

Mild exercise increases dihydrotestosterone in hippocampus providing evidence for androgenic mediation of neurogenesis

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Contributed by Bruce S. McEwen, June 20, 2012 (sent for review December 10, 2011)

Mild exercise activates hippocampal neurons through the glutamatergic pathway and also promotes adult hippocampal neurogenesis (AHN). We hypothesized that such exercise could enhance local androgen synthesis and cause AHN because hippocampal steroid synthesis is facilitated by activated neurons via *N*-methyl-*D*-aspartate receptors. Here we addressed this question using a mild-intense treadmill running model that has been shown to be a potent AHN stimulator. A mass-spectrometric analysis demonstrated that hippocampal dihydrotestosterone increased significantly, whereas testosterone levels did not increase significantly after 2 wk of treadmill running in both orchidectomized (ORX) and sham castrated (Sham) male rats. Furthermore, analysis of mRNA expression for the two isoforms of 5 α -reductases (*srd5a1*, *srd5a2*) and for androgen receptor (AR) revealed that both increased in the hippocampus after exercise, even in ORX rats. All rats were injected twice with 5'-bromo-2'-deoxyuridine (50 mg/kg body weight, i.p.) on the day before training. Mild exercise significantly increased AHN in both ORX and Sham rats. Moreover, the increase of doublecortin or 5'-bromo-2'-deoxyuridine/NeuN-positive cells in ORX rats was blocked by s.c. flutamide, an AR antagonist. It was also found that application of an estrogen receptor antagonist, tamoxifen, did not suppress exercise-induced AHN. These results support the hypothesis that, in male animals, mild exercise enhances hippocampal synthesis of dihydrotestosterone and increases AHN via androgenic mediation.

neurosteroid | exercise-induced neurogenesis | low intensity exercise | de novo synthesis | paracrine

Gonadal hormones enhance neuronal plasticity, including adult hippocampal neurogenesis (AHN), which occurs in the dentate gyrus throughout life in mammals (1, 2)—as well as having neuroprotective effects (3–6). In particular, the hippocampus is known to be a target of androgen actions (5, 7, 8). Testosterone (T) and dihydrotestosterone (DHT) exert neuroprotective effects through androgen receptors (ARs) in the hippocampus (5, 7, 9), although T can also be converted to estradiol (E2) via aromatase in the brain (10, 11). Intriguingly, current studies revealed that androgens can be synthesized in the hippocampus (10, 12, 13), in contrast to the widespread notion that the testes provide androgens to the brain via the circulatory system. The possibility of local androgen synthesis should be supported by the fact that immunoreactivity for both 5 α -reductase 1 and 2 is observed in the dentate granule cell layer of the hippocampus (14). If locally produced by a physiological stimulus such as exercise, then formation of DHT, with its high affinity for ARs, could have beneficial effects on neuronal plasticity, including AHN.

Indeed, we have demonstrated that hippocampal steroid synthesis is facilitated via *N*-methyl-*D*-aspartate receptors (15). Furthermore, mild exercise increases regional cerebral blood flow, an index of neuronal activity, through the glutamatergic pathway, and continuous mild exercise enhances AHN (16, 17). These results led

us postulate that exercise would enhance androgen synthesis, which may in turn mediate exercise-induced AHN, even though the possible role of androgens in hippocampus plasticity has not been extensively studied, particularly in relation to the effects of exercise.

The mechanism of exercise-induced AHN is still unknown, although some of the beneficial effects of exercise on cognition and mood may be mediated by exercised-induced AHN (18). The participation of several trophic factors such as brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) has been proposed (18), and their possible androgenic mediation has been reported in adult canaries (19). The dentate gyrus, which expresses ARs (20, 21), is also a target of androgens, and one study has shown that androgens enhance AHN via increased cell survival in an androgen-dependent manner (9). Taking these findings into consideration, androgens may play a significant role in the regulation of exercise-induced AHN.

Furthermore, regarding exercise conditions that activate AHN, much research has demonstrated that both wheel (22) and treadmill (23, 24) running enhance AHN (*SI Text, Mild-Intense Treadmill Running Model and Exercise-induced AHN*). We used treadmill running, which can be controlled for speed, and developed a mild-intensity treadmill exercise model below the lactate threshold (LT, about 20 m/min) with less blood lactate accumulation and minimal stress response; the LT is a physiologic index of moderate exercise intensity established in rats (25, 26) as in humans (27). We have also shown that hippocampal neuronal activity and neurogenesis is induced by the mild-intensity exercise (16, 17, 28).

Here we address the possibility that hippocampal androgen formation is increased by mild exercise under the LT, which may, in turn, mediate the exercise-induced AHN through ARs. For this purpose, we applied derivatization methods to improve the sensitivity of liquid chromatography with tandem mass spectrometry (LC-MS/MS) (12). We confirmed that mild exercise training for 2 wk below the LT enhances AHN whereas AHN is not enhanced above the LT (*SI Text, Mild-Intense Treadmill Running Model and Exercise-induced AHN* and Fig. S1). We thus examined the effects of 2 wk of mild exercise on androgen levels in hippocampus and AHN in male rats.

Results

Experiment I: Levels of DHT, T, and E2 in the Hippocampus and Plasma After Exercise. On the basis of the LT, the rats were subjected to mild treadmill exercise at a running speed of 13.5 m/min, five

Author contributions: M.O., B.S.M., and H.S. designed research; M.O., K.I., and T.M. performed research; Y.H. and S.K. contributed new reagents/analytic tools; M.O., Y.H., S.K., B.S.M., and H.S. analyzed data; and M.O., B.S.M., and H.S. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1210023109/-DCSupplemental.

