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Full Paper

Role of neurosteroid allopregnanolone on age-related differences in exercise-induced hypoalgesia in rats

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ABSTRACT

The beneficial effects of physical activity for pain are denominated exercise-induced hypoalgesia (EIH). Here, we examined the age-related change and potential role of the neurosteroid allopregnanolone (ALLO) on EIH in rats. Adult and aged rats were randomly divided into one of three groups; non-exercise control, Low-exercise, and High-exercise. The animals in the Low- and High-exercise groups were subjected to a 10-minute treadmill workout at 40% and 80% maximum oxygen intake intensity, respectively. In the Low-exercise groups, a significant EIH response was observed in aged but not in adult rats. The pre-treatment with ALLO synthesis inhibitor finasteride, but not opioid-receptor antagonist naloxone, inhibited the Low-exercise induced EIH response in aged rats. Furthermore, the Low-exercise increased brain ALLO levels in aged animals compared with controls, which was correlated with the mechanical pain sensitivity. On the other hand, High-exercise could induce EIH response in both adult and aged animals, but it was more effective in adult rats. The pre-treatment with naloxone, but not finasteride, reduced the EIH observed after High-exercise in both adult and aged rats. Our findings demonstrated that effective EIH can be achieved even by mild-intensity exercise in aged animals *via* an increase of the brain ALLO levels.

1. Introduction

Evidence suggests that physical exercise is an effective non-pharmacological pain intervention.[1,2] This endogenous pain modulation is currently termed as exercise-induced hypoalgesia (EIH). Taking into account the relatively safe and effective profile, EIH may be a more suitable alternative for geriatric pain management. However, a recent study in healthy older and younger adults revealed that EIH can be induced in both, but its effectiveness was lower in older adults.[3] Although preclinical studies indicated that multiple pain-processing pathways in EIH development, including an endogenous opioid system,[4–6] the age-specific mechanisms remain largely under-investigated.

Neurosteroids, which are synthesized within the central nervous system (CNS), influence the excitability of the CNS *via* non-genomic mechanisms.[7] Among the neurosteroids, allopregnanolone (ALLO), a metabolite from progesterone (PROG), is the most potent endogenous activator of γ-aminobutyric acid (GABA) transmission, and hence, has anti-stress, neuroprotective, and analgesic properties.[8–10] Previous studies found that the ALLO concentration in the brain declines with age and due to neurodegenerative diseases.[9] Based on these earlier findings, we hypothesized that the underlying mechanisms of the age-related EIH difference involve changes in ALLO biosynthesis after exercise.

To address our hypotheses, we compared the pain sensitivity and the brain ALLO levels after exercise between adult and aged rats.

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2. Materials and methods

2.1. Animals

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Kochi Medical School. Adult (2–3 months old; 160–230 g body weight) and aged (19–24 months old; 540–680 g) male Wistar rats were used. All rats were housed with a standard 12-h dark–light cycle, and access to food and water *ad libitum*.

2.2. Maximal exercise test and experimental designs

Twenty-four hours after their last familiarization, The % maximum oxygen consumption ($VO_{2\ max}$) for all rats was individually measured using the maximum exercise test as previously described. [11] Each rat ran on a treadmill in the metabolic cage at a low initial speed with a 0-degree incline. Then, the speed was increased at a rate of 3.0 m/min every 2 min. The $VO_{2\ max}$ was determined when oxygen consumption reached a plateau and was not affected by increasing the workload.

After the maximum exercise tests, the animals of each age were randomly assigned to three groups according to the intensity of exercise: 1) the non-exercise control group; 2) the low-intensity exercise (Low-exercise) group; and 3) the high-intensity exercise (High-exercise) group. The animals in the Low- and High-exercise groups were subjected to a 10-min treadmill workout at a speed corresponding to approximately 40% and 80% $VO_{2\ max}$, respectively. The rats in the control groups were only manipulated and placed on the treadmill without movement during the identical time periods. The animals were further divided into three sets of experiments. Experiment-1 was conducted to assess the effects of the exercise procedure on pain-related behaviors. In Experiment-2, ALLO and opioid-related antagonist experiments were performed. Experiment-3 was designed to analyze the effects of the exercise protocols on neurosteroid levels, 5α-reductase activity, and peripheral stress responses.

2.3. Measurement of exercise-induced analgesia

Following an exercise regimen, animals were placed in Plexiglas boxes (30 × 28 × 28 cm) with a wire grid floor on an elevated platform, and allowed to rest and acclimatize for 10 min prior to examination. The reflex-based withdrawal and pain-related learned behaviors were then assessed as a measure of pain sensitivity according to previous studies, [4,12] with some modifications. A cross-over within-subject counterbalance design was applied with 2 days of rest between the sensory tests.

