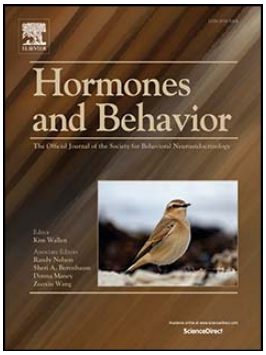

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Restricted vs. unrestricted wheel running in mice: effects on brain, behavior and endocannabinoids

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Abstract

Beneficial effects of voluntary wheel running on hippocampal neurogenesis, morphology and hippocampal-dependent behavior have widely been studied in rodents, but also serious side effects and similarities to stereotypy have been reported. Some mouse strains run excessively when equipped with running wheels, complicating the comparability to human exercise regimes. Here, we investigated how exercise restriction to 6 hours/day affects hippocampal morphology and metabolism, stereotypic and basal behaviors, as well as the endocannabinoid system in wheel running C57BL/6 mice; the strain most commonly used for behavioral analyses and psychiatric disease models. Restricted and unrestricted wheel running had similar effects on immature hippocampal neuron numbers, thermoregulatory nest building and basal home-cage behaviors. Surprisingly, hippocampal gray matter volume, assessed with magnetic resonance (MR) imaging at 9.4 Tesla, was only increased in unrestricted but not in restricted runners. Moreover, unrestricted runners showed less stereotypic behavior than restricted runners did. However, after blockage of running wheels for 24h stereotypic behavior also increased in unrestricted runners, arguing against a long-term effect of wheel running on stereotypic behavior. Stereotypic behaviors correlated with frontal glutamate and glucose levels assessed by ^{1}H-MR spectroscopy. While acute running increased plasma levels of the endocannabinoid anandamide in former studies in mice and humans, we found an inverse correlation of anandamide with the daily running distance after long-term running. In conclusion, although there are some diverging effects of restricted and unrestricted running on brain and behavior, restricted running does not *per se* seem to be a better animal model for aerobic exercise in mice.

Keywords: Endocannabinoids, anandamide, exercise, physical activity, neurogenesis, stereotypy, MR volumetry, MR spectroscopy, hippocampus, C57BL/6 mice

Introduction

Wheel running in rodents is a model for aerobic exercise training in humans and one of the mostly studied animal models in general (Richter et al. 2014). It is often used to demonstrate mechanisms for the beneficial effects of exercise training (Coleman and Rager 1993; Dishman et al. 1995). In contrast to forced models of physical activity (e.g., treadmill running), wheel running rodents are allowed to run on a voluntary basis and thus wheel running has often been referred to as an animal model for human physical exercise (Richter et al. 2014). However, some mouse strains perform wheel running excessively and run almost throughout their entire active phase, covering up to 12 kilometers per day (Fuss et al. 2010b; Lightfoot et al. 2004). It is thus obvious that the degree of wheel running highly exceeds common human exercise regimens and it could be argued that the excessive amount of wheel running in mice may limit its comparability to aerobic exercise in humans. Some researchers have therefore favored forced exercise models, as the standardized and time-restricted protocol appears more comparable to 'human workout' (Burghardt et al. 2004; Leasure and Jones 2008). On the other hand, forced exercise can be stressful for rodents and can have detrimental rather than protective effects on mortality (Cook et al. 2013).

Concerning neurobiological consequences of exercise, the hippocampus is the brain structure that is probably most highly affected by wheel running in rodents. At a cellular level, voluntary wheel running increases hippocampal neurogenesis in the dentate gyrus (DG) of mice (Fuss et al. 2010b; van Praag et al. 1999). At a macroscopic level, hippocampal gray matter volume measured with magnetic resonance (MR) imaging was found to be increased after wheel running in mice (Biedermann et al. 2012; Fuss et al. 2014). An increase of hippocampal volume was also found after exercise in humans (Demirakca et al. 2014; Erickson et al. 2011). This increase highly correlates with immature hippocampal neuron numbers (Biedermann et al. 2016) and glutamate levels in mice (Biedermann et al. 2012). When hippocampal neurogenesis is ablated, hippocampal gray matter increase is absent after running (Biedermann et al. 2016; Fuss et al. 2014).

All these rodent studies, however, were performed with voluntarily running mice. Therefore, it is an important question whether an exercise regimen which is more comparable to exercise in humans (e.g. running restriction to several hours per day) has the same or different impact on these findings within the hippocampal structure.

In the present study, we were thus aiming to study restricted running in mice. In restricted runners, running wheels were only accessible for 6 hours per day. We hypothesized that this might elicit a different neurobiological and behavioral response in comparison to forced or voluntary running, as it does not allow excessive running while mice still run voluntarily. Since the hippocampus seems to be the most critical brain structure to detect effects of wheel running, we hypothesized that restricted

running might differently affect hippocampal gray matter as compared to unrestricted running. Since running-related hippocampal gray matter increase depends on adult neurogenesis (Biedermann et al. 2016), we also studied how running restriction affects the number of newborn hippocampal neurons. Moreover, we expected altered hippocampal-dependent behaviors as a consequence of running induced changes in brain structure. In an exploratory attempt, we thus performed assessments of stereotypic and basal home-cage behaviors (Latham 2006; Pawlowicz et al. 2010; Richter et al. 2008; Bult et al. 1993; Bult et al. 1992; Carter et al. 2000). As it was shown that endocannabinoids (i) mediate some of the beneficial effects of acute exercise (Fuss et al. 2015), (ii) seem to be crucial for the motivation to perform wheel running in the long-term (Dubreucq et al. 2013; Fuss and Gass 2010; Fuss et al. 2015; Raichlen et al. 2012; Sparling et al. 2003) and (iii) may play a role in the regulation of hippocampal neurogenesis (Hill et al. 2006), we also measured plasma endocannabinoids in an exploratory design.

In our study, C57BL/6J mice with running restriction to 6 hours /day (restricted runners=RR) were compared to unrestricted runners (R) and sedentary controls (C) after long-term running.

Materials and methods

Animals

A total of 30 C57BL/6J male mice were obtained at the age of 4 weeks from Charles River (Sulzfeld, Germany). Mice were acclimatized for four weeks and subsequently single-housed in Macrolon type III cages. At the age of 10 weeks, all mice were divided into runners (R), restricted runners (RR) and sedentary controls (C). Runners were given free access to a running wheel, restricted runners were given six hours per day access to running wheels beginning at 9 am. Wheels were blocked with a small piece of wire at 3 pm each day. Controls were supplied with blocked wheels as described before (Fuss et al. 2013b). Running distance was measured with a cycle computer attached to the cage lid (Digi Speed 5, Cycling Data System, Atech Scientific Measurement Ltd, Hongkong). Mice were individually housed in a temperature and humidity controlled room, on a 12 h dark-light cycle with lights on at 9 pm. Water and food were available ad libitum. Handling, behavioral testing and MR imaging of the mice were done during the dark phase. Full details of the study had been approved by the German animal welfare authorities (Regierungspräsidium Karlsruhe) and the principles of laboratory animal care (NIH publication No. 86–23, revised 1985) were followed.

