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Rats bred for low intrinsic aerobic exercise capacity link obesity with brain inflammation and reduced structural plasticity of the hippocampus

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ABSTRACT

Background: Increasing evidence shows obesity and poor metabolic health are associated with cognitive deficits, but the mechanistic connections have yet to be resolved. We studied rats selectively bred for low and high intrinsic aerobic capacity in order to test the association between low physical fitness, a genetic predisposition for obesity, and brain health. We hypothesized that low-capacity runner (LCR) rats with concurrently greater levels of adiposity would have increased hippocampal inflammation and reduced plasticity compared to the more physically fit high-capacity runner (HCR) rats.

Methods: We examined markers for inflammation and brain plasticity in the hippocampi of LCR rats and compared them to HCR rats. The effect of age was determined by studying the rats at a young age (8 weeks) and later in life (40 weeks). We used western blots and immunohistochemistry to quantify the expression of target proteins.

Results: Our study showed that the number of adult-born new neurons in the hippocampus was significantly lower in LCR rats than it was in HCR rats already at a young age and that the difference became more pronounced with age. The expression of synaptic proteins was higher in young animals relative to older ones. Brain inflammation tended to be higher in LCR rats than it was in the HCR rats, and more prominent in older rats than in young ones.

Conclusion: Our study is the first to demonstrate that low intrinsic aerobic fitness that is associated with obesity and poor metabolic health is also linked with reduced hippocampal structural plasticity at a young age. Our results also suggest that inflammation of the brain could be one factor mediating the link between obesity and poor cognitive performance.

1. Introduction

The human population is getting out of shape, as the worldwide number of adults with obesity has reached 2 billion and is constantly increasing (WHO, 2017). At the same time, the risk for developing metabolic diseases has multiplied. This association has negative consequences for both the body and the brain, especially with age. It is well known that high cardiovascular fitness, and thus likely a lean phenotype associates with reduced risk of mental and physiological illness as well as with better cognitive ability (Hillman et al., 2008; Barak et al., 2015).

Abbreviations: **AHN**, **adult hippocampal neurogenesis**; **GFAP**, **glial fibrillary acidic protein**; **HCR**, **High-capacity runner**; **IBA1**, **ionized calcium-binding adaptor molecule 1**; **IHC**, **Immunohistochemistry**; **IL-1β**, **interleukin 1 beta**; **LCR**, **low-capacity runner**; **SYN-1**, **Synapsin 1**; **SYP**, **Synaptophysin**; **TLR**, **Toll-like receptor**; **TLR3**, **Toll-like receptor 3**; **WB**, **Western blot**.

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In addition to the effects of lifestyle, such as diet and physical activity, genetic background in itself has an important impact on obesity and cardiovascular fitness (Fall and Ingelsson, 2014). Genotype has been shown to influence brain health (Lett et al., 2020) and to increase the susceptibility to neurodegeneration even at a young age (Niemann and Jankovic, 2019). For instance, an obesity-causing variant of the fat mass and obesity-associated gene (FTO) is associated with impulsive behavior and reduced functions of some brain areas in humans (Chuang et al., 2015). However, it is not clear whether the same genotype could explain the variance in both cardiovascular fitness and brain health, and if so, at what age would the effects of the genotype on the brain start to manifest.

A major mechanism that could possibly mediate the effects of obesity and low fitness on the brain is inflammation (Almeida-Suhett et al., 2017; Yang and Zhang, 2020). However, this mechanism has not been widely studied. Immune surveillance in the brain relies mainly on microglia (Norris and Kipnis, 2019) but also involves other glial cells such as astrocytes. Astrocytes are known to secrete proteins, lipids and small molecules that bind to neural receptors to promote synaptogenesis and regulate synaptic connectivity (Baldwin and Eroglu, 2017; Chung et al., 2015). Microglia, in turn, are involved in synaptic pruning, a process necessary for normal development of the brain that continues until adulthood (Mota, et al., 2019; Paolicelli et al., 2011). When faced with an injury or pathogen, astrocytes start to secrete different cytokines and chemokines that mediate the inflammatory reactions in the brain (Colombo and Farina, 2016). Microglia, astrocytes and neurons express several members of the toll-like receptor (TLR) family that participate in the responses of these cells to injury and infection (Hanke and Kielian, 1979). TLRs recognize pathogen-associated molecular patterns, which are small molecular motifs conserved within a class of microbes. In addition to mediating inflammation, for example, TLR3 negatively regulates the proliferation of neural progenitor cells (Lathia et al., 2008), axonal and dendritic growth (Chen et al., 2017), and constrains adult hippocampal neurogenesis (AHN) (Okun et al., 2010). However, it is not known how the genetic predisposition to obesity affects this important player of brain inflammation and development. To conclude, while a functional neuroinflammatory system seems to be crucial for normal development of the brain, and the purpose of the inflammatory response is mainly to protect the brain, its aberrant activation during early development can increase the risk for neurodevelopmental deficits and neuronal disorders (Chen et al., 2019).

The hippocampus is responsible for the memory of what, where, and when (Scoville and Milner, 2000) and in dementia it is this area that is degraded. AHN is a continuous process through which the cells proliferate in the dentate gyrus of the mammalian hippocampus, mature into granule cells, and ultimately become incorporated into neuronal networks (Kozareva et al., 2019). Numerous studies in rodent models indicate that exercise can increase AHN (Nokia et al., 2016; Vivar and van Praag, 2017), while it is reduced by prolonged neuroinflammation (Valero et al., 2017) and obesity brought about by a high-fat diet (Robison et al., 2020). In rodents, adult-born hippocampal neurons seem crucial for a variety of adaptive behaviors, such as responses to stress (Snyder et al., 2011). Adult neurogenesis also exists in the human brain, although its role in cognition remains unclear (Beckervordersandforth et al., 2017). Interestingly, energy restriction in obese humans might have a positive effect on cognitive functions that in mice are associated with AHN (Kim et al., 2020). It appears, therefore, that diet-induced obesity might be associated with impaired AHN, which could further impair cognition, but to our knowledge studies on the effects of inherited obesity on AHN are lacking.

In this study we aimed to determine whether and how a genotype that disposes to low cardiovascular fitness and further to the development of obesity is associated with neuroinflammation and hippocampal structural plasticity. To this end, we utilized a well-described polygenic rat model, initially developed for studying metabolic diseases (Koch and Britton, 2018). The idea behind the animal model originates from the energy transfer hypothesis, which states that the "variation in capacity for energy transfer is the central mechanistic determinant of the divide between disease and health" (Koch et al., 2012). The capacity for energy transfer can also be referred to as aerobic fitness, and as a test for the energy transfer hypothesis, Koch and Britton used a two-way selection of rats based on high- and low-intrinsic aerobic capacity. The outcome of this artificial selection supports the energy transfer hypothesis, as disease risk segregates with selection for low-capacity runners (LCR), whereas high-capacity runners (HCR) are resistant to risk for disease. We were especially interested in whether the LCR rats developing obesity and metabolic disease also showed an increase in neuroinflammation as well as a decrease in expression of synaptic proteins and AHN and, if so, whether these effects were visible at a young age. Our experiment will provide, for the first time, the ability to test the hypothesis that a genetic susceptibility for metabolic dysfunction alters the development of the brain by involving increased inflammation and decreased plasticity already at a young age. This study sheds light on the development rate and the extent of the possible brain pathology that follows from a less-than-optimal fitness-related genotype.