1) Reflex-based withdrawal behaviors: A train of 20 mechanical stimuli with a 60-g von Frey filament was applied alternately on the left and right hind paws at the rate of approximately 0.2 Hz. The withdrawal response was scored either as none or positive if the paw was removed. The degree of pain sensitivity was expressed as the percentage of positive responses. The responses to mechanical stimuli were evaluated just before exercise (baseline), and at 10, 20, 30, 40, 50, and 60 min post-exercise. The 60-g mechanical stimulation was sufficient to elicit a reflex response in both adult and aged rats, *i.e.*, the average paw withdrawal threshold in adult and aged rats was 23.7 ± 2.1 g and 25.8 ± 2.9 g, respectively, in our preliminary experiment.

2) Pain-related learned behaviors: An aversive stimulus provides reinforcement for avoidance learning in animals, and thus the passive avoidance paradigm has been used as a non-reflex pain measurement of the motivational component. During testing, the rat was placed on a platform located in same place as the sensory equipment for mechanical withdrawal testing. The latency to step down from the platform was measured (cutoff 150 s). Immediately after stepping down from the platform, the rat received 3 mechanical stimuli with a 60-g von Frey filament on the hind paw at each trial. This procedure was repeated for each rat for 9 learning trials at 5 min intervals.

2.4. Drugs

To assess the involvement of endogenous ALLO and opioid in the EIH, finasteride (Sigma–Aldrich, St. Louis, MO; 50 mg/kg), an inhibitor of the main rate-limiting enzyme 5α-reductase in ALLO synthesis, and naloxone (Sigma–Aldrich; 10 mg/kg), an opioid-receptor antagonist, were used as the pharmacological tools in Experiment-2. Both drugs were injected intraperitoneally (*i.p.*) 30 min prior to exercise. Finasteride was suspended in 5% Tween 80 (Sigma–Aldrich) and diluted with 0.9% saline. The finasteride dose was selected based on our pilot experiment (Supporting data 1). Naloxone was dissolved in 0.9% saline solution, and saline solution alone was administered as a control.

2.5. Sample preparation for analysis of neuroactive steroids and 5α-reductase activity

To assess the effects of exercise on the brain levels of the neuroactive steroids, the animals were euthanized under deep isoflurane (4–5%) anesthesia 20 min after exercise or the control procedure. The reflex-based withdrawal behaviors, as mentioned above, were also tested just prior to anesthesia. The entire brain and lumbar spinal cord (L4–L6) were rapidly removed, and stripped of meninges. Each sample was frozen on dry ice and stored at −80 °C until analysis. Trunk blood was collected into heparinized tubes, and centrifuged at 3000 rpm for 20 min at 4 °C to separate plasma and kept frozen until assaying. The plasma samples were also used to assess the stress response to exercise intervention. The levels of PROG and ALLO, as well as 5α-reductase activity, were analyzed by chromatography-mass spectrometry (GC–MS) analyses as described in detail in Supporting data 2. Plasma levels of corticosterone and norepinephrine were measured by enzyme-linked immunosorbent assays (ELISA) using the corticosterone EIA Kit from Enzo Life Sciences (NY, USA) and the norepinephrine ELISA kit from MyBiosource (San Diego, California), respectively.

2.6. Statistical analysis

All data were expressed as the mean ± standard deviation (SD). For each dependent variable, group and/or other main effect(s) were tested by repeated measures ANOVA. Whenever ANOVA demonstrated significance of a main effect, post hoc comparisons between the groups were performed in a pairwise manner by the Wilcoxon–Mann–Whitney test with Bonferroni correction. All data were analyzed using SPSS (versions 11; SPSS Inc, Chicago, IL). $p < 0.05$ was considered significant.

3. Results

All animals tolerated the testing procedure without mortality or apparent clinical signs. To assess whether post-exercise muscle fatigue influenced subsequent behavioral outcomes, additional satellite rats (n = 5 in each age group) were evaluated for muscle performance using a standard grip strength task. For both adult and aged rats, there were no significant differences in the grip strength when baseline and post-exercise were compared (Supporting data 3).

