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The efficacy of voluntary exercise and branched chain amino acids in preventing obesity-induced pathological changes in brain and behavior

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Obesity poses substantial societal risks, affecting public health and threatening the economy. Importantly, obesity-induced changes as found in the periphery also extend to brain structure and function, promoting premature cognitive dysfunction. Voluntary exercise and dietary supplements such as branched chain amino acids (BCAA) each demonstrate promising treatment opportunities. Following a longitudinal paradigm, the preventative effect of voluntary exercise in combination with BCAA against obesity-related changes in brain structure and function is investigated using male diet-induced obese (Ldlr-/-.Leiden) mice. Measurements included physiological parameters and a battery of behavioral tests to investigate cognition and motor skill. In addition, a neuroimaging protocol was performed to assess structural and functional brain connectivity (rs-fMRI and ASL). Individual locomotion and running wheel activity was measured using a digital ventilated cages system. Results demonstrated increased body weight in all animals in response to high fat (+BCAA) diet. While running activity decreased over time, we found that animals on BCAA preserved running endurance during nighttime. However, obesity-related motor impairments could not be prevented by exercise and BCAA supplementation. Moreover, functional MRI was not influenced by exercise and BCAA supplementation, however, exercise normalized CBF under normal conditions relative to baseline. Overall, this study demonstrated that exercise has beneficial effects on obesity-induced pathological changes in brain and behavior, while the synergistic effect of exercise and BCAA remains unseen in early disease state.

Keywords: Exercise, Branched chain amino acids, Obesity, Cognition, Animal model, MRI

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1. Introduction:

Steadily increasing global rates of obesity are posing a substantial threat to the economy and public health. As a more severe form of being overweight, obesity is characterized by a body mass index (BMI) of 30 kg/m^2 or higher. Obesity has a strong impact on the periphery, including strong associations with diabetes, hypertension, and cardiovascular disease. In addition, obesity is a major risk factor for pathological changes in the brain, affecting both structure and function. As such, obesity-induced morphological changes in terms of whole brain volume [1] and decreased gray matter (GM) volume [2] were found across the adult life span and independent of disease state. In addition, clinical studies consistently associate a higher BMI with increased rates of hippocampal atrophy (HP; [3]), a crucial brain structure for memory, as well as whole brain reductions in white matter (WM) integrity [4]. Moreover, obesity is associated with lower regional cerebral flood flow (CBF), which is associated with cognitive impairments in both humans and animals, as well as a greater risk for dementia, including Alzheimer's disease and vascular dementia [3, 5]. Crucially, this association between reduced CBF and cognitive decline pertains when controlling for cognitive aging, which is a normal process of the aging brain resulting in cognitive decline through changes in brain structure and function [6, 7]. Reductions in cognitive performance are also prominent at earlier ages, long before clinical dementia onset. For example, previous studies in healthy and obese adults found a linear association between BMI and impaired cognitive function across age groups, including language, memory, attention, processing speed, and visuomotor performance, as well as reduced executive functioning [8, 9].

There is a great scientific consensus on the role of other modifiable risk factors for cognitive decline and dementia besides obesity and its comorbidities, such as lack of exercise [6]. Exercise has several beneficial metabolic effects [10]. In addition, exercise has a beneficial effect on the brain, including CBF [5, 11] and consequently structural and functional network connectivity, often referred to as brain plasticity. Previous experimental evidence links exercise to a decreased rate in cognitive aging via reduced brain atrophy, and hence lower risk for cognitive decline, Alzheimer's disease, and dementia in general [10]. Besides reductions in neuronal death, exercise benefits axons and dendritic arborization, increases brain-derived neurotrophic factor (BDNF; [11]), regulates synaptic plasticity [12], and stimulates neurogenesis, i.e., neuronal birth [6]. In addition, physical activity positively impacts functional connectivity via enhanced structural WM fiber integrity in a stroke mouse model [13], promotes vascularization, and facilitates maintenance of cognitive function throughout

aging [10]. As such, beneficial effects of physical activity are reflected on the behavioral level as improvements in learning [10, 11]. For example, murine studies demonstrate that running improves spatial learning in healthy mice as reflected by improved performance on the Morris water maze task [12]. Running also prevented spatial learning deficits in western diet-induced obese mice in the spontaneous alteration task and the novel spatial recognition test [6].

Dietary habits are another crucial risk factor for both metabolic disease and cognitive dysfunction that can easily be modified, for example through supplementation of certain nutrients. Branched-chain amino acids (BCAA: leucine, isoleucine, and valine) are the most abundant essential amino acids and have several functions, including glucose homeostasis, protein synthesis, energy production, and synthesis of amine neurotransmitters such as dopamine, serotonin, and noradrenaline [14]. In addition, diets rich in BCAA have been found beneficial to the aging process, increase the life span, and aid in regulation of body weight [14-17]. Thus, there are several indications that BCAA supplementation may also have a variety of beneficial effects on obesity [18], metabolism [16, 19], and cognition [14, 15]. BCAA can be burnt in the muscles, and this combustion is promoted with movement [15, 16, 18]. As the depletion of BCAA in muscle and plasma during exercise can result in muscle fatigue and central fatigue, respectively, BCAA supplementation is commonly found in athletes to promote their physical, but also mental, performance [14]. In fact, BCAA-enriched diets improve physical endurance, maintain muscle fiber size, and benefit motor coordination in mice [16], and these effects on muscle functional capacity were promoted by exercise [15]. With respect to neurotransmitter modulation, studies have shown that BCAA ingestion alone improves cognitive performance through a cascade of biochemical processes in both healthy subjects and individuals with metabolic disease states [14]. That is, rapidly rising BCAA levels in the plasma cause increased passage of such through the blood brain barrier. This increased uptake of BCAAs in the brain in turn decreases levels of aromatic amino acids and hence leads to reduced levels of serotonin, noradrenaline, and dopamine [14]. Interestingly, both serotonin and dopamine are involved in controlling food intake [20, 21], and low levels of serotonin have further been associated with central fatigue [14]. In addition, while noradrenaline is one of the major agents of the autonomic nervous system [22], dopamine plays an important role in the brain's reward system following physical exercise [23]. The beneficial effect of BCAA supplementation on cognition is also supported by a murine study in which BCAA supplementation rescued brain-injury-induced impairments in cognitive performance [15]. Interestingly, also negative effects of increased BCAA levels have been reported. For example, metabolic disorders such as obesity, type 2 diabetes mellitus, and insulin resistance are strongly

associated with increased BCAA levels due to impaired catabolism of these amino acids. In support of this, a recent study has found BCAA supplementation in combination with forced treadmill running to impair insulin sensitivity and promote lipogenesis in diet-induced obese mice [18]. Moreover, in respective human risk populations, higher levels of BCAA are predictive of type 2 diabetes mellitus, and correlate with increased risk for cardiovascular disease [15]. With respect to brain function, oral BCAA supplementation effectively restored regional reductions in relative CBF in patients with liver cirrhosis [24].

To date, only a few studies have examined exercise and BCAA on metabolic recovery in athletes, but to our knowledge no studies have examined the preventative effect of voluntary exercise in combination with BCCA against obesity-related changes in brain structure and function. Given our knowledge, it is of inevitable importance to promote lifestyle modifications that stimulate metabolic health and hence reduce the obesity-induced risk for premature cognitive decline. Crucially, animal research allows for reductionist mechanistic understanding and proof of causality on multiple levels such as molecular, cellular, neural circuit level, as well as the behavioral level. Low density lipoprotein receptor-deficient Leiden (Ldlr-/-.Leiden) mice are trans-genetically modified in such as to develop not only obesity but also further pathological changes, including atherosclerosis, hypertension in conjunction with brain dysfunction, hyperinsulinemia, dyslipidemia, adipose tissue inflammation, nonalcoholic steatohepatitis, and liver fibrosis in a short period of time, within approximately 30 days on high-fat diet (HFD) [27]. This mouse model closely mimics diet-induced cardiometabolic comorbidities and subsequent pathological brain changes, which are the most important risk factors of obesity-induced cognitive dysfunction [6, 25]. Consequently, using diet-induced obese Ldlr-/-.Leiden mice as a model for human cardio-metabolic disease, the preventative effect of long-term voluntary exercise in combination with oral supplementation of BCAA on brain health is therefore being investigated in this study. In addition, we will look at the effects of treatment on metabolic health, motor skills, and behavior. Based on current knowledge, it is predicted that exercise will have beneficial effects on body weight and brain health of diet-induced obese mice. The combination of BCAA supplementation with voluntary exercise is expected to affect metabolism and brain health in a dose-dependent manner. Because of dysfunctional catabolic and anabolic signaling in obesity, worse outcomes of BCAA supplementation in combination with exercise are expected as compared to obese exercising mice without BCAA supplementation if plasma BCAA levels exceed what can be metabolized.

