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Physical exercise increases adult hippocampal neurogenesis in male rats provided it is 
aerobic and sustained

Running title: Aerobic exercise promotes adult neurogenesis

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Key points summary

• Aerobic exercise such as running enhances adult hippocampal neurogenesis (AHN) in rodents.

• Little is known about the effects of high-intensity interval training (HIT) or of purely anaerobic resistance training on AHN.

• Here, compared to a sedentary lifestyle, we report a very modest effect of HIT and no effect of resistance training on AHN in adult male rats.

• We find most AHN in rats that were selectively bred for an innately high response to aerobic exercise that also run voluntarily and - increase maximum running capacity.

• Our results confirm that sustained aerobic exercise is key in improving AHN.
Abstract

Aerobic exercise, such as running, has positive effects on brain structure and function, for example, adult hippocampal neurogenesis (AHN) and learning. Whether high-intensity interval training (HIT), referring to alternating short bouts of very intense anaerobic exercise with recovery periods, or anaerobic resistance training (RT) has similar effects on AHN is unclear. In addition, individual genetic variation in the overall response to physical exercise likely plays a part in the effects of exercise on AHN but is less studied. Recently, we developed polygenic rat models that gain differentially for running capacity in response to aerobic treadmill training. Here we subjected these Low Response Trainer (LRT) and High Response Trainer (HRT) adult male rats to various forms of physical exercise for 6 to 8 weeks and examined its effects on AHN. Compared to sedentary animals, the highest number of doublecortin-positive hippocampal cells was observed in HRT rats that ran voluntarily on a running wheel while HIT on the treadmill had a smaller, statistically non-significant effect on AHN. AHN was elevated in both LRT and HRT rats that endurance trained on a treadmill compared to those that performed RT by climbing a vertical ladder with weights, despite their significant gain in strength. Furthermore, RT had no effect on proliferation (Ki67), maturation (doublecortin) or survival (BrdU) of new adult-born hippocampal neurons in adult male Sprague-Dawley rats. Our results suggest physical exercise promotes AHN most if it is aerobic and sustained, and especially when accompanied by a heightened genetic predisposition for response to physical exercise.

Keywords: aerobic exercise, resistance training, high-intensity interval training, doublecortin immunohistochemistry, dentate gyrus
Abbreviations. AHN, adult hippocampal neurogenesis; BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; HIT, high-intensity interval training; HRT, high response trainer; IGF-1, insulin-like growth factor 1; LRT, low response trainer; RW, running wheel; Sed, sedentary.
Introduction

Adult hippocampal neurogenesis (AHN) is a continuous process through which cells proliferate in the subgranular zone of the dentate gyrus, mature into granule cells, and ultimately become incorporated into hippocampal neuronal networks [for review see (Aimone et al., 2014)]. In rodents, adult-born hippocampal neurons seem crucial for a variety of adaptive behaviors such as learning (Shors et al., 2001), pattern separation (Clelland et al., 2009), and responses to stress (Snyder et al., 2011). Aerobic exercise, e.g. running, increases AHN and improves cognitive performance in both male and female adult rodents (Creer et al., 2010, Fardell et al., 2012, van Praag et al., 2005, Marlatt et al., 2012, van Praag et al., 1999). The increase in AHN in response to running is reported to be in part due to an increase in the number of surviving neuronal precursor cells (type 2) rather than to the shortening of the cell cycle (Fischer et al., 2014). There are also studies indicating that running increases the survival and incorporation of newly divided hippocampal cells, born days before commencing training, to increase net neurogenesis (Castilla-Ortega et al., 2014, Lee et al., 2013). The increase in AHN is considered to be mediated by an up-regulation of factors including brain-derived neurotrophic factor (BDNF) (Marlatt et al., 2012, Farmer et al., 2004, Li et al., 2008) and insulin-like growth factor 1 (IGF-1) (Trejo et al., 2001, Carro et al., 2000). In addition to hippocampus, exercise also increases adult neurogenesis in the subventricular zone (Bednarczyk et al., 2009, Chae et al., 2014) and in the hypothalamus (Niwa et al., 2015), suggesting that the neurogenic effect of exercise might span throughout the brain.

Examining the effects of exercise on AHN in animal models has been, for the most part, limited to studying the effects of running. Among humans, different forms of anaerobic exercise such as resistance training, modeled in rats by using a progressive training regimen on a vertical ladder with weights attached to the base of the tail (Hornberger and Farrar,
as well as combinations of aerobic and anaerobic exercise, like high-intensity interval training [HIT, for review see (Gibala et al., 2012)], are gaining in popularity. HIT in rodents refers to a regimen of alternating brief bouts of very intense anaerobic exercise, such as running on a treadmill at ~85-90% of maximum speed, with short recovery periods and repeating this cycle to accomplish a training session of up to an hour in duration [see for example (Haram et al., 2009)]. HIT can increase maximal oxygen uptake (VO$_2$max), a commonly used measure of aerobic fitness, in rodents similar to, or even more than moderate sustained aerobic exercise (Haram et al., 2009). A recent study conducted on healthy adult male rats reported a larger increase in inducers of neuroprotective factors (H$_2$O$_2$ and tumor necrosis factor alpha), as well as in BDNF and glial cell line-derived neurotrophic factor in whole-brain samples in response to HIT compared to continuous training (Afzalpour et al., 2015). To our knowledge, the effects of HIT directly on AHN have not been studied and few reports exist on the effects of purely anaerobic exercise on AHN. In adult male rats, progressive resistance training on a vertical ladder increased IGF-1, but not BDNF, expression in the hippocampus (Cassilhas et al., 2012a). Resistance training is also reported to promote hippocampal cell proliferation (Novaes Gomes et al., 2014) and to improve spatial learning (Cassilhas et al., 2012a, Cassilhas et al., 2012b). It should be noted that forced exercise usually involves the use of a reward or punishment to motivate animals to perform. For example, mild electrical shocks are routinely used to encourage rats to keep running on a treadmill. These shocks likely cause negative stress to the animals. It is well-documented that especially prolonged and unpredictable stress inhibits adult neurogenesis [for a recent review, see (Lucassen et al., 2015)] whereas mild stress can enhance AHN (Parihar et al., 2011).

In addition to the type of physical exercise, the effects of exercise on AHN are likely influenced in part, by individual variation in responsiveness to exercise training. While aerobic exercise is, on average, beneficial for health, its effects vary between individuals
presumably due to considerable genetic variance. For some, aerobic training provides substantial gain in maximal aerobic capacity (VO\textsubscript{2}max) and metabolic health whereas for others the same amount of training results in little or even negative change (Bouchard and Rankinen, 2001) [see also (Timmons, 2011, Bouchard et al., 2012)]. Several clinically supervised exercise training studies report that up to 20% of participants fail to increase VO\textsubscript{2}max and can be considered non-responders. Studies examining predictors of non-response show that groups that exercise at greater volumes (longer durations at same relative intensity) associate with a higher probability of responding (Sisson et al., 2009).

Recently, we developed a contrasting rat model system for low (LRT) and high (HRT) response to aerobic exercise training (Koch et al., 2013). Starting with a founder population of genetically heterogeneous rats (N/NIH stock), we applied two-way artificial selection based on the magnitude of change in running capacity after completing 8 weeks of standardized aerobic treadmill training. After 15 generations of selection, rats bred as HRT increased maximal treadmill running distance from 646 m to 869 m (Δ 223 ± 20 m) while rats bred as LRT went from 620 m to 555 m (Δ- 65 ± 15 m) after completing the same absolute amount of training (Koch et al., 2013). Note that only rats that complete the exercise regimen are used for breeding to ensure no underlying difference in motivation to exercise develops between the two rat lines. As a result of selection, the vast majority of HRT rats show a clear increase in a number of markers of cardiorespiratory fitness such as running capacity, VO\textsubscript{2}max and cardiac muscle cell function (Wisloff et al., 2015). In contrast, these same indices of cardiorespiratory fitness do not change, or even decrease in LRT rats, despite successfully completing the training regimen. Importantly, no baseline difference in cardiorespiratory fitness exists between the LRT and HRT, i.e. in the non-trained condition, both LRT and HRT rats have comparable exercise capacities (Wisloff et al., 2015).

