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Effects of exercise on adolescent and adult hypothalamic and hippocampal neuroinflammation

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Abstract

Adolescence is a period of significant brain plasticity that can be affected by environmental factors, including the degree of physical activity. Here we hypothesized that adolescent rats would be more sensitive to the beneficial metabolic and anti-inflammatory effects of voluntary exercise than adult rats, whose more mature brains have less capacity for plasticity. We tested this by giving adolescent and adult Wistar rats four weeks' voluntary access to running wheels. At the end of this period we assessed metabolic effects, including weight and circulating leptin and ghrelin, as well as performance in a novel object recognition test of memory and central changes in neuronal proliferation, survival, synaptic density, and inflammatory markers in hippocampus. We found exercise reduced fat mass and circulating leptin levels in both adults and adolescents but suppressed total weight gain and lean mass in adults only. Exercise stimulated neuronal proliferation in the suprapyramidal blade of the dentate gyrus in both adults and adolescents without altering the number of mature neurons during this time frame. Exercise also increased dentate microglial numbers in adolescents alone and microglial numbers in this region were inversely correlated with performance in the novel object recognition test. Together these data suggest that adolescent hippocampal microglia are more sensitive to the effects of exercise than those of adults, but this leads to no apparent improvement in recognition memory.

Introduction

Regular exercise has clear beneficial effects on an array of physiological functions, including maintaining healthy heart rate, blood pressure, metabolism, and body weight (Ryan & Nolan, 2015), lowering the risk of high cholesterol, hypertension and heart attack (Stults-Kolehmainen, 2013). Recently, exercise has also been identified as having significant positive effects on the brain, including to increase hippocampal neurogenesis and improve cognition, while suppressing the neuroinflammation that can be detrimental to both (Ryan & Nolan, 2015). For instance, in mice, voluntary wheel running stimulates dentate gyrus neuronal proliferation and survival and improves spatial memory and learning (van Praag, Christie, Sejnowski & Gage, 1999; van Praag, Kempermann & Gage, 1999). In humans, exercise programs lead to improved blood flow to the hippocampus and better declarative memory compared with non-exercising controls (Pereira et al., 2007).

While exercise at any phase of life is likely to be beneficial, there are particular stages when an individual may be especially sensitive to its effects. The *in utero* period, when the brain and peripheral organs are undergoing substantial development, is one critical window of sensitivity. The period around puberty is a second, although currently less well investigated, window. During puberty, hormonal changes stimulate significant neuronal plasticity and brain development (Romeo & McEwen, 2006; Sisk & Zehr, 2005). The adolescent human and rodent brain is particularly sensitive to environmental factors that may influence these hormonal changes (Andersen, 2003; Dahl, 2004; Romeo & McEwen, 2006; Spear, 2000). Adolescents are more sensitive to the detrimental effects of alcohol than adults (Broadwater & Spear, 2013a; Broadwater & Spear, 2013b; Broadwater, Liu, Crews & Spear, 2014; Broadwater & Spear, 2014; Vetreno, Broadwater, Liu, Spear & Crews, 2014). They have different molecular strategies for responding to hippocampal injury (McPherson, Aoyama &

Harry, 2011). They are highly sensitive to the long-term effects of stress (Chaby, Cavigelli, Hirrlinger, Lim, Warg & Braithwaite, 2015; Wong & Marinelli, 2015), and, if maintained on a high fat diet, they have exacerbated emotional memory of negative events, while adults do not (Boitard et al., 2015). Encouragingly, it has also recently been demonstrated that exercise during adolescence can have a lasting positive effect. Thus, four weeks of voluntary exercise in the rat improved memory retention in the novel object recognition task so that the exercised but not the adolescent sedentary rats retained recognition memory when tested two weeks after the first phase of the study. Adults showed no such learning and memory improvement with the same exercise protocol (Hopkins, Nitecki & Bucci, 2011). At least part of this effect may be due to exercise mitigating the brain's neuroinflammatory profile (Ryan & Nolan, 2015). Thus, even very mild exercise in aged rats suppressed the ageing-related microglial sensitization and completely reversed the ageing-related cognitive deficits caused by an immune challenge (Barrientos et al., 2011). Exercise can also positively influence metabolic factors, improving adiposity, leptin levels, and insulin sensitivity (Schroeder, Shbiro, Gelber & Weller, 2010; Yu et al., 2016). These factors can independently affect both brain inflammation and cognition (Miller & Spencer, 2014). Thus, clinical and pre-clinical studies show high fat diet and obesity are associated with poorer learning, memory and executive function (Cournot et al., 2006; Elias, Elias, Sullivan, Wolf & D'Agostino, 2003; Elias, Elias, Sullivan, Wolf & D'Agostino, 2005; Sabia, Kivimaki, Shipley, Marmot & Singh-Manoux, 2009) and with neuroinflammation that may be a driver of these effects (Miller & Spencer, 2014).

Despite evidence of adolescent sensitivity to environmental factors, very few studies have appropriately assessed adolescent parameters in direct comparison to those of adults. In the present study we therefore hypothesized that adolescent rats, i.e. those of peripubertal age,

would be more sensitive to the beneficial metabolic and anti-inflammatory effects of voluntary exercise than adult rats whose more mature brains have less capacity for plasticity.

This hypothesis is particularly important to test given recent trends for adolescents in the developed world to lead highly sedentary lifestyles (Saunders, Chaput & Tremblay, 2014). Australian teenagers are now watching onscreen media (television and leisure time computer activity) an average of 3-4 hours per day (Straker, Smith, Hands, Olds & Abbott, 2013). Despite current recommendations that adolescents should accumulate at least an hour of moderate to intensive exercise daily, 23% of Australian teens do no exercise and 50% exercise for only 4.5 hours per week (ABS, 2009). In England, only 8% of girls in the 13-15 age range and 14% of boys meet the recommendations for physical activity levels (BHF, 2015). The increasing use of electronic media for entertainment suggests this trend of sedentary behaviour is likely to continue unless we develop a good understanding of its detrimental effects. In the present study we examined the effects of four weeks voluntary running wheel exercise in adolescent (peripubertal) and adult male Wistar rats on metabolic and central pro-inflammatory parameters. To test the potential interplay between the metabolic and cognitive effects of exercise, we examined weight and fat mass changes, circulating satiety and inflammatory markers and hypothalamic and hippocampal indices of inflammation, including expression of pro-inflammatory genes and changes in microglia. We also examined numbers of hippocampal doublecortin (DCX)- and neuronal nuclei (NeuN)-immunoreactive cells as an index of neuronal proliferation and survival, as well as density of synaptophysin as an index of synaptic density, anticipating these would be associated with improved memory performance with exercise.