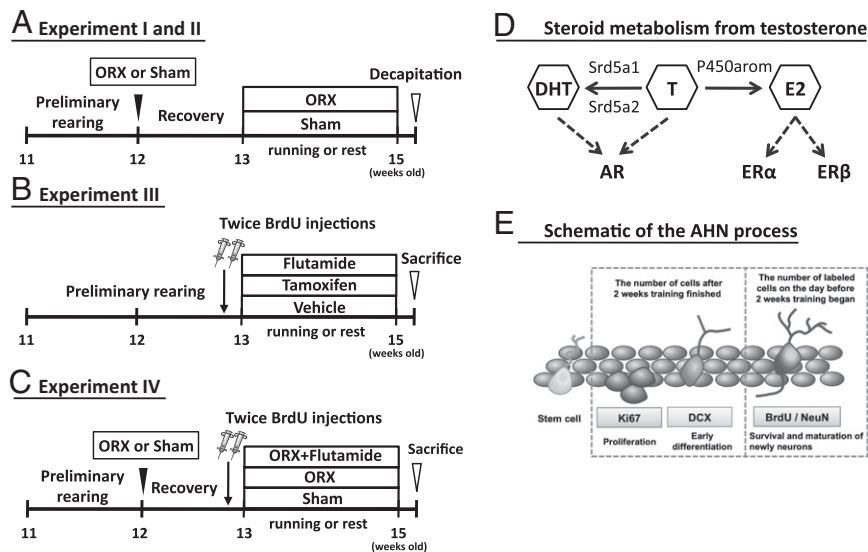


Fig. 1. Experimental design. To exclude acute effects of treadmill running, sample collection was performed 2 d after the last training session. (A and C) ORX or Sham was performed 1 wk before exercise training, at which time the rats were 12 wk old. (B and C) On the day before the 2-wk training began, all of the animals were injected twice i.p. (at 8:00 AM and 8:00 PM) with BrdU (50 mg/kg, B.W.) as a short cell-survival marker in this study. Also, rats were administered the flutamide (30 mg/kg, B.W, s.c.) or the estrogen receptor antagonist tamoxifen (1 mg/kg, B.W., s.c.) suspended in sesame oil 2 h before every exercise session. Rats in the control group were injected with sesame oil only. (D) The enzyme 5 α -reductases (Srd5a1,2) convert T into DHT. The effects of T and DHT caused by binding to the AR. T is also converted to estradiol (E2) by the enzyme aromatase (P450arom) and the effect of E2 caused by binding to the estrogen receptors (ER α , β). (E) We have divided adult hippocampal neurogenesis into three primary phases: proliferation, differentiation, and survival of cells. Sham: sham castrated; ORX: orchidectomized; flutamide: an androgen receptor antagonist; tamoxifen: an estrogen receptor antagonist.

times per week for 30 min at a time (Fig. 1A). We measured postexercise changes in the hippocampal gonadal hormones using HPLC and LC-MS/MS (Fig. 1A). Table 1 shows the levels of androgens and estradiol in the hippocampus and plasma after treadmill running. Mass-spectrometric analysis of DHT, T, and E2 levels demonstrated that treadmill running significantly increased hippocampal DHT levels in both the sham castrated (Sham) and orchidectomized (ORX) groups (effect of treadmill exercise: $F_{(1,33)} = 5.06, P < 0.05$; effect of ORX: $F_{(1,33)} = 110.7, P < 0.0001$; interaction of treadmill exercise and ORX: $F_{(1,33)} =$

3.18, $P = 0.08$) without any changes in T levels. The levels of androgens in hippocampus and plasma after ORX are lower than Sham, with the decrease after ORX being more pronounced in plasma than in the hippocampus, so that the ratios of hippocampus to plasma DHT concentrations are as follows: Sham-sedentary, 23:1; Sham-exercise, 32.3:1; ORX-sedentary, 71.4:1; ORX-exercise, 220:1. We also investigated a possible contribution of estrogens. However, hippocampal E2 levels were unchanged with exercise. Note that plasma levels of DHT

Table 1. Effects of treadmill running on hippocampal or plasma gonadal hormone levels

	Sham		ORX	
	Sedentary	Exercise	Sedentary	Exercise
Hippocampus				
DHT (nM)	2.30 \pm 0.3	3.24 \pm 0.2*	0.10 \pm 0.01	0.22 \pm 0.03*
DHT (ng/g wet weight)	0.67 \pm 0.1	0.94 \pm 0.1*	0.03 \pm 0.002	0.06 \pm 0.01*
Testosterone (nM)	20.5 \pm 2.2	19.5 \pm 1.6	0.77 \pm 0.05	0.90 \pm 0.07
Testosterone (ng/g wet weight)	5.9 \pm 0.7	5.6 \pm 0.5	0.22 \pm 0.02	0.26 \pm 0.02
Estradiol (nM)	0.68 \pm 0.1	0.88 \pm 0.1	0.46 \pm 0.05	0.33 \pm 0.08
Estradiol (ng/g wet weight)	0.19 \pm 0.03	0.22 \pm 0.04	1.29 \pm 0.15	0.92 \pm 0.24
Plasma				
DHT (nM)	0.10 \pm 0.04	0.10 \pm 0.03	0.0014 \pm 0.0005	0.0010 \pm 0.0002
DHT (pg/mL)	29.62 \pm 11.04	30.25 \pm 8.32	0.40 \pm 0.14	0.30 \pm 0.05
Testosterone (nM)	5.11 \pm 1.8	4.6 \pm 1.2	0.00046 \pm 0.0001	0.00069 \pm 0.0002
Testosterone (pg/mL)	1472.5 \pm 523.7	1326.3 \pm 353.4	0.13 \pm 0.03	0.20 \pm 0.60

The steroid levels of Sham rats and ORX rats are shown. The steroids were separated into fractions of DHT, T, and E2 using a normal-phase HPLC system with a silica gel column, and then each steroid was measured using LC-MS/MS. After 2 wk of training, hippocampal DHT levels had increased in both Sham and ORX groups. There were no significant changes in hippocampal T and E2 levels or in plasma T and DHT levels. To compare the hippocampal level of steroids with plasma steroids, we converted to nanomolar concentration via the following estimation. First, 1 mL of plasma (93% is water) is assumed to have 1 g weight, as 1 mL of water is 1 g. Second, we assume that the hippocampal tissue having 1 g of wet weight has an approximate volume of 1 mL, as nearly 78% of the brain tissue consists of water (29). Data represent the mean \pm SEM ($n = 6$ –14 rats in each group).

* $P < 0.05$ in comparison with sedentary rats (two-way ANOVA). Sham: sham castrated; ORX: orchidectomized.

and T were also unchanged with exercise in sham rats and were extremely low after ORX.

Experiment II: Expression of mRNA for Steroidogenic Enzymes and Steroid Receptors in the Hippocampus After Exercise. We determined postexercise changes in the mRNA expression of steroid-related enzymes and receptors in the hippocampus using a real-time quantitative PCR (Fig. 1A). For the sequences of the oligonucleotide, see Table S1. Fig. 1D shows steroid metabolism from testosterone. Both of the two isoforms of the enzyme 5 α -reductases (*srd5a1*, *srd5a2*) convert T into DHT. The enzymatic activity of *srd5a2* is higher than that of *srd5a1* (30). Both synthase genes increased with mild exercise [*srd5a1*: effect of treadmill exercise— $F_{(1,17)} = 44.93$, $P < 0.0001$ (Fig. 2A); *srd5a2*: effect of treadmill exercise— $F_{(1,18)} = 7.12$, $P < 0.05$ (Fig. 2B)]. Regarding *srd5a1*, the increase with exercise was lower in the ORX than in the Sham group. There was a significant interaction between treadmill exercise and ORX [interaction: $F_{(1,17)} = 7.076$, $P < 0.05$], suggesting that the exercise-induced *srd5a1* mRNA expression was attenuated by ORX. The increase of aromatase (*P450arom*) mRNA expression with exercise was completely blocked by ORX [effect of treadmill exercise; $F_{(1,18)} = 1.63$, $P = 0.22$; effect of ORX: $F_{(1,18)} = 0.47$, $P = 0.5$; interaction of treadmill exercise and ORX: $F_{(1,18)} = 5.88$, $P < 0.05$ (Fig. 2C)]. The Bonferroni post hoc test from two-way ANOVA showed that Sham-exercised rats had significantly higher mRNA expression of *P450arom*, the enzyme that converts T into E2, than those that were sedentary ($P < 0.05$). In both Sham and ORX groups, treadmill exercise enhanced mRNA expression of ARs [effect of treadmill exercise: $F_{(1,18)} = 9.9$, $P < 0.01$ (Fig. 2D)]. Exercise also significantly up-regulated mRNA expression of *ER α* [effect of treadmill exercise: $F_{(1,18)} = 11.25$, $P < 0.01$ (Fig. 2E), but not of *ER β* (Fig. 2F)] in both groups. These data imply that mild exercise promotes hippocampal DHT synthesis independently of circulating androgens. Moreover, although the increase in *P450arom* thus depends on circulating gonadal hormones, the *ER α* mRNA increase does not.

