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Exercise exerts an anxiolytic effect against repeated restraint stress through 5-HT_{2A}-mediated suppression of the adenosine A_{2A} receptor in the basolateral amygdala

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ABSTRACT

Repeated or chronic stressful stimuli induce emotion- and mood-related abnormalities, such as anxiety and depression. Conversely, regular exercise exerts protective effects. Here, we found that exercise recovered anxiety-like behaviors, as measured using the open field and elevated plus maze tests in an anxiety mouse model. In addition to behavioral improvement, exercise enhanced the synaptic density of the 5-hydroxytryptamine 2A receptor (5-HT_{2A}R), but not the 5-HT_{1A}R in the basolateral amygdala (BLA) region in this mouse model. Furthermore, global treatment with a selective 5-HT_{2A}R antagonist (MDL11930) generated an anxiety phenotype. Thus, synaptic recruitment of 5-HT_{2A}R in BLA neurons may mediate the anxiolytic effects of exercise. The exercise regimen also reduced adenosine A_{2A} receptor (A_{2A}R)-mediated protein kinase A (PKA) activation, and the anxiolytic effect of the exercise was blunted by local activation of A_{2A}R within the BLA using CGS21680, a selective A_{2A}R agonist. Particularly, A_{2A}R-mediated PKA activity was shown to be dependent on 5-HT_{2A}R signaling in the BLA. These results imply that repeated stress upregulates A_{2A}R-mediated adenosine signaling to facilitate PKA activation, whereas regular exercise inhibits A_{2A}R function by increasing 5-HT_{2A}R in the BLA. Accordingly, this integrated modulation of 5-HT and adenosine signaling, via 5-HT_{2A}R and A_{2A}R respectively, may be a mechanism underlying the anxiolytic effect of regular exercise.

1. Introduction

Stress is a critical factor in the etiology of mental abnormalities. Repeated stress induces hypersecretion of adrenaline and glucocorticoids, which may lead to mental illnesses, such as anxiety disorder and depression (de Kloet et al., 2005; McEwen and Gianaros, 2010). The amygdaloid complex is a pair of nuclei within the temporal lobe that are connected to stress-response structures including the hippocampus (Osório et al., 2017; Russo et al., 2012). Particularly, hyperactivity of the basolateral amygdala (BLA) principle neurons contributes to the disruption of mood and emotions such as fear and anxiety (Hubert et al., 2014; Padival et al., 2013; Vyas et al., 2004).

Additionally, serotonergic system dysfunction in the brain is strongly associated with the generation of aversive emotional and behavioral consequences, such as anxiety and panic (Canteras and Graeff, 2014; Sah et al., 2003). 5-hydroxytryptamine 1A receptor (5HT_{1A}R) knockout models showed higher levels of anxiety than did wild-type controls, and mice lacking 5HT_{1A} autoreceptors (but not heteroreceptors) displayed an increased serotonergic tone and anxious profile according to their different genetic backgrounds (Richardson-Jones et al., 2011; Żmudzka et al., 2018). A more recent study has revealed enhanced basal anxiety phenotypes measured using open field test (OFT) and elevated plus maze (EPM) tests in serotonin transporter $(SERT)^{-/-}$, but not $SERT^{+/-}$ rats, along with altered expression of 5-HT receptor subtypes in the BLA area (Johnson, et al., 2019). In the same study, the panic vulnerability, manifested as the respiration rate following the exposure to 7.5% CO_2 was heightened in the $SERT^{-/-}$ rat relative to that of $SERT^{+/-}$ and wild type rats (Johnson, et al., 2019). Moreover, cortical restoration of 5HT_{2A}R signaling reversed the conflict anxiety behavior in 5$HT_{2A}R^{-/-}$ mice (Weisstaub et al., 2006). These results collectively suggest that brain 5-HT neurotransmission plays a crucial role in the development of anxiety-related disorders. Hence,

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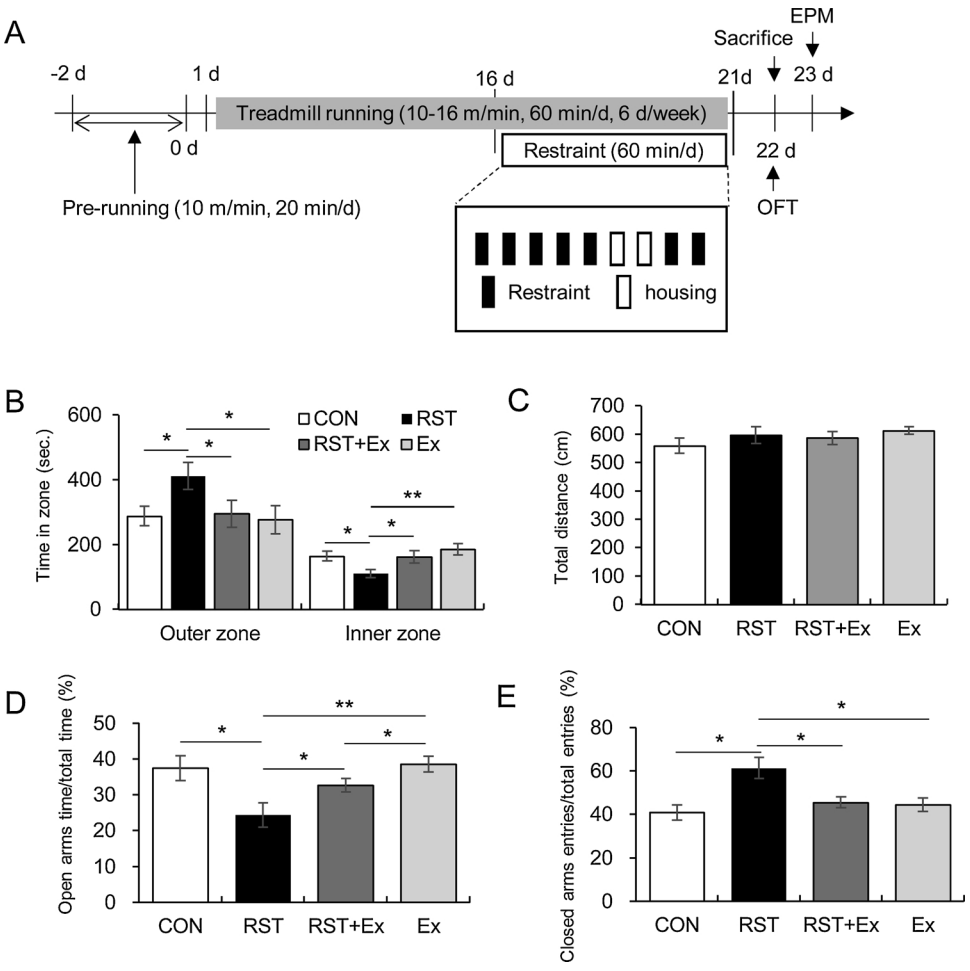