To test this, a longitudinal design has been chosen to investigate effects under consideration of disease progression. As such, all mice have undergone baseline measurements,

including structural and functional MRI imaging, and behavioral tests. At W=0 the animals were matched in the respective diet and exercise groups: (1) Reference group (chow diet), (2) High-fat diet (HFD) + no exercise group (blocked running wheel), (3) HFD + exercise group (Running Wheel, RW), and (4) HFD+BCAA and exercise group (RW). Mice have undergone regular measurements of physiological parameters (body weight, food intake, systolic blood pressure, and blood sampling). In addition, motor skill measurements have been taken at various time points, i.e., grip strength measurements and rotarod. Moreover, two different cognitive tests, the object recognition test and the (reverse) Morris water maze have been performed. MRI was used for the analysis of markers reflecting cerebrovascular changes, cerebral blood flow as well as structural and functional connectivity (rs-fMRI).

2. Materials and Methods

2.1 Ethical statement

All experiments performed involving animal care and treatment were described according to the ARRIVE guidelines and carried out in accordance with international European ethical standards (European Directive 2010/63/EU) and guidelines of the Dutch federal regulations for animal protection. Approval for these experiments was acquired by the Veterinary Authority of Radboud university medical center, Nijmegen, The Netherlands, and the Animal Experiment Committee (Dierenexperimentencomissie (DEC), (2021-001-003)) of the Radboud University, Nijmegen, the Netherlands.

2.2 Experimental animals

For this study, male Ldlr-/-.Leiden mice (2 months of age) from a pathogen-free breeding stock were used as a model for obesity. From weaning until group allocation at W=0, all animals were fed a standard chow diet (58% kcal carbohydrates, 33.0% kcal protein, and 9.0% kcal fat, Sniff R/M-H diet V1530, Sniff Spezialdiäten GmbH, Soest, Germany). At W=0, diet was switched in the respective experimental groups to either HFD (36.0% kcal carbohydrates, 18.0% kcal protein, and 46.0% kcal fat, D12451, Research Diets Inc, New Brunswick, USA) or BCAA-enriched HFD (36.0% kcal carbohydrates, 18.0% kcal protein, and 46.0% kcal fat, D12451, Research Diets Inc, New Brunswick, USA). All experimental animals were obtained from TNO Metabolic Health Research (Leiden, the Netherlands). Mice were pair-housed at the Animal Research Facility, Radboud university medical center Nijmegen, the Netherlands, within the Preclinical Imaging Center (PRIME) in digital ventilated cages (DVC; Tecniplast

SPA, Buguggiate (VA), Italy) containing standard corn-based bedding material (Bio Services, Uden, Netherlands), wood wool sizzle material (Bio Services, Uden, Netherlands), and a mouse igloo (Plexx, Elst, Netherlands). These cages allow for 24hours/day locomotor-activity-tracking of the mice. Temperature (21°C±1°C) and relative humidity (50 - 60%) within the animal rooms was maintained at constant levels. Food and water (autoclaved) were provided *ad libitum*, and light cycle was set to 7 a.m. - 7 p.m. (lights on at 7 a.m.). All behavioral and neuroimaging experiments were performed solely by female researchers between 7 a.m. and 18 p.m. within the facilities of PRIME. Animals were given 30 minutes undisturbed habituation time in the experimental room within their home cages before each behavioral test.

2.3 Study design

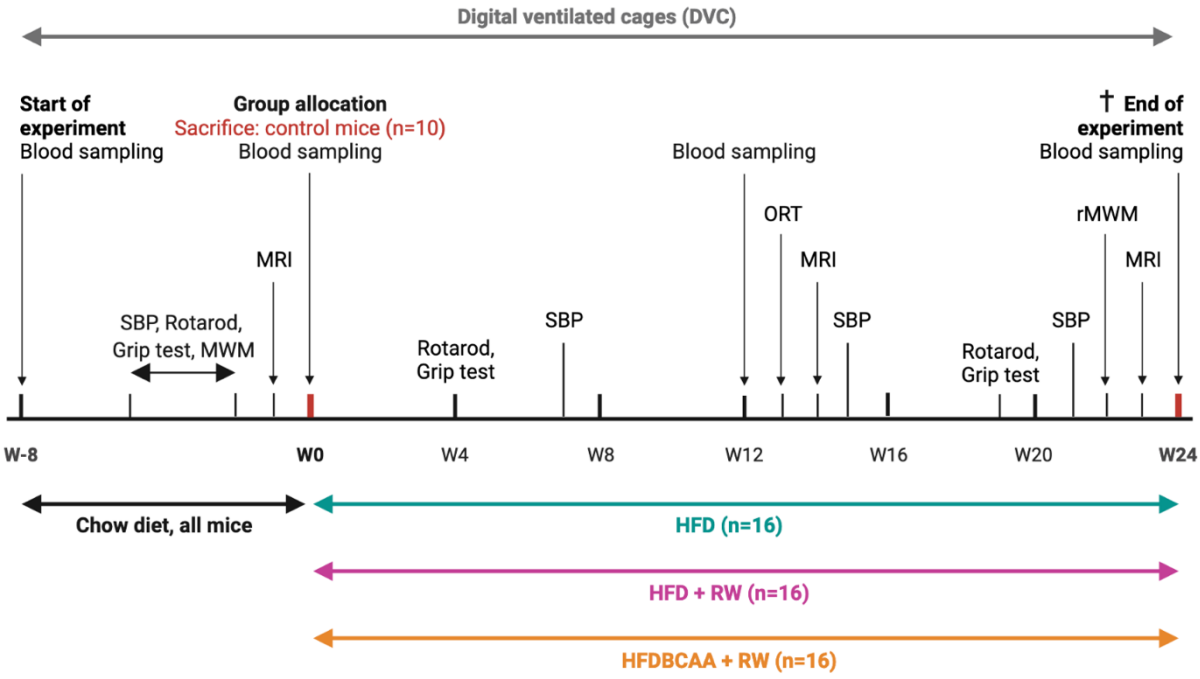

Figure 1 | Experimental Timeline. W = timepoints (in weeks) within the experimental timeline, SBP = Systolic blood pressure, (r)MWM = (reverse) Morris Water Maze, MRI = Magnetic resonance imaging, ORT = Object recognition test, HFD = High fat diet, RW = Running wheel, BCAA = Branched chain amino acids.

This was a double-blinded (blinded for investigators and outcome assessors) and randomized controlled study. The experiment was divided into animal cohorts due to time consuming procedures (MRI, behavioral tests), which hence were performed in a time-shifted manner. Mice were housed in digital ventilated cages (DVC) for the entire duration of the experiment to track locomotor and running wheel (GYM500 activity wheel, Tecniplast S.p.A., Buguggiate (VA), Italy) (RW; as of week 0) activity 24hours/day. During acclimatization and baseline physiological measurements (bodyweight, systolic blood pressure (SBP)), motor skill

assessment (Rotarod, Grip strength test), spatial memory performance (Morris water maze (MWM)), and neuroimaging measurements (Magnetic resonance imaging (MRI)), all mice were fed a chow diet. Following baseline testing, blood sampling was performed to ensure similar average levels of triglyceride, cholesterol, and glucose levels across groups. Based on this, mice were randomly divided into 4 experimental groups: Control group (chow diet; n=10), HFD with no exercise (n=16), HFD with exercise (n=16), and HFD+BCAA with exercise (n=16). At this timepoint, indicated as week 0, the control group was sacrificed via transcardial perfusion and brains were harvested for additional analyses. The other groups received the respective (blocked) RW and dietary interventions. For simplicity, timepoints within the experimental timeline are indicated in weeks before and after group allocation; they do not refer to the age of the animals. Physiological parameters were monitored on a regular basis (food intake weekly, body weight monthly, SBP in weeks 7, 15 and 21, and blood sampling in weeks 12 and 24). Motor skills (Rotarod, Grip strength test) were repeatedly tested in weeks 4 and 19, and non-invasive neuroimaging experiments (MRI) were performed at two additional timepoints, in weeks 14 and 23, to measure functional connectivity (rs-fMRI) and cerebral blood flow (CBF). In addition, recognition memory was examined using the object recognition test (ORT) in week 13, and spatial memory was tested via the reverse Morris water maze (rMWM) in week 22. In week 24, mice were sacrificed immediately after the last blood sampling via transcardial perfusion, and brains and other tissues were harvested for further analyses.

2.4 Physiological parameters

2.4.1 Body weight & caloric intake

Body weight and caloric intake of the mice was monitored on regular basis. For this, caloric intake was assessed by weekly weighing procedures of food intake per cage (corrected for the number of mice per cage), whereas body weight was measured monthly.