Compared to HRT rats, LRT rats demonstrate impaired skeletal muscle angiogenesis, have
altered signal transduction for JNK and p38 mitogen-activated protein kinase (Lessard et al., 2013), and are lower for mitochondrial biogenesis regulating factors (PGC1-α, NRF1, and TFAM) (Marton et al., 2015), suggesting diminished exercise-induced plasticity in the muscle. A microarray of RNA from skeletal muscle indicates large differences in transcriptional responses between LRT and HRT to the same exercise bout (Lessard et al., 2013). Indeed, the purpose for development of the HRT and LRT rats was to provide contrasting polygenic models to further explore in more mechanistic detail the so-called Energy Transfer Hypothesis (Koch and Britton, 2008): According to this hypothesis the capacity for energy transfer, as measured typically through maximal aerobic exercise capacity, could be the central mechanistic determinant underlying the divide between complex metabolic disease and health.

Here, using this unique contrasting rat model system of HRT and LRT rats, we aimed to test if HIT (Experiment 1) and resistance training (Experiments 2 and 3) are comparable to aerobic exercise (wheel running and endurance treadmill training) in terms of promoting AHN in male rats. Based on the literature reviewed above, we expected HIT to promote AHN. In terms of resistance training, we anticipated to see an increase in the number of proliferating cells in the hippocampus (Novaes Gomes et al., 2014). We hypothesized that there would be no effect of genetic predisposition on baseline AHN, but that compared to sedentary controls exercise activity, in general, would promote AHN more in HRT rats relative to LRT.
Methods

Ethical approval

All the experimental procedures were implemented in accordance with the directive 2010/63/EU of the European Parliament and approved by the National Animal Experiment Board, Finland.

Subjects and groups

All animals were housed on the premises of the animal research unit at the University of Jyväskylä. Food and water were freely available, and room temperature and humidity were controlled at 21 ± 2 °C and 50% ± 10 percentage units, respectively. All rats had aspen chips (Tapvei, Kaavi, Finland) at the bottom of the cage as bedding material. Rats in cages without running wheels were provided wooden toys. Rats were maintained on a 12/12 hour light/dark cycle, with lights on at 8 am. All procedures were conducted during the light portion of the cycle.

Experiment 1

The outline of the experiment is depicted in Figure 1A. The subjects were 88 adult male LRT and HRT rats representing the 17th generation of these rat lines developed by selective breeding and maintained at the University of Michigan (Michigan, USA) (Koch et al., 2013). All rats were singly housed and were ~8 months old at the beginning of the experiment. The average bodyweight in HRT rats was 388 ± 6 g (mean ± SEM) and 403 ± 6 g in LRT rats. The animals were not phenotyped for their training response in order to keep them naïve to exercise training. Animals from both LRT and HRT lines were divided into four different treatment groups: 1) Sedentary (Sed) rats were not subjected to any physical exercise and spent the entire time in their home cage (Tecniplast 1354, Italy; size: 595 x 380 x 200 mm). 2) Control (C) rats were tested for aerobic capacity at the beginning and end of the 7 week
experiment. Half of the animals in the C-group were housed in standard cages and half in cages (Tecniplast 2154; 480 x 265 x 210 mm) fitted with a disabled running wheel. 3) Running (RW) rats were housed in cages fitted with active running wheels for the duration of the exercise intervention (7 weeks). Animals in the RW group were also tested for aerobic capacity before and after exposure to running wheels. 4) High intensity interval training (HIT) rats were housed in regular cages and trained on a treadmill 3 times a week. Rats in the HIT group were tested for aerobic capacity once a week to keep the training parameters optimal (please see the details in the section on Tests of exercise capacity and training regimes below). As a result, 8 groups were formed: LRT-Sed (n = 8), LRT-C (n = 12), LRT-RW (n = 12), LRT-HIT (n = 12), HRT-Sed (n = 8), HRT-C (n = 12), HRT-RW (n = 12), and HRT-HIT (n = 12).

Experiment 2

The outline of the experiment is depicted in Figure 2A. The subjects were 28 adult male LRT and HRT rats representing the 18th generation of these rat lines. Upon arrival to University of Jyväskylä, the rats were allowed to acclimate for 4-5 weeks. After this, when the rats were ~6 months old, they were tested for their response to aerobic exercise. All rats were subjected to an 8-week exercise regime during which they were trained on a treadmill 3 times a week (see section Tests of exercise capacity and training regimes for details). The rats were then divided into four training groups matching for body weight and capacity for aerobic training response and training was either continued as aerobic treadmill endurance or changed to resistance ladder climb training: 1) LRT rats subjected to resistance training (LRT-Res = 6; 417 ± 17 g, adaptive response to 8 weeks of aerobic training (see details below): 9% ± 5 percentage units), 2) LRT rats subjected to aerobic endurance training (LRT-End, n = 8; 421 ± 14 g; 10% ± 6), 3) HRT rats subjected to resistance training (HRT-Res, n = 6; 386 ± 15 g,
24% ± 16), and 4) HRT rats subjected to aerobic endurance training (HRT-End, n = 8; 359 ± 9 g, 24% ± 16).

Experiment 3
The outline of the experiment is depicted in Figure 3A. The subjects were 20 outbred Sprague-Dawley male rats obtained from Harlan (Netherlands). The rats were first allowed to acclimate for 2 weeks after which experiments were started. The rats were 9 weeks old at the onset of experiments and weighed on average 276 ± 2 g. The rats were housed in pairs: One animal in each cage was subjected to resistance training (Res, n = 10) and the other was kept sedentary (Sed, n = 10).

Tests of exercise capacity and training regimes
All protocols for testing maximum running capacity and for endurance training, conducted on a treadmill, were adapted from the protocol developed for selective breeding purposes (Koch et al., 2013). Across 15 generations (n = 3,114), we find over 90% of phenotyped rats are successful in completing this type of training program. Further details are presented below.

Experiment 1
Exercise test for maximal running capacity
The exercise test for maximum running speed was conducted before and after the training period. For all experiments, a custom-made treadmill was used for testing maximum running capacity. The running space for each rat was 9 x 70 cm and a 9 x 9 cm electrified grid was located at the end of the lane. The electrified grid at the end of the lane delivered a mild electrical shock (adjustable between 0.2-2 mA, usually set to 1 mA) and was used to help educate rats to run on the treadmill and to evaluate maximal running capacity. If a rat slid off the treadmill belt and stayed on the electrified grid for more than 5 s rather than continued running, the running test was stopped and the rat was removed from the treadmill.
Unfortunately, the treadmill did not include a counter for frequency of shock delivery. At the completion of each session time spent on the treadmill running was recorded. The test protocol is presented in the following: Rats were first allowed to warm-up for 5 minutes at a speed of 8-9 m/min. The test itself began at a speed of 10 m/min after which the speed was increased by 2.4 m/min every other minute. The test was conducted three times, on consecutive days, and the best result (speed measured as m/min) obtained for each rat was considered the best estimate of maximal running capacity as previously described (Koch and Britton, 2001).

Voluntary training on a running wheel (RW)

Rats had free access to a RW in the home cage for 7 weeks. The distance run was recorded continuously by a computerized system using custom-made software. Data collection started when the RW started moving and data were stored in 2 min bouts. No motivational punishments or rewards were used in conjunction with voluntary running on a running wheel.

High-intensity interval training (HIT)

Rats were subjected to HIT three times a week, with 1-2 days of recovery between each session, for 7 weeks. Each HIT session started with 5 minutes on the treadmill at a speed corresponding to 50-60% of the rat’s individual maximum speed in the maximum capacity test. After that, each rat ran 3 minutes at a speed corresponding to 85-90% of its maximum and then again for 2 minutes at a speed equaling to 50% of its maximum. This 5-min HIT-trial was conducted a total of 3 times per session. That is, the duration of the session was 20 minutes altogether. The inclination of the treadmill was 10° uphill during the entire training period. To keep the parameters for the HIT training regime up to date, maximal running capacity was tested once a week (one test session, substitute for HIT training, please see above). During HIT the electrified grid at the end of the treadmill lane was used to encourage rats to keep running (see also Exercise test for maximal running capacity above). However, to
minimize the use of shocks rats were gently pushed by hand to help avoid stepping off the treadmill belt and onto the grid. If a rat repeatedly slid off the treadmill belt and stayed on the electrified grid for more than 5 s rather than continued running, the training was stopped and the rat was removed from the treadmill.