3.1. Reflex-based withdrawal behaviors after exercise

The withdrawal frequencies at the pre-exercise baseline were 70% or higher among all rats in the experiments, and there were no significant differences between groups. Without exercise, both adult and aged control rats exhibited no change in the withdrawal frequencies throughout the observation period. In adult animals, Low-exercise did not cause any analgesic effects. In contrast, in aged rats, the withdrawal frequencies significantly decreased at 10–30 min after Low-exercise compared with those in the control group. On the other hand, the withdrawal frequencies significantly decreased after High-exercise compared with those in the control group at 10–20 min in the adult group and at 20 min in the aged group. However, these High-exercise-induced analgesic effects at 20 min after exercise were more pronounced in the adult group (33.1 ± 15.7%) than in the aged group (49.7 ± 10.4%; $p < 0.05$) (see Fig. 1).

3.2. Pain-related learned behaviors after exercise

Without mechanical stimulation, the satellite rats exhibited no change in step-down latency across trials in both adult and aged groups (n = 5 in each, data not shown). With needle stimulation, one-way ANOVA with repeated measures revealed that both adult ($p < 0.05$, Fig. 2A) and aged ($p < 0.05$, Fig. 2B) animals in the non-exercise control group developed prolonged latency during the trial sequence. In adult rats, there was no difference in latency across trials between control and Low-exercise groups ($p = 0.73$), whereas the prolongation in the High-exercise group (24.8 ± 10.2 s at the 9th trial) was significantly shorter than that in the control group (42.0 ± 8.4 s; $p < 0.05$, Fig. 2A). In contrast, in aged rats, latency across trials was not significantly different between the control and High-exercise groups ($p = 0.75$), whereas the prolongation in the Low-exercise group (17.5 ± 8.3 s at the 9th trial) was significantly shorter than that in the control group (41.5 ± 7.1 s; $p < 0.05$, Fig. 2B).

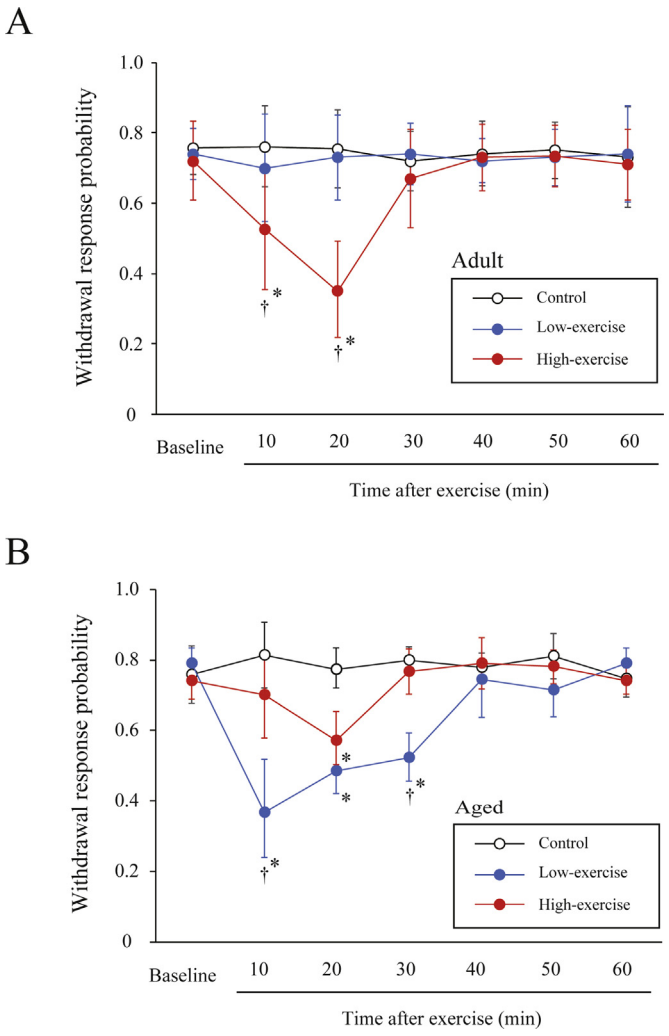

Fig. 1. Time course of the mechanical pain behavior after non-exercise control, Low-exercise, or High-exercise intervention. The paw withdrawal frequencies from 20 trials with a 60-g von Frey filament were assessed in adult (**A**) and aged (**B**) rats. The sensory tests were performed before intervention (baseline), and at 10, 20, 30, 40, 50, and 60 min after intervention. Each vertical bar represents the mean ± SD (n = 8 in each group). *$p < 0.05$ *vs.* baseline, †$p < 0.05$ *vs.* corresponding time points in the control group.