2.4.2 Digital ventilated cages

In this study, running wheel (RW; GYM500 activity wheel, Tecniplast S.p.A., Buguggiate (VA), Italy) activity and home cage locomotor activity were monitored 24 hours/day by housing mice pairwise in digital ventilated cages (DVC). That is, via 12 electrodes placed underneath each home cage, activity was monitored as described elsewhere [26]. RW distance was calculated per cage for daytime and night-time separately (12-hour intervals). For analysis,

only data gathered on weekends was used, as this is the most reliable measure of undisturbed DVC activity.

2.4.3 Blood sampling: Tail artery incision

The animals' blood was collected to measure glucose, triglyceride, and cholesterol levels. Food was removed from cages 5 hours prior to blood sampling. During sampling, 3 cages at a time, holding 2 mice each, were placed under a heating lamp to ensure artery widening for a faster and easier blood collection. After approximately 10 minutes, animals were individually placed in a restrainer and a small incision in the artery of the tail was made. From this, 200µl blood was collected in a EDTA tube, and glucose levels were measured using one drop of blood. Plasma triglyceride and cholesterol levels were measured enzymatically using kits number 11489437 and 11488872 (Roche Diagnostics, Almere, The Netherlands).

2.4.4 Systolic blood pressure: Tail-cuff plethysmography

Systolic blood pressure (SBP) was examined using a warmed tail cuff plethysmography device (IITC Life Science Instruments, Woodland Hills, CA, USA). Mice were placed in a preheated restrainer from Plexiglas. According to the size of the animals, a head gate was positioned in front of their heads and their tails were attached to cuffs with tail gate pulse sensors. Thereafter, mouse restrainers were placed into a warming chamber (38°C) where the animals had time to acclimatize at least 5 minutes before the measurements started. Blood pressure was measured in 10 trials of 30s twice, once in the morning and once in the afternoon, and recorded by the software BPMonWin (IITC Life Science Instruments, Woodland Hills, CA, USA). The SBP was analyzed manually by determining at which pressure of the tail cuff the first tail pulse became visible in the recorded plot. Plots showing noise caused by tail movements were excluded.

2.5 Behavioral tests

2.5.1 Rotarod

This behavioral test was used to evaluate balance and motor coordination. The animals were placed on a rotatable rod (3.18 cm in diameter: IITC Inc., Woodland Hills, CA, USA) with accelerating speed (4-40 rpm). Three animals were tested simultaneously on separate lanes – lane 1, 3 and 5, with one empty lane (lane 2 and lane 4, respectively) in between two animals to reduce possible distraction. All mice were walking forwards (comparable to a human

treadmill). Rotation of the rod was initiated manually each trial once all mice of a given trial were placed on the rod. After each trial, before placing the next mouse, the rod and each ground plate were cleaned with water to remove and prevent formation of odors caused by urine or feces of the previous mouse. The latency to fall (s), as well as speed (rpm) and distance travelled (m) were recorded as a measure of their ability to stay on the device. All trials had a maximum duration of 300s with an intertrial duration of at least 20 min. In total, 4 trials were performed on each mouse.

2.5.2 Grip Strength Test

The Grip Strength Test is used to evaluate the forelimb muscle strength as well as the total limb muscle strength. Therefore, a Grip Strength Meter (Grip strength meter, 47200, Ugo Basile, Gemonio (VA), Italy) was adjusted horizontally on a table and the measuring device computing the peak force was connected a trapeze or a grid. To examine the forelimb muscle strength, mice were lifted individually by the base of their tail and hovered over the measuring device, allowing them to grasp to the bar of the trapeze with the two forepaws. Maximum muscle strength was recorded by pulling the mice gently at the base of their tail until they released the bar. Half an hour later, total muscle strength was determined in a similar way with the distinction of allowing the mice to grasp a grid with all four paws instead of the trapeze. In total, each mouse was tested 5 times at the trapeze and grid respectively. The first measurement was discarded per mouse as a habituation trial of the respective experimental day. Muscle strength was determined by averaging the measured peak strength (gf) from all remaining valid trials (at least 3 valid measurements per mouse). Trials in which the animals did not grasp either with two paws (trapeze) or four paws (grid) were considered as invalid, as well as trials in which the trapeze or grid disconnected from the measuring device because of vertical rather than horizontal pulling of the mice.

2.5.3 Object Recognition Test

Nonspatial learning and (recognition) memory was assessed using the Object Recognition Test (ORT). On 3 consecutive days, mice were placed individually in a square open field (45 cm x 45 cm x 30 cm) with a flat white surface and transparent Plexiglass walls. Each trial had a duration of 4 minutes and was video-taped with a camera. Object recognition was assessed using two types of trials, explorative trial, and novel object trial. During explorative trials, mice were exposed to two similar objects placed in the center of the field at a similar distance from the wall and each other. In the novel object trial, one of these objects was exchanged with a

dissimilar object (hence 1 familiar and 1 novel object). In total, 4 different object types were used: A calcium egg, a small glass cup, a yellow plastic cone, and a sand-filled bottle. The order of mice, object type, and whether the left or right object was exchanged in the novel object trial was randomized daily. The field and all objects were cleaned with 70% alcohol between each trial. On day 1, mice first underwent a habituation trial in which the open field was left empty. In the second haft of the first day, mice were tested with a 30 min delay between trials (explorative trial and novel object trial). On day 2, the delay between trials was increased to 1 hour, and on day 3 the inter-trial delay was set for 2 hours. Locomotion, overall exploratory behavior within the field, and time spent at each object were automatically assessed with EthoVision XT16 (Noldus, Wageningen, The Netherlands). Object recognition was measured by calculating several indexes in both trial types. For the novel object trial, a discrimination index (DI), indicative of intact discrimination between objects, was calculated as the as the time spend around N1 minus F3, divided by the time spend around both objects (DI = (N1 − F3) / (N1 + F3)). The resulting score ranges from -1 to +1 and reflects more time spend around F3 or N1, respectively, with 0 indicating no difference in time spend at each object. In a next step, recognition memory was measured by calculating a recognition index (RI) as the time spend around N1 as a fraction of the time spend around both objects (RI = N1/ (N1 + F3)). Finally, a preference index (PI) was calculated to measure preference for either object as the as time spend around N1 (or F3) as a percentage of the time spend around both objects (PI = 100 × ([N1 or F3] / (N1 + F3)). Preference is indicated by the percentage relative to the chosen numerator, with 50% indicating no preference. With N1 as numerator, preference for the novel object is indicated by a score closer to 100%, while a score below 50% reflects preference for F3 (vice versa if F3 is the numerator).

2.5.4 Morris Water Maze

The Morris Water Maze (MWM) and reverse MWM (rMWM) were used to measure long-term spatial learning (acquisition) and memory (probe). That is, mice were trained to find an escape platform (8 cm diameter) that was hidden (submerged 1 cm below water surface level) in the northeast quadrant of a white circular pool (108 cm diameter) filled with opaque water (water mixed with milk powder, temperature of 21-22°C). Four distinct visual cues were attached to the walls around the maze (0.5 m distance from the pool, one on each cardinal point) for orientation purposes. At baseline, the standard MWM was performed. On 4 constitutive acquisition days (learning phase), mice were individually placed in the pool four times each (120 s maximal swimming time; 30 s on the platform; 1-hour minimal inter-trial interval),

starting from different cardinal points (south, north, west, east). If the platform was not found within 120 s, mice were manually placed and held on the platform for 30 s. To test spatial memory, a single probe trial (starting point: south) was performed on day 4 following the last acquisition, in which the escape platform was fully removed from the pool to measure the entry frequency and total time spent in the platform quadrant and platform zone. During the second experimental time point (per cohort), a rMWM protocol (duration of 2 consecutive days instead of 4) was used. To assess whether mice remembered the platform location from the standard MWM protocol (baseline memory), a probe trial was performed on day 1 prior to the first acquisition. For acquisition, the platform location was changed from the previous one, and mice thus had to learn the new location within two days (learning). On day 2, following the last acquisition, another probe was performed to assess whether mice remembered the new platform location (memory). All trials were video-taped with a ceiling-camera and the escape latency (s), total distance moved (cm), and swim speed (cm/s) per mouse were calculated automatically using EthoVision XT16 (Noldus, Wageningen, The Netherlands).