**Sedentary**

Rats in the sedentary groups (C and Sed) did not exercise. Rats in the C group were tested for maximal running capacity (see above) before and after the 7-week intervention. Rats in the Sed group were not tested for maximal running capacity nor given any type of physical training during the study.

**Experiment 2**

**Test for adaptive response to aerobic training**

The protocol included four phases: 1) familiarization, 2) baseline maximal running capacity test before training, 3) 8 weeks of aerobic training on a motorized treadmill, and 4) maximal running capacity test after training as previously described (Koch et al., 2013). In short, all the rats were familiarized to running on a treadmill for at least 3 days, 10-20 minutes at a time. Treadmill speed was mildly increased in each session so that each rat was able to run at a speed of 10 m/min in the end of familiarization. After that, the rats had at least one day of rest before they performed the pre-training maximal running capacity test. Speed-ramped treadmill running tests were conducted three times to acquire a reliable measure of the baseline performance level. Each test was conducted with a 15° inclination, starting with a speed of 10 m/min without a warm-up. The running speed was increased by 1 m/min every other minute until the rat was exhausted. Note that this test result reflects the baseline maximum running capacity of the rats and no difference was expected (or observed, please see Results) between the HRT and the LRT rat lines.
Two to four days after the last baseline test, aerobic training was started. On the first
day the rats ran at a speed of 10 m/min for 20 min. Speed was gradually increased (1 m/min)
every other session up to a maximum speed of 20 m/min, if the rat was able to keep the speed
during the training session. Also the duration of training was increased 0.5 min every session
up to 30 min per session. Training was done 3 times a week (Mon, Wed, and Fri) for 8
weeks. The treadmill inclination was kept at a constant 15°. Similar to HIT, pushing by hand
and mild electric shocks were used to motivate animals to keep running on the treadmill.

After training, the maximal running capacity was again tested with a procedure identical to
that used during the baseline tests and maximal running speed recorded. Note that at this
point, the HRT rats were expected (and observed, please see Results) to have increased their
maximal running capacity more in comparison to the LRT rats, in which little or no increase
was expected (and observed, please see Results and Figure 2B).

Endurance training

Training on a motorized treadmill was conducted three times per week for 6 weeks, with at
least one day of rest between each of the training sessions. The inclination of the belt was
kept constant at 15° uphill. The first training session lasted 25 minutes and the duration of the
session increased every week by 1 min. The starting speed for training was 60% relative to
the average maximal running speed in each rat line and increased 1 m/min every week.

During each individual training session the treadmill speed was kept constant. Maximal
running capacity was tested once every two weeks and in the end of intervention.

Resistance training

The resistance training protocol was a modification from one used by Hornberger and Farrar
(2004) and the training lasted 6 weeks. The HRT and LRT rats were familiarized to a custom-
made vertical ladder (height x width: 90 x 15 cm, 2 cm separation between steps, 85° incline)
on three occasions during the first week. On the first day the rats climbed without an extra
load. On the next two days a load pouch containing lead weights corresponding to <50% of
the rat’s body weight was fixed in the proximal part of the tail with a double-sided tape and
Velcro strap. Next, the rats began a progressive resistance training period. The training
sessions took place three times a week (Mon, Wed, Fri). The first load was 75% of the body
weight of a rat. When the rat successfully accomplished the climb with this load (1 trial), it
was increased by 30 g and the test was repeated. The load was increased by 30 g increments
until the rat could no longer reach the top of the ladder. The highest load the rat successfully
carried to the top of the ladder was considered as the maximal carrying capacity for that
session. Subsequent training sessions consisted of 9 trials: During the first three climbs, 50%,
75% and 90% of the previous maximal load was used. Then the load was increased by 30 g
until a new maximal load was reached. Three trials were then attempted to conduct with this
new maximal load. Between the climbing trials the rats were allowed to rest for 90 seconds in
an open chamber (length x width x height: 30 x 15 x 11 cm) located at the top of the ladder.
Note that the rats were not punished or rewarded to motivate them to climb.

Experiment 3

Resistance training of young male Sprague-Dawley rats was carried out as explained above
but the duration of the regime was increased to 8 weeks. Sedentary cage mates were placed in
a custom-made plywood box (20 x 30 x 25 cm) with a Plexiglas wall and ceiling, and placed
in the training room for the duration of the training session.

Immunohistochemistry

Adult neurogenesis was studied using doublecortin (all experiments), bromodeoxyuridine
(Experiment 3) and Ki67 (Experiment 3) as markers.
Bromodeoxyuridine (BrdU) injections

BrdU (Sigma, # B5002) was injected i.p. at a dose of 200 mg/kg (15 mg/ml, diluted in physiological saline) to mark dividing cells. BrdU-injections were performed during the first week of training, 2-6 hours prior to the resistance training session. Thus, a total of three injections per rat were administered, once every other day. The dose is comparable to that used in several previous studies to mark dividing cells in the hippocampus, in order to study AHN [see for example (Gould et al., 1999, Nokia et al., 2012)] and no adverse effects of the injections were observed. The dose of 200 mg/kg is high enough to label the majority of dividing cells and yet low enough not to be toxic (Cameron and McKay, 2001). Based on a number of previous reports, ~80% of the hippocampal dividing cells labeled with BrdU mature into neurons indicated by double-labeling for BrdU and neuronal markers such as doublecortin (Dalla et al., 2009), NeuN (Leuner et al., 2010) or TuJ1 (Leuner et al., 2010, Cameron and McKay, 2001).

Tissue preparation

Forty-eight hours after the last training session/exposure to running wheels, animals were euthanized by exposure to a rising concentration of CO₂ and death verified by cardiac puncture. The brain was immediately extracted and post-fixed in 4% paraformaldehyde in PBS solution (pH = 7.4) at +4 °C for 48 h after which the paraformaldehyde solution was replaced by 0.1 M PBS (pH = 7.4). Next, coronal sections (40 µm) were cut through the entire dentate gyrus of the left or right (randomized) hippocampus with a vibratome (Leica VT 1000 S). Every 12th section was collected into a tube filled with cryoprotectant solution (30% sucrose + 30% ethylene glycol in 0.1 M PB, pH = 7.6). The samples were then stored at -20 °C until staining.
Staining

Immunohistochemical staining was performed using free-floating samples. It was made sure that samples from each group to be later compared were stained at the same time. First, the cryoprotectant solution was washed out with 0.1 M PBS (pH = 7.6; 3 x 15 min washes).

Then, necessary steps for blocking and DNA denaturation were performed: For Doublecortin, samples were first subjected to citric acid solution (pH = 6) at +80 °C for 30 minutes and then blocked in H2O2. For Ki67, samples were also boiled in citric acid and then blocked with both H2O2 and normal goat serum. For BrdU staining, samples were first soaked in 2 M HCl for 15 minutes at +37 °C and then blocked with H2O2 and normal goat serum. After these pre-treatments, samples were washed (3 x 5 min) in TBS supplemented with 0.3% Triton X-100 (TBS-T, pH = 7.6). Primary anti-BrdU antibody was obtained from Becton-Dickinson (BD Pharmingen, #347580, made in mouse) as was also the primary antibody for Ki67 (#556003, mouse). A 1:100 dilution was used for BrdU and a dilution of 1:500 for Ki67. Doublecortin staining was performed using an anti-doublecortin antibody from Santa Cruz (sc-8066, goat, dilution: 1:250). Antibodies were diluted into TBS-T (doublecortin) or PBS-T (Ki67 and BrdU) and supplemented with 2% normal goat serum when using a secondary antibody made in goat (see below). Free floating sections were incubated with primary antibodies overnight at room temperature. The next day, samples were first washed with the buffer solution. Then, corresponding biotinylated secondary antibodies (BrdU and Ki67: Abcam, ab98691, goat anti-mouse, dilution: 1:500; doublecortin: Vector Labs, BA-5000, rabbit anti-goat, dilution: 1:500) were diluted into the buffer solution and samples were incubated at room temperature for 2 hrs. Next, samples were again washed with the buffer solution and then incubated in HRP labeled streptavidin (GE Healthcare, RPN1231, dilution: 1:1000) diluted in the buffer solution for 2 hrs at room temperature. Again, samples were washed with the buffer solution. To visualize the immunostaining, samples were immersed in diaminobenzidine solution.
(DAB, D5905, Sigma-Aldrich; 0.25 mg/ml in Tris-buffer, pH = 7.6) supplemented with 0.075% H₂O₂. Reaction was stopped after a maximum of 30 minutes with 0.1 M PB (pH = 7.6) and sections were then mounted on slides using a gelatin solution and dried at +37 °C overnight. Last, samples mounted on slides were counterstained with 0.1% cresyl violet, cleared in xylene, and coverslipped (Depex) [for details on cresyl violet staining see (Nokia et al., 2012)].