Nest building test

The nest building test was performed after 4 weeks of running as described previously (Deacon 2006). Animals were housed for 1 week without the standard nesting material (paper tissue). At the

beginning of the dark phase, a Nestlet of 3 g compressed cotton (Ancare, Bellmore, NY) was placed in the middle of the cage. After 5h and after 24h the nests were assessed on a rating scale of 1 – 5 by two independent raters: 1 = Nestlet > 90 % intact, 2 = Nestlet 50-90 % intact, 3 = Nestlet mostly shredded but no identifiable nest site, 4 = particles in round shape but flat nest, 5 = crater-shaped nest. Inter-rater reliability was calculated using the single-measure intraclass correlation coefficient (ICC) with the two-way random effects model absolute agreement type. We found an excellent interrater reliability after 5 hours (0.974) and a good interrater reliability after 24 hours (0.691) according to Fleiss (Fleiss 1981). For statistical analyses the mean of both raters was used.

Home cage behavior

Undisturbed behavior in home cages was monitored by video observation. Each cage was recorded for 23 h after 5 weeks of wheel running. Unrestricted runners were studied twice: First with open access to wheels (R) and second with blocked wheels (withdrawn runners = WR). Restricted runners and controls were studied only once. We used 8 VC-FCYW-WIDE-RS485 cameras (VC Germany) simultaneously and recorded on 24-channel VC-recorders. Subsequently, all behavioral observations were analyzed manually in real time by the same researcher using Mangold Interact 9 software. Focal animal sampling (Martin 1993) was used throughout the analysis to record the frequencies or durations, or both, of all elements of laboratory mouse behavior. Each mouse was observed continuously according to the following time schedule: four sessions of 7.5 min each were analyzed in the light phase (starting at 11 pm, 2 am, 5 am, and 7 am) and six sessions of 15 min each during the dark phase (starting at 10 am, 12 am, 2 pm, 4 pm, 6 pm, and 8 pm), thus yielding 120 min undisturbed home cage activity per mouse in each condition (C, RR, R, WR). All observed behaviors were categorized according to established protocols (Abou-Ismail et al. 2010; Fuss et al. 2013a) with minor modifications (Suppl. Table 1). We analyzed 1) general activities like feeding, drinking, grooming, climbing, rearing for food, and other rearings; 2) bedding material manipulation (=burrowing), 3) running wheel directed behaviors like running, route-tracing, wheel climbing, wheel sitting, wheel cradling; 4) sleeping, and 5) stereotypic behaviors like bar-mouthing, circular jumping (=twirling), and circular climbing (=circling).

Magnetic resonance imaging and spectroscopy procedures

All animals were measured after the running/sedentary period of ~9 weeks in a 9.4 T horizontal bore animal scanner (Bruker, Rheinstetten, Germany) equipped with a two element anatomically shaped cryogenic mouse surface coil cooled to 28 K. Mice were anesthetized by a gas mixture of O2: 50 % and air: 50 % with approximately 1.8 % isoflurane. Respiration rate was monitored throughout the

experiment. Body temperature was maintained at 37°C by warm water circulation using an external coil-heater and verified by a rectal thermosensor. High resolution 3D structural data were acquired using a T2-weighted RARE-sequence (Rapid Acquisition with Refocused Echoes, RARE factor 16) with a resolution of 78 × 78 × 156 µm³ at TE = 50 ms and TR = 1.2 s.

For MR-volumetric analyzes a group template of tissue classification maps (gray matter, white matter and cerebrospinal fluid) was created from all 3D images involving post processing with SPM8 (Wellcome Department of Cognitive Neurobiology, University College of London, UK) and FSL (Smith et al. 2004) as described before (Biedermann et al. 2012). Afterwards the individual images were post processed, segmented and normalized (for protocol see (Biedermann et al. 2012)). Hippocampal gray matter volume was calculated with the help of a mask using the image calculation function in SPM8 resulting in an estimated value given in mm^3 (for detailed description please see (Biedermann et al. 2012)).

^{1}H-MR spectra were acquired using a PRESS (point resolved spectroscopy) sequence (echo time (TE) = 10 ms; repetition time (TR) = 4 s; 256 averages) from a 3.2 µl volume (2.2×1.2×1.2 mm³) placed in the right hippocampus and from a 2.5 µl volume (1.6 × 1.2 × 1.3 mm³) placed in the area corresponding to the right prefrontal cortex with a scan time of 17 min per spectra. To exclude partial volume the voxel was angulated. To minimize chemical shift displacement artifacts the PRESS sequence was modified to deliver the slice selective excitation and refocusing pulses with a frequency shift of −2 ppm referred to the water peak. Using LCModel (LCModel, Provencher, ver. 6.2-0R), 16 ^{1}H-MRS metabolites were quantified and referenced to an unsuppressed water signal acquired from the same voxel with no frequency shift. Metabolites were further corrected for spatial tissue compartment using an in-house algorithm as described in (Auer et al. 2015). We focused on the following metabolites: N-acetylaspartate + N-acetylaspartylglutamate (summed as total NAA = tNAA), creatine + phosphocreatine (summed as total creatine = tCr), glycerophosphocholine + phosphocholine (summed as total choline = tCho), glutamate (Glu), glutamine (Gln), the sum of Glu + Gln (Glx), taurine (Tau), γ-aminobutyric acid (GABA), myoInositol (mI), glucose (Glc) and lactate (Lac). One frontal ^{1}H-MR spectrum from one mouse of the running group was excluded due to bad spectra quality. Results of the ^{1}H-MRS are depicted in table 1.

Immunohistochemistry and quantification of immature hippocampal neurons and proliferating cells

Directly after MR imaging mice were anesthetized by i.p. injection of ketamine and xylazine, blood for plasma analysis was sampled from the right heart ventricle and mice were perfused transcardially. Brains were removed, postfixed for 8 h in 4 % paraformaldehyde, and kept in PBS

overnight. Coronal sections (40 µm) were cut on a vibratome and kept at -20°C in cryo protection solution until further processing. Every twelfth section was processed free floating. To evaluate cell proliferation and immature hippocampal neuron numbers, a primary rabbit polyclonal anti-Ki67-antibody (1:5000; NCL-Ki67p, Novocastra, Newcastleupon Tyne, UK) and a primary goat polyclonal anti-DCX-antibody (Doublecortin; 1:1000; sc-8066, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

Primary antibodies were visualized using biotinylated secondary antibodies followed by incubation with horseradish peroxidase–avidin–biotin complex (Vectastain ABC Elite Kit, Vector Laboratories) and developing for peroxidase activity in 0.05 % 3,3-diaminobenzidinehydrochloride (Sigma) as described earlier (Bisler et al. 2002; Gass et al. 1995). For hematoxylin counterstaining of DCX cell nuclei, sections were mounted on glass slides and dried overnight at room temperature, and then defatted in descending ethanol concentrations and rinsed in H2O. Slides were then incubated in Mayer's hematoxylin stock solution (Merck, Darmstadt, Germany) diluted 1:1 in H2O for 5 min, differentiated in 1 % acetic acid and 96 % ethanol, dehydrated in 99 % ethanol, cleared with xylol, and cover slipped.