Materials and Methods

Animals

We ordered male Wistar rats from the Animal Resources Centre, WA, Australia to arrive at the RMIT Animal Facility on either postnatal day (P)22 or P63, when they were immediately allocated to their experimental groups. We maintained all the rats at 22 °C on a 12 hr light / dark cycle (0700 – 1900 hr) with pelleted rat chow and water available *ad libitum*. All procedures were conducted in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals and were approved by the RMIT Animal Ethics Committee. We weight-matched the pairs (so the heaviest rat was paired with the next heaviest and so forth) and then randomly assigned them to one of four treatment groups n=8 per group: Adult, sedentary (AS); Adult, exercised (AE); Adolescent ("teen"), sedentary (TS); Adolescent, exercised (TE).

Exercise

We allocated 50% of the pairs to standard home cages (40 x 27 x 16 cm dimensions with the standard plastic tube "hide" as an enrichment). The other 50% were placed in Lafayette Rat Activity Wheel Cages (41 x 51 x 21cm; Lafayette Instruments, IN, USA). To provide them with maximum opportunity for voluntary exercise, we gave them free access to the running wheels 24 hr per day. This protocol has been previously described (Barrientos et al., 2011; Lee, Rakwal, Shibato, Inoue, Chang & Soya, 2014). We also gave these rats the standard plastic tube "hide" as an enrichment. We recorded voluntary wheel usage with a Lafayette Activity Wheel Monitor (Lafayette). The rats were housed in pairs to avoid the stress associated with protracted isolated housing. The distance ran and food intake measurements are therefore per pair and each pair is counted as an "n" of one for these measurements. We chose home cages over locked running wheels as controls because rodents climb in the locked wheels, thereby potentially getting both exercise and enrichment (Koteja, Garland,

Sax, Swallow & Carter, 1999; Smith & Pitts, 2012). Our exercise paradigm therefore includes elements of both exercise and enrichment as, indeed, would be comparable to the human experience when newly introduced to exercise.

Experimental design

We kept the rats under experimentation (i.e. sedentary or exercised) for four weeks. We measured food intake on three consecutive days weekly to calculate an average daily food intake for that week. We also measured weight gain weekly. On day 20 and 21 we habituated the rats to the novel object recognition test arena and on day 22 tested them in the novel object recognition test of memory (see below). On day 27 we conducted an EchoMRI scan on each rat to assess fat and lean mass. On day 30, we removed the food from all the rats at lights-on, ensuring each rat was fasted for at least 2 hr prior to cull to standardize satiety levels without inducing negative energy balance. We then deeply anaesthetised the rats with Lethabarb (~150 mg/kg pentobarbitone sodium, i.p.), quickly extracted blood, epididymal fat samples and whole brains. We collected the blood samples on EDTA over ice and subsequently centrifuged them and stored the plasma supernatant at -20 °C until assayed. Fat samples were snap-frozen in liquid nitrogen. We hemisected the brains sagitally through the midline and dissected and snap-froze the hippocampus and hypothalamus from the right hemisphere. We immersion-fixed the left hemispheres in 4% paraformaldehyde in phosphate buffered saline (PBS; 4 °C, pH 7.4) for 24 hr before placing them in cryoprotectant with 20% sucrose in PBS (4 °C). We subsequently cut forebrains into 30 µm coronal sections using a cryostat. We conducted all behavioral tests and dissections between 1100 and 1300 hr to limit potential effects of circadian rhythms on any parameters measured.

Novel object recognition

To examine effects on recognition memory, we tested the rats in the novel object recognition task (Ennaceur & Delacour, 1988). This test exploits the rats' attraction to novelty. We first gave the rats one session of 3 min habituation to an empty arena (a black plywood box, 60 cm x 60 cm x 50 cm) on each of the two days preceding the test. On the testing day, we gave the rats a 3 min acquisition trial in the same arena with two identical objects. Objects were placed in the centre of the box equidistant from the sides. Following a 4 hr inter-trial interval, we returned the rats to the arena with one familiar object and one novel object for a 3 min retention test. The rats were tested in cage-mate pairs in two separate arenas simultaneously and we utilized two pairs of objects. One familiar object from the acquisition trial was used as the novel object for the retention trial in the alternate arena thus counterbalancing the objects. The arena was cleaned with 70% ethanol between each phase and each rodent. All sessions were filmed and an experimenter blinded to treatment group scored the videos for time spent interacting with each object using Ethovision 11.5 (Noldus Information Technology; Wageningen, The Netherlands). Results from the retention phase were expressed as a discrimination index calculated as time spent interacting with novel object minus time spent interacting with familiar object divided by the overall exploration time of the two objects in seconds (TN-TF)/(TN+TF). A discrimination ratio of zero indicates no preference while a discrimination ratio above zero indicates a preference for the novel object over the familiar.

Immunohistochemistry

Sections through the hypothalamus and hippocampus were immunolabelled for ionized calcium-binding adapter molecule-1 (Iba-1; a marker for microglia/macrophages). Sections through the hippocampus were also immunolabelled for DCX (a marker for developing neurons), NeuN (a marker for total neuron numbers) or synaptophysin (a pre-synaptic

terminal marker). Randomly selected sections from each treatment group were processed at the same time in batches. Briefly we incubated a single one in five series of sections from each animal in primary antibody overnight at 4 °C; (Iba-1: 1:1000; rabbit; Wako Chemicals, USA Inc., Richmond, VA, USA. DCX: 1:500; goat; Santa Cruz Biotechnology Inc., Dallas, TA, USA. NeuN: 1:5000, rabbit, 4 °C, Abcam, Cambridge, England, UK. Synaptophysin: 1:1000, mouse, RT, Sigma-Aldrich, St Louis, MO, USA. This was followed by secondary antibody (Iba-1, NeuN: 1.5 hr; 1:200; biotinylated anti-rabbit; Vector Laboratories, Burlingame, CA, USA. DCX: 1:500; biotinylated anti-goat; Vector. Synaptophysin: 2 hr 1:500, Alexa Fluor goat anti-mouse 488, Life Technologies, Carlsbad, CA, USA). For Iba-1, NeuN and DCX we used an avidin-biotin horseradish peroxidase (HRP) complex (ABC; 45 min; Vector Elite kit; Vector) followed by diaminobenzidine (DAB) to visualize the HRP activity, seen as amber staining for Iba-1 or with nickel colbalt intensification seen as black staining for DCX and NeuN. We stopped the reaction when the contrast between specific cellular and non-specific background labelling was optimal. We slide-mounted and air-dried the brain sections, dehydrated them in a series of alcohols, cleared them in histolene and coverslipped. For synaptophysin we used DAPI as a nuclear counterstain (10 min; 1:2000 from 5 mg/mL stock;), followed by immediate coverslipping with Dako fluorescence mounting medium (Dako, Glostrup, Denmark).