2. Methods

2.1. Ethical approval

All the experimental procedures were approved by the Animal Experiment Board of Finland (license ESAVI/7647/04.10.07/2014) and implemented in accordance with directive 2010/63/EU of the European Parliament and of the Council on the care and use of animals for research purposes.

2.2. Animals

We utilized a polygenic rat model (Koch and Britton, 2018) developed by two-way selective breeding for intrinsic aerobic running capacity, which also divides the rats for their susceptibility to obesity and metabolic disease. This long-term selective breeding program has produced two lines of rats, HCR and LCR. Now, decades later, the LCR rat model performs well as a model for obesity, associated metabolic syndrome, aging, cognitive decline and numerous other diseases (Koch and Britton, 2018). Because the rat model is based on genetically heterogeneous stock, it is suggested to be well translatable to humans in comparison to inbred or transgenic models, for example (Koch et al., 2012).

Phenotyped adult male and female HCR and LCR rats (Koch and Britton, 2001) were shipped from the University of Michigan, Ann Arbor and bred at the University of Jyväskylä, Finland, to provide the male rats of generation 36 used in these experiments. The rats were housed at the Laboratory Center of the University of Jyväskylä in groups of two or three per cage (Macrolon IV, Techniplast, Italy). Aspen chips (Tapvei, Estonia) were used as bedding and a plastic toy was provided in each cage. Housing conditions were controlled with temperature at $21 \pm 2\,^{\circ}C$, and humidity at $50 \pm 10\%$. The rats were kept in a 12-hr light–dark cycle, with lights on from 8:00 am to 8:00 pm. Food (R36; Labfor, Lantmännen, Stockholm, Sweden) and water were available *ad libitum*. To study the effect of aging, both HCR and LCR siblings were divided as equally as possible into *young* and *old* groups to minimize the effect of genetic variation on the variables studied between the age groups. At the time of sacrifice, the younger rats were 8 weeks old (young) while the older rats were 40 weeks old (old). Rats in the old groups were in fact middle-aged (Sengupta, 2013), but we use the terms *young* and *old* for clarity. During the study, to avoid the possible effects of difference in running on the studied variables, all animals were kept sedentary, meaning we did not phenotype them for running capacity and they had no access to a running wheel.

2.3. Blood analyses and tissue collection

The non-fasted rats were stunned with CO_2 and death was ensured by

open chest cardiac puncture. The blood was collected from the heart, glucose was measured with HemoCue Glucose (201+; HemoCue, Ängelholm, Sweden), then the blood was centrifuged to separate serum, which was stored at −80 °C. Serum cytokines were analyzed with a rat cytokine inflammation 9-plex ELISA kit (Quansys biosciences, #111649RT) according to the manufacturer's instructions and imaged with a Q-view imager (Quansys biosciences, Logan, UT, USA). The detection limits for the cytokines were as follows: interleukin (IL)-1a, 4.80 pg/mL; IL-1β, 2.90 pg/mL; IL-2, 8.70 pg/mL; IL-4, 3.60 pg/mL; IL-6, 74.0 pg/mL; IL-10, 12.5 pg/mL; IL-12, 82.0 pg/mL; interferon (IFN)-γ, 5.30 pg/mL and tumor necrosis factor (TNF)-α, 33.0 pg/mL. However, most of the samples were under the detection limits of the kit, so results are not reported for the data which could not be analyzed. Serum insulin was measured with rat insulin ELISA kit (Mercodia, #10–1250-01) according to the manufacturer's instructions using Dynex DS2 automated ELISA system (Dynex technologies, VA, USA). The brain was removed from the skull and the left and right hemispheres were separated. The hippocampus was extracted from the left hemisphere and slices (2 × 1 mm thick) were taken from both the dorsal and ventral parts. These slices were snap-frozen in liquid nitrogen and used later for western blot (WB) analysis. The right hemisphere was post-fixed with 4% paraformaldehyde solution and kept at + 4 °C for 48 h and then transferred into 0.1 M phosphate-buffered saline (PBS, pH 7.4). The brains were prepared for sectioning by cryoprotecting them in 30% sucrose solution for 48 h at room temperature (RT). After that, the hemisphere was embedded in dry ice and cut into 40 μm thick coronal sections with a sliding microtome (Leica SM 2000 R Microtome, Leica Instruments GmBH, Germany). The slices were collected into 12 tubes filled with the cryoprotectant solution, so that each tube contained a set of 9 to 10 slices representing the whole hippocampus. The sections were stored at −20 °C until staining.

2.4. Immunohistochemistry

Immunohistochemical (IHC) stainings were performed using the free-floating method, as previously described (Nokia et al., 2016). We chose to stain the hippocampal sections using antibodies for double-cortin (DCX) and ionized calcium-binding adaptor molecule 1 (IBA1). DCX is a marker for migrating, immature neurons (Ayanlaja et al., 2017), and serves well to quantify the number of newborn neurons, whereas IBA1 was used as a marker for microglia (Norden et al., 2016).

The samples were kept on a shaker plate during all washes and incubations. First, the cryoprotectant was removed from the sections by washing with 0.1 M PBS. Antigen retrieval was performed by heating the samples in + 80 °C 0.01 M citric acid (pH 6.0) solution. The samples were cooled down in 0.1 M Tris-buffered saline supplemented with 0.3% Triton X-100 (TBS-T). Next, they were treated with 3% H_2O_2 (30 min, RT, diluted to 0.1 M PB) to block endogenous peroxidases, and with 10% rabbit serum to block unspecific binding (1 h, diluted to TBS-T). The samples were washed between the pretreatment steps with TBS-T. Polyclonal goat anti-DCX (Santa Cruz, sc-8066) was used as a primary antibody at 1:2000 dilution in TBS-T, supplemented with 1% rabbit serum. The samples were incubated with the primary antibody over night at + 4 °C. The primary antibody was washed out and followed by a 2 h incubation with the secondary antibody, biotinylated rabbit anti-goat IgG (Vector laboratories, BA-5000) using a 1:500 dilution in TBS-T. The samples were washed with TBS-T, followed by 2 h incubation with a tertiary antibody, streptavidin horseradish peroxidase (GE Healthcare, RPN1231), diluted at 1:1000 in TBS-T. The sections were then washed again with TBS-T. The visualization of the staining was done with 3,3′ -Diaminobenzidine (DAB, Sigma-Aldrich) solution (0.25% DAB in Tris-buffer) supplemented with 0.0225% H_2O_2. The stain was let to develop for approximately 4 min, after which the staining reaction was stopped by replacing DAB with Tris-buffer. The sections were mounted on a clean glass microscope slide (Thermo Fischer) using 0.4% gelatin solution and left to dry at RT.