Positive cells were counted throughout the section thickness but excluding cells in the uppermost focal plane (for an illustration of Ki67 and DCX staining see Fig. 2A and 2B, respectively). The total cell number was calculated by multiplying the number of cells counted by the inverse of the section sampling fraction, i.e., 12. Ki67-immunoreactive cells were counted along the subgranular zone of the DG using a 100 oil-immersion objective as described earlier (Fuss et al. 2010a). DCX cells were counted in the DG and were estimated using the optical fractionator (StereoInvestigator, Microbrightfield, Williston, VT, USA) with a 100 x oil immersion objective. Counting frames (45 x 35 µm) were placed over the DG (for DCX) at given intervals (135 µm along the x-axis and 105 µm along the y-axis). Two animals were excluded from analysis due to poor tissue quality (both from the restricted runners group).

Endocannabinoid Extraction and Quantification

For endocannabinoids (eCBs) extraction, plasma samples were placed on ice and to each tube 300 µL of ice-cold ethylacetate: n-hexane (9:1, v/v) and 50 µL ice-cold acetonitrile containing deuterated internal standards (anandamide-d_4 (AEA-d4), 2-arachidonoyl glycerol-d_5 (2-AG-d_5), oleoyl ethanolamide-d_2 (OEA-d_2), palmitoyl ethanolamide-d_4 (PEA-d_4), 1-arachidonoyl glycerol-d_5 (1-AG-d_5), and arachidonic acid-d_8 (AA-d8)) were added. Samples were vortexed for 10 seconds, followed by centrifugation for 15 min, at 16000 g and 4°C. Afterwards, samples were frozen for 20 min at -20 °C. The organic phase was recovered, evaporated to dryness and reconstituted in 50 µL acetonitrile

water, 1:1, v/v for LC injection. The LC/MRM parameters and conditions were the same as reported in Lomazzo et al. (2015). The eCBs values were normalized to the plasma volume.

Statistical analyses

Statistical analysis was carried out using IBM SPSS Statistics 22.0 (IBM Corp., Armonk, NY). Normal distribution was assessed using Kolmogorov-Smirnov test.

Differences between groups were detected using univariate analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) for parametric data with 'running condition' as factor. Non-parametric data was assessed using Kruskal-Wallis test. Both tests were followed by Tukey's or Bonferroni's post hoc analysis where appropriate. The difference before and after wheel blockage in unrestricted runners was studied using paired t-test or Wilcoxon rank-signed test, where appropriate (see results section for details). We calculated an index of stereotypic behavior summing up the duration of all stereotypic behaviors that were shown in the observation period (stereotypic behavior = circling + twirling + bar-mouthing). Associations of this behavior with the results of the ^{1}H-MRS metabolites in the hippocampal and prefrontal cortex were investigated with an exploratory correlation analysis using Pearson correlation. The level of significance was set at $p < 0.05$.

Effect sizes (eta squared = η^2) for one-way ANOVAs were calculated by dividing the between-group effect (between-groups sum of squares) by the total amount of variance in the data (total sum of squares). Thereby, $\eta^2 \geq 0.02$ is considered a small, $\eta^2 \geq 0.13$ a medium and $\eta^2 \geq 0.26$ a large effect.

Results

Hippocampal voxel-based morphometry in runners and restricted runners

In a paired t-test there was no significant difference between left and right hippocampal gray matter volume (p=.126). One-way ANOVA was used to evaluate the effect of running-condition on hippocampal gray-matter volume. A significant effect of running was seen for the sum of right and left hippocampal gray matter ($F_{2,27} = 4.52$; $p = 0.020$, $\eta^2=0.25$), right ($F_{2,27} = 5.34$, $p = 0.011$, $\eta^2=0.28$) and on trend-level for left ($F_{2,27} = 3.29$, $p = 0.052$, $\eta^2=0.2$) hippocampal gray matter volume (Fig. 1). Post-hoc statistics using Tukey-correction showed significantly higher total ($p = 0.019$) and right ($p = 0.010$, Fig 1) hippocampal gray matter volumes in running compared to sedentary mice. This effect was seen for the left hippocampus on trend level only ($p = 0.051$, Fig 1). We also found a trend for higher right hippocampal gray matter volume in runners compared to restricted runners ($p = 0.077$). There were no significant differences between sedentary mice and restricted runners as well as between restricted and unrestricted runners.

Immature hippocampal neuron numbers in runners and restricted runners

The number of newborn neurons stained with Doublecortin was significantly increased in both running groups compared to controls ($F_{2,25}$ = 4.82, p = 0.017, η^2= 0.278, Fig. 2C). Post-hoc analysis with Tukey correction revealed no difference between both running groups (p = 1.0), while restricted (p = 0.043) and unrestricted runners (p = 0.028) had significantly more DCX-positive cells compared to controls. In contrast, the number of cells expressing the proliferation marker Ki67 was not affected by running ($F_{2,25}$ = 0.55, p = 0.59).

Hippocampal and frontal ^{1}H-MRS in unrestricted and restricted runners

The MANOVA for ^{1}H-MRS values in the right hippocampus as well as in the frontal cortex did not reveal any significant effects of running on these metabolites. We tested the correlation of hippocampal GM volume and glutamate with one-sided partial correlation, due to the before detected correlation of glutamate and hippocampal GM volume shown in (Biedermann et al. 2012). We found an inverse correlation of glutamate and hippocampal gray matter volume not reaching trend level (R = -0.24, p = 0.108).

In an exploratory correlation analysis with the sum of the duration of stereotypic behaviors and hippocampal as well as frontal ^{1}H-MRS metabolites we found no correlations of hippocampal ^{1}H-MRS metabolites and stereotypic behaviors. However, a significant correlation of stereotypic behavior with frontal glucose (R = 0.44; p = 0.031) and inverse correlations with frontal glutamate (R = -0.44; p = 0.018), and frontal tNAA (R = -0.40; p = 0.032) were seen.

In a further analysis for the different stereotypic behaviors using partial correlation with running behavior as control variable, glutamate in the frontal cortex correlated negatively with twirling (R = -0.53, p = 0.004), whereas glucose in the frontal cortex correlated with bar-mouthing (R = 0.49, p = 0.019). In a further exploratory analysis we found a significant association of eating behavior and frontal glucose (R = 0.47, p = 0.024; for scatterplots see Fig. 3).