Sections were assessed by an experimenter blinded to treatment groups for numbers and density of cells positive for Iba-1 (hippocampus and hypothalamus) and for numbers or density of cells positive for DCX or NeuN (hippocampus). For Iba-1 and NeuN, we assessed these as we have previously described (Beynon & Walker, 2012; Radler, Hale & Kent, 2014; Ziko et al., 2014). Briefly, we used the thresholding method on photomicrograph images imported into image analysis software Image J (National Institutes of Health, Bethesda, MD,

USA). For each region we selected a sub-region of interest, identified according to the Paxinos and Watson Rat Brain Atlas (Paxinos & Watson, 2009). For NeuN, cell number was assessed in the hilus, but the relatively high cell density in the subgranular region meant there was not good separation between the cells so we assessed area density here. For DCX, we manually counted DCX-positive cells in the subgranular / granule cell region of the dentate gyrus. No DCX-immunoreactive cells were visible elsewhere. For the hippocampus we analysed six (Iba1) or four (DCX, NeuN) sections 120 μm apart between 2.04 and 3.09 mm caudal to bregma per animal. For the hypothalamus we analysed two sections 120 μm apart between 1.56 and 1.92 mm caudal to bregma. We saw no differences between the rostrocaudal levels for any of the hippocampal or hypothalamic regions, so we took the summed counts and mean density of the assessed images as our sampled result. For synaptophysin, we employed the thresholding method on regions of interest through the hilus (three sections 120 μm apart between 2.76 and 3.48 mm caudal to bregma) to determine the intensity of immunofluorescence (NIS-Elements AR software; Nikon, USA).

To provide an estimation of relative hemispheric volumes for the various hippocampal structures we also analysed these using the Cavalieri point-counting principle on the NeuN-stained tissue. Thus, we took images of eight hippocampal sections 120 μm apart from a one in five series across the hippocampus from each animal with a 4x objective. Image J was used to superimpose at random a grid of systematic uniform test points 169 μm apart. Each point represented an area (a) of 28.75 mm^2 in the section plane. The number of points hitting each hippocampal subdivision (ΣP) was multiplied by the area associated with each point (a) to obtain an unbiased estimate of sectional area of each hippocampal subdivision. The sum of sectional areas of each subdivision for each image was used to estimate hippocampal subdivision volume (V) from the relationship V := T.a.ΣPi, where := represents estimates, T

represents the intersection thickness, and ΣPi is the sum of the number of points hitting the region of interest in every 'ith' section (Dorph-Petersen & Lewis, 2011).

ELISAs

To determine circulating leptin and Cxcl10 concentrations, we performed a standard leptin (Millipore, Billerica, MA) or Cxcl10 (Abnova Corp., Taipei, Taiwan) ELISA, following the manufacturer's instructions. The intra- and inter-assay variabilities for leptin were 1.9-2.5% and 3.0-3.9% (Cxcl10, not given). Lower limits of detection were 0.04 ng/mL for leptin and 16 pg/mL for Cxcl10. We assayed the samples in duplicate using a Polarstar Optima plate reader.

For ghrelin, blood was treated on collection with pefabloc (1 mg/mL final concentration) in a tube that contained no anticoagulant. Blood was left to clot at room temperature for 30 min then centrifuged at 2500 x centrifugal force g for 15 min at 4 ± 2 °C. Serum was transferred into a fresh tube then 0.5 M HCl (final concentration 0.05 M HCl) was added. The samples were mixed, aliquoted, and stored at -20 °C avoiding freeze-thaw cycles. To determine serum acyl and desacyl ghrelin concentrations, we performed standard ghrelin ELISAs for total and acyl ghrelin (Millipore, Billerica, MA, USA) following the manufacturer's instructions. Intra-assay variability was 0.3-7% and 0.7-1.3% CV, inter-assay variability 1-10% and 1.8-4.5% CV, and lower limit of detection 0.8 pg/mL and 0.04 ng/mL for total and acyl ghrelin respectively. Samples were assayed in duplicate. Acyl ghrelin concentrations were subtracted from total ghrelin concentrations to derive a value for serum desacyl ghrelin (Hosoda et al., 2004).

Real time reverse transcriptase polymerase chain reaction (rt-PCR) analysis

We isolated RNA from brain and epididymal fat using QIAzol and an RNeasy purification kit (Qiagen, Valencia, CA, USA). RNA (1 µg) was transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturers' instructions. Real-time rt-PCR was performed using Taqman Gene Expression Assays (Applied Biosystems, Mulgrave, Vic, Au). The specific primer details are shown in Table 1. Fold differences in target mRNA expression were measured using the delta-cycle threshold method by comparison with the housekeeping gene, β-actin (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008), which did not differ between groups, and was expressed as mRNA relative fold change as described previously (Mouihate, Galic, Ellis, Spencer, Tsutsui & Pittman, 2010; Ziko et al., 2014).

DNA methylation

DNA methylation within the toll-like receptor 4 (*Tlr4*) and C-X-C motif chemokine 10 (*Cxcl10*) promoter regions was quantified by bisulfite pyrosequencing. A conserved CpG site -127 base pair (bp) upstream from the transcriptional start site (TSS) was analysed within the proximal *Cxcl10* promoter. Two CpG sites, -27 bp (CpG1) and -1 bp (CpG2), upstream from the TSS were analysed within the *Tlr4* promoter region.

DNA was extracted from hippocampi and hypothalami using QIAzol reagents and purified with DNeasy Blood and Tissue kit (Qiagen). Initially, 1 µg genomic DNA was bisulfite-converted using the EpiTect Bisulfite Conversion kit (Qiagen) according to the manufacturer's instructions. Approximately 10 ng of bisulfite-converted DNA was used to amplify the region of interest in a PCR reaction with the use of the PyroMark PCR reagents (Qiagen). PCR and sequencing primers were designed using PyroMark Assay Design software (Qiagen). *Tlr4* forward: GGTTAGATGATTTTTTGGGATGAAAG, reverse:

biotin-AAAATCCCTAAAACCCCTACC, sequencing TTTTGTTGTTTTTTTAGAAGTTG, *Cxcl10* forward: GGAAAGAGGGAAATTTTAAGTTTATGG, reverse: biotin-CCCTCCCTAAATCTTAATTAACTAACTT, sequencing: GTTATTATAAATATAAGTAGTGTTT (Thermo Fischer Scientific, UK). The PCR program was as follows: 95 ^{o}C (15 min), [94 ^{o}C (30 s), 56 ^{o}C (30 s), 72 ^{o}C (30 s)] 50 cycles, 72^{o} C (10 min).