Staining of the free-floating samples was performed in a similar manner for studying neuroinflammation and neuronal activation. Microglia were stained using a rabbit anti-human IBA1 (ThermoFischer, PA5-27436) primary antibody, diluted at 1:500 in PBS-T with 2% goat serum. A biotinylated goat anti-rabbit IgG (Vector laboratories, BA-1000) was used as the secondary antibody at 1:500 dilution in PBS supplemented with Triton X-100 (PBS-T). The tertiary antibody treatment was the same as with the DCX staining and the DAB visualization was the same but with the addition of nickel intensification. To confirm the specificity of the staining, "no primary antibody" controls were included for IBA1.

The slices stained for DCX were also counterstained with 0.1% cresyl violet after they had dried on the slides. The slides were cleared in rising alcohol concentration and xylene after which they were covered with Depex (VWR International, Radnor, PA, USA) and cover glasses (VWR). For IBA1, the slides were cleared with xylene and coverslipped without cresyl violet staining.

2.5. Microscopic analyses

All the microscopic analyses were performed blinded. For neurogenesis, the number of DCX positive cells was counted from the granule cell layer of the hippocampus with a light microscope (Olympus BX50, Olympus, Japan) with 400x magnification. Every 12th unilateral section throughout the dentate gyrus was analyzed. Altogether, 8 to 10 slices representing one hippocampus per animal were included in the analysis. The number of slices was taken into account when calculating the estimation of the total number of adult-born cells. Finally, the number of cells was multiplied by 2 to get an estimation of the total number of DCX labelled cells in both hippocampi. Representative images of the staining were taken with an Olympus BX50 microscope equipped with a color view II camera and UplanFLN 10x/0.30 objective. For greater magnification, a 40x/0.75 objective was used.

For IBA1, the stained sections were scanned in the Central Finland Central Hospital with a NanoZoomer microscope (Hamamatsu, Japan; 40x resolution). The images were then analyzed with open source bioimage analysis software QuPath (Bankhead et al., 2017) to obtain the number of stained cells based on their shape and color intensity. To start, the no primary antibody control sections were confirmed to be without staining. Automatic cell detection with specified parameters (detection based on optical density sum with requested pixel size 2 μm, background radius and cell expansion 8 μm) was used to count the IBA1 positive cells from the four middle hippocampal sections of each brain (approximately sections from −3.0 mm to −5.0 mm relative to bregma (Paxinos and Watson, 2007). For each rat, the threshold used for cell detection was determined based on the background by calculating the mean optical density (OD mean, background staining) of the sections, the value of which was then used as a threshold intensity value in QuPath analysis. If the background between sections was uneven, then the intensity threshold was calculated for each section separately. The areas of interest were CA1, CA3, the hilus and the granule cell layer (GCL). For CA1 and CA3, OD was measured from an area of 200 × 200 μm rectangle and for hilus from 100 × 100 μm rectangle, approximately from the same spots in all sections. The chosen areas do not represent the whole area but are representative samples of the selected regions. The granule cell layer was manually selected by the experimenter from the image using the brush tool in QuPath. To get an estimate of microglia expression, average cell counts and densities (number of cells per mm^2) per section from each area were calculated for each animal and used in statistical analyses.

2.6. Western blot

WB analysis was used to quantify inflammation and synaptic proteins in hippocampal tissue. Inflammatory markers TLR3 and interleukin 1-beta (IL-1β) were selected based on the knowledge that

activation of TLR3 increases inflammation, and they are related to neurodegeneration and decreased neurogenesis (Field et al., 2010; Vetreno et al., 2018). Furthermore, the activation of TLR3 leads to an increased transcription and production of IL-1β (Lopez-Castejon and Brough, 2011). The expression of synapsin (SYN-1) and synaptophysin (SYP) were analyzed to quantify the amount of synaptic proteins (Menegon et al., 2006; Sarnat and Born, 1999). Both SYP and SYN-1 are abundant proteins of synaptic vesicles (Greengard et al., 1993) and thus can be used as markers for synaptic density and synaptic plasticity. Glial fibrillary acidic protein (GFAP) and IBA1 were analyzed to quantify astrocytes and microglia, respectively (Norden et al., 2016). GFAP is an intermediate filament protein, used for motility and structure maintenance in astrocytes (Norris and Kipnis, 2019).

The hippocampal tissue sample was homogenized in 200 µl of ice-cold lysis buffer (20 mM HEPES (pH 7.4), 5 mM EGTA, 1 mM EDTA, 0.2% sodium deoxycholate, 10 mM $MgCl_2$, 2 mM DTT, 1% NP-40, 1% protease phosphatase inhibitor (Halt Protease and Phosphatase Inhibitor Cocktail 100X, Thermo Scientific, Waltham, MA, USA), 1 mM Na3VO4, 100 mM β-glycerophosphate) using Qiagen TissueLyzer II (Hilden, Germany). After homogenizing for 2 × 2 min at 20 Hz, the soluble proteins were obtained by centrifugation for 10 min at 10,000 × g. The final protein concentration was measured using bichintronic acid method (BCA kit, Thermo Scientific). For the WB, 30–40 µg of total protein were run in 4% to 20% CriterionTM TGX Stain-FreeTM Precast Gels (Bio-Rad, Hercules, CA, USA). After that, the gels were ultraviolet activated with ChemiDoc™ imaging system (Bio-Rad) to allow stain-free visualization of the blots afterwards. Then, the proteins were blotted onto nitrocellulose membranes using Trans-Blot® Turbo™ RTA Midi Nitrocellulose Transfer Kit (Bio-Rad) and Trans-Blot® Turbo™ Transfer System (Bio-Rad). After blotting, the membranes were imaged with ChemiDoc™ imaging system (BioRad) using default settings for the stain-free blots to be used later for total protein normalization. After that, the membranes were cut horizontally in order to separate the molecular weights corresponding to the proteins of interest for incubation with primary antibodies. Before that, the membranes were blocked for 1 h at RT with OdysseyR Blocking buffer (LI-COR 927–40000, Lincoln, NE, USA) and then incubated overnight at + 4 °C with primary antibodies against synaptic markers SYN-1 (ANR-014, Alomone labs, Jerusalem, Israel, 1:1000 dilution) and SYP (ANR-013, Alomone labs, Jerusalem, Israel, 1:1000), glial marker GFAP (ab7260, Abcam, Cambridge, UK, 1:5000 dilution) and microglial marker IBA1 (PA5-27436, ThermoFisher Scientific, Rockford, IL, USA, 1:700 dilution). To study brain inflammation, we used antibodies against TLR3 (SAB2900405-50UG, Sigma-Aldrich, Saint Louis, MO, USA, 1:500 dilution) and IL-1β (AB1832P, Merck, Darmstad, Germany, 1:500 dilution). As a secondary antibody Goat anti-Rabbit IRDye® 800CW (Licor Biosciences, Lincoln, NE, USA) was used at a dilution of 1:20,000. The membranes were imaged using ChemiDoc™ imaging system (BioRad) with a 60-second exposure time. The band intensities of the proteins of interest were quantified using Image Lab 6.0 (Bio-Rad) and normalized first to the amount of total protein from stain-free images and then to the average score of the first group (young HCR rats) on each gel to minimize the effect of different runs. The stain-free method uses total protein amounts for data normalization, providing more accurate western blotting results than the use of housekeeping proteins. The stain-free method enables the detection of small fold differences in protein expression compared to housekeeping proteins, which expression may differ depending on rat line or treatment (Ferguson et al., 2005). In the stain-free method, the total density for each lane is quantified and a lane profile is obtained. Using 4% to 20% CriterionTM TGX Stain-FreeTM Precast Gels this means that all proteins ranging from 10 to 200 kDa of molecular weight are visualized and quantified. Image Lab 6.0 (Bio-Rad) can interpret the data from the lanes in three dimensions, and the background is adjusted by subtracting the total background from the sum of the density of all the bands in each lane.