Fig. 1. The anxiolytic effect of regular exercise against repeated restraint stress. (A) The experimental procedure. (B) Quantitative analysis of the time traveled in the inner zone ($F_{3, 30} = 4.12$, $p < 0.05$) and the outer zone ($F_{3, 30} = 2.84$, $p < 0.05$) in the open field test (OFT). (C) Quantitative analysis of the total distance traveled in the arena in the OFT ($F_{3, 30} = 0.87$, $p > 0.05$). (D) Quantitative analysis of the time spent in the open arm in the EPM test ($F_{3, 30} = 5.59$, $p < 0.01$). (E) Quantitative analysis of the time spent in the closed arm in the EPM test ($F_{3, 30} = 5.27$, $p < 0.01$). Data are presented as the mean ± standard error of the mean. *$p < 0.05$, **$p < 0.01$. RST: restraint stress treatment.

many therapeutic approaches to mood- and emotion-related psychosocial stress have focused on serotoninergic transmission. Particularly, 5-HT receptors affect anxiety behavior and gamma aminobutyric acid (GABA)-mediated inhibitory activity in the BLA. For example, 5-HT receptor agonists exert anxiolytic effects and 5-HT induces GABA-mediated inhibitory potential in the BLA against chronic stress (Donatti and Leite-Panissi, 2009; Jiang et al., 2009). Thus, serotonergic input in the BLA region affects emotional behaviors such as anxiety under pathophysiological stress.

The adenosine neuromodulation system, through inhibitory adenosine A_1 receptors (A_1Rs) and facilitatory A_{2A} receptors (A_{2A}Rs), controls neuronal excitability, synaptic plasticity, and synaptic neurotransmission (Cunha, 2008; Fredholm et al., 2005). Particularly, A_{2A}Rs are abundant in the neuronal synapses of the hippocampus and the BLA region (Costenla et al., 2011; Simões et al., 2016). The crucial role of neuronal A_{2A}R in synaptic plasticity and behavior is typified in the hippocampus, as indicated by conflicting outcomes that depend on its activity in response to stress (Batalha et al., 2013; Kaster et al., 2015; Pagnussat et al., 2015). Several studies have demonstrated the regulatory role of brain A_{2A}R in anxiety-related behaviors (Reviewed in Yamada et al., 2014). For example, $A_{2A}R^{-/-}$ mice had more anxiety than did wild-type mice in the EPM, dark/light box, and novel OF tests (Ledent et al., 1997; López-Cruz et al., 2017). On the contrary, treatment with low doses of caffeine, a nonselective A_1/A_{2A}R antagonist, ameliorated the anxiety phenotype, whereas high-dose caffeine treatment worsened anxiety-related symptoms in rodents (Hughes et al., 2014; Hughes and Hancock, 2016; Sweeney et al., 2016). Caffeine treatment ameliorated chronic stress-provoked anxiety-like behavior by suppressing A_{2A}R function (Kaster et al., 2015). As mentioned above, the role of A_{2A}R in anxiogenic and anxiolytic effects is rather controversial and remains unclear. With regard to synaptic plasticity in the BLA, pharmacologic manipulation of A_{2A}R activity in the BLA affects long-term potentiation (LTP) and slow afterhyperpolarization (sAHP) amplitude, which are associated with intrinsic excitability of BLA neurons (Rau et al., 2015; Simões et al., 2016). Based on these previous studies, the altered phenotype of A_{2A}R in the BLA may be linked to the development of anxiety disorder in response to chronic stress.

Exercise improves brain function, influencing both cognitive function and mood. Particularly, neuronal metabolic activity, including the adenosine monophosphate/adenosine triphosphate ratio and adenosine content, and neurotransmitters such as 5-HT are dynamically altered in response to exercise (Hackney, 2006; Nybo and Secher, 2004). However, the mechanism underlying the anxiolytic effect of regular exercise remains elusive in terms of the cooperative regulation of 5-HT and adenosine neuromodulation in the BLA region. Therefore, we explored the modulatory effects of regular exercise on 5-HT and adenosine receptors with repeated stress in the BLA region, specifically by focusing on 5-HT_{2A}R and A_{2A}R. Furthermore, we investigated whether the exercise-mediated alteration of 5-HT_{2A}R and A_{2A}R function controls anxiety-like behaviors, in addition to the contribution of 5-HT_{2A}R activity to A_{2A}R function in the BLA area under stressful conditions.