Experiment III: Suppressive Effects of an AR Antagonist on Exercise-Induced AHN. To investigate whether androgens have a role in exercise-induced AHN, rats received sesame oil or flutamide [an AR antagonist; 30 mg/kg body weight (BW), s.c.] during 2 wk of

mild treadmill exercise (Fig. 1B). We measured the weight of seminal vesicles and prostate to check the effect of the antagonist. The seminal vesicular and prostate weights of flutamide-treated rats were significantly lower compared with those of vehicle rats (Table S2). We have already demonstrated that this exercise protocol is sufficient to increase AHN for mice (16) and rats (Fig. S1). Treadmill running is a forced exercise for rodents, but, when carefully and systematically done after a preconditioning, acclimatization regimen, it causes several beneficial effects in rodents. Indeed, a relatively mild-intensity exercise has already been shown to enhance AHN, with neurogenesis increased by 40–60% compared with control (23, 24), which is similar to our present study (54%).

We assessed the impact of exercise on three primary phases of neurogenesis (proliferation, differentiation, and survival of cells) in the hippocampal dentate gyrus (Fig. 1E). To estimate neuronal survival and maturation, all of the animals were given two (at 8:00 AM and 8:00 PM) i.p. injections of 5-bromo-2-deoxyuridine (BrdU, 50 mg/kg B.W.) on the day before training (Fig. 1B). To monitor cell proliferation and differentiation after 2 wk of training, proliferation was assessed using a specific marker (Ki67, a proliferation marker for neurons and other cell types), and early neuronal differentiation was measured by doublecortin (DCX) immunoreactivity (immature neuron marker) (Fig. 1E). Exercise increased the number of Ki67 immunoreactive cells compared with respective sedentary rats [effect of treadmill exercise: $F_{(1,40)} = 39.65$, $P < 0.0001$ (Fig. 3A)], an effect that was unaltered with flutamide treatment. However, the exercise-induced increases of DCX-positive cells (Fig. 3B) and BrdU/NeuN (a neuron survival marker) double-positive cells (Fig. 3C) were blocked by flutamide [DCX: effect of treadmill exercise— $F_{(1,38)} = 13.52$, $P < 0.001$; effect of antagonists— $F_{(2,38)} = 4.37$, $P < 0.05$; interaction of treadmill exercise and antagonists— $F_{(2,38)} = 4.76$, $P < 0.05$; BrdU: effect of treadmill exercise— $F_{(1,40)} = 10.95$, $P < 0.01$; effect of antagonists— $F_{(2,40)} = 3.7$, $P < 0.05$; interaction of treadmill exercise and antagonists— $F_{(2,40)} = 3.7$, $P < 0.05$]. In contrast, the administration of the estrogen-receptor inhibitor tamoxifen (1 mg/kg B.W.) had no effect on the increased number of exercise-induced DCX-positive and BrdU/NeuN double-positive cells (Fig. 3).

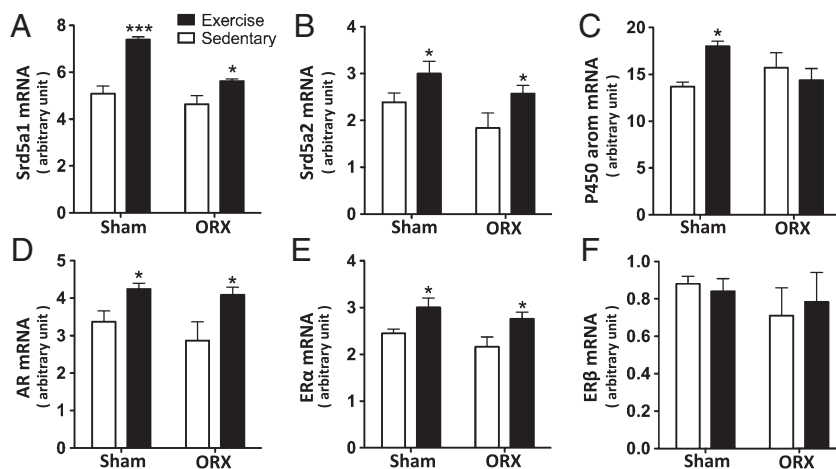


Fig. 2. The effect of treadmill running on hippocampal sex steroidogenesis-related enzymes and steroid receptor mRNA. The mRNA expressions were quantified by real-time PCR. (A, B, and D) Analysis data of mRNA expression for the two isoforms of 5 α -reductases (*srd5a1*: $P < 0.0001$; *srd5a2*: $P < 0.05$), which convert T into DHT, and for ARs ($P < 0.01$) revealed that both increased in Sham and ORX rats. (C) *P450arom*, which converts T into E2, mRNA expression was increased in Sham rats only. (E and F) Treadmill exercise increased the mRNA expression of *ER α* ($P < 0.05$), but not *ER β* , in both Sham and ORX groups. Expressions of mRNA were normalized by GAPDH housekeeping gene as an internal standard. Data represent the mean \pm SEM ($n = 5$ –6 rats in each group). * $P < 0.05$ and *** $P < 0.0001$ in comparison with sedentary rats (two-way ANOVA). Sham: sham castrated, ORX: orchidectomized.