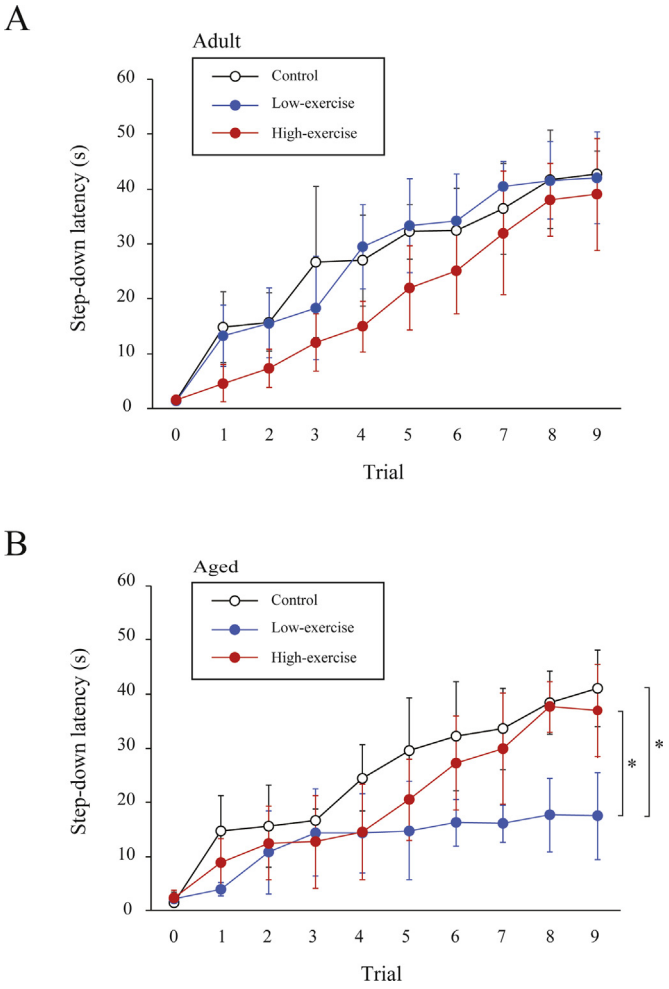

Fig. 2. Step-down latency following mechanical stimulation after non-exercise control, Low-exercise, or High-exercise intervention. Adult (**A**) and aged (**B**) rats received 3 mechanical stimuli with a 60-g von Frey filament immediately after stepping down from a platform during 9 consecutive trials at 5 min intervals. Each vertical bar represents the mean ± SD (n = 8 in each group). *$p < 0.05$, difference between groups at the 9th trial.

3.3. Effects of naloxone and finasteride on EIH

There was no significant difference in the paw withdrawal frequencies to mechanical stimulation between adult or aged non-exercise control rats receiving naloxone or finasteride, or vehicle alone (Supporting data 4). In adult rats with Low-exercise, neither naloxone nor finasteride influenced the withdrawal frequencies compared with the control (Fig. 3A). However, naloxone, but not finasteride, exerted significant alleviative effects on the High-exercise-induced hypoalgesia (H-EIH) observed 10 min after exercise in adult rats (Fig. 3B). In aged rats, the pre-treatment with finasteride significantly inhibited the Low-exercise-induced hypoalgesia (L-EIH; Fig. 4A, $p < 0.05$), whereas it failed to exert significant analgesic effects on H-EIH (Fig. 4B, $p = 0.44$). On the other hand, the H-EIH, but not L-EIH, response was significantly inhibited by pre-administration of naloxone ($p < 0.05$ for H-EIH, $p = 0.87$ for L-EIH).

3.4. Levels of the two endogenous neurosteroids PROG and ALLO after exercise

The levels of PROG and its metabolite ALLO in the brain, spinal cord, and plasma were measured 20 min after either control, Low-, or High-exercise intervention. As shown in Fig. 5A, among the adult rats, there was no difference in brain PROG levels between the groups ($p = 0.76$), whereas the brain ALLO levels in the High-, but not in the Low-, exercise group was significantly higher than that in the control group ($p < 0.05$ for High, $p = 0.35$ for Low). In the aged control group, brain levels of PROG and ALLO were significantly lower by 28.5% and 15.3%, respectively, compared with those in adult controls. Among the aged rats, although there were no significant group differences in brain PROG levels ($p = 0.26$), significant differences were found in brain ALLO levels ($p < 0.05$). Specifically, post-hoc comparisons revealed a significant increase in ALLO brain levels in the Low-exercise group of more than 3.5-fold compared with the control ($p < 0.05$). However, such an increase in the brain ALLO level was not found in aged rats in the High-exercise group ($p = 0.47$). On the other hand, neither a within-group difference nor a between-age group difference was detected in the spinal cord (Fig. 5B) or plasma (Fig. 5C) levels of PROG or ALLO. Taking all experimental groups within each age group together, the withdrawal frequencies to mechanical stimulation were negatively correlated with the brain levels of ALLO in aged animals ($R^2 = -0.41$, $p < 0.05$), but were not correlated in adult rats ($R^2 = -0.08$, $p = 0.64$, Supporting data 5). In addition, the pre-