Pyrosequencing was performed on the PyroMark Q24 system (Qiagen) using PyroMark Q24 Advanced CpG sequencing reagents (Qiagen). Briefly, 10 µL PCR product was mixed with 40 µL binding buffer, 29 µL ddH_2O and 1 µL Streptavidin Sepharose High Performance beads (GE Healthcare). Samples were mixed for 10 min before being processed on the PyroMark Q24 workstation; 70% ethanol (5 s), denaturation buffer (5 s) and wash buffer (10 s). Samples were then mixed with 0.3 µM sequencing primer and heated to 80^{o}C for 5 min before being sequenced. A bisulfite conversion control was included to confirm complete bisulfite conversion of DNA. DNA methylation quantification was performed using Q24 Advanced software (Qiagen).

Data analysis

We compared exercise, weights, food intake, and caloric efficiency using an analysis of variance (ANOVA) with repeated measures, with age and exercise as between factors and time as the repeated measure. When a significant interaction was found we used Tukey *post hoc* comparisons. We compared novel object recognition, rt-PCR, DNA methylation and immunohistochemistry results using ANOVAs with Tukey *post hocs*. We also performed Pearson's correlation analysis with a Bonferroni correction for the novel object recognition discrimination ratio to assess if there were any correlations between novel object recognition

performance and those parameters significantly affected by exercise or age. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was assumed when $p < 0.05$.

Results

Voluntary exercise in adolescent and adult rats

As expected, all the rats did most of their exercise in the dark phase (Fig. 1A, B). There were no differences between the groups in the light phase and the distance ran during this time was minimal (Fig. 1A). Adult rats ran significantly further in the dark-phase than adolescents in weeks three and four (significant time by age interaction: $F_{(3,15)} = 12.26$, $p < 0.001$; Fig. 1B).

Effects of exercise on adolescent and adult weight gain

To assess if voluntary exercise could modulate weight gain in adolescent and adult rats, we first examined weight gain and lean and fat mass in rats given free access to exercise wheels compared with sedentary controls. As expected, adults weighed more than adolescents when the experiment began and weekly thereafter, but there were no significant differences in the weights between the two adolescent groups or the two adult groups at the start of the experiment. By the second week, exercise had suppressed weight gain in the adults but had no effect in adolescence (significant time by age by exercise interaction: $F_{(4,112)} = 8.44$, $p < 0.001$; Fig. 1C).

After four weeks we assessed lean and percentage fat mass in the EchoMRI. Adults had more lean mass than adolescents and this was also suppressed with exercise in the adults (significant age by exercise interaction: $F_{(1,28)} = 5.09$, $p = 0.032$; Fig. 1D). There was no difference between adolescents and adults in their percentage fat mass and exercise

suppressed fat mass at both ages (significant effect of exercise: $F_{(1,27)} = 55.13$, $p < 0.001$; Fig. 1E).

Absolute food intake was subtly affected by age and exercise (significant time by exercise interaction: $F_{(4,44)} = 3.84$, $p = 0.023$; Fig. 1F). *Post hoc* tests indicated that after week one, adults, both exercised and sedentary, ate more than adolescents. At weeks two and four exercise stimulated food intake in the adults without affecting it in adolescents.

As is reflected in total food intake, at week one caloric efficiency was suppressed in the adults compared with adolescents and in the adult exercised group compared with all the other groups (significant time by age interaction: $F_{(3,33)} = 4.24$, $p = 0.024$; Fig. 1G). At weeks two, three, and four, caloric efficiency was lower in the adult exercised than in the adolescent groups, again reflecting the need to consume more calories to maintain body weight.

Effects of exercise on adolescent and adult satiety and peripheral inflammatory markers

Neither age nor exercise influenced basal ghrelin levels (Fig. 1H-J). However, leptin was strongly suppressed by exercise in both the adolescent and adult groups (significant effect of age: $F_{(1,27)} = 10.33$, $p = 0.033$ and exercise: $F_{(1,27)} = 90.26$, $p < 0.001$ Fig. 1K). We saw no effect of age or exercise on circulating levels of the inflammatory marker Cxcl10 nor adipose tissue expression of *Tlr4* nor *Cxcl10* (Fig. 1L-N).

Effects of exercise on adolescent and adult hypothalamic inflammatory markers

To assess if exercise was able to suppress expression of inflammatory markers in a key feeding- and metabolism-regulatory region, the hypothalamus, we examined expression of a

suite of pro-inflammatory genes in the region as well as changes in microglial number and morphology. We found no differences in expression of the free fatty acid / lipopolysaccharide receptor *Tlr4* or chemokine *Cxcl10* (Fig. 2A, D) and no differences in percentage methylation of CpG sites for *Tlr4* or *Cxcl10* (Fig. 2B, C, E). There were also no differences in any of the downstream cytokines assessed (tumour necrosis factor (*Tnf)α*, Fig. 2F; interleukin (*Il)-1β*, Fig. 2G) except that hypothalamic *Il-6* was elevated in the adult exercised group compared with the adolescent exercised (significant effect of age: $F_{(1,27)} = 14.63$, $p = 0.001$; Fig. 2H).

Adult age led to reduced microglial numbers in the PVN (significant effect of age: $F_{(1,26)} = 4.32$, $p = 0.048$) and elevated microglial density in the ARC (significant effect of age: $F_{(1,27)} = 5.74$, $p = 0.024$) and adult age interacted with exercise to affect ARC microglial numbers (significant age by exercise interaction: $F_{(1,27)} = 4.48$, $p = 0.044$). However, there were no *post hoc* differences between the groups in hypothalamic microglial numbers or density (Fig. 2I-L).

Effects of exercise on adolescent and adult hippocampal inflammatory markers

Due to reports showing exercise may improve function in brain regions involved in cognition (Barrientos et al., 2011; Hopkins, Nitecki & Bucci, 2011), we next examined if exercise was able to suppress expression of inflammatory markers in the hippocampus. Adult age led to a suppression of *Cxcl10* (significant effect of age: $F_{(1,27)} = 9.83$, $p = 0.004$; Fig. 3D). Hippocampal *Cxcl10* percentage CpG1 methylation was also suppressed with age (significant effect of age: $F_{(1,27)} = 6.39$, $p = 0.018$; Fig. 3E), indicating a potential for enhanced transcription of the gene, paradoxical with the suppressed gene expression. There were no significant differences in *Tlr4*, *Tnfα*, or *Il-6*, but *Il-1β* expression was suppressed with age, being significantly lower in sedentary adults relative to adolescents, but not in the exercised

adults (significant effect of age: $F_{(1,25)} = 6.78$, $p = 0.015$; Fig. 3G).