**Microscopic analysis**

From the stained slices, estimates of total numbers of BrdU-, Ki67- or doublecortin-labeled cells were obtained with a modified unbiased stereology protocol. The experimenters were unaware of the experimental conditions when counting the cells. In essence, the number of labeled cells in the granule cell layer and the hilus were counted using a light microscope from every 12th unilateral section throughout the dentate gyrus [(one slide per rat, a total of 9 slices, 6.3–1.8 mm posterior to bregma (Paxinos G., 1998)]. The number of cells was multiplied by 24 to obtain an estimate of the total number of labeled cells in the hippocampus. As can be seen from Figure 3 panels C, D and E, the new cells were loosely packed and few so counting them at 400x was deemed justified. As for the fractionator method, even though it might lead to some clusters of cells being overlooked, this should not be systematic in any way, and should thus not affect the results of group comparisons between cell counts. In addition, we were interested in the relative difference between the experimental groups, not so much in the absolute number of cells.

**Statistical analyses**

All statistical analyses were conducted using IBM SPSS Statistics version 22. Repeated measures analysis of variance (ANOVA) was used for analyzing changes across sessions/time. One-way and univariate ANOVA was used for simple group comparisons. Bonferroni corrected p-values were used for post-hoc comparisons. Pearson’s correlation
coefficient (r) was used in analyzing connections between variables. When the sample size was small (< 5), correlations were probed using a nonparametric measure of dependence, the Spearman’s rank correlation coefficient ($r_s$).

Results

Experiment 1. Adult hippocampal neurogenesis was most abundant in HRT rats in response to voluntary wheel running.

Only animals with successful sectioning and staining for doublecortin were included in the analyses. In addition one animal in the LRT-RW group was dropped as it was observed to rotate the RW only with its fore paws thus producing unreliable data on running distance.

Final group sizes are reported below and in each figure. There was no difference between the HRT and LRT rat lines for baseline maximum running speed (one-way ANOVA: $F[1, 61] = 0.57, p = .454$). Accordingly, rats of both lines assigned to the RW groups (HRT-RW: n = 9, LRT-RW: n = 11) ran an equal distance on the first day (one-way ANOVA: $F[1, 18] = 0.763, p = .394$). During the first week of training (7 days, see Figure 1B) running distance increased more in HRT-RW than LRT-RW rats (repeated measures ANOVA: main effect of day: $F[6, 108] = 9.27, p < .001$; main effect of rat line: $F[1, 18] = 17.13, p = .001$; interaction: $F[6, 108] = 5.05, p = .009$). This was to be expected based on the inherently better response to aerobic exercise training in HRT rats. Mean daily running distances across the whole training period for animals in the RW groups are presented in Figure 1C. There was no statistically significant difference for wheel running between the LRT and HRT rat lines (repeated measures ANOVA: main effect of week: $F[6, 108] = 13.4, p < .001$; interaction of rat line and week: $F[6, 108] = 1.38, p = .264$; main effect of rat line: $F[1, 18] = 3.96, p = .062$). Running distance increased in both rat lines across the first few weeks and
then leveled off, in a quadratic manner ($p < .001$). The average total running distance (sum of 7 weeks) was $183 \pm 18$ km for HRT rats and $106 \pm 32$ km for LRT rats.

On average, HRT rats subjected to HIT completed $89\% \, (\pm 4 \, \text{percentage units})$ and LRT rats completed $95\% \, (\pm 1 \, \text{percentage units})$ of training. There was no difference between rat lines (one-way ANOVA: $F [1, 18] = 2.18, p = .157$). Consistently, the total running distances across the 7 weeks of HIT training in HRT and LRT rats were comparable ($F [1, 18] = 0.04, p = .844$) at $6733 \pm 454$ m and at $6824 \pm 241$ m, respectively (note the difference in scale compared to running distances in RW groups). To summarize, LRT and HRT rats exercised to a comparable degree when subjected to HIT.

Consistent with the above, both HIT and voluntary running on a wheel increased running capacity in both rat lines (Figure 1D), whereas little change occurred in the control group (HRT-C: $n = 11$, LRT-C: $n = 12$) (univariate ANOVA: main effect of rat line: $F [1, 57] = 2.19, p = .145$, main effect of training type: $F [2, 57] = 14.24, p < .001$, interaction of rat line and training type: $F [2, 57] = 2.56, p = .086$). Maximal running speed increased the most in response to voluntary wheel running (Bonferroni-corrected post-hoc tests: RW vs. HIT: $p = .048$, RW vs. C: $p < .001$) but there was also a significant improvement in the HIT groups compared to the control groups ($p = .037$). The maximum running speed increased by $3\% \, (\pm 9 \, \text{percentage units})$ in the HRT-C group and by $7\% \, (\pm 6)$ in the LRT-C group. The corresponding percentages for the HRT-HIT and LRT-HIT groups were $29\% \, (\pm 11)$ and $26\% \, (\pm 8)$, respectively. For the HRT-RW and the LRT-RW groups the corresponding change in maximum running speed were $67\% \, (\pm 12)$ and $34\% \, (\pm 8)$, respectively. Planned comparisons between rat lines within each exercise group using one-way ANOVA indicated a significant difference in training response between the HRT-RW and LRT-RW groups ($F [1,18] = 5.74, p = .028$) but not between the HRT-HIT and LRT-HIT groups ($F [1,18] = 0.34, p = .857$). Further, planned comparisons between the two exercise groups within each rat line indicated...
a higher training response in the HRT-RW compared to the HRT-HIT group (F [1, 16] = 5.59, p = .031). A similar difference was not evident in the LRT rats (F [1, 20] = 0.57, p = .459). In conclusion, voluntary running was more effective for demonstrating a differential response to training between LRT and HRT rat lines and for increasing running capacity in HRT rats (see Figure 1D).

Next, we studied possible differences between groups in AHN measured as the number of doublecortin positive cells in the hippocampus. To avoid confounding effects related to time of staining, comparisons were either limited to groups stained at the same time (HRT-C vs. LRT-C), or the number of neurons was standardized relative to the sedentary control group (Sed vs. HIT vs. RW). Figure 1E shows that the number of doublecortin-positive cells in the hippocampi of HRT-C and LRT-C was comparable (one-way ANOVA: F [1, 21] = 0.01, p = .933). That is, LRT and HRT selected lines did not show a difference in AHN in the baseline (i.e. non-trained) condition. Figure 1F, however, shows that HIT and voluntary running affected neurogenesis in differing ways in the HRT and LRT rats (univariate ANOVA, main effect of rat line: F [1, 47] = 27.68, p < .001; main effect of exercise type: F [2, 47] = 28.34, p < .001; interaction: F [2, 47] = 24.81, p < .001). Specifically, there were more doublecortin positive cells in the hippocampi of animals subjected to voluntary running compared to those subjected to HIT (Bonferroni-corrected post-hoc test: p < .001) or those kept sedentary (Sed, p < .001; HIT vs Sed, p = .057). More importantly, compared to HIT or sedentary, there were more doublecortin positive cells in the hippocampi of HRT rats subjected to voluntary running (one-way ANOVA: F [2, 22] = 41.04, p < .001; Bonferroni-corrected post-hoc: RW vs. Sed: p < .001, RW vs. HIT: p < .001, HIT vs. Sed: p = .242). This effect was not present in the LRT rats (one-way ANOVA: F [2, 25] = 1.32, p = .285). That is, voluntary wheel running was most effective in producing a divide between LRT and HRT rats for running capacity (Figure 1D) and also for increasing AHN (Figure 1F).
The relationship between running capacity and AHN was also evaluated. First, we calculated correlations without separating HRT and LRT rat lines. Then, each rat line was analyzed separately. The standardized measure of AHN was used, to avoid any confounding effects related to differences in staining date between rat lines. There was no correlation between post-training maximum running capacity and AHN in rats subjected to HIT (rat lines combined: \( r = .188, p = .427, n = 20 \)). A separate analysis for each rat line confirmed this result (HRT-HIT: \( r = .443, p = .232, n = 9 \); LRT-HIT: \( r = -.146, p = .668, n = 11 \)). There was also no correlation between AHN and the change (%) in maximum running capacity in rats subjected to HIT (combined: \( r = -.309, p = .185, n = 20 \); HRT-HIT: \( r = -.147, p = .706, n = 9 \); LRT-HIT: \( r = -.579, p = .062, n = 11 \)). However, a significant positive correlation was found between the total distance ran during HIT and AHN (rat lines combined: \( r = .538, p = .014, n = 20 \)) (see Figure 4A). Further, this correlation was statistically significant only in the HRT rats (\( r = .797, p = .010, n = 9 \)) and not in the LRT rats (\( r = .100, p = .771, n = 11 \)), when rat lines were analyzed separately (see Figure 4A). That is, the amount of forced aerobic exercise predicted the outcome on AHN only in HRT rats but not in the LRT rats.