2.7. Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics version 24 (Chicago, IL, USA). The results are visualized as box plots unless otherwise stated. Shapiro-Wilk's test was used to test for normality. Most of the data was not normally distributed, and therefore non-parametric Mann-Whitney *U* test was used to compare the groups. However, for the normally distributed data, an independent samples *t*-test was applied. A p-value of 0.05 was set as the threshold for statistical significance. For technical reasons the number of subjects in all analyses is not equal, but it is important to note that no samples were left out from the analysis as outliers.

3. Results

3.1. Body weight and blood analyses

The LCR rats (n = 19) weighed about 20 g more than the HCR rats (n = 20) already at young age (Fig. 1) (mean 188.05 ± 18.73 g vs 165.05 ± 23.15 g, p = 0.002). The difference was even more pronounced in adult rats, with the LCR rats (n = 19) being about 100 g heavier than the HCR rats (n = 20) (mean 481.89 ± 48.26 g vs 375.55 ± 42.28 g, p < 0.001). We did not perform a full body composition analysis because previous studies have already shown that the LCR rats have more body fat compared to HCR rats (Stephenson et al., 2012). Serum insulin concentration was approximately three times higher in older rats than in the young ones in both lines (young HCR vs old HCR: Mdn = 31.32 vs 87.87 µU/mL, U = 12.5, p < 0.001, young LCR vs old LCR: Mdn = 24.36 vs. 71.05 µU/mL, U = 42.0, p < 0.001, see Table 1). There was no difference between the rat lines. In contrast, serum glucose concentration was higher in young rats than it was in the old rats in both lines (young HCR vs old HCR: Mdn = 8.05 vs. 7.20 mmol/L, U = 111.5, p = 0.021, young LCR vs old LCR: Mdn = 8.30 vs. 7.40, U = 102.0, P = 0.022, see Table 1), but again, no differences was seen between the rat lines. We have reported serum lipids at both ages previously and the results indicate better metabolic health in HCR rats than in LCR rats (Pekkala et al., 2017).

3.2. Neuroinflammation

We analyzed several inflammatory markers from hippocampal tissue samples using WB. First, we measured the expression of TLR3 to reflect

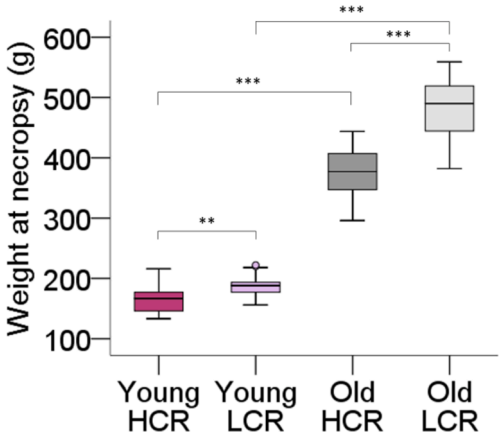

Fig. 1. The body weights (g) of the rats at necropsy show that rats bred for low capacity for running (LCR) were heavier than those bred for high capacity for running (HCR) already at the age of 8 weeks (young) but especially as they aged (10 months). Interpretation of the boxplot: The box represents the interquartile (IQ) range, that is, the middle 50% of the records. The whiskers represent the highest and lowest values within 1.5 times the IQ range. Median is shown as a line across the box. Independent samples *t*-test. *** $p \leq 0.001$.

Table 1
Serum insulin and glucose concentrations at necropsy.

Group	Insulin μU/mL, median (IQR)	Glucose mmol/L, median (IQR)
Young HCR	31.32 (15.08) [a]) ***	8.05 (1.30) [a]) *
Young LCR	24.36 (19.72) [b]) ***	8.43 (1.70) [b]) *
Old HCR	87.87 (45.68) [a]) ***	7.20 (1.03) [a]) *
Old LCR	71.05 (37.70) [b]) ***	7.40 (1.30) [b]) *

IQR = Interquartile Range.
[a] = difference between Young HCR and Old HCR.
[b] = difference between Young LCR and Old LCR.
Mann-Whitney *U* test, * $p < 0.05$, *** $p \leq 0.001$.
HCR = high-capacity runner, LCR = low-capacity runner.

the level of inflammation present in the hippocampus. We found that TLR3 expression was 1.8-fold lower in young LCR rats and 3.1-fold lower in young HCR rats than in the old rats (LCR: Mdn = 8.70 vs. 15.66, n = 10 vs. n = 10, U = 19, p = 0.019; HCR: Mdn = 4.10 vs. 12.87n = 8 vs. *n* = 11, U = 0, $p < 0.001$, see Fig. 2A). Furthermore, the LCR rats expressed twice as much TLR3 as HCR rats did at young age (Mdn = 8.70 vs 4.10, U = 9, p = 0.006) and 1.2 times more at old age (Mdn = 15.66 vs 12.87, U = 25, p = 0.036), indicating that the genotype affected hippocampal expression of TLR3 regardless of age as it was generally higher in LCR rats.

Next, we quantified IL-1β, a pro-inflammatory cytokine that is produced upon activation of TLR3. IL-1β is known to be expressed in different regions of the brain, most abundantly in the hippocampus, and to be present in a healthy state, whilst also playing a role in various diseases (Fogal and Hewett, 2008). We found that the young HCR rats (n = 8) expressed less than half of IL-1β than the old HCR rats did (n = 10) (Mdn = 1.47 vs. 3.27, U = 2, p = 0.001, see Fig. 2B), but there was no age difference in LCR rats (Mdn = 2.60 vs. 3.92, n = 10 vs. n = 10, U = 32, p = 0.174). The genotype affected the expression of IL-1β at younger age, the young LCR rats having 1.8 times higher expression of IL-1β than the young HCR rats did (Mdn = 2.60 vs. 1.47, U = 7, p = 0.003), but no significant difference was seen between the genotypes at the older age (Mdn HCR = 3.27 vs. LCR = 3.92, U = 33, p = 0.199). To recap, IL-1β expression doubled with age, but only in HCR rats. The obese phenotype contributed to an almost 2-fold difference in IL-1β expression, but only at younger age, although the same tendency of the genotype remained at older age.