Examination of hippocampal microglial numbers and morphology also revealed an unexpected anti-inflammatory effect of age and pro-inflammatory effect of exercise. In the CA1 region, microglial density was suppressed with age, with *post hoc* tests revealing significant suppression in the sedentary adults relative to adolescents but not in the exercised group (significant effect of age: $F_{(1,28)} = 10.34$, $p = 0.003$; Fig. 4A, B). There were no effects on the CA3 (Fig. 4C, D) or hilus (not shown). In the subgranular / granule cell layer exercise increased microglial numbers in the adolescents and the exercised adults had fewer microglia than the exercised adolescents (significant age by exercise interaction: $F_{(1,26)} = 5.57$, $p = 0.026$; Fig. 4E, I). There was a p value of 0.058 for the effect of age on subgranular / granule microglial density (Fig. 4F, I). In the molecular region age again suppressed microglial numbers, in this case with the sedentary adults having fewer microglia than sedentary adolescents (significant effect of age: $F_{(1,26)} = 7.01$, $p = 0.014$; Fig. 4G, I).

Exercise and age both significantly influenced neuronal proliferation. The adolescent and adult exercised groups had more DCX-immunoreactive cells in the suprapyramidal blade of the dentate gyrus than their sedentary counterparts, and adolescents had more DCX-positive cells than adults (significant effect of age: $F_{(1,24)} = 11.32$, $p = 0.003$; and exercise: $F_{(1,24)} = 19.19$, $p < 0.001$; Fig. 5A, I). There was also a significant main effect of age of DCX-immunoreactive cells in the infrapyramidal blade, with *post hoc* tests indicating the adolescent exercised group had more DCX cells than the adult exercised and sedentary groups (significant effect of age: $F_{(1,24)} = 12.6$, $p = 0.002$; Fig. 5B, I). There were, however, no differences in the number of mature neurons in any of the hippocampal regions (NeuN; Fig. 5C-G) and no differences in the density of synaptophysin (Fig. 5H). We found no

differences in estimated volume in any of the subdivisions of the hippocampus or in total hippocampus (Table 2).

Effects of exercise on adolescent and adult recognition memory

To assess if these effects of voluntary exercise on hippocampal inflammatory markers, microglia and neuronal proliferation could impact memory function we next tested these rats in a novel object recognition test for memory. There were no differences between the groups in the time spent exploring both the familiar objects in the trial phase (Fig. 6A). Neither was there an effect of age or exercise on absolute novel object exploration in the test phase (Fig. 6B). There was an age effect on the ability to discriminate the two objects in the test phase, with adolescents having relatively poor discrimination, but there were no differences between the groups with *post hoc* tests (significant effect of age: $F_{(1,24)} = 6.98$, $p = 0.014$; Fig. 6C), indicating the exercise-induced changes within the hippocampus were insufficient to support improvement in this memory test. While the adolescents did not have ratios significantly different from zero (indicating they did not recognize or prefer the novel object), both groups of adults showed significant novel object preference (AS: $t_{(6)} = 3.36$, $p = 0.015$; AE: $t_{(6)} = 4.15$, $p = 0.006$). Novel object recognition performance (discrimination ratio) was significantly inversely correlated with microglial numbers in the dentate gyrus subgranular / granule cell layer such that those rats with fewer microglia in this region performed better in this task ($R^2 = 0.24$; $p = 0.0095$).

Discussion

In terms of metabolic effects, exercise can undoubtedly be beneficial. It improves insulin sensitivity and glucose homeostasis and can serve to maintain healthy body weight (Bajpeyi et al., 2009). It also balances energy utilisation in skeletal muscle, building muscle mass

during early life and preserving it in ageing (Christianson & Shen, 2013). In addition, exercise can improve cardiovascular fitness, cognitive performance, and mood (Ryan & Nolan, 2015). As such, physical inactivity has been listed as the fourth leading risk factor for mortality globally (Neufer et al., 2015). Here we see both fat mass and circulating leptin levels are decreased in adolescents and adults relative to sedentary controls, indicating a beneficial effect of exercise on fat mass and metabolic signalling. However, exercise to the degree where lean (muscle) mass is affected is potentially detrimental. In humans excessive cardiovascular exercise can result in lean mass loss (Beavers et al., 2014; Chaston, Dixon & O'Brien, 2007). A reduction in lean mass is also seen with severe calorie restriction, including in activity based anorexia models of anorexia nervosa (Achamrah et al., 2016; Chaston, Dixon & O'Brien, 2007). Although we did not see differences in ghrelin to indicate continuing negative energy balance (Lockie & Andrews, 2013), the reduced caloric efficiency and relative reduction of lean mass compared with sedentary controls suggest our exercised adults need to eat more to maintain the same body mass, potentially making them less competitive than the non-exercised controls. The same effect is not seen in adolescence, with lean mass and caloric efficiency being maintained in this group despite effective loss of fat mass. We also did not find notable differences between the groups in hypothalamic inflammatory markers, indicating the effects of exercise and age on feeding and metabolism are probably independent of microglial activity here.

Previous investigations have shown exercise can dampen central inflammation and potentially has positive effects on cognitive function by doing so. Thus, physical activity can improve the decreases in brain plasticity and cognitive function caused by long-term high fat diet (Woo, Shin, Park, Jang & Kang, 2013), while longer-term (24 hr) retention of the novel object recognition task is improved in adolescence after four weeks exercise (Hopkins,

Nitecki & Bucci, 2011). In ageing animals, exercise improves hippocampal neurogenesis and memory, and does so in association with neuroimmune modulation (Speisman, Kumar, Rani, Foster & Ormerod, 2013). Similarly, in a model of traumatic brain injury, exercise is effective at combatting cognitive dysfunction by suppressing the associated inflammatory response (Piao et al., 2013). In the present study we hypothesized adolescent rats would be more sensitive to the beneficial metabolic and anti-inflammatory effects of moderate voluntary exercise. We found these hypotheses largely not supported in our model. We found exercise does not affect inflammatory markers in the hypothalamus. In the hippocampus, moderate voluntary exercise increased microglial numbers in the subgranular / granule cell region of the dentate gyrus in adolescents but not adults and, interestingly, microglial numbers in this region were significantly inversely correlated with novel object recognition performance. Exercise also increased numbers of DCX-immunoreactive cells in the suprapyramidal blade of the dentate gyrus in adolescents such that it was greater than that seen in adults. However, we saw no differences in numbers of mature neurons in the hippocampus, indicating exercise does not contribute to incorporation of mature granule cells, at least within the time frame assessed. Our lack of changes in numbers of mature neurons may reflect timing in neuronal maturation relative to the exercise task in that the exercise period was necessarily relatively short, leaving little time for neurons born in the later stages to fully mature. The effect of exercise on cell proliferation was also not reflected in memory performance. These findings differ somewhat from the few other studies showing exercise in adolescence can have beneficial effects on memory greater than those seen in adulthood (Hopkins, Nitecki & Bucci, 2011), potentially a facet of study design that suggests exercise duration or intensity may need to be greater than that achieved by a voluntary *ad libitum* effort in order to produce notable central effects, or that the type of memory test is important. Notably, in Hopkins et al's study, they used a similar task, but much longer ITI

(24 hr) making the task more difficult and assessing longer-term memory retention, which might also explain the differences between our findings (Hopkins, Nitecki & Bucci, 2011). There has been extensive work to try to determine the respective roles of the hippocampus, perirhinal cortex and other regions in object recognition memory and there is still some disagreement in the field. However, studies with temporary lesions of the hippocampus reveal a lesion-induced impairment of object recognition, suggesting the hippocampus is crucially involved in object recognition memory if the time between initial exposure and the recognition trial is longer than 10 min (Cohen & Stackman, 2015). Previous findings that the hippocampus has no detectable role in object recognition memory after permanent lesions may be explained by the lesions leaving some hippocampal structures intact to perform the task and by compensatory remodelling resulting in another region completing a normally hippocampally-dependent task (Cohen & Stackman, 2015). We should also note that the rat brain does display some lateralisation and in the present study we used the right hypothalamus and hippocampus for molecular work and the left for immunohistochemistry. It is possible the differences (or absence of differences) we see between our groups is not reflected in the opposite side.