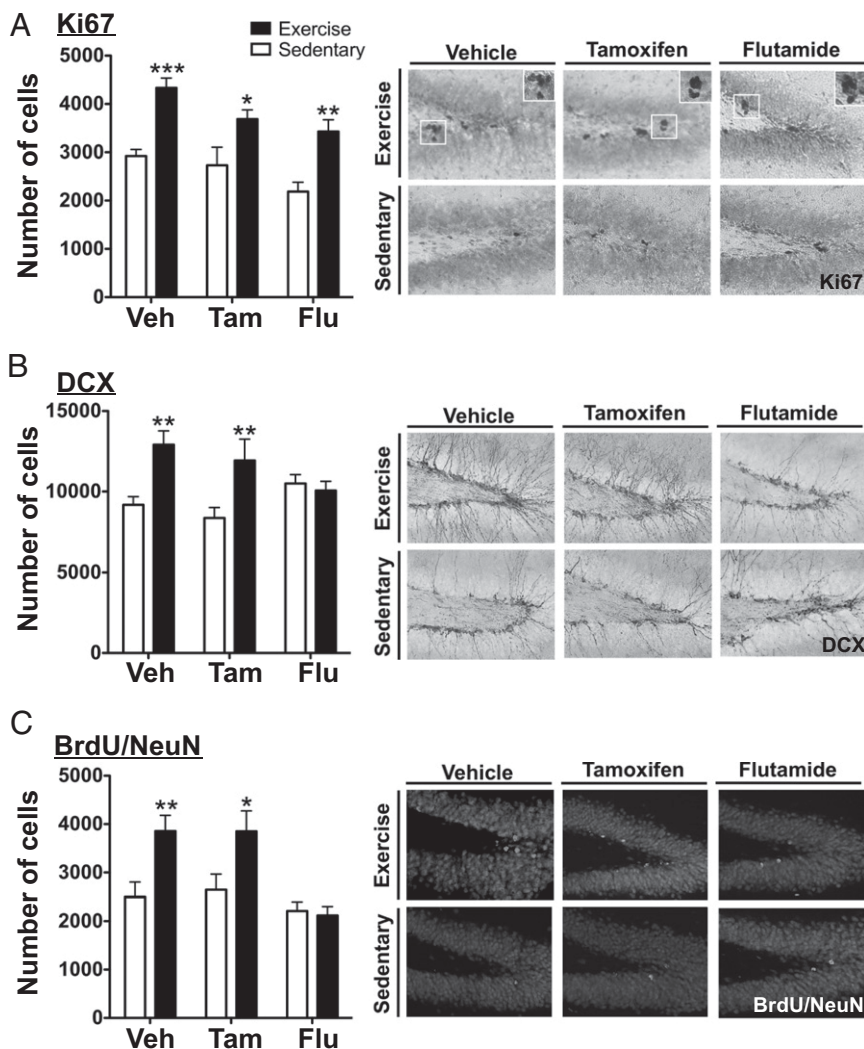


Fig. 3. Effects of sex steroid receptor antagonists on exercise-induced AHN of male rats. (A) Mild exercise significantly increased the number of Ki67⁺ cells (proliferation marker) in Veh-, Flu-, and Tam-treated rats ($P < 0.0001$). (B and C) Flu, but not Tam, blocked the increase of the number of DCX⁺ (immature neuron marker, $P < 0.001$) and BrdU⁺/NeuN⁺ (cell survival and mature neuron marker, $P < 0.01$) cells with mild exercise. In immunostaining, the Upper and Lower panels show the cells of exercise rats and sedentary rats. (Left) Veh rats. (Center) Tam-treated rats. (Right) Flu-treated rats. Data represent the mean \pm SEM ($n = 7-8$ rats in each group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ in comparison with sedentary rats (two-way ANOVA and Bonferroni post hoc tests). Veh: vehicle; Tam: tamoxifen; Flu: flutamide.

Experiment IV: Effects of Orchidectomy on Exercise-Induced AHN. To determine whether testis-derived androgens have a role in exercise-induced AHN, rats were either sham-operated (Sham), orchidectomized (ORX), or ORX+flutamide (ORX+Flu) and divided into sedentary and exercise groups (Fig. 1C). The seminal vesicle and prostate weights of the ORX and ORX+Flu rats were significantly lower compared with those of vehicle rats (Table S2). As in sham rats, exercise increased the number of Ki67 immunoreactive cells in the ORX dentate gyrus compared

with respective sedentary rats, but this effect was not blocked by flutamide [effect of treadmill exercise: $F_{(1,39)} = 39.83$, $P < 0.0001$ (Fig. 4A)]. However, exercise-induced increases of DCX-positive cells (Fig. 4B) and BrdU/NeuN double-positive cells (Fig. 4C) were prevented by ORX+Flu, but not by ORX (DCX: effect of treadmill exercise— $F_{(1,34)} = 44.2$, $P < 0.0001$; effect of treatments— $F_{(2,34)} = 13.95$, $P < 0.0001$; interaction of treadmill exercise and treatments— $F_{(2,34)} = 13.95$, $P < 0.0001$; BrdU: effect of treadmill exercise— $F_{(1,33)} = 17.66$, $P < 0.01$; effect of

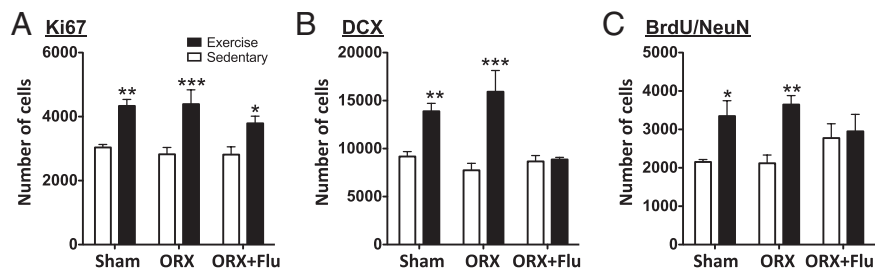


Fig. 4. Effects of orchidectomy on exercise-induced AHN of male rats. (A) Mild exercise significantly increased the number of Ki67⁺ cells in Sham, ORX, and ORX+Flu rats ($P < 0.0001$). (B and C) Mild exercise increased the number of DCX⁺ and BrdU⁺/NeuN⁺ cells in ORX rats depleted of circulating androgens, and the effects were blocked by Flu. Data represent the mean \pm SEM ($n = 6-8$ rats in each group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$ in comparison with sedentary rats (two-way ANOVA and Bonferroni post hoc tests). Sham: sham castrated; ORX: orchidectomized; ORX+Flu: orchidectomized+flutamide.

treatments— $F_{(2,33)} = 3.32, P < 0.05$; interaction of treadmill exercise and treatments— $F_{(2,33)} = 3.32, P < 0.05$).

Discussion

This report tests the hypothesis that hippocampal DHT synthesis is stimulated by mild exercise and contributes to exercise-induced AHN through the AR. We found that 2 wk of mild running resulted in increased hippocampal DHT levels, but not in those of T or E2, independently of circulating androgens. Furthermore, we demonstrated, using the AR antagonist, flutamide, that androgens stimulate exercise-induced AHN. In particular, orchidectomized rats with depleted circulating androgens still showed enhanced levels of AHN, which was completely blocked by flutamide, but not by tamoxifen, an estrogen receptor antagonist. These results provide evidence supporting the hypothesis that hippocampal androgen synthesis is stimulated by mild exercise and contributes to stimulation of AHN.

We demonstrated that hippocampal DHT levels and syntheses (*srd5a1,2*) (Fig. 1D) are both increased with mild exercise and found this to be the case after ORX, which depleted circulating androgens. These results support the hypothesis that DHT could be synthesized in the hippocampus. This hypothesis was also supported by the fact that plasma androgens levels were unchanged with exercise with or without ORX. Furthermore, in the hippocampus, DHT is rapidly metabolized to $3\alpha, 5\alpha$ -androstanediol and inactivated by 3α -HSD (12). Therefore, the hippocampal DHT in the current study is very unlikely to have accumulated from peripheral sources, but was most likely synthesized *de novo* in the hippocampus.

The source of DHT should not always be restricted to the hippocampus, although dehydroepiandrosterone (DHEA) as adrenal androgen cannot be produced in rats due to their lack of adrenal Cyp17 (31), and both testosterone and DHT levels have been shown to be undetectable in the adrenals. It is possible that pregnenolone or progesterone, for example, coming from the adrenals could serve as substrates for hippocampal DHT. There is the possibility that DHEA produced in the brain mainly during early development but persisting in the adult brain (32) could be a source of androgen. Thus, hippocampal DHT could be synthesized from peripheral androgen precursors and/or synthesized *de novo* in the hippocampus.

