NEUROMODULATION OF EXERCISE: IMPACT ON DIFFERENT KINDS OF BEHAVIOR

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NEUROMODULATION OF EXERCISE: IMPACT ON DIFFERENT KINDS OF BEHAVIOR

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EDITORIAL
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Editorial: Neuromodulation of Exercise: Impact on Different Kinds of Behavior

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Editorial on the Research Topic

Neuromodulation of Exercise: Impact on Different Kinds of Behavior

The physiology and anatomy of the brain adapts to changing demands by modulating its functional and structural properties (Budde et al., 2016). Convergent evidence from both human (8 studies in this issue) and animal studies (2 studies in this issue) suggests that enhanced physical exercise facilitates this neuromodulation of certain brain structures and as a result behavioral responses.

This special issue wants to enhance our understanding of the neurobiological mechanisms of a variety of physical activities (see Budde et al., 2015b; Wegner et al., 2020 for differentiating issues). The outcome variables referred to cognitive and motor performance measures, white matter volume as well as growth factors, lactate and cortisol.

Kujach et al. found that acute sprint interval exercise (SIE) increases both the cognitive functions and peripheral neurotrophic factors and discussed the possible involvement of lactate in humans, which is a further mechanistic step of previous study that high-intensity interval exercise improves cognitive function (Kujach et al., 2018). Acute SIE shortened response times for both the Stroop task and TMT A and B. In response to acute SIE, blood lactate levels significantly increased and correlated with increased levels of BDNF, IGF-1, and VEGF. Furthermore, cognitive functions and BDNF are found to be correlated. Therefore, the improvement in cognitive performance following SIE may result from the synthesis or release of neuroprotective proteins modulated by high post-exercise blood lactate concentration.

Wegner et al. investigated the "Effects of Different Types of Exercise Training (ET) on the Cortisol Awakening Response (CAR) in Children." The acute effects of exercise on cortisol have been evaluated in the past (Wegner et al., 2014a,b,c; Budde et al., 2015a). In a longitudinal study for 10 weeks 71 children (9–10 years old) were randomly assigned to a cardiovascular exercise group ($n = 27$), a motor exercise group ($n = 23$), or a control group ($n = 21$). They trained for 45 min., three times a week. Children who enhanced their cardiovascular fitness over the course of the intervention showed an increased CAR after the intervention time, whereas children who underwent a motor exercise intervention and at the same time gained in motor fitness exhibited a decreased CAR after intervention.

Also in the saliva, Caserta et al. measured proNGF and proBDNF levels in 24 subjects before and after two training interventions of 12 weeks. Taken together "Influence of Quadrato Motor Training on Salivary proNGF and proBDNF" suggest that the two neurotrophins undergo a complex modulation, likely related to the different pathways by which they are regulated. Since variations of these neurotrophins have been previously linked to depression, stress and anxiety

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(Helmich et al., 2010), this study may have practical implications and aid in understanding the possible physiological mechanisms that mediate improved well-being, and the dynamic change of neurotrophins as a result of training.

The study by Matsui et al. entitled "Tyrosine as a Mechanistic-Based Biomarker for Brain Glycogen Decrease and Supercompensation with Endurance Exercise in Rats: A Metabolomics Study of Plasma," used a rat model of endurance exercise. They detected 186 metabolites in the plasma, and 110 metabolites changed significantly during and following exhaustive exercise. Brain glycogen levels correlated negatively with plasma glycogenic amino acids (serine, proline, threonine, glutamate, methionine, tyrosine, and tryptophan). In particular, plasma tyrosine as a precursor of brain noradrenaline might be a valuable mechanistic-based biomarker to predict brain glycogen dynamics in endurance exercise.

Also in an animal model "Nerve Growth Factor (NGF) is Responsible for Exercise-Induced Recovery of Septohippocampal Cholinergic Structure and Function" Hall et al. showed that exercise-induced enhancement of NGF within the septohippocampal pathway represents a key avenue for aiding failing septo-hippocampal functioning and therefore has significant potential for the recovery of memory and cognition in several neurological disorders.

The study "The Choice of Sports Affects Mental Rotation Performance in Adolescents" by Pietsch et al. investigates mental rotation performance of adolescent female dancers and soccer players in object-based and egocentric mental rotation tasks using human body stimuli. Contrary to the literature, they didn't find significant higher reaction times and error rates for stimuli presented in front view compared to back view in general but only for egocentric transformations. The results of this study show that specific sports affect individual aspects of mental rotation performance.

Van den Berg, Saliasi, Groot et al. showed in their randomized controlled trail "Improving Cognitive Performance of 9–12 Years Old Children: Just Dance?" that daily 10-min exercise breaks in the classroom for 9 weeks did not improve, nor deteriorate cognitive performance in children, comparable with Ludyga et al. (2019).

Van den Berg, Saliasi, Jolles et al. investigated: Exercise of Varying Durations: No Acute Effects on Cognitive Performance in Adolescents. In sum, contrary to literature (Budde et al., 2010; Niemann et al., 2013) acute exercise bouts with a duration of 10, 20, or 30 min did not improve, but neither deteriorate cognitive performance of young adolescents compared to a sedentary control condition.

A group around Terentjeviene et al. performed a study named: Prefrontal Cortex Activity Predicts Mental Fatigue in Young and Elderly Men During a 2 h "Go/NoGo" Task. They did not use exercise as an intervention but measured cognitive stress and their effect on motor functions and concluded: Because of the greater mental load and (possibly) greater activation of prefrontal cortex during the 2 h "Go/NoGo" task, there was greater mental and neuromuscular performance fatigue in young men than in elderly men. However, contrary to this results Wegner et al. (2014a) found an improving effect of acute psychosocial stress on fine motor skills in High School students.

Findings from the ActiveBrains ($n = 100$) and FITKids2 Projects ($n = 242$) named: Physical Fitness, White Matter Volume and Academic Performance in Children by Esteban-Cornejo et al. showed in a cross-sectional design that cardiorespiratory fitness may positively relate to white matter volume in overweight/obese children, and in turn, academic performance.

The results of this special issue suggest that physical exercise triggers neuromodulation and thereby, enhances an individual's capacity to respond to new demands with behavioral alterations (Gronwald and Budde, 2019). Besides the need for more RCT studies we believe that it will become more and more necessary to implement an extra sham condition in future studies to be able to prove that one exercise intervention is more effective than another (Budde et al., 2018).

AUTHOR CONTRIBUTIONS

HB and HS conceptualized and drafted the initial manuscript. HB, BV, PR, and HS reviewed and revised the manuscript. All authors have read and approved the final version of the manuscript.

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ORIGINAL RESEARCH
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Prefrontal Cortex Activity Predicts Mental Fatigue in Young and Elderly Men During a 2 h "Go/NoGo" Task

Asta Terentjeviene[1], Edita Maciuleviciene[2], Kazys Vadopalas[3], Dalia Mickeviciene[1,3], Diana Karanauskiene[2], Dovile Valanciene[1], Rima Solianik[1,3], Arunas Emeljanovas[2], Sigitas Kamandulis[1] and Albertas Skurvydas[1,3]*

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Background: Although the effects of mental fatigue on cognitive–motor function and psychological state in young adults are well-documented, its effects in the elderly are not completely understood. The aim of this study was to estimate the effect of prolonged cognitive load on the indicators of psychological, cognitive, and motor functions.

Methods: Fifteen young and 15 elderly men were asked to perform a 2 h "Go/NoGo" task. Psychological state (mood and motivation), cognitive (prefrontal cortex activity and cognitive performance), and motor (motor cortex excitability and grip strength) functions were measured before and after the task. During the 2 h task, both groups had a significantly similar increase in the number of "Incorrect NoGo" errors. Only in young men reaction time (RT) of "Incorrect NoGo" and intraindividual variability of RT of "Incorrect NoGo" significantly increased during task. After the task, handgrip strength decreased for the young men, whereas latency of motor evoked potentials prolonged both groups. Nevertheless, both groups indicated that they felt fatigue after the 2 h task; we observed that mental demand increased, whereas intrinsic motivation and mood decreased only in young men. Prolonged task decreased the switching/rest ratio of oxygenated hemoglobin for the young and the elderly men; however, greater for elderly than young men. Interestingly, the more the prefrontal cortex was activated before the 2 h task during the switching task, the fewer of "Incorrect NoGo" errors made by the young men and the greater the number of errors made by the elderly men.

Conclusion: Because of the greater mental load and (possibly) greater activation of prefrontal cortex during the 2 h "Go/NoGo" task, there was greater mental and neuromuscular performance fatigue in young men than in elderly men.

Keywords: aging, motor fatigue, mental fatigue, prefrontal cortex, executive function

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INTRODUCTION

Functional limitations, disabilities, mortality, and other adversative consequences in the elderly can be highly predicted by fatigue (Skurvydas et al., 2011; Ishii et al., 2014). Mental fatigue is first of all the subjective feeling of a worsened ability to engage in mental activities, but it can also be measured objectively by decreased performance (Lorist, 2008; Solianik et al., 2016; Skurvydas et al., 2017;

Chuang et al., 2018). There is a lack of understanding of the cognitive mechanisms of mental fatigue origin. It is still not clear whether the decreased performance due to mental fatigue is caused by a continuous deterioration of the cognitive properties (e.g., attention and memory) (Baumeister, 2014) or by a scarce enrolment of intact cognitive processes, caused by the decrease in motivation (Chaudhuri and Behan, 2000). Botvinick and Braver (2014) suggest that mental fatigue has a control mechanism that expels people from lengthy tasks and pushes them to newer and supposedly more rewarding activities. They established that after mental fatigue, increasing extrinsic motivation recovered the level of performance which was before the fatigue, and maintained that this provided evidence in support of a fatigue-induced detachment from the task (Botvinick and Braver, 2014). Chaudhuri and Behan (2000) suggest that mental fatigue might be caused by decreased motivation to take part in self-initiated activities and it is a consequence of changes in the motivational brain circuits, together with the basal ganglia.

The self-control mechanism is highly dependent on the so-called executive function, which operates effectively if concentration on the required object and inhibition of unnecessary objects (temptations), working memory, and flexible switching of attention work well (Diamond, 2013). The main controller of the executive function (and self-control) is localized in the prefrontal cortex of the brain, which is responsible for the management of cognitive tasks and emotions (Buschman and Miller, 2014). One of the most important characteristics of self-control is the ability to inhibit undesirable stimuli, such as temptations (Baumeister, 2014). Cognitive fatigue was caused by continued performance of tasks which were cognitively demanding (compared with controls) (Klaassen et al., 2014). Results showed that age affected the left dorsolateral prefrontal and superior parietal cortex activation during working memory encoding; also greater activation was more pronounced among middle-aged than young adults irrespective of the load of the working memory or the condition of fatigue (Klaassen et al., 2014).

"Go/NoGo" and "Stroop" test exercises are widely used for inducing cognitive fatigue (Netz et al., 2016; Solianik et al., 2018). These and similar exercises usually last from 30 min to several hours and cause a decrease in mental working capacity (Shigihara et al., 2013). It has been proven that the "Go/NoGo" task requires self-control skills, the main goal of which is to inhibit redundant tasks (Brown et al., 2015; Netz et al., 2016).

The speed of processing, working memory, inhibitory function, and long-term memory decline with age, similarly, the brain structure size and white matter integrity decrease (Park and Reuter-Lorenz, 2009). The age-related compensatory recruitment of prefrontal cortex during cognitive (Emery et al., 2008; Park and Reuter-Lorenz, 2009) and motor controls (Seidler et al., 2010) tasks has been established. Older adults demonstrate greater activation when they perform tasks that engage executive functions, episodic memory, and working memory tasks, compared with young adults (Emery et al., 2008). Older adults show more widespread involvement of brain regions responsible for motor control than young adults, principally the prefrontal cortex and basal ganglia networks (Seidler et al., 2010). These regions are the most susceptible to age-related effects, which results in a disparity between "supply and demand." However, other elements of these compensatory mechanisms and their findings reflecting cognitive decline must be thoroughly investigated in the future.

The main aim of our studies was to test the following hypothesis. As older adults more highly activate executive, cognitive, and association brain regions aiming at performing the tasks (Emery et al., 2008; Park and Reuter-Lorenz, 2009; Seidler et al., 2010; Klaassen et al., 2014), (1) elderly men should have greater fatigue in a "Go/NoGo" task lasting 2 h than younger men; (2) they should make more errors, there should be a greater increase in the variability of task performance, a greater decrease in neuromuscular function, a greater increase in the subjective feeling of fatigue and greater effort during exercise, a greater decrease in cognitive function, while motivation during the task should not be different between the young and elderly men; and (3) objective and subjective indicators of fatigue both in young and elderly men should depend on prefrontal cortex activity as measured by functional near-infrared spectroscopy (fNIRS).

MATERIALS AND METHODS

Subjects

Thirty healthy participants took part in this study: 15 young [age: 22.2 ± 2.7 years, height: 180.1 ± 6.3 cm, body mass index (BMI): 23.5 ± 2.5 kg/m^2] and 15 elderly (age: 72.7 ± 5.7 years, height: 176.3 ± 4.8 cm, BMI: 25.1 ± 3.1 kg/m^2) men. Young and elderly participants were healthy, non-smokers, and right-handed (confirmed using the Edinburgh Handedness Inventory). Participants were asked not to consume caffeine and alcohol-containing products 12 h before the experiment, and were told to come another time if they were ill or did not sleep well the night before the experiment. Written informed consent was obtained from all participants after explaining to them all the details of the experimental procedures as well as potential discomforts and risks. The studies were approved by the local Ethics Committee (The Kaunas Regional Ethics Committee, No. BE-2-40), performed in accordance with the Declaration of Helsinki.

Rationale of Experiment

Participants came to the laboratory three times. During the first and the second visit (day), participants were familiarized with the experimental procedures (cognitive and motor performance tests, assessment of motor cortex excitability during TMS). Besides, on the second day the subjects filled in a self-assessment questionnaire: the Schutte Self-Report Emotional Intelligence test (SSREIT). The third day protocol is given in **Figure 1**. Each participant accomplished all three visits during 2 weeks with a minimum of 48 h recovery period between each visit. Before the start of the experiment (the third day), the participants were asked to sleep for 8 h the night before the experiment, and to avoid ingesting alcoholic beverages, caffeine, and sedating antihistamines for 48 h and from heavy exercise for at least 24 h

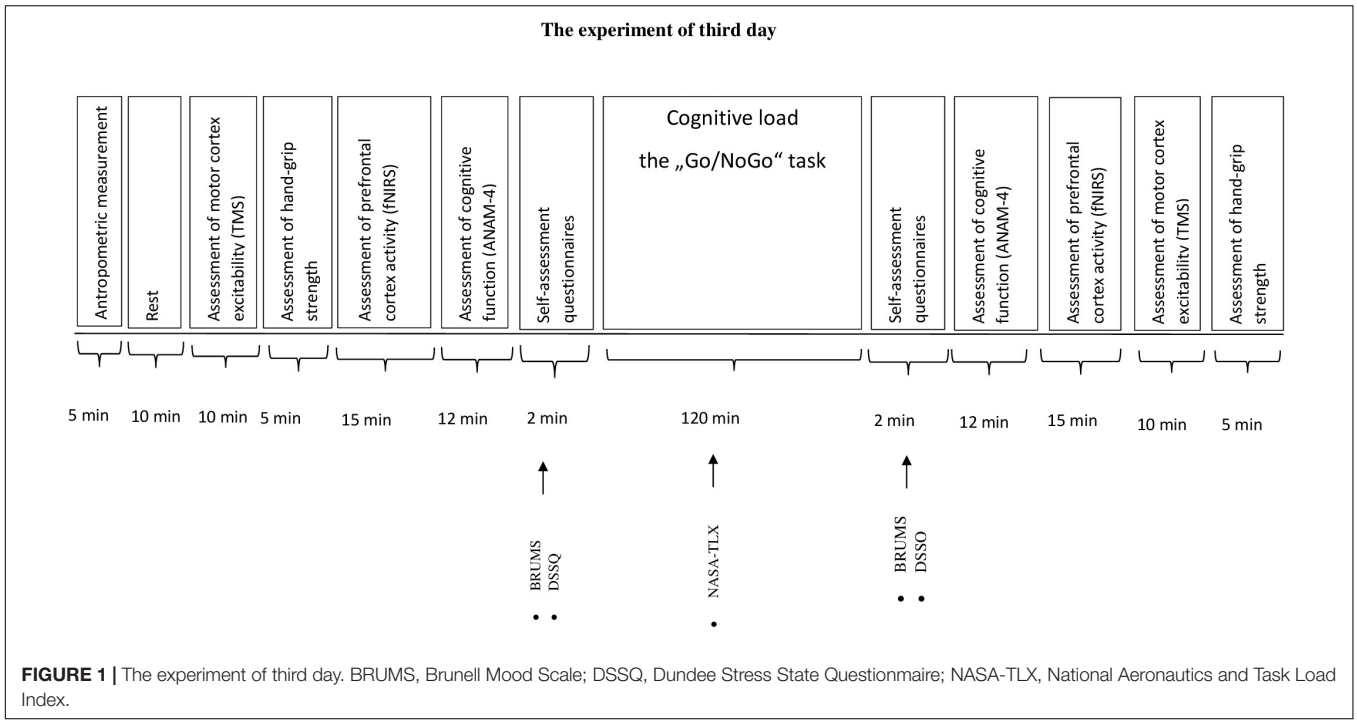

FIGURE 1 | The experiment of third day. BRUMS, Brunell Mood Scale; DSSQ, Dundee Stress State Questionnaire; NASA-TLX, National Aeronautics and Task Load Index.

before the experiment. The experiment of the third day was performed at 8:00 in the morning.

Measurements

Assessment of Prefrontal Cortex Activity

The fundamentals of fNIRS are described in detail in other sources (Ferrari and Quaresima, 2012). Assessment of brain activity was performed on a continuous wave system (fNIR Imager 1100, fNIR Devices LLC, Potomac, MA, United States) using a flexible 16 optode probe set. The sensor has a temporal resolution of 500 ms per scan with a 2.5 cm source-detector separation allowing for approximately 1.25 cm penetration depth and 16 measurement locations on a rectangular grid covering the forehead region, designed to observe dorsal and inferior frontal cortical areas (Ayaz et al., 2013). Two different wavelengths (730 and 850 nm) are used by the system, and its frequency is controlled for wavelengths and channels to avoid cross talk. COBI Studio software was used for data acquisition (Ayaz et al., 2013). The signals of all channels were verified before recording. Data analysis was performed using fNIRSoft analysis software (BIOPAC Systems, Inc., United States). Oxygenated hemoglobin (OxyHb) values were calculated from raw data by solving a modified Beer–Lambert equation. Data were filtered to remove physiological and other artifacts. The changes of OxyHb were acquired from all the participants in all 16 channels and the data were averaged. Prefrontal cortex activity was assessed during the 5 min rest and during the Switching Task.

Assessment of Cognitive Function

Cognitive function was assessed using a computerized Automated Neuropsychological Assessment Metric, version 4 (*ANAM-4;* Center for the Study of Human Operator Performance University of Oklahoma, Norman, OK, United States), a reliable screening instrument designed for repeated evaluations (Reeves et al., 2007). The test battery took about 12 min to perform. The chosen tests measured the task accuracy (percent of correct responses) and mean response time (mean reaction time, RT).

The Switching Task (ST)

This task measures the ability of mental flexibility and shifting set (Reeves et al., 2007). It is a combination of the Mathematical Processing Task (MPT) and the Manikin Task (MT). The MT is located on the left side of the computer screen and the MPT is located on the right side of the computer screen, and the user is guided by means of a red arrow at the bottom of the screen to respond to the problem on the left or the right side. Responses are inserted by using the keyboard, when the left hand is used for the MT and the right hand is used for the MPT. This test consisted of 64 trials.

The Simple Reaction Time Task (SRTT)

This test measures Simple Reaction Time (SRT) by providing the participant with a series of "*" symbols on the display. The participant is instructed to respond as quickly as possible by pressing a button as soon as the stimulus appears. This test consisted of 40 trials. Results of this test are used as a measure of attention, visuo-motor response planning, and timing.

The Code Substitution and Code Substitution Delayed Tasks (CSIT and CSDT)

This test measures attention, concentration, and learning. During this test, nine symbols and nine numbers are paired with a unique number located below a specific symbol. The participant is instructed to try to remember the symbol–number pairs because they will be asked to recall them later. During the learning

phase, the participant indicates whether or not the pairings at the bottom match the key and receives feedback for incorrect responses, if the pair is correct, the participant presses the left mouse button; if incorrect, the right mouse button. During the recall phase, there is no key at the top and the participant must indicate if the pairings appearing at the bottom are correct or incorrect from memory. This test consisted of 40 trials.

The Two-Choice Reaction Time Task (TCRTT)

This task measures ability to shift mental set (mental flexibility). During this test, one of two stimuli is presented on the screen ("*" or "o") with a variable interstimulus. The participant is instructed to respond as quickly as possible by pressing the left mouse button each time the "*" stimulus is presented or the right mouse button each time the "o" stimulus is presented. This test consisted of 40 trials. Results of this test are used as a measure of processing speed and alternating attention with a motor speed component.

The Mathematical Processing Task (MPT)

This task measures working memory. During this test, an arithmetic problem requiring an addition and subtraction of three single-digit numbers is displayed (e.g., "5 – 2 +3 = "). The participant is instructed to respond as quickly as possible by pressing the left mouse button if the answer to the equation is greater than 5 or the right mouse button if the answer is less than 5. The correct answer may be any number from 1 to 9 except 5. This test consisted of 20 trials. Results of this test are used as an index of basic computational skills, concentration, and working memory.

The Matching Grids Task (MGT)

This task measures visuospatial discrimination. During this test, two 4 × 4 grids are displayed side by side on the screen; however, one 4 × 4 pattern is rotated. The participant is instructed to indicate as quickly as possible if the grids are exactly the same, except for a possible rotation, and to click the left mouse button and the right mouse button if the grids are different. This test consisted of 20 trials. Results of this test are used as an index of visuospatial processing.

The Pursuit Tracking Task (PTT)

This task measures visuomotor control. During this test, the participant is instructed to move the computer mouse so that the cursor tracks a moving target with a "+" symbol inside. The mouse cursor is required to remain inside the box and be kept as close to the symbol as possible as it moves across the screen in a circular pattern for 2 min. The path and the accuracy of movement are established.

The Manikin Task (MT)

This task measures spatial orientation ability. During this test, a figure of a man is presented holding a ball in one hand and a cube in the other hand, and a ball or a cube is displayed at the bottom of the screen. The figure of the man appears in various orientations: standing upright or upside down and either facing toward the test taker or away. The participant is instructed to indicate as quickly as possible which of the man's hands is holding the object displayed at the bottom on the screen and to press the left mouse button if the answer is left and the right mouse button if right. This test consisted of 32 trials. This test assesses three-dimensional spatial rotation ability, left–right orientation, problem solving, and attention.

The Memory Search Task (MST)

This task is an adaptation of Sternberg's memory search/serial reaction time task, which measures verbal working memory. During this test, a string of six letters is presented for memorization. The participant is instructed to press the space bar once the string has been memorized; then, it disappears from view and individual letters are presented one at a time. The participant is instructed to indicate as quickly as possible whether the letter belongs to the memorized set and press the left mouse button for letters included in the memory set and the right mouse button for those not. This test consisted of 40 trials. Results of this test are used as an indicator of verbal working memory, immediate recognition, and attention.

Cognitive Load – The "Go/NoGo" Task

The *"Go/NoGo" task* measures response inhibition (Chikazoe, 2010). During this test, a participant is required to respond to a go stimulus as quickly as possible, but is required to withhold a response to a no-go stimulus. During this test, 5400 stimuli appeared on the computer screen for each research participant: 4320 of them were "Go" stimuli and 1080 – "NoGo" stimuli ("Go" stimuli occurred in 80% of trials, with "NoGo" stimuli occurring in 20%.). As soon as the participant reacted to the stimulus on the screen, a new stimulus appeared immediately. The duration of the test was 120 min, 8 series, 15 min each (2–3 s between series). We established the following indicators: "Correct Go" RT, "Incorrect NoGo" RT, "Correct Go after Correct NoGo" RT, number of correct and incorrect responses ("Incorrect Go"), intraindividual variability [coefficient of variation (CV)] of "Correct Go," "Incorrect NoGo," "Correct Go after Correct NoGo." During 2 h "Go/NoGo" task the participant filled in the National Aeronautics and Space Administration Task Load Index (NASA-TLX) questionnaire every 30 min. It took about 2 min.

Assessment of Motor Cortex Excitability During Transcranial Magnetic Stimulation (TMS)

EMG was recorded from the abductor pollicis brevis (APB) muscle with a motor evoked potentials (MEP) monitor (MagVenture A/S, Denmark) with 26 mm diameter pregelled disposable Ag/AgCl electrodes (FIAB, Italy) placed on clean skin. EMG recordings were band-pass filtered (20–5000 Hz), sampled at 100 ks/s, digitized, and stored in a computer for analysis. A Magpro X100 transcranial magnetic stimulator (MagVenture A/S, Denmark) with a handheld figure-of-eight coil (95 mm D-B80 Butterfly Coil; MagVenture A/S) was used to elicit MEPs in the right ABP muscle. Single pulses (biphasic waveform, pulse width 280 μs) were delivered manually. The optimal coil position was determined before the experiment as follows. Using a slightly suprathreshold stimulus intensity, the coil was moved to determine the optimal point on the left primary motor cortex for stimulation, from which maximal amplitude MEPs were elicited in the ABP muscle. The coil was placed tangentially to the scalp with the handle pointing backward and laterally at a 45° angle away from the midline. Then the position of the coil was marked

on a latex swimming cap for correct repositioning. The stimulator output intensity was adjusted to 130% of resting motor threshold (rMT), which was defined as the minimal intensity of stimulator output that produces MEPs with amplitudes of at least 50 mV with 50% probability (Rossini et al., 1994). The subjects were constantly monitored to ensure absence of voluntary APB muscle contraction. A total of 10 stimuli were collected at approximately 10 s intervals, and averaged for analysis. The most important indicators were latency and amplitude of MEP.

Assessment of Hand-Grip Strength

In the study, a hydraulic hand dynamometer (Jamar, Lafayette Instrument Company, United States) was used to measure isometric hand-grip strength. After the maximal hand grip, maximal hand-grip strength (0–90 kg) was shown on the screen. For each subject, the device handle was adapted according to the size of their hand. With the dominant hand (all subjects were right handed) the subject performed the testing with Jamar hydraulic hand dynamometer twice: before "Go/NoGo" task and after the 2 h "Go/NoGo" task. The subject was seated in a chair with their arm pointing to the front, the elbow bent at 90°. The subject was allowed three trials, the best result was recorded.

Self-Assessment Questionnaires

The Schutte Self-Report Emotional Intelligence Test (SSREIT)

To measure the emotional intelligence of participants, the SSREIT (Schutte et al., 1998) was used. The SSREIT is a self-report inventory consisting of 33 items scored on a five-point Likert scale (1 = strongly disagree, 2 = disagree, 3 = neither disagree nor agree, 4 = agree, 5 = strongly agree). All these items can be divided into four subscales: perception of emotion (e.g., "When I am faced with obstacles, I remember times I faced similar obstacles and overcame them"), managing own emotions (e.g., "I am aware of the non-verbal messages I send to others"), managing others' emotions (e.g., "I know when to speak about my personal problems to others"), and utilization of emotions (e.g., "When my mood changes, I see new possibilities"). Total scores can range from 33 to 165, where a higher score indicates a higher quality of emotional intelligence.

The Brunel Mood Scale (BRUMS)

Current mood ("How do you feel right now?") was assessed with the Brunel Mood Scale (BRUMS) (Terry et al., 2003). This questionnaire contains 24 items (e.g., "angry," "uncertain," "miserable," "tired," "nervous," and "energetic") divided into six respective subscales: anger, confusion, depression, fatigue, tension, and vigor. The items were answered on a five-point Likert scale (0 = not at all, 1 = a little, 2 = moderately, 3 = quite a bit, 4 = extremely), and each of subscales with four items can summed for a total score of 0 to 16. Higher scores on each subscale represent a greater current mood extreme (anger, confusion, depression, fatigue, tension, or vigor) of the participants.

The Dundee Stress State Questionnaire (DSSQ)

The Dundee Stress State Questionnaire (DSSQ) thinking content and motivation scales were used (Matthews and Desmond, 2002). The thinking content scale related to task performance was measured on subscales of the DSSQ: task-related interference (eight items) and task-irrelevant interference (eight items). The scale consists of 16 items (e.g., "I thought about how I should work more carefully" and "I expect the content of the task will be interesting") scored on a five-point Likert scale (0 = not at all, 1 = a little bit, 2 = somewhat, 3 = very much, 4 = extremely). Therefore, total scores for each thinking content subscale range between 0 and 32, where a higher score indicates higher thinking content. The DSSQ motivation scale was used to assess motivation related to "Go/NoGo" task performance. The motivation scale comprises 15 items that include groups of three-dimensional questions: questions about success motivation (want to perform actions good or better than others), questions about intrinsic motivation (to be interested), and one question about overall motivation. The scale consists of two subscales: success motivation (seven questions) and intrinsic motivation (seven questions) (e.g., "I wanted to succeed on the task" and "I felt apathetic about my performance"). The scale is scored on a five-point Likert scale (0 = not at all, 1 = a little bit, 2 = somewhat, 3 = very much, 4 = extremely). Therefore, total scores for each motivation scale range between 0 and 28, where a higher score indicates higher motivation.