The same analyses applied to rats subjected to voluntary wheel running indicated a significant correlation between post-training maximum running capacity and AHN (rat lines combined: \( r = .649, p = .002, n = 20 \)) (see Figure 4B) but not between the change (%) in maximum running capacity and AHN (\( r = .378, p = .100, n = 20 \)). Further examination of each rat line individually revealed no statistically significant correlations between post-training maximum running capacity and AHN (HRT-RW: \( r = .504, p = .167, n = 9 \); LRT-RW: \( r = .545, p = .083, n = 11 \)) (see Figure 4B) or between the change (%) in maximum running capacity and AHN (HRT-RW: \( r = -.481, p = .190, n = 9 \); LRT-RW: \( r = .371, p = .261, n = 11 \)). We also calculated the correlation between the total running distance across 7 weeks and AHN: There was a significant correlation overall (rat lines combined: \( r = .463, p = .014, n = 20 \)).
.040, n = 20, see Figure 4C) but not when each rat line was examined separately (HRT-RW: r = .256, p = .506, n = 9; LRT-RW: r = .219, p = .518, n = 11) (see Figure 4C). Thus, the significant correlation between maximum running capacity and AHN as well that between running distance and AHN reflected the divide in training response between the two rat lines: HRT and LRT.

Experiment 2. More AHN was observed in rats subjected to endurance training compared to those subjected to resistance training

Only animals with successful sectioning and staining for doublecortin were included in the following analyses (final group sizes reported below). All animals in this experiment were first subjected to 8-weeks of a standardized absolute amount of exercise training on a treadmill to measure adaptive response to aerobic training (Koch et al., 2013). Before any training, HRT and LRT animals were able to run at comparable speeds (one-way ANOVA: F [1, 23] = 2.02, p = .168). In response to 8 weeks of training on a treadmill, the maximum running speed increased more in HRT compared to LRT rats (see Figure 2B, change: 15.85% ± 1.35 percentage units vs. 5.43 ± 1.01 percentage units, respectively; one-way ANOVA: F [1, 23] = 37.47, p < .001).

Half of the LRT and HRT rats were then subjected to sustained endurance training on a treadmill for 6 additional weeks performed at 60% relative to maximal running speed. The HRT rats started with a speed of 17 m/min and LRT rats with a speed of 14 m/min (63.5% ± 1.6 percentage units and 58.3% ± 1.4 percentage units of the maximal running speed, respectively). The velocity was increased 1 m/min every week so that at the end of the training HRT rats ran at a speed of 22 m/min and LRT rats ran at a speed of 19 m/min. Thus, the HRT ran a total running distance of 8016 ± 276 m in the HRT rats (n = 8) whereas the LRT rats ran 6972 ± 258 m (n = 7). That is, HRT rats trained longer distances than LRT rats (one-way ANOVA: F [1, 13] = 8.82, p = .011). However, with this relative adjustment, both
rat lines were able to complete the training sessions to an equivalent degree (HRT: 99% ± 1 percentage units vs. LRT: 95% ± 3 percentage units; F [1, 13] = 3.13, p = .100). Endurance training resulted in similar improvements in the maximum running capacity of both HRT and LRT rats (see Figure 2C, rm ANOVA, main effect of exercise: F [1, 13] = 22.75, p < .001; interaction of rat line and exercise: F [1, 13] = 0.07, p = .792). On average HRT rats improved their running capacity by 18.04% (± 6.33 percentage units). The corresponding increase in running capacity for the LRT rats was 17.48% (± 3.43). Overall, maximum running capacity during endurance training was higher in HRT compared to LRT rats (main effect of rat line: F [1, 13] = 4.67, p = .050). This overall difference was due to a difference in relative running speed at the start of the training period (one-way ANOVA: F [1, 13] = 5.37, p = .037). Both HRT and LRT rats ran at comparable speeds during the post-training test (F [1, 13] = 1.87, p = .195).

The other half of the rats that received resistance training on a vertical ladder for 6 weeks demonstrated an increase in strength (measured as the maximum load (g) the rat was able to carry to the top of the ladder) in both LRT and HRT rat lines (see Figure 2D, repeated measures ANOVA: main effect of exercise, pre vs. post training: F [1, 8] = 797.73, p < .001). However the increase in strength was more prominent in the HRT (n = 5) compared to the LRT (n = 5) rats (interaction of rat line and exercise: F [1, 8] = 22.99, p = .001, main effect of rat line: F [1, 8] = 8.19, p = .021). The increase in strength was on average 201% (± 13 percentage units) in the HRT rats whereas in the LRT rats the respective change was on average 147% (± 17).

We also tested if the individual variation in the adaptive response to training during the initial 8-week absolute training period predicted the outcome during subsequent relative endurance or resistance training. We found no correlation between the increase in maximal running speed (%) during the initial absolute training with the increase in running capacity
during continued relative endurance training ($r = -0.044$, $p = 0.875$, $n = 15$) when both rat lines were included in the analysis. However, further analysis done for each rat line separately indicated a significant correlation between the change (%) in maximal running speed during initial training and the increase (%) in running capacity during subsequent endurance training in the LRT rats ($r = 0.942$, $p = 0.002$, $n = 7$) but not in the HRT rats ($r = -0.459$, $p = 0.252$, $n = 8$). This suggests that genetic factors selected for in the high response to aerobic exercise are different from the genetic factors selected for in the low response line.

For resistance training, we found a significant positive correlation between response to training during the initial 8-week absolute training period and the increase in strength (%) during resistance training (rat lines combined: $r = 0.740$, $p = 0.014$, $n = 10$). However, there was no correlation between initial training and resistance training when each rat line was analyzed separately (HRT: $r_s = 0.359$, $p = 0.553$, $n = 5$; LRT: $r_s = 0.400$, $p = 0.505$, $n = 5$). This suggests that while the rat’s performance during aerobic training predicts performance during strength training, the genetic factors selected for within low and high response to aerobic training do not uniquely associate with a response to resistance training.

Last, we examined the effects of endurance vs. resistance training on AHN (see Figures 2E and 2F). Animals subjected to relative endurance training had significantly more doublecortin-positive hippocampal cells compared to animals subjected to resistance training, regardless of rat line (univariate ANOVA, main effect of exercise type: $F [1, 21] = 5.72$, $p = 0.026$; interaction of exercise type and rat line: $F [1, 21] = 0.11$, $p = 0.743$; main effect of rat line: $F [1, 21] = 0.23$, $p = 0.636$). Using correlation analysis we found no relationship between maximum running speed after endurance training and the number of new neurons (rat lines combined: $r = 0.156$, $p = 0.579$, $n = 15$; HRT: $r = 0.212$, $p = 0.614$, $n = 8$ and LRT: $r = 0.082$, $p = 0.861$, $n = 7$) or between the change (%) in maximum running speed due to endurance training and the number of new neurons (rat lines combined: $r = 0.057$, $p = 0.840$, $n = 15$; HRT: $r =
Although the relative training was different for the LRT and HRT, there was also no correlation between the total distance during endurance training and the number of new hippocampal neurons (rat lines combined: \( r = -.334, p = .684, n = 7 \)). In addition, there was no correlation between the absolute strength after resistance training and the number of new neurons (\( r = -.029, p = .917, n = 15 \); HRT: \( r = -.123, p = .771, n = 8 \) and LRT: \( r = .205, p = .659, n = 7 \)). There was no correlation between the absolute strength after resistance training and the number of new neurons (rat lines combined: \( r = -.029, p = .917, n = 15 \); HRT: \( r = -.123, p = .771, n = 8 \) and LRT: \( r = .205, p = .659, n = 7 \)). Separate analyses for each rat line yielded similar results (absolute strength x neurogenesis, HRT/LRT: \( r_s = .600/.791, p = .285/.111, n = 5/5 \) and change in strength x neurogenesis, HRT/LRT: \( r_s = .600/.700, p = .285/.188, n = 5/5 \)). To conclude, endurance training enhanced AHN compared to resistance training independent of the inherited predisposition for low or high response to aerobic exercise.