Behavioral consequences of restricted and unrestricted running

On average, restricted runners ran significantly less compared to unrestricted runners (RR = 5.6 km/day, R = 9.6 km / day, p < 0.001). Nine weeks of wheel running did not affect body weight in either group in a univariate ANOVA (C = 31.5 ± 1 g, RR = 29.8 ± 0.7 g, R = 29.9 ± 0.6 g, $F_{2,27}$ = 1.423, p = 0.258).

Nest building. In the nest building test, controls built more complex nests compared to both running groups. The difference in nest building performance was present 5 hours ($F_{2,27}$ = 6.044, p = 0.007) and 24 hours ($F_{2,27}$ = 3.407, p = 0.048) after nesting material had been introduced (Fig. 4).

Undisturbed home-cage behavior. Interestingly, the observation of undisturbed home-cage behavior revealed that diet-related basal activities like feeding ($F_{2,27}$ = 0.32, p = 0.73), drinking (Kruskal Wallis test = 1.7, df = 2, p = 0.42), rearing for food ($F_{2,27}$ = 0.37, p = 0.69), and other rearings (Kruskal Wallis test = 4.69, df = 2, p = 0.096) e.g. toward the side of the cage were not different between groups (Fig. 5). In contrast, climbing at the cage top, food hopper or side walls (Kruskal Wallis test= 15.21, df = 2, p < 0.001) was significantly reduced in unrestricted (p<0.001) and restricted runners (p=0.021) compared to controls. Sleeping time was also significantly different between groups ($F_{2,27}$ = 4.25, p = 0.025) and the difference was also significant between controls and unrestricted runners in a post-hoc test (p = 0.02). The frequency of burrowing behavior was in contrast not affected by running ($F_{2,27}$= 0.44, p = 0.65).

Analysis of running wheel directed behaviors showed a significant effect for wheel running duration (Kruskal Wallis test = 24.17, df = 2, p < 0.001) with restricted runners spending less time in the wheel than unrestricted runners (p = 0.044). In contrast, climbing at the wheel (Kruskal Wallis test = 18.66, df = 2, p < 0.001) was significantly lower in unrestricted compared to restricted runners (p = 0.022) and controls (p < 0.001). While sitting in the wheel (Kruskal Wallis test = 20.04, df = 2, p < 0.001) was higher in controls compared to unrestricted (p < 0.001) and restricted runners (p = 0.009). Route tracing (Kruskal Wallis test = 7.83, df = 2, p = 0.02) was also significantly different between groups and post-hoc comparison revealed more route tracing in restricted runners compared to controls (p = 0.016), while cradling in the wheel was comparable between groups (Kruskal Wallis test = 3.60, df = 2, p = 0.17).

Stereotypic behavior. Bar-mouthing frequency (Kruskal Wallis test = 15.91, df = 2, p < 0.001) and duration (Kruskal Wallis test = 14.26, df = 2, p = 0.001) were significantly different between groups and significantly lower in runners (p < 0.001 for frequency and p = 0.001 for duration) compared to controls. Moreover, circular jumping (= twirling) was also significantly different between groups concerning frequency (Kruskal Wallis test = 14.26, df = 2, p = 0.001) and duration (Kruskal Wallis test = 14.26, df = 2, p = 0.001). Interestingly, post-hoc restricted runners exhibited a higher frequency (p = 0.005) and duration (p = 0.003) of twirling than runners. The duration and frequency of circular climbing (= circling) and self-grooming, another complex, repetitive, sequentially patterned and self-directed behavior was not significantly different between groups.

Behavioral consequences of wheel-blocking in unrestricted runner. We next blocked the running wheels of runners (withdrawn runner= WR, dotted bar in Fig. 5) for 24 hours and again investigated undisturbed home cage behavior in these mice (right panel, Fig. 5). As a consequence of blocking the wheels, running behavior was absent. When behaviors of runners before and after blockage were compared by a paired t-test or Wilcoxon signed-ranks test (for non-parametric data), food-related

activities like feeding (t(9)=0.71, p = 0.50), drinking (Z = 14, p = 0.313), and rearing for food (t(9)= 0.47, p = 0.652) remained constant, while other activities like rearing (Z = 52, p = 0.013) and climbing (Z = 48, p = 0.037) increased significantly. Sleeping time also significantly increased in withdrawn runners (t(9)=-3.47, p = 0.007). Interestingly, stereotypic twirling increased after wheel blockage (Z = 26, p = 0.043) as well as the duration of self-grooming (t(9)=-2.93, p = 0.017). Although the average duration of bar-mouthing increased from 2.3 s to 29.7 s, this difference did not reach statistical significance in Wilcoxon signed-ranks test (Z = 29, p = 0.12). Stereotypic circling was also comparable after wheel blockage (Z = 2, p = 0.655) as well as route tracing (p = 0.066), wheel sitting (p = 1), cradling (p = 0.109), and burrowing behaviors (t(9)=-0.57, p =0.58).

Long-term running reduces plasma levels of anandamide

Plasma levels of the endocannabinoid anandamide were significantly affected by long-term running ($F_{2,27}$ = 7.36, p = 0.003, Fig. 6A) and lower in runners (p = 0.003) and restricted runners (p = 0.04) as compared to controls. Interestingly, the average daily running distance was negatively correlated with plasma anandamide levels (AEA; R = - .68, p < 0.001; Fig. 7). Partial correlation with running condition as control variable was also significant (R = -.52, p = 0.024). In contrast, basal plasma levels of the endocannabinoid 2-arachidonoylglycerol (2-AG; $F_{2,27}$ = 0.53, p = 0.60), and the endocannabinoid-like substances 1-arachidonoylglycerol (1-AG; $F_{2,27}$ = 0.76, p = 0.48), oleoylethanolamide (OEA; $F_{2,27}$ = 1.30, p = 0.29), palmitoylethanolamide (PEA; $F_{2,27}$ = 2.35, p = 0.12), and arachidonic acid (AA; $F_{2,27}$ = 1.22, p = 0.31) were not affected by long-term running (Fig. 6B-F).

Discussion

The present study was designed to investigate how exercise restriction in wheel running mice affects the hippocampal micro- and macrostructure. Moreover, we were interested how this may affect hippocampus-dependent stereotypic and basal behaviors as well as the endocannabinoid system. We found that only unrestricted running increased hippocampal gray matter volume, although the number of immature hippocampal neurons was increased in restricted and unrestricted runners. Concerning most basal and homecage behaviors, restricted and unrestricted runners performed comparably. Of note, however, we observed more stereotypic twirling in restricted compared to unrestricted runners. Interestingly, when unrestricted runners were also exposed to blocked wheels for 24 hours, they exhibited significantly more stereotypic twirling.