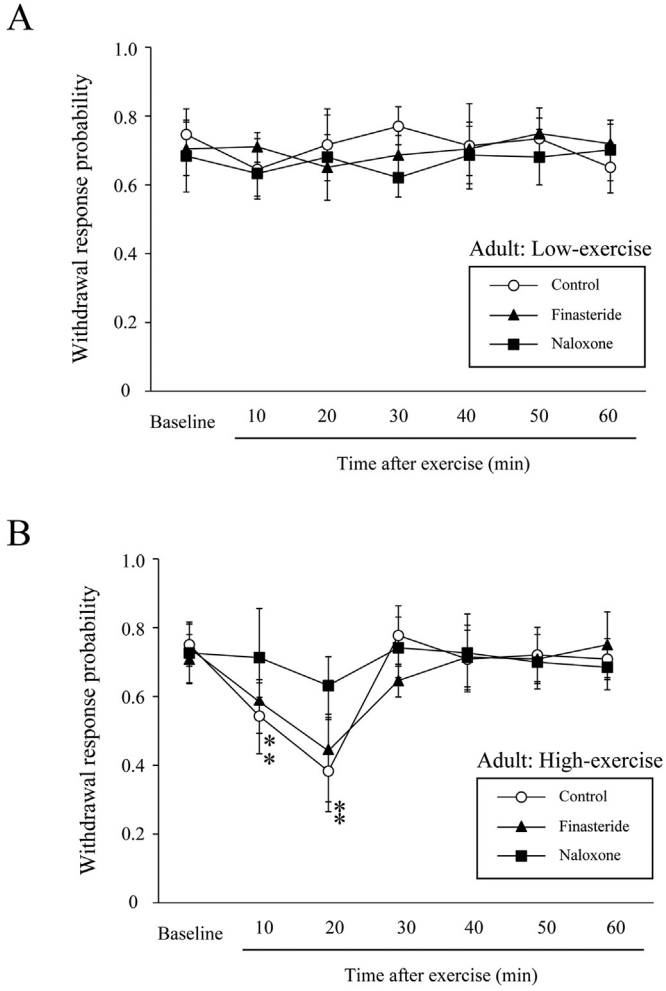

Fig. 3. Effects of antagonists on the mechanical pain behaviors in adult rats. An inhibitor of the main rate-limiting enzyme of allopregnanolone synthesis, finasteride, an opioid-receptor antagonist, naloxone, or vehicle was pre-treated 30 min before Low-exercise (**A**) or High-exercise (**B**) intervention. Each vertical bar represents the mean ± SD (n = 8 in each group). *$p < 0.05$ *vs.* baseline.