The National Aeronautics and Space Administration Task Load Index (NASA-TLX)

To evaluate the subjects' perceived workload and performance during 2 h "Go/NoGo" task, participants were asked to respond to the NASA-TLX questionnaire, which includes six dimensions: mental demand ("How much mental and perceptual activity was required?"), physical demand ("How much physical activity was required?"), temporal demand ("How much time pressure did you feel due to the rate or pace at which the tasks or task elements occurred?") and perceived performance ("How successful do you think you were in accomplishing the goals of the task set by the experimenter?"), effort ("How hard did you have to work to accomplish your level of performance?") and frustration ("How insecure, discouraged, irritated, stressed and annoyed versus secure, gratified, content, relaxed and complacent did you feel during the task?") (Hart and Staveland, 1988). The participants scored each of the items on a scale divided into 20 equal intervals anchored by a bipolar descriptor (e.g., high/low). This score was multiplied by 5, resulting in a final score between 0 and 100 for each of the subscales, where a higher score indicates higher overall workload.

Statistical Analysis

The data were tested for normal distribution using the Kolmogorov–Smirnov test, and all data were found to be normally distributed. A two-way mixed analysis of variance (ANOVA) with age as a between-group factor and with time as a within-group factor was taken. If significant effects were found, Sidak's *post hoc* adjustment was used for multiple comparisons across a set of conditions within each repeated-measures ANOVA. Statistical significance was defined as $p < 0.05$. Together with this, calculations for statistical power [observed power (*OP*)] were performed and the partial eta squared (η_p^2) was estimated as a measure of the experimental trial effect size. Pearson correlation

coefficients (r) were used to identify relationships between variables. Statistical analyses were performed using IBM SPSS Statistics software (v. 22; IBM Corporation, Armonk, NY, United States).

RESULTS

"Go/NoGo" Task

There was a significant increase in the number of "Incorrect NoGo" errors in the young men and the elderly men during the "Go/NoGo" task ($F_{(7,196)} = 3.43$; $p = 0.003$; $OP = 0.95$; $\eta_p^2 = 0.22$) (**Figure 2**). However the effect of age was not significant ($F_{(1,29)} = 0.26$; $p = 0.619$; $OP = 0.076$; $\eta_p^2 = 0.021$). The "Incorrect Go" error depends neither on task performance ($F_{(7,196)} = 0.66$; $p = 0.708$; $OP = 0.27$; $\eta_p^2 = 0.05$), nor age ($F_{(1,29)} = 3.34$; $p = 0.094$; $OP = 0.38$; $\eta_p^2 = 0.21$). "False alarm" error did not change significantly during 2 h "Go/NoGo" task ($F_{(7,196)} = 0.64$; $p = 0.719$; $OP = 0.26$; $\eta_p^2 = 0.05$) and there were no significant differences between young and elderly men ($p > 0.05$) in all cases interaction effect of task and age was not significant ($p > 0.05$).

The RT of "Correct Go" did not significantly depend on the task ($F_{(7,196)} = 0.71$; $p = 0.667$; $OP = 0.29$; $\eta_p^2 = 0.06$), but it significantly depended on age ($F_{(1,29)} = 6.77$; $p = 0.024$; $OP = 0.69$; $\eta_p^2 = 0.36$) (**Figure 3**). The RT of "Incorrect NoGo" significantly depended on the task ($F_{(7,196)} = 2.47$; $p = 0.02$; $OP = 0.85$; $\eta_p^2 = 0.18$) and on age ($F_{(1,29)} = 2.98$; $p = 0.012$; $OP = 0.55$; $\eta_p^2 = 0.19$). Only in young men RT of "Incorrect NoGo" significantly increased during 2 h "Go/NoGo" task ($p < 0.05$). The after "Correct NoGo" RT of "Correct Go" did not significantly depend on the task ($F_{(7,196)} = 1.57$; $p = 0.154$; $OP = 0.52$; $\eta_p^2 = 0.11$), but it depended on age ($F_{(1,29)} = 9.78$; $p = 0.009$; $OP = 0.82$; $\eta_p^2 = 0.45$). Moreover, the ratio of RT of "Correct Go" to "Incorrect NoGo" depended significantly neither on the task nor on age ($p > 0.05$). We found that the ratio of RT of "Correct Go" "after correct NoGo" to "Correct Go" did not significantly depend on the task ($F_{(7,196)} = 6.71$; $p = 0.137$; $OP = 0.49$; $\eta_p^2 = 0.12$), but it depended on age ($F_{(1,29)} = 4.97$; $p = 0.041$; $OP = 0.69$; $\eta_p^2 = 0.29$) In all cases the interaction effect of age and task was not significant ($p > 0.05$).

The effect of task on CV of RT of "Incorrect NoGo" was significant ($F_{(7,196)} = 3.13$; $p = 0.005$; $OP = 0.93$;$\eta_p^2 = 0.2$), however, the effect of age was not significant ($F_{(1,29)} = 0.78$; $p = 0.39$; $OP = 0.12$; $\eta_p^2 = 0.06$) (**Figure 4**). Only in young men CV of RT of "Incorrect NoGo" significantly increased during PCL ($p < 0.01$). We established that there was not significant effect of task and effect of age on CV of RT both "Correct Go" and "Correct Go" after "Correct NoGo" ($p > 0.05$). In all cases the interaction effect of age and task was not significant ($p > 0.05$).

Effect of the 2 h "Go/NoGo" Task on Motivation

The performance of the 2 h "Go/NoGo" task significantly decreased the intrinsic motivation from 23.2 (5.1) to 20.2 (5.8) ($p < 0.05$) in young men, whereas intrinsic motivation remained unchanged in elderly men [24.6 (4.4) to 22.5 (4.3); $p > 0.05$]. The prolonged performance of the task did not affect the success and overall motivation in young men [16.5 (5.5) to 17.4 (6.4) for success motivation, and 3.2 (0.8) to 3.1 (0.8) for overall motivation; $p > 0.05$] and elderly men [17.2 (3.3) to 16.5 (4.1) for success motivation, and 3.1 (1.1) to 3.5 (0.5) for overall motivation; $p > 0.05$]. There were no significant differences in success, intrinsic, and overall motivation variables between young men and elderly men.

Effect of the 2 h "Go/NoGo" Task on Mood State

There were no significant differences in mood variables before the "Go/NoGo" task between the young men and the elderly men (**Table 1**). Subjective fatigue, tension, and confusion significantly ($p < 0.05$) increased, and vigor decreased after the "Go/NoGo" task in the young men. Subjective fatigue significantly ($p < 0.05$) increased after the "Go/NoGo" task in the elderly men.

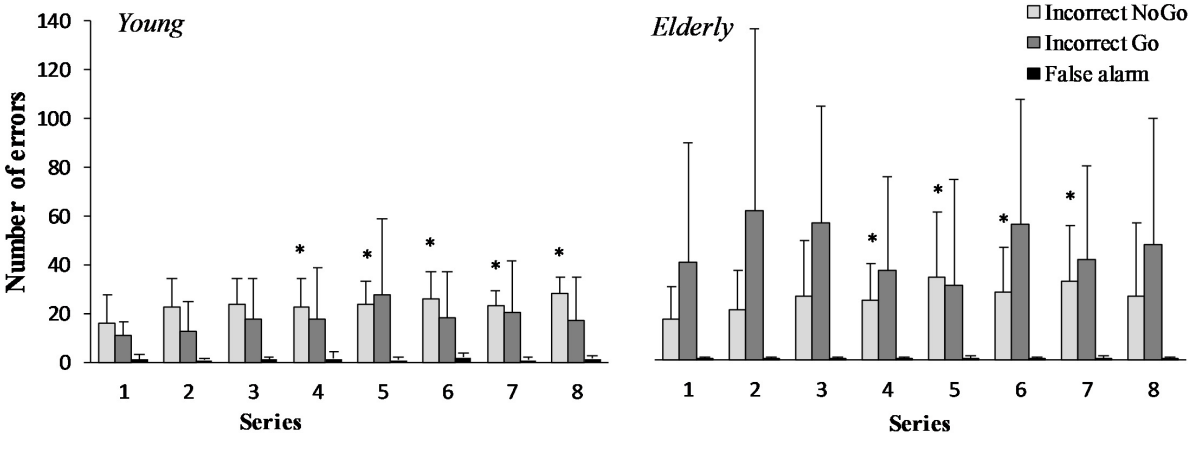

FIGURE 2 | "Incorrect NoGo," "Incorrect Go," "False alarm" errors during 2 h "Go/NoGo" task in young and elderly men. *$p < 0.05$ compared with the first series.

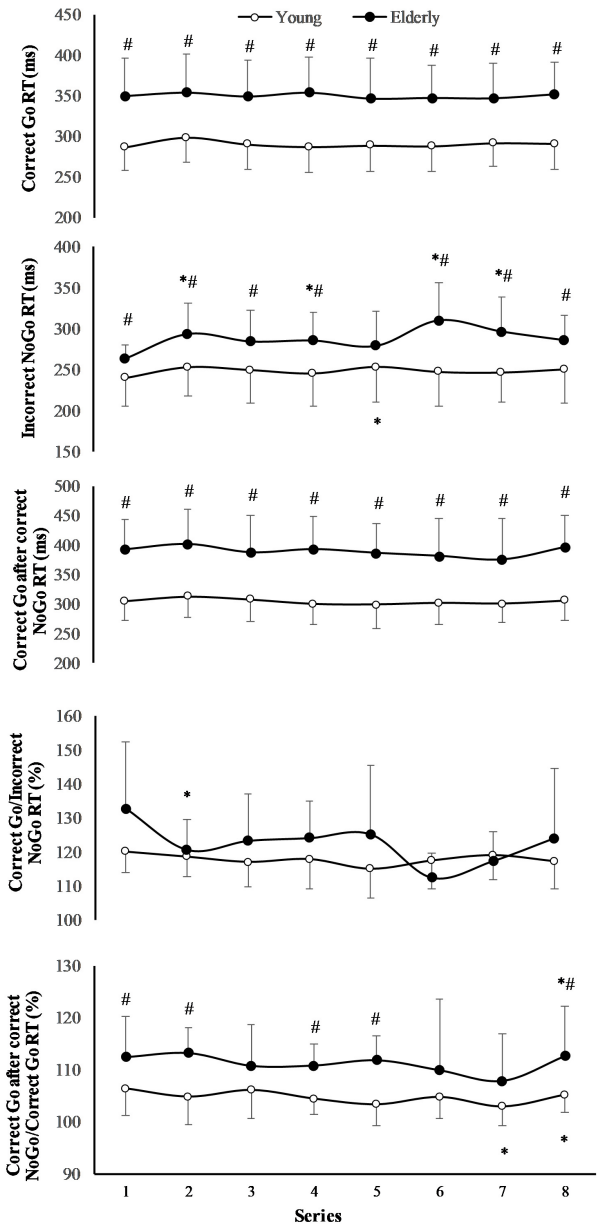

FIGURE 3 | Reaction time (RT) during 2 h "Go/NoGo" task. *$p < 0.05$ compared with the first series; #$p < 0.05$ compared with the young men.

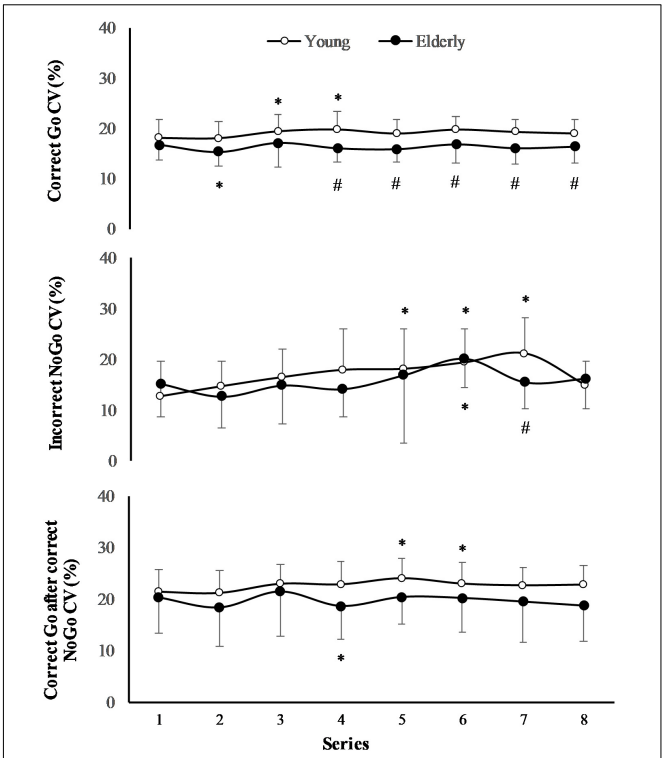

FIGURE 4 | Coefficient of variation (CV) of RT during 2 h "Go/NoGo" task. *$p < 0.05$ compared with the first series; #$p < 0.05$ compared with the young men.

TABLE 1 | Mood state before and after a 2 h "Go/NoGo" task.

	Young		Elderly	
	Before	After	Before	After
Parameter	Go/NoGo task		Go/NoGo task	
Anger	4.4 (0.9)	4.6 (0.7)	4.3 (0.5)	5.0 (2.5)
Confusion	4.5 (1.2)	6.7 (3.0)*	4.9 (1.5)	5.9 (2.5)
Depression	4.5 (1.5)	5.4 (2.4)	4.4 (0.5)	5.5 (3.0)
Fatigue	6.3 (2.9)	12.1 (4.7)*	5.4 (1.8)	7.6 (3.1)*
Tension	4.8 (1.1)	5.8 (1.8)*	6.3 (2.0)	5.6 (2.4)
Vigor	15.1 (3.2)	10.5 (3.5)*	14.8 (2.4)	14.5 (3.3)

*Data are presented as mean (standard deviation). *$p \leq 0.05$ compared with the values before the task.*

Effect of the 2 h "Go/NoGo" Task on Cognitive Performance

In the young men, the RT and error rate of Memory Search Task (MST) significantly increased ($p < 0.05$), while the accuracy of ST increased and the accuracy of CSDT decreased in the elderly men after the 2 h "Go/NoGo" task ($p < 0.05$) (**Table 2**). The RT variable of all cognitive tasks was significantly greater in the elderly men ($p < 0.05$; $OP > 0.90$). The accuracy of ST, MT, and CSDT was greater, and the distance from the target of the Pursuit Tracking Task (PTT) was less in young men than in elderly man ($p < 0.05$) before and after the 2 h "Go/NoGo" task.

Effect of the 2 h "Go/NoGo" Task on Prefrontal Cortex Activity

The switching/rest ratio of oxygenated hemoglobin before "Go/NoGo" task in the elderly men was significantly greater than in the young men ($p = 0.01$) (**Figure 5**). The 2 h "Go/NoGo" task significantly decreased the switching/rest ratio of oxygenated hemoglobin for the young and the elderly men ($p < 0.05$). This ratio significantly more decreased for elderly compared to young men ($p < 0.01$). The ratio of oxygenated hemoglobin during ST after 2 h "Go/NoGo" to ST before the "Go/NoGo" task was 144.4 ± 4.2% in the young men and 33.5 ± 35.7% in the elderly

TABLE 2 | Cognitive performance before and after a 2 h "Go/NoGo" task.

Parameter	Young		Elderly	
	Before	After	Before	After
	Go/NoGo task		Go/NoGo task	
Simple Reaction Time Task				
Reaction time (ms)	296.1 (34.1)	301.4 (42.9)	376.1 (82.5)#	373.4 (82.5)#
Two-Choice Reaction Time Task				
Reaction time (ms)	416.8 (51.6)	423.8 (51.8)	601.0 (11.3)#	637.2 (104.1)#
Accuracy (%)	95.9 (3.0)	95.9 (3.8)	97.8 (2.5)	98.8 (1.9)
Switching Task				
Reaction time (ms)	2059.2 (481.8)	1982.5 (286.1)	4204.8 (592.3)#	4061.8 (581.0)#
Accuracy (%)	93.4 (4.4)	93.8 (2.8)	78.7 (17.9)#	86.3 (6.2)#
Matching Grid Task				
Reaction time (ms)	1254.2 (287.6)	1233.3 (277.9)	2488.2 (409.8)	2643.9 (453.3)
Accuracy (%)	95.5 (5.0)	96.5 (5.3)	95.0 (6.5)	97.5 (2.7)
Mathematical Processing Task				
Reaction time (ms)	1789.1 (382.4)	1823.3 (435.5)	2626.9 (707.0)#	2824.8 (863.8)#
Accuracy (%)	92.5 (8.9)	94.0 (5.2)	95.0 (5.3)	96.3 (4.4)
Manikin Task				
Reaction time (ms)	1375.6 (454.9)	1340.8 (375.3)	3205.3 (754.4)#	3414.4 (650.5)#
Accuracy (%)	93.4 (6.2)	95.0 (1.6)	79.7 (13.5)#	84.4 (9.7)#
Memory Search Task				
Reaction time (ms)	749.3 (110.4)	875.6 (212.6)*	1453.3 (434.4)#	1463.4 (371.4)#
Accuracy (%)	97.0 (2.3)	91.0 (7.0)*	94.7 (6.7)	94.7 (3.6)
Code Substitution Immediate Task				
Reaction time (ms)	993.6 (213.2)	1031.7 (215.3)	2331.5 (775.8)#	2089.3 (386.4)#
Accuracy (%)	98.0 (25.1)	98.1 (1.9)	92.9 (13.0)	97.7 (2.9)
Code Substitution Delayed Task				
Reaction time (ms)	953.5 (190.4)	969.2 (165.7)	2154.1 (702.7)#	2432.6 (517.1)#
Accuracy (%)	94.7 (5.6)	95.3 (5.6)	80.9 (14.2)	75.0 (14.0)*
Pursuit Tracking Task				
Distance from target (mm)	6.8 (3.2)	6.4 (1.9)	11.9 (3.9)#	11.9 (3.7)#
Time on target (%)	99.7 (0.4)	99.7 (0.6)	96.4 (4.4)	96.6 (4.8)

*Data are presented as mean (standard deviation). *$p \leq 0.05$ compared with values before the task; #$p \leq 0.05$ compared with young men.*

men, respectively ($p = 0.001$; between the young men and the elderly men).

Effect of the 2 h "Go/NoGo" Task on Motor Cortex Excitability

Latency increased significantly after the "Go/NoGo" task in both groups of young and elderly men ($p < 0.05$); latency was significantly greater in the elderly men before the "Go/NoGo" task (**Table 3**). The "Go/NoGo" task did not affect the MEP amplitude ($p > 0.05$) there was no difference between the MEP amplitudes of the young and the elderly men ($p > 0.05$).

Effect of the 2 h "Go/NoGo" Task on Muscle Strength

Muscle strength in the young men before and after "Go/NoGo" task was respectively 51.4 (11.4) kg and 47.6 (10.6) kg ($p = 0.004$); elderly men, 44.3 (10.6) kg and 44.9 (10.3) kg ($p = 0.031$). There were significant differences before and after the 2 h "Go/NoGo" task between young men and elderly men ($p < 0.05$).

Emotional Intelligence

There were no significant differences in emotional intelligence items of four subscales (perception of emotion, managing own emotions, managing others' emotions and utilization of emotions) between the young men and the elderly men ($p > 0.05$) (**Table 4**).

Thinking Content During the 2 h "Go/NoGo" Task

According to the DSSQ score task-related score was 22.5 ± 5.3 for young men, and 21.6 ± 4.0 for elderly men ($p > 0.05$); task-irrelevant score was 15.0 ± 5.6 for young men, and 12.0 ± 4.1 for elderly men ($p > 0.05$). Thus, there were no significant differences in thinking content during the "Go/NoGo" task between the young men and the elderly men ($p > 0.05$).

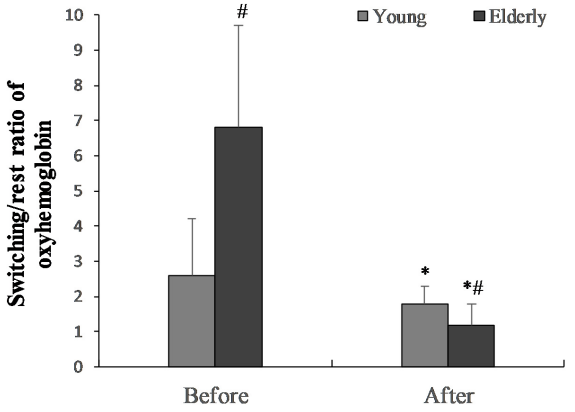

FIGURE 5 | The switching/rest ratio of oxygenated hemoglobin before the "Go/NoGo" task in the young and elderly men. *$p < 0.05$ compared with before the "Go/NoGo" task; #$p < 0.05$ compared with the young men.

Effect of the 2 h "Go/NoGo" Task on Perceived Workload

Physical demand increased significantly ($p < 0.05$) in the young and the elderly men during the "Go/NoGo" task; mental demand increased significantly only in the young men during "Go/NoGo" task. However, temporal demand and load effort were significantly ($p < 0.05$) greater in the young men than in the elderly men during the "Go/NoGo" task (**Table 5**).

Correlation Relationships Between Variables

The average number of "Incorrect Go" errors significantly correlated with an increase in prefrontal cortical activation during ST before the "Go/NoGo" task, inversely for the young men ($r = -0.71$; $p < 0.05$), and directly for the elderly men ($r = 0.95$; $p < 0.05$); the average number of "Incorrect NoGo" errors was respectively correlated ($r = 0.77$ and $r = 0.99$; $p < 0.05$). The increase in prefrontal cortex activity after exercise during the ST, significantly correlated ($p < 0.05$) with the correct cognition task performance after the "Go/NoGo" task, directly for the young men ($r = 0.89$, $p < 0.05$) and inversely ($r = -0.89$; $p < 0.05$) for the elderly men. In addition, the internal motivation significantly correlated with the overall increase in the number of errors ($r = -0.77$; $p < 0.05$) for the young men. The percentage increase in the total number of errors for the young men correlated strongly with tension ($r = 0.89$; $p < 0.05$) and vigor ($r = -0.77$; $p < 0.05$) before exercise.

TABLE 3 | The effect of the 2 h "Go/NoGo" task on the motor cortex excitability.

	Amplitude (mV)		Latency (ms)	
	Before	After	Before	After
	Go/NoGo task		Go/NoGo task	
Young	3.1 (1.3)	3.8 (1.4)	23.5 (1.2)	24.4 (1.0)*
Elderly	2.2 (1.3)	2.6 (1.3)	24.2 (1.4)	24.8 (1.5)*

*Data are presented as mean (standard deviation). *$p \leq 0.05$ compared with values before the task.*

TABLE 4 | The Schutte Self-Report Emotional Intelligence Test.

	Young	Elderly
Perception of emotion	35.1 (5.9)	34.6 (2.5)
Managing own emotions	31.8 (4.5)	32.8 (3.1)
Managing others' emotions	39.1 (4.1)	43.0 (3.4)
Utilization of emotion	14.0 (1.5)	15.8 (2.0)
All	120 (11.6)	126.4 (8.2)

DISCUSSION

To our knowledge, this is the first study that has addressed the following research question: what are the differences between the young men and the elderly men in brain response (prefrontal cortex activity) and neuromuscular function (grip strength, motor control, and TMS), cognitive function (attention, executive function, memory and fast learning, and response inhibition control), and psychological variables (mood, motivation, sense of cognitive load, and thinking during exercise) during and after a PCL (2 h "Go/NoGo" task).

The first finding of our study is that during the 2 h "Go/NoGo" task, both the young men and the elderly men had a significantly similar increase in the number of "Incorrect NoGo" (inhibition) errors (the effect of age was not significant), but the number of "Incorrect Go" errors was unchanged. The RT of "Correct Go" did not significantly depend on the PCL task, but it significantly depended on age (for the elderly it was longer). Only in young RT of "Incorrect NoGo" as well as CV of RT "Incorrect NoGo" significantly increased during PCL ($p < 0.05$). Thus, contrary to our expectations, men young men showed more signs of cognitive fatigue than the elderly.

There is no doubt that during the PCL, executive function and its main elements such as concentration of attention, working memory, inhibition control, and executive flexibility were especially overloaded. This is consistent with findings

TABLE 5 | Perceived workload state during the first and the last 30 min of the 2 h "Go/NoGo" task.

	Young		Elderly	
	0–30	90–120	0–30	90–120
Mental demand	51.4 (22.3)	65.9 (29.8)*	33.0 (24.3)	45.6 (20.4)
Physical demand	26.8 (20.4)	53.2 (28.8)*	28.8 (14.8)	45.0 (16.7)*
Temporal demand	75.9 (17.0)	77.3 (16.8)	65.0 (12.5)	56.9 (8.8)#
Load effort	68.2 (20.1)	70.0 (23.6)	56.3 (14.1)	56.9 (11.6)#
Frustration	45.0 (24.5)	47.0 (26.6)	22.6 (15.8)	33.1 (17.5)
Performance	55.0 (14.0)	55.5 (16.8)	48.8 (2.3)	52.5 (8.5)

*Data are presented as mean (standard deviation). *$p \leq 0.05$ compared with first 30 min; #$p \leq 0.05$ compared with young men.*

by Verbruggen and Logan (2009) that response inhibition tasks require concentration of attention, working memory, and flexibility of executive function. The main target of fatigue during our PCL was the prefrontal cortex because it is the most responsible for the control of the aforementioned mechanisms (Diamond, 2013). The growing literature suggests that prefrontal contributions to executive functions cannot be analyzed in isolation from the effects of more distributed gray and white matter in healthy older adult subjects (Bettcher et al., 2016). However, it is not clear what the precise mechanisms of the origin of fatigue are because their potential contribution is considerable. For example, the neural mechanisms of mental fatigue related to cognitive task performance are more complex than previously thought and mental fatigue is not only caused by impaired activity in task-related brain regions. There is substantial evidence to support the existence of mental facilitation and inhibition systems (Ishii et al., 2014). A number of hypotheses on the mechanisms of mental fatigue origin (including self-control) have been proposed (Van der Linden et al., 2003; Ishii et al., 2014). Some of these hypotheses argue that during prolonged mental exercise, self-control resources are exhausted (Baumeister, 2014) activities of executive function and decision-making are impaired, and inhibiting processes appear in the brain (Ishii et al., 2014). Other researchers maintain that mental fatigue and self-control are highly dependent on the specifics of motivation and the reward of the task performed, especially when compared with the size of the input (Boksem et al., 2006). High internal (and perhaps external) motivation and a big reward can compensate for the manifestation of mental fatigue (Boksem et al., 2006; Braver et al., 2014) and can switch the voluntary control of task performance to be automatic (Lorist, 2008).

After a 2 h "Go/NoGo" task, handgrip strength decreased for the young men, and latency of MEPs significantly increased for both the young men and the elderly men. This clearly shows that mental fatigue caused fatigue in the neuromuscular system. However, the negative effect of mental fatigue on perception of effort reflects no greater development of central or peripheral fatigue (Pageaux et al., 2015).

Task performance strategy depends not only on the task prediction, but also on the current situation (Verbruggen and Logan, 2009). For example, if there has been an error in the case of task inhibition, then this is taken into consideration during the following trial (Verbruggen and Logan, 2009). This is consistent with our finding that after inhibition of the error, RT for the other correctly performed task was significantly longer than that under the normal conditions. Moreover, this phenomenon was exhibited more prominently by the elderly men (**Figure 3**). The second main finding of our study was that despite the motivation level at the beginning of exercise not differing between the young men and the elderly men, the young men felt more fatigue, lack of energy, and mental and temporal demand during exercise, and their intrinsic motivation was more decreased during exercise. Taken together, psychological strain in the PCL was higher for the young men than for the elderly men. This is consistent with a study by Wascher et al. (2016) that found young people experienced more fatigue from monotonous cognitive exercise than the elderly. Thus, impairment of the intrinsic motivation of young people could be a key factor in why young people felt a greater psychological fatigue because it has been clearly established that when the subjects are involved in mental activity and engage with it, they experience less mental fatigue (Shigihara et al., 2013).

When people become tired, it is more likely that they continue to perform a task using automatic control (Verbruggen and Logan, 2009). As our findings show, internal motivation decreases. We found that the smaller the internal motivation for young men, the more the number of errors increased for them in the performance of PCL. Although the young men in our study reduced their intrinsic motivation during exercise, they did not change their extrinsic motivation; we believe that they moved from a strategy of "I want" to "I have to." This is consistent with the finding of other researchers that the "I have to" task performance strategy is more tiring than the "I want" strategy (Inzlicht et al., 2014). Moreover, this coincides with a popular principle of brain activity, namely the minimum energy (mental effort) required achieving the goal (Shenhav et al., 2013). Gergelyfi et al. (2015) concluded that mental fatigue in healthy subjects is not caused by changes in the task engagement (motivation), but is likely to be a result of a decrease in the effectiveness, or availability, of cognitive resources.