Effects of restricted running on hippocampal volume and immature neuron numbers

Newborn neuron numbers assessed with DCX-staining were comparable between restricted and unrestricted runners and significantly higher compared to sedentary mice (Fig. 2). This finding is of interest, as a recent report found an attenuated increase of DCX-positive cells after restricted running (Dostes et al., 2016). Importantly, however, running was restricted to 3 hours / day over 3 weeks in the Dostes et al. study, which indicates that either longer daily (we chose 6 hours / day) or a longer absolute time (9 weeks in the present study in contrast to 3 weeks) or a combination of both is required to reach matching levels of immature hippocampal neurons in restricted and unrestricted runners. Moreover, it is possible that we found no difference between restricted and unrestricted runners because hippocampal neurogenesis is strongly declining with age (Ben Abdallah et al. 2010). When newborn neurons were assessed in the present study, mice were 19 weeks old and thus had much lower newborn neuron levels and less variability as compared to younger mice. Although we found no difference in cell counts, it would be interesting to study in the future if the dendritic morphology of DCX-positive cells (Oomen et al. 2010) is differently affected by restricted and unrestricted running as earlier studies found an effect of running on the morphology of immature neurons (Naylor et al. 2008).

Surprisingly yet, we did not find an increase of hippocampal gray matter volume in restricted runners (Fig. 1). Only unrestricted runners showed increased hippocampal gray matter volume compared to controls. This result is in contrast to our findings in (Biedermann et al. 2016) where the number of newborn neurons significantly correlated with hippocampal gray matter volume in wheel running and sedentary mice. As it has been suggested before (Biedermann et al. 2016; Czeh and Lucassen 2007) it is very likely that other neurobiological mechanisms that co-occur with neurogenesis are relevant for a significant hippocampal volume change in wheel running mice. One such mechanism, which was not investigated in the present study, is hippocampal vasculature. Clark et al. found that wheel running in mice increases angiogenesis in the dentate gyrus, but not in the total hippocampus (Clark et al. 2009). Nevertheless, this seems not to lead to an increase of hippocampal vessels, as neither we (Biedermann et al. 2016) nor others (Van Praag et al. 2005) found an increase of the number of hippocampal vessels after running. However, it is possible that an alteration of vascularity, especially the diameter and surface of vessels as shown by Van Praag et al. (2005) can play a role in hippocampal volume alterations through wheel running.

An important influencing factor to explain differences between restricted and unrestricted runners that also affect vasculature could be the hypothalamic-pituitary-adrenal (HPA) axis. The daily manipulation of running wheels or the simple fact that mice could not run due to the blockage may have represented a significant stressor in the present study. Importantly, increase of corticosterone by acute or chronic stressors decreases the proliferation rate in the adult dentate gyrus (for review

see Lucassen et al. (2015) or Fitzsimmons et al. (2016)) and corticosterone can also affect endothelial cells in environmentally enriched and running rats (Ekstrand et al. 2008). Moreover, a single dose of hydrocortisone in humans decreased hippocampal volume in an earlier work (Brown et al. 2015). It was shown that physical exercise also influences the HPA axis (Stranahan et al. 2008; Fuss et al. 2010b). Importantly, long-term exercise not only alters baseline levels of corticosterone and ACTH but also HPA activity in response to an acute stressor (Droste et al. 2003). Different running regimes may thus differently affect the HPA axis. Strikingly, an increase of HPA activity through exercise does not lead to a decrease of neuroplasticity including neurogenesis. This seems to be regulated by mechanisms involving secretion of peripheral molecules such as vascular endothelial growth factor (VEGF) (Fabel et al. 2003), brain-derived neurotropic factor (BDNF) (Marlatt et al. 2012), or cathepsin B (Moon et al. 2016) after long-term running. An important limitation of the present study therefore is that we did assess neither HPA activity nor peripheral growth factors such as BDNF or VEGF, which may show a relation with histological markers such as angiogenesis. This could have helped to explain why restricted runners showed no hippocampal volume change. However, because we did neither find significant differences in hippocampal volume between sedentary and restricted as well as between restricted and unrestricted runners it is as well possible, that our study was underpowered to detect more discrete differences. Thus, it may be that hippocampal volume in restricted runners falls between the other two groups.

Behavioral effects of restricted running

Since most rodent species perform wheel running, it is an interesting question how wheel running behavior can be categorized (Sherwin 1998). It was argued previously that it might share features with stereotypic behaviors given that it is seemingly goalless, repetitive, and invariant (Mason 1991a; Mason 1991b; Richter et al. 2014). Stereotypic behaviors typically develop when animals are living in captivity and are unable to adjust behaviorally. Therefore, it may be an indication of poor animal welfare. Just like stereotypic behavior, voluntary wheel running gradually develops after introduction of running wheels to captive rodents (Fuss et al. 2010b). Moreover, wheel running performed on a daily and voluntary basis can have serious side effects in long-term experiments such as deformation of the tail and spine (Richter et al. 2014) and induce withdrawal like symptoms (Hoffmann et al. 1987; Kanarek et al. 2009). However, when running wheels are placed in a natural environment, wild mice also regularly visit the running wheel covering distances comparable to captive mice (Meijer and Robbers 2014). It is yet unclear, if these wild mice are representative for other wild rodents. In the laboratory, wheel running in rats was even established as a reinforcement model where rats pressed a lever to get access to a running wheel (Iversen 1993).

In the present study, we found that stereotypic behaviors in sedentary controls, like bar-mouthing, twirling and circling (Howerton et al. 2008; Richter et al. 2008), were almost absent in unrestricted runners. In contrast, restricted runners exhibited twirling and bar-mouthing. Interestingly, after blockage of running wheels, stereotypic twirling and bar-mouthing also appeared in (formerly) unrestricted runners. This argues against any significant long-term effect of wheel running on stereotypy. In line, Pawlowicz and colleagues (Pawlowicz et al. 2010) found no effect of previous wheel-running on stereotypic behaviors in a novel testing environment. This indicates that mice show less stereotypic behavior only as long as running wheels are present. Accordingly, we found that restricted runners (which are also exposed to blocked wheels) exhibited significantly more stereotypic twirling than unrestricted runners (Fig. 5). This may also indicate that repeated termination of wheel access is a stressor for mice. Basal activities, like eating and drinking as well as rearing for food were not affected by wheel running. Of note, however, sleeping time of unrestricted runners was significantly lower compared to controls and increased after blockage of the wheel. This might be important given the important impact of sleep on endocrinology.

Moreover, nest building was impaired in runners. A negative relation between thermoregulatory nest building and wheel running has been described before (Bult et al. 1993; Bult et al. 1992; Carter et al. 2000). Since nest building is highly correlated with maternal nest building and pup weaning success, this impact of wheel running on nest building may affect reproductive success in female mice. However, because we investigated male mice in the present study, further studies with female mice under pup weaning circumstances would be necessary to better understand the effect of restricted wheel running on reproductive aspects.