Then, we analyzed GFAP in hippocampal protein extracts using WB. The reason to quantify GFAP was that astrocytes, like microglia, are activated due to inflammatory stimulus. Therefore, GFAP can be used to estimate the amount and activation of astrocytes (Norden et al., 2016). We observed almost four times higher expression of GFAP in the hippocampus in the young HCR rats (n = 8) than in the old HCR rats (n = 11) (Mdn = 11.86 vs 3.06, U = 0, $p < 0.001$, see Fig. 2C). In addition, the expression was more than 2-fold higher in the young LCR rats (n = 10) than in the old LCR rats (n = 10) (Mdn = 10.78 vs. 4.50, U = 16, p = 0.010), indicating an effect of age on GFAP regardless of genotype. The old HCR rats expressed 1.5 times less GFAP than the old LCR rats (Mdn = 3.06 vs. 4.50, U = 17, p = 0.007). However, no genotype-dependent difference was observed in the young animals (Mdn HCR = 11.86 vs. LCR = 10.78, U = 37, p = 0.790). To conclude, GFAP expression was highest in the young animals. The obese phenotype also increased the expression of GFAP at older age, as old HCR rats had less GFAP expression than their LCR counterparts did.

Next, we measured IBA1 expression to reflect the amount of microglia and their activation. It is known that microglia activate as a result of different inflammatory stimuli or trauma, and once activated they secrete a variety of pro-inflammatory molecules (Norden et al., 2016). An increase in IBA1 expression has been previously linked to microglia activation, although it does not reflect an early state of activation, but rather a state of chronic inflammation (Norden et al., 2016). Two different methods to study IBA1 were used, IHC and WB. The IHC analysis enabled us to investigate the regional differences in protein expression in the hippocampus. In the CA3 sub-region, the old rats had significantly more microglia than the young rats did, the difference being almost 3-fold in HCR rats and 2.5-fold in LCR rats (young vs. old, HCR: Mdn = 196.88 vs. 568.74, n = 9 vs. n = 11, U = 17, p = 0.026; LCR: Mdn = 165.63 vs. 407.29, n = 10 vs. n = 7, U = 16, p = 0.034, see Fig. 3A and B). No genotype-dependent differences were observed at either age (young: HCR vs LCR, Mdn = 196.88 vs. 165.63, U = 34.5, p = 0,625, old: Mdn = HCR 168.75 vs. LCR 211.46, U = 31, p = 0.283). In the hilus, the old HCR rats had 2.8 times more microglia than the young HCR rats did (Mdn = 450.0 vs. 162.5, U = 13, p = 0.009), but no effect of age was seen in the LCR rats (Mdn = young 187.5 vs old 404.15, U = 22.5, p = 0.119). No genotype-dependent differences were present

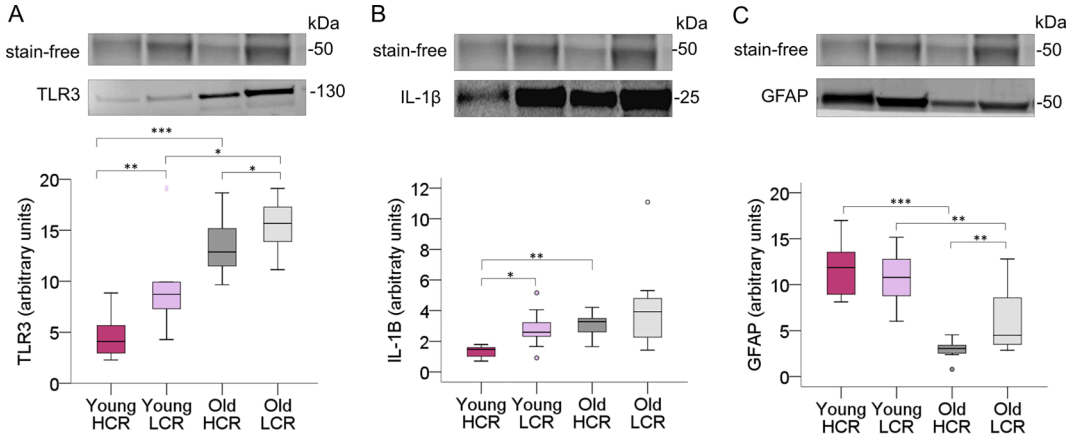

Fig. 2. Western blot analyses were used to study the expression of Toll-like receptor 3 (TLR3), interleukin 1-beta (IL-1β) and Glial fibrillary acidic protein (GFAP) in hippocampal homogenates of rats bred for high capacity for running (HCR) and low capacity for running (LCR). A representative western blot of (A) TLR3, (B) IL-1β and (C) GFAP. The membrane was cut horizontally to separate TLR3, IL-1β and GFAP according to their molecular weights, which are shown with the representative blot image. The graphs show the amount of the proteins normalized to the total protein as analyzed from the stain-free blot. From the stain-free blot only the band of approximately 50 kDa is shown. Mann-Whitney *U* test. * $p \leq 0.050$, ** $p \leq 0.010$ and *** $p \leq 0.001$.

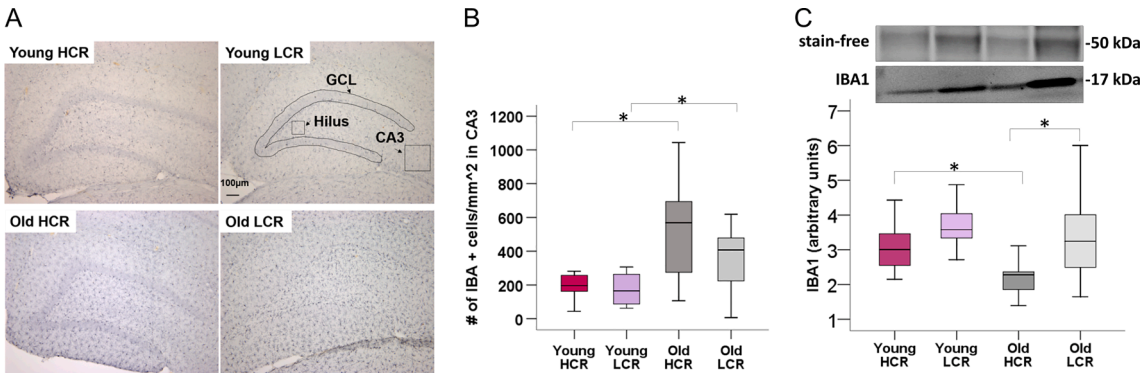

Fig. 3. To quantify microglia, ionized calcium-binding adaptor molecule 1 (IBA1) expression was analyzed using immunohistochemistry in different areas of the hippocampus and western blotting in rats bred for high capacity for running (HCR) and low capacity for running (LCR). (A) Representative bright-field light microscopy images from the hippocampi of the HCR and LCR rats at young and old. IBA1 was visualized by nickel-DAB and positive cells are black. Cells were counted from different areas of the hippocampus: hilus, CA3, CA1 (not visible in the image) and the granule cell layer (GCL). Scale bar 100 µm. (B) IBA1-positive cells were counted by computer-based image analysis. The graph shows the average density (cells per mm^2) of IBA1-positive cells in CA3. (C) IBA1 expression was analyzed from hippocampal samples also by western blotting. A representative western blot of IBA1 together with its molecular weight. The graphs show the amount of the IBA1 normalized to the total protein as analyzed from the stain-free blot. From the stain-free blot only the band of approximately 50 kDa is shown. The boxes and whiskers are the same as in Fig. 1. Mann-Whitney *U* test. * $p \leq 0.050$.