Our third main finding was that although this was unexpected, the elderly men performed some cognitive tests better (by about 10%) after PCL; they made fewer mistakes in the ST and CSIT. In sum, because many cognitive functions did not deteriorate in either the young men or the elderly men, we cannot claim that there is a significant difference between age groups. In our opinion, after "the task and motivation switch," most cognitive tasks "recover." This is consistent with data from other studies that manifestation of cognitive fatigue is specific, i.e., it depends not only on fatigue, but also on the specificity of tests establishing mental working capacity (Cook et al., 2014). Botvinick and Braver (2014) suggest that fatigue consists of a control mechanism that discourages individuals away from lengthy tasks and toward newer, possibly more satisfying activities. They found that after fatigue, increasing extrinsic motivation recovers to the level of performance before fatigue, and argued that this provides evidence in favor of a fatigue-induced disengagement from the task.

The fourth finding of our study was that prefrontal cortex activity increased during the ST more in the elderly men than in the young men before the "Go/NoGo" task. However, after the "Go/NoGo" task, the prefrontal cortex activity of the elderly men decreased, while in the young men it increased during the ST. The average number of "Incorrect NoGo" errors during 2 h "Go/NoGo" task significantly and directly correlated with an increase in prefrontal cortical activation during ST before the "Go/NoGo" task both in young and elderly man. However, the more the prefrontal activity increased during the ST after exercise, the greater the number of inhibition errors made by the elderly men, and the fewer the number made by the young men.

Seidler et al. (2010) showed that older adults progressively relied on cognitive brain processes for motor control ("cognitive

demand") because of structural and functional deteriorations in the motor cortical regions, cerebellum, and basal ganglia pathways. At the same time, attentional capability and other relevant cognitive resources ("cognitive supply") are reduced because of differential degradation of the prefrontal cortex and anterior corpus callosum. This is consistent with our finding that, before the exercise, the elderly men had a more activated prefrontal cortex during a cognitive task, but after the 2 h "Go/NoGo" task, the activity decreased.

Mattay et al. (2002) found that, compared with young adults, older adults recruited additional cortical and subcortical areas for the performance of a simple RT task. Heuninckx et al. (2008) found that during isolated rhythmical hand/foot movements performed in the same direction or in opposite directions, executive, cognitive, and association brain regions were more highly activated by older adults to perform tasks that young adults performed with more automated processes. In this framework, the age-related compensatory recruitment of the prefrontal cortex, in terms of executive system, has been established (Sugiura, 2016).

Cabeza et al. (2002) established that low-performing older adults employed a similar prefrontal network as young adults, but used it uneconomically, whereas high-performing older adults responded to neural decline related to age through more recruited prefrontal cortex. However, our findings apparently contradict those of Cabeza et al. (2002), who concluded that because the prefrontal cortex is more activated before exercise than during the ST, more inhibition errors were made by the elderly men.

Greater activation of the left dorsolateral prefrontal and superior parietal cortex during working memory was more evident in middle-aged than in young adults regardless of working memory load or fatigue condition (Klaassen et al., 2014). This contradicts our findings because the activation of the prefrontal cortex of the elderly men in our study decreased after the "Go/NoGo" task.

CONCLUSION

We found that young men showed greater signs of cognitive fatigue than elderly men during a PCL, young men felt more fatigue after exercise than elderly men, and elderly men performed some cognitive tests better after PCL than young men. Because of the greater mental load and (possibly) greater recruitment (mobilization) of prefrontal cortex during a 2 h "Go/NoGo" task (PCL), there was greater mental and neuromuscular performance fatigue in young compared with elderly men. Prolonged task performance decreased the switching/rest ratio of oxygenated hemoglobin for the young and the elderly men; however, greater decrease was observed for elderly than young men. Finally, baseline prefrontal cortex activity during the switching task predicted mental performance changes during demanding mental load as the more highly the prefrontal cortex was activated, the better was the inhibitory control observed in young men, and the poorer was the inhibitory control observed in elderly men.

ETHICS STATEMENT

All procedures performed in studies involving human participants were in accordance with the ethical standards of the local Ethics Committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

INFORMED CONSENT

Written informed consent was obtained from all participants included in the study.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

AS: conception and design of the study, acquisition, analysis, and interpretation of the data, and drafting the study. AT, EM, KV, DM, DK, DV, RS, and AE: collection, analysis, and interpretation of the data. AS and SK: drafting the study and revising it critically.

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Exercise of Varying Durations: No Acute Effects on Cognitive Performance in Adolescents

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Participation in structured physical activity is assumed to have a positive effect on cognitive and academic performance. A single bout of moderate to vigorous exercise has been found to have a small acute positive effect on the cognitive performance of children and adolescents. However, the dose-response effects of exercise duration are largely unknown. Therefore, the current study examined the acute effects of moderate-to-vigorous exercise with a duration of either 10, 20, or 30 min on selective attention and working memory performance of young adolescents. One hundred and nineteen adolescents (11–14 years old) participated in a randomized, controlled crossover study. Adolescents were assigned to one of the three exercise durations, each paired with a sedentary control session of the same duration. Cognitive performance was measured before and immediately after the exercise and control condition. The Attention Network Test and n-back task were used to measure selective attention and working memory, respectively. There were no significant exercise effects on selective attention (i.e., alerting, orienting, or executive control) or working memory performance measured immediately after the exercise bouts. Furthermore, there were no differential effects of exercise duration. In sum, acute exercise bouts with a duration of 10, 20, or 30 min did not improve, but neither deteriorate cognitive performance of young adolescents compared to a sedentary control condition.

Keywords: physical activity, cognitive performance, selective attention, working memory, exercise duration, dose-response, children, adolescents

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INTRODUCTION

The maturation of the adolescent brain is guided by an interaction between genetic and environmental factors (Rosenzweig, 2003; Lenrootand Giedd, 2008). Among these factors, physical activity (PA) has been well studied, in particular because its potential beneficial effects on cognitive functioning and academic achievement. Two systematic reviews concluded that *overall*, single bouts of PA have small positive acute effects on cognitive performance of children and adolescents (Donnelly et al., 2016; Ludyga et al., 2016). In addition, a recent meta-analysis concluded that PA

can have acute positive effects on attention and inhibition in pre-adolescent children (de Greeff et al., 2018). Besides evidence on the acute effects, meta-analyses of longitudinal studies have shown that engaging in structured PA sessions can have a neutral or positive effect on cognitive functioning in children, and certainly does not harm children's performance (e.g., Li et al., 2017; Watson et al., 2017; de Greeff et al., 2018).

Despite the positive effects of PA on mental (Biddle and Asare, 2011) and physical health (Janssen and Leblanc, 2010), and its assumed effect on cognitive functioning, there is ample evidence that many children and adolescents do not meet PA guidelines (WHO, 2010; Health Council of the Netherlands, 2017). Schools are seen as the most appropriate setting to enforce structural opportunities to increase PA levels in children and adolescents as they spend a substantial amount of their time at school (WHO, 2010, 2014; Webster et al., 2015). However, time constraints are a frequently mentioned barrier that hinders implementation of PA in schools (e.g., Howie et al., 2014b; McMullen et al., 2014; Naylor et al., 2015; Stylianou et al., 2015; van den Berg et al., 2017). Therefore, teachers have indicated that it would only be feasible to implement short PA bouts in the school curriculum, with a maximum of 5 (Howie et al., 2014b) or 10 min per session (van den Berg et al., 2017).

Although studies have consistently shown that the intensity of acute PA needs to be of at least moderate to vigorous intensity to gain most cognitive benefits (McMorris and Hale, 2012; Peruyero et al., 2017), the optimal duration of acute PA is still unclear and needs further investigation (Janssen et al., 2014b; Verburgh et al., 2014; Donnelly et al., 2016). Previous studies have shown that acute exercise bouts with a duration of 30 or more minutes can improve children's and adolescent's performance in inhibition and shifting (Ellemberg and St-Louis-Deschênes, 2010; Chen et al., 2014), working memory (Pontifex et al., 2009; Chen et al., 2014), selective attention (Gallotta et al., 2012), free-recall memory (Pesce et al., 2009), planning (Pirrie and Lodewyk, 2012), and executive attention (Kubesh et al., 2009). However, studies by Pirrie and Lodwyk (2012, information processing and selective attention) and (Kubesh et al. (2009), working memory and cognitive flexibility) reported no effects. The effects of a medium exercise duration (i.e., 20 min) on cognition are also inconclusive, with some studies showing improved performance in inhibitory control (Hillman et al., 2009; Drollette et al., 2012, 2014), comprehension (Hillman et al., 2009), and selective attention (depending on time of the day; Altenburg et al., 2016), and others showing no effects on inhibitory control (Stroth et al., 2009), working memory (Drollette et al., 2012), and broad measures of executive functioning (Howie et al., 2015). Also, the effects of exercise of a shorter duration of 10–15 min are inconclusive: beneficial effects have been reported on selective attention (Budde et al., 2008; Niemann et al., 2013; Janssen et al., 2014a), working memory (depending on exercise intensity and performance level) (Budde et al., 2010), as well as on broad measures of executive functioning (Cooper et al., 2012, 2016; Benzing et al., 2016), while no effects on selective attention and information processing (van den Berg et al., 2016), visuo-spatial memory and general psychomotor speed (Cooper et al., 2016), sustained attention (Wilson et al., 2016), and executive functioning (Howie et al., 2015) have been reported. Studies examining the effects of 5-min exercise sessions found no effects (Kubesh et al., 2009; Howie et al., 2014a, 2015). In sum, the evidence on the acute effects of relatively short, medium, and long exercise bouts on cognitive performance is inconclusive and the differences in cognitive outcome measures across studies make it particularly difficult to compare the effects of exercise bouts with different durations with each other. Therefore, dose-response studies are needed to be able to elucidate the acute effects of exercise duration on cognitive performance.

To date, only few studies investigated the acute dose-response effects of exercise duration on cognitive performance in children and adolescents. Two studies of Howie and colleagues (Howie et al., 2014a, 2015) investigated whether the cognitive performance of children (aged 9–12 years) differed after 5, 10, and 20 min of moderate to vigorous classroom-based exercise compared to 10 min of sedentary activities (i.e., listening to a lesson about exercise science). The authors reported higher math fluency scores after 10 and 20 min of exercise compared to the sedentary condition (Howie et al., 2015), and improved on-task behavior after 10 min, but not after 5 and 20 min of exercise (Howie et al., 2014a). While these studies investigated the effect of different exercise durations, the authors conducted separate analyses and did not compare the effects of 5, 10, and 20 min exercise with each other. Recently, two studies in young male adults (20–23 years old) examined the dose-response relation between exercise duration and cognitive performance on a Color-Word Stroop task (Chang et al., 2015; Tsukamoto et al., 2017). Chang and colleagues found that 20 min moderate intensity exercise on a cycle ergometer resulted in larger improvements in cognitive performance than 10 or 45 min of exercise (Chang et al., 2015). In contrast, Tsukamoto and colleagues reported no difference in the positive effects of 10, 20, or 40 min moderate intensity cycle ergometer exercise on cognitive performance (Tsukamoto et al., 2017).

In the current study, we examined the dose-response effects of exercise duration (10, 20, or 30 min) on selective attention and working memory of young adolescents (11–14 years). We conducted a randomized controlled cross-over study in the school setting and assessed effects on attention and working memory as these cognitive functions are associated with academic achievement (Stevens and Bavelier, 2012; van der Ven et al., 2013). Selective attention is defined as "the differential processing of simultaneous sources of information" (Johnston and Dark, 1986, p. 44). In other words, it determines which stimuli are relevant and which are irrelevant and should be suppressed (Ellemberg and St-Louis-Deschênes, 2010). Working memory is a cognitive function with limited capacity that allows individuals to temporarily store and actively manipulate information over a brief period of time (Baddeley, 2003). Based on the results of earlier studies, we hypothesize that moderate to vigorous exercise bouts of different durations will have a neutral or positive acute effect on selective attention and working memory performance of young adolescents.

METHODS

Sample Size Calculation

An independent statistician performed a sample size calculation based on the effect size (partial $\eta^2 = 0.12$) of an earlier study using a similar research design and cognitive tasks (Drollette et al., 2012). A sample of ∼62 adolescents was needed to detect within-subjects effects (i.e., exercise effects on cognition), and ∼105 participants to detect within-between interaction effects (i.e., differential effects of exercise duration) with 80% power, 2-sided testing at $\alpha = 0.05$.

Participants

We invited a convenience sample of three elementary schools and one secondary school to participate with all apparently healthy adolescents attending the last grade of elementary school (11–12 years) or the first grade of secondary school (12–13 years). First, we provided detailed information on the study to the school staff. After obtaining their consent, we provided adolescents and their parents with written information about the procedure and the scope of the study. Written informed consent was obtained from a parent/caregiver, and adolescents who were 12 years or older. Adolescents with a confirmed medical condition that could affect memory or concentration (e.g., ADHD, epilepsy) were identified by the school staff and were not included in the statistical analyses. Adolescents received a small present for their participation after the study. The Medical Ethical Committee of the VU University Medical Center in Amsterdam, the Netherlands, concluded that the study does not fall within the scope of the Medical Research Involving Human Subjects Act and approved the study protocol [2014.363].

Design and Randomization

We conducted a randomized controlled trial with a crossover design, including two within- and one between-subjects variables. The first within-subjects variable was "intervention session": all adolescents performed one control and one exercise session of the same duration (i.e., either 10, 20, or 30 min), thereby acting as their own control. The second within-subjects variable was "test": we conducted cognitive tests before (*pretest*) and after (*posttest*) the control and exercise session, to control for intra-individual differences in test performance. The between-subject variable was the "duration" of the exercise/control session: 10, 20, or 30 min. The order of the control and exercise session was counterbalanced, i.e., half of the adolescents started with the control session, and the other half with the exercise session.

We applied a block-random selection procedure to determine duration (i.e., 10, 20, or 30 min) and sequence (i.e., order of exercise and control session) using two online software programs (http://www.randomizer.org/form.htm and http://www.graphpad.com/quickcalcs/randomize2/). We stratified by sex in the randomization procedure.

Procedure

We visited the schools on four separate occasions within a period of 3 weeks (see **Figure 1**). The first and the second visit were generally scheduled within the same week. The first visit consisted of a familiarization session in which adolescents received detailed information on the experiment and practiced with the two cognitive tasks (see section Cognitive Measures). During the second visit, we assessed their maximum heart rate and fitness by means of a Shuttle Run test. The third and fourth visit consisted of the experimental days, which were scheduled 1 week apart at the same time of the day (between 08:00 and 11:45 a.m.). We asked the adolescents to keep their bedtime, breakfast, and transport mode to school similar before each experimental day. We invited groups of four to six students to the testing location, which was a private area within the school. Each day had the same standardized routine: (1) all adolescents practiced with the two cognitive tasks and self-reported their sleep, breakfast, mode, and duration of transportation to school; (2) in the exercise session we measured height and weight of the adolescents and provided them with a Polar RS800cx heart rate monitor. After adolescents laid on their back comfortably for 5 min, with legs and arms positioned along the body, the resting heart rate was measured to calculate the heart rate zone corresponding to moderate to vigorous intensity (see section Exercise Bout); (3) adolescents performed two cognitive tasks on a laptop (pretest); (4) during the exercise session adolescents cycled for 10, 20, or 30 min, whereas during the control session they worked on educational materials (e.g., puzzles, questionnaires, worksheets) seated for either 10, 20, or 30 min; (5) adolescents performed the two cognitive tasks again (posttest) on the same laptop.

Exercise Bout

The exercise bout followed a bicycle ergometer protocol. The adolescents biked at moderate to vigorous intensity for a duration of 10, 20, or 30 min. The first minute and a half served as warming-up [workload = 0 kilopond (Kp)]. After this period, the workload increased until adolescents biked within the predetermined boundaries of their moderate to vigorous intensity level. The maximum heart rate and resting heart rate were used to calculate the lower (40%) and upper (60%) boundary of the heart rate reserve, corresponding to a moderate to vigorous intensity level of exercise. The boundaries were calculated as follows: 40% = [(maximal heart rate – resting heart rate) * (40/100) + resting heart rate]; 60% = [(maximal heart rate – resting heart rate) * (60/100) + resting heart rate] (ACSM, 2010). Adolescents who did not reach the specified heart rate zone were excluded from the data analyses (i.e., if the mean exercise intensity was below 40% or above 60% of the heart rate reserve). To facilitate biking in a steady state manner, the number of flywheel revolutions per minute was matched to a metronome with 120–160 beats per minute. The last minute served as cooling down, in which the flywheel revolutions per minute were progressively reduced (workload = 0 Kp).

MEASURES AND MEASUREMENT INSTRUMENTS

Anthropometrics

Height (cm) and weight (kg) were measured in sport clothing using a Seca weighing scale (Seca Instruments, Frankfurt,

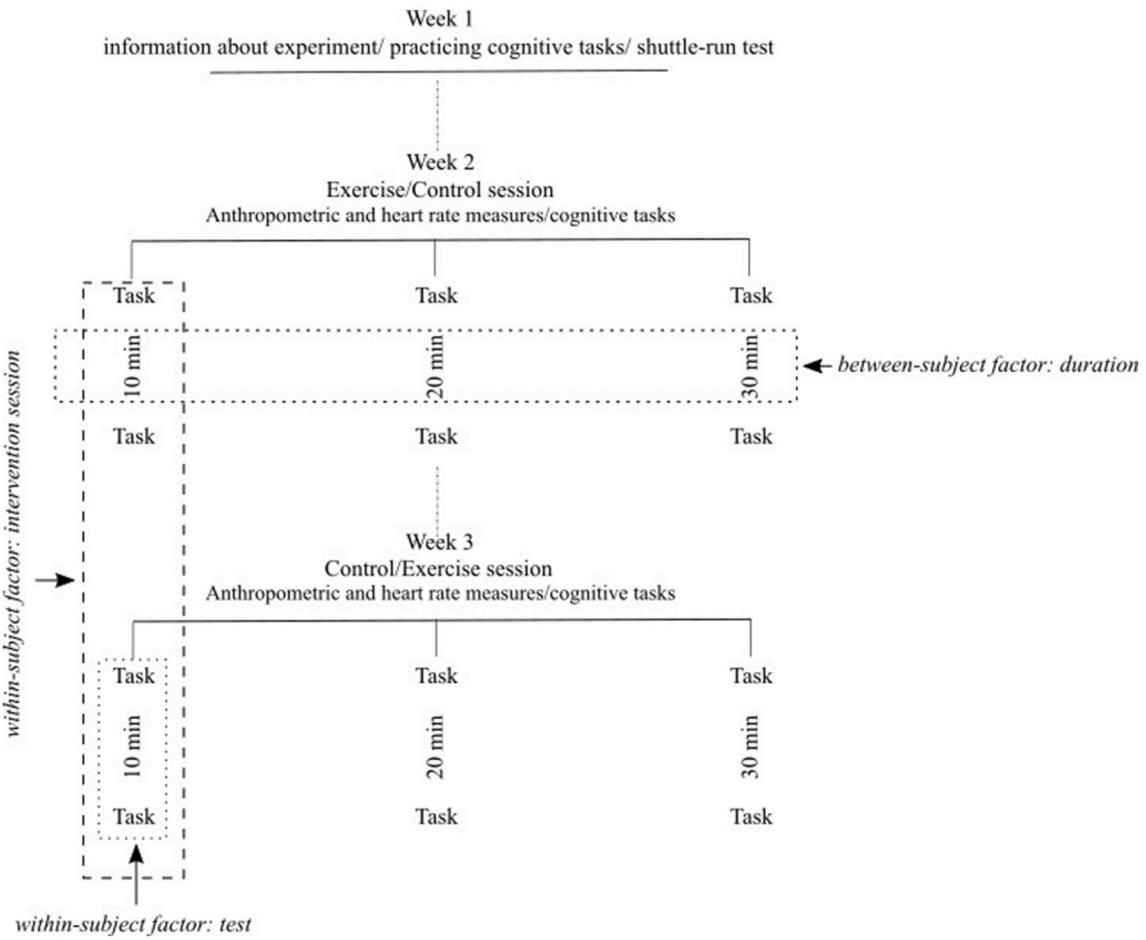

FIGURE 1 | Schematic representation of the experimental design.

Germany) and a Leicester Height Measure Mk II (Harlow Healthcare, UK). Body Mass Index (BMI) was calculated by dividing weight (kg) by height squared (m).

Shuttle Run Test

We administered a Shuttle Run test to assess the maximum heart rate and cardiovascular fitness (VO_2 max) of the adolescents. All participants wore a Polar H7 heart rate monitor that was connected to the Polar Team App (Polar Electro Oy, Finland), in which the heart rate data was stored. The test was performed during a regular physical education lesson, under the supervision of the physical education teacher. All adolescents were familiar with this test and were encouraged by their teacher and the research team to exert maximum performance.

Due to the dimensions of the sports halls, students in the elementary schools performed an 18 m instead of 20 m Shuttle Run, while secondary school students performed the standard 20 m test. The test had an initial running speed of 8.0 km/h that progressively increased with 0.5 km/h in 1 min stages in the 20 m test. This corresponded with an initial running speed of 7.2 km/h increasing with 0.45 km/h in 1 min stages in the 18 m test. We recorded the highest completed stage with an accuracy of half a stage and calculated VO_2max (ml/kg/min) (Léger et al., 1988).

Cognitive Measures

We used two computerized cognitive tasks: the Attention Network Test (ANT) and the n-back task. Both tasks have been shown to have optimal criterion validity, good statistical dependencies and adequate factorial structure, suggesting that these tasks are valid measures of cognitive performance in children (Forns et al., 2014). The tasks were programmed using E-Prime 1.2 software (Psychology Software Tools, Pittsburgh, PA), which was also used for stimulus generation and response registration. To minimize interference during the tasks, a maximum of two adolescents were seated at one working desk, facing each other supervised by a member of the research team. We asked them to work quietly and individually and to focus on their task the entire time. The order in which the cognitive tasks were performed was randomized and counterbalanced between participants (first ANT and then n-back, or vice versa).

Practice Trials

During the familiarization session, the adolescents practiced 57 trials (nine trials with feedback) of the ANT and 150 trials (60 trials with feedback) of the n-back task. Given the complexity of the current n-back task, we incorporated a loop, which allowed them to repeat any part of the task (instructions or task blocks) until the task was fully understood. In addition, they made a few practice trials of both tasks at the start of each experimental day.

Attention Network Test

We used the short-version (Fan et al., 2007) of the ANT (Fan et al., 2002) to assess three attentional networks: alerting (i.e., achieving and maintaining an alert state), orienting (i.e., selection of information from sensory input), and conflict/executive control (i.e., resolving conflict among responses). The stimuli of this task were sets of five horizontal black arrows presented on a white background. The middle arrow pointing either to the left or to the right was the target, flanked by two lateral arrows on the left and the right. The flanker arrows pointed either in the same direction of the target arrow (congruent flanker condition: >>>>> or <<<<<) or in the opposite direction of the target arrow (incongruent flanker condition: >><>> or <<><<). We instructed the adolescents to respond as fast and as accurately as possible by pressing the left mouse button if the target arrow was pointing to the left and by pressing the right mouse button if the target arrow was pointing to the right. Adolescents were asked to focus on the fixation cross that was presented in the middle of the screen. A warning cue in the form of an asterisk sign (*) appeared in 66.7% of the trials for a duration of 200 ms in the center ("center cue" condition), or above or below the fixation cross ("spatial cue" condition), while being absent in the remaining trials ("no cue" condition). Details on the task parameters can be found elsewhere (Fan et al., 2007).

The total task lasted ∼12 min and consisted of three blocks of 48 trials, with 1 min breaks between the blocks. We calculated accuracy rates (proportion of correct responses) and mean reaction times of the correct responses for the three attentional networks by the following formulas: Alerting = (Score_no cue – Score_center cue); Orienting = (Score_center cue – Score_spatial cue); Conflict/executive control = (Score_incongruent – Score_congruent) (Fan et al., 2007). Responses with reaction times faster than 200 ms were considered as incorrect (Fan et al., 2007). For the alerting and orienting scores, a larger value for the difference in reaction time and a larger negative value for the difference in accuracy means better performance. For the conflict/executive control score, a smaller value for the difference in reaction time and a smaller negative value for the difference in accuracy means better performance. The task was downloaded from the website of the Sackler Institute for Developmental Psychobiology (www.sacklerinstitute.org/cornell/assays_and_tools/).

n-Back Task

We assessed working memory using a visual n-back task. After the task instructions, a continuous stream of letters (consonants displayed in 40 points Arial) was presented. The letters appeared one by one in the middle of the screen for a duration of 500 ms. The time between the stimuli varied randomly between 1,000 and 2,000 ms. The distance between the adolescent and the screen was ∼65 cm. The visual angle of the stimuli was ∼1.25°.

The task had three load conditions: the 0-back, 1-back, and 2-back load. In the 0-back load, the target was the letter "X." In the 1-back load, the target was any letter identical to the letter presented in the last trial preceding it. In the 2-back load, the target was any letter identical to the letter presented two trials preceding it. We asked the adolescents to respond as fast and as accurately as possible by manually pressing a "green button" for targets and a "red button" for non-targets. For half of the adolescents, the green and the red button corresponded, respectively, to the key 1 and 2 on the left side of the keyboard. For the other half, these buttons were reversed.

The total task contained three blocks of 60 trials each and lasted ∼10 min. Each block contained one load condition and the order of blocks was semi-randomized and counterbalanced between participants. Adolescents could differentiate the load of the block only through the instructions that were displayed on screen between the blocks. In each block, targets occurred randomly in ∼37% of the trials. We calculated accuracy rates (proportion of correct responses) and mean reaction times of the correct responses for each load condition. Responses faster than 200 ms were considered as incorrect. Faster reaction times and higher accuracy rates indicate better working memory performance.

Data Analysis

All analyses were performed in the SPSS version 22.0 (IBM Corp. Released 2013. Armonk, NY: IBM Corp.). We examined differences in demographic measures between the 10-, 20-, and 30-min exercise groups by means of univariate ANOVA (see **Table 1**). For both cognitive tasks, reaction time and accuracy scores were separately analyzed by means of repeated measures (RM) ANOVAs, with intervention session (exercise vs. control) and test (pretest and posttest) as within-subjects factors and duration (10, 20, and 30 min) as between-subjects factor. In addition, the factor load (0-, 1-, and 2-back) was entered as a within-subject factor for the analyses of the n-back task. Baseline reaction times for the n-back task and the ANT conflict/executive control score differed significantly between the exercise and control condition. Therefore, we included the pretest score as covariate in the respective RM ANOVA models. We report interactions between the factors intervention session, test, and duration in the Results section. Estimated effect sizes are reported using eta-squares ($?^2$). Statistical significance was set at $p < 0.05$.

Adolescents with accuracy rates lower than chance level, indicating that they did not appropriately understood or followed the task instructions, were excluded from the respective analysis. For the n-back task, we only excluded adolescents with a lower accuracy rate than chance level in the 0-back and 1-back loads due to the difficulty of the 2-back load condition for which lower accuracy scores can be expected.

Between-Tests Timing

We aimed to assess the differential effect of exercise duration on cognitive performance. The groups did not only differ in terms

TABLE 1 | Baseline characteristics of the total study sample, as well as each of the subgroups according to the duration of each intervention session (means and SD).

	Selective attention (ANT)					Working memory (n-back task)				
	Total sample		10 min	20 min	30 min	Total sample		10 min	20 min	30 min
N (included/excluded)	99	20	34 8	30 8	35 4	92	27	30 12	28 10	34 5
Age (years) (included|excluded)	12.3 (0.6)	12.6 (0.6)	12.2 (0.7)	12.4 (0.7)	12.4 (0.5)	12.3 (0.6)	12.6 (0.7)	12.3 (0.6)	12.3 (0.6)	12.3 (0.5)
Sex (n; male/female) (included|excluded)	47/52	10/10	17/17	13/17	17/18	42/50	16/11	14/16	12/16	16/18
BMI[a] (kg/length^2)	17.9 (2.5)		17.8 (2.6)	17.5 (1.9)	18.2 (2.7)	17.7 (2.5)		18.0 (2.7)	17.4 (1.9)	18.1 (2.8)
VO_2 max score[b] (ml/kg/min)	47.6 (4.9)		47.7 (4.8)	47.5 (5.7)	47.7 (4.4)	47.7 (4.7)		47.4 (4.8)	47.9 (4.9)	47.8 (4.5)
Maximum HR (beats per minute)	207.0 (8.2)		208.8 (6.7)	206.4 (9.0)	205.6 (8.7)	206.9 (8.3)		208.8 (7.0)	206.7 (8.8)	205.5 (8.9)
Resting HR (beats per minute)	73.3 (9.8)		73.9 (9.0)	74.7 (8.1)	71.5 (11.6)	73.5 (9.4)		73.9 (7.9)	75.3 (7.7)	71.6 (10.6)
Average HR exercise (beats per minute)	134.6 (7.6)		132.9 (7.7)*	137.0 (6.4)	134.8 (8.1)	134.5 (7.7)		131.8 (7.9)*	137.2 (6.5)	134.8 (7.8)
40% HRR (beats per minute)	126.7 (7.7)		127.7 (6.9)	127.4 (7.2)	125.1 (8.8)	126.8 (7.6)		127.7 (7.2)	127.9 (6.8)	125.2 (8.5)
60% HRR (beats per minute)	153.4 (7.3)		154.6 (6.3)	153.7 (7.5)	151.9 (8.1)	153.4 (7.3)		154.6 (6.7)	154.1 (7.1)	151.9 (8.0)

*Descriptives are rounded to the first decimal. ANT, [a]BMI values are based on 89 participant [10 min (31), 20 min (24) and 30 min (34)]; [b]VO_2 max values are based on 93 participants [10 min (33), 20 min (29) and 30 min (31)]; n-back: [a]BMI values are based on 83 participant [10 min (27), 20 min (23) and 30 min (33)]; [2]VO_2 max values are based on 85 participants [10 min (29), 20 min (27), and 30 min (29)]. *Significant group difference in average HR during exercise, HR in the 10 min group is lower than HR in the 20 min.*

of exercise duration, but also in terms of the timing between the pre- and posttest (i.e., between-tests timing). In order to exclude any possible influence of the between-tests timing on the effect of exercise on reaction time and accuracy, we additionally examined the interaction between test (pre- and post-test) and duration for each cognitive task.