^{1}H-MR Spectroscopy and stereotypic behavior

In contrast to our earlier finding of decreased hippocampal glutamate in wheel running mice compared to sedentary controls (Biedermann et al. 2012), we did not find a significant effect of running on hippocampal glutamate in the current study. The previously detected correlation of glutamate and hippocampal gray matter volume (Biedermann et al. 2012) was not significant neither. Importantly, ^{1}H-MRS detectable glutamate seems to be state dependent. Recently, functional glutamate imaging has been developed for visual cortex (Apšvalkaa et al. 2015; Mullins et al. 2005) and anterior cingulate cortex (Taylor et al. 2015) in humans. Importantly, increased hippocampal activation, reflected by an increase of c-Fos positive cells, was shown in wheel-running mice before (Biedermann et al. 2016; Fuss et al. 2010a). These circumstances could partially explain diverging findings in this and our latter study. In the latter study, we performed the MR experiments in the inactive phase of animals, with lights on in the morning (Biedermann et al. 2012). In contrast, we

now performed behavioral experiments and housed mice on reversed circle, thus performing MR-imaging in the active phase.

Interestingly, we found associations between certain metabolites and stereotypic behaviors in the present study. Stereotypic circular jumping (twirling) was negatively correlated with glutamate levels in the right frontal cortex. Moreover, bar-mouthing behavior positively correlated with glucose levels in the same area. Interestingly, along with the correlation of glucose levels and bar-mouthing behavior we found a correlation between frontal (but not hippocampal) glucose levels and eating behavior. Although we excluded spectra with Cramér-rao lower bounds (CRLB) > 20 leading to a mean CRLB for glucose of 14.63 ± 2.92 (SD) (for comparison CRLB for frontal glutamate were 3.38 ± 0.62), it has to be noted that glucose spectroscopy is not well established in ^{1}H-MRS research though it belongs to the basis metabolite set used in LC-Model. Due to its overlap with other metabolites, it is rather hidden in the noise of the spectrum. Another limiting factor is that we correlated behaviors measured in awake animals with brain metabolites in anesthetized mice, making it more difficult to draw direct conclusions.

Nevertheless, we think the associations of ^{1}H-MRS detectable metabolites with stereotypic behavior are worth mentioning, although they need to be replicated. Stereotypic behavior has been associated with alterations in the prefrontal cortex before (for review (Langen et al. 2011)) and thus studying frontal glutamate and glucose metabolism may help to understand the biological underpinnings of stereotypic behaviors.

Endocannabinoids

Acute exercise in humans and mice activates the endocannabinoid system (Fuss et al. 2015; Sparling 2003). Disruption of the endocannabinoid system by deletion of cannabinoid CB1 receptors (Dubreucq et al. 2013; Fuss and Gass 2010) reduces wheel running in mice profoundly. More specifically, a recent study by Dubreucq and colleagues identified cannabinoid CB1-receptors on forebrain GABAergic neurons to be crucially involved in wheel running behavior (Dubreucq et al. 2013). We found that the beneficial consequences of acute exercise – often termed as “a runner’s high” – depend on cannabinoid CB1 receptors (Fuss et al. 2015). The effects of long-term exercise on the endocannabinoid system have yet been studied less. Therefore, we thought it would be interesting to investigate how long term running affects baseline levels of endocannabinoids. In the present study, long-term running significantly reduced plasma anandamide levels. Interestingly, the average daily running distance negatively correlated with anandamide levels. This indicates that baseline anandamide plasma levels are dose-dependently influenced by long-term physical exercise and running restriction thus modulates this down-regulation. Given that endocannabinoids are

important regulators of emotional and feeding behaviors the relation between individual differences in wheel-running activity and plasma anandamide levels may represent an important exploratory finding. So far, exercise-brain-behavior relations have predominantly focused on adult hippocampal neurogenesis, which in contrast was not related to the individual amount of exercise in the present study. Our findings may thus stimulate research into the relation between long-term running, anandamide and brain and behavior.
Nevertheless, an important limitation is that plasma sampling was performed after anesthesia. Our endocannabinoid data thus await replication in mice and regularly exercising humans.

In conclusion, running restriction does not seem to be a better model for voluntary exercise in mice. In the present study, it was associated with more stereotypic behavior and less neurobiological effects as compared to unrestricted running. Thus, we hypothesize that the daily running restriction may rather be stressful for mice. However, more studies are needed to better explain our partially diverging findings in restricted and unrestricted runners.

Figure Legends

Fig. 1: MR volumetry: Higher gray matter volume in the left (below; p = 0.020) and right (above; p = 0.004) hippocampus in unrestricted runners (R) compared to sedentary controls (C) and restricted runners (RR; left p=0.031; right as trend: p=0.078).* indicates a significant difference compared to the other groups. Boxplots represent median, middle 50% of scores, upper and lower quartile, small dots represent outliers.

Fig. 2: Hippocampal immature neuron numbers stained with DCX (illustrated in B) are increased in unrestricted (R) and restricted runners (RR) compared to sedentary controls (C), while cell proliferation stained with Ki67 (A) is comparable between groups. * indicates a difference compared to controls at a significance level of $p \leq 0.05$. Boxplots represent median, middle 50% of scores, upper and lower quartile.

Fig. 3: Frontal glucose and behavior: Scatterplot depicting correlations between glucose in the frontal cortex and stereotypic behaviors (left; R=0.403, p=0.056), bar mouthing (middle; R = 0.485, p=0.019), and eating behavior (right; R=0.470, p=0.024) in runners (R), restricted runners (RR) and sedentary mice (S).

Fig. 4: Nest building test: Sedentary controls (C) built significantly more complex nests compared to unrestricted runners (R) and restricted runners (R). Boxplots represent median, middle 50% of scores, upper and lower quartile, small dots represent outliers.

Fig. 5: Home cage behavior: Behavioral components recorded by undisturbed home cage observation in sedentary controls (C) unrestricted (R) and restricted runners (RR). Boxplots represent median, middle 50% of scores, upper and lower quartile, small dots represent outliers.. # indicates a significant difference ($p<0.05$) in post-hoc tests between C, RR and R. + indicates a significant difference between WR and R.

Fig. 6: The influence of long-term running on plasma levels of endocannabinoids. Significant lower anandamide in unrestricted (R) und restricted running mice (RR). *, ** indicate a difference compared to controls at a significance level of $p < 0.05$ and $p < 0.01$, respectively. Boxplots represent median, middle 50 % of scores, upper and lower quartile, dots represent outliers.

Fig. 7: Scatterplot showing the inverse correlation between the endocannabinoid anandamide and the average daily running distance of mice ($R = -.677$, $p < 0.001$).

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Table 1: ^{1}H-Magnetic resonance spectroscopy (MRS) values of unrestricted running (R), restricted running (RR) and sedendary (S) mice in the right hippocampus and frontal cortex given in mean ± standard deviation (STD). Glx (glutamate + glutamine), total creatine (creatine + phosphocreatine), total choline (glycerophosphocholine + phosphocholine), tNAA (N -acetylaspartate + N -acetylaspartylglutamate), γ-aminobutyric acid (GABA).