2. Materials and methods

2.1. Experimental mice

Male 7-week-old C57BL/6 mice were obtained from Daehan Biolink, Inc. (Eumsung, Chungbuk, Korea) and housed in clear plastic cages

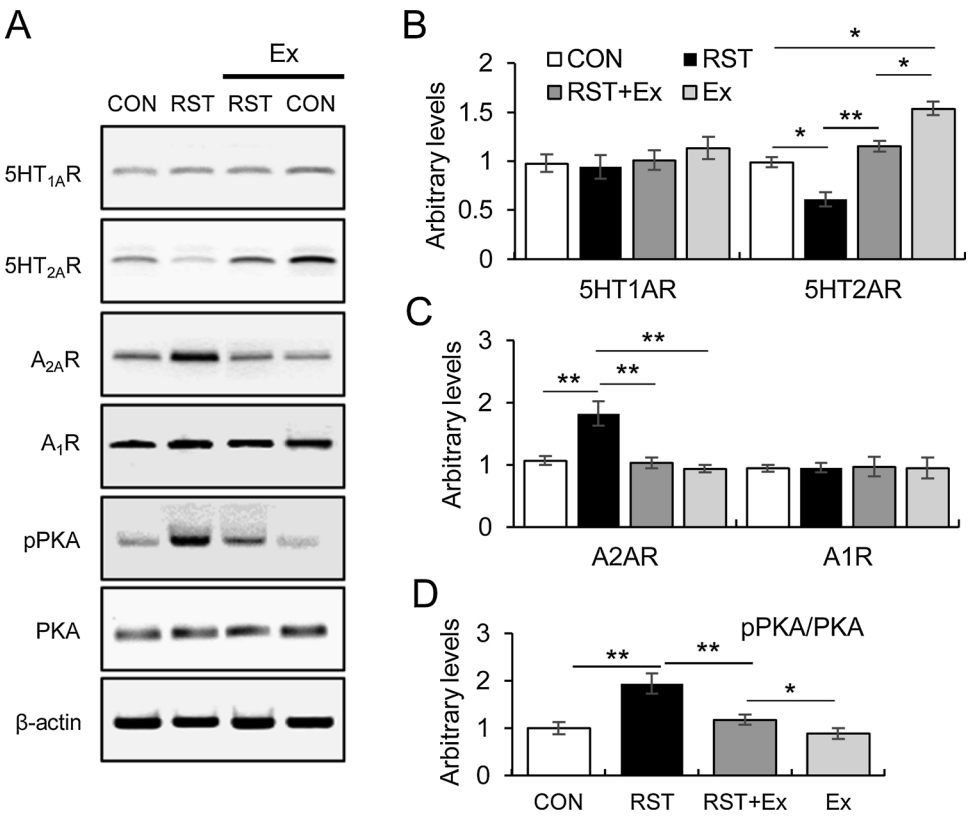

Fig. 2. The effect of exercise on the expression of synaptic 5-hydroxytryptamine (5-HT) and adenosine receptors, and phospho-protein kinase A (p-PKA) activity in the basolateral amygdala against repeated restraint stress. (A) Representative images for western blot data. (B) Quantitative analysis of 5-HT_{1A}R (5-HT_{1A}R: $F_{3,\ 8} = 0.78$, $p > 0.05$) and 5-HT_{2A}R ($F_{3,\ 8} = 38.71$, $p < 0.01$). (C) Quantitative analysis of A_{2A}R ($F_{3,\ 8} = 35.53$, $p < 0.01$) and A_1R ($F_{3,\ 8} = 0.02$, $p > 0.05$). (D) Quantitative analysis of the ratio of p-PKA to PKA ($F_{3,\ 8} = 30.68$, $p < 0.01$). Data are presented as the mean ± standard error of the mean. *p < 0.05, **p < 0.01.

under specific pathogen-free conditions with a 12:12-h light–dark cycle (lights on at 08:00 and off at 20:00). Mice had free access to standard irradiated chow (Purina Mills, Seoul, Korea). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Ewha Womans University, and conducted in accordance with the guidelines of the National Institute of Heath's Guide for the Care and Use of Laboratory Animals.

2.2. Experimental design

In experiment 1 (Fig. 1), the mice were divided into four groups (control: CON, repeated stress: RST, repeated stress with treadmill running: RST + Ex, and treadmill running: Ex; each group N = 8–9). Mice were subjected to treadmill running for 21 days and restraint stress was applied for 9 days within the period of the exercise regimen. Behavior tests were conducted 1 day after the last exercise treatment. The EPM test was implemented approximately 24 h after the OFTs. In experiment 2 (Fig. 2), the procedure designed to assess synaptosomal protein levels was the same as that of experiment 1 except for the behavior test (each group N = 8). In experiment 3 (Fig. 3), to explore the anxiogenic role of the 5-HT_{2A}R antagonist, mice were intraperitoneally injected with MDL11939 (0–1 mg/kg; Tocris Bioscience, Bristol, UK). OFTs were subsequently performed 90 min after drug treatment, followed by the EPM test 30 min later (each group N = 7). To assess synaptosomal protein levels, mice were decapitated 30 min after the EPM test. In experiment 4 (Fig. 4), to explore the anxiogenic role of A_{2A}R agonist, mice were assessed using OFTs and EPM tests (with the EPM tests implemented approximately 30 min after the OFTs) 120 min after stereotaxic injection with CGS21680 (0–40 ng/side; Sigma-Aldrich, St. Louis, MO, USA), an adenosine A_{2A}R agonist, bilaterally into the BLA (each group N = 7). In experiment 5 (Fig. 5), to explore the role of A_{2A}R in the anxiolytic effect of exercise, mice were subjected to treadmill running for 21 days and restraint stress was applied for 9 days within the period of the exercise regimen. A 1.0-μL solution containing 20 ng of CGS21680 or a vehicle was bilaterally injected into the BLA 1 day after the last exercise regimen. Behavior tests (with EPM tests implemented approximately 30 min after the OFTs) were conducted 120 min after the drug treatment (each group N = 8).

2.3. Repeated stress administration

The repeat stress procedure adopted in this study was modified from a previous method (Padival et al., 2013). To induce repeated stress, 8-week-old mice were individually placed into a restrainer for 7 out of 9 days (60 min/session, one session/day). Stress was administered at set times from 10:00 to 11:00 for a duration of 60 min. Control mice remained undisturbed in their home cages.

2.4. Exercise protocol

Mice were acclimated to pre-exercise for 3 days (12 m/min, 20 min/day, 0% grade) prior to the principal exercise. Mice were subjected to treadmill running for 60 min (12 m/min for 10 min, 16 m/min for 40 min, 12 m/min for 10 min, 0% grade, 6 days/week) at 16:00–18:00 for 21 days.