RESULTS

Participants

A total of 119 students participated. Data from 99 adolescents in the ANT and 92 adolescents in the n-back task were included in the statistical analyses (see **Table 1**). One adolescent achieved accuracy scores below chance level in the ANT and seven adolescents performed below chance level in the n-back task (0-back and 1-back). Data from 17 adolescents were incomplete [i.e., participated in only one test session ($n = 15$)] or data was lost due to technical problems [n =2, one in the ANT and one in the n-back task]. Two adolescents were diagnosed with a medical condition and one adolescent exercised at a mean exercise intensity below 40% HRR.

Descriptive Characteristics

Characteristics of the total sample and the subgroups according to exercise duration are presented in **Table 1**. Adolescents in the 10, 20, and 30 min duration had similar age, sex ratio, BMI, VO_2max, maximal HR, resting HR, 40% HRR, and 60% HRR values. Adolescents in the 10 min exercise group had lower average HR scores within the moderate to vigorous intensity zone than those in the 20 min group but not the 30 min group, whereas average HR in the 20 and 30 min group was similar.

Cognitive Performance

ANT

For all three attentional networks of the ANT, accuracy rates were not significantly different between the exercise and control condition, or between the 10-, 20-, and 30-min exercise groups. Likewise, reaction time was not significantly different between the exercise and control condition for alerting and orienting, nor for conflict/executive control after controlling for pretest score. We found no differences in reaction time performance between the 10-, 20-, or 30-min exercise groups. Pre- and post-test scores and F-statistics of the RM ANOVA models can be found in **Tables 2**, **3**, respectively. There were no interactions between the factors test and duration, indicating that the results were not influenced by the time between the tests.

n-Back

For accuracy, we found no significant differences between the exercise and control condition or between the 10-, 20-, or 30-min exercise groups. Likewise, after controlling for pretest scores, there were no significant differences between the exercise and control condition for reaction time performance, nor between the 10-, 20-, and 30-min exercise groups. Pre- and post-test scores and F-statistics of the RM ANOVA can be found in **Tables 4**, **5**. In line with the ANT data, we observed no significant between-test time differences.

DISCUSSION

This study investigated the acute effects of 10, 20, and 30 min of moderate to vigorous intensity exercise on selective attention and working memory performance in 11–14 years old adolescents. In addition, we explored possible dose-response effects of exercise duration on cognitive performance.

We found no acute effects of exercise on selective attention and working memory performance and no differential effects of exercise duration, measured immediately after the exercise bouts.

Our results are in line with some earlier studies that neither found acute effects on selective attention after long (45 min; Pirrie and Lodewyk, 2012), medium (20 min; Stroth et al., 2009), or short (12 min; van den Berg et al., 2016) bouts of exercise. Other studies, however, did report positive effects on selective attention performance. For example, Budde et al. (2008), Gallotta et al. (2012), Niemann et al. (2013), and Janssen et al. (2014a) reported acute effects of single exercise bouts on selective attention in children and adolescents. A difference between above-mentioned studies and our study was the administration of a paper-and-pencil task (d2 test of attention) vs. a computerized Flanker task. However, the use of a different cognitive task might not fully explain the differences in the results, as our findings are also inconsistent with the results of studies that used comparable computerized Flanker tasks. The studies that assessed the acute effects of exercise with comparable Flanker tasks reported positive effects on children's and adolescent's reaction time (Kubesh et al., 2009; Chen et al., 2014) and accuracy scores (Hillman et al., 2009; Drollette et al., 2012). In contrast to our study, in which cognitive performance was measured immediately after the cessation of exercise, cognitive performance in the studies of Hillman et al. (2009), Kubesh et al. (2009), Drollette et al. (2012), Niemann et al. (2013), and Chen et al. (2014) was measured with a delay of ∼5–38 min after the exercise session ended. All of the before mentioned studies reported positive effects of the exercise bouts on selective attention. Chang et al. (2012) reported in their meta-analysis that acute exercise effects on cognition are largest when cognitive tests are assessed 11–20 min after the exercise bout (Chang et al., 2012). Although we found no acute effects of exercise, it might be that exercise related effects on cognitive performance exist, but only become detectable sometime after cessation of the exercise bout. The timing of the cognitive task administration is therefore an important factor to consider in future "exercise-cognition" research. We recommend future research to gain more insight in the timing of the posttest measurements, for example by comparing children's cognitive performance immediately as well as with a delay after a single exercise bout. In addition, it would be interesting to include multiple follow-up measures or to compare effects of different posttest timings (e.g., after 10, 45, and 60 min) to see how long potential exercise-related effects remain. This type of research has however a considerable participant burden reducing the feasibility in the school setting. Another potential reason for the differences in results might be the differences with regard to the control conditions. For example, Hillman et al. (2009) and Drollette et al. (2012) used passive control conditions, i.e., seated rest in which children performed no

TABLE 2 | ANT data: pre- and posttest scores in the control and exercise condition (means, standard errors and 95% confidence intervals).

	Alerting		Orienting		Conflict/Executive control	
	Control	Exercise	Control	Exercise	Control	Exercise
REACTION TIME (ms)						
Pretest	19.0 (2.8) [13.5; 24.5]	20.4 (2.4) [15.6; 25.2]	63.2 (2.9) [57.3; 69.1]	59.9 (2.5) [54.9; 64.9]	78.3 (3.3) [71.7; 84.9]	86.8 (2.9) [81.1; 92.6]
Posttest	22.3 (2.8) [16.8; 27.7]	23.0 (2.6) [17.7; 28.2]	57.4 (3.1) [51.3; 63.6]	60.4 (2.9) [54.7; 66.1]	72.0 (3.3) [65.5; 78.5]	70.1 (2.8) [64.6; 75.6]
ACCURACY (%)						
Pretest	1.1 (0.6) [−0.1; 2.2]	1.6 (0.4) [0.8; 2.5]	−2.2 (0.5) [−3.2; −1.1]	−1.9 (0.5) [−2.9; −0.9]	−7.7 (0.9) [−9.5; −5.9]	−7.8 (0.9) [−9.5; −6.0]
Posttest	1.1 (0.6) [0.0; 2.2]	1.7 (0.7) [0.3; 3.0]	−2.0 (0.5) [−3.0; −1.1]	−3.2 (0.5) [−4.2; −2.3]	−8.3 (0.7) [−9.8; −6.9]	−7.5 (0.9) [−9.2; −5.7]

TABLE 3 | F-statistics of the RM ANOVA model for the ANT data (reaction time and accuracy).

ANT	**Reaction time**			**Accuracy**		
RM ANOVA: factor interactions	**Statistics**			**Statistics**		
	***F* (df1, df2)**	***p***	η^2	***F* (df1, df2)**	***p***	η^2
ALERTING						
Intervention session*test	0.023 (1,96)	0.880	0.000	0.000 (1,96)	0.987	0.000
Intervention session*test* duration	0.133 (2,96)	0.876	0.003	0.090 (2,96)	0.914	0.002
ORIENTING						
Intervention session*test	1.892 (1,96)	0.172	0.019	2.586 (1,96)	0.111	0.026
Intervention session*test* duration	0.155 (2,96)	0.856	0.003	0.777 (2,96)	0.463	0.016
CONFLICT/EXECUTIVE CONTROL						
Intervention session*test	0.616 (1,96)	0.434	0.007	1.397 (1,96)	0.240	0.014
Intervention session*test* duration	0.463 (2,96)	0.631	0.010	0.247 (2,96)	0.782	0.005

activities, whereas the children in the control condition in our study were working on school-related tasks, i.e., more cognitively engaging activities. It has been hypothesized that performing any cognitive activities during the control condition, such as watching an educational video or reading a book, may cause acute effects in cognitive performance which may be absent in case of a completely passive control condition in which children are not allowed to do anything (Best, 2010). In this respect, there could have been too little contrast between the exercise and control condition in our study to detect subtle exercise related effects. Future research with various sedentary control conditions is needed to further explore this issue. Lastly, inconsistencies in results between our and other studies might be attributed to differences in the age of the participants, i.e., 11–14 years old adolescents in our study vs. 8–11 years old children in the studies of Hillman et al. (2009), Drollette et al. (2012), Gallotta et al. (2012), Chen et al. (2014), and Janssen et al. (2014a). In this respect, a recent meta-analysis found the largest effects of acute aerobic exercise bouts on reaction time measures of executive functioning in preadolescent children (6–12 years), as compared to older adolescents (13–19 years) (Ludyga et al., 2016).

Working memory performance was neither affected by exercise bouts of 10, 20, or 30 min, which is in line with previous studies that used a similar n-back task (Drollette et al., 2012; Soga et al., 2015), as well as studies that used a Sternberg task (Cooper et al., 2013) or a mixed dot task (Kubesh et al., 2009). In contrast to our results, a study of Chen et al. (2014) found faster reaction times after exercising compared to the control session on a n-back task (Chen et al., 2014). In their study, only the 2-back load of the n-back was used and their stimuli remained on screen for a considerably longer time than in our study (i.e., 2,000 vs. 500 ms). Hence, the n-back version we used in our study could be considered as more difficult due to a shorter memory trace. The difficulty of the n-back version we used in the current study could have contributed to the fact we were not able to detect the subtle effects of exercise

TABLE 4 | n-back data: pre- and posttest scores in the control and exercise session (means, standard errors, and 95% confidence intervals).

	Reaction time (ms)		Accuracy (%)	
	Control	Exercise	Control	Exercise
Pretest	501.9 (8.4) [485.3; 518.5]	495.6 (7.5) [480.7; 510.5]	86.3 (0.8) [84.8; 87.8]	85.7 (0.8) [84.2; 87.3]
Posttest	497.0 (8.0) [481.1; 512.8]	501.4 (8.3) [484.8; 517.9]	84.7 (0.9) [82.8; 86.5]	84.9 (0.8) [83.2; 86.6]

TABLE 5 | F-statistics of the RM ANOVA model for the n-back data (reaction time and accuracy).

n-back	Reaction time			Accuracy		
RM ANOVA: factor interactions	**Statistics**			**Statistics**		
	***F* (df1, df2)**	***p***	**η^2**	***F* (df1, df2)**	***p***	**η^2**
Intervention session*test	2.478 (1,89)	0.119	0.028	1.398 (1,89)	0.240	0.015
Intervention session*test* duration	2.205 (2,89)	0.116	0.048	0.749 (2,89)	0.476	0.017
Intervention session*test*load	0.627 (2,178)	0.536	0.007	0.888 (2,178)	0.413	0.010
Intervention session*test*load*duration	1.037 (4,178)	0.390	0.023	0.969 (4,178)	0.426	0.021

on working memory performance. There were also differences in age and exercise activities (8–11 years old children and group-based running exercises in Chen et al., 2014), but not in the sample size tested ($N = 92$ in our study and $N = 98$ in Chen et al., 2014). The n-back task is often used to train cognitive performance, as it engages multiple executive functions at once which allows little room for employing automatic processes or task-specific strategies to optimally perform this task (Jaeggi et al., 2008). In retrospect, the n-back task is highly susceptible to intra-individual differences and might not be the best choice to investigate the subtle effects of exercise on performance.

Another purpose of our study was to assess the possible dose-response effects of 10, 20, and 30 min of moderate to vigorous intensity exercise on cognitive performance. As we found no acute effects of any exercise duration, we cannot make statements on a "longer duration—better performance" type of function between exercise and cognitive performance. This is in line with the study of Howie and colleagues who found no improvement in executive functioning after exercise bouts of either 5, 10, or 20 min (Howie et al., 2015). In contrast, they did report higher math fluency scores after 10 and 20 min of exercise compared to a 10-min sedentary control condition (Howie et al., 2015). However, the math fluency test measures other domains of cognitive/academic performance and is therefore difficult to compare with our findings. Although we recruited∼15% more participants than needed based on the sample size calculation ($n = 105$ to detect differential effects of exercise duration), the final number of adolescents included in the data analyses was 92 in the n-back task and 99 in the ANT analyses. However, given the small effect sizes and large p-values that we found ($\eta^2 = 0.002$–0.017 and $p = 0.463$–0.914), we expect that the lack of significant effects is not due to lack of power. We measured cognitive performance immediately before and after our 10-, 20-, and 30-min condition. Although we found no differences related to the time between pre- and posttest across the three conditions, further research on dose-response effects should consider to use comparable time frames between testing (e.g., group A: sitting for 20 min followed by 10 min of exercise, group B: sitting for 10 min followed by 20 min of exercise, and group C: exercising for 30 min).

Although we found no positive acute effects of exercise on selective attention and working memory measured immediately after the exercise bouts, adolescent's performance did not deteriorate after exercising for 10, 20, or 30 min compared to working on school related tasks. This is in line with the (sub)conclusions of several recently published systematic reviews and meta-analyses on the acute effects of exercise on cognition in children and adolescents (e.g., Donnelly et al., 2016; Li et al., 2017; Daly-Smith et al., 2018; de Greeff et al., 2018). Hence, implementing single exercise bouts of moderate to vigorous intensity throughout the school day does not seem to harm cognitive performance, and may help to increase the overall physical activity levels of children and adolescents (WHO, 2011; Bassett et al., 2013). The relevance of implementing exercise bouts for academic achievement in the long term needs further study. Therefore, we recommend researchers to investigate whether the longer term implementation of single exercise bouts may result in improved cognitive and academic performance of children and adolescents. Repeated exercise bouts may also increase enjoyment of school lessons and thereby improve cognitive and academic performance.

CONCLUSION

In summary, acute moderate to vigorous exercise bouts with a duration of 10, 20, and 30 min did not improve nor deteriorate selective attention and working memory performance of young adolescents immediately after exercising, compared to a control condition in which they worked on school-related tasks. We found no differential effects of exercise bouts of relatively long, medium, and short duration.

AUTHOR CONTRIBUTIONS

VvdB, ES, JJ, RdG, MC, and AS conceived and designed the study; VvdB and ES performed the data acquisition and analyzed the data; VvdB, JJ, RdG, MC, and AS contributed to the data interpretation and presentation; ES wrote the initial manuscript; VvdB, JJ, RdG, MC, and AS made critical revisions on several drafts of the manuscript; VvdB revised the initial manuscript and wrote the final version. All authors approved the final version of the manuscript.

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ORIGINAL RESEARCH
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Nerve Growth Factor Is Responsible for Exercise-Induced Recovery of Septohippocampal Cholinergic Structure and Function

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Exercise has been shown to improve or rescue cognitive functioning in both humans and rodents, and the augmented actions of neurotrophins within the hippocampus and associated regions play a significant role in the improved neural plasticity. The septohippocampal circuit is modified by exercise. Beyond an enhancement of spatial working memory and a rescue of hippocampal activity-dependent acetylcholine (ACh) efflux, the re-emergence of the cholinergic/nestin neuronal phenotype within the medial septum/diagonal band (MS/dB) is observed following exercise (Hall and Savage, 2016). To determine which neurotrophin, brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF), is critical for exercise-induced cholinergic improvements, control and amnestic rats had either NGF or BDNF sequestered by TrkA-IgG or TrkB-IgG coated microbeads placed within the dorsal hippocampus. Hippocampal ACh release within the hippocampus during spontaneous alternation was measured and MS/dB cholinergic neuronal phenotypes were assessed. Sequestering NGF, but not BDNF, abolished the exercise-induced recovery of spatial working memory and ACh efflux. Furthermore, the re-emergence of the cholinergic/nestin neuronal phenotype within the MS/dB following exercise was also selectively dependent on the actions of NGF. Thus, exercise-induced enhancement of NGF within the septohippocampal pathway represents a key avenue for aiding failing septo-hippocampal functioning and therefore has significant potential for the recovery of memory and cognition in several neurological disorders.

Keywords: NGF, BDNF, acetylcholine, exercise, basal forebrain, hippocampus, nestin

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INTRODUCTION

Exercise improves an array of health outcomes, including the enhancement of learning and memory, particularly under pathological conditions. Upregulation of neurotrophins is a crucial mechanism by which exercise affects cognitive function (Neeper et al., 1996; Cotman et al., 2007), and each neurotrophin has a unique role in improving cognitive outcome. Brain-derived neurotrophic factor (BDNF) is considered to play an essential role in mediating the pro-cognitive effects of exercise via the enhancement of hippocampal neurogenesis, dendritic complexity, and synaptic plasticity (Vaynman et al., 2004; Stranahan et al., 2007; Wrann et al., 2013; Vivar and van Praag, 2017). Nerve growth factor (NGF) is essential for normal development as well as the

functioning of mature cholinergic neurons in the basal forebrain. Across the lifespan, NGF is retrogradely transported from the hippocampus to MS/dB cholinergic neurons where it influences structure (both soma size and dendritic complexity) and modulates the activities of choline acetyltransferase and acetylcholinesterase (Conner et al., 2009; Isaev et al., 2017). The loss of NGF leads cholinergic neuronal atrophy (Koh et al., 1989; Sofroniew et al., 1990). In preclinical models of neurological disease, NGF treatment reverses the effects of lesions and age-related degeneration of basal forebrain cholinergic neurons, including the recovery of learning and memory (Tuszynski and Blesch, 2004).

Although the expression of NGF is amplified in the MS/dB and hippocampus following exercise (Neeper et al., 1996; Chae and Kim, 2009; Hall and Savage, 2016), its unique role in brain plasticity following exercise has not been determined. This is surprising given that NGF is critical for maintaining and promoting cholinergic neuron survival and specifically upregulates ChAT+ phenotypic expression in the basal forebrain (Fischer et al., 1987; Gustilo et al., 1999; Tuszynski et al., 2015), and that forebrain cholinergic neurons are imperative for learning and memory (Conner et al., 2009; Easton et al., 2012; Ballinger et al., 2016).

Exercise increases muscarinic receptor density and high affinity choline uptake in the hippocampus (Fordyce and Farrar, 1991) and increases the number of neurons expressing choline acetyltransferase (ChAT+) in the horizontal diagonal band (Ang et al., 2006). One month of voluntary wheel running also increased the afferent input from medial septum to newly born hippocampal neurons (Vivar et al., 2016). Furthermore, we demonstrated (Hall and Savage, 2016) that voluntary wheel running recovered both spatial working memory and behaviorally stimulated hippocampal ACh efflux, as well as rescued degenerating MS/dB cholinergic neurons in an animal model of alcohol-related amnestic syndrome, or Korsakoff Syndrome. Interestingly, the restorative effect of exercise was primarily seen in the unique phenotype of ChAT+ neurons that also express nestin. About 30% of the forebrain ChAT+ neurons express nestin and these mature neurons are more responsive to stimulation (Gu et al., 2002; Hendrickson et al., 2011; Zhu et al., 2011). We hypothesized that the ChAT/nestin neuronal phenotype in the MS/dB are a malleable type of cholinergic neuron that is very responsive to neurotrophin modulation and are influential in driving activity-dependent hippocampal ACh release and associated behaviors (Hall and Savage, 2016).

However, given that both BDNF and NGF have durable trophic effects, such as rescuing atrophied forebrain ChAT+ neurons (Morse et al., 1993; Tuszynski and Gage, 1995), it is unclear which neurotrophin is instrumental for the exercise-dependent rescue of the septohippocampal cholinergic system. Exercise has been shown to increase both BDNF and NGF levels in the hippocampus in normal rats (Neeper et al., 1996), and exercise recovers BDNF and NGF neurotrophin deficits in rats made amnestic by thiamine deficiency (Hall and Savage, 2016). Our laboratory employs the pyrithiamine-induced thiamine deficiency (PTD) model of Korsakoff Syndrome, the alcohol-related amnestic disorder, to study system level interactions in neuropathology, neurochemical dysfunction and behavioral impairment. Beyond the traditional thalamic pathology associated with thiamine deficiency, there are also reductions in cortical and hippocampal behaviorally activated acetylcholine (ACh) efflux that has been linked to the loss of forebrain cholinergic populations (see Savage et al., 2012).

In the present study, we blocked the actions of either BDNF or NGF with microbeads coated with either TrkB-IgG or TrkA-IgG antibodies (Vaynman et al., 2004; Griesbach et al., 2009), during the voluntary exercise bout. This sequestering technique has been effectively used to block neurotrophin action for weeks throughout the hippocampus (Vaynman et al., 2003, 2004, 2006; Miladi-Gorji et al., 2011). To determine the functional output of voluntary exercise, with and without sequestered neurotrophins, spontaneous alternation performance, activity-dependent ACh efflux and unbiased stereological assessment of MS/dB cholinergic neuronal phenotypes were assessed at a critical time point after exercise (see Hall et al., 2014; Hall and Savage, 2016).

The data revealed that the nestin cholinergic neuronal phenotype was the most responsive to both pathology and exercise. Furthermore, sequestering the actions of NGF, throughout exercise, blocked both the functional and structural cholinergic improvements advanced by exercise in amnestic rats. In control rats, sequestering NGF and BDNF lead to small but significant decreases in spatial alternation, but only inhibiting the actions of NGF suppressed activity-dependent ACh efflux after exercise. The nestin cholinergic phenotype in the intact brain also responded to the sequestering of NGF. Such results suggest that exercise modulates the cholinergic forebrain through an NGF-dependent mechanism and the nestin cholinergic phenotype is exceptionally reactive to NGF levels.

MATERIALS AND METHODS

Subjects

For the behavioral assessment, ACh efflux measures and cell counting procedures, adult male Sprague-Dawley rats (N = 133), weighing between 275 and 300 g (Envigo, Indianapolis, IN, United States) were used throughout this experiment. The goal was to conclude with eight rats per group, so additional rats were included to account for attrition due to treatment or surgery. There was a low level of attrition, thus some groups contain more than eight rats (see below). Rats were placed in a temperature-controlled vivarium (20–22°C), and maintained on a 12-h light/dark cycle with light onset at 07:00 h. All procedures followed full accordance with the Institutional Animal Care and Use Committee of Binghamton University and the National Institute of Health: Guide for the Care and Use of Laboratory Animals (9th ed., National Academies Press, 2014). Additionally, these rats were all pair-housed, all had standard bedding in clear plastic cages and had access to an enrichment wood chew block for the entire duration of the study.

A separate cohort (N = 42) of adult male Sprague-Dawley rats (275–300 g, Envigo, Indianapolis, IN, United States) were used to initially determine whether delivery of unilateral or

bilateral TrkA-IgG and TrkB-IgG coated microbeads abolished the exercise-induced increase in neurotrophin protein levels, and whether this suppression persisted throughout exercise.

Pyrithiamine-Induced Thiamine Deficiency (PTD)

The details of the standard Pair-fed (PF) and PTD treatment have been described extensively in our previously published studies (see Roland and Savage, 2009; Hall et al., 2014; Hall and Savage, 2016). Briefly, pyrithiamine hydrobromide injections (0.25 mg/kg; Sigma-Aldrich Corp., St. Louis, MO, United States) were given for 14–16 days in conjunction with *ad libitum* thiamine-deficient chow, until the appearance of severe neurological symptoms, at which rats were given a large bolus injection of thiamine. This standard treatment induces the neuropathology similar to KS. PF control rats received pyrithiamine hydrobromide equivalent to the amount consumed by PTD-treated rats in addition to thiamine hydrochloride (0.4 mg/kg; Sigma-Aldrich Corp., St. Louis, MO, United States) in order to replete thiamine levels. Following PTD and PF treatment all rats were placed back onto a normal diet consisting of Purina rat chow for a 10-day recovery period prior to surgery. An experimental overview and timeline of the study can be seen in **Figure 1**.

Microbead Preparation

To scavenge available BDNF and NGF, during exercise, fluorescent microbeads (Green Retrobeads^{TM} IX; LumaFluor Inc., Durham, NC, United States) were treated with either recombinant human TrkB Chimeric Fusion Protein (Fc chimera; Cat: 688-TK-100, R&D Systems, Minneapolis, MN, United States) or reconstituted recombinant TrkA Fc chimera (Cat: 175-TK-050; R&D Systems) (see Vaynman et al., 2004, 2006; Ying et al., 2008). These microbeads slowly release the coated substances.

Fluorescent latex microbeads were added to a centrifuge tube containing 1.0 mL of 0.1% BSA (in PBS) at a ratio of 1:5, respectively (100 μL green fluorescent microbeads to 500 μL recombinant human TrkB/Fc or 100 μL green fluorescent microbeads to 1,000 μL recombinant human TrkB/Fc chimera). To prepare the PBS control microbeads, 1.0 mL of the 0.1% bovine serum albumin (BSA) solution (in PBS) was added to a centrifuge tube, and 100 μL of the green fluorescent microbeads were added in addition to 500 μL PBS (0.1M from above), at the 1:5 respective ratio. This control was shown to be effective by Ying et al. (2008). The solutions were mixed and stored at 4°C overnight. The next day, each solution was centrifuged for 30 min at 14,000 rpm. Supernatant was removed, and the pellet was re-suspended with 10 μL of sterile nanopure dH_2O.

Microbead Infusion and Cannulation Surgeries

Hippocampal cannulations (8 mm guide cannula; Synaptech Technology Inc., Marquette, MI, United States) were performed on all rats using the following coordinates from bregma [(AP) = −0.53 mm, (ML) = −0.51 mm, (DV) = −0.42 mm; see Savage et al., 2011; Hall and Savage, 2016]. Microbead solutions were delivered as previously described (see Vaynman et al., 2004, 2006). Pilot data indicated that blockade of NGF required TrkA-IgG coated microbeads to be delivered bilaterally to both hemispheres. To block BDNF, we added a bilateral TrkB-IgG group to address the issue of laterality with blocking NGF, despite that a unilateral dose of TrkB-IgG coated microbeads were sufficient at reducing both the contralateral and ipsilateral hemispheres. Infusions of TrkB-IgG or TrkA-IgG coated microbead solution, 2 μL, was inserted into a hemisphere, which sequesters BDNF or NGF for 14 days of exercise (see Ying et al., 2008). The respective microbead solution was delivered to the dorsal hippocampus [(AP) = −0.38 mm, (ML) = +0.26 mm, (DV) = −0.37 mm; relative to bregma] over a period of 15 min. Immediately following surgery rats were placed in pairs (two rats/cage) in the exercise running wheel chamber apparatuses for the remainder of the experiment, and the two rats per cage shared one running wheel (locked or unlocked, see below).

Exercise Paradigm

Identical exercise parameters were adapted from our previous published studies (Hall et al., 2014; Hall and Savage, 2016). Microbead and cannulated PTD- and PF-treated rats were randomly assigned into one of two exercise conditions: (1) a voluntary exercise condition (VEx) in which a running wheel (Lafayette Instrument Company, Lafayette, IN, United States) was attached to the home cage or (2) a sedentary condition with a locked exercise wheel (Stat). Rats were pair-housed and food restricted to 16 g/rat/day. Food restriction has been shown to increase wheel running (see Sherwin, 1998; Lee et al., 2002). Furthermore, rats remained in the wheel running apparatus for a period of 2-weeks, with daily running (cumulative m/day) recorded via AWM® software (Lafayette Instrument Company).

BDNF and NGF Western Blot Expression

To determine the degree and persistence of the treated microbeads to suppress BDNF and NGF levels following exercise, non-treated rats were into the following conditions: Stat + PBS coated microbeads (n = 6), VEx + PBS coated microbeads (n = 6), VEx + unilateral TrkB-IgG coated microbeads (n = 6), VEx + bilateral TrkA-IgG (n = 6), VEx + unilateral TrkA-IgG (n = 6), VEx + bilateral TrkA-IgG coated microbeads (n = 6). These rats were sacrificed on day 14 of VEx.