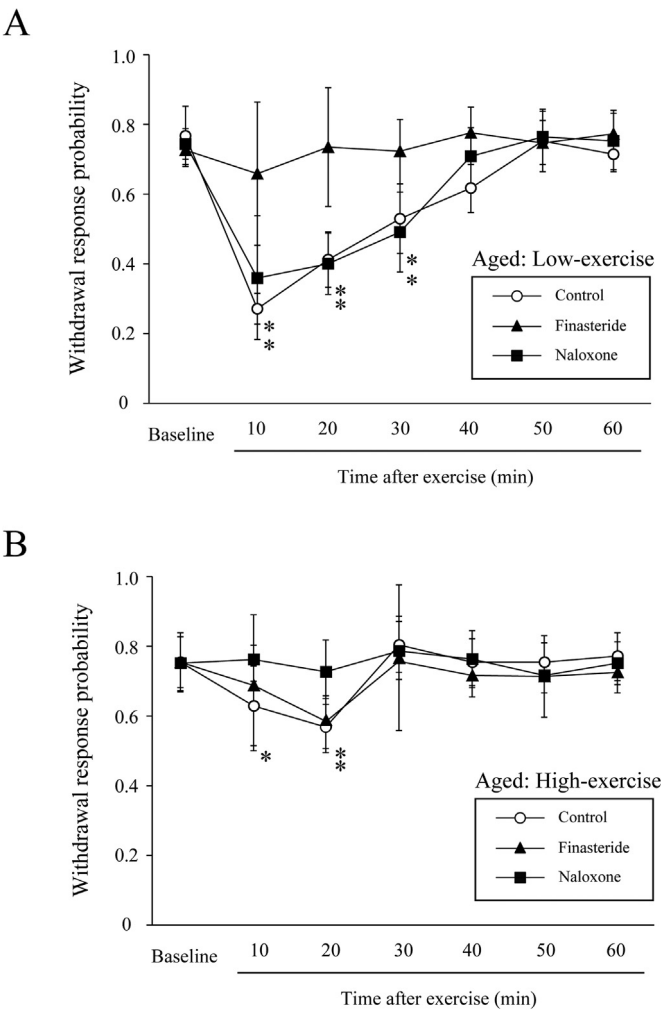

Fig. 4. Effects of antagonists on the mechanical pain behaviors in aged rats. An inhibitor of the main rate-limiting enzyme in allopregnanolone synthesis, finasteride, an opioid-receptor antagonist, naloxone, or vehicle was pre-treated 30 min before Low-exercise (**A**) or High-exercise (**B**) intervention. Each vertical bar represents the mean ± SD (n = 8 in each group). *$p < 0.05$ *vs.* baseline.

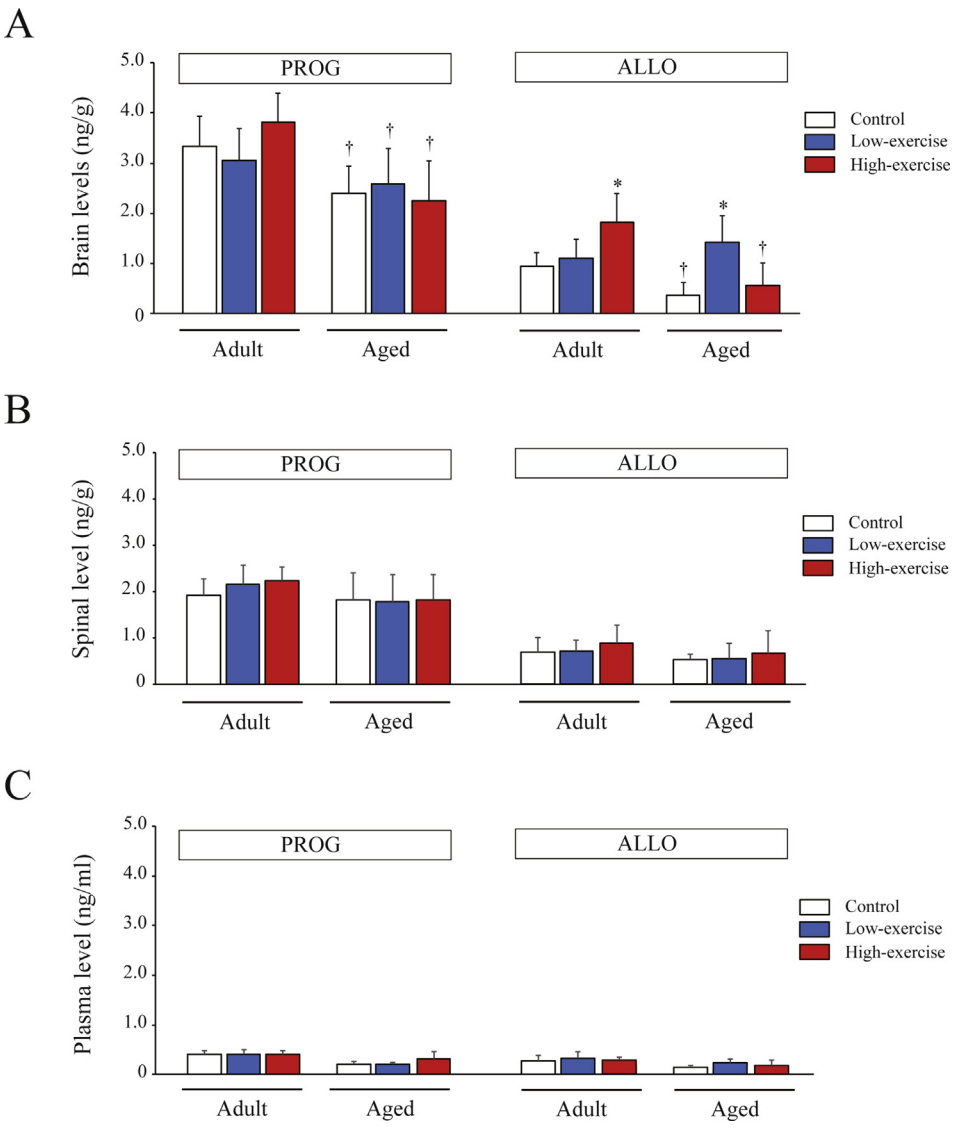

Fig. 5. Changes in levels of neurosteroids after non-exercise control, Low-exercise or High-exercise intervention. The levels of progesterone (PROG) and allopregnanolone (ALLO) in the brain (**A**), spinal cord (**B**), and plasma (**C**) in both adult and aged rats are shown. Each vertical bar represents the mean ± SD (n = 8 in each group). *$p < 0.05$ *vs.* control, †$p < 0.05$ *vs.* corresponding group of adult rats.

treatment with exogenous ALLO (10 mg/kg, *i.m*) significantly prolonged the duration of L-EIH compared with that in the vehicle-treated Low-exercise group, *i.e.*, the withdrawal frequencies significantly decreased at 10–50 min after Low-exercise (Supporting data 6).