Interestingly, our study revealed a clear effect of age on some markers of inflammation and on microglial number and density, particularly in the hippocampus. In the hippocampus there was an age-associated suppression of microglial number as well as an age-associated improvement in recognition memory in a novel object recognition test. Age has previously been shown to be pro-inflammatory, with ageing rats having hippocampal microgliosis, elevated pro-inflammatory gene expression and central cytokine levels, and exacerbated sickness responses to an immune challenge (Barrientos et al., 2011). Aged rats are also at risk of poorer cognitive function than their younger counterparts, showing ageing-related memory

decline and being more susceptible to cognitive impairment by immune or other challenges (Barrientos et al., 2006; Spiegel, Sewal & Rapp, 2014). Anti-inflammatories can ameliorate some of these effects, improving memory and hippocampal functional connectivity in humans and rats (Kodali, Parihar, Hattiangady, Mishra, Shuai & Shetty, 2015; Witte, Kerti, Margulies & Floel, 2014). However, these studies compared young-adult rats with those of advancing age (approximately equivalent to 65+ in human years) (Sengupta, 2013). In the present study our "adult" rats and our effect of age should not be confused with "old" rats or the effects of old age or advanced ageing, our adults having only reached sexual maturity a few weeks prior to the start of the experiment. Thus, the microglial suppression in adults compared with adolescents is perhaps not surprising, reflecting the brain's maturation into adulthood at this time.

In summary, exercise influences weight gain and lean mass in adults only, but reduces fat mass and circulating leptin levels in both adults and adolescents. Exercise stimulates neuronal proliferation in the suprapyramidal blade of the dentate gyrus at both ages, but this is not reflected in an improvement in performance in the novel object recognition test. Finally, adolescence was associated with an unexpected significant increase in hippocampal microglial number and density relative to adults. Together these data suggest exercise has a beneficial effect on metabolic parameters, particularly in adolescents and can simulate neuronal proliferation and changes in hippocampal microglia without influencing memory in a recognition task.

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Table 1. Primer details for rt-PCR.

Target Gene	NCBI Reference Sequence	TaqMan Assay ID	Product Size
Actb	NM_031144.2	4352340E	91
Cxcl10	NM_139089.1	Rn01413889_g1	106
Il-1β	NM_031512.2	Rn00580432_m1	74
Il-6	NM_012589.2	Rn01410330_m1	121
Tlr4	NM_019178.1	Rn00569848_m1	127
Tnfα	NM_012675.3	Rn01525859_g1	92

Table 2. Volume estimations for hemispheric hippocampus and subdivisions. Estimated volume (mm^3). T = adolescent, A = adult, S = sedentary, E = exercised.

Region	TS	TE	AS	AE
CA1	14.3 ± 0.9	15.4 ± 1.0	14.7 ± 1.3	16.1 ± 0.9
CA3	10.0 ± 0.6	11.2 ± 0.6	11.3 ± 0.5	11.2 ± 0.6
DG Suprapyramidal	5.9 ± 0.2	6.5 ± 0.4	5.9 ± 0.3	6.3 ± 0.5
DG Infrapyramidal	3.3 ± 0.3	3.9 ± 0.4	3.5 ± 0.2	4.2 ± 0.5
Total hippocampus	33.6 ± 0.8	37.1 ± 1.6	35.4 ± 1.6	37.9 ± 1.6

Figure Legends

Fig. 1. Effects of exercise on adolescent and adult weight gain, satiety signals and peripheral inflammatory markers. A) Average daily distance travelled. B) Average nightly distance travelled. * Significant *post hoc* difference at weeks 3 and 4. C) Weekly weight gain. # Adults weighed more than adolescents at all stages with *post hoc* tests. * Adult sedentary rats weighed more than adult exercised at weeks 1, 2, 3, and 4. D) Lean mass after four weeks. E) % fat mass after four weeks. F) Average food intake calculated weekly. # Adults ate more than adolescents with *post hoc* tests at week 1. * Adult exercised rats ate more than the other three groups at weeks 2 and 4. G) Average calorie efficiency calculated weekly. Adults had lower caloric efficiency than adolescents with *post hoc* tests at week 1. * Adult exercised rats had lower caloric efficiency than the adolescent groups at weeks 2, 3, and 4. H) Total serum ghrelin. I) Serum acyl ghrelin. J) Serum desacylated ghrelin. K) Plasma leptin. L) Plasma Cxcl10. M) Adipose expression of toll-like receptor 4 (*Tlr4*). N) Adipose expression of *Cxcl10*. Data are mean + SEM. $p < 0.05$. D, E, K: different letters indicate differences between the groups. T = adolescent, A = adult, S = sedentary, E = exercised.

Fig. 2. Effects of exercise on adolescent and adult hypothalamic inflammatory markers. A) Hypothalamic expression of toll-like receptor 4 (*Tlr4*) mRNA. B) Percentage methylation at *Tlr4* CpG1. C) Percentage methylation at *Tlr4* CpG2. D) *Cxcl10* expression. E) Percentage methylation at *Cxcl10* CpG1. F) Tumour necrosis factor α (*Tnfα*) expression. G) Interleukin 1β (*Il1β*) expression. H) *Il6* expression. I) Number of microglia (Iba-1 immunoreactive cells) in the paraventricular nucleus of the hypothalamus (PVN). J) Microglial density in the PVN. K) Microglial numbers in the arcuate nucleus of the hypothalamus (ARC). L) Microglial density in the ARC. Data are mean + SEM. $p < 0.05$. E: different letters indicate differences between the groups. I, K and L: * indicates significant main effect of age. # indicates significant age by exercise interaction. T = adolescent, A = adult, S = sedentary, E = exercised.