2.6 Neuroimaging Protocol: ASL and rs-fMRI

All neuroimaging measures were operated on Paravision 6.0.1 software (Bruker, Karlsruhe, Germany) using an 11.7 T BioSpec Avance III small animal MR system (Bruker BioSpin, Ettlingen, Germany) equipped with an actively shielded gradient set of 600mT/m, an actively decoupled mouse brain quadrature surface coil for signal reception, and a circular polarized volume resonator for signal transmission (Bruker BioSpin). During MRI scan acquisition, motion restraint was achieved using a stereotactic. Anesthesia with Isoflurane (Abbott Animal Health, Abbott Park, IL, USA) was induced (3.5%) and maintained (1.7 - 2.2%) with in an oxygen and air mixture (1:2, with maintenance dosage dependent on respiration frequency (~100 bpm) as measured by a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc., NY, USA). Body temperature was monitored using a rectal probe and controlled via a warming pad placed on top of the mouse (~37°C). Following standard adjustments and before each experimental scanning procedure, gradient images were acquired in three orthogonal directions (sagittal, coronal, axial) for anatomical reference. All MRI parameters were motion corrected. Cerebral blood Flow (CBF) levels were measured using an established Arterial spin labeling (ASL) method with the flow-sensitive alternating inversion recovery (FAIR) technique from a series of echo planar images (EPI). Cerebral vasoreactivity, i.e., the ability of the cerebrovasculature to adapt from normal to vasoconstrictive conditions,

was assessed using CBF values from normal and vasoconstrictive conditions (normal CBF – vasoconstriction CBF / (normal CBF + vasoconstrictive CBF). Vasoconstriction CBF was measured by switching the normal oxygen-air mixture (1:2) to pure oxygen (3:0 oxygen-air mixture). In addition to the total brain, CBF was also measured in three different regions of interest (ROI, Bregma: -1.94): the cerebral cortex, hippocampus (important for memory), and thalamus (involved in relaying motor signals to the cortex). Functional connectivity between specific ROI supporting cognitive and motor processes was calculated from the blood oxygen level-dependent (BOLD) time series of resting-state functional MRI (rs-fMRI) acquisitions. ROI included dorsal hippocampus, ventral hippocampus, auditory cortex, motor cortex, somatosensory cortex, and visual cortex. MRI imaging data (e.g., cerebral blood flow, and rs-fMRI) were analyzed using MATLAB. To analyze rs-fMRI, partial correlations were used which attenuate the direct connectivity between two ROI while regressing the temporal BOLD signal from all other ROI, Specifics on calculations of regional CBF and functional connectivity analyses performed are described elsewhere [27, 28].

2.7 Statistics

The statistical analysis was performed using IBM SPSS Statistics 25 software (IBM Corporation, New York, NY, USA). All experimental animals were split up in respective experimental groups (1 = HFD, 2 = HFD + RW, 3 = HFDBCAA + RW). To analyze the effects of diet and exercise over time, repeated measures GLM with post-hoc Bonferroni corrections were performed on physiological (body weight, caloric intake, DVC, Rotarod, and grip strength), cognitive parameters (MWM and rMWM), and neuroimaging parameters (ASL and rs-fMRI) with experimental group as between-subject factor. Because these measures (except for DVC and (r)MWM) were performed at baseline and one or more times after group allocation, all immediate and long-term effects were analyzed by calculating all timepoints post group allocation as relative scores (in percentages) to baseline measurements. DVC data was analyzed using true scores, as RW were introduced at time of group allocation, hence no baseline measurements could be performed. With respect to (r)MWM measures, absolute scores were analyzed for each test individually. In addition, effects of diet and exercise on recognition memory (ORT) were analyzed using a multivariate GLM with post-hoc Bonferroni corrections and experimental group as between-subject factor on each of the three indices that were calculated to investigate object discrimination, object recognition, and percentage of time

spent around the novel object. Statistical significance was set at $p < 0.05$ and trends at $0.05 < p < 0.08$. All data are represented as mean ± SEM.

3. Results

3.1 Body weight & caloric intake

All experimental animals showed an increase in body weight over time (figure2A, $F(5, 195) = 262.042$, $p < 0.001$), and HFDBCAA + RW mice gained less body weight than both HFD + RW and HFD mice (figure2A, $p = 0.003$ and $p = 0.023$, respectively). There was a main effect of caloric intake over time in all groups (figure2B, $F(5, 95) = 12.134$, $p < 0.001$), with an initial decrease in caloric intake between the first two timepoints (figure2B, $p = 0.002$). However, there was no difference in caloric intake over time in either group when comparing first and last timepoints (figure2B, $p = 0.128$).

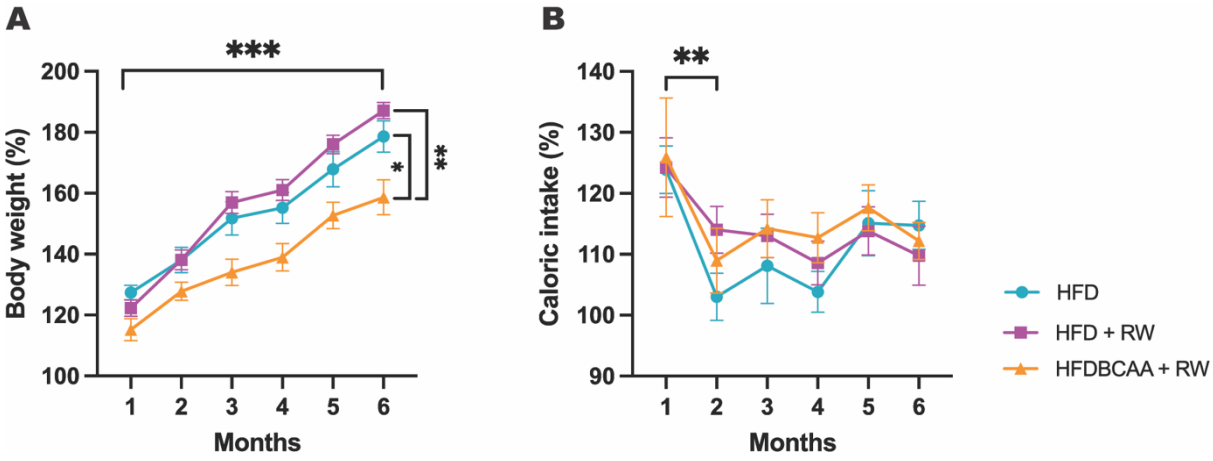

Figure 2 | Body weight and caloric intake. (A) Increase in body weight was found in all experimental groups ($p < 0.001$). HFDBCAA + RW were lighter than both HFD + RW ($p = 0.003$) and HFD mice ($p = 0.023$). (B) There was an initial decrease in caloric intake between the first two timepoints ($p = 0.002$), but no difference in caloric intake from first to last timepoint ($p = 0.128$). Data are presented as percentages relative to baseline measurements (mean ± SEM, **P < .01 or ***P < .001).

3.2 Digital ventilated cages

Analysis of running wheel activity of experimental groups 2 (HFD + RW) and 3 (HFDBCAA + RW) was performed for daytime and nighttime activity separately per cage. Running distance overall decreased during daytime in both groups (figure3A, $F(14, 168) = 9.772$, $p < 0.001$). As for nighttime activity, this change over time differed by experimental groups (figure3B, $F(14, 168) = 1.886$, $p = 0.031$). The running wheel distance in the HFD + RW group decreased ($F(1, 14) = 5.645$, $p < 0.001$), while remaining stable in the HFDBCAA + RW animals.

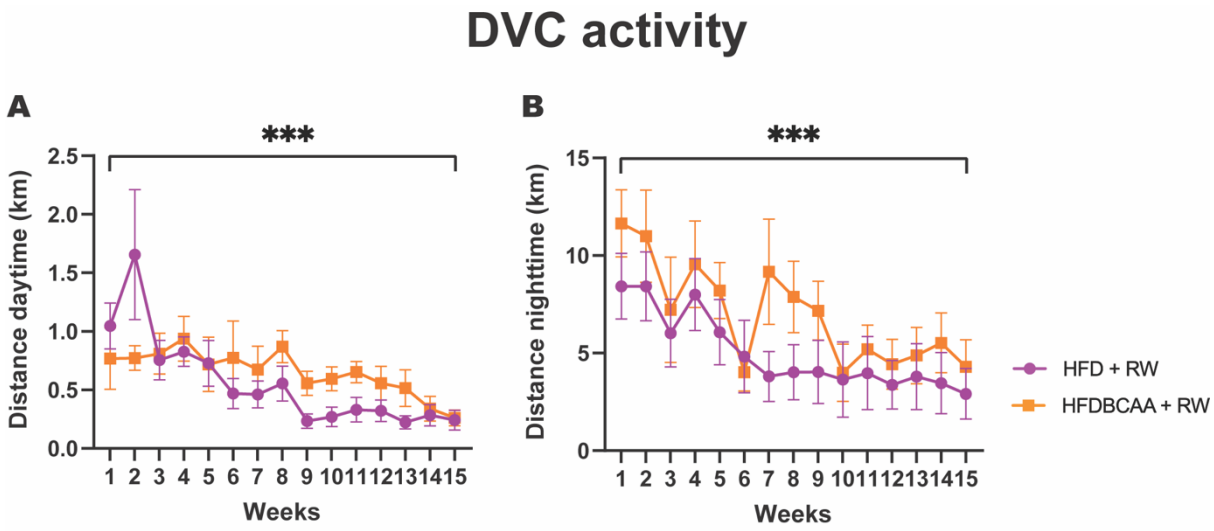

Figure 3 | DVC activity. (A) Running distance decreased over time during daytime ($p < 0.001$). (B) During nighttime, running distance decreased in HFD+RW mice ($p < 0.001$).