The 5α-reductase activity was not significantly different between groups in adult rats (Fig. 6, $p = 0.18$). The activity in the aged control group was lower than that in the adult control group ($p < 0.05$), and significant differences were found between groups of aged rats ($p < 0.05$), *i.e.*, the 5α-reductase activity in the Low-exercise group ($p < 0.05$), but not the High-exercise group ($p = 0.21$), was significantly higher than that in the controls. Furthermore, plasma corticosterone and noradrenaline levels in both adult and aged animals in the Low-exercise group were comparable (Table 1, $p > 0.05$ in each pair-wise comparison), whereas those in the High-exercise group were significantly higher than those in the control group ($p < 0.05$ in each pair-wise comparison).

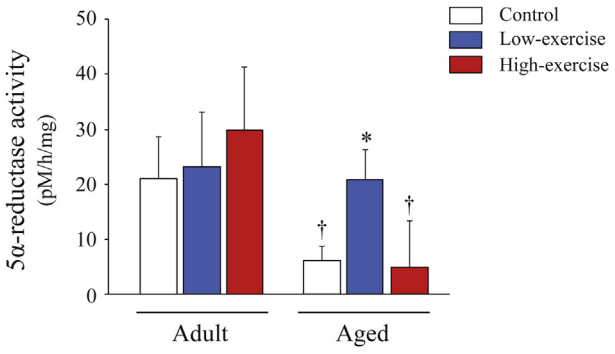

Fig. 6. Changes in brain 5α-reductase activity after non-exercise control, Low-exercise or High-exercise intervention. The activity of 5α-reductase in both adult and aged rats is shown. Each vertical bar represents the mean ± SD (n = 8 in each group). *$p < 0.05$ *vs.* control, †$p < 0.05$ *vs.* corresponding group of adult rats.

Table 1
Plasma levels of corticosterone and norepinephrine 20 min after exercise.

	Corticosterone (ng/ml)	Norepinephrine (ng/ml)
Adult		
Control	43.0 ± 13.8	39.4 ± 14.7
Low-exercise	37.3 ± 16.7	41.8 ± 23.3
High-exercise	86.5 ± 34.8 *	80.3 ± 43.0 *
Aged		
Control	2.86 ± 1.77	2.64 ± 1.70
Low-exercise	2.71 ± 1.60	2.48 ± 0.89
High-exercise	5.41 ± 1.90 *	43.0 ± 3.88 *

*$p < 0.05$ *vs.* control.

4. Discussion

In the present study, we demonstrated the age-dependent EIH responses following a single bout of aerobic exercise in rats. It should be noted that these endogenous analgesic effects were dependent on the exercise intensity. Specifically, Low-exercise induced subsequent analgesic effects in aged rats but not in adult rats. In contrast, High-exercise led to EIH in both adult and aged animals, whereas more potent effects were observed in adult rats.

Our findings further revealed that L-EIH observed in aged animals may be associated with the increase in the brain ALLO level after exercise. Endogenous ALLO concentrations in the brain were inversely correlated with the number of withdrawal responses to mechanical stimulation in aged animals (Supporting data 5). Unlike for aged animals, Low-exercise had no effect on endogenous brain ALLO concentrations in adult animals (Fig. 5A). These findings suggest that the rapid increase in *de novo* synthesized ALLO in the brain is a causal mechanism for age-related L-EIH. On the other hand, the H-EIH responses in both age groups may be mediated mainly *via* the endogenous opioid system rather than the changes in brain ALLO levels.