As to the role of androgens in stimulating AHN, there are three principal stages of neurogenesis from progenitor cells in the hippocampus that can be distinguished using markers (33): proliferation, differentiation, and survival (Fig. 1E). Regarding differentiation and survival, we found that exercise stimulated expression of DCX, an early marker of neuronal differentiation, and that flutamide completely blocked exercise stimulation of DCX. We found the same for BrdU/NeuN, which, together, are markers of cell survival and neuronal maturation, indicating androgenic modulation of cell differentiation and survival in exercise-induced AHN. As one of the mechanisms, we considered Wnt/ β -catenin signaling. Androgen receptors interact with β -catenin (34), which can enter the cell nucleus and then, via Wnt/ β -catenin signaling, trigger the expression of NeuroD1 to promote neuronal differentiation in hippocampal neural progenitors (35). In the hippocampus, ARs thus may act via β -catenin in enhancing AHN. Future studies should address this mechanism.

We found that mild exercise increased Ki67, a proliferation marker, even in flutamide-administrated rats. The results are in agreement with a report by Spritzer and Galea showing that T and DHT, but not E2, enhanced cell survival without affecting cell proliferation in the dentate gyrus of adult male rats (9). The lack of a flutamide effect on Ki67 expression is likely explained by concurrent glial cell or progenitor cell proliferation that is androgen independent and might be stimulated by other mediators, such as IGF-I (36). Because Ki67 labels cells undergoing proliferation within 24 h (31), we gave no exercise for about 2 d

(36 h) before euthanasia to exclude the acute effects of the last bout of exercise (37). Thus, the Ki67 results have no bearing on the effects of acute exercise, and we suggest that our results of increased Ki67-positive cells indicate a sustained effect of mild exercise training on enhancement of cell proliferation.

Importantly, we confirmed that the mild exercise significantly increases AHN even in rats with ORX that have depleted circulating androgens. Furthermore, with the administration of flutamide to ORX rats, the effects of exercise on DCX or BrdU/NeuN-positive cells were completely blocked. These results strongly suggest a paracrine/autocrine effect on AHN by hippocampus-synthesized DHT. Androgen might increase BDNF and VEGF (19), resulting in enhancement of neurogenesis. Collectively, these findings imply a stimulatory role for hippocampal DHT in the cascade of molecular mechanisms that underlie exercise-induced AHN.

It is important to consider possible indirect effects of AR on the hippocampus via afferent inputs because circulating testosterone may modulate hippocampal acetylcholine release (38), which is one of the factors that enhance AHN (39). Any contribution of acetylcholine neurons, however, may be excluded, because running-enhanced neurogenesis is unchanged in mice with partial cholinergic denervation (40) and in ORX rats where circulating androgens were eliminated (the current study). There does remain a possibility that hippocampal DHT could stimulate acetylcholine release from terminals in hippocampus via non-genomic AR (21).

Our finding that mild exercise increases hippocampal androgen levels, detected by a sensitive method, may be relevant for the prevention and slowing down of neurodegenerative diseases, such as Alzheimer's disease, especially in people with a lower level of physical fitness (41). Because the injection of testosterone has been shown to reduce deposits of β -amyloid protein *in vitro* through the enhanced effects of neprilysin (42), it might be that mild exercise, in mediating paracrine effects through androgens and/or DHT, may, in turn, also reduce the deposits of β -amyloid protein and protect cognitive functions.

In conclusion, androgens, which modulate hippocampal synaptic plasticity (5), including spinogenesis (43) and enhance cognitive function (44), also modulate AHN stimulated by mild exercise below the lactate threshold. The current observations should encourage further research into hippocampal androgens as physiological mediators of neuronal plasticity as well as neuroprotection.

Materials and Methods

For a full description of all materials and methods, see [SI Materials and Methods](#). Each experimental design is shown in Fig. 1.

Animals. Eleven-week-old adult male Wistar rats (SEASCO Co.) were maintained on a 12-h light/dark schedule (light on at 7:00 AM) and given *ad libitum* access to food and water. All of the experimental protocols were performed in accordance with the University of Tsukuba Animal Experiment Committee guidelines. Animals were acclimatized to ambient rearing conditions for 7 d (two to three rats per cage) and then randomly assigned to the treadmill running or sedentary control groups.

Exercise Training Protocol. Our previous studies revealed that the LT of rats is at a running speed of ~ 20 m/min (25, 26). On the basis of the LT, the rats were subjected to mild treadmill exercise at a running speed of 13.5 m/min. We previously showed that hippocampal activation is induced by exercise intensities below the LT (17). The rats were habituated to the treadmill apparatus for 10 min (KN-73; Natsume) before training, and then they were subjected to 2 wk of training, five times per week for 30 min at a time. The training of exercised rats included gradual adaptation to the running. Sedentary rats remained sitting on the treadmill without running for the same amount of time. On the day before training, all of the animals were given two (at 8:00 AM and 8:00 PM) *i.p.* injections of BrdU (50 mg/kg B.W.) as a short cell-survival marker in this study.

Drug Administration. Rats were s.c. administered with the androgen receptor antagonist flutamide (30 mg/kg B.W.) or the estrogen receptor antagonist tamoxifen (1 mg/kg B.W.) suspended in sesame oil 2 h before every exercise session. Rats in the control group were injected with sesame oil only. Because rats were subjected to treadmill exercise five times per week for 2 wk, the injections were given 10 times.

Sample Collection. To exclude the acute effects of treadmill running, sample collection was performed 2 d after the last training session. In the experiment to assess adult hippocampal neurogenesis, the rats were deeply anesthetized with pentobarbital and transcardially perfused with 0.9% saline. Brains were carefully removed and fixed overnight at 4° C with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer and equilibrated in 30% (wt/vol) sucrose.

Mass-Spectrometric Assay of Steroids. To examine how much androgen and estrogen were synthesized in the hippocampus after exercise, we performed a mass-spectrometric analysis. Detailed procedures are described elsewhere (12) and in the *SI Materials and Methods, Mass-Spectrometric Assay of Steroids*. Hippocampi were homogenized for steroid extraction using a hexane:ethylacetate = 2:3 mixture. The steroid extracts were applied to a C₁₈ Amprep solid-phase column (Amersham Biosciences). The androgen or estrogen fraction was purified from the eluted steroids using a normal-phase HPLC system (Jasco) with a silica gel column. For induced ionization, T and DHT were derivatized into T or DHT-picolinoyl (12). To increase the evaporation efficiency, E2 was derivatized into estradiol-3-pentafluorobenzoxy-17-

picolinoyl-ester (estradiol-PFBz-picolinoyl). The LC-MS/MS system, which consisted of a reverse-phase LC coupled with an API 5000 triple-stage quadrupole mass spectrometer (Applied Biosystems), was operated with electron spray ionization in the positive-ion mode. The LC chromatographic separation for T, DHT-picolinoyl, or estradiol-PFBz-picolinoyl was performed on a Cadenza CD-C₁₈ column (Imtakt). The MS/MS processes monitored the *m/z* transition from 396 to 203 for DHT-picolinoyl, from 394 to 253 for T-picolinoyl, and from 558 to 339 for estradiol-PFBz-picolinoyl. DHT-picolinoyl-d₃, T-picolinoyl-d₃, and ¹³C₄-estradiol-PFBz-picolinoyl were used as internal standards to measure the recovery of steroids and to calibrate the retention time.