Hippocampal tissue was dissected and each sample was diluted (1:10) and normalized to the same protein level (50 μg). Total protein was determined with a BCA protein assay (Thermo Fisher, cat# 23227, Waltham, MA, United States). Samples along with the standard ladder and manufacturer recommended positive controls were loaded into Novex® pre-cast 16% polyacrylamide gels, and electrophoresed (125 V; 2.5 h). Western blots were chosen to measure extracellular NGF and BDNF protein levels since enzyme-linked immunosorbent assay (ELISA) analysis was not possible because the hippocampal tissue contained fluorescent latex microspheres that would directly interfere with BDNF quantification because the beads would fluoresce at the specific wavelengths. Western blots for BDNF and NGF were run on separate gels with the standard ladder,

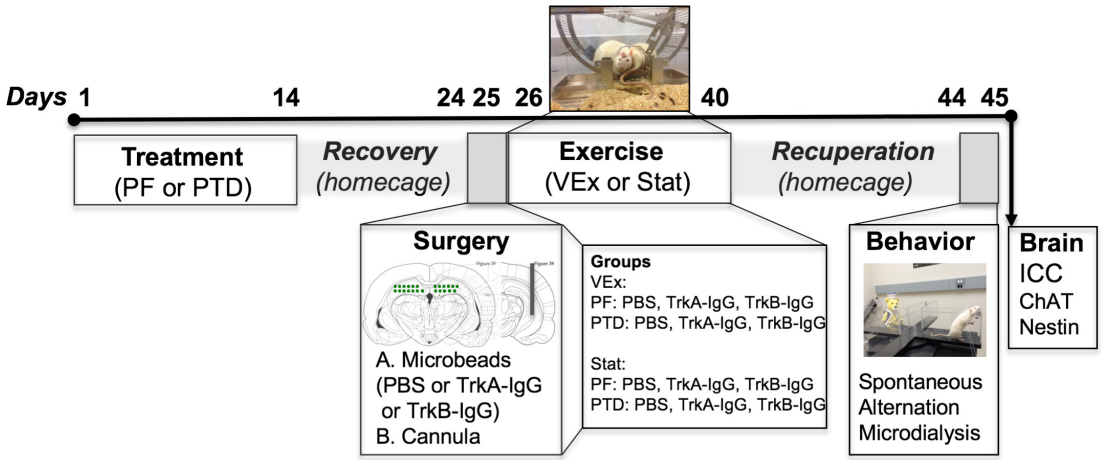

FIGURE 1 | Experimental timeline indicating that the subjects were randomly split between PTD and PF treatment. After treatment recovery, rats received a dorsal hippocampal infusion of either TrkB-IgG-, TrkA-IgG- or saline-coated microbeads, in addition to a cannulation to measure ACh efflux. Immediately following surgery rats were split into two groups, either a VEx (voluntary exercise) group where rats received running wheels attached to a home-cage for 2-weeks, or a Stat group (stationary) that had exposure to immobile wheels for this 2-week duration. Following the exercise exposure, rats were placed back into normal homecages and were tested 2-weeks later on a spontaneous alternation task with microdialysis to collect hippocampal ACh dialysate samples, followed by perfusion and assessment of ChAT and nestin cellular morphology in the MS/dB. The images indicating microbead delivery and hippocampal cannulations were adapted from Paxinos and Watson (Paxinos and Watson, 2014).

and were initially tested for their respective positive control (BDNF: glioblastoma human whole cell lysate, cat# U-87; NGF: mouse brain extract, cat# sc-2253; Santa Cruz Biotechnology, Santa Cruz, CA, United States). Blots were blocked for 60 min with 5% BSA in TBS+Tween. Next, blots were incubated into respective primary antibodies at a dilution of 1:500 in 5% BSA overnight at 4°C (BDNF: affinity purified rabbit polyclonal, cat# sc-546, Santa Cruz Biotechnology; NGF: rabbit polyclonal, cat# sc-548, Santa Cruz Biotechnology). The following day, blots were incubated for 60-min in secondary antibody (1:1000; bovine anti-rabbit IgG-HRP secondary antibody, cat# sc-2370, Santa Cruz Biotechnology), prior to development of film using Pierce ECL Western blotting detection reagents (Thermo Fisher Scientific). Hypoxanthine phosphoribosyltransferase-1 (HPRT-1; goat polyclonal, 1:1000, Santa Cruz Biotechnology) was used as a housekeeper, for within-subject normalization of BDNF/NGF per HPRT-1 values. Analysis of NGF/BDNF bands were performed using the program ImageJ (Gómez-Pinilla and Ying, 2010; Ding et al., 2011) to determine size of bands in comparison to the housekeeper HPRT-1.

Behavioral *in vivo* Microdialysis and Maze Testing

Given that the Western blot data demonstrated equal effectiveness for unilateral and bilateral placement of TrkB-IgG, but not TrkA-IgG, in sequestering the corresponding neurotrophin, both the PF and PTD groups contained the following microbead conditions: (1) VEx TrkB-IgG (bilateral + unilateral; PF $n = 15$; PTD $n = 15$); (2) Stat TrkB-IgG (bilateral + unilateral; PF $n = 14$; PTD $n = 14$); (3) VEx TrkA-IgG (bilateral; PF $n = 8$; PTD $n = 8$), (4) Stat TrkA-IgG (bilateral; PF $n = 8$; PTD $n = 9$); (5) VEx + PBS (bilateral + unilateral; PF $n = 11$; PTD $n = 12$) and (6) Stat + PBS (bilateral + unilateral; PF $n = 10$; PTD $n = 8$). Bilateral PBS conditions were added as well as unilateral conditions to ensure delivery would not affect behavior or neurochemical analyses.

Rats were returned to standard pair-housing conditions after VEx/Stat for 9 days prior to being handled daily for an additional 5 days prior to behavioral testing as a 14-day period has been demonstrated to be needed to see exercise-induced changes in the forebrain cholinergic system (see Hall and Savage, 2016). Spontaneous alternation testing procedures, in conjunction with microdialysis collection of hippocampal ACh efflux, was conducted with procedures published in our previous work (see Anzalone et al., 2010; Hall and Savage, 2016; Fernandez and Savage, 2017). Spontaneous alternation is a spatial task that is sensitive to the cholinergic system as well as the functioning of the hippocampus and basal forebrain (for review, see Lalonde, 2002). Microdialysate samples were collected every 6-min during baseline (total: 18-min), during spontaneous alternation (total: 18-min), and post-baseline (total: 18-min). An alternation was defined as entry into four different arms in overlapping successive sequences of four arm entries (for example, in the successive arm entries of B, A, D, C, A, D, C, A, D, B, C, D, B, A; the first sequence of BADC was an alternation, but the next 4-arm sequence ADCA was not). The percent alternation score is equal to the ratio of actual alternations to possible alternations (total alternations/[trial number $-$ 3] $\times$ 100).

Tissue Preparation and Immunohistochemistry

Rats were transcardially perfused with 4% methanol-free formaldehyde in PB. Brains were extracted, kept in formalin for 24-h, and placed into 30% sucrose for 5 days prior to coronal

sectioning (40 μM) on a freezing microtome (Lecia, Instruments, Wetzler, Germany). Slices from the HPC coordinates were mounted, coverslipped, and viewed under fluorescence to determine Trk(A/B)-IgG location. Furthermore, we examined cannula placement within the hippocampus on Nissl stained sections and sequential sections were used to identify cholinergic phenotypes (see below).

Immunohistochemistry—ChAT/Nestin and Unbiased Stereological Cellular Quantification

Since the latex fluorescent microbeads retrogradely label neurons in the MS/dB, a brightfield stain was employed to allow for analysis the co-localization of Nestin with ChAT (see **Figure 2**). Thus, we developed a brightfield co-labeling stain to assess the Nestin/ChAT populations of neurons as well as the overlap in the Nestin and ChAT neuronal populations. Six sections from every subject (sampling every 5th section) spanning the MS/DB were treated with a standardized ICC protocol (see Roland and Savage, 2009; Hall and Savage, 2016; Fernandez and Savage, 2017). Following rinsing (PB), quench and blocking steps, sections were first processed with the ChAT primary antibody (1:200, goat polyclonal anti-ChAT, cat# AB144P; Millipore, Billerica, MA, United States), and where then incubated in a secondary antibody (biotinylated anti-goat IgG 1:100, cat# BA-5000; Vector Laboratories, Burlingame, CA, United States). This was followed a PB rinse and then incubation in an avidin-biotin complex (cat# PK-6100; Vector Laboratories). Finally, ChAT was developed in an ImmPACT NovaRED solution kit (cat# SK-4805; Vector Laboratories), which marks cholinergic neurons as reddish brown under a brightfield microscope. Next, the stained ChAT tissue was treated with the Nestin primary antibody (1:200; mouse monoclonal anti-Nestin, cat# MAB353; Millipore). The next day, tissue was rinsed (PB) and incubated into a pre-made secondary antibody solution (ImmPRESS AP anti-mouse IgG-alkaline phosphatase, cat# MP-5402, Vector Laboratories). Lastly, the tissue was rinsed and developed in a Vector blue substrate solution (cat# SK-5300, Vector Laboratories).

Unbiased stereological assessment is commonly employed to assess the cholinergic basal forebrain neuronal population (see Yeo et al., 1997; Gritti et al., 2006; Roland et al., 2014) and allows for a better assessment of neuronal quantification since this measure is unbiased and is currently the most validated and widely used method (see West et al., 1991; Naumann et al., 2002). We used unbiased stereology to estimate ChAT+ and Nestin+ cell populations in the MS/DB (see Yoder and Pang, 2005; Savage et al., 2007; Anzalone et al., 2010; Hall and Savage, 2016; Fernandez and Savage, 2017). Slides were coded to ensure that the experimenter that performed cell counts was blind to all experimental conditions. A Carl Zeiss Microscope (Zeiss Axioscope 2-Plus, Thornwood, NY, United States) with an attached digital camera (DVC-1310; DVC Company, Austin, TX, United States) containing a motorized stage was used in combination with Stereo Investigator software (MicroBrightField Bioscience, Williston, VT, United States) on a computer containing a Windows XP operating system. As depicted in **Figure 2**, for cholinergic (ChAT) staining ChAT+/Nestin− cells appear reddish while Nestin+/ChAT− cells appear bluish. However, since > 95% of Nestin+ neurons are also ChAT+ neurons (Nestin+/ChAT+), nearly all the Nestin+ neurons co-express ChAT+ and therefore are purple. Quantification of neuronal phenotypes were performed using a 40× dry-objective lens, and counting was performed using the optical fractionator function, as previously described (see Hall and Savage, 2016; Fernandez and Savage, 2017).

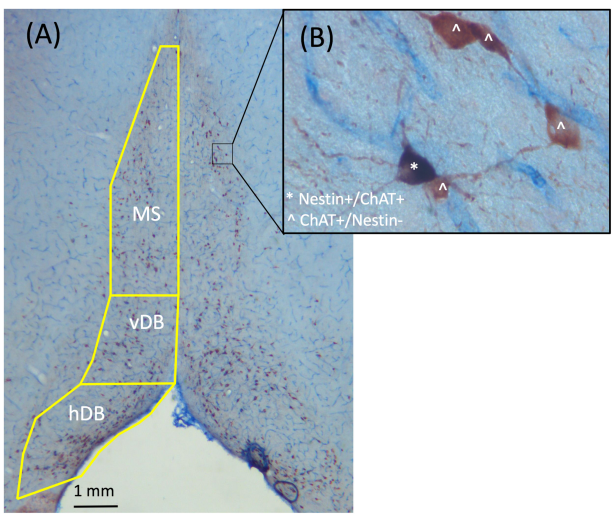

FIGURE 2 | Representative images taken from the MS/dB with 2.5× **(A)** and 40× **(B)** objectives of ChAT+/Nestin+ dual brightfield immunohistochemical stain. ChAT+/Nestin– cells were stained red, while Nestin+/ChAT+ neurons were stained purple. Cells labeled with ^ indicate they are ChAT+/Nestin– while cells labeled with * indicate they are Nestin+/ChAT+.

High Performance Liquid Chromatography (HPLC)

Microdialysis samples were submitted to HPLC (HTEC-500, Eicom USA, San Diego, CA, United States) to assess ACh content (see Savage et al., 2011; Hall and Savage, 2016; Fernandez and Savage, 2017). The detection of the system is 5 fmol and sample fmol value calculations were performed via the software Envision® (Eicom).

Statistical Procedures and Data Availability

All data were expressed as means ± SEM. All data were analyzed using the statistical program SPSS Statistics for Macintosh (Version 21.0; IBM Corp., Armonk, NY, United States). Cumulative distance data was analyzed as a mixed model repeated measures ANOVA with the one between-subjects factors Treatment (PF vs. PTD) and one within-subjects factor Day (across 14 days). For spontaneous alternation, ChAT and Nestin populations, we employed a three-factor between-subjects ANOVA with Treatment (PF vs. PTD), Exercise (VEx vs. Stat)

and Microbeads (TrkB-IgG, TrkA-IgG, PBS) as between-subjects variables. Hippocampal ACh efflux was analyzed as a mixed-model repeated measures ANOVA with Treatment, Exercise, and Microbeads as between subjects factors, with Sample Time (blocks 1–3) collapsed within Phase (baseline, maze, after) as two within-subjects factors. Furthermore, in cases of any significant main effects or interactions, we ran *post hoc* tests for type of Microbead (TrkB-IgG, TrkA-IgG, PBS) using Scheffe's test. The raw numerical data supporting this manuscript will be made available by the authors, without undue hesitation, to any qualified researcher.

RESULTS

Both Unilateral or Bilateral Hippocampal Infusions of TrkB-IgG Effectively Blocked Exercise-Induced Amplification of BDNF, Whereas Bilateral Infusion of TrkA-IgG Was More Effective Than Unilateral Infusion of TrkA-IgG at Blocking the Exercise-Induced Rise of NGF

An initial experiment was conducted to ensure that the microbead procedures (unilateral vs. bilateral hippocampal implantation of microbeads coated with either TrkA-IgG or TrkB-IgG) could inhibit available NGF and BDNF for an extended time period. Representative blots for NGF and BDNF are shown in **Figure 3A**, along with the housekeeper HPRT1 bands. Whereas bilateral hippocampal implantation of TrkA-IgG microbeads was more effective at reducing available NGF than unilateral implantation of TrkA-IgG microbeads [$F(1,10) = 4.54$, $p = 0.045$, see **Figure 3C**], this was not the case for of the implantation of TrkB-IgG microbeads, as unilateral and bilateral implantation did not differ in reducing available BDNF [$F(1,8) = 0.058$, $p = 0.816$], similar to previous studies indicating that injection of unilateral TrkB-IgG microbeads are sufficient to reduce BDNF levels in the HPC (see Vaynman et al., 2004; Griesbach et al., 2009). As shown in **Figure 3B**, both unilateral and bilateral hippocampal implantation of TrkB-IgG microbeads were both similarly effective at reducing available BDNF [$F(1,30) = 140.210$, $p = 0.000$; see also Vaynman et al., 2006]. Since injections of TrkB-IgG delivered unilaterally and bilaterally led to a similar blockade of BDNF, the two TrkB-IgG conditions were combined. To demonstrate the selectivity of the microbead procedures, levels of the alternative growth factor were also measured. The TrkB-IgG treated microbeads selectively blocked the exercise-induced increase in BDNF, but did not sequester NGF, which significantly rose (about 30%) with exercise [$F(1,30) = 14.87$, $p = 0.001$]. Furthermore, the TrkA-IgG treated microspheres, while blocking the exercise-induced rise in NGF, did not inhibit the exercise-induced rise in BDNF [$F(1,30) = 27.31$, $p = 0.000$]. Importantly, we observed no differences between the unilateral and bilateral groups on behavior, ACh efflux or cell counts, were detected as a function of unilateral or bilateral implantation of TrkB-microbeads (all p's > 0.201). However, given that the unilateral condition suppressed NGF less than the bilateral condition, only the bilateral Trk-A condition was used for behavioral, ACh and cellular analyses.

PTD-Treated Rats Run Less Than PF-Treated Control Rats and Microbead Infusion Does Not Impact Wheel Running

Because sphericity was violated for the within-subjects factor of Day [$\chi^2(90) = 3241.33$, $p = 0.0001$], a Greenhouse–Geisser correction was applied for the analyses. As shown in **Figure 4**, a main effect of Treatment indicated that PF-treated control rats ran more compared with PTD-treated rats [$F(1,63) = 6.37$, $p = 0.014$]. Analysis at each day indicated that PTD-treated rats ran less than PF-treated rats from days 3 to 14 [all F's$(1,69) < 6.00$, $p < 0.02$]. Similar to our previous data (see Hall et al., 2014; Hall and Savage, 2016), we observed the greatest difference toward the end of exercise exposure as indicated by a Treatment × Day interaction [$F(1.102,69.43) = 5.60$, $p = 0.018$]. Importantly, there was no effect of the microbeads on the cumulative distance that rats ran [$F(2,63) = 0.70$, $p = 0.50$].

The Exercise-Induced Enhancement of Spontaneous Alternation Behavior in PTD-Rats Was Selectively Blocked by Sequestering of NGF, but Not BDNF

As previously reported, **Figure 5** depicts that PTD-treatment impairs spontaneous performance [$F(1,121) = 16.25$, $p = 0.0001$], but exercise did recover this deficit [$F(1,121) = 8.253$, $p = 0.005$]. Although exercise did not enhance spontaneous alternation performance in PBS-microbead treated PF rats, [$F(1,19) = 1.471$, $p = 0.240$], the effect of exercise was significant in PBS treated PTD rats [$F(1,18) = 12.953$, $p = 0.002$]. Furthermore, exercise fully recovered the behavioral impairment in PTD-treated rats: PTD rats exposed to VEx with PBS-treated microbeads did not differ from PF rats [$F(1,20) = 0.464$, $p = 0.504$].

Importantly, the sequestering neurotrophins with microbeads was critical for the exercise-induced recovery of spontaneous alternation performance [main effect of microbeads: $F(1,121) = 8.541$, $p = 0.005$], and follow-up analyses revealed that the action of NGF were necessary to for exercise to recover alternation behavior in PTD-treated rats (**Figure 5A**). As depicted, PTD-treated rats with TrkA-IgG microbeads, to inhibit the action of NGF, did not show any improvement in alternation performance as a function of exercise [$F(1,15) = 0.49$, $p = 0.828$]. Additionally, in PF VEx rats the TrkA-IgG microbeads caused a significant impairment in alternation performance [$F(1,17) = 8.264$, $p = 0.011$], compared with PF VEx rats that were administered PBS-treated microbeads. No significant effect of TrkA-IgG was seen in either PF rats [$F(1,17) = 4.204$, $p = 0.06$] or PTD rats [$F(1,15) = 0.044$, $p = 0.837$] without exercise.

In contrast, the TrkB-IgG microbeads did not block the actions of exercise (see **Figure 5B**) in the PTD group. The spontaneous alternation performance of PTD VEx rats that received TrkB-IgG microbeads did not differ from PTD VEx

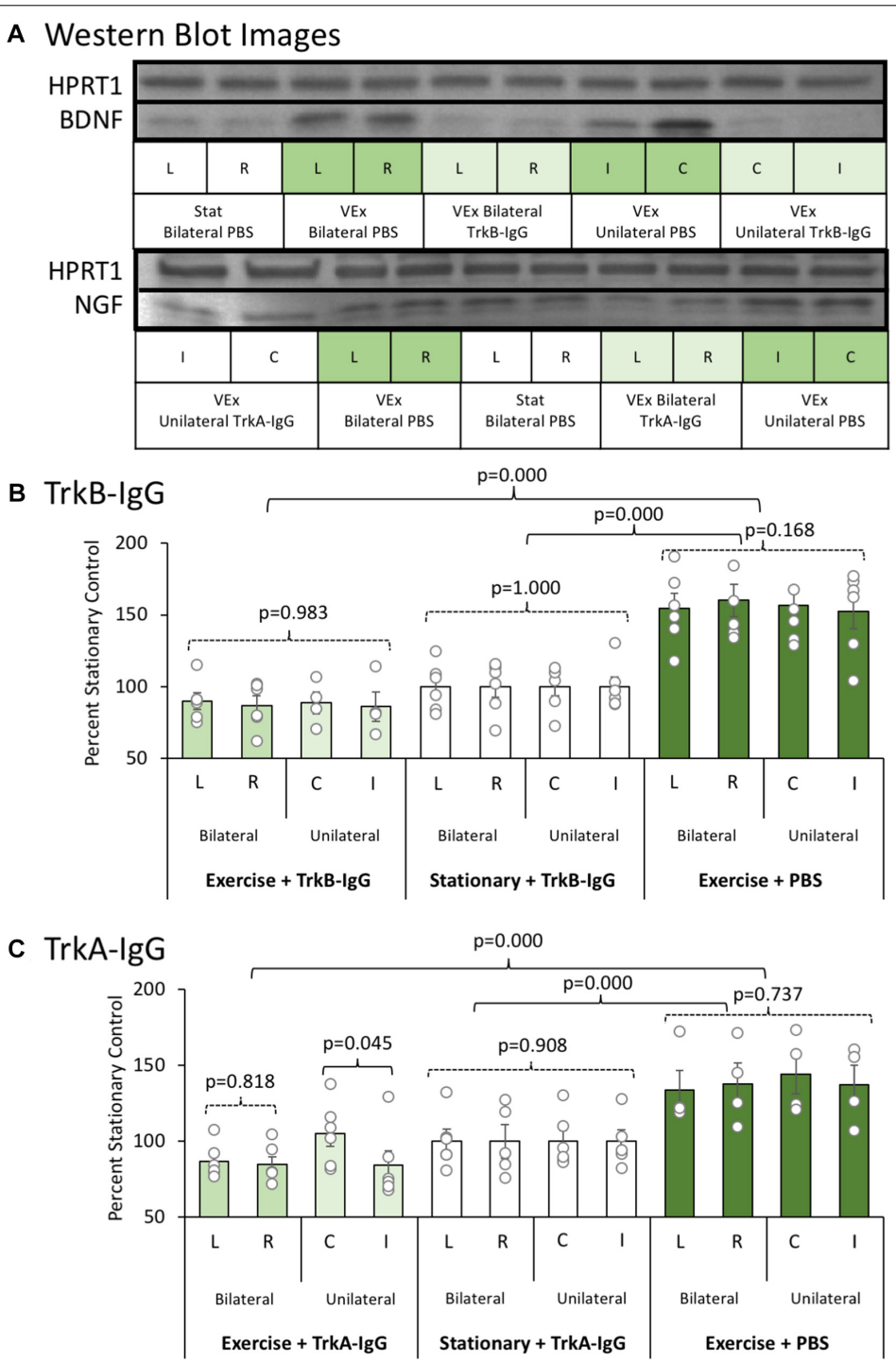

FIGURE 3 | (A) Representative Western blot images for BDNF and NGF proteins as a function of exercise condition (VEx = Exercise; Stat = Stationary control wheels), substance coating the microbeads (PBS, TrkB-IgG; TrkA-IgG), and whether the beads were implanted on both sides of the dorsal hippocampus (bilateral) or only on one side (unilateral) in control rats. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as a housekeeper. **(B)** The mean ± SEM of percent control of BDNF with values normalized to HPRT1 levels. Relative to the stationary wheel condition (Stat), control rats that voluntarily exercised had persistent increased BDNF levels. However, both bilateral and unilateral hippocampal infusion of TrkB-IgG coated microspheres completely suppressed the exercise-induced increase in BDNF such that they were comparable to the BDNF level in sedentary rats. **(C)** The mean ± SEM of percent control of NGF with values normalized to HPRT1 levels. Relative to the stationary wheel condition (Stat), rats that voluntarily exercised had persistent increased NGF levels. However, unlike TrkB IgG, the infusion of TrkA IgG coated microspheres differentially suppressed exercise-induced increases in NGF as a function of whether the infusion was bilateral or unilateral within the hippocampus. Specifically, in rats that exercised, only bilateral infusion of TrkA-IgG coated microspheres led to a significant suppression of NGF, while the unilateral infusion of TrkA-IgG coated microspheres did not significantly decrease NGF levels in the hippocampus in the contralateral hemisphere.

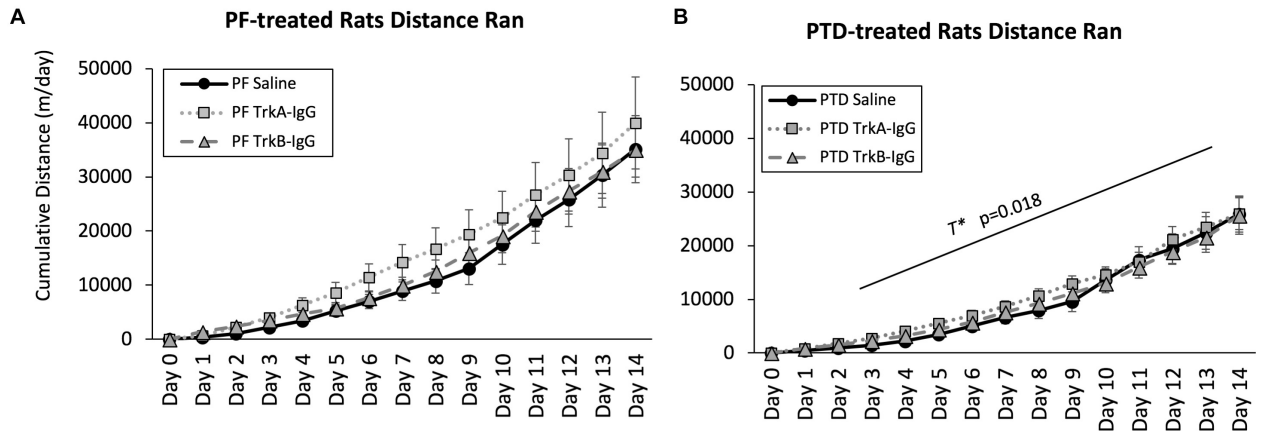

FIGURE 4 | Cumulative distance ran throughout the 2-weeks of wheel running for PF rats **(A)** and PTD rats **(B)**. Distance traveled is expressed as meters per day ± SEM. PTD rats ran less than control PF rats from days 3 to 14. There were no differences observed in running as a function of blocking BDNF (TrkB-IgG) or NGF (TrkA-IgG). T* indicates a significant effect of Treatment.

rats with PBS-treated microbeads [$F(1,25) = 0.856$, $p = 0.364$]. Additionally, the TrkB-IgG microbeads had no effect in PTD Stat rats in comparison to PTD stat rats with PBS-treated microbeads [$F(1,20) = 0.209$, $p = 0.652$].

Although exercise did not improve alternation performance in PF rats, the sequestration of BDNF decreased spontaneous alternation performance selectively in PF rats regardless of exercise condition. PF VEx rats treated with TrkB-IgG microbeads did have a reduced spontaneous alternation performance relative to PF VEx rats with PBS-coated microbeads [$F(1,24) = 4.28$, $p = 0.05$]. Even the alternation scores of stationary PF rats were affected sequestering the neurotrophins: Blocking the actions of BDNF in sedentary PF rats decreased alternation behavior [$F(1,24) = 5.053$, $p = 0.035$]. Thus, it appears that control and amnestic rats have differential sensitivity to blocking the actions of BDNF and NGF, with BDNF appearing critical for normal spatial working memory performance in control rats, while NGF appears critical for the exercise-induced rescue of spatial working memory in PTD-treated rats.

Arm Entries During Spontaneous Alternation Were Not Affected by PTD Treatment, Exercise or Microbeads Treatment

Overall, we did not observe any effect of PTD Treatment, Exercise, or Microbeads manipulation on the number of arms entered during spontaneous alternation testing [Treatment: $F(1,121) = 1.76$, $p = 0.187$, Exercise: $F(1,121) = 0.002$, $p = 0.963$, Microbeads: $F(1,121) = 1.502$, $p = 3.14$; Means ± SEM: PBS Microbeads: PF-VEx: 29.90 ± 3.15, PF-Stat: 35.60 ± 3.29, PTD-VEx: 28.42 ± 2.50, PTD-Stat: 36.38 ± 6.13; TrkA-IgG Microbeads: PF-VEx: 37.63 ± 5.68, PF-Stat: 25.00 ± 2.91, PTD-VEx: 22.88 ± 2.42, PTD-Stat: 26.11 ± 3.96; TrkB-IgG Microbeads: PF-VEx: 32.67 ± 3.66, PF-Stat: 32.14 ± 3.46, PTD-VEx: 33.07 ± 3.50, PTD-Stat: 28.71 ± 3.00]. Furthermore, there were no interactions between the variables on arm entries.

Basal Levels of Hippocampal ACh Are Reduced by the Sequestration of NGF in PTD Rats

A main effect of PTD treatment indicated that there was a reduction in basal levels of ACh compared with PF control rats [$F(1,121) = 7.40$, $p = 0.01$], while exercise did not affect basal ACh levels [$F(1,121) = 3.14$, $p = 0.079$]. As shown in **Figure 6** (insets), basal hippocampal ACh levels were reduced by microbead delivery [$F(2,121) = 12.06$, $p = 0.01$], and Scheffe's *post hoc* test indicated that these levels were significantly reduced by approximately 15% with the sequestration of NGF, via TrkA-IgG coated microbeads, in the PTD VEx group ($p = 0.01$), but not via blockade of the actions of BDNF ($p = 0.09$).