2.5. Behavioral assessment

To measure the degree of anxiety-related behavior, the EPM test was conducted using an apparatus consisting of four arms (30 × 7 cm) composed of black acrylic. The apparatus was elevated 50 cm above the ground. Two of the arms had 20-cm-high walls (enclosed arms) and the others had no walls (open arms). For the OFT, mice were placed in the center of a clear Plexiglas box made of white non-porous plastic (50 cm × 50 cm × 38 cm). The total distance traveled and the time spent in each zone within 5 min were measured using the SMART 3.0 program (Panlab, SLU, Barcelona, Spain) on a computer connected to a ceiling-mounted camera directly above the arena. A series of 10 × 10 cm zones within the arena were identified and used to evaluate mouse tracks. The outer zone was defined by 16 blocks around the

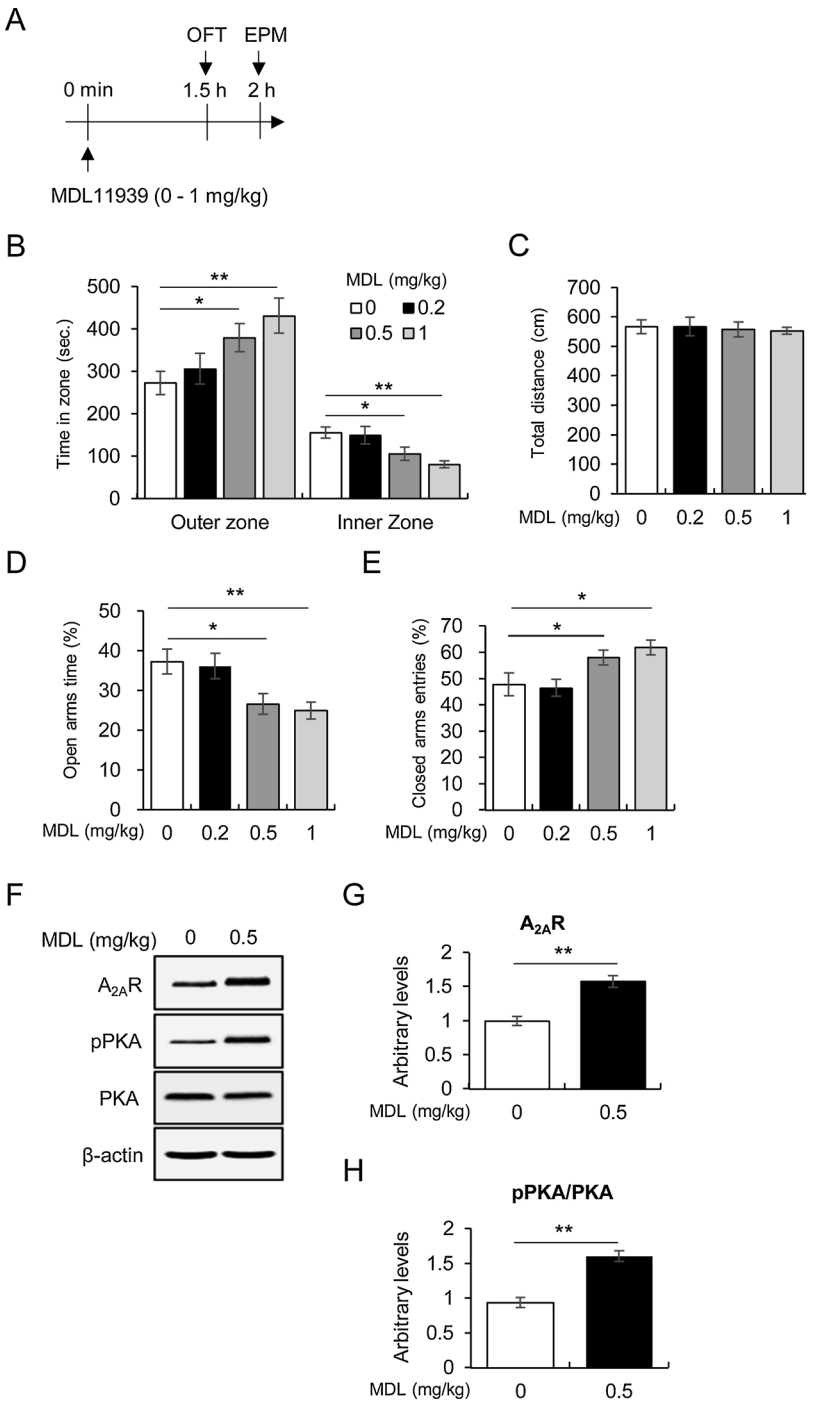

Fig. 3. The effects of MDL11939 on anxiety-like behaviors and basolateral amygdala synaptic $A_{2A}R$ and p-PKA levels in naïve mice. (A) The experimental procedure. (B) Quantitative analysis of the time traveled in the inner zone ($F_{3, 24} = 5.79$, $p < 0.01$) and the outer zone ($F_{3, 24} = 4.19$, $p < 0.05$) in the open field test (OFT). (C) Quantitative analysis of total distance traveled in the arena in OFT ($F_{3, 24} = 0.079$, $p > 0.05$). (D) Quantitative analysis of the time spent in the open arm in the EPM test ($F_{3, 24} = 5.13$, $p < 0.01$). (E) Quantitative analysis of the time spent in the closed arm in the EPM test ($F_{3, 24} = 6.33$, $p < 0.01$). (F) Representative images for western blot data. (G) Quantitative analysis of $A_{2A}R$ ($t_4 = -9.38$, $p < 0.01$). (H) Quantitative analysis of the ratio of p-PKA to PKA ($t_4 = -11.07$, $p < 0.01$). Data are presented as the mean ± standard error of the mean. *$p < 0.05$, **$p < 0.01$.

circumference of the arena and the inner zone was defined by 9 blocks inside the outer zone.

