Supporting information

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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 ~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 μm 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 diaminobenzidin-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 ^{3}H-steroids were added to homogenates as internal standards. The steroid extracts were applied to a C_{18} 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; Nacalai 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-d_3, DHT-d_3, $^{13}C_4$-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 picolinoic acid solution and 20 μL of triethylamine for 0.5 h at room temperature. The product of the subsequent reaction was purified with the C_{18} 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 picolinoic acid solution [2% (wt/vol) picolinoic 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 C_{18} 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 N_2 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-d_3), 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 ± 4%, 73 ± 5%, and 89 ± 8%, respectively, after derivatization, purification, and MS/MS detection. Total recovery during all of the steps was determined via ^{3}H-labeled steroids in step i and isotope-labeled steroids in step ii.

The limits of quantification for steroids were measured with blank samples, prepared alongside hippocampal samples throughout the extraction, fractionation, and purification procedures. The limits of quantification for T, DHT, and E2 were 1 pg, 1 pg, and 0.3 pg per 0.1 g of hippocampal tissue or 1 mL of plasma, respectively. From the calibration curve using standard steroids dissolved in blank samples, good linearity was observed.

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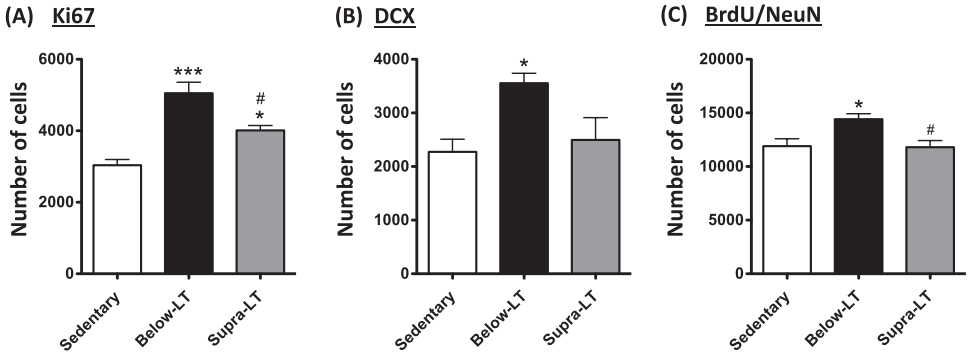

Fig. S1. Effects of different exercise intensities on adult neurogenesis of male rats. Sedentary (0 m/min), Below-LT (13.5m/min), Supra-LT (28 m/min). (*A*) Number of Ki67+ cells. (*B*) Number of DCX+ cells. (*C*) Number of BrdU+/NeuN+ cells in the dentate gyrus. Data represent the mean ± SEM (n = 7 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	*Ar*	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′
ERα	*Esr1*	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′
ERβ	*Esr2*	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	*Cyp19a1*	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′
5α-reductase 1	*Srd5a1*	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′
5α-reductase 2	*Srd5a2*	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	*Gapdh*	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 ± 5.3	423.1 ± 5.0	391.8 ± 4.7	468.3 ± 7.7	432.5 ± 18.9	448.0 ± 8.11
Seminal vesicle (mg/g B.W.)	5.04 ± 0.14	2.09 ± 0.26***	2.90 ± 0.09***	3.02 ± 0.13	0.4 ± 0.02$^{###}$	0.36 ± 0.02$^{###}$
Prostate (mg/g B.W.)	1.53 ± 0.07	1.94 ± 0.24	0.8 ± 0.06**	2.15 ± 0.05	0.42 ± 0.06$^{###}$	0.47 ± 0.03$^{###}$

Data are mean ± 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 (Dunnet's post hoc).