3.3 Rotarod

Rotarod measures balance and motor coordination and is assessed using the parameters speed (rounds per minute), latency to fall (seconds), and distance (meters). In all experimental groups, performance decreased equally on all three measurements over time (figure5A-C, speed $F(1, 39) = 54.908$, $p < 0.001$, latency to fall $F(1, 39) = 87.849$, $p < 0.001$, distance $F(1, 39) = 58.787$, $p < 0.001$).

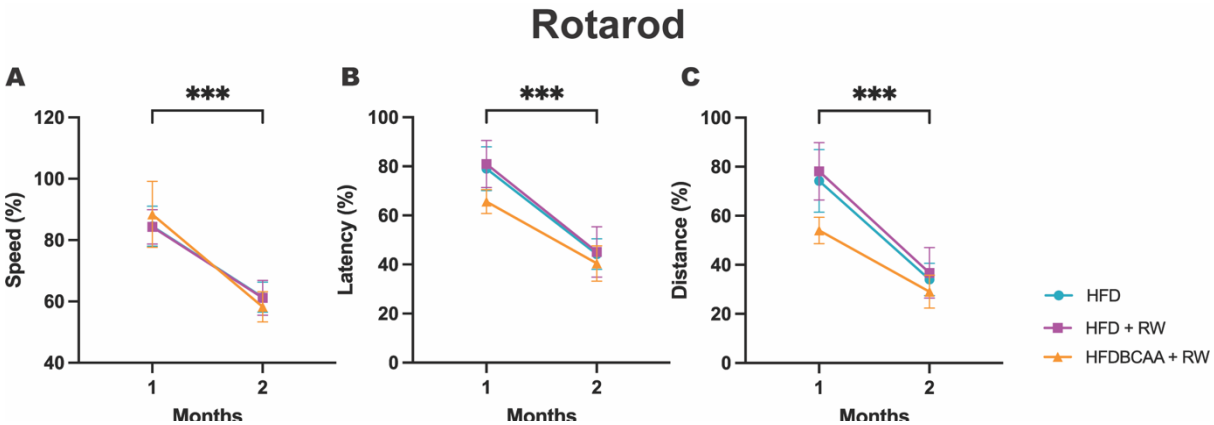

Figure 5 | Rotarod. (A-C) Performance decreased in all groups equally over time (A: speed $p < 0.001$, B: latency to fall $p < 0.001$, C: distance $p < 0.001$). Data are presented as percentages relative to baseline measurements (mean ± SEM, ***P < .001).

3.4 Grip Strength Test

Forelimb muscle strength and the total limb muscle strength were assessed using either a trapeze or grid, respectively, measuring average and peak force. Forelimb muscle strength decreased over time in all animals (figure6A-B, average force $F(1, 37) = 12.721$, $p = 0.001$, peak force $F(1, 37) = 7.136$, $p = 0.011$). However, there was no difference in total limb muscle

strength in either group between the two timepoints (average force F(1, 37) = 0.377, p = 0.543, peak force F(1, 37) = 0.708, p = 0.406).

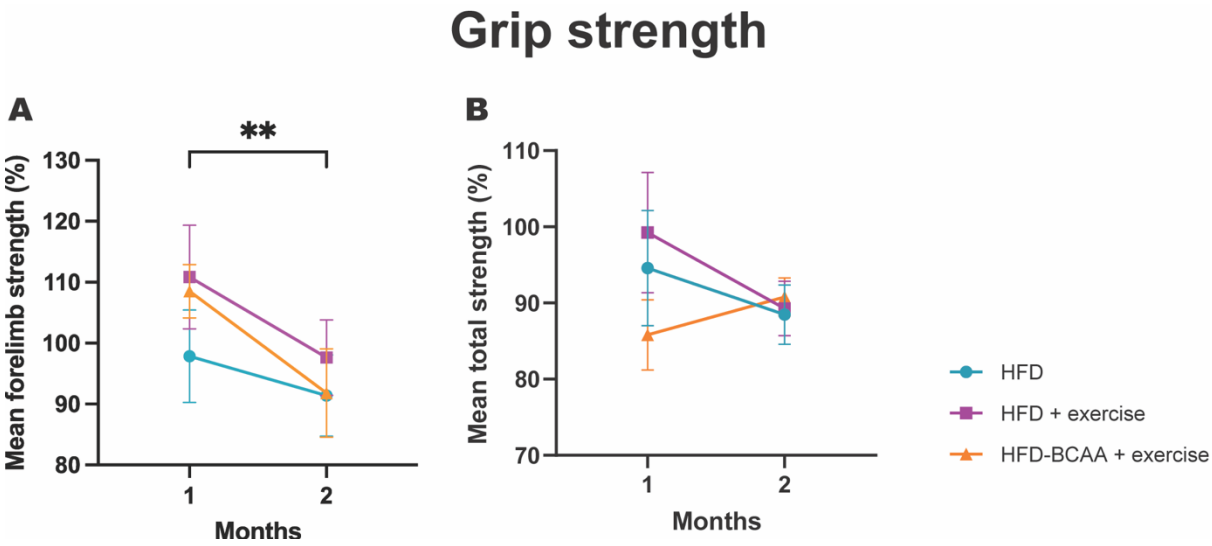

Figure 6 | Grip Strength test. (A-B) Forelimb muscle strength decreased over time in all animals (A: average force p = 0.001, B: peak force p = 0.011). Data are presented as percentages relative to baseline measurements (mean ± SEM, *P < .05 or **P < .01).

3.5 Object Recognition Test

Approximately halfway through the experiment (week 13 after group allocation), the ORT was performed to assess group differences in nonspatial learning and (recognition) memory. There was no evidence of group differences with respect to object discrimination, object recognition, or percentage of time spent around the novel object (Figure 7A-B, 30 min delay F(16, 68) = 1.288, p = 0.230, 60 min delay F(16, 68) = 1.070, p = 0.400, 120 min delay (F(16, 68) = 0.952, p = 0.517).

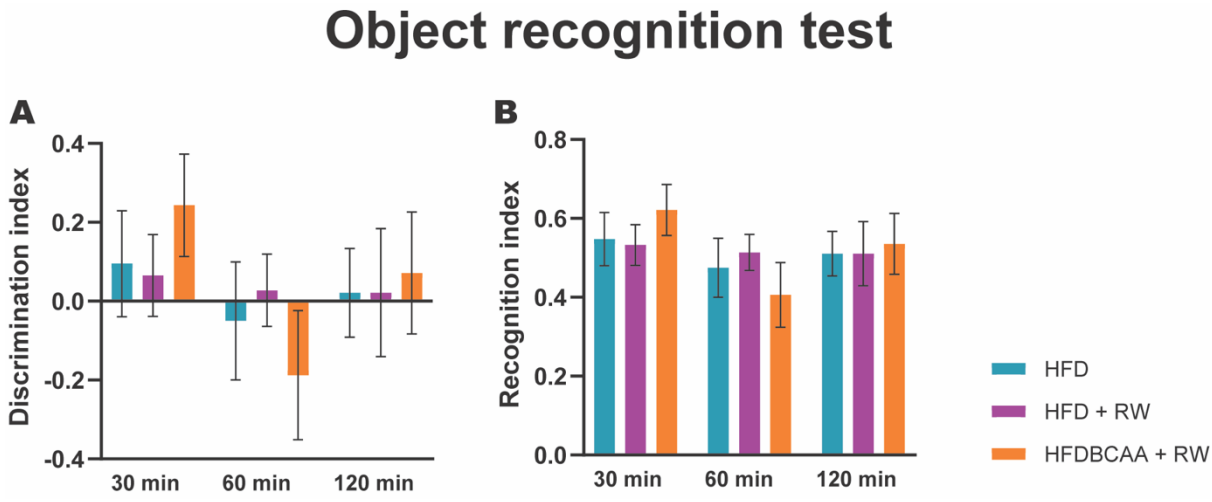

Figure 7 | Object recognition test. (A-B) There was no evidence of group differences for either amount of delay (30 min p=0.230, 60 min p=0.400, 120 min p=0.517). Data are presented as mean ± SEM.