**Experiment 3. Adult hippocampal neurogenesis in rats subjected to resistance training did not differ from that observed in their sedentary counterparts.**

Eight weeks of resistance training in young adult Sprague-Dawley male rats increased strength by approximately 250% (± 8 percentage units) (see Figure 3B, paired-samples test: \( t(9) = 58.55, p < .001 \)). Note that the 10 animals subjected to resistance training increased absolute carrying capacity similar to the HRT but performed within a much narrower range both before and after training (see Figure 3B). Compared to sedentary control rats, rats subjected to resistance training gained less bodyweight during the 8-week training period (repeated measures ANOVA: interaction of group and time: \( F[1, 18] = 10.44, p = .005 \); main effect of group: \( F[1, 18] = 4.33, p = .052 \); main effect of time: \( F[1, 18] = 444.26, p < .001 \)). The mean bodyweight in rats subjected to resistance training increased from 293 ± 2 g to 386 ± 7 g whereas in the sedentary control group bodyweight increased from 295 ± 4 g to 421 ± 12 g.
The effects of resistance training on proliferation (Ki67), maturation (doublecortin) and survival (BrdU) of new adult-born hippocampal neurons are presented in Figure 3 panels C, D and E, respectively. Analysis of Ki67-positive cell counts indicated no difference in cell proliferation between the sedentary and the trained group (one-way ANOVA: F [1, 18] = 0.12, P = .736). The same was true for the number of immature doublecortin-positive neurons (F [1, 18] = 0.71, p = .412). This result was corroborated by the fact that there was also no difference in the number of hippocampal BrdU-positive cells between the groups (F [1, 16] = 0.35, p = .563) indicating no effect of resistance training on the survival of adult-born new cells in the hippocampus. Note that one animal in each group had to be excluded from the BrdU-analysis due to nonexistent staining. Similar to what was reported in Experiment 2, there was no consistent correlation between the change in strength (%) and neurogenesis (Ki67/doublecortin/BrdU: r = -.262/-0.036/-0.253, p = .465/.922/.512, n = 10/10/9). Due to the very small variation in absolute strength after training (see Figure 3B), correlations between it and neurogenesis were not calculated. To summarize, compared to sedentary controls, 8 weeks of anaerobic resistance exercise on a vertical ladder had no effect on AHN in young adult male Sprague-Dawley rats.

Discussion
Adult hippocampal neurogenesis is a continuous process that contributes to a variety of adaptive behaviors such as learning [for review see (Aimone et al., 2014)]. A well-demonstrated means of promoting AHN in rodents is aerobic exercise, namely running (van Praag et al., 1999). Here, we studied whether the effects of high-intensity interval training (HIT) or resistance training are comparable to aerobic exercise for promoting AHN in male rats. In line with previous work, our studies show the number of adult-born hippocampal neurons was higher in animals subjected to sustained aerobic exercise, namely voluntary wheel running or treadmill endurance running compared to sedentary control animals.
Contrary to previous findings (Novaes Gomes et al., 2014), resistance training did not promote AHN compared to sedentary controls, while HIT had a smaller than expected effect on AHN, that did not reach statistical significance. Our development of a rat model system for low (LRT) and high (HRT) response to aerobic exercise training (Koch et al., 2013) also provided us an opportunity to study whether a genetic predisposition for adaptive capacity for aerobic trainability relates to the beneficial effects of physical exercise on AHN. Our data indicate highest numbers of adult-born hippocampal neurons in rats selectively bred for a high response to aerobic exercise that ran voluntarily on running wheels. That is, AHN is highest in animals born with a tendency for a higher response to exercise training and that engage in a high amount of voluntary aerobic activity.

*A rat model system of low and high response to training*

In Experiment 1 summarized in Figure 1, we discovered that when provided unlimited access to voluntary wheel exercise HRT rats increased running distance and improved running speed more than the LRT rats. Accordingly, the amount of AHN was significantly higher in HRT rats compared to LRT rats. This was expected based on the inherently differential responses to aerobic exercise training in the two rat lines (Koch et al., 2013). The LRT and HRT rats were not different in their capacity to complete HIT training or in the effects of HIT on AHN. Experiment 2 (see Figure 2) shows that with 8 weeks of endurance treadmill training using a standard absolute speed-ramped aerobic protocol, the HRT rats gain significantly more capacity for exercise compared to the LRT rats. Further, when resistance trained using a load of 50-90% relative to maximal load, performed 3 days a week for 6 weeks, the HRT rats gained significantly more strength than the LRT rats. Interestingly, an equivalent increase in maximum running capacity in both rat lines was observed in response to relative endurance training on a treadmill performed at ~60% of maximum running capacity and conducted following the initial 8-week training period. This suggests animals with an inherently low
response to training are capable of improving their performance if aerobic training is
continued for an extended period of time and accommodated to their performance level. With
this training set-up, more AHN was observed in rats subjected to sustained endurance training
versus those performing resistance training. Thus, we demonstrate that the HRT-LRT rat
model system provides a unique substrate for investigating the physiological and molecular
connections between variation in response to exercise training and AHN.

HIT had a smaller than expected effect on AHN
To our knowledge, our study is the first to address whether an exercise increasing in
popularity among humans, namely HIT [see for example (Gibala et al., 2012)], has effects on
AHN. Although HIT training is shown in some cases to be superior for increasing
cardiovascular fitness over moderate endurance training (Afzalpour et al., 2015, Haram et al.,
2009), HIT had a smaller than expected effect on AHN in our current study. Despite HIT
improving running capacity for both LRT and HRT rats, it did not affect AHN to a
statistically significant degree, although Figure 1E suggests a trend towards an increase in the
number of new neurons for both LRT and HRT rats. This is somewhat surprising taken that
in Experiment 2 we did find increased AHN in animal subjected to endurance training on a
treadmill when compared to that observed in animals subjected to anaerobic resistance
ing training (please see next section). It might be that if we had used a longer training session
during HIT, we would have observed bigger improvements also in AHN. For example Haram
et al. (Haram et al., 2009) used 60-minute sessions of HIT and 2-hr sessions of continuous
running on a treadmill in their study reporting greater benefits of HIT compared to
continuous exercise in reducing a number of cardiovascular risk factors.

Our current results on the (lack of) effects of HIT on AHN comply with a recent report
of greater improvements in AHN in terms of maturation of new neurons in response to 6
weeks (60 min/day, 5 days a week) of mild (speed: 15 m/min) rather than intense (40 m/min)
forced running on a treadmill in adult male Wistar rats (Inoue et al., 2015). Inoue and colleagues (2015) propose their results might be explained by differences between the two forms of exercise in activating certain genes related to lipid metabolism, protein synthesis and inflammation in the hippocampus and also by the higher level of stress induced by intense exercise compared to mild exercise. In a recent study, the same group studied the effects of mild (15 m/min) versus intense (30 m/min) running on a treadmill (30 min/day, 5 days a week) in adult male C57BL/6J mice and found elevated plasma corticosterone levels in the intensely trained group compared to a sedentary control group (Okamoto et al., 2015). More importantly, the same group reports observations of increased hippocampal BDNF transcription (Soya et al., 2007) and AHN (Okamoto et al., 2015) only in response to mild but not intense exercise. Interestingly, blocking mineralocorticoid and glucocorticoid receptor function seems to attenuate the increase in AHN induced by mild exercise suggesting some level of corticosterone action is needed to support elevations in AHN (Okamoto et al., 2015). This is in agreement with an observation of enhanced AHN in response to long-term predictable mild stress (Parihar et al., 2011).