at either age (young: Mdn = HCR 162.5 vs. LCR 187.5, U = 34, p = 0.593, old: Mdn = HCR 450.0 vs. LCR 404.15, U = 38.5, p = 0.650). No significant differences were observed in CA1 area, neither between the genotypes (HCR vs. LCR, young: Mdn = 131.24 vs. 143.75, U = 38.0, p = 0.897, old: Mdn = 168.75 vs. 211.46, U = 40.5, p = 0.772) nor between the age groups (young vs. old, HCR: Mdn = 131.25 vs. 168.75, U = 33.0, p = 0.363, LCR: Mdn = 143.75 vs. 211.46, U = 24.0, p = 0.155) in the number of microglia. Finally, no differences were seen in the number of microglia in the GCL area (young HCR vs. young LCR: Mdn = 674.90 vs. 697.77, U = 41.0, p = 0,780, young HCR vs. old HCR: Mdn = 674.90 vs. 994.30 U = 42.5, p = 0,254, old HCR vs old LCR: Mdn = 994.30 vs. 813.17, U = 55.0, p = 0.525 and young LCR vs old LCR: Mdn = 697.77 vs 813.17, U = 44.0, p = 0.710).

The expression of IBA1 in hippocampal protein extracts analyzed with WB was 1.3-fold higher in the young HCR (n = 8) rats compared to the old HCR (n = 11) rats (Mdn = 3.01 vs. 2.28, U = 11, p = 0.006, see Fig. 3C), but no significant effect of age was seen in the LCR rats (n = 10 vs. n = 10, Mdn = young 3.58 vs. old 3.24, U = 36, p = 0.290). At the older age, there was a difference between the genotypes as the old HCR rats expressed 1.4 times less IBA1 than the old LCR rats (Mdn = 2.28 vs. 3.24, U = 19, p = 0.011). There was no significant difference between the genotypes at young age (Mdn = HCR 3.01 vs LCR 3.58, U = 21, p = 0.091). To summarize, IBA1 expression, analyzed with WB, decreased in the HCR rats with age while no such change was observed in the LCR rats. However, at old age the low aerobic capacity was associated with higher expression of IBA1.

3.3. Synaptic proteins

Hippocampal plasticity at the level of synapses was studied using WB. Regarding the synaptic proteins we measured SYN-1 and SYP. SYN-1 is present at the nerve endings, where it regulates the function of the pool of synaptic vesicles (Menegon et al., 2006). The young LCR rats (n = 10) expressed 1.6 times more SYN-1 than did the young HCR rats (n = 8) (Mdn = 18.90 vs. 11.47, U = 14, p = 0.021, see Fig. 4A). However, no difference was seen between the genotypes at old age (LCR vs. HCR; n = 10 vs. n = 11, Mdn = 3.43 vs. 3.04, U = 54, p = 0.944). In the LCR rats, SYN-1 expression decreased more than five times with age (Mdn = young 18.90 vs old 3.43, U = 0, p < 0.001) while no difference was seen between the young HCR rats and the old HCR rats (Mdn = 11.47 vs. 3.04, U = 22, p = 0.069). Thus, the hippocampal expression of SYN-1 was most pronounced in the young LCR rats.

The expression of SYP, a marker that reflects the density of synapses (Sarnat and Born, 1999), was 1.6-fold higher in the HCR rats than it was in the LCR rats at both young age (n = 8 vs. n = 10, Mdn = 4.99 vs 3.04, U = 11, p = 0.010, see Fig. 4B) and old age (n = 11 vs. n = 10, Mdn = 2.80 vs. 1.69, U = 2, p < 0.001). In addition, the young rats had over 1.5-fold higher hippocampal expression of SYP than did the adults in both HCR (Mdn = young 4.99 vs. old 3.27, U = 9, p = 0.004) and LCR (Mdn = young 3.04 vs. old 1.69, U = 2, p < 0.001). Therefore, synapses seemed to be denser in young compared to old rats and in HCR compared to LCR rats showing. This suggests an effect of age and genotype on SYP.

3.4. Adult hippocampal neurogenesis

The number of new adult-born neurons was quantified with DCX staining. We used DCX as a marker of AHN, since it is an important protein in cell proliferation in neurogenesis and promotes cell migration. DCX is specific for newly generated neurons, and it is not expressed in neural stem cells or glial precursor cells (Couillard-Despres et al., 2005). DCX does not require *in vivo* labelling, and is therefore a commonly used marker for neurogenesis (Ayanlaja et al., 2017). DCX-positive cells were counted from the granule cell layer of the dentate gyrus. The number of DCX-positive cells was significantly higher in the HCR rats than in the LCR rats in both young (n = 18 vs. n = 19; Mdn = 5834.50 vs. 4659.0, U = 88, p = 0.012, see Fig. 5) and old age (n = 19 vs. n = 18; Mdn = 778.0 vs. 505.55, U = 20, p < 0.001). The difference was 1.25-fold at young age but grew to 1.5-fold at old age. However, independent of the genotype, the young rats had significantly more DCX-positive cells than the old rats did, the difference being 7.5-fold in HCR and 9.2-fold in LCR rats (HCR: Mdn = young 5834.50 vs. old 778.0, U = 0, p < 0.001) (LCR: Mdn = young 4659.0 vs. old 505.55, U = 0, p < 0.001). To summarize, AHN was more prominent in young rats than it was in old ones, and in HCR rats than it was in LCR rats. This indicates an effect of age and genotype on AHN.

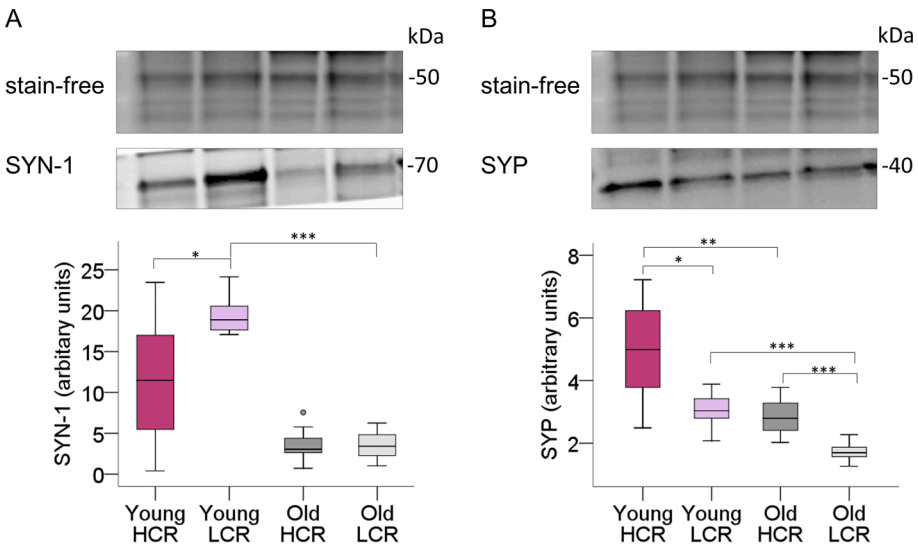

Fig. 4. Older rats expressed less synaptic proteins (arbitrary units) in the hippocampus compared to young rats and the difference is more drastic in low-capacity Runner (LCR) rats than in high-capacity runner (HCR) rats. Western blot analyses of the expression of (A) synapsin (SYN-1) and (B) synaptophysin (SYP) in hippocampal homogenates of HCR and LCR rats at the age of 8 weeks (young) and 10 months (old). The membrane was cut horizontally to separate SYN-1 and SYP according to their molecular weights, which are shown with the representative blot image. The graphs show the amount of the proteins normalized to the total protein as analyzed from the stain-free blot. From the stain-free blot only the band of approximately 50 kDa is shown. The boxes and whiskers are the same as in Fig. 1. Mann-Whitney *U* test. * $p \leq 0.050$, ** $p \leq 0.010$ and *** $p \leq 0.001$.