	Right Hippocampus ^{1}H-MRS						***Frontal Cortex ^{1}H-MRS***					
	R n=10		**RR n=10**		**S n=10**		**R n=9**		**RR n=10**		**S n=10**	
	mean	± STD	mean	± STD	mean	± STD	mean	± STD	mean	± STD	mean	± STD
total creatine	10,49	± 0,49	10,52	± 0,72	10,27	± 0,75	9,45	± 0,50	9,28	± 0,36	9,27	± 0,89
glutamine	3,33	± 1,10	3,05	± 0,88	2,79	± 0,31	3,47	± 0,86	3,29	± 1,11	2,98	± 0,39
glutamate	7,54	± 0,74	7,61	± 1,04	7,40	± 0,69	9,77	± 0,60	9,48	± 0,77	9,47	± 1,16
Glx	10,87	± 1,42	10,66	± 1,34	10,19	± 0,72	13,24	± 0,90	12,78	± 1,34	12,44	± 1,26
total choline	1,65	± 0,37	1,89	± 0,26	1,57	± 0,22	2,48	± 0,27	2,49	± 0,15	2,47	± 0,43
lactate	5,56	± 1,55	6,29	± 1,62	5,10	± 1,80	4,52	± 1,57	5,22	± 1,55	4,82	± 2,58
myoInositol	7,26	± 0,92	6,78	± 0,55	7,02	± 0,48	9,34	± 0,82	8,71	± 0,83	8,66	± 0,99
total NAA	9,48	± 1,18	9,96	± 0,62	9,31	± 0,92	9,47	± 0,44	9,74	± 0,34	9,50	± 1,01
taurine	10,22	± 0,82	9,96	± 1,12	10,39	± 1,05	11,00	± 0,98	11,34	± 0,74	11,35	± 1,47
GABA	3,22	± 0,27	3,11	± 0,25	3,03	± 0,47	2,96	± 0,29	3,01	± 0,27	3,02	± 0,52
	n=6		**n=10**		**n=9**		**n=5**		**n=10**		**n=9**	
glucose	3,32	± 0,70	3,23	± 1,01	4,14	± 0,84	2,88	± 1,29	2,42	± 1,60	3,91	± 0,62