Based on the above, stress might have dampened the effects of forced treadmill training, especially HIT, on AHN in our study [for a recent review see (Lucassen et al., 2015)]. Actually, the number of cells positive for doublecortin in our current experiments overall was relatively low compared to other reports of neurogenesis in adult rats using the same primary antibody and counting protocol [see for example (Winocur et al., 2014)]. To summarize, it seems that sustained (mild) aerobic exercise rather than HIT increases AHN in HRT and LRT rats, and the role of stress warrants further investigation.

**Resistance training does not promote AHN**

Our findings using the LRT and HRT rat lines as well as our results testing commercially available Sprague-Dawley rats are contrary to a previous finding (Novaes Gomes et al., 2014)
indicating that resistance training promotes AHN. Our current data indicate that resistance
training does not affect the proliferation, neuronal maturation or the survival of adult-born
new cells in the hippocampus although an improvement in strength is evident. This is in
direct contrast to a recent study reporting a higher number of proliferating cells in the
hippocampi of male Wistar rats subjected to 4 weeks of resistance training according to a
regime similar to that used in our current study (Novaes Gomes et al., 2014). It is possible
that the effects of resistance training are transient in nature, perhaps only increasing cell
proliferation early in training. This could explain why no effects were evident when
neurogenesis was evaluated after 6 (Experiment 2) or 8 (Experiment 3) weeks of training in
our current study. Note that in our current experiments we did not use any external motivators
to encourage rats to perform resistance training. Thus, it is unlikely that stress alone would
explain why we did not observe an increase in AHN in response to resistance training [for a
recent review see (Lucassen et al., 2015)]. This applies especially to Experiment 2 where
comparisons were made between rats trained on a treadmill (with shocks) and those trained
on a vertical ladder (without shocks). It may in fact be that mild stress during the forced
treadmill training may have contributed towards an increase in AHN (Parihar et al., 2011).
The role of stress should be further investigated in future experiments of similar kind.

Our results on the effects of resistance training on AHN comply with and could also in
part be explained by the previously reported effects of anaerobic exercise on neural growth
factors: Resistance training on a vertical ladder 5 times per week (8 climbs per session) did
not increase BDNF expression in the hippocampus compared to sedentary controls as
measured after 8 weeks of training (Cassilhas et al., 2012a). As it turns out, BDNF has a
crucial role in regulating AHN (Scharfman et al., 2005, Sairanen et al., 2005) and normal
BDNF-function appears to be a requisite for the exercise-induced increase in AHN (Li et al.,
2008). In our current study (Experiment 2) 30 minutes of forced aerobic endurance training
on a treadmill 3 times a week led to higher numbers of new immature hippocampal neurons
compared to that observed in animals engaged in resistance training, according to the same
schedule. Thus, it seems that aerobic exercise is more efficient in persistently increasing
AHN than anaerobic exercise, at least when carried out for extended periods.

All in all, and in accordance with previous knowledge, our results imply that in order to
maximally promote AHN, exercise should be aerobic and sustained. Previous studies
reviewed above further suggest that the exercise induced increases in AHN are dependent on
enhanced BDNF function. In the future, to further clarify exercise effects on AHN,
comparable dose-response studies with different training regimens should be performed.

The amount of running correlates with AHN

In accordance with several previous reports on the beneficial effects of running on AHN and
cognition in rodents [for a review see (Vivar et al., 2013)], in our current study forced
endurance training on a treadmill as well as voluntary running in a running wheel led to a
higher number of immature adult-born hippocampal neurons compared to that observed in
animals not engaged in aerobic exercise. Furthermore, we find daily voluntary running on a
running wheel increased AHN considerably more than 30 minutes of forced endurance
training on a treadmill three times a week. This is not surprising considering the positive
correlation between distance run and AHN (Allen et al., 2001), a link also evident in our
current data (Experiment 1, see Figure 4). Note that running on a treadmill at a steady speed
of 40 m/min (the maximum speed for some of the best runners in our experiments) for 30
minutes amounts to 1200 meters, whereas animals typically run several kilometers daily
when given free access to a running wheel. To summarize, the amount of aerobic exercise
may be crucial to its effect on AHN.

The correlation between running distance and AHN might be explained by considering
the consequences of running (in a more naturalistic setting): The further an individual travels,
the more likely it is to encounter new environments and stimuli from which it must make
sense rapidly. It could be that the act of running per se, even if performed in a wheel or on a
treadmill, primes the brain to take on the changes in external environment by, for example,
increasing AHN. This notion is supported by findings indicating that new hippocampal
neurons are especially important for learning (Shors et al., 2001) and pattern separation
(Clelland et al., 2009), animals performing worse in these tasks if AHN is disrupted.

One should also note that the positive effects of prolonged aerobic exercise on adult
neurogenesis are not limited to just the hippocampus as increases in adult neurogenesis have
been reported also in the subventricular zone (Bednarczyk et al., 2009, Chae et al., 2014) and
in the hypothalamus (Niwa et al., 2015). These anatomically widespread increases in adult
neurogenesis in response to sustained aerobic exercise suggest that in addition to mechanisms
local to the hippocampus, such as increased androgenic function (Okamoto et al., 2012), a
common mediator, the most obvious candidate being increased blood flow, must exist to
support AHN. A large proportion of proliferating hippocampal cells is located in the vicinity
of small capillaries (Palmer et al., 2000). In addition, aerobic exercise increases blood flow
specifically in the dentate gyrus in both humans and in mice (Pereira et al., 2007). This
increase in blood flow likely increases both metabolic and trophic support to the neurogenic
niche. In fact, previous studies on the hippocampus indicate that in rodents running distance
also correlates positively with BDNF expression [see for example (Johnson et al., 2003)] and
anaerobic exercise fails to upregulate BDNF [(Cassilhas et al., 2012a), see also (Soya et al.,
2007)]. Further, BDNF expression and function is also elevated by increased neuronal
activity [for a recent review see (Rothman and Mattson, 2013)]. Related, the effect of
learning on the survival of new adult-born hippocampal neurons (Gould et al., 1999) appears
to depend on the amount of training: Generally, the more effort learning a certain task takes,
and, presumably, the longer the hippocampal engagement in the learning process, the more
new hippocampal neurons survive in response to learning [for a review see (Shors et al., 2012), see also (Sisti et al., 2007)].

Taken together, it seems that training (whether cognitive or physical) should be sustained to most effectively promote AHN. However, the underlying fundamental biological mechanism of how exercise enhances AHN is controversial. The precise regulatory mechanisms where the gene variants and exercise inputs interact are unknown but likely involve networks of modifier gene interactions that link increases in blood flow, signaling pathways, neuronal activity and neurotrophic action.

Limitations

Some limitations of our current study warrant further discussion. We conducted studies exclusively on adult male rats. However, as previous studies in females (van Praag et al., 1999, Marlatt et al., 2012) and in aged animals (van Praag et al., 2005) indicate an enhancement of both AHN and cognition in response to running, our results could be assumed to apply to both sexes and to older rodents as well. Our decision to test males only was a direct result of the high workload required for animal exercise training studies and our interest in conducting a comparable study for AHN in response to resistance training that was reportedly done in adult male rats. Of course, in future studies both sexes and animals of different ages should preferably be tested. Second, in terms of inter-species applicability, several studies have reported larger hippocampal volume and better cognitive ability in physically active/fit older adult humans (Varma et al., 2015, Erickson et al., 2011, Erickson et al., 2009). In a recent study conducted in mice, hippocampal gray matter volume correlated strongest with the number of hippocampal doublecortin-positive cells (Biedermann et al., 2014) suggesting that larger hippocampal volume might be for the most part a product of enhanced AHN. Tentatively, it would seem that physical exercise affects hippocampal structure and function in humans in a manner resembling that observed in laboratory rodents.
[see also (Hauser et al., 2009)]. Last, although we did not directly measure motivation to exercise in our current experiments, its role in mediating the beneficial effects of physical activity on the brain should not be overlooked. In terms of forced training and testing maximal performance capacity, we did not observe any difference between the HRT and LRT rat lines in their motivational behavior on the treadmill or the vertical ladder.