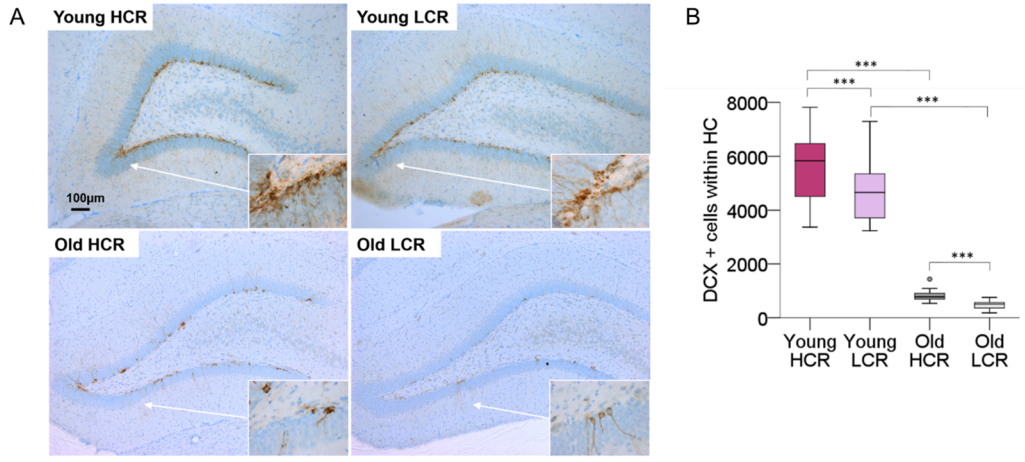

Fig. 5. There were more adult-born neurons in the hippocampus in high-capacity runner (HCR) than in low-capacity runner (LCR) rats and more in young rats than in old rats. (A) Representative bright-field light microscopy images from the hippocampi of the HCR and LCR rats at young (8 weeks) and old (10 months). The newly generated doublecortin (DCX) positive cells are brown. Scale bar 100 µm. (B) Counts of DCX positive cells from the granule cell layer of the hippocampus. The boxes and whiskers are the same as in Fig. 1. Mann-Whitney *U* test. *** $p \leq 0.001$.

4. Discussion

In the present study, we assessed the effects of low intrinsic aerobic capacity, which is associated with obesity and metabolic syndrome, on inflammation and the structural plasticity of the hippocampus. In addition, we studied whether the possible differences due to the low intrinsic aerobic capacity develop already at a young age or only appear upon aging, secondary to the existing obesity and metabolic defects in various tissues in the LCR rats (Karvinen et al., 2016). Here we demonstrate that the LCR rats had less neurogenesis and fewer synaptic proteins in the hippocampus than the HCR rats did, independent of age. In addition, compared to the HCR rats, the LCR rats had increased expression of some inflammatory markers, such as IL-1β and TLR3, in the hippocampus, the difference being visible already at a young age. To our knowledge, this study is the first to show that poor intrinsic aerobic capacity, which translates into a risk for obesity and metabolic syndrome, disturbs the brain at a young age. Furthermore, as expected, aging affected both neuronal plasticity and inflammation, and the poor inherited aerobic capacity accelerated the decrease of neurogenesis and the up-regulation of inflammation-related markers as a function of age. However, although aging increased the expression of some inflammatory markers, the expression of others was higher at a younger age. This might be due to the complex and changing role of the inflammatory system at different ages.

TLRs are important regulators of innate immunity and respond to both pathogens and tissue damage–associated molecular patterns, which, when activated, lead to the production of inflammatory cytokines such as IL-1β (Okun et al., 2010; Field et al., 2010). In our study, higher expression of TLR3 was linked to poor aerobic capacity in both young and older rats (see Fig. 2). An increase in the expression of TLR3 has previously been reported in relation to neurodegenerative diseases (Bsibsi et al., 2002). Further, TLR3 is suggested to impair hippocampal plasticity, as TLR3-deficient mice are described to have increased neurogenesis (Okun et al., 2010), which is in line with our results showing lower AHN in LCR rats that expressed more TLR3. Overall, TLR activation has been associated with low-grade inflammation and a sedentary lifestyle, which usually go hand in hand with obesity and metabolic syndrome. However, to the best of our knowledge, there is no previous research conducted on how physical fitness affects TLR3 expression in the brain. In the current study, ageing also increased the expression of TLR3 in the hippocampus of both HCR and LCR rats. This is in agreement with an extensive human study by Cribbs et al., in which TLR3 expression was upregulated upon aging in the hippocampus (Cribbs et al., 2012). In addition, one human study found noticeable TLR3 immunoreactivity in aged brains, which increased if Alzheimer plaques were present (Walker et al., 2018). However, contradictory results have also been published by Letiembre et al., who found an increase in multiple TLRs, but not in TLR3 in aging mice (Letiembre et al., 2007).

The LCR rats expressed almost twice as much IL-1β in the hippocampus than the HCR rats did at younger age (Fig. 2). However, the

difference between the genotypes disappeared at older age. Elevated IL-1β is known to associate with neurodegenerative diseases such as Alzheimer's and Parkinson's (Kim and Joh, 2006), which are characterized by loss of neurogenesis. In agreement, our results indicated a simultaneous increase in IL-1β and a decrease in AHN. Exercise has been shown to reduce the expression of IL-1β in a mouse model of Alzheimer's disease (Nichol et al., 2008), and therefore intrinsic aerobic capacity and a lean phenotype might have an effect similar to physical activity since the aerobically fit HCR rats expressed less IL-1β than the less fit LCR rats. Here, the expression of IL-1β increased with age in the HCR but not in LCR rats, which is to be expected because aging has been associated with an increase in neuroinflammation (Sanada et al., 2018).