Behaviorally Stimulated Hippocampal ACh Efflux Is Blunted by PTD-Treatment, Which Can Be Selectively Restored by Exercise, but Is Dependent Upon the Actions of NGF

Overall, PTD treatment blunted behaviorally stimulated hippocampal ACh efflux [Treatment × Phase interaction: $F(2,242) = 11.095$, $p = 0.0001$] and exercise ameliorated this deficit [see **Figures 6B,D**; Exercise × Phase interaction: $F(2,242) = 3.63$, $p = 0.028$]. Overall, the there was a main effect of microbeads on ACh efflux [$F(2,121) = 16.753$, $p = 0.0001$] and a Microbeads × Phase interaction on ACh efflux [$F(4,242) = 15.572$, $p = 0.0001$]. Specifically, blocking NGF appeared to be critical to ACh efflux, as Scheffe's *post hoc* analysis revealed that rats with TrkA-IgG coated microbeads had an attenuated effect of ACh efflux during spontaneous alternation testing, compared with rats treated with PBS-coated microbeads ($p = 0.001$). Furthermore, Scheffe's *post hoc* analyses revealed that in PTD-treated rats, the exercise-induced rise in ACh efflux was abolished with the application of TrkA-IgG coated microbeads ($p < 0.01$). In PF rats (**Figures 6A,C**), despite no significant effect

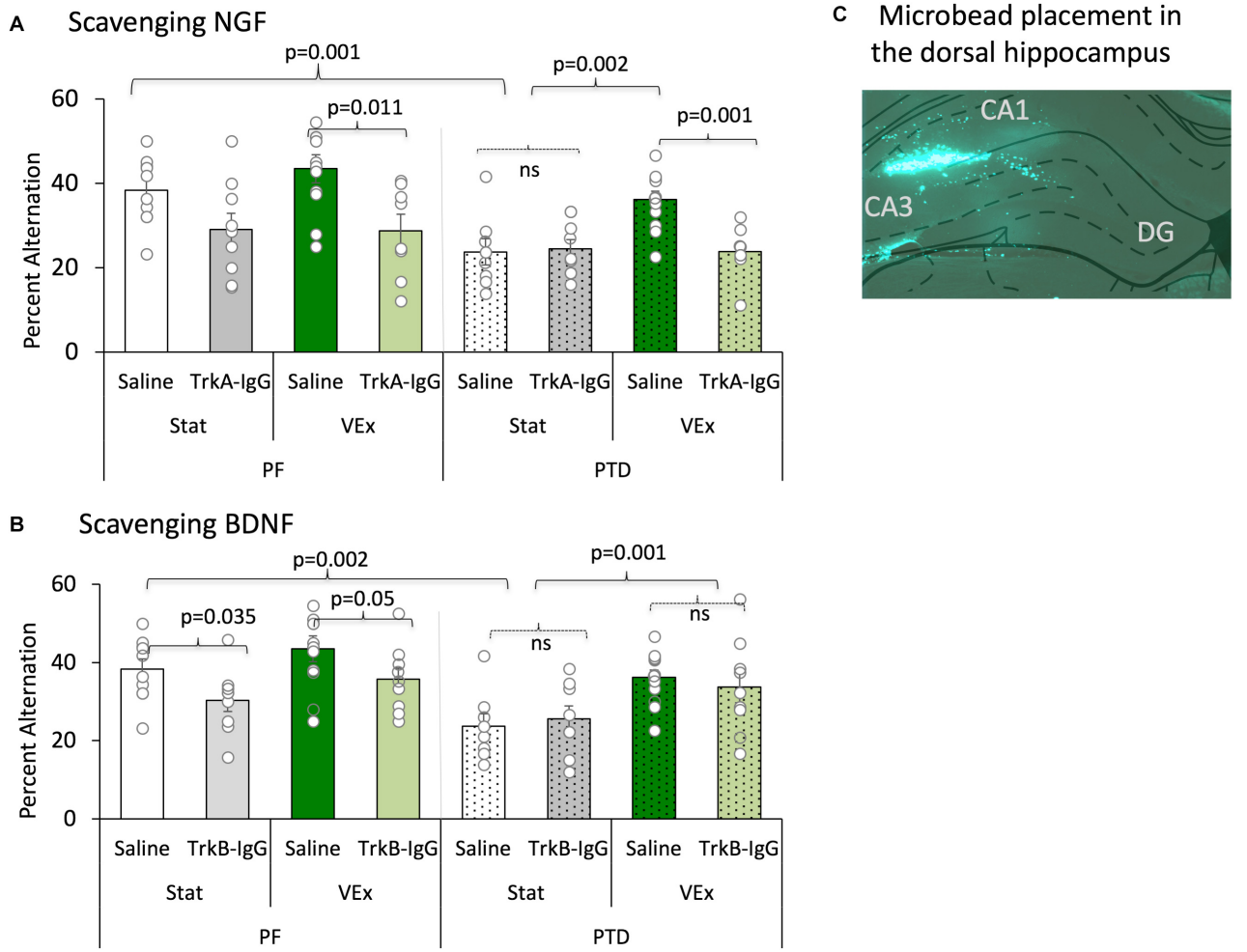

FIGURE 5 | Spontaneous alternation is displayed with the sequestering of NGF [**(A)** (TrkA-IgG) and BDNF **(B)** (TrkB-IgG)], and is expressed as mean percent alternation ± SEM. Exercise (green bars) completely ameliorated the spontaneous alternation impairment in PTD-treated rats; conversely, blocking NGF (light green bars) completely abolished the exercise related recovery in PTD rats, and diminished performance in PF rats. Blocking BDNF impaired performance in PF control rats, regardless of exercise status. In contrast, inhibiting the actions of BDNF had no effect on the behavior of PTD-treated rats. Microbead placement is observed from a green fluorescent image taken of the dorsal hippocampus with 5× objective lens **(C)**.

of exercise on hippocampal ACh efflux (all p's > 0.06), Scheffe's test revealed that the TrkA-IgG coated microbeads reduced the ACh efflux (p's < 0.05). In contrast, sequestering of BDNF, by TrkB-IgG coated microbeads, did not influence ACh efflux in PF rats ($p = 0.16$) or PTD rats ($p = 0.21$).

Exercise Rescues the PTD-Induced Loss of the Nestin+/ChAT+ Phenotype, and NGF Is a Critical Mediator of This Cellular Recovery

The analyses from the overall population of cholinergic neurons revealed that PTD-treated rats had a decreased expression of cholinergic markers compared with PF-treated rats [$F(1,121) = 14.621$, $p = 0.001$; see **Table 1A**] and that exercise resulted in a higher number of cholinergic markers being expressed, compared with sedentary rats [$F(1,121) = 46.639$, $p = 0.0001$]. *Post hoc* analyses indicated that in PTD-treated rats, exercise led to a higher expression of ChAT neurons, compared to what was observed in stationary rats [$F(1,60) = 34.843$, $p = 0.0001$]. Interestingly, the effect of exercise to increase the number of neurons that expressed ChAT, relative to stationary wheel conditions, was also true for PF rats [$F(1,67) = 13.675$, $p = 0.001$].

To understand the extent of neuronal remodeling in the basal forebrain that occurs with exercise, we assessed the profile counts for the two cholinergic phenotypes: Nestin+/ChAT+ and ChAT+/Nestin−. There were a negligible number of Nestin+ cells that did not co-localize with ChAT (Nestin+/ChAT−; < 3 cells/mm^2); and these non-co-localized cells did not vary in response to any manipulation, PTD treatment, exercise or microbead delivery (all p's > 0.20). Importantly, the co-localization rates in our saline-treated rats did not differ from our previous measures employing confocal microscopy (see

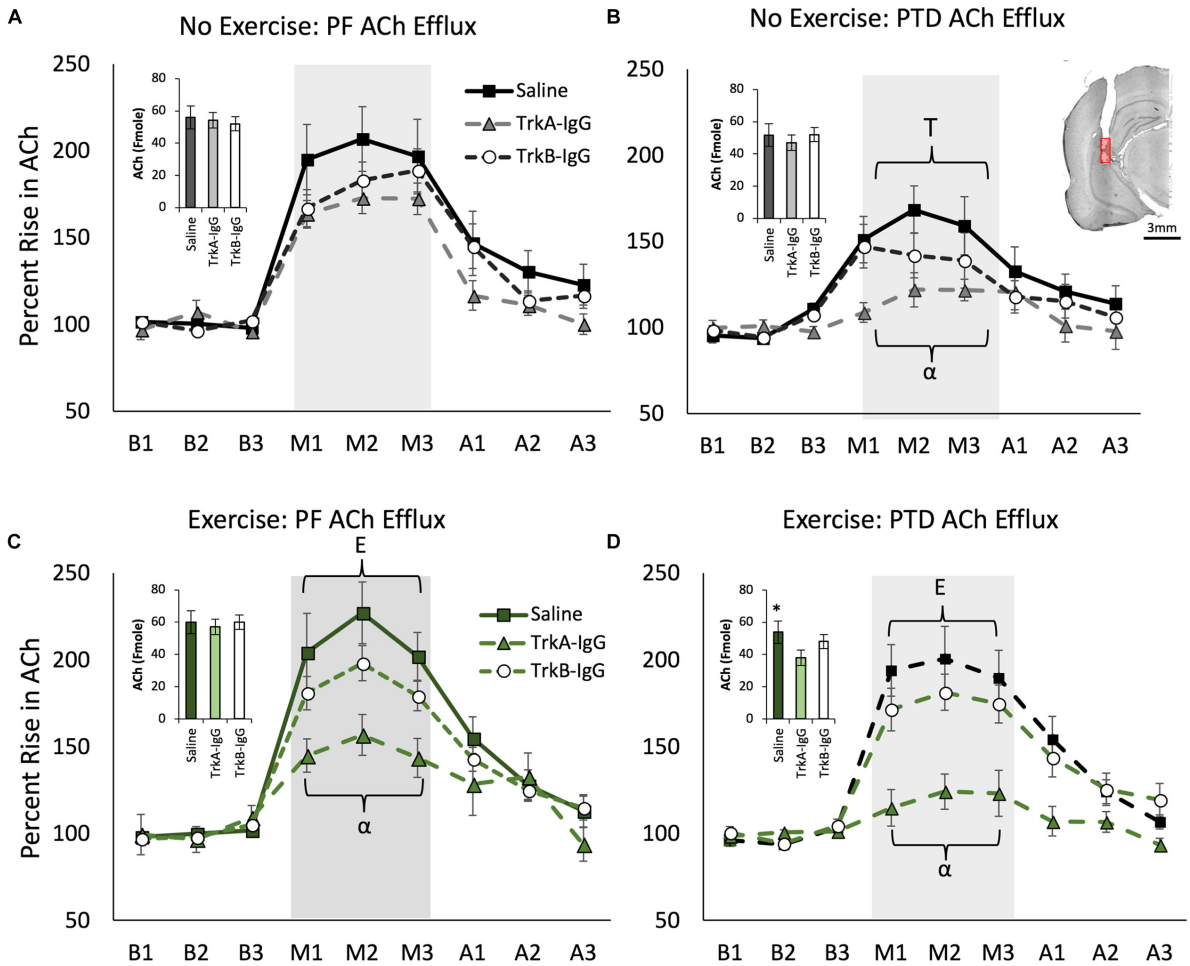

FIGURE 6 | Hippocampal acetylcholine (ACh) efflux measured throughout baseline (B1–B3), spontaneous alternation (M1–M3) and post-baseline (A1–A3) phases. Inset graphs represent basal ACh levels in femtomoles. PTD-treatment reduced ACh efflux during maze testing **(A)** vs. **(B)**. In the no exercise condition (Stat), sequestering NGF with TrkA IgG reduced ACh efflux during maze testing in PTD rats **(B)**. Exercise increased ACh efflux in both PF control and PTD-rats **(C,D)**. In PTD VEx rats **(D)**, exercise rescued the impaired ACh levels to the level of control rats. However, sequestering NGF suppressed behaviorally activated ACh efflux from exercise in both PF and PTD rats **(C,D)**. **E** indicates a significant effect of exercise; **T** indicates a significant effect of treatment, * indicates a significant effect of TrkA-IgG microbeads on basal ACh and **α** indicates a significant effect of blocking NGF. Within **(B)** is an illustrative example hippocampal placement of the microdialysis cannula. The red region represents the probe membrane.

Hall and Savage, 2016). The overall ANOVA indicated that PTD treatment reduces the number of neurons expressing the Nestin+/ChAT+ phenotype [$F(1,121) = 11.499$, $p = 0.001$]. Exercise increased the expression of the Nestin+/ChAT+ phenotype [$F(1,121) = 36.086$, $p = 0.0001$], and this was evident in both PF [$F(1,61) = 19.93$, $p = 0.001$] and PTD-treated rats [$F(1,60) = 16.65$, $p = 0.001$]. Importantly, the Nestin+/ChAT+ neurons were sensitive to the sequestering of NGF during exercise exposure. There was main effect of microbead treatment [$F(2,121) = 7.275$, $p = 0.001$] and an Exercise × Microbead treatment interaction [$F(2,121) = 3.29$, $p = 0.041$]. Follow-up analyses, using Scheffe's *post hoc* test, revealed that sequestering the actions of NGF inhibited the exercise-induced re-emergence of Nestin+/ChAT+ phenotype ($p = 0.0001$), while blocking BDNF had no effect ($p = 0.100$). Specifically, in PTD-treated rats there was a main effect of Exercise [$F(1,60) = 16.651$, $p = 0.001$] and a Exercise × Microbead interaction [$F(2,60) = 5.354$, $p = 0.007$], such that PTD VEx rats had a higher number of neurons expressing Nestin+/ChAT+, compared to PTD Stat rats ($p = 0.005$). In contrast, sequestration of BDNF did not block the effect of exercise to recover the Nestin+/ChAT+ phenotype in PTD rats ($p = 0.104$). In PF control rats, there was a main effect of microbeads [$F(2,61) = 3.384$, $p = 0.04$] and Scheffe's *post hoc* test indicated that there was a trend for TrkA-IgG coated microbeads to decrease the Nestin+/ChAT+ expression in the stationary condition ($p = 0.055$). However, TrkB-IgG coated microbeads did not alter the Nestin+/ChAT+ neuronal expression in PF rats ($p = 0.519$).

Overall, inhibiting the actions of the neurotrophins was exclusive to the Nestin+/ChAT+ expressing neurons, since for ChAT+/Nestin− neurons there was no main effect of microbead application [$F(2,121) = 0.552$, $p = 0.577$]. However,

TABLE 1 | (A) Unbiased estimations of the number of cells/mm^2 for total ChAT neurons, Nestin+/ChAT− neurons, ChAT+/Nestin+ neurons, within the MS/DB.

(A) Estimated cell number		Total ChAT++ cells mm^2	ChAT++/Nestin- cells mm^2	Nestin++/ChAT++ cells mm^2
PF Stat	PBS	174.36 ± ±9.41	109.74 ± ±6.17	64.62 ± ±4.18
	TrkB-IgG	178.98 ± 6.94	114.13 ± ±5.31	64.85 ± 3.52
	TrkA-IgG	172.37 ± ±6.89	117.62 ± ±7.17	54.75 ± 1.97
PF VEx	PBS	209.11 ± 13.25^	128.02 ± 10.23	81.09 ± ±3.87^
	TrkB-IgG	206.16 ± ±9.14^	132.81 ± ±7.21^	73.35 ± ±3.64
	TrkA-IgG	199.65 ± 10.16^	129.18 ± 10.01	70.47 ± 2.75^
PTD Stat	PBS	140.21 ± 6.77*	88.08 ± 8.74	52.12 ± ±2.85*
	TrkB-IgG	147.92 ± 9.62*	96.84 ± 7.00	52.08 ± ±3.93*
	TrkA-IgG	140.33 ± ±9.15*	86.09 ± 7.46*	54.24 ± ±3.65
PTD VEx	PBS	203.44 ± 11.41^	123.05 ± ±8.25^	80.39 ± 5.01^
	TrkB-IgG	197.71 ± ±9.66^	127.39 ± ±8.19^	70.32 ± ±4.39^
	TrkA-IgG	177.18 ± ±8.61^	125.16 ± ±7.01^	52.02 ± 3.12α
(B) Estimated cell size		**ChAT++/Nestin-μm^2**	**Nestin++/ChAT++μm^2**	
PF Stat	PBS	178.89 ± ±9.84	191.45 ± 11.62	
	TrkB-IgG	182.78 ± ±4.72	187.39 ± ±6.67	
	TrkA-IgG	174.94 ± 7.49	189.33 ± 14.04	
PF VEx	PBS	183.03 ± ±8.34	209.60 ± ±9.30	
	TrkB-IgG	180.01 ± ±5.66	197.61 ± ±9.34	
	TrkA-IgG	176.04 ± 12.39	177.87 ± ±9.42	
PTD Stat	PBS	170.93 ± 12.36	178.91 ± 10.45	
	TrkB-IgG	175.16 ± ±8.29	183.82 ± 10.95	
	TrkA-IgG	175.41 ± 11.75	170.24 ± 14.41	
PTD VEx	PBS	179.92 ± ±9.96	214.59 ± ±7.75	
	TrkB-IgG	184.17 ± ±8.20	199.12 ± ±9.66	
	TrkA-IgG	176.67 ± ±6.30	176.17 ± ±7.01	

PTD treated decreased the number of cholinergic neurons regardless of phenotype. Exercise increased both populations of cholinergic neurons. ***(B)*** *Soma size of Nestin+/ChAT− and ChAT/Nestin+ neurons within the MS/DB. Overall, exercise increased the Nestin+/ChAT+ soma size in both PF and PTD rats, whereas TrkA-IgG reduced the soma size. Pink represents an overall main effect of Treatment; Red* indicates a significant post hoc effect of Treatment; Light green represents a main effect of Exercise, ^ with dark green shading indicates a significant effect of Exercise within treatment conditions; Gray represent a main effect of microbeads, α with a black background indicates a significant effect of blocking NGF.*

for ChAT+/Nestin− neurons, there was an effect of exercise [$F(1,121) = 29.649$, $p = 0.0001$], such that exercise led to an increase in the number of ChAT+/Nestin− neurons, and this effect was seen in both PF [$F(1,61) = 6.302$, $p = 0.015$] and PTD rats [$F(1,60) = 26.176$, $p = 0.0001$].

Exercise-Induced Hypertrophy of the Nestin+/ChAT+ Phenotype Is Abolished With Blockade of NGF

We assessed somatic area as this is mediated by NGF through the tyrosine kinase A receptor (TrkA-R; van der Zee and Hagg, 2002; Huh et al., 2008). Similar to our previously published data, PTD treatment had no effect on the somatic area on cholinergic neurons that did not express nestin [$F(1,121) = 0.192$, $p > 0.66$; see **Table 1B**]. Furthermore, neither exercise [$F(1,121) = 0.503$, $p > 0.47$], microbeads [$F(2,121) = 0.303$, $p > 0.73$], nor the interaction of these variables, altered the size of ChAT+/Nestin− neurons. In contrast, exercise increased the somatic area in the Nestin+/ChAT+ neurons [$F(1,221) = 4.094$, $p = 0.04.5$]. Importantly, there was a significant main effect of microbead treatment [$F(2,121) = 3.338$, $p = 0.039$], with *post hoc* analyses indicating that the blockade of NGF abolished the hypertrophy of the cholinergic neurons that expressed nestin ($p = 0.02$), but blockade of BDNF did not affect soma size ($p = 0.48$).

DISCUSSION

Exercise is neuroprotective and can lead to behavioral recovery following brain damage. The current data revealed that in the pathological brain, with cholinergic phenotype reduction, exercise revives the distinct subset of cholinergic neurons that co-express nestin. This exercise-induced cellular change is associated with a recovery of activity-dependent hippocampal ACh efflux and improved spatial memory performance. Furthermore, if exercise-induced changes in NGF are inhibited, the cellular and functional recovery of the septohippocampal cholinergic system is blocked. In contrast, exercise-induced changes in BDNF appear not to be critical for septohippocampal remodeling, as inhibiting the actions of BDNF does not obstruct exercise-induced recovery of cholinergic neurons, hippocampal ACh efflux, or spatial behavior.

However, in the intact brain, inhibiting the actions of BDNF does lead to a small, but significant, suppression of spatial behavior, regardless of exercise status. However, this deficit was not mirrored by changes in hippocampal ACh efflux or altered expression of cholinergic phenotypes. Baseline ACh levels were decreased in intact PF rats only with TrkA-IgG coated microspheres, further emphasizing that NGF is critical in modulating septohippocampal cholinergic tone, whereas BDNF may modulates behavioral outcome in intact animals through a non-cholinergic mechanism, such as effective synaptic plasticity or neurogenesis (see Vivar and van Praag, 2017). Furthermore, blocking the actions NGF in the intact brain impaired spatial behavior and suppressed ACh efflux, and without exercise, reduced expression of the cholinergic/nestin phenotype. It is well known that even in the intact brain that NGF, through activation of the Trk-A receptor, activates the cholinergic gene locus (Berse et al., 1999), to influence ChAT enzyme activity, vesicular ACh transporter (Berse et al., 1999), and enhanced high affinity choline uptake (Williams and Rylett, 1990), which modulate the ability of the neuron to express the cholinergic phenotype and release ACh. Interestingly, following exercise, the inhibition of NGF's actions no longer suppressed the cholinergic/nestin phenotype.

Although there have been studies which demonstrated that exercise influenced the septohippocampal cholinergic system (Fordyce and Farrar, 1991; Ang et al., 2006; Hall and Savage, 2016; Vivar et al., 2016), none revealed which biochemical features of exercise modified the system. This series of studies revealed that NGF is a critical modulator of exercise-induced changes in the cholinergic forebrain system, in particular under pathological conditions. This matches the extensive literature that NGF has the capacity to rescue degenerating cholinergic neurons and drive remodeling of the septohippocampal circuit. Cholinergic neuronal identity (ChAT+), survival and cell size is regulated by NGF (Tuszynski and Gage, 1995; Naumann et al., 1997; Niewiadomska et al., 2002). Our data suggest that phenotypic plasticity of the cholinergic/nestin phenotype is also modulated by NGF.

Loss of Cholinergic Neurons, Cognitive Dysfunction, and Recovery

Cholinergic cell loss is often not to be the sole pathology prompting cognitive impairment in neurodegenerative disorders, but it is still a key-inciting factor that contributes to behavioral dysfunction (Douchamps and Mathis, 2017). This is the scenario for the alcohol-related amnestic disorder caused by thiamine deficiency: The primary damage is thalamic cell loss (Mair, 1994; Savage et al., 2011), but we have demonstrated that exercise does not recover thalamic loss in the PTD rat model (Hall and Savage, 2016). Exercise produced robust improvements in spatial behavior and hippocampal ACh efflux, which were related to a recovery of the MS/dB cholinergic/nestin phenotype in amnestic PTD rats. These results further demonstrate that the loss of the forebrain cholinergic phenotype directly impacts learning and memory success, even when it is secondary pathology. Future studies need to demonstrate that direct NGF application into the hippocampus alone could fully recover the cholinergic phenotypes and recover the associated activity-dependent ACh efflux in amnestic rats.

After a neurotoxic event, cholinergic neurons enter an atrophic quiescent state in which they do not express the enzymes to maintain the cholinergic phenotype or transmission (Hagg et al., 1988; Tuszynski and Gage, 1995; Nagahara et al., 2009). However, a significant portion of cholinergic neurons (30–40%) can be rescued from this pathological state with timely and repeated exposure to NGF (Naumann et al., 1997; Gustilo et al., 1999). Cognitive recovery after NGF exposure requires 2–3 weeks (Markowska et al., 1996; Frick et al., 1997), which suggests the several behavioral effects of NGF are mediated by structural changes in cholinergic neurons. We previously found that recovery of the ChAT/nestin phenotype following 2-weeks of exercise required an additional 2-week restoration period (Hall and Savage, 2016). Nestin participates in the dynamic remodeling of cells throughout development. The expression of nestin in neural progenitor cells is regulated by other growth factors (EGF and FGF2; Pollard et al., 2006; Sun et al., 2008). Such findings support that idea that growth factors positively regulate nestin expression within some cells, and nestin modulates cell survival, which may or may not involves nestin's critical role in the cytoskeleton integrity (Park et al., 2010). In the adult nervous system nestin appears to exert a cytoprotective function (Huang et al., 2009).

In 2002, Nestin+ cells were discovered in the MS/dB (Gu et al., 2002) that selectively co-localized with ChAT neurons, as no co-localization occurs in glia or other neuronal populations (GABAergic or glutamatergic; Wang et al., 2006; Guo et al., 2010; Hendrickson et al., 2011). Cholinergic/nestin neurons make up 35–45% of the total cholinergic neuronal population in the MS/dB in both humans and rats (Hendrickson et al., 2011), and these are mature neurons that have a higher excitability and received stronger spontaneous excitatory synaptic inputs than ChAT neurons that do not express nestin (Zhu et al., 2011). Finally, although ChAT/nestin neurons, relative to ChAT neurons without nestin, initially show greater sensitivity to colchicine-induced neurotoxicity, a larger proportion of ChAT/nestin neurons eventually recover (2–4 weeks) from neurotoxicity (Yu et al., 2011).

Although the precise role of nestin within ChAT neurons is unknown, the limited data suggest that nestin neurons within the basal forebrain are sensitive to aging and neuro-degeneration (Li et al., 2008; Hall and Savage, 2016; Fernandez and Savage, 2017). Li et al. (2008) have shown that in rats, Nestin+ neurons within the MS/dB decline with age, and that rat that displayed age-related memory impairment on Morris water maze had the lowest number of Nestin+ neurons. Furthermore, Nestin+ neurons in aged rats show reduced complexity with respect to dendritic arborization and dendritic length (Li et al., 2008). Such data support our hypothesis that the Nestin+ cholinergic phenotype is the more responsive cell population to neurotrophins.

The co-localization of nestin with ChAT might serve a neural recovery function that is mediated through the NGF-Ras-ERK signaling pathway (Huang et al., 2009). Our data suggest that a key difference between Nestin+ and Nestin− cholinergic neurons is a NGF-regulated type of plasticity that influences

cholinergic marker proteins, cell shrinkage, neuronal survival, as well as synaptic efficiency, which ultimately influences attention, learning and memory performance.

Different Roles of BDNF and NGF in Modulating Neuronal Plasticity, Learning, and Memory

Although there is evidence that BDNF and NGF have some overlapping trophic functions (see Castrén and Antila, 2017), their actions and timing within the septohippocampal circuit are different. General actions of BDNF occur on a constrained time scale (minutes to hours) and are activity-dependent at local synapses (Mcallister et al., 1996). For example, BDNF influences the induction of hippocampal LTP (Messaoudi et al., 2002), as well as transform early-phase LTP into late-phase LTP (Lu et al., 2008; Lynch et al., 2008). We recently demonstrated that BDNF rescues dysfunctional hippocampal LTP in the PTD-amnestic model (Vedder and Savage, 2017). The early effects of BDNF result from phosphorylation of synaptic proteins, whereas later effects mainly arise from transcriptional changes at the synapse (Leal et al., 2014). However, in the hippocampus, as well as other brain regions, changes in BDNF can persist through altered transcriptional control (Bramham and Messaoudi, 2005). Furthermore, blocking BDNF action with TrkB-IgG abolishes exercise-induced improvements in spatial learning (Vaynman et al., 2004). Although the TrkB receptor has high affinity for several neurotrophins, a more selective inhibition of BDNF signaling, via use of a BDNF siRNA, prevents enhancements of spatial learning seen following exercise (Intlekofer et al., 2013). Blocking the actions of acute BDNF via a TrkB antagonist or antibodies does impair the development of late LTP (Kang et al., 1997; Korte et al., 1998) and hinder consolidation and reconsolidation of inhibitory avoidance memory (Blank et al., 2016). It addition, acute blocking of TrkB receptors also blocks exercise-induced improvements in spatial memory (Korol et al., 2013). Thus, BDNF's action on hippocampal function appears to be activity dependent. Although TrkB is also the receptor for NT-3 and NT-4 (Chung et al., 2013), blocking the TrkB receptor did not influence cholinergic recovery, thus the actions of NT-3 and NT-4 are also not likely to influence cholinergic recovery. Rather, TrkA-IgG was the important modulator, suggesting NGF is critical for the exercise-induced recovery in medial septal cholinergic function and release.

In contrast, although NGF is not capable of inducing hippocampal LTP, having NGF at the synapse can facilitate LTP (Kelly et al., 1998; Conner et al., 2009). Indeed, NGF is retrogradely transported from the hippocampus to the MS/dB, where it activates a series of transcriptional events that maintains the cholinergic phenotype (see Alderson et al., 1996), as well as the cholinergic tone within the septohippocampal circuit (for review, see Johnson et al., 1987; Mufson et al., 1999; Ito and Enomoto, 2016). While TrkA has been thought to be important for survival and differentiation, some cholinergic neurons also express the p75 receptor, which has been linked to cholinergic neuronal degeneration, and has an atrophic role in cholinergic basal forebrain neurons (Boskovic et al., 2014). This may suggest that p75 receptor expression is likely altered between nestin-expressing and non-expressing cholinergic neurons, as perhaps receptor distribution contributes to the exercise-induced cholinergic recovery. In a previous study (Hall and Savage, 2016), we found that exercise-induced enhancements of the cholinergic/nestin phenotype took weeks to emerge. In the degenerating human brain, viral delivery of NGF slowly promoted axonal sprouting and hypertrophy in cholinergic neurons that persists across months (Tuszynski et al., 2015). Thus, it appears that NGF regulates the effectiveness of the forebrain cholinergic system on a protracted time frame (Conner et al., 2009). Although BDNF can also improve cholinergic function and structure, it is not as effective at doing so as NGF (Morse et al., 1993). Our data revealed that exercise-induced increases in BDNF are not involved in the boosting of cholinergic structure or function within the septohippocampal circuit.