Fig. 3. Effects of exercise on adolescent and adult hippocampal inflammatory gene expression and DNA methylation. A) Hippocampal expression of toll-like receptor 4 (*Tlr4*) mRNA. B) Percentage methylation at *Tlr4* CpG1. C) Percentage methylation at *Tlr4* CpG2. D) *Cxcl10* expression. E) Percentage methylation at *Cxcl10* CpG1. F) Tumour necrosis factor α (*Tnfα*) expression. G) Interleukin 1β (*Il1β*) expression. H) *Il6* expression. Data are mean + SEM. $p < 0.05$. D and E: * indicates significant main effect of age. G: different letters indicate differences between the groups. T = adolescent, A = adult, S = sedentary, E = exercised.

Fig. 4. Effects of exercise on adolescent and adult hippocampal microglia. A) Number of microglia (Iba-1 immunoreactive cells) in the CA1 region of the hippocampus. B) Microglial density in the CA1. C) Microglial numbers in the CA3. D) Microglial density in the CA3. E) Microglial numbers in the subgranular (SG) / granule cell region of the dentate gyrus (DG).

F) Microglial density in the SG / granule cell region of the DG. G) Microglial numbers in the molecular region of the DG. H) Microglial density in the molecular region of the DG. I) Representative photomicrographs of Iba-1 labelling. Scale bar = 200 μm. Data are mean + SEM. $p < 0.05$. B, E, G: different letters indicate differences between the groups. T = adolescent, A = adult, S = sedentary, E = exercised.

Fig. 5. Effects of exercise on adolescent and adult hippocampal neurogenesis. Numbers of doublecortin (DCX)-immunoreactive cells in the A) suprapyramidal and B) infrapyramidal blades of the dentate gyrus (DG). Numbers of neuronal nuclei (NeuN)-immunoreactive cells in the C) CA1, D) CA3, E) DG hilus, F) DG suprapyramidal, G) DG infrapyramidal hippocampus. H) Representative photomicrographs of DCX labelling (NeuN counterstain). Scale bar = 200 μm. Data are mean + SEM. $p < 0.05$. Different letters indicate differences between the groups. T = adolescent, A = adult, S = sedentary, E = exercised.

Fig. 6. Effects of exercise on adolescent and adult novel object recognition memory. A) Object exploration in the training trial. B) Novel object exploration in the test trial. C) Novel object discrimination. Data are mean + SEM. $p < 0.05$. * indicates significant main effect of age. T = adolescent, A = adult, S = sedentary, E = exercised.