Activation of microglia increases with age and in metabolic diseases (Maldonado-Ruiz et al., 2017), while physical exercise shifts microglia towards a neuroprotective phenotype (Kohman et al., 2013; Vukovic et al., 2012). Therefore surprisingly, we found genotype differences in the expression of IBA1 only in the WB analysis, the old LCR rats expressing more IBA1 than the old HCR rats (Fig. 3). As the samples for WB included only a small piece of the hippocampus, with the different sub-regions (Cornu Ammonis and Dentate Gyrus) mixed together, it might therefore slightly differ from the IHC findings, which represent the whole hippocampus and allowed analyses of the different sub-regions. However, it seems that the poor genotype itself associates with microglia activation, and the healthy genotype protects against it. Regardless of the genotype, the older rats had more IBA1 positive cells in the CA3 region than younger ones did. The CA3 region is known for its extensive interconnections and importance for spatial short-term memory (Kesner, 2007). However, the differences seen in our study were relatively small, which might be due to the fact that our old rats were only 10 months old, which roughly corresponds to middle age in humans (Sengupta, 2013). Therefore, not all aging-related disruptions might be visible at that age.

Astrocytes interact with neurons to maintain healthy energy metabolism and to clean neurotoxins in the brain (Mason, 2017). Conversely, oxidative stress, ammonia toxicity and astrocyte swelling, for example, lead to a reduction in GFAP expression, while in neurodegenerative diseases, like Parkinson's, the number of astrocytes is increased, and in Alzheimer's astrocytes undergo hypertrophy around neuritic plaques (Kesner, 2007). The expression of GFAP, marker of astrocytes, was higher in older LCR rats than it was in older HCR rats (Fig. 2). We suspect that this might be due to the need for additional neuroprotection caused by the "poor" genotype (Sidoryk-Wegrzynowicz et al., 2011). In our study, GFAP expression was also higher in younger rats than in older rats, which may be due to the complex role of astrocytes throughout life. Astrocytes are known to support neuronal growth and synapses, but are also involved in synaptic pruning (Mason, 2017; Lee and Chung, 2019). Altogether, our results showing more microglia and astrocytes in LCR rats suggests that poor aerobic fitness increases inflammation in the hippocampus.

Previous studies have demonstrated that exercise increases the expression of SYP (Hescham et al., 2009; Lambert et al., 2005) and SYN-1 (Vaynman et al., 2006). Our study shows that the rats with low intrinsic aerobic capacity expressed less SYP in the hippocampus than the fit HCR rats did, independent of age (Fig. 4). Thus, it might be that in addition to exercise, SYP also responds to physical fitness. However, the amount of SYN-1 was *lower* in young HCR rats than it was in the young LCR rats, while there was no difference between the genotypes at older age. This is in contrast to the previous findings regarding the effects of exercise on SYN-1 expression (Hescham et al., 2009). Consistent with previous studies, the amount of synaptic proteins dropped in both genotypes with aging. That was to be expected because aging is known to decrease hippocampal synaptic plasticity (Ojo et al., 2012). The decrease in the synaptic vesicle proteins might play a role in the loss of plasticity, as rats with a synapsin deficiency are susceptible to age-induced cognitive impairment (Corradi et al., 2008). Here, the fit phenotype of the HCR rats did not protect the brain from the loss of SYN-1 and SYP during aging. However, the old HCR rats maintained SYP at a higher level than the old LCR rats did. Overall, the SYP levels seemed to be positively affected by the fit phenotype, whereas SYN-1 levels were not. The effects of genotype and age on synaptic plasticity, and especially on functional plasticity, should be studied in more detail.

Exercise is known to increase AHN, especially in those individuals that respond well to exercise (Nokia et al., 2016; van Praag et al., 1999). However, it remains unknown to what extent the positive effects of exercise are a result of increased fitness and lean phenotype, and how big a role the physiological effects of exercise play. The results of the present study indicated that a genotype predisposing to a lean and fit, healthy phenotype itself is enough to promote adult hippocampal neurogenesis (Fig. 5). Furthermore, neurogenesis has been linked to improved learning (Shors et al., 2002; van Praag et al., 2005). At these ages, differences in cognition were variable, although HCR rats tended to be more curious and active in the behavioral tasks and had better auditory information processing than the LCR rats did (Wikgren et al., 2021). In a previous study, HCR rats have also demonstrated more flexible cognition than LCR rats (Wikgren et al., 2012). According to our current results, this might relate to the differences in AHN, synaptic proteins and neuroinflammation between the rat genotypes. Numerous studies have demonstrated that aging drastically reduces the level of neurogenesis (Heine et al., 2004), but exercise interventions, such as running, enhance neurogenesis even in aged individuals (van Praag et al., 2005). Similar phenomena were observed in our study, as the number of newborn neurons in the old rats dropped to a tenth of what they were in young rats. In addition, we found that high aerobic fitness and the lean phenotype of the HCR rats protected against brain inflammation and maintained their AHN at a higher level than the LCR rats did. In agreement with our study, previous studies have shown that inflammation suppresses AHN (Ekdahl et al., 2003). Thus, the LCR rats display signs of impaired brain metabolism, which can create a harmful feedback loop between bioenergetics, inflammation, and AHN that might explain the difference in AHN between the HCR and LCR rats, and also the difference between young and old animals. These findings are also in agreement with the energy transfer hypothesis: The aerobically fit HCR rats demonstrate an overall healthier hippocampus than their low fit LCR counterparts do. Most importantly, we demonstrated for the first time that intrinsic low aerobic capacity and the co-segregated trait for obesity is linked to increased inflammation and plasticity deficits in the hippocampus, already at a young age.

4.1. Limitations

Overall, although we conducted our study on rats, the HCR-LCR model is rather well translatable to humans because it is polygenic, like humans with obesity often are. Both LCR and HCR rats represent the extreme ends of a continuum of genotypes. At this stage of selective breeding especially the HCR rats might have spun off the continuum of normal variation: Their running ability has rapidly increased over generations whereas the running capacity of the LCR rats has stayed relatively stable over time, possibly due to a floor effect (Koch et al., 2012). Finally, our study was conducted on male rats only due to lack of resources, despite the fact that sex differences in AHN, synaptogenesis and inflammation are known to exist. In fact, sex and hormone levels have been shown to greatly influence hippocampal neurogenesis (Duarte-Guterman et al., 2015). Interestingly, aging is shown to increase inflammation in different areas in males compared to females (Cribbs et al., 2012). In addition, the response to inflammatory signals seems to vary between sexes, as females are more likely to express inflammation-related sickness behavior and fatigue than males are (Lasselin et al., 2018). For this reason, both male and female rats should be included in further studies.

5. Conclusions

Our findings demonstrate that inherited low aerobic capacity that predisposes to obesity, poor metabolic health and poor fitness associated with negative impacts on brain health. Interestingly, some of these effects, such as reduced AHN, were evident already at a young age (eight-weeks). The genotype for poor aerobic fitness also resulted in increased hippocampal inflammation, which could accelerate brain and cognitive aging. Conversely, the genotype producing a lean and fit phenotype leads to a healthier hippocampus, an effect similar to that observed in response to physical exercise. This work will be an excellent foundation for future investigations. For example, it would be important to study how exercise and/or physical performance affects the factors studied here, and whether exercise could reverse the poor impact of obesity on brain, cognition and overall health.

6. Ethics approval and consent to participate

See methods section.

7. Availability of data and materials

Original data and all material are available from corresponding author upon reasonable request.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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