3.6 Reverse Morris Water Maze

Baseline measurement (MWM) were performed as described to assess baseline learning of all animals prior to group allocation. Respective data is not shown in this report. Analysis on the effects of diet and exercise on spatial learning and memory was performed on rMWM scores, hence post group allocation.

3.6.1 Acquisition

Intact long-term spatial learning was assessed using measurements that were collected on 2 consecutive acquisition days. All animals demonstrated long-term spatial learning. Over time, the total swim distance and mean distance to the platform decreased equally in all groups (figure8A-B, total swim distance $F(1, 39) = 8.854$, $p = 0.005$, mean distance to platform $F(1, 39) = 6.223$, $p = 0.017$) and there was a similar trend with respect to the total distance to the platform ($F(1, 39) = 3.847$, $p = 0.057$). Importantly, the latency to find the platform decreased equally in all experimental groups (figure8C, latency $F(1, 39) = 5.343$, $p = 0.026$). All animals swam slower over time (figure8D, velocity $F(1, 39) = 18.528$, $p < 0.001$). Noteworthy, HFD + RW mice, but not HFDBCAA + RW mice, had a trend towards less reduction in speed over time, i.e., swimming faster on the second day, than HFD mice (figure8D, $p = 0.057$).

3.6.2 Probe

Long-term spatial memory was assessed during Probe trials. Analysis showed no evidence of baseline memory with respect to the platform location used during the standard MWM protocol in any group (frequency of entering the platform quadrant $F(2, 38) = 0.090$, $p = 0.914$, time spent in the platform quadrant $F(2, 38) = 0.473$, $p = 0.626$). Intact spatial memory was found for the new platform location (figure8E, $F(3, 114) = 2201.595$, $p < 0.001$). There was no evidence of group differences in spatial memory for the new platform location as measured by frequency of entering the platform quadrant or time spent in the platform quadrant ($F(2, 38) = 0.073$, $p = 0.930$, and $F(2, 38) = 0.657$, $p = 0.524$, respectively). However, there was a significant difference with respect to platform zone crossings across groups (figure8F, $F(2, 38) = 3.975$, $p = 0.027$), with HFD + RW mice having a trend of crossing the platform zone more frequently than HFDBCAA + RW mice (figure8F, $p = 0.051$).

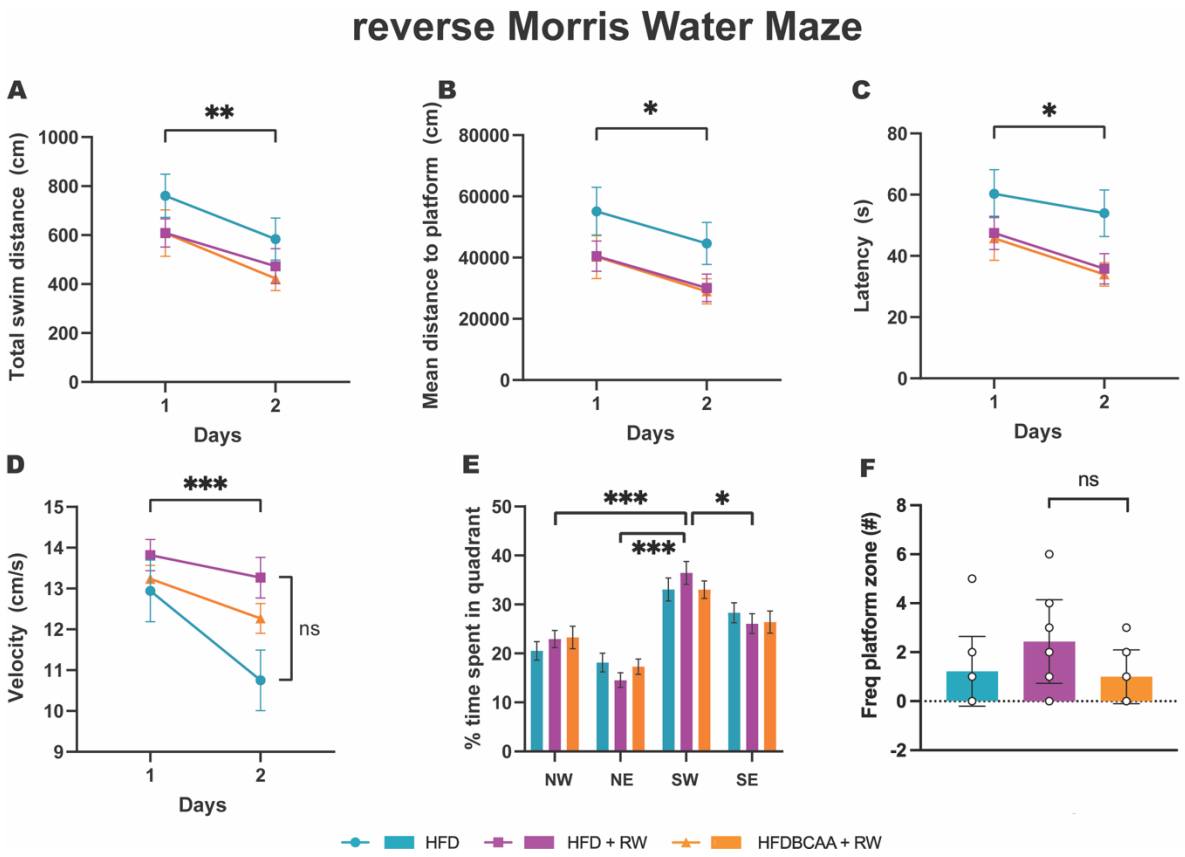

Figure 8 | Reverse Morris Water Maze. (A-B) The total swim distance and mean distance to the platform decreased equally in all groups over time (A: total swim distance p = 0.005, B: mean distance to platform p = 0.017). (C) The latency to find the platform decreased equally in all experimental groups over time (p = 0.026), (D) as did the swim velocity (p < 0.001), and HFD + RW mice had a trend of swimming faster than HFD mice (p = 0.057). (E) All mice spent more time in the new platform quadrant as compared to the other quadrants. (F) The number of platform zone crossings differed across groups (p = 0.027), with a trend of more crossings in HFD + RW mice than HFDBCAA + RW mice (p = 0.051). Data are presented as mean ± SEM, *P < .05 or **P < .01 or ***P < .001

3.7 Neuroimaging

3.7.1 ASL

Changes in CBF under normal and vasoconstrictive conditions, as well as vasoreactivity, were investigated using ASL in three regions of the brain, namely the cortex, hippocampus, and thalamus. Under normal conditions, HFD mice had increased CBF as compared to HFD + RW mice in both cortex and hippocampus (figure9A-B, cortex p = 0.038, and hippocampus p = 0.015). In addition, CBF significantly changed over time in the hippocampus and thalamus in all groups under normal conditions (figure9B, hippocampus $F(1, 32) = 7.03$, $p = 0.012$, figure9C thalamus $F(1, 32) = 5.55$, $p = 0.025$). There was no evidence of changes in vasoconstriction or vasoreactivity over time, or between experimental groups, in either ROI.