Consistent with previous reports,[9] brain ALLO levels in aged controls were lower than those in adult rats (Fig. 5A). The present study also revealed that brain ALLO levels in aged animals markedly increased after Low-exercise intervention, becoming comparable to those in the adult Low-exercise group. On the other hand, PROG, another neurosteroid, was not influenced by exercise intervention. PROG serves as a precursor for biosynthesis of ALLO within the brain, being converted by the rate-limiting enzyme 5α-reductase to 5α-DHP, after which 3α-hydroxysteroid dehydrogenase catalyzes the conversion of 5α-DHP to ALLO.[7–10] We found that 5α-reductase activity was lower in aged brains than in adult brains (Fig. 6), suggesting that aging inhibits the reactions converting PROG into ALLO. However, the age-related impaired 5α-reductase activity and low ALLO levels in aged brains was significantly reversed by Low-exercise. In addition, pre-treatment with finasteride, a 5α-reductase inhibitor, attenuated the L-EIH response in aged rats (Fig. 4A). These findings imply that brain 5α-reductase activity is a modifiable key determinant of age-related differences in ALLO-associated brain functions.

In addition to being synthesized in the brain, ALLO can be produced at the periphery, *i.e.*, by gonads and adrenal glands.[9,10] However, our analyses indicated that plasma levels of ALLO did not increase at 20 min after Low-exercise (Fig. 5C), the time point that was observed for L-EIH. In agreement with previous animal studies, the brain levels of ALLO increased in response to acute stress even in adrenalectomized and gonadectomized animals. [13] These findings indicate that the increased brain ALLO after Low-exercise originate *de novo* within the CNS. In contrast, serum ALLO levels were reported to be inversely correlated with self-reported pain symptoms [14] However, these studies assessed chronic pain conditions. One plausible explanation for this discrepancy is that the peripheral ALLO levels may have a delayed window to reflect the brain levels. Therefore, serum ALLO concentrations may be inappropriate as a marker of acute EIH response.

Exercise not only benefits physical health, but it is also a well-known stressor that stimulates the hypothalamus-pituitary-adrenal (HPA) axis in an intensity-dependent manner. [15] In humans, acute exercise stimulates the HPA axis after a duration of 10 min or more at approximately 60% of the VO_2 max or greater. [16] In fact, stress responses were induced following a bout of High-exercise, but not Low-exercise, comparably in adult and aged animals (Table 1). It has been reported that acute exercise increases the peripheral levels of endogenous opioids, corresponding to changes in HPA axis hormones. [16] Consistent with this, the findings from previous studies, as well as our results from the High-exercise groups, demonstrate the link between EIH and stress-induced activation of the endogenous opioid system; *i.e.*, naloxone sensitive. This may also explain why endogenous opioid system was not involved in Low-EIH in aged animals; *i.e.*, naloxone insensitive. Moreover, the function of the CNS opioid system has been reported to decrease with aging. [17] These age-related changes may underlie the H-EIH efficiency difference between adult and aged animals.

In addition to the endogenous opioid system, ALLO is released in the brain in response to stress and regulates HPA axis function. [18] Therefore, brain ALLO levels in adult rats after High-exercise increased likely due to the stress response, which may be independent of brain 5α-reductase activity. However, the H-EIH response observed in adult animals was not influenced by finasteride (Fig. 3B), suggesting a lack of ALLO involvement. Since brain ALLO levels in adult rats is relatively high even in non-exercise control condition, increases in ALLO levels after High-exercise may fail to induce additional analgesic effects. Furthermore, it is possible that endogenous analgesic processes mediated by the opioid system are more potent than the ALLO-associated mechanism. On the contrary, in aged rats, brain ALLO concentrations were not influenced by High-exercise, suggesting that aging leads to a decline in neurosteroid responses to acute stressors.

Of note, our results in aged animals demonstrated that both the analgesic effects and duration of L-EIH were superior to those of H-EIH. Specifically, Low-exercise, *e.g.*, walking or gardening, is safe and tolerable for elderly people. In addition, we found that the systemic administration of ALLO could prolong the duration of L-EIH in aged rats (Supporting data 6), representing a novel pharmacological strategy for treating the geriatric pain syndrome. However, in order to address the detailed role of ALLO in central pain processing, the analgesic effect of ALLO administered intracerebroventricularly should be examined. The underlying mechanisms of the age-dependent stimulating effect of exercise on brain 5α-reductase activity has yet to be elucidated. Furthermore, a number of important translational questions remain such as what are the best types of exercise, frequency, and gender differences for the elderly EIH phenomenon. Therefore, future studies are needed before our findings can be translated into clinical practice.

5. Conclusion

We demonstrated that effective EIH can be achieved even by Low-exercise in aged, but not adult, animals. Our observations further indicated that the rapid restoration of age-related brain ALLO depletion may contribute to the development of L-EIH. Our findings suggest that brain ALLO represents an intrinsic and modifiable target for EIH, especially in elderly patients.

Conflicts of interest

All authors have no financial or scientific conflict of interest regarding the research described in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2018.11.009.

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