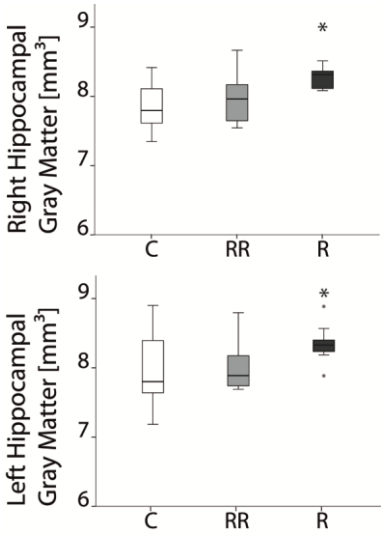

Figure 1

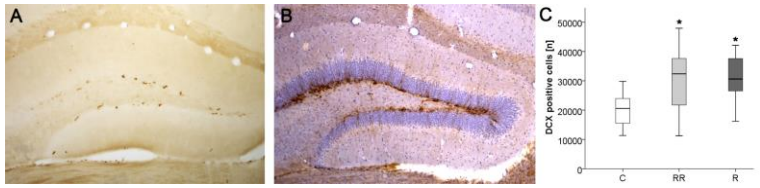

Figure 2

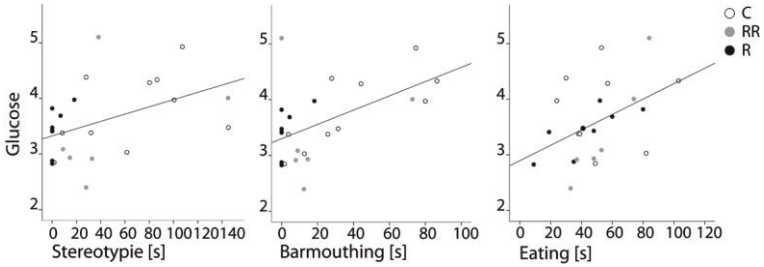

Figure 3

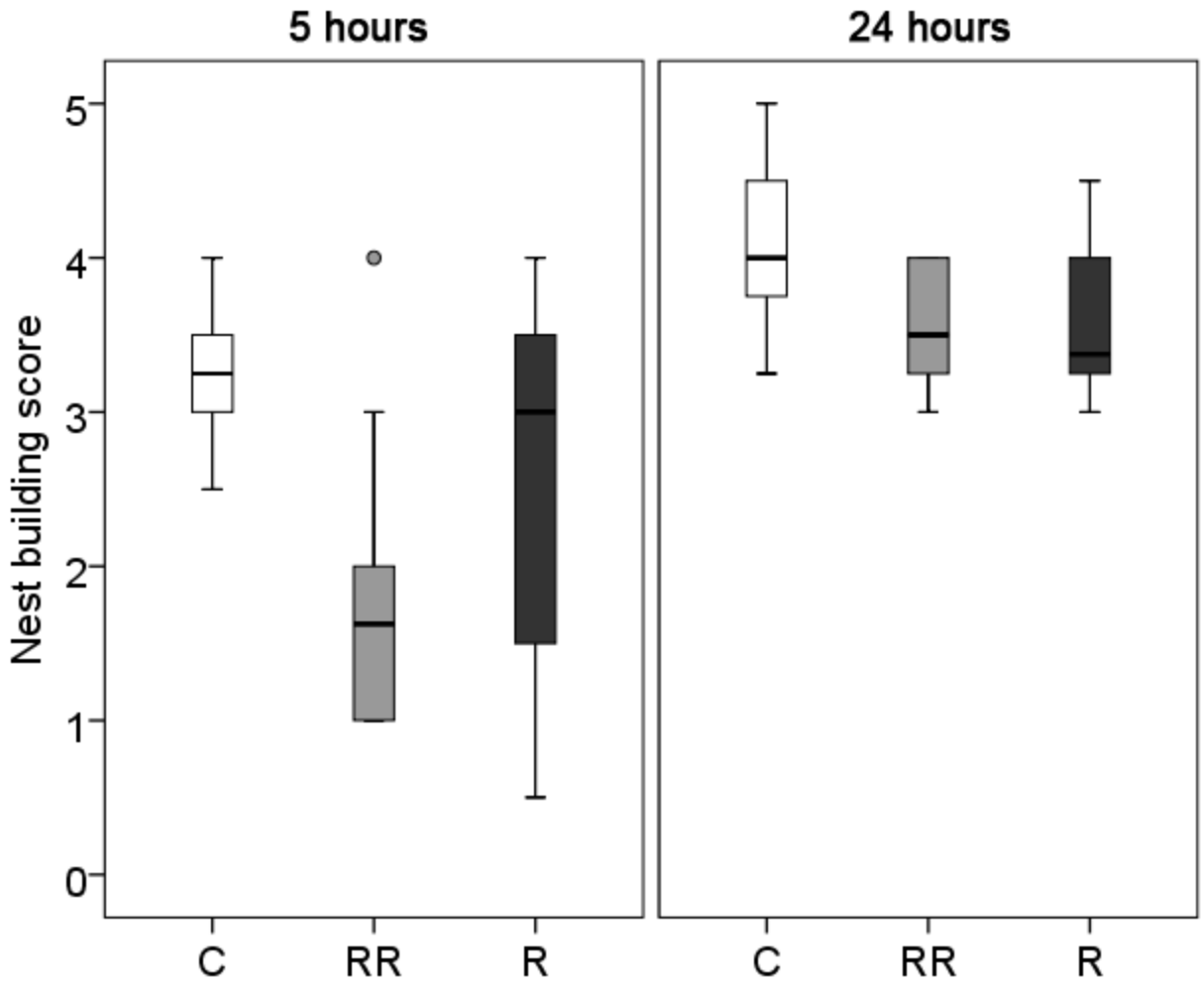

Figure 4

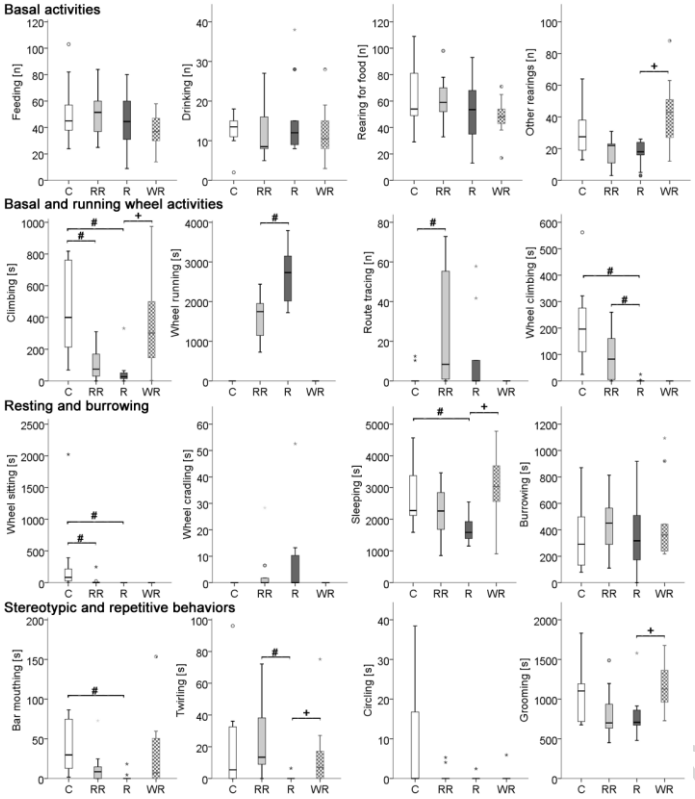

Figure 5

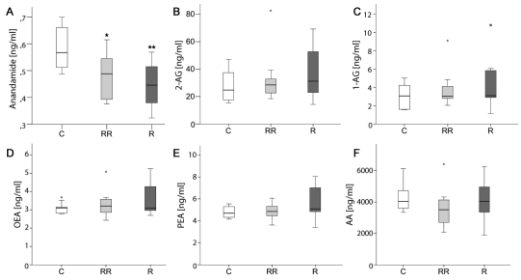

Figure 6

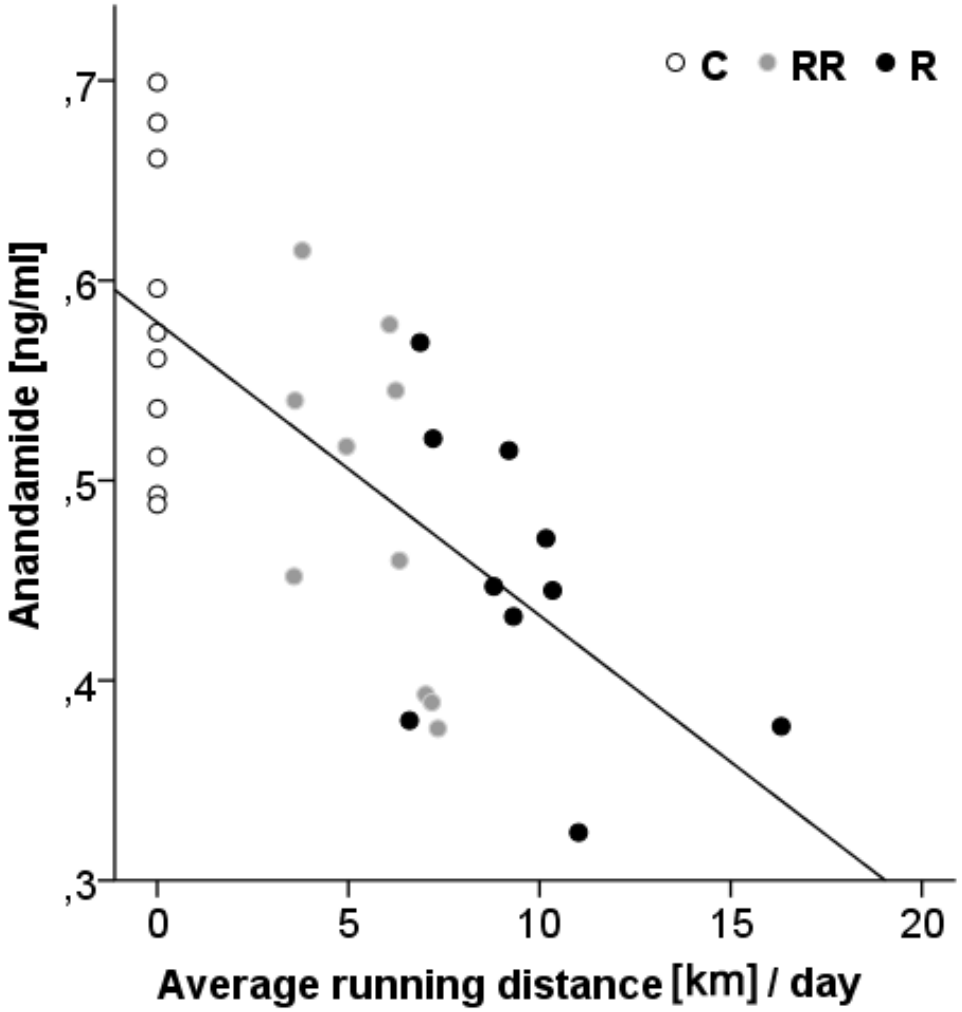

Figure 7

Highlights:

- Restricted and unrestricted wheel running increase immature hippocampal neuron numbers.
- Hippocampal gray matter volume is only increased after unrestricted running.
- Long-term running reduces the endocannabinoid anandamide in a dose-dependent manner.
- Unrestricted running decreases stereotypic behaviors, correlating with frontal glutamate and glucose levels.
- Acute running wheel blockage increases stereotypic behavior