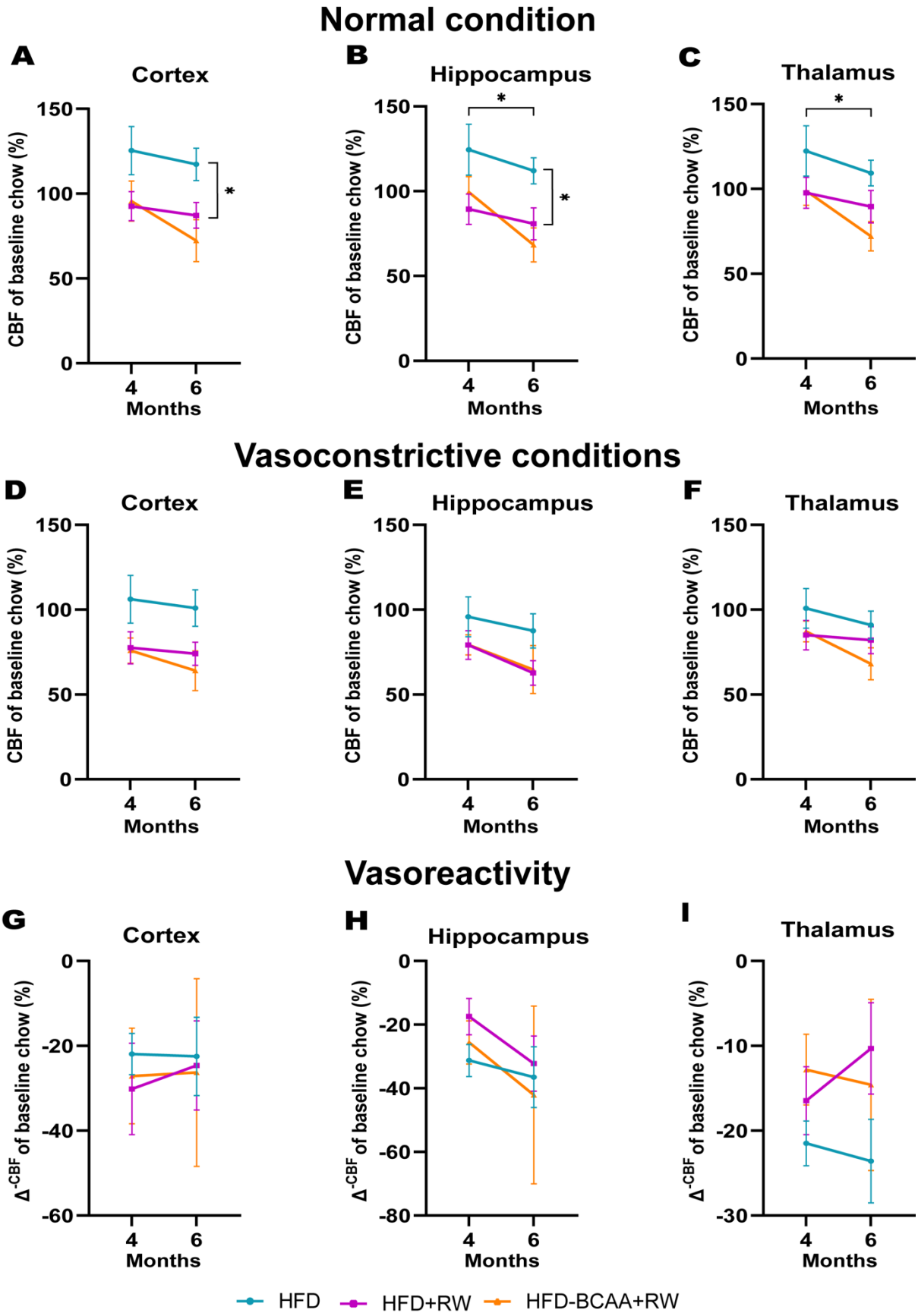

Figure 9 | Cerebral blood flow. (A-B) HFD mice had increased CBF as compared to HFD+RW in cortex ($p = 0.038$) and hippocampus ($p = 0.015$). (B-C) Under normal conditions, CBF changed over time in hippocampus (p

= 0.012) and thalamus (p = 0.025). (D-I) There was no difference in vasoconstriction (D-F) or vasoreactivity (G-I) over time or between groups, in either ROI.

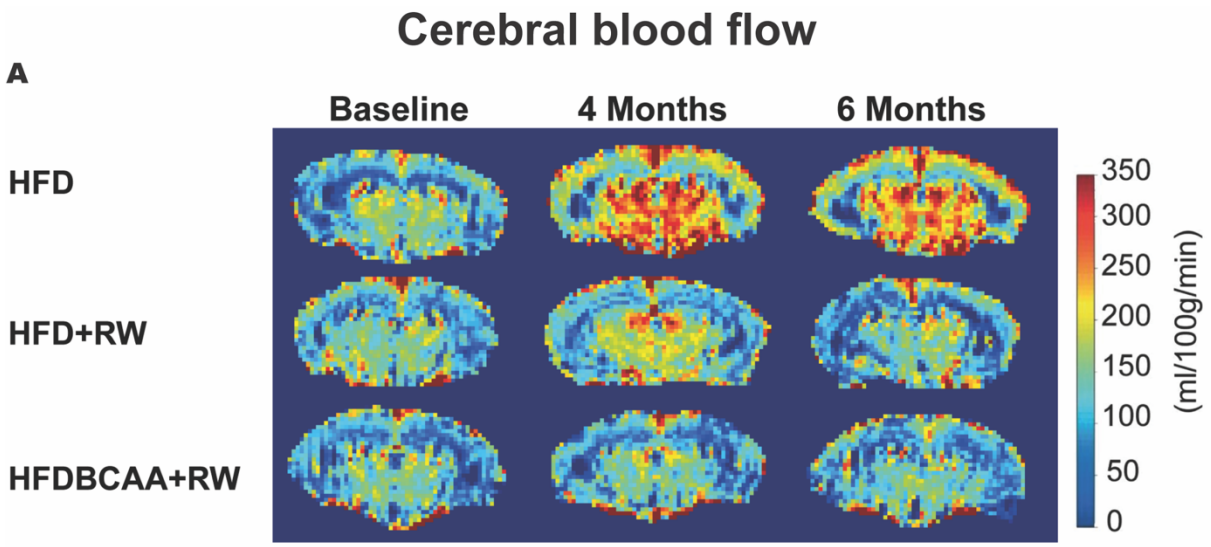

Figure 10 | Cerebral blood flow. (A) Representative high-resolution voxel-wise CBF images at 3 different time points under normal condition.

3.7.2 Rs-fMRI

Brain function was investigated using BOLD time series of rs-fMRI acquisitions of predefined ROI supporting cognitive and motor processes. There was no evidence of group differences in functional connectivity for either ROI through total or partial correlation (Figure11).

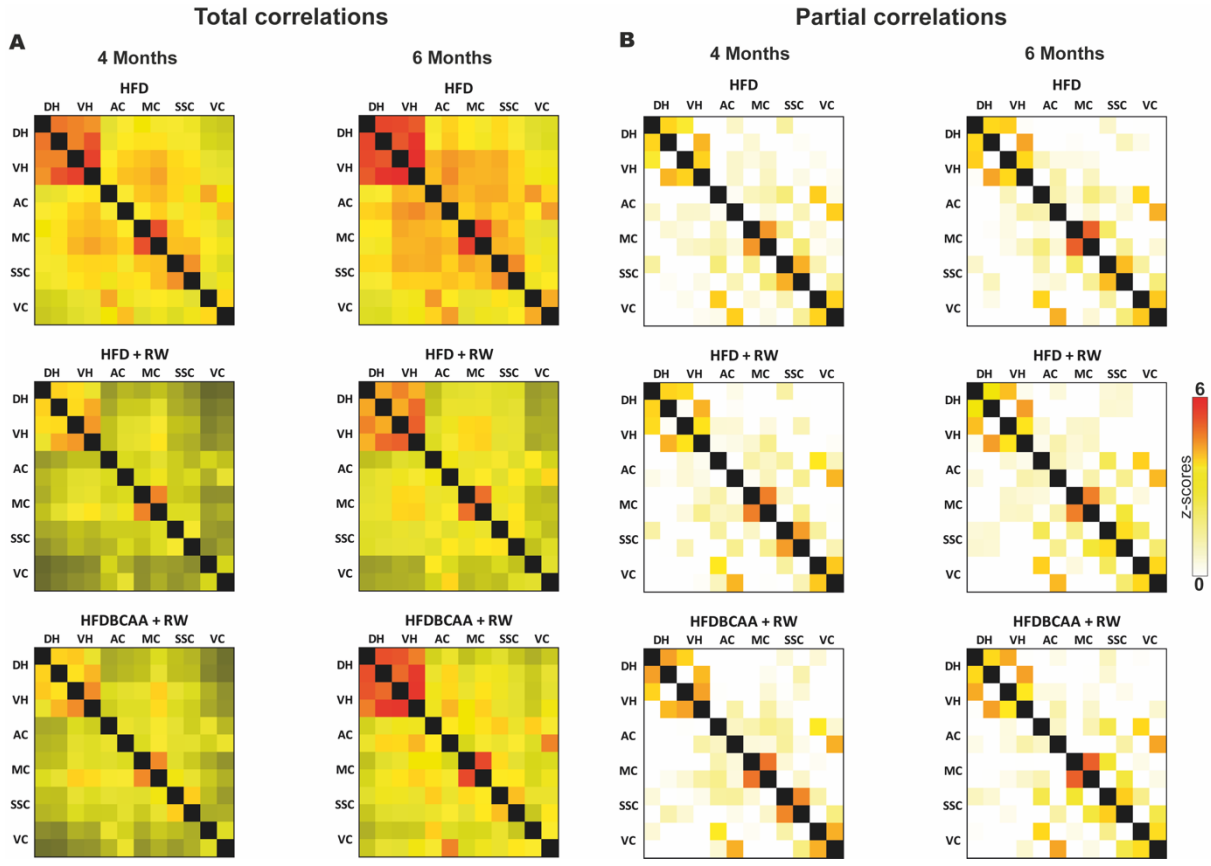

Figure 11 | Resting-state functional connectivity. (A-B) Based on total (A) and partial (B) correlation analyses. Total correlation matrixes of the HFD, HFD+RW, and HFDBCAA+RW experimental groups. The selected brain regions (dorsal hippocampus (DH), ventral hippocampus (VH), auditory cortex (AC), motor cortex (MC), somatosensory cortex (SSC) and visual cortex (VC) are subdivided in left hemisphere (first row) and right hemisphere (second row). A higher Z-score (red) indicates a stronger functional connectivity.