Statistical Analysis. Values are expressed as mean ± SEM and were analyzed using Prism 5 (MDF Co.). Data were analyzed with one-way ANOVAs followed by Dunnett's post hoc or two-way ANOVAs followed by Bonferroni post hoc tests. Statistical significance was assumed at *P* values < 0.05.

ACKNOWLEDGMENTS. We thank M. Iemitsu and K. Aizawa for technical support of real-time quantitative PCR. This work was supported in part by Japan Society for the Promotion of Science (Grants-in-aid for Scientific Research B 20300214 and A 23240091) and for the Body and Mind Integrated Sports Sciences Project (2010–2013) of the Ministry of Education, Culture, Sports, Science and Technology, Japan; and by a grant-in-aid for the Japan Society for the Promotion of Science Fellows (11J01243). Masahiro Okamoto and Takashi Matsui are both Research Fellows of the Japan Society for the Promotion of Science.

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Supporting information

Okamoto et al. 10.1073/pnas.1210023109

SI Text

Mild-Intense Treadmill Running Model and Exercise-Induced AHN. There are numerous reports on exercise-induced adult hippocampal neurogenesis (AHN) using wheel running, although both wheel (1–4) and treadmill exercise enhanced AHN (5–8). The conditions of voluntary running, however, are not always available in detail: only running distance can be assessed, and thus it is still undetermined which exercise conditions (intensity and frequency, etc.) activate AHN, and thus no studies are directly applicable to humans as “translational research.” Furthermore, stress adaptation has been induced in mice that underwent voluntary running (9). Accordingly, we used a treadmill, which allowed us to control the speed, and, on the basis of lactate threshold (LT), developed the mild exercise model without any stress response.

Effects of high corticosterone (CORT) on brain functions are well known; CORT also dampened AHN. However, we have already shown that our mild exercise model, which maintains an intensity below the LT, does not elicit a stress response (10–12). When exercise intensity (running speed) exceeds the LT (50–60% of maximum oxygen intake in rats and humans), both the blood levels of lactate and adrenocorticotropic hormone (ACTH) (typical systemic markers in response to biological stress) rise rapidly, leading to the release of glucocorticoids (13). Thus, we will regard exercise over the LT (moderate or intense exercise) as stress-inducing (exercise stress), whereas exercise under the LT (mild or light intensity) is stress-free, much as in humans. In this regard, we believe that the influence of CORT will probably be negligible in the development of AHN in this study. Indeed, we have already demonstrated that mild exercise enhances AHN (14).

A growing number of studies have revealed that exercise is a robust AHN inducer (4). The mechanism is still controversial, although BDNF, VEGF, or IGF-I may play a role (8, 15, 16). The involvement of androgens is suggested by the report that testosterone (T) implants resulted in increased BDNF and VEGF (17, 18) and, as noted, BDNF and VEGF are reported to play a role in neurogenesis in the songbird brain (17, 18).

Neurodifferentiation and Neuroprotection Effects of Androgens on AHN. Our results suggest possible androgen effects on cell differentiation and survival in exercise-induced AHN. The results in this study are in agreement with a report by Spritzer and Galea showing that systemic T and dihydrotestosterone (DHT), but not E2, enhanced cell survival without affecting cell proliferation in the dentate gyrus of adult male rats (19). Androgens can promote neuron survival against cell death induced by β -amyloid protein, which is likely the key mediator of Alzheimer's disease (20, 21). It is suggested that the mechanisms underlying the neuroprotective properties of androgens include the activation of mitogen-activated protein kinase/extracellular signal-regulated kinase, leading to inactivation of proapoptotic protein (20) or DHT-induced CREB phosphorylation via protein kinase C signaling (22). DHT may promote dentate gyrus neuron survival through this signaling.

SI Materials and Methods

Animals. Eleven-week-old adult male Wistar rats (SEASCO Co.) were maintained on a 12-h light/dark schedule (light on at 7:00 AM) and given ad libitum access to food and water. All of the experimental protocols were performed in accordance with the University of Tsukuba Animal Experiment Committee guidelines. Animals were acclimatized to ambient rearing conditions

for 7 d (two to three rats per cage) and then randomly assigned to the treadmill running or sedentary control groups.

Surgery. Surgeries were performed 1 wk before exercise training, at which time the rats were 12 wk old. Under intraperitoneally administered pentobarbital sodium anesthesia, males were either bilaterally castrated or underwent sham castrations. For castrations, the testicles were exposed through a small scrotal incision, and the ductus deferens was isolated and ligated with a silk suture. Then the testicles were removed bilaterally, and the incision was closed and sutured. The sham operation involved the exposure of the testicles without isolation. Immediately after surgery, each rat was given a s.c. antibiotic injection of Mycillinsol.

Exercise Training Protocol. We used an original treadmill exercise protocol for rats. When exercise intensity (running speed) exceeds the LT (50–60% of maximum oxygen intake in human), both the blood levels of lactate and ATCH rise rapidly, leading to the release of glucocorticoid. Thus, we shall regard exercise over the LT as stress (exercise stress), and exercise under the LT as stress-free exercise (mild intensity). Our previous studies revealed that the LT of rats is at a running speed of \sim 20 m/min (10–12) and showed that hippocampal activation is induced by exercise intensities below the LT, which lead to increased regional blood flow through neurovascular coupling (23, 24). In a preliminary experiment, we found that mild exercise below the LT 13.5 m/min increased adult hippocampal neurogenesis more than high-intensity exercise supra LT (28 m/min) (Fig. S1). Thus, we used the mild exercise model in this study. On the basis of the LT, the rats were subjected to mild treadmill exercise at a running speed of 13.5 m/min. The rats were habituated to the treadmill apparatus for 10 min (KN-73; Natsume) before training, and then they were subjected to 30 min of treadmill exercise five times per week for 2 wk. The training of exercised rats included gradual adaptation to the running. Sedentary rats remained on the treadmill without running for the same amount of time.

Drug Administration. On the day before training began, all of the animals were given two (at 8:00 AM and 8:00 PM) i.p. injections of 5-bromo-2-deoxyuridine (BrdU; 50 mg/kg B.W.) as a short cell-survival marker. Also, rats were s.c. administered with the androgen receptor antagonist flutamide (30 mg/kg BW) or the estrogen receptor antagonist tamoxifen (1 mg/kg BW) suspended in sesame oil 2 h before every exercise session. Rats in the control group were injected with sesame oil only.