CONCLUSION

We revealed an association between the exercise-related improvement of spatial memory and the improvement of septohippocampal cholinergic system. Augmentation of neurotrophin levels is a key feature of exercise that leads to improved cognitive outcomes. We determined that exercise-induced changes in NGF are critical for restoring waning cholinergic/nestin neurons, blunted hippocampal ACh efflux and impaired spatial behavior following thiamine deficiency. Furthermore, in the intact brain inhibiting the actions of NGF reduces the effectiveness of exercise on behavior and hippocampal ACh efflux, without changes in the cholinergic/nestin phenotype. Interestingly, exercise-induced changes in BDNF are not critical for restoring/elevating septohippocampal function. However, our data demonstrate an important avenue (beyond neurogenesis) by which exercise changes cognitive outcome: modulation of the cholinergic system, particularly in the pathological brain. Given that cholinergic function is the most documented neural substrate of cognition, and that cholinergic dysfunction is commons in an array of neurodegenerative diseases (Schliebs and Arendt, 2011), exercise should be considered as a therapeutic for disorders of cholinergic function.

AUTHOR CONTRIBUTIONS

JH and LS designed and planned the study, analyzed and interpreted the results, and contributed toward the final version of the manuscript. FG-P assisted in developing the manuscript. JH carried out the experiment with support and assistance from FG-P.

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frontiers in Psychology

ORIGINAL RESEARCH
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Improving Cognitive Performance of 9–12 Years Old Children: Just Dance? A Randomized Controlled Trial

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Exercise is assumed to have positive effects on children's cognitive performance. However, given the inconclusive evidence for the long-term effects of exercise, it is difficult to advice schools on what specific exercise programs can improve children's cognitive performance. In particular, little is known about the effects of small exercise programs that may be feasible in daily school practice. Therefore, we assessed the effects of a 9-weeks program consisting of daily exercise breaks on children's cognitive performance, aerobic fitness and physical activity levels. We conducted a cluster-randomized controlled trial in 21 classes of eight Dutch primary schools. A total of 512 children aged 9–12 years participated. The exercise intervention had a duration of 9 weeks and consisted of a daily 10-min classroom-based exercise break of moderate to vigorous intensity. Before and after the intervention, we used four cognitive tasks (i.e., the Attention Network Test, Stroop test, d2 test of attention and Fluency task) to measure children's cognitive performance in domains of selective attention, inhibition and memory retrieval. In addition, we measured aerobic fitness with a Shuttle Run test and physical activity during school hours by accelerometers. We analyzed data using mixed models, adjusting for baseline scores, class and school. After 9 weeks, there were no intervention effects on children's cognitive performance or aerobic fitness. Children in the intervention group spent 2.9 min more of their school hours in moderate to vigorous physical activity as compared to the children in the control group. In conclusion, daily 10-min exercise breaks in the classroom did not improve, nor deteriorate cognitive performance in children. The exercise breaks had no effect on children's fitness, and resulted in 2.9 min more time spent in moderate to vigorous physical activity during school hours. Daily exercise breaks can be implemented in the classroom to promote children's physical activity during school time, without adverse effect on their cognitive performance.

Keywords: physical activity, exercise, selective attention, inhibition, memory, aerobic fitness, MVPA, children

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Abbreviations: MVPA, moderate to vigorous physical activity; PA, physical activity.

INTRODUCTION

The assumed positive relationship between exercise and cognitive performance is widely used to advocate in favor of increasing exercise opportunities in schools (e.g., Erwin et al., 2012; Webster et al., 2015; Savina et al., 2016). In particular, since cognitive performance in domains such as selective attention, inhibition, working memory and cognitive flexibility, has been shown to be important for children's academic performance (Stevens and Bavelier, 2012; Jacob and Parkinson, 2015). However, recent systematic reviews and meta-analyses have shown that the evidence for the long-term effects of structured exercise programs on children's cognitive performance is inconclusive; some studies report positive effects, while others report no effects (see for reviews Donnelly et al., 2016; Li et al., 2017; Watson et al., 2017; Singh et al., 2018). Nevertheless, it can be concluded that increasing the time spent on exercise in school at the cost of academic lessons does not negatively impact children's cognitive performance (Donnelly et al., 2016; Singh et al., 2018).

Recently, an international expert panel indicated that there is a need for more well-designed, randomized controlled (RCT) trials to gain better insight in the causal effects of exercise on cognition (Singh et al., 2018). In addition, the experts highlighted the importance of elucidating the characteristics of exercise interventions that may improve cognitive performance. Due to substantial heterogeneity in interventions (e.g., duration, frequency, content), it is difficult to advise schools on the optimal form of exercise interventions to improve children's cognitive performance (Donnelly et al., 2016; Watson et al., 2017; Singh et al., 2018).

The vast majority of studies that examined the long-term effects of exercise on cognitive performance of children have implemented extensive exercise interventions with durations of 30–60 min per session, mostly delivered three to five times a week (Donnelly et al., 2016; Alvarez-Bueno et al., 2017; Singh et al., 2018). However, it seems unlikely that such time-consuming programs will be implemented on a large scale in real-life daily school practice. Several qualitative studies have reported that time constraints are perceived as a major barrier that limit the opportunities for physical activity and exercise in schools (Howie et al., 2014b; McMullen et al., 2014; Stylianou et al., 2015; Dinkel et al., 2017; van den Berg et al., 2017). In addition, teachers indicate that it would only be feasible to implement short exercise bouts with a maximum duration of 5–10 min (Howie et al., 2014b; van den Berg et al., 2017).

Previous studies have focused on the *acute,* or immediate, effects of relatively short exercise bouts on cognitive performance, such as attention, inhibition and working memory (e.g., Niemann et al., 2013; Howie et al., 2015; van den Berg et al., 2016). Several systematic reviews and meta-analyses concluded that *overall*, single moderate to vigorous exercise bouts with a minimum duration of 10 min can have small to moderate acute positive effects on children's classroom behavior (i.e., time-on-task) (Watson et al., 2017; Daly-Smith et al., 2018), selective attention (Chang et al., 2012; Janssen et al., 2014; de Greeff et al., 2018), and executive functioning (Chang et al., 2012; Verburgh et al., 2014; Donnelly et al., 2016; Ludyga et al., 2016). However, it is still unclear whether these acute effects accumulate over time, i.e., if implementing short exercise bouts on a regular basis can improve children's cognitive performance after weeks or months.

Several potential mechanisms underlying the effects of exercise on cognition have been discussed in the literature. For example, acute effects of exercise have been related to increased blood flow (Ogoh and Ainslie, 2009), increased release of neurotrophic factors, such as brain derived neurotropic factor (BDNF) and insulin-like growth factor-1 (Piepmeier and Etnier, 2015), increased arousal levels (McMorris and Hale, 2015), and increased activity in certain brain areas (Budde et al., 2008). Mechanisms of chronic exercise effects include increased availability of growth factors (e.g. BDNF), development of new blood vessels and neurons, changes in brain volume, increased efficiency of neural networks, and increased physical fitness (see for reviews Hillman et al., 2008; Huang et al., 2014; Fernandes et al., 2017). In addition, chronic exercise is suggested to improve self-control, which is important for self-regulation and functioning of higher cognitive functions (Audiffren and André, 2015). Some of the above-mentioned mechanisms may explain cumulative effects of acute exercise. For example, acute exercise-induced elevations of BDNF have been shown to be augmented by repeated exercise, resulting in increased resting levels of BDNF important for cognitive improvements and brain changes (see for a review Huang et al., 2014). Furthermore, several acute and long-term studies have shown that cognitively demanding exercise (e.g., coordinative exercise, team games) can improve cognitive performance to a higher extent than mere repetitive aerobic exercise (e.g., Budde et al., 2008; Koutsandreou et al., 2016; Schmidt et al., 2016), likely due to the inherent motor and cognitive demands (Best, 2010; Tomporowski et al., 2015). Acute cognitively demanding exercise requires high cognitive effort due to exercise complexity and changing circumstances, which may provide long-term improvement of self-control capacities and cognitive functioning (Best, 2010; Audiffren and André, 2015). This type of exercise could also result in higher intervention compliance, since challenge and variety seem important for children's exercise motivation (e.g., Martins et al., 2015).

To the best of our knowledge, no previous studies investigated the long-term effects of short exercise breaks (i.e., 10 min) in the classroom on cognitive performance of preadolescents (aged 9–12 years). Costigan et al. (2016) examined the effects of two 8-week interventions, in which 8- to 10-min exercise bouts consisting of (1) high intensity aerobic exercises or (2) high intensity combined aerobic and strength exercises were implemented three times per week in 14–16 years old adolescents. The exercise bouts were implemented once a week during recess and twice a week as part of the regular physical education (PE) classes. The authors found no significant differences in executive functioning between the intervention groups and the control group that followed the regular PE classes (Costigan et al., 2016). Little contrast in the amount of additional exercise in the three groups and the absence of measures to compare adolescent's physical activity levels limit conclusions about the exercise related effects on cognitive performance. Furthermore, the authors indicated that the relatively small sample (N = 65)

from one secondary school limits the generalizability of their results (Costigan et al., 2016).

To fill this gap, we conducted a cluster RCT trial to investigate the effects of a 9-week exercise break program on cognitive performance of 9–12 years old Dutch primary school children. The intervention consisted of one daily, classroom-based 10-min exercise break in which children were asked to mimic dance movements (i.e., aerobic exercise with coordinative and cognitive demands). The intervention was implemented within the school curriculum, as it has been shown that curricular exercise programs can result in stronger effects on cognition compared to programs that are implemented outside school hours (Alvarez-Bueno et al., 2017). Moreover, Dutch teachers have indicated that classroom-based physical activity is most feasible in daily school practice (van den Berg et al., 2017).

We examined the effects of the intervention on selective attention, inhibition, and semantic memory retrieval, since these cognitive domains are associated with children's academic performance (Rueda et al., 2010; Stevens and Bavelier, 2012). As secondary outcomes, we measured children's aerobic fitness and their physical activity levels during school hours. Given the earlier reported *acute* effects of short exercise bouts, we hypothesized that implementing a daily exercise break will have a positive effect on children's cognitive performance after 9 weeks.

MATERIALS AND METHODS

Sample Size Calculation

We used G*power 3.1.9.2 (Faul et al., 2007) to calculate the required sample size. In line with earlier studies and meta-analytic findings, we expected to find a small to medium effect of our exercise intervention on children's cognitive performance (e.g., Schmidt et al., 2015; Costigan et al., 2016; Vazou et al., 2016). The sample size calculation revealed that we needed to include a total of 404 participants (N = 202 per group) to detect a small to medium effect (f = 0.18) of the intervention on children's cognitive performance, with a power of 95% (two-sided testing at $\alpha = 0.05$).

Recruitment of Participants

We approached a convenience sample of regular primary schools from the network of our research group by email and personal contact. Twenty-three schools across the Netherlands received an information letter and were asked to respond if they were interested to participate. We included schools that were willing to participate with a minimum of two classes. For feasibility reasons, we decided to stop the inclusion after eight schools agreed to participate. Two schools declined due to busy school schedules and one school was excluded since they had only one class available. Twelve schools did not respond, but were neither followed-up since we reached the required sample size with schools that responded to our first invitation.

All children in grades 5 and 6 (N = 549) were invited to participate. Children and their parents/caregivers received an information letter about the study, including an informed consent form. In consultation with the schools it was decided that all children participated in the intervention/control program as part of the regular school curriculum. Permission of at least one parent/caregiver and children of 12 years and older was required to participate in the measurements. We received informed consent of 512 children (93%), who were included in the study. The study was approved by the Medical Ethical Committee of the VU University Medical Center Amsterdam [2014.363].

Study Design, Randomization and Blinding

We conducted a cluster RCT. An independent statistician randomly assigned the participating classes to the intervention (N = 11) or control group (N = 10). Randomization was performed in R using block randomization with blocks of size 2. The randomization was stratified by school and grade for the schools in which multiple classes of the same grade took part. Randomization for the remaining schools was done by randomly assigning the 5th grade to one of the two conditions (with the 6th grade automatically receiving the alternative). This procedure ensured that in each school there were both control and intervention classes and that number of control and intervention classes was balanced between the two grades. The randomization took place after the pretest measurements to ensure that all children, teachers and researchers were blinded. Two members of the research team remained blinded the entire experiment and acted as test administrators at the posttest measurements.

Procedure

Before the experiment, we trained the research team to conduct the measurements following a standardized protocol. We visited each class six times (see **Figure 1**). The first visit consisted of a familiarization session in which we introduced the study and explained all measurement procedures. Children received detailed instructions about four cognitive tasks and practiced all tasks to make sure they understood them well. Furthermore, children filled out a demographics questionnaire and we measured their baseline height, weight, and fitness. During the second visit, we conducted the baseline measurements (pretest) in which the cognitive tasks and a questionnaire were administered. We randomly assigned the children in each class to group A and B, stratified by gender (10–15 children per group, depending on class size). Group A started in the classroom where two paper-and-pencil cognitive tasks and the questionnaire were administered, while group B started in a separate room where two computerized cognitive tasks were administered on laptops. Halfway the test session, the groups switched rooms and continued with the other half of the measurements. In one school (N = 2 classes) there was no private room available, so we administered the paper-and-pencil tasks with the entire class, and divided the classroom in two testing areas for the laptop tasks and questionnaire. The week after the baseline measures, the classes started with the 9-week intervention/control program. The third and fourth visit were scheduled during the intervention period to: (1) hand out accelerometers in a subgroup of children, and (2) measure the exercise intensity of one exercise break in the

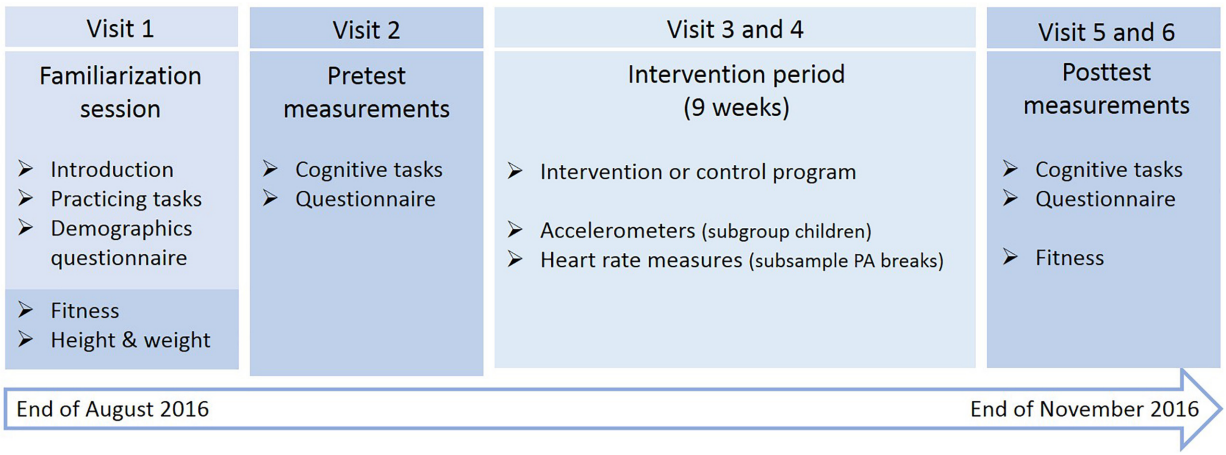

FIGURE 1 | Overview and timeline of the study.

intervention classes. After the intervention period, we conducted the post-intervention measurements (fifth visit: posttest), which were identical to the pretest and scheduled at the same day of the week and time of the day. To avoid contamination of the effects by possible acute exercise effects, we instructed all teachers not to perform an exercise break on the measurements days. During the sixth visit we measured children's fitness again. After the experiment, all children received a small symbolic present for their participation.

Intervention Program

The intervention lasted 9 weeks and consisted of one moderate to vigorous intensity exercise break per school day. The intervention duration was chosen for feasibility reasons. A period of 9 weeks best fitted the school's year schedules and was relatively short, by which we aimed to keep compliance high during the entire intervention period.

Each exercise break lasted approximately 10 min and consisted of three 'Just Dance' videos (Ubisoft, free available via YouTube). In the videos, a figure performs a dance which the children are asked to mimic. Our choice for Just Dance videos and the exercise duration of 10 min was based on the acute exercise literature combined with the preferences of Dutch teachers and children in the upper grades of primary school. The exercise literature indicates that moderate-to-vigorous exercise bouts need to have a duration of at least 10 min to exert acute cognitive improvements (Howie et al., 2014a, 2015; Daly-Smith et al., 2018). Teachers have indicated that they prefer additional PA in school to be classroom-based, easy to implement (i.e., requiring little preparation time) and up to a maximum of 10 min (van den Berg et al., 2017). Accordingly, many teachers in the Netherlands already use Just Dance in the classroom setting. A recent study of our group revealed that one of the ideas of children to increase PA in school, that matched the preferences of teachers, is to implement short exercise breaks during classroom time, for example Just Dance (van den Berg et al., 2018). Teachers received an instruction sheet with a password to access the exercise breaks via a secured webpage. The exercise breaks were performed in the classroom and we asked teachers to make sure that all children kept moving. The exercise break program was performed in addition to the regular PE classes.

The exercise breaks were selected based on a pilot study, in which we tested 83 Just Dance videos once (100%) or twice (67%) in 31 grades 5 and 6 of 14 primary schools (unpublished data). Children (N = 766) wore heart rate monitors to determine the mean exercise intensity of each video and we asked them to rate the videos on being fun and difficult, respectively. In addition, we observed the feasibility of performing the dances in the classroom. The pilot resulted in the selection of 55 suitable videos that were used to compose 45 different exercise breaks used in the current study. To ensure variety in the program, each exercise break consisted of a unique combination of three videos. Each video returned two or three times during the 9 weeks period, each time combined with two other videos.

Control Program

The control program consisted of nine educational lessons, lasting 10–15 min, one for each week of the experiment. The lessons were unrelated to the core school curriculum. We composed the lessons using information and educational videos on topics related to the body, exercise and/or sports for 9–12 year olds (free available online; see for example NTR, 2012). During six lessons children watched a short 5-min video (e.g., about agility in gymnasts, the role of balance in sports, or endurance in runners) and were asked to answer four to five questions based on what they learned from watching the video. In three lessons, children were asked to read one page of written information, for example about bones, joints and muscles in the body or about the respiratory system, and to answer five questions based on this information. The teacher discussed the answers with the entire class at the end of each lesson. Teachers received an instruction card including a password to access a secured webpage with the instructions, videos, worksheets and answers for each lesson. During the

experiment, children in the control group followed the regular PE classes.

Measures and Measurement Instruments

Demographics and Anthropometrics

Children self-reported their birth date, gender and sports participation. The questions on sports participation were derived from the ENERGY-child questionnaire, showing good to excellent test-retest reliability (ICC's: 0.68–1.00) and moderate to excellent construct validity (ICC's: 0.51–1.00) (Singh et al., 2011). The Dutch version of the Harter's Self Perception Profile for Children was administered to measure children's perceived competence in five domains (scholastic, social, athletic, physical appearance, behavioral conduct) and their perceived global self-worth (Veerman et al., 1997). This questionnaire has been shown to have sufficient construct validity and good test–retest reliability (ICC's $\geq$ 0.84) in 8–14 years old Dutch children (Muris et al., 2003; Egberink and Vermeulen, 2018a). Parents self-reported their highest completed educational level, which was used as a proxy measure of socio-economic status.

We asked teachers to provide standardized test scores of the children on reading comprehension, orthography and arithmetic. Scores were obtained from the standardized and norm-referenced CITO test battery (Hollenberg and Van Der Lubbe, 2017), which most schools in the Netherlands administer twice a year to assess and track children's academic performance. After the experiment, teachers provided information on children with special educational needs (e.g., ADHD, autism spectrum disorders, learning disorders).

We measured children's body height (cm) and weight (kg) in sport clothes without shoes, using a Leicester Height Measure Mk II (Harlow Healthcare, United Kingdom) and a Seca weighting scale (Seca Instruments, Frankfurt, Germany). The Body Mass Index (BMI) of each child was calculated with the formula: [weight (kg)/height (m)^2].

Intervention Integrity

Each class received a calendar-poster that was attached to the classroom wall and remained visible during the intervention. We asked teachers and children to put a sticker on the poster each time they performed an exercise break (intervention group) or an educational lesson (control group). The poster served as a reminder to implement the program, as well as a measure of intervention integrity. We calculated the percentage of exercise breaks that were conducted, with 45 exercise breaks equaling 100% implementation. Halfway the intervention, we asked teachers to report potential implementation problems. In case of problems, we gave advice and encouraged teachers to implement as many exercise breaks or educational lessons as possible.

Exercise Break Intensity

We assessed the intensity of a subsample of exercise breaks by monitoring heart rate (11 exercise breaks; one per intervention class). All children were fitted with a Polar H7 Bluetooth heart rate monitor that was connected to the Polar Team App (Polar Electro Oy, Finland) in which the mean heart rate of each child was stored. Exercise intensity was calculated as percentage of the maximum heart rate: (mean heart rate/maximum heart rate)*100. The maximum heart rate was measured during the Shuttle Run test (see Aerobic Fitness).

Primary Outcomes: Cognitive Performance

We measured cognitive performance with two paper-and-pencil tasks, i.e., the d2 Test of Attention and the Fluency Task, and two computerized tasks, i.e., the Stroop Color-Word task and Attention Network Task (ANT) using E-prime 1.2 Software (Psychology Software Tools, Pittsburgh, PA, United States). During the pre- and posttest, children received standardized verbal and written instructions and made a few practice trials (d2 test, Stroop, ANT). Two trained and blinded test instructors gave task instructions for all tests and kept track of time in case of the d2 test and Fluency task. During the tests, two to three members of the research team each supervised a small group of children and made notes. We instructed the children to work quietly, individually, and as fast and accurately as possible. The order in which the tests were administered was counterbalanced and randomized, stratified by gender, grade and intervention/control group. The order of tests was identical during the pre- and posttest and each child made all tests on the same laptop.

d2 Test of Attention

The d2 test was used to measure selective attention (Brickenkamp and Oosterveld, 2012). The construct validity of the d2 test has been rated as sufficient (Egberink and Vermeulen, 2018c). The reliability has been rated as good, with moderate to high test-retest reliability in 10–13 years old Dutch children (r = 0.79–0.83) (Brickenkamp and Oosterveld, 2012; Egberink and Vermeulen, 2018c).

The d2 test consists of one page with fourteen lines, each consisting of 47 characters 'd' and 'p' with one to four dashes displayed above and/or below. We instructed the children to mark as much letters 'd' with a total of two dashes ('d2') as possible, while ignoring the other characters. They had to work from the left to the right, with a time limit of 20 s per line. The test instructor gave a signal when to continue with the next line. The total test lasted 4 min and 40 s.

We used the concentration performance (i.e., number of correctly marked d2's minus the number of incorrectly marked characters) as dependent variable, since this is an objective measure of selective attention (Brickenkamp and Oosterveld, 2012).

Fluency Task

We used a paper-and-pencil version of the Verbal Fluency task (Mulder et al., 2006) to measure semantic memory retrieval performance. The validity and reliability of the Verbal Fluency task has been shown sufficient in children and adolescents (Korkman et al., 1998; Egberink and Vermeulen, 2018b).

Children were instructed to write down as many words as possible in the category 'animals' within 60 s. The total number of correct words was used as dependent variable.

Attention Network Task

We used the short version of the ANT to assess the efficiency of three attentional networks: alerting (i.e., achieving and maintaining an alert state), orienting (i.e., selection of information from sensory input) and executive control (i.e., resolving conflict among responses) (Fan et al., 2002, 2007). Several studies have recommended the use of the ANT in children, as it has been shown a valid instrument to measure their attentional performance (Rueda et al., 2004; Forns et al., 2014). The task was downloaded from the website of the Sackler Institute for Developmental Psychobiology (Sackler Institute for Developmental Psychobiology [SIDP], 2016).

Sets of five horizontal black arrows pointing to the right or left were presented on a white 15-inch laptop screen. Children were instructed to identify the direction of the middle arrow (the 'target'), by pressing the right mouse button for the right direction and the left mouse button for the left direction. The central target was 'flanked' by two lateral arrows on the left and on the right, pointing either in the same direction (congruent; >>>>> or <<<<<) or in the opposite direction (incongruent; >><>> or <<><<). A fixation cross remained visible in the middle of the screen during the task. In two-third of the trials, a warning cue (*) was presented for 200 ms either above or below (spatial cue) or at the place of the fixation cross (center cue) before the stimuli appeared. The total task lasted approximately 12 min and contained three blocks of 48 trials, with 1-min breaks in between.

We calculated the mean reaction time (correct responses only) and accuracy (proportion of correct responses) by the formulas of Fan et al. (2007): Alerting effect = (SCORE no cue – SCORE center cue); Orienting effect = (SCORE center cue - SCORE spatial cue); Conflict effect (executive control) = (SCORE incongruent - SCORE congruent). Larger reaction time scores indicate better alerting and orienting performance, while a smaller value indicates better conflict performance. For accuracy, a larger value indicates better alerting performance, a larger negative value better orienting performance, and a smaller negative value better conflict performance. Reaction times faster than 200 ms were considered as incorrect and excluded from the data analysis (Fan et al., 2007).

Stroop Color-Word Task

We used a computerized Stroop Color-Word task to assess children's inhibitory performance. Computerized versions of the Stroop have been shown to have moderate to good test-retest reliability in children (r = 0.50–0.80) (Penner et al., 2012).

During the task, a color-word (the Dutch word for BLUE, GREEN, or RED) was presented on a 15-inch white laptop screen. In the congruent conditions, the color-word was displayed in a similar text color as the meaning of the word (e.g., the word BLUE displayed in a blue text color). In the incongruent conditions, the text color differed from the meaning of the color-word (e.g., GREEN written in a red text color). Children were instructed to press the button '1,' '2' or '3' at the left side of the key board that corresponded to the text color of the color-word. A fixation cross was presented for 1000 ms, followed by the color-word that was presented for 2500 ms. After a child responded, the color-word disappeared. The inter stimuli interval was 4000 ms. The answer options, 1 = GREEN, 2 = BLUE, 3 = RED, remained visible at the bottom of the screen. The task consisted of 105 trials and lasted approximately 9 min.

We calculated the interference score as dependent variable by subtracting the scores of the incongruent from the congruent conditions for both reaction time (correct responses only) and accuracy rates. A smaller interference score indicates better inhibition.

Secondary Outcomes

Aerobic Fitness

We conducted a Shuttle Run test to assess children's aerobic fitness (Léger et al., 1988). Due to the limited dimensions of the sports halls, all children performed the test over a distance of 18 m instead of 20 m. The highest completed stage was recorded with an accuracy of a half stage and was used to estimate children's VO_2max (Léger et al., 1988). All children were familiar with the test and were encouraged by the research team to exert maximum performance. Children wore heart rate monitors (Polar H7, Polar Team App) to determine their maximum heart rate.

Physical Activity Levels

We measured children's PA during the intervention period with GT3x ActiGraph accelerometers (De Vries et al., 2009). In each class, we randomly selected a subgroup of 11–19 children that were asked to wear the device during waking hours for seven consecutive days, including the weekend (mean of 15 children per class; total n = 330). We gave children verbal instructions on how to wear the device and provided them and their parents/caregivers with an information sheet including a web-link to an online instruction video. ActiLife 6.13.3 software (ActiGraph, LCC.) was used to initialize the accelerometers and for processing the data (epoch = 15 s).

We calculated children's PA levels during school hours only. We included children in the data analysis when they wore the accelerometer at least 4 week days (Yildirim et al., 2011). We created a time filter for each school to extract only the exact school hours for analysis (e.g., 08:30 a.m. to 15:00 p.m.). Recess time was included in the analyses, because this is part of a regular school day for both intervention and control group. Non-wear time was defined as having 20 min consecutive zero's (Yildirim et al., 2011). We used the cut points of Evenson (Evenson et al., 2008) to estimate the time spent in sedentary (0–100 cpm), light (101–2295 cpm), moderate (2296–4011 cpm) and vigorous intensity activity (>4012 cpm), which have been shown to most accurately classify PA intensity levels in children and adolescents (Trost et al., 2011).