2.6. Synaptosome extraction and western blot analysis

To determine the synaptosome fraction, tissues dissected from the BLA of the mice, which were isolated using tissue punches (1.25-mm diameter), were pooled (2–3 tissues). The procedure for determining the synaptosomal fraction of the tissues was as previously described (Yi et al., 2017). Protein samples were electrophoretically separated on 10% polyacrylamide gels and then incubated overnight with primary antibodies in a blocking buffer at room temperature. The membranes were washed in the buffer and incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG 1:3000) for 2 h. The optical density of each band was measured using Image J (National Institutes of Health, Image Engineering, Bethesda, MD, USA). Anti-protein kinase A (PKA) (1:2,000) and anti-phospho-PKA (1:1,000) were obtained from Cell Signaling Tech. Inc. (Danvers, MA, USA). Anti-5-$HT_{1A}R$ (1:500), anti-5-$HT_{2A}R$ (1:500), and anti-β-actin (1:5,000) were purchased from Abcam (Cambridge, UK). Anti-$A_{2A}R$ (1:1,000) and anti-A_1R (1:500) were obtained from Millipore (Billerica, MA, USA).

2.7. Stereotaxic injections into the BLA

Mice were anesthetized with tribromoethanol at 250 mg/kg body weight. A 1-μL solution of CGS21680 dissolved in sterile saline with 0.5% dimethyl sulfoxide or a vehicle was bilaterally microinjected into the BLA at a speed of 0.3 μL/min using a 31-gauge needle

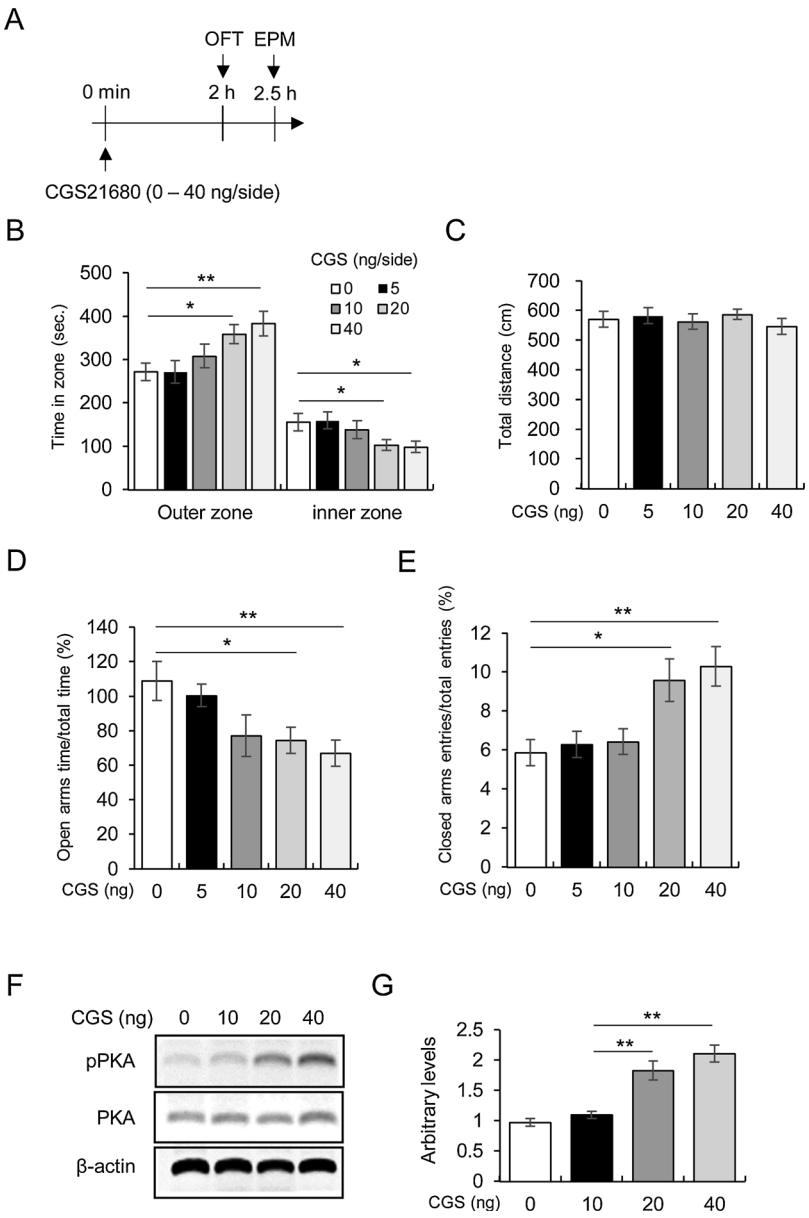

Fig. 4. The effects of CGS21680 on anxiety-like behaviors and basolateral amygdala synaptic p-PKA levels in naïve mice. (A) The experimental procedure. (B) Quantitative analysis of the time traveled in the inner zone ($F_{4, 30} = 2.64$ $p < 0.05$) and the outer zone ($F_{3, 30} = 4.11$, $p < 0.01$) in the open field test (OFT). (C) Quantitative analysis of the total distance traveled in the arena in the OFT ($F_{4, 30} = 0.43$, $p > 0.05$). (D) Quantitative analysis of the time spent in the open arm in the EPM test ($F_{4, 30} = 3.80$, $p < 0.05$). (E) Quantitative analysis of the time spent in the closed arm in the EPM test ($F_{4, 30} = 6.05$, $p < 0.01$). (F) Representative images for western blot data. (G) Quantitative analysis of the ratio of p-PKA to PKA ($F_{3, 12} = 23.45$, $p < 0.01$). Data are presented as the mean ± standard error of the mean. *p < 0.05, **p < 0.01.

(anteroposterior, -1.40 mm; mediolateral, ± 3.1 mm; dorsoventral, -4.9 mm; Stoelting Co, Wood Dale, IL, USA).

2.8. Statistical analysis

Significant differences between groups were determined using independent t-tests, one-way analyses of variance, and the Statistical Package for the Social Sciences for Windows, version 23 (SPSS Inc., Chicago, IL, USA). Post hoc comparisons were made using least significant difference tests. All values are reported as the mean ± standard deviation or error of the mean. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Treadmill running alleviated repeated-stress-induced anxiety behaviors

To investigate the protective effect of exercise on anxiety phenotypes, mice were subjected to treadmill running prior to and during exposure to repeated restraint stress. The exercise regimen alleviated the restraint-stress-induced increase in the time traveled in the outer zone and decrease in that in the inner zone in OFTs (Figs. 1A and B). The total traveled distance in the arena did not differ between the groups during the tests (Fig. 1C). Furthermore, the exercise regimen ameliorated the repeated-stress-induced decrease in time spent in the open arms (Fig. 1D) and increase in entrances into the closed arms in the EPM test (Fig. 1E).