**Conclusions**

Our results add to the body of literature indicating sustained aerobic exercise increases AHN and advances this field of study in several ways. First, we tested several different forms of physical exercise to study their effects on AHN. We also took advantage of a newly developed genetically heterogeneous contrasting rat model system that we selectively bred for low and high response to aerobic training to take into account genetic variation for training responsiveness. According to our findings, anaerobic resistance training does not affect AHN in the studied animals, despite its overall positive effects on physical fitness. Second, the effects of exercise on AHN depend, at least to some extent, on sustained aerobic activity as HIT did not have statistically significant effect on AHN. Lastly, the highest numbers of adult-born hippocampal neurons were observed in rats selectively bred for a high response to aerobic exercise that ran voluntarily on running wheels. Thus, for all reasons combined, AHN is highest in animals born with a tendency for a higher response to exercise training engaging in a high amount of voluntary aerobic activity.
References


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Competing interests

The authors state no competing interests.

Author contributions

The experiments were performed at the Department of Psychology and Department of Biology of Physical Activity at the University of Jyväskylä.

MSN acquired, analyzed and interpreted the data and wrote the report. SL designed the work, acquired and analyzed the data and revised the report. JPA designed the work, acquired the data and revised the report. PPJ acquired and analyzed the data and revised the report.

LGK designed the animal model, interpreted the data and wrote the report. SLB designed the animal model, interpreted the data and revised the report. HK designed the work and revised the report.

All authors have approved the final version of the manuscript, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Figure 1. Experiment 1 indicated that the number of doublecortin-positive new neurons was highest in rats genetically predisposed to a high response to training and in which aerobic fitness consequently increased significantly in response to voluntary wheel running. A) Outline of the experiment. IHC = Immunohistochemistry. B) Running in rats allowed access to running wheels (RW) across the first 7 days. HRT rats increased running more compared to LRT rats. C) Across the whole 7-weeks training period running distance was comparable in both rat lines and changed in a quadratic manner. D) Running speed increased in both LRT and HRT rats in response to either HIT or RW. The biggest improvement was seen in HRT rats voluntarily training on a running wheel. E) There was no baseline difference in adult hippocampal neurogenesis in the two rat lines. F) However, when allowed to run freely on a wheel in the home cage for 7 weeks, the HRT rats showed a higher number of new neurons in the hippocampus compared to rats of the same rat line either not trained at all (Sed) or subjected to HIT 3 times a week. G) Representative examples of doublecortin-positive cells (brown) in the dentate gyrus in each group of rats are illustrated. The background stain (cresyl violet) shows cell bodies of mature neurons in light blue. The hilus and the granule cell layer (gcl) are indicated in the top-left panel. The scale bar length is 50 microns and the scale is the same in all panels (400x). Insets depict cell groups positive for doublecortin in 1000x. Arrows point to these cells in the 400x photos. In panels B-F, vertical lines depict standard error of mean. Asterisks refer to statistically significant effects/differences: *** p < .001, ** p < .01, * p < .05.

Figure 2. Experiment 2 indicated more doublecortin-positive new hippocampal neurons in rats subjected to endurance training on a treadmill compared to rats subjected to resistance training on a vertical ladder. A) Outline of the experiment. IHC = Immunohistochemistry. B) HRT rats showed a greater adaptive response to aerobic training,
i.e. their (%) change in maximum running speed increased more compared to LRT rats. C) Subsequent endurance training on a treadmill resulted in a comparable increase in maximum running speed in both rat lines. However, HRT outperformed LRT rats overall. D) Resistance training lead to a bigger improvement in strength in HRT compared to LRT rats. E) Rats subjected to endurance training (regardless of rat line) had a greater number of new neurons in the hippocampus. F) Representative examples of doublecortin-positive cells (brown) in the tip of the dentate gyrus in each group of rats. The background stain (cresyl violet) shows cell bodies of mature neurons in light blue. The hilus and the granule cell layer (gcl) are indicated in the top-left panel. The scale bar length is 50 microns and the scale is the same in all panels (400x). Insets illustrate the doublecortin-positive cells pointed to by black arrows in 1000x.

In panels B-E, vertical lines depict standard error of mean. Asterisks refer to statistically significant differences: * p < .05, *** p < .001.

Figure 3. Experiment 3 indicated no effect of 8 weeks of resistance training on a vertical ladder on adult hippocampal neurogenesis in male Sprague-Dawley rats. A) Outline of the experiment. IHC = Immunohistochemistry. B) Strength increased in all rats subjected to resistance training (Res). Asterisks refer to a significant difference (p < .001). Vertical lines depict standard error of mean. C) The proliferation of cells in the hippocampus was similar in the Res and the sedentary (Sed) groups. Black arrows mark the locations of clusters of Ki67-positive cells (400x) illustrated in the insets (1000x). The background stain (cresyl violet) shows cell bodies of mature neurons in light blue. The hilus and the granule cell layer (gcl) are indicated in the top panel. The scale bar length is 50 microns and it applies to all the 400x photos. D) Animals subjected to resistance training and those kept sedentary had a similar number of immature adult-born new neurons in the hippocampus. Doublecortin-positive cells in brown, mature neurons in light blue. E) The survival of adult-born neurons in the
hippocampus was also not affected by resistance training. BrdU-positive cells in brown, mature neurons in light blue.

**Figure 4. Post-training maximum running capacity and total running distance correlate with adult hippocampal neurogenesis in adult male rats.** All data in this figure are from Experiment 1. A) The greater distance an animal ran during HIT training on the treadmill across 7 weeks of training, the more doublecortin-positive new neurons were present in the hippocampus at the end of the experiment. B) The higher the maximum running capacity of a given rat subjected to voluntary running on a running wheel (RW) was at the end of the training period, the more new neurons were found in the hippocampus. C) The greater distance an animal voluntarily ran in the running wheel across the 7 weeks of training (RW), the more doublecortin-positive new neurons were present in the hippocampus at the end of the experiment.
A) 7 weeks
- Sedentary (C and Sed)
- High-intensity interval training (HIT)
- Running wheel (RW)

B) Running (km)
- HRT-RW, n = 9
- LRT-RW, n = 11

C) Running (km/day)
- HRT-RW, n = 9
- LRT-RW, n = 11

D) Maximum running speed (m/min)
- HRT-C
- HRT-HIT
- HRT-RW
- LRT-C
- LRT-HIT
- LRT-RW

E) # of Doublecortin+ cells
- LRT-C
- HRT-C

F) # of Doublecortin+ cells relative to sedentary controls (Sed)
- LRT
- HRT

G) Images of Doublecortin+ cells in different conditions:
- LRT-C
- LRT-Sed
- LRT-HIT
- LRT-RW
- HRT-C
- HRT-Sed
- HRT-HIT
- HRT-RW
**A**

HRT and LRT male rats, ~6 mo

8 weeks

Running on a treadmill to determine response to aerobic exercise

6 weeks

Endurance training (End)

Resistance training (Res)

Hippocampus: IHC for Doublecortin

---

**B**

Change (%) in maximum running speed

<table>
<thead>
<tr>
<th>Rat line</th>
<th>LRT</th>
<th>HRT</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>13</td>
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</tbody>
</table>

***

**C**

Maximum running speed (m/min)

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre Endurance training</th>
<th>Post Endurance training</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRT-End, n=8</td>
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<td>36</td>
</tr>
<tr>
<td>LRT-End, n=7</td>
<td>29</td>
<td>32</td>
</tr>
</tbody>
</table>

**D**

Maximum weight carried (g)

<table>
<thead>
<tr>
<th>Group</th>
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<th>Post Resistance training</th>
</tr>
</thead>
<tbody>
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<td>HRT-Res, n=5</td>
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<td>1800</td>
</tr>
<tr>
<td>LRT-Res, n=5</td>
<td>1200</td>
<td>1400</td>
</tr>
</tbody>
</table>

---

**E**

Number of Doublecortin+ cells

<table>
<thead>
<tr>
<th>Rat line</th>
<th>Endurance training</th>
<th>Resistance training</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

* ***

**F**

Images showing Doublecortin+ cells in different regions (gcl, hilus) for LRT-End, LRT-Res, HRT-End, HRT-Res.