coping ability. *Exp Neurobiol* 24:156-168.
 36. David DJ, Klemenhagen KC, Holick KA, Saxe MD, Mendez I, Santarelli L, Craig DA, Zhong H, Swanson CJ, Hegde LG, Ping XI, Dong D, Marzabadi MR, Gerald CP, Hen R (2007) Efficacy of the MCHR1 antagonist N-[3-(1-{[4-(3,4-difluorophenoxy)phenyl]methyl}(4-piperidyl))-4-methylphenyl]-2-methylpropanamide (SNAP 94847) in mouse models of anxiety and depression following acute and chronic administration is independent of hippocampal neurogenesis. *J Pharmacol Exp Ther* 321:237-248.
 37. McEwen BS, Eiland L, Hunter RG, Miller MM (2012) Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. *Neuropharmacology* 62:3-12.
 38. Ke Z, Yip SP, Li L, Zheng XX, Tong KY (2011) The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: a rat brain ischemia model. *PLoS One* 6:e16643.
 39. Hare BD, Beierle JA, Toufexis DJ, Hammack SE, Falls WA (2014) Exercise-associated changes in the corticosterone response to acute restraint stress: evidence for increased adrenal sensitivity and reduced corticosterone response duration. *Neuropsychopharmacology* 39:1262-1269.