3.2. Treadmill running reversed repeated-stress-induced changes in synaptosomal 5-HT_{2A}R, A_{2A}R, and p-PKA protein levels in the BLA

To elucidate the molecular mechanisms underlying the beneficial exercise effects in the anxiety mouse model, synaptosomal 5-HT receptor (5-HT_{1A}R and 5-HT_{2A}R) and adenosine receptor subtype (A_1R and A_{2A}R) levels was measured in the BLA. We found that exercise recovered the repeated-stress-induced decrease in synaptosomal 5-HT_{2A}R levels. Additionally, the exercise regimen itself enhanced 5-HT_{2A}R levels relative to that of the control BLA, but not 5-HT_{1A}R levels (Figs. 2A and B). Exercise normalized the repeated-stress-induced synaptosomal enhancement of A_{2A}R, but not A_1R levels (Figs. 2A and C). Moreover, exercise down-regulated the repeated-stress-evoked increase in synaptosomal PKA activity (the pPKA to PKA ratio) (Figs. 2A and D;

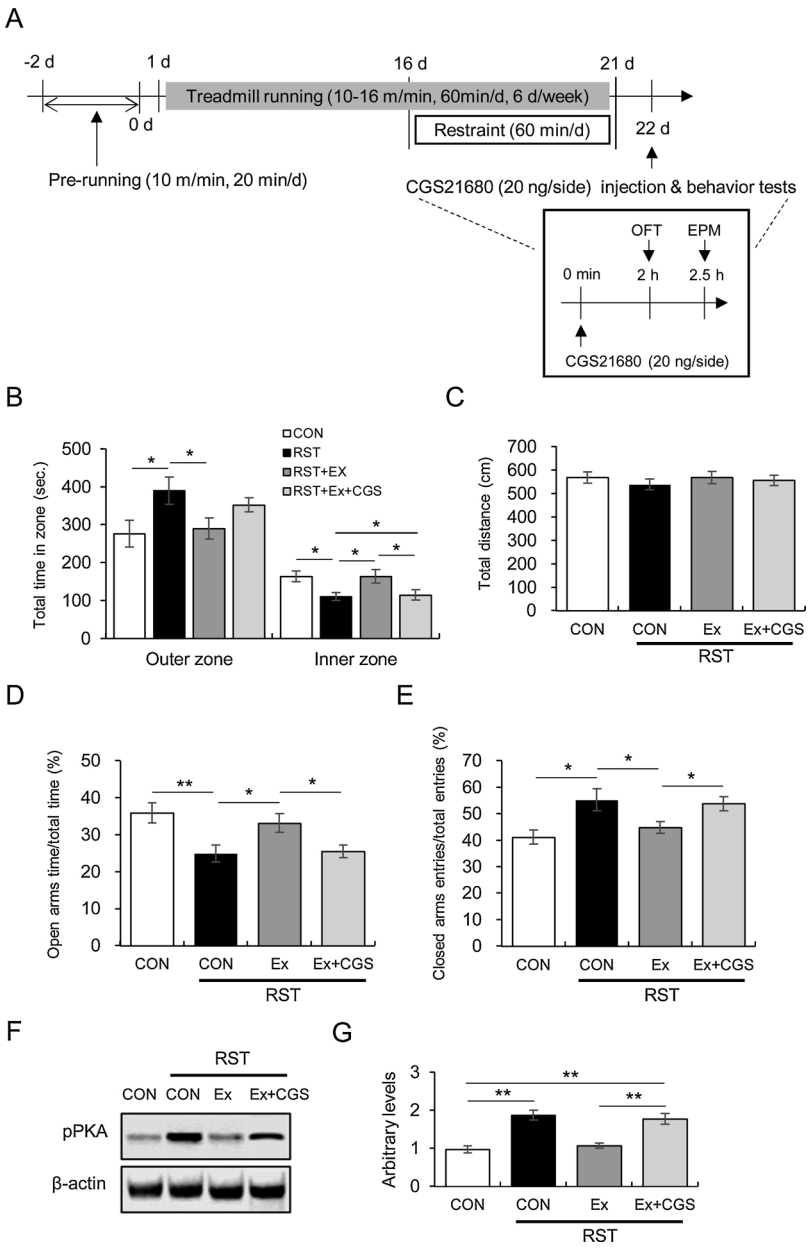

Fig. 5. The effects of CGS21680 on anxiolytic effect of exercise and basolateral amygdala synaptic p-PKA levels in stressed mice. (A) The experimental procedure. (B) Quantitative analysis of the time traveled in the inner zone ($F_{3,\ 28}$ = 4.21, $p < 0.01$) and the outer zone ($F_{3,\ 28}$ = 3.13, $p < 0.05$) in the open field test (OFT). (C) Quantitative analysis of the total distance traveled in the arena in the OFT ($F_{3,\ 28}$ = 0.34, $p > 0.05$). (D) Quantitative analysis of the time spent in the open arm in the EPM test ($F_{3,\ 28}$ = 5.49, $p < 0.01$). (E) Quantitative analysis of the time spent in the closed arm in the EPM test ($F_{3,\ 28}$ = 5.13, $p < 0.01$). (F) Representative images for western blot data. (G) Quantitative analysis of the ratio of p-PKA to PKA ($F_{3,\ 12}$ = 17.98, $p < 0.01$). Data are presented as the mean ± standard error of the mean. *$p < 0.05$, **$p < 0.01$.

$F_{3,\ 8}$ = 30.68, $p < 0.01$).