Figure 1

Soch et al 2016

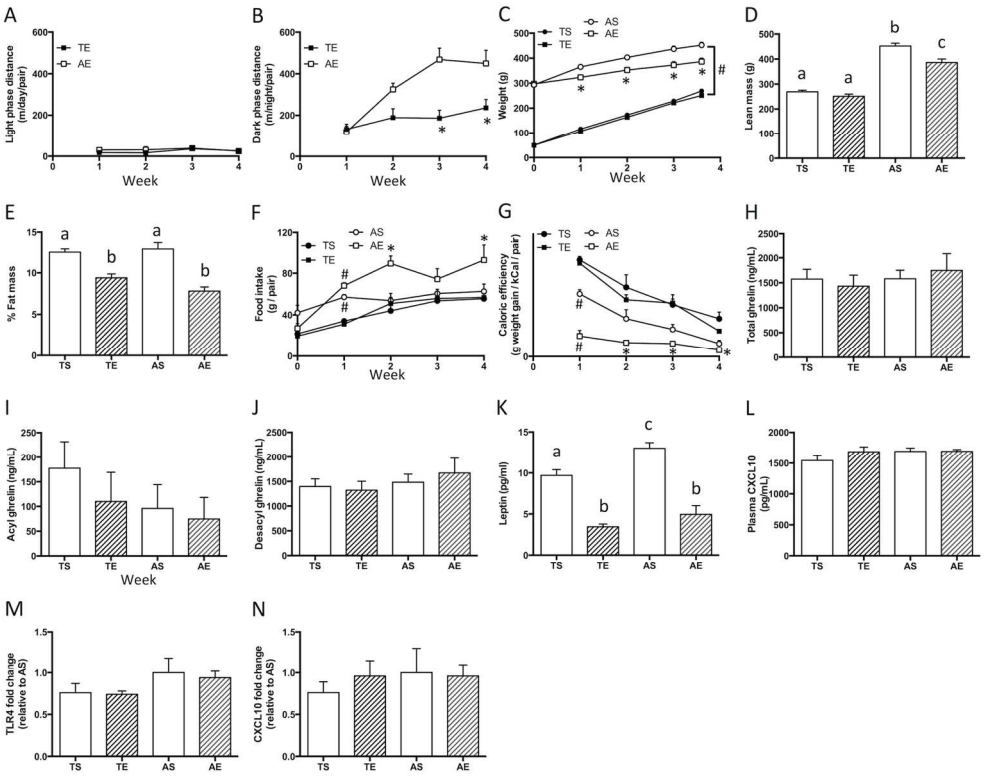

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Figure 2 Soch et al 2016

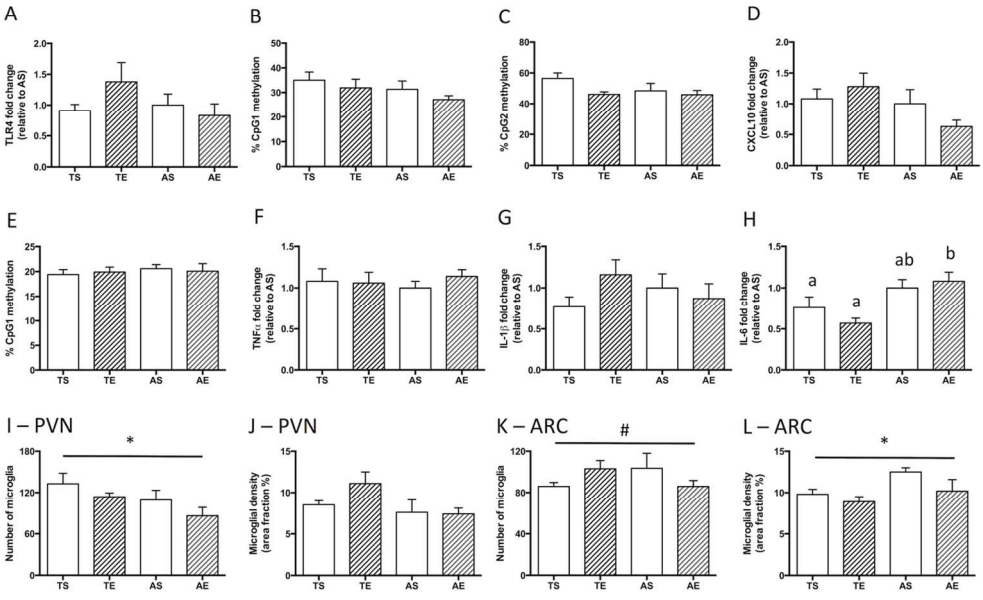

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Figure 3

Soch et al 2016

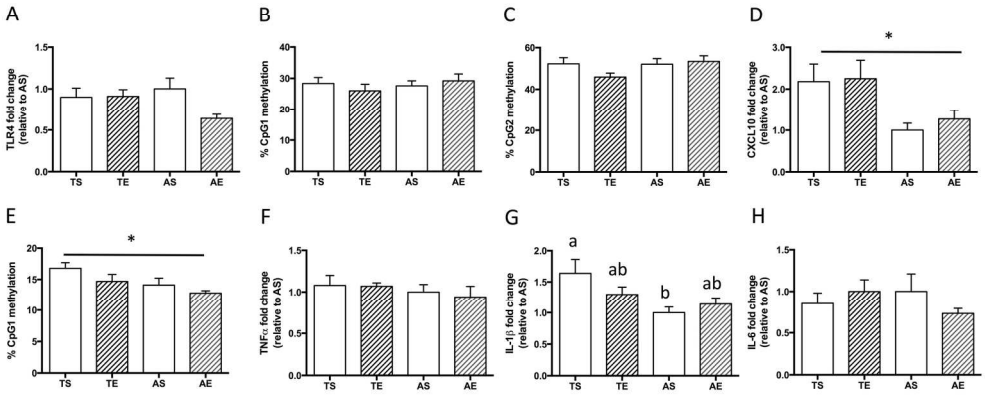

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Figure 4 Soch et al 2016

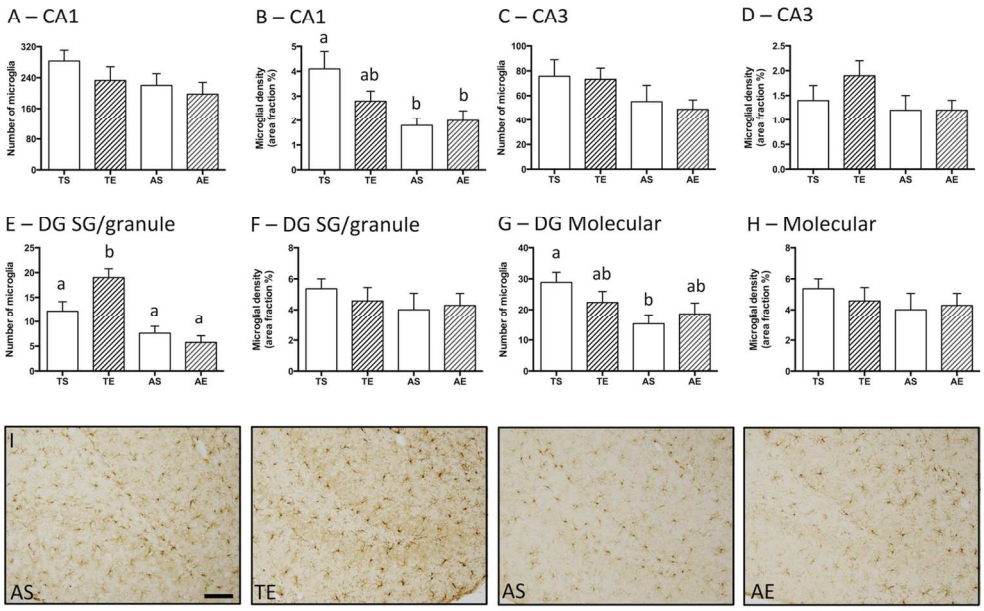

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Figure 5 Soch et al 2016

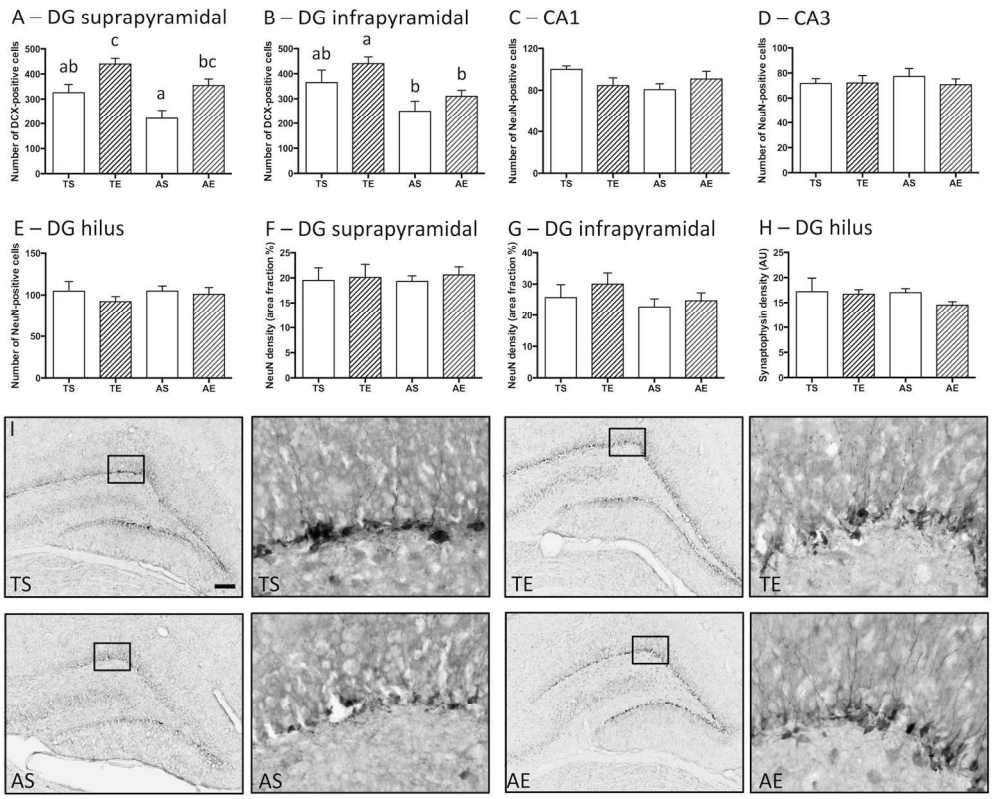

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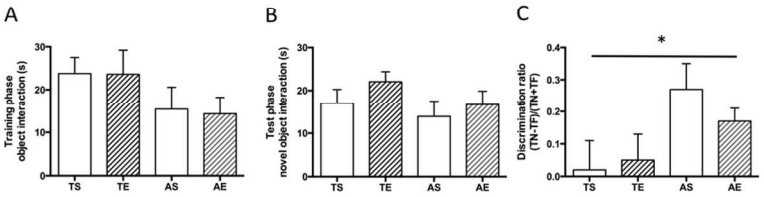
Figure 6 Soch et al 2016



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