4. Discussion

Using a diet-induced obese transgenic mouse model, the present study investigated the preventative effect of voluntary exercise in combination with dietary supplementation of BCAA on obesity-induced pathological changes in brain structure and function. In addition, we examined the effects of this combined treatment on metabolic health and cognition. Exercise was expected to reduce body weight and benefit brain health, while the combination of BCAA and exercise was expected to affect metabolism and brain health in a dose-dependent manner. Following a longitudinal paradigm, physiological, behavioral, and neuroimaging measurements were performed at baseline and post intervention initiation. Obesity-related motor impairments could not be prevented by exercise and BCAA supplementation. Interestingly, exercising animals on BCAA preserved running endurance and attenuated weight gain. We found no evidence of exercise or diet effects on cognition or functional connectivity, however, exercise normalized CBF under normal conditions relative to baseline.

4.1 Physiological parameters

In line with previous research, all animals showed increased body weight within one month on HFD [25], and continued to gain weight over the course of the study. Importantly, Zhang et al. found BCAA supplementation to promote lipogenesis in diet-induced obese mice during exercise [18], which is supported by continued weight gain over time that was found in this study. However, mice in the combined BCAA + exercise group gained the least amount of weight. This contrasts their finding that BCAA impairs the beneficial effect of exercise on obesity [18], as only the combination of exercise with BCAA resulted in attenuated weight gain. Thus, our findings support research suggesting beneficiary effects of exercise and BCAA supplementation on body weight [15, 16]. Not surprisingly, we found a significant reduction in caloric intake between the first two timepoints, which reflects the diet switch from chow to HFD or HFD with BCAA. That is, while switching the diet caused increased food intake in response to novelty, this increase attenuated quickly and balanced over time, resulting in no difference in caloric intake when comparing first to last timepoint in either group. Assessment of DVC data provided proof of concept in terms of actual utilization of RW, and further

revealed a decrease in running distance over time during both day- and nighttime as expected based on contemporaneous increase in body weight. Based on studies showing combined BCAA and exercise interventions to improve muscle functional capacity [15, 16], higher running activity was presumed in exercising mice fed HFD with BCAA. Indeed, HFDBCAA + RW mice preserved running endurance as compared to HFD + RW mice, who showed a reduction in running activity. Additional physiological measurements were performed on blood plasma and systolic blood pressure on a regular basis as described in the methodology. Acquired data is still being analyzed, wherefore results of these procedures are not shown and possible insights cannot be discussed in the present paper. However, it is known from previous studies that triglyceride and cholesterol are increasing with HFD feeding [27]. Based on studies examining the effect on exercise on HFD-induced obesity, it is speculated that obesity-induced changes in the blood plasma will be attenuated through exercise and BCAA supplementation [29]. Our findings provide evidence for the suitability of the chosen model for obesity, as well as the applicability of DVC systems for monitoring running activity.

4.2 Behavioral tests: motor skills and cognition

Effects of treatment on balance and motor coordination as assessed using the Rotarod showed equal reduction in performance over time in all experimental groups, suggesting that neither exercise alone nor the combination of exercise and BCAA could counteract the negative effects of obesity on motor skills. Similarly, forelimb muscle strength as measured by the grip strength test decreased in all animals over time, once again highlighting the severity of obesity-induced reductions in muscle function. Noteworthy, there was no decrease in total limb strength over time in either group, as would be expected due to the positive association between body mass and strength [30]. It should be tested in following analyses whether such a correlation is indeed present in the current data. Interestingly, there was no evidence of differences in nonspatial learning and recognition memory between experimental groups in the object recognition test. This result opposes previous findings that suggest preventative effects of exercise on learning and (recognition) memory in humans [31] and (obese) mice [11, 32]. Because this test was administered on one timepoint only, however, it is arguable that possible changes over time could have differed between groups. Thus, we found no evidence of preventative effects of diet and/or exercise on obesity-induced decline in motor skill.

Learning and memory are consistently found to improve following exercise [10-12]. We found intact long-term spatial learning in all animals, irrespective of experimental group,

indicating that mice were in early disease stage which is not yet affecting cognition. While there was an overall decrease in swim velocity over time in all groups, HFD + RW mice showed a trend of swimming faster than HFD mice on the second day of acquisition. As expected, no mice had memory of the old platform location used during the standard MWM protocol administered 24 weeks prior. Intact long-term spatial memory was found for the new platform location, but no group differences were found. Based on these results, we suggest that cognition was not yet impaired due to early disease stage, and hence possible effects of diet and exercise are not prominent. Noteworthy, HFD + RW mice showed a trend of better memory for the new platform location than HFDBCAA + RW mice, suggesting that exercise only might benefit spatial memory more than the combination of exercise and BCAA. Additional consideration of baseline measurements from the standard MWM protocol could help elucidate the presumed obesity-induced change in cognition and the extend of the effects that diet and exercise had. Future research therefore should focus on the bigger picture by considering intervention effects in relation to a baseline. Overall, there was no deficits in spatial learning and memory in either group, hence no evidence of improved nonspatial learning and memory following exercise or exercise with BCAA was found either. Interestingly, exercise, but not exercise with BCAA, had a trend of boosting spatial memory.

4.3 Neuroimaging: cerebrovasculature and functional connectivity

Analysis of data assessing effects of voluntary exercise and BCAA on CBF in the cortex, hippocampus, and thalamus under normal and vasoconstrictive conditions revealed group differences in both conditions. It is often found that regional CBF in the prefrontal cortex is reduced in obese individuals due to imbalance of adipokines, and CBF velocity reductions have been directly associated to cognitive decline [5]. Another study found supporting evidence of reduced CBF in the hippocampus and thalamus of HFD-fed Ldlr-/- mice as compared to chow-fed and HFD with butyrate-fed mice [33]. Similarly, we found an overall decrease in CBF in the hippocampus and thalamus under normal conditions as would be expected in consequence of aging and progression of obesity. However, we also found that HFD mice showed increased CBF in the cortex and hippocampus under normal conditions as compared to mice in the HFD and exercise group. These findings are in line with Arnoldussen et al., who found increased CBF in mice fed a diet high in fat and carbohydrate content [34]. Together, these findings suggest a compensatory mechanism in obese animals in which CBF increases to provide sufficient blood supply prior to reduction in CBF with disease progression. Crucially, we found

exercise, but not the combination of exercise and BCAA, to prevent this pathological change by normalizing such increase in CBF under normal conditions relative to baseline. Based on this result, we speculate that BCAA might counteract the beneficial effect of exercise on CBF. It would be interesting to test this hypothesis in future research by introducing an additional control group receiving BCAA supplementation on HFD without exercise. Interestingly, there was no evidence of changes in vasoconstriction or vasoreactivity in either group over time, or between experimental groups in either ROI. It is possible that further progression into pathology is required to elucidate effects on vasoconstriction and vasoreactivity.

Obesity alters functional connectivity in multiple brain regions of various resting-state networks [35]. Such altered network function can be normalized through exercise [36]. In contrast to our expectations, neither exercise alone nor exercise in combination with BCAA supplementation influenced functional connectivity. It is suggested that these findings are a consequence of increased CBF at this early stage of disease, and that with prolonged disease progression effects might become apparent. Future studies should therefore look at later disease stages to validate these presumed compensatory mechanisms in brain function prior to degeneration of connectivity.

5. Conclusion

BCAA supplementation was found to have a positive effect on body weight. Obesity-induced impairments in motor skill could not be prevented by exercise and BCAA supplementation. Exercise and BCAA supplementation did not influence functional MRI, however, exercise prevented obesity-induced CBF changes under normal conditions. Findings remain mixed insofar as to which intervention approach promises better outcomes. To better elucidate possible preventative effects of exercise and BCAA, future studies should focus on the influence of exercise and BCAA supplementation on neuroinflammation, small vessel health, and white matter integrity. Furthermore, gene expression analysis could provide additional insights into pathology progression, as well as possible preventative effects of exercise and BCAA on the genetic level that are not yet reflected in behavioral change. Overall, it is suggested that exercise promises long-term benefits to attenuating obesity-related pathological changes in the brain and should thus be integrated into accessible lifestyle-modification interventions for obese individuals. With the synergistic effect of exercise and BCAA remaining unseen in early disease state, we advise on additional research to validate presumed benefits of exercise and BCAA in later disease state prior to initiating clinical interventions.

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