3.3. The 5-HT_{2A}R antagonist resulted in anxiogenic behavior and enhanced synaptosomal A_{2A}R and p-PKA levels in the BLA

To elucidate the anxiolytic effect of 5-HT_{2A}R signaling, mice were treated with MDL11930 (a selective 5-HT_{2A}R antagonist) and anxiety-like behaviors were evaluated (Fig. 3A). MDL11930 (0–1 mg/kg) injected intraperitoneally, increased the time spent in the outer zone and reduced the time in the inner zone in a dose-dependent manner in the OFTs (Fig. 3B). Total traveled distance did not differ significantly between the groups (Fig. 3C). MDL11930 also reduced the time spent in the open arms and increased the entrances into the closed arms in a dose-dependent fashion in the EPM tests (Figs. 3D and E). Furthermore, MDL11930 (0.5 mg/kg) treatment significantly enhanced synaptosomal A_{2A}R levels and PKA activity (Fig. 3F-H). The results suggest that 5-HT_{2A}R signaling is an upstream negative modulator of A_{2A}R levels and PKA activity.

3.4. The local activation of A_{2A}R in the BLA produced anxiogenic behavior and enhanced PKA activity in the BLA

To explore the role of A_{2A}R activation in anxiety-like behaviors, behaviors were measured after CGS21680, a selective A_{2A}R agonist, was micro-infused in the BLA (Fig. 4A). CGS21680 (0–40 ng/side) in the BLA enhanced the time traveled in the outer zone and reduced the time traveled in the inner zone in a dose-dependent manner in the OFTs (Fig. 4B). Total traveled distances between the groups did not differ significantly (Fig. 4C). In the EPM tests, CGS21680 also reduced the time spent in the open arms and increased the entrances into the closed arms in a dose-dependent fashion (Figs. 4D and E). Moreover, CGS21680 enhanced PKA activities in the BLA region in a dose-dependent fashion (Figs. 4F and G).

3.5. A_{2A}R inhibition in the BLA is required for the anxiolytic effect of exercise against repeated stress

To investigate whether A_{2A}R activity in the BLA is involved in the

anxiolytic effect of exercise, mice were administrated CGS21680 and behavior tests were performed (Fig. 5A). The exercise-elicited enhancement of the time traveled in the inner zone was abrogated by CGS21680 (20 ng/side), whereas time in the outer zone was not significantly altered by repeated stressful stimuli in the OFTs (Figs. 5B and C). In the EPM tests, the exercise-elicited increase in the time spent in the open arms and decrease in the time spent in the closed arms were also abolished by CGS21680 (Figs. 5D and E). Moreover, the exercise-mediated PKA activity reduction was reversed by CGS21680 in the BLA under stress (Figs. 5F and G). Thus, the data suggest that $A_{2A}R$ inhibition is crucial for the anxiolytic effects of exercise.

4. Discussion

This study demonstrated the anxiolytic effects of exercise against repeated restraint stress and its underlying mechanisms. Exercise improved the repeated restraint stress-induced anxiety-like symptoms as measured using the OF and EPM tests, and reversed the decreased $5HT_{2A}R$, the enhanced $A_{2A}R$, and the increased p-PKA levels in the BLA. In addition, using $5HT_{2A}R$ antagonist and $A_{2A}R$ agonist, these receptors were found to directly contribute to anxiety phenotype in normal mice. Finally, we confirmed that $5HT_{2A}R$-modulated inhibition of $A_{2A}R$ and PKA functions in the BLA was an important mechanism underlying the anxiolytic effect of exercise against repeated stress, as proved by the lack of the anxiolytic effect of exercise in the BLA that was locally administered with an $A_{2A}R$ agonist.

The amygdaloid complex, which comprises a battery of deep nuclei located within the temporal lobe innervates stress-response circuits (de Kloet et al., 2005; McEwen and Gianaros, 2010; Sah et al., 2003). Enhanced excitatory and plastic BLA subpopulation contributes to developing mood- and emotion-related diseases such as anxiety disorder (Hubert et al., 2014; Padival et al., 2013; Vyas et al., 2004; Yi et al., 2017). Aberrant serotoninergic transmission contributes to the ontogeny of emotional abnormalities, such as anxiety and panic. Several studies have described the modulatory role of 5-HT neurotransmission in anxiety-related disorders using the genetic manipulation of 5-HT signaling-linked genes, including $5HT_{1A}R^{-/-}$, $5HT_{2A}R^{-/-}$, and $SERT^{-/-}$ (Johnson, et al., 2019; Richardson-Jones et al., 2011; Weisstaub et al., 2006; Żmudzka et al., 2018). However, these studies are thought to be somewhat insufficient to fully elucidate the region-specific role for 5-HT receptor function, especially the BLA that is a key structure linked to the stress response. Here, we found that synaptic 5-$HT_{2A}R$ transmission in the BLA was crucial in anxiety symptoms in response to stress and exercise, proved by the exercise-elicited recovery of synaptic receptor levels in the repeatedly-stressed BLA and 5-$HT_{2A}R$ antagonist-induced anxiety-like behaviors in naïve mice. Although the systemic treatment with $5HT_{2A}R$ antagonist might affect diverse brain regions, $5H_{2A}R$ antagonist worked dominantly in BLA, thereby affecting anxiety-related behaviors and molecules expression because anxiety-related behaviors is dominantly controlled in BLA region. Furthermore, exercise alone enhanced synaptic 5-$HT_{2A}R$ levels relative to those of controls. These results suggest that exercise improves anxiety-related symptoms despite noxious stimuli by increasing 5-$HT_{2A}R$-mediated serotonergic input in the BLA. This interpretation has supporting evidence; for example, the anxiolytic effects of the 5-$HT_{2A}R$ agonist was abolished by pretreatment with a $GABA_A$ receptor antagonist (Donatti and Leite-Panissi, 2009). The 5-HT-induced increase in spontaneous inhibitory postsynaptic currents is suppressed by the 5-$HT_{2A}R$ antagonist in the BLA, alongside reduced 5-$HT_{2A}R$ expression in the BLA under stress (Jiang et al., 2009). Furthermore, reduced 5-$HT_{2A}R$ levels are involved in post-traumatic stress disorder (PTSD) (Mellman et al., 2009). Results from previous studies in combination with our data suggest that exercise may facilitate $5HT_{2A}R$-mediated GABAergic inhibitory input in the BLA, thereby exerting an anxiolytic effect against psychological stress.