Sample Collection. To exclude the acute effects of treadmill running, sample collection was performed 2 d after the last training session. In the experiment to assess adult hippocampal neurogenesis, the rats were deeply anesthetized with pentobarbital and transcardially perfused with 0.9% saline. Brains were carefully removed and fixed overnight at 4° C with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer and equilibrated in 30% (wt/vol) sucrose. Sequential coronal sections (50 μ m thick) throughout the entire hippocampus were collected individually in 96-multiwell culture plates. One in 10 random serial sections were collected for immunohistochemistry, and the brain slices were preincubated in phosphate buffer (PB) with 1.0% Triton X-100 and 1.0% BSA. In the experiment to measure hippocampal sex steroids and to detect mRNA related to these steroids, rat hippocampi were removed immediately after decapitation and homogenized to quantify sex hormones or frozen in liquid ni-

trogen to evaluate mRNA expression. To measure androgen levels in plasma, trunk blood was collected after decapitation.

Immunohistochemical Analysis. Immunofluorescence staining for BrdU and NeuN was performed on one series of brain sections selected at random, as described previously (8). Briefly, a 1-in-10 series of sections were used for cell counting (25). The sample was pretreated with 2 N HCl at 37 °C for 30 min to denature the DNA. Then the free-floating slices were incubated for 2 d at 4 °C with the primary antibodies diluted with 0.1 M PB containing 1% BSA and 1% Triton X-100 (PB-T). Rat monoclonal anti-BrdU antibody (1:500; AbD Serotec) and mouse monoclonal anti-NeuN (1:500; Chemicon) were used as the primary antibodies. The slices were then incubated for 24 h at 4 °C with an appropriate secondary antibody: Cy3 donkey anti-rat (1:500; Jackson ImmunoResearch) and aminomethylcoumarin acetate (AMCA) donkey anti-mouse (1:250; Jackson ImmunoResearch). To visualize Ki67⁺ and DCX⁺ cells, respectively, another series of sections was subjected to immunoperoxidase staining as performed previously but with minor modifications (10). Briefly, the sections were rinsed three times in PB-T, then soaked in 0.3% hydrogen peroxide for 30 min to quench endogenous peroxidase activity, and rinsed again three times (10 min each) with PB-T. The sections were then placed in PB-T containing 2% (vol/vol) normal goat serum for Ki67 and 2% (vol/vol) normal rabbit serum for DCX for 30 min. Sections were then incubated in a primary antibody for 24 h at 4 °C. For diaminobenzidine-based detection, the primary antibodies were rabbit monoclonal anti-Ki67 (1:250; Abcam) or goat polyclonal anti-DCX (1:250; Santa Cruz). After washes, the sections were incubated in biotinylated rabbit or goat IgG (1:200; Vectastain Elite ABC Kit, Vector Laboratories) for 2 h. Next, sections were rinsed three times (10 min each) in PB-T and incubated with ABC solution (1:50; Vectastain Elite ABC Kit) for 90 min. After serial rinsing in PB-T and 0.1 M acetate buffer, the sections were made visible by incubating with 0.025% 3,3-diaminobenzidine tetrahydrochloride, 0.08% ammonium chloride, 0.4% glucose, and 0.03% glucose oxidase (10,000 IU) in PB-T for 5–10 min at room temperature. The sections were mounted on slides and air-dried, and then a subset of sections from each condition were counterstained with Nissl staining, dehydrated in a graded ethanol series (60–100%), delipidated in xylene, and coverslipped with Mount-Quick (Daido Sangyo).

The sections were mounted on gelatin-covered slides and analyzed with a Leica DMRB optical microscope (Leica). Estimates of immunolabeled BrdU⁺ cells were determined using the Cavalieri method (26). Labeled cells on every 10th unilateral section throughout the dentate gyrus were counted. The total cell numbers were obtained by multiplying the neuronal density by the total volume of the dentate area. To estimate dentate gyrus volume, Nissl staining was performed in another series of sections.

Real-Time Quantitative PCR. The mRNA expression was measured with quantitative real-time PCR (ABI-PRISMA 7700 Sequence Detector, PerkinElmer Applied Biosystems), as performed previously (27). The hippocampus was homogenized in Isogen to isolate total tissue RNA. Total RNA was extracted with chloroform, precipitated with isopropanol, and washed with 75% (vol/vol) ethanol. Total RNA was treated with an RNase-free DNase Kit (QIAGEN) and further purified with an RNeasy mini Kit (QIAGEN). Single-strand cDNA from prepared RNA (2 µg) was synthesized with Omniscript RT (QIAGEN) using an oligo(dT) primer at 37 °C for 60 min. The mRNA expression levels of *srd5a1,2*, an androgen receptor, *P450arom*, estrogen receptors α , β , and GAPDH in the hippocampus were analyzed using real-time quantitative PCR with a TaqMan probe. The real-time quantitative PCR was performed according to the method described previously with minor modifications (27). Gene-specific

primers and TaqMan probes were synthesized according to the published cDNA sequences for each of the following. The sequences of the oligonucleotides were as in Table S1. The expression of GAPDH mRNA was determined as an internal control. The PCR mixture (25 µL total volume) consisted of 450 nM of both forward and reverse primers for each target gene, 200 nM of fluorescein-aminohexyl (FAM)-labeled primer probes (Perkin-Elmer Applied Biosystems), and TaqMan Universal PCR Master Mix (Perkin-Elmer Applied Biosystems). Each PCR amplification was performed in duplicate, using the following profile: 1 cycle at 95 °C for 10 min and 40 cycles at 94 °C for 15 s and 60 °C for 1 min. The quantitative values of target genes were normalized against those of GAPDH mRNA expression.

Mass-Spectrometric Assay of Steroids. Step i: Purification of steroids from hippocampi with normal-phase HPLC. The preparation of hippocampal homogenates from slices and the extraction of steroids were performed as described (28). The ³H-steroids were added to homogenates as internal standards. The steroid extracts were applied to a C₁₈ Amprep solid-phase column (Amersham Biosciences) to remove contaminating fats. Then steroids were separated into fractions of T, DHT, and E2 using a normal-phase HPLC system (Jasco) with a silica gel column (Cosmosil 5SL; Nacal Tesque). Plasma was prepared by centrifugation from trunk blood collected from decapitated rats (29).

Step ii: Derivatization of HPLC-purified steroids before application to liquid chromatography with tandem mass spectrometry. First, 500 pg of isotope-labeled steroids (T-d₃, DHT-d₃, ¹³C₄-E2) were added to steroid extracts prepared via step i (30, 31). For preparation of T-17-picolinoyl-ester and DHT-17-picolinoyl-ester, evaporated steroid extracts from the hippocampus or from plasma were combined with 50 µL of picolinic acid solution and 20 µL of triethylamine for 0.5 h at room temperature. The product of the subsequent reaction was purified with the C₁₈ column using 80% (wt/vol) acetonitrile. The purified T or DHT derivative was dissolved in an elution solvent for LC. For preparation of E2-PFBz-picolinoyl, evaporated E2 extracts from the hippocampus or evaporated total steroid extracts from plasma were combined with 5% pentafluorobenzyl bromide/acetonitrile under KOH/ethanol for 1 h at 55 °C. After evaporation, the products were combined with 100 µL of picolinic acid solution [2% (wt/vol) picolinic acid, 2% (wt/vol) of 2-dimethylaminopyridine, 1% 2-methyl-6-nitrobenzoic anhydride in tetrahydrofuran] and 20 µL of triethylamine for 0.5 h at room temperature. The products of the subsequent reactions were dissolved in 1% acetic acid and purified by a Bond Elute C₁₈ column (Varian). The dried sample was dissolved in an elution solvent for LC (30, 31).

Step iii: Determination of the concentration of T, DHT, and E2 using LC-MS/MS. The LC-MS/MS system, which consists of a reverse-phase LC (Agilent 1100, Agilent Technologies) coupled with an API 5000 triple-stage quadrupole mass spectrometer (Applied Biosystems) was operated with electrospray ionization in the positive-ion mode.

Derivatized steroids were first separated into an LC column. In the multiple reaction monitoring mode, the instrument monitored the *m/z* transition from 394 to 253 for T-picolinoyl, from 396 to 203 for DHT-picolinoyl, and from 558 to 339 for E2-PFBz-picolinoyl, respectively. In MS/MS procedures, the mother ion (DHT derivative, *m/z* = 396) is first selected using a first-stage mass spectrometer. This DHT derivative is then broken by collision with N₂ gas, and the fragmented ion (*m/z* = 203) is selected using a second-stage mass spectrometer and detected.

To examine the specificity of LC-MS/MS analysis, samples were spiked with steroid isotopes as internal standards. Although the *m/z* transitions were different between intact steroids (e.g., from *m/z* = 396–203 for DHT) and their isotopes (e.g., from *m/z* = 399–206 for DHT-d₃), their retention times were the same because the affinity of intact steroids for an LC column is the same as that for their isotopes.

Isotope-labeled steroid derivatives were used for internal standards to measure the recovery of steroids as well as to calibrate the retention time. By monitoring isotope steroids, the recovery of T, DHT, and E2 were determined as $75 \pm 4\%$, $73 \pm 5\%$, and $89 \pm 8\%$, respectively, after derivatization, purification, and MS/MS detection. Total recovery during all of the steps was determined via ^3H -labeled steroids in step i and isotope-labeled steroids in step ii.

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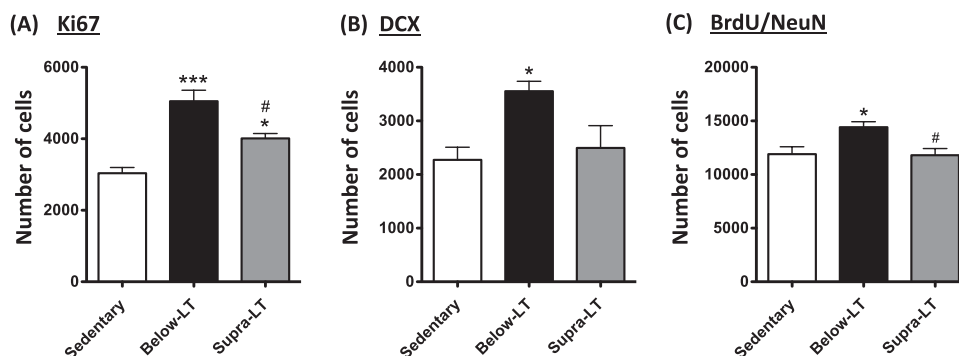


Fig. S1. Effects of different exercise intensities on adult neurogenesis of male rats. Sedentary (0 m/min), Below-LT (13.5m/min), Supra-LT (28 m/min). (A) Number of Ki67+ cells. (B) Number of DCX+ cells. (C) Number of BrdU+NeuN+ cells in the dentate gyrus. Data represent the mean \pm SEM ($n = 7$ rats in each group). $*P < 0.05$ and $***P < 0.0001$ in comparison with sedentary; $\#P < 0.05$ inn comparison to Below-LT (Tukey's post hoc tests).

Table S1. Primer and probe sequences

Enzyme/protein name	Gene name	Accession no.	Direction	Primer and probe sequences
AR	<i>Ar</i>	NM_01502	Forward	5'-AGT ACC AGG GAC CAC GTT TTA C-3'
			Reverse	5'-CAC AGA TCA GGC AGG TCT TCT-3'
			Probe	5'-CCA TCG ACT ATT ACT TCC CAC CC-3'
ER α	<i>Esr1</i>	NM_012689	Forward	5'-GCC CGC AGC TCA AGA TG-3'
			Reverse	5'-CTT GCT GTT GTC CAC GTA CAC-3'
			Probe	5'-CCC AGA GCC CTC TCC ATG-3'
ER β	<i>Esr2</i>	NM_012754	Forward	5'-GCG GAG GCA GAG AAG TTC TAG-3'
			Reverse	5'-CAT GCC CAC CGT TTC TCT TG-3'
			Probe	5'-TTG CTC AGG CAG TGT ACC-3'
P450arom	<i>Cyp19a1</i>	NM_017085	Forward	5'-CGA GAT CGA AAT TCT GGT GGA AAA G-3'
			Reverse	5'-TGC AAA ATC CAT ACA GTC TTC CAG TT-3'
			Probe	5'-CTG CTG AGG AAA CTT T-3'
5 α -reductase 1	<i>Srd5a1</i>	NM_017070	Forward	5'-CTG ATC CAC TAC GTG CAA AGG A-3'
			Reverse	5'-GGG CTT CCC TCC CCT GAT-3'
			Probe	5'-CTG GTT TTC CCG GTT CTG-3'
5 α -reductase 2	<i>Srd5a2</i>	NM_022711	Forward	5'-ACC GCC CGG GAA TGT C-3'
			Reverse	5'-CCC TCT TGT GAG CAA CGA GTA AAT A-3'
			Probe	5'-CTT CTC TGC ACA TTA CTT CC-3'
GAPDH	<i>Gapdh</i>	NM_017008	Forward	5'-GTG CCA AAA GGG TCA TCA TCT C-3'
			Reverse	5'-GGT TCA CAC CCA TCA CAA ACA TG-3'
			Probe	5'-TTC CGC TGA TGC CCC-3'

AR, androgen receptor; ER, estrogen receptor.

Table S2. Body weight, seminal vesicle weight, and prostate weight of male rats treated with sex-steroid receptor antagonists or orchidectomy

	Antagonist experiment			Orchidectomy experiment		
	Vehicle	Tamoxifen	Flutamide	Sham	ORX	ORX+Flu
Body weight (g)	406.9 \pm 5.3	423.1 \pm 5.0	391.8 \pm 4.7	468.3 \pm 7.7	432.5 \pm 18.9	448.0 \pm 8.11
Seminal vesicle (mg/g B.W.)	5.04 \pm 0.14	2.09 \pm 0.26***	2.90 \pm 0.09***	3.02 \pm 0.13	0.4 \pm 0.02###	0.36 \pm 0.02###
Prostate (mg/g B.W.)	1.53 \pm 0.07	1.94 \pm 0.24	0.8 \pm 0.06**	2.15 \pm 0.05	0.42 \pm 0.06###	0.47 \pm 0.03###

Data are mean \pm SEM for seven to eight rats in each group. ** P < 0.01 and *** P < 0.0001 compared with vehicle; ### P < 0.0001 compared with Sham (Dunnett's post hoc).