Adenosine receptors modulate neuronal excitability, synaptic plasticity, and synaptic neurotransmission through inhibitory A_1 receptors (A_1Rs) and facilitatory A_{2A} receptors ($A_{2A}Rs$) in some limbic structures (Costenla et al., 2011; Cunha, 2008; Fredholmet al., 2005). Brain $A_{2A}R$ contributes to modulating anxiety-related behaviors. Caffeine treatment at a low dose has been shown to improve anxiety profile, but not at a high dose, depending on the rodent strain, test methodology, and the sex (Hughes et al., 2014; Sweeney et al., 2016). A recent study has described the anxiolytic effect of caffeine through blocking of the $A_{2A}R$ function of hippocampal glutamatergic neurons in chronically stressed mice (Kaster et al., 2015). On the contrary, mice globally lacking $A_{2A}R$ exhibit more anxiety-like characteristics than do their wild-type littermates (Ledent et al., 1997; López-Cruz et al., 2017). These studies provide considerable insight that $A_{2A}R$ signaling may elicit region-specific differential effects in anxiety-related behavior. Hence, the behavioral consequences may also be associated with $A_{2A}R$-mediated neuronal excitability and synaptic plasticity depending on the brain region involved, such as the hippocampus or the BLA.

The controlling role of $A_{2A}R$ in synaptic plasticity under noxious brain conditions such as chronic stress has been well-studied in the hippocampus. For example, chronic stress upregulates synaptic $A_{2A}R$ levels and antagonists such as caffeine, SCH58261, and KW6002 reverse the stress-induced decrease in LTP amplitude, alongside altered stress-associated behaviors (Costenla et al., 2011; Pagnussat et al., 2015; Rau et al., 2015). Thus $A_{2A}R$-mediated signal transduction is involved in synaptic plasticity in hippocampal excitatory synapses. In general, the synaptic plasticity-associated changes in the hippocampus appear to differ from that of the amygdala in response to psychological stress. Unlike the hippocampus, robust excitability of the amygdala both at rest and in neurological response to stress occurs in patients with PTSD and anxiety disorder (Agoglia and Herman, 2018; Padival et al., 2013). We showed that exercise recovered the repeated-stress-induced synaptic abundance of $A_{2A}R$, but not A_1R, in the BLA, and local $A_{2A}R$ activation of the BLA subpopulation evoked anxiety-like behaviors. Several studies have described that $A_{2A}Rs$ regulate excitability and the long-term plastic processes of BLA glutamatergic synapses in response to stimuli with strong emotional valence. For example, synaptic $A_{2A}R$ levels in the lateral amygdala were enhanced in fear conditioned mice, in which global treatment with SCH58261, a selective $A_{2A}R$ antagonist, and local $A_{2A}R$ knockdown within the BLA reduced LTP amplitude triggered by a high-frequency stimulation train, but not DPCPX, an A_1R antagonist (Simões et al., 2016). Furthermore, AHP via the calcium-gated potassium channel contributed to intrinsic neuronal excitability, and its suppression enhanced neuronal firing (Bond et al., 2004; Power et al., 2011). $A_{2A}R$ activation enhanced intrinsic excitability by reducing sAHP, as indicated by voltage response reduction by several microseconds induced by CGS21680, a selective $A_{2A}R$ agonist, after cessation of hyper- and depolarizing current steps (Rau et al., 2015). The previous study indicated that $A_{2A}R$ differentially modulated the neuronal excitability and synaptic plasticity between the hippocampus and the BLA, thereby dissimilarly affecting anxiety-related phenotype. These previous findings in combination with our results suggest that an exercise regimen may alleviate the synaptic $A_{2A}R$-modulated excessiveness of plastic processes and hyperexcitability of BLA principal neurons in response to anxiety-associated cues.

The activation of $A_{2A}R$, which is a G protein-coupled receptor liberating the G_s protein, leads to cAMP-mediated PKA signaling (Abbracchio et al., 2009; Faber and Sah, 2002). Here, we showed that $A_{2A}R$-mediated adenosine signaling activates PKA activity. Furthermore, we previously described the critical role of PKA activity in synaptic plasticity in the BLA under chronic stress (Yi et al., 2017). This cellular episode is likely to play a crucial role in BLA activity. For example, CGS21680-induced increase in intrinsic excitability was blocked by PKA inhibitor treatment in BLA neurons (Rau et al., 2015). Notably, the exercise-regimen-elicited anxiolytic effects and decrease in PKA activity was abrogated by the $A_{2A}R$ agonist in the BLA population. This cellular alteration of the BLA may be required for anxiolytic effects of exercise against an aberrant psychiatric status. Moreover, we showed

that A_{2A}R-mediated PKA activation was modulated by $5H_{2A}$R activity within the BLA subpopulation in this study using a selective $5H_{2A}$R antagonist, suggesting that A_{2A}R function is dependent on $5H_{2A}$R signal transduction in the BLA subpopulation. Thus, exercise regimen suppressed the A_{2A}R- mediated PKA activation of BLA neurons by enhancing 5-HT_{2A}R-mediated serotonergic activity under stressful conditions.

5. Conclusion

The current study demonstrated that the regular exercise has an anxiolytic effect through $5HT_{2A}$ receptor-mediated facilitation of serotonergic activity in BLA region. This serotonergic input suppressed A_{2A}R-mediated PKA activity in BLA subpopulation, which may be a potential mechanism underlying the anxiolytic effect of regular exercise. Therefore, regular exercise can effectively manage and alleviate psychopathological manifestations such as anxiety disorder under repeated aversive stimuli.

Author contributions

Y. H. Leem and H. S. Kim designed the experiments and wrote the paper; Y. H. Leem, J. H. Jang, and J. S Park performed the experiments and analyzed the data.

Conflicts of interest

There are no conflicts of interest to declare.

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