Data Analysis

We performed all statistical analyses in SPSS version 22.0 (IBM SPSS Statistics). Independent t-tests and Chi-square tests were used to compare baseline values of the control and intervention group. To test the effect of the intervention, we conducted a separate mixed-model analysis for each cognitive outcome and for aerobic fitness (VO_2max). The mixed-model included the cognitive outcome or VO_2max as dependent variable and group (i.e., control or intervention) as fixed factor. Class and

school were included as random intercepts. Covariates were the pretest score on the dependent variable, age and/or arithmetic performance. The latter two were included because of group differences at baseline and their expected relationship with the dependent variables. Differences in PA levels between the intervention and control group were also analyzed by mixed-models, with group as fixed factor, class and school as random intercepts, and total wear time as covariate. The level of significance was set at $\alpha < 0.05$.

We used an intention-to-treat approach, including all children that participated in the study in the data analyses. However, children with a missing pre- or posttest score of the dependent variables or with a missing score on a covariate, were excluded from the respective analysis. In addition, children who did not fully understand or follow the test instructions [i.e., having accuracy rates below chance level (<50%) in the ANT or Stroop task, or indicated by a note of the researchers] were excluded from the respective analysis.

RESULTS

Study Population and Descriptive Characteristics

A total of 510 children between 9 and 12 years old completed the trial (n = 2 lost to follow-up). The number of children included in the data analyses ranged from 448 to 467, depending on the outcome variable (**Figure 2**). A flow diagram including the numbers and reasons for exclusion can be found in **Figure 2**. In addition to common reasons for exclusion (e.g., absence during the pre- or posttest, missing arithmetic score), we excluded 13 children from the d2 test analysis due to a technical mistake in the test administration by one of the test instructors.

Baseline characteristics of the control and intervention group were similar, except for age and arithmetic performance (see **Table 1**). There were no significant differences between the groups in pretest scores on any of the outcome variables.

Intervention Integrity and Exercise Break Intensity

The median of implemented exercise breaks was 89%, which corresponds to 4.4 exercise breaks per week during the 9-week intervention (range: 49–98% across classes). The mean exercise intensity of the subsample of tested exercise breaks was 60% (SD 8.5) of the maximum heart rate.

Intervention Effects: Cognitive Performance and Fitness

We found no significant differences between the intervention and control group in any of the cognitive outcomes, after controlling for pretest score, age, arithmetic performance, class and school. Children in both groups showed similar patterns of change from pre- to posttest. Thus, the exercise intervention did not improve cognitive performance of the children as compared to the control group. We found no intervention effect on aerobic fitness either. An overview of the mean scores, regression coefficients, 95% confidence intervals, and p-values can be found in **Table 2**.

PA Levels

A total of 312 children (95%) had valid wear time and were included in the data analysis. Children in the intervention group spent on average significantly more minutes of their school hours in moderate PA (1.7 min) and moderate to vigorous PA (MVPA; 2.9 min) per day as compared the control group, adjusted for total wear time, class and school (see **Table 3**).

DISCUSSION

Daily exercise breaks did not improve nor harm children's selective attention, inhibition and semantic memory retrieval performance as compared to the control group. Likewise, there were no effects on children's aerobic fitness. Children that followed the intervention spent about 3 min more of their school hours in moderate to vigorous PA per day than the children in the control group.

Our results are in line with the study of Costigan et al. (2016) who assessed the effect of two 8-week exercise interventions, consisting of short exercise bouts that were implemented three times a week, on executive functioning in adolescents. Although there were several differences between our study and the study of Costigan and colleagues, such as the sample size (512 versus 65), setting in which the exercise bouts were implemented (classroom versus during recess and PE), exercise intensity (moderate versus high) and age of the participants (9–12 years versus 14–16 years), there were also similarities. In both studies, the exercise intervention lasted approximately 2 months and consisted of bouts of approximately 10 min. Our findings do not confirm our hypothesis that acute effects of short exercise bouts on cognition accumulate over time. It is possible that exercise sessions of longer duration are needed to have beneficial effects on cognitive outcomes. In this respect, Ludyga and colleagues, who evaluated an 8-week school-based exercise program in which children performed a daily 20-min exercise bout, reported improvements in working memory (Ludyga et al., 2018b) and inhibition (Ludyga et al., 2018a). Furthermore, a longer intervention period than 9 weeks might be needed to find effects of 10-min exercise bouts.

We also found no effects on children's aerobic fitness, which may be explained by our minimal exercise intervention. This finding is in line with several systematic reviews reporting that school-based exercise interventions with long durations and high frequencies are needed to improve children's aerobic fitness (e.g., Kriemler et al., 2011; Dobbins et al., 2013; Braaksma et al., 2018). Another reason for the lack of cognitive effects might be due to the coordinative requirements of our exercise breaks. Our exercise breaks may have been (too) difficult for the children, thus limiting the time they were active at moderate-to-vigorous intensity, which has been suggested to be important to exert cognitive effects (Chang et al., 2012; McMorris and Hale, 2012). Furthermore, high difficulty levels might have led to substantial cognitive demands/effort during the exercise breaks, depleting children's cognitive resources and hindering improvements in

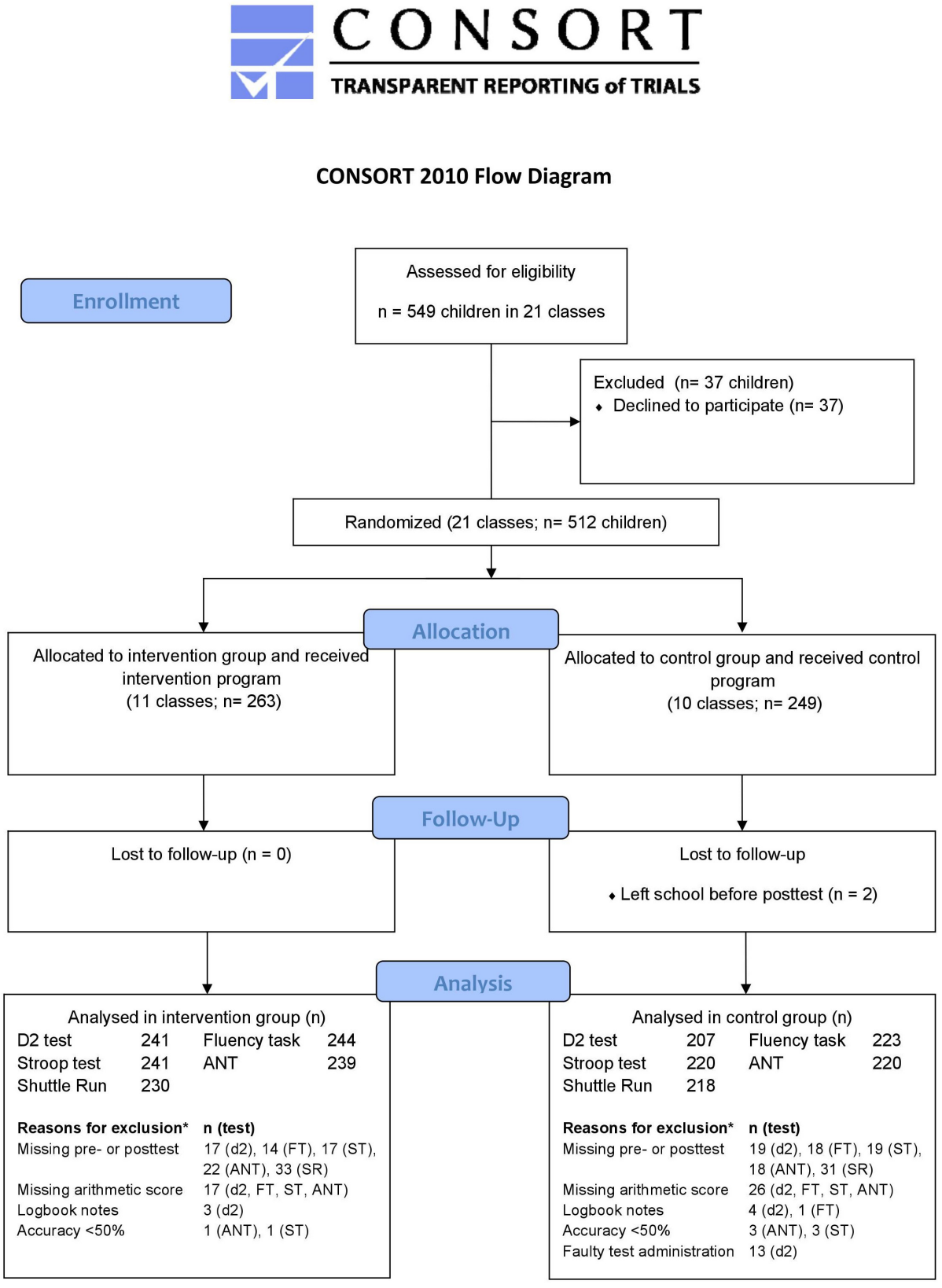

FIGURE 2 | CONSORT flow diagram: progress of participants through the trial. *Children can be excluded for one or more reasons; e.g., a child with a missing arithmetic score can also have a missing pretest score. d2, d2 test; FT, Fluency task; ST, Stroop test; ANT, Attention Network Test; SR, Shuttle Run test.

TABLE 1 | Descriptive characteristics (means and standard deviations) and group differences.

	Control group (*n* = 249)	Intervention group (*n* = 263)	*p*-value
Age (years)	10.9 (0.7)	10.8 (0.6)	0.01*
Special educational needs (%)	13	19	0.06
Sex (%, boys/girls)	53/47	54/46	0.79
Parental educational level (%)	(*n* = 228)	(*n* = 244)	0.25
-low	0	0.8	
-low to medium	3.6	1.9	
-medium	24.9	28.9	
-high	63.1	61.2	
Height (cm)	(*n* = 244)	(*n* = 259)	
	148.7 (7.2)	147.5 (7.6)	0.06
Weight (kg)	(*n* = 238)	(*n* = 258)	
	38.4 (6.9)	37.7 (7.3)	0.26
BMI	(*n* = 238)	(*n* = 258)	
	17.3 (2.3)	17.2 (2.3)	0.78
Academic performance	(*n* = 242/244/238)	(*n* = 255/258/258)	
-reading comprehension	42.6 (15.1)	40.7 (16.2)	0.17
-orthography	140.2 (7.4)	140.1 (7.0)	0.88
-arithmetic	102.1 (14.7)	98.0 (13.9)	0.00*
Sports participation (hours per week)	(*n* = 244)	(*n* = 258)	
	3.4 (2.0)	3.3 (1.9)	0.78
Self-competence	(*n* = 236/237)	(*n* = 255/257)	
-scholastic	17.3 (3.8)	16.9 (3.5)	0.22
-social	19.1 (3.5)	18.8 (3.4)	0.36
-athletic	19.1 (3.5)	18.9 (3.4)	0.53
-physical appearance	20.0 (3.7)	19.9 (3.8)	0.98
-behavioral conduct	18.3 (3.0)	18.3 (2.9)	0.79
-self-worth	20.7 (3.1)	20.5 (3.6)	0.46
VO_2max, pretest (ml/kg/min)	(*n* = 236)	(*n* = 253)	
	48.1 (5.0)	48.0 (5.0)	0.84
d2 test, pretest	(*n* = 237)	(*n* = 258)	
	133.9 (22.9)	132.1 (22.2)	0.38
Fluency, pretest	(*n* = 240)	(*n* = 258)	
	10.7 (3.3)	10.8 (3.3)	0.80
Stroop interference, pretest	(*n* = 236)	(*n* = 254)	
-reaction time (ms)	46.2 (69.2)	41.1 (80.2)	0.45
-accuracy (%)	−1.0 (5.4)	−1.7 (5.2)	0.14
ANT pretest, reaction time (ms)	(*n* = 236)	(*n* = 253)	
-Alerting	25.6 (37.3)	24.6 (35.5)	0.78
-Orienting	58.0 (36.5)	61.1 (39.6)	0.37
-Conflict	108.4 (45.4)	105.3 (41.5)	0.43
ANT pretest, accuracy (%)	(*n* = 236)	(*n* = 253)	
-Alerting	0.7 (5.1)	0.8 (4.9)	0.80
-Orienting	−1.5 (4.3)	−1.3 (4.7)	0.70
-Conflict	−5.3 (5.0)	−5.9 (5.8)	0.27

**Significant group-difference, $p < 0.05$.*

cognition after exercise. This claim finds support in some earlier acute studies that reported no effects of classroom-based cognitive demanding exercise bouts on selective attention (van den Berg et al., 2016), updating and inhibition (Jäger et al., 2015; Egger et al., 2018), and even negative effects on shifting (Egger et al., 2018) as compared to low cognitive demanding aerobic exercise in children and young adolescents. In contrast, recent meta-analyses reported positive chronic effects of cognitively demanding exercise programs (e.g., Alvarez-Bueno et al., 2017; de Greeff et al., 2018). Therefore, we recommend future research to examine how exactly the effects of acute and chronic cognitively demanding exercise relate to each other. Furthermore, research is needed to gain more insight in the optimal dose of the cognitive demands, taking into account children's motor- and cognitive

TABLE 2 | Test performance (means, standard errors, and [95% confidence intervals]) and statistics of the mixed model analyses for cognitive performance and fitness.

Dependent variable (posttest)	Control group	Intervention group	Regression coefficient (*SE*)	95% confidence interval	*p*
d2 test	151.2 (0.86)	152.5 (0.80)	1.3 (1.5)	−1.9 – 4.4	0.42
(*n* = 448)	[149.5; 152.9]	[151.0; 154.1]			
Fluency	11.9 (0.17)	11.7 (0.16)	−0.2 (0.2)	−0.7 – 0.2	0.33
(*n* = 467)	[11.6; 12.2]	[11.4; 12.0]			
Stroop interference, reaction time (ms)	41.1 (4.4)	33.4 (4.2)	−7.7 (6.0)	−19.6 – 4.1	0.20
(*n* = 461)	[32.6; 49.7]	[25.2; 41.6]			
Stroop interference, accuracy (%)	−1.8 (0.4)	−1.3 (0.3)	0.5 (0.5)	−0.5 – 1.5	0.29
(*n* = 461)	[−2.5; −1.1]	[−2.0; −0.6]			
ANT alerting, reaction time (ms)	23.5 (2.0)	23.5 (1.9)	0.0 (2.8)	−5.4 – 5.4	0.99
(*n* = 459)	[19.6; 27.4]	[19.7; 27.2]			
ANT orienting, reaction time (ms)	61.6 (2.1)	61.5 (2.0)	1.9 (3.3)	−4.5 – 8.3	0.57
(*n* = 459)	[57.5; 65.6]	[57.6; 65.4]			
ANT conflict, reaction time (ms)	95.6 (2.3)	92.3 (2.2)	−3.9 (3.7)	−11.1 – 3.4	0.29
(*n* = 459)	[91.1; 100.1]	[88.0; 96.7]			
ANT alerting, accuracy (%)	1.0 (0.3)	1.2 (0.3)	0.1 (0.5)	−0.8 – 1.0	0.85
(*n* = 459)	[0.4; 1.7]	[0.5; 1.8]			
ANT orienting, accuracy (%)	−1.5 (0.3)	−1.4 (0.3)	0.1 (0.5)	−0.8 – 1.1	0.77
(*n* = 459)	[−2.2; −0.9]	[−2.0; −0.7]			
ANT conflict, accuracy (%)	−6.4 (0.4)	−5.9 (0.3)	0.6 (0.5)	−0.4 – 1.6	0.24
(*n* = 459)	[−7.1; −5.7]	[−6.6; −5.2]			
VO_2max (ml/kg/min)	48.8 (0.2)	48.9 (0.2)	0.1 (0.3)	−0.6 – 0.7	0.77
(*n* = 448)	[48.4; 49.2]	[48.5; 49.3]			

TABLE 3 | Physical activity levels during school hours (means, standard deviations, [95% confidence intervals]) and statistics of the mixed-model analyses.

	Control (*n* = 144)	Intervention (*n* = 168)	Regression coefficient	95% confidence interval	*p*
Sedentary (minutes/day)	228.9 (2.0)	224.6 (1.8)	−2.6 (4.3)	−11.9 – 6.7	0.56
	[225.1; 232.8]	[221.0; 228.2]			
Light PA (minutes/day)	98.2 (1.6)	99.4 (1.5)	−0.3 (3.5)	−7.9 – 7.4	0.95
	[95.0; 101.4]	[96.5; 102.4]			
Moderate PA (minutes/day)	12.6 (0.4)	14.4 (0.3)	1.7 (0.7)	−0.3 – 3.2	0.02*
	[11.8; 13.3]	[13.7; 15.1]			
Vigorous PA (minutes/day)	8.1 (0.4)	9.3 (0.3)	1.2 (0.7)	−0.2 – 2.6	0.09
	[7.3; 8.8]	[8.7; 10.0]			
MVPA (minutes/day)	20.6 (0.7)	23.8 (0.6)	2.9 (1.3)	0.2 – 5.6	0.04*
	[19.3; 22.0]	[22.5; 25.0]			

**Significant, $p < 0.05$; PA, physical activity; MVPA, moderate to vigorous physical activity. PA levels include the exercise break time in the intervention group.*

development, and exercise characteristics such as difficulty, duration and intensity (Pesce et al., 2013; Egger et al., 2018).

Although the exercise breaks in our study did not result in improvements in cognitive domains of attention, inhibition and memory retrieval, it might be that short exercise bouts contribute to improved academic performance (e.g., maths or language scores) in the long-term via increasing children's learning efficiency and academic engagement (e.g., improved classroom behavior, motivation) in the lessons following the exercise bouts (Owen et al., 2016). Long-term effects of exercise interventions with a relatively short bout duration on academic performance in children are inconsistent. A recent study of Fedewa et al. (2018) reported small improvements in reading performance of 8–11 year olds who participated in two 5-min aerobic exercise breaks per day for a period of 9 months as compared to children who performed two 5-min exercise bouts in which academic content was integrated (Fedewa et al., 2018). On the other hand, Ahamed et al. (2007) found that implementing a daily 15-min classroom-based exercise break for 16 months did not improve academic performance in children aged 9–11 years. Given the inconsistent findings, more insights need to be gained on the relevance of implementing short exercise breaks for academic purposes. Therefore, we recommend researchers to (1) combine acute as well as long-term measures; (2) include cognitive- as well as academic outcomes; and (3) include an inactive control group.

Our results further revealed that children who participated in the exercise breaks spent 2.9 min more of their school

hours in MVPA per day compared to children in the control group. Our findings are in line with an earlier study that found that implementing three 5-min classroom-based exercise breaks per day, increased schoolchildren's MVPA levels (Drummy et al., 2016). Hence, these results suggest that implementing short exercise breaks in the classroom are one promising way to promote PA in children. The additional time spent in MVPA during school hours in our study, however, does not equal the length of the exercise breaks, i.e., 10 min MVPA per day. This might be due to an underestimation of MVPA during dance movements using accelerometers (van Ekris, personal communication). On the other hand, it could be that children were not (moderate to vigorously) active the entire exercise break. In this respect, our heart rate data showed that the mean intensity of the exercise breaks was at the lower boundary of MVPA (i.e., 60% HR max), indicating that it may be difficult to reach or sustain moderate to vigorous intensity levels in a classroom setting. The low exercise intensity could also be a reason for not finding improvements in cognitive performance (McMorris and Hale, 2012).

Our results have several implications for practice and future research. First, it is important to be aware of the apparent gap between research and practice. Although we found no effects of daily exercise breaks on children's cognitive performance, teachers have indicated that they experience improved classroom behavior and performance when using short exercise breaks in the classroom throughout the school year (e.g., Carlson et al., 2015). Therefore, it might be important to consider using more ecological valid measurement instruments, such as systematic observations, teacher logs and/or tasks that mimic curricular activities as a more appropriate representation of classroom-related performance (Khan and Hillman, 2014). In addition, measures of academic engagement and enjoyment of academic lessons can provide important additional information as these factors may have a role in the relationship of exercise and cognitive/academic performance (Owen et al., 2016). Second, the number of exercise breaks implemented in our study was relatively high (median of 4.4 per week), suggesting that 10-min exercise breaks in the classroom are feasible to implement in school practice. However, the controlled setting and reminders during the experiment (i.e., poster-calendar, email contact and visits by the researchers) have likely influenced these outcomes. Future studies should therefore evaluate the feasibility of the long-term implementation of short exercise breaks in real-life school practice. Third, it is important to notice that children's cognitive performance did not deteriorate either. We can therefore conclude that implementing exercise breaks on a daily basis, instead of devoting this time to academic tasks, has no adverse effect on children's cognitive performance. Lastly, an increase of 3 min MVPA induced by the exercise breaks is small. However, implementing short exercise breaks can be a relatively feasible and easy manner to start increasing PA opportunities in school. In order to increase minutes of MVPA during a school day, we recommend to complement exercise breaks in the classroom with other short and feasible exercise interventions in school, e.g., during recess or integrated in academic lessons. Furthermore, PA is suggested to have beneficial effects for mental health (e.g., depression), well-being, mood, self-esteem, motivation, and social connectedness (Lubans et al., 2016; Biddle et al., 2018). However, the effects of short exercise bouts on before mentioned outcomes is still unknown (Poitras et al., 2016). Therefore, we recommend including these outcome measures in future research on the effects of short exercise bouts. As such, we can gain deeper insight in the benefits of short exercise bouts on several domains important to children's (academic) development, and thereby strengthening the relevance of short exercise bouts in school.

Our study has several strengths, such as the RCT design, substantial sample size, blinded test administrators, use of objective measurement instruments, high compliance and a high implementation rate. Though, our study had also some limitations. Our population consisted of children of parents with a relatively high educational level, which limits the generalizability of the results. In addition, we have no baseline accelerometer-based measure of PA. However, the intervention and control classes were equally distributed within each school, i.e., representing a similar population, and did not differ on important descriptive characteristics, such as sports participation, aerobic fitness and parental educational level. Another limitation is that we did not assess children's PA behavior outside school hours. It could be that exercise breaks influenced children's PA behavior outside school, for example if children liked the Just Dance videos they could have decided to perform them during leisure time as well. Lastly, for practical reasons we used a paper-and-pencil version of the verbal fluency task which adds a motor component to the task. Therefore, the test outcomes also depend on writing speed and the length of the chosen words. In addition, we used a computerized Stroop task which measures interference effects to a somewhat lower extent than interference effects measured by the original oral version of the Stroop task (Penner et al., 2012).

CONCLUSION

In sum, we found that implementing a daily 10-min exercise break for a period of 9 weeks in the classroom had no effects on cognitive performance and aerobic fitness of 9–12-year old children. The exercise breaks brought about 3 min more MVPA during school hours. Therefore we conclude that schools can implement the seemingly feasible daily exercise breaks in the classroom to promote PA in children without adverse effects on their cognitive performance.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Medical Ethical Committee of the VU University Medical Center, with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was

approved by the Medical Ethical Committee of the VU University Medical Center.

AUTHOR CONTRIBUTIONS

VvdB, ES, RdG, MC, and AS conceived and designed the study. VvdB and ES acquired the data. VvdB analyzed the data. VvdB, MC, and AS interpreted the data analyses. VvdB drafted the manuscript. ES, RdG, MC, and AS contributed to critical revision of the draft. All authors read and approved the final manuscript.

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Influence of Quadrato Motor Training on Salivary proNGF and proBDNF

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Previous studies demonstrated exercise-induced modulation of neurotrophins, such as Nerve Growth Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF). Yet, no study that we are aware of has examined their change as a function of different training paradigms. In addition, the understanding of the possible training-induced relationship between NGF and BDNF change is still lacking. Consequently, in the current study we examined the effect of a Walking Training (WT) and of Quadrato Motor Training (QMT) on NGF and BDNF precursors (proNGF and proBDNF). QMT is a specifically structured sensorimotor training that involves sequences of movements based on verbal commands, that was previously reported to improve spatial cognition, reflectivity, creativity as well as emotion regulation and general self-efficacy. In addition, QMT was reported to induce electrophysiological and morphological changes, suggesting stimulation of neuroplasticity processes. In two previous independent studies we reported QMT-induced changes in the salivary proNGF and proBDNF levels. Our present results demonstrate that following 12 weeks of daily QMT practice, proNGF level increases while proBDNF showed no significant change. More importantly, while no correlation between the two neurotrophins prior to training was detectable, there was a significant correlation between change in proNGF and proBDNF levels. Taken together the current results suggest that the two neurotrophins undergo a complex modulation, likely related to the different pathways by which they are produced and regulated. Since variations of these neurotrophins have been previously linked to depression, stress and anxiety, the current study may have practical implications and aid in understanding the possible physiological mechanisms that mediate improved well-being, and the dynamic change of neurotrophins as a result of training.

Keywords: Quadrato Motor Training, proNGF, proBDNF, neuroplasticity, neurotrophins, well-being

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INTRODUCTION

The understanding of the relationship between a healthy body and mind is of crucial relevance, but the physiological mechanisms underlying this relationship are still far from being clarified. Many studies have addressed this topic, highlighting that physical exercise can contribute to improved cognition and well-being, by directly affecting neuroplasticity (for a review see Voss et al., 2013b; Budde et al., 2016). Neuroplasticity is mediated by neurotrophic factors, among which Nerve

Growth Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF). Neurotrophins are involved in the regulation of synaptic connectivity, fiber guidance and dendritic morphology in the peripheral and central nervous system (Bibel and Barde, 2000).

These factors are synthesized as precursors, proNGF and proBDNF, and released in the synaptic space, where they undergo cleavage and maturation, following which the mature forms are internalized via the binding to specialized receptors (Chao, 2003; Lu et al., 2005). Several studies have also underlined a relevant role for the precursor proteins in mediating axonal development and synaptic plasticity (Costa et al., 2018). Different types of environmental enrichment, many of which are based on physical activity and exercise, were found to be associated with modulation of proBDNF (Neeper et al., 1995; Ploughman, 2008; Rasmussen et al., 2009; Baroncelli et al., 2010; Sigwalt et al., 2011; Voss et al., 2013a,b). Research related to training-induced dependent changes of neurotrophins is not abundant (Neeper et al., 1996; Hong et al., 2015; Okudan and Belviranli, 2017; Arvidsson et al., 2018).

Very few studies, mostly conducted in animals, examined exercise-related proNGF changes (Chae et al., 2014; Ando et al., 2016). A rare example in human are the studies on the Quadrato Motor Training (QMT), a structured sensorimotor training, that combines motor and cognitive functions, such as reflectivity and spatial cognition (Dotan Ben-Soussan et al., 2013; Ben-Soussan et al., 2014) as well as emotional well-being (Paoletti et al., 2017; Piervincenzi et al., 2017). In addition, proNGF salivary levels of adults and children were found to decrease following 4 weeks of practice. Interestingly, decreased proNGF correlated with increased ideational flexibility and creativity (Venditti et al., 2015). To the best of our knowledge QMT is the only paradigm, so far investigated in humans, for which change in the level of NGF was observed. Moreover, proBDNF salivary levels were shown to increase following 12 weeks of daily QMT. Parallel MRI examination of the subjects involved in this study, revealed significant correlation of higher proBDNF levels with increased functional connectivity and neuronal arborization (Ben-Soussan et al., 2015b).

Nonetheless, to date, a unified picture of the effects of QMT on both proNGF and proBDNF is still lacking. Consequently, in the present work, we set up a more comprehensive study, in which we conducted the molecular analysis of both proNGF and proBDNF in the same group of subjects, before and after 12 weeks of practice. The aim of the current study is to improve the understanding of neurotrophins modulation induced by QMT compared to a simple walking training (WT), and to infer the possible different involvement of the two neurotrophins in response to practice.

MATERIALS AND METHODS

Participants and Procedure

A total of 40 right handed female participants were enrolled in the study. All were healthy with no emotional or behavioral disorders, general cognitive disorders, or developmental coordination disorders or medical history that might affect their performance. This study was approved by the CNR Research Ethics and Bioethics Advisory Committee, Protocol AMMCNT-CNR n. 0080953, November 26th, 2015.

In the first visit the participants were introduced to the entire procedure, adequate understanding was tested, and the informed written consent was obtained, in accordance to the Declaration of Helsinki. The participants were randomly allocated to either the QMT or the control WT groups. The subjects were then explained the QMT or WT procedure and were first asked to donate a sample of saliva in triplicate for molecular assessments. They were also informed of the option of interrupting the training and dropping-out from the study at any time for any reasons, including changes of the clinical status that would impede continuation, refusal to continue and personal needs. The subsequent sessions of QMT or WT, were conducted at home. To check with the compliance to the exercise, subjects were asked to fill up a personal diary on daily bases to collect information about their practice and habits during the period of exercise. The collection of saliva samples was repeated in the laboratory after 12 weeks of daily training. Although the initial group size was identical ($n = 20$), the final number of participants finishing the 12 weeks of training was $n = 13$ for QMT and $n = 11$ for WT. Thus, the analyses were conducted on a total of 24 subjects finishing the 12 weeks training (mean age $\pm$ SD: 48.5 $\pm$ 10.6).

Training Groups

Quadrato Motor Training (QMT)

The QMT requires standing at one corner of 0.5 $\times$ 0.5 m square and making movements in response to a verbal command given by an audio recording. In the QMT space there are three optional directions of movement (**Figure 1**). At each corner there are three possible directions of movement, thus the training consists of 12 possible movements (3 directions $\times$ 4 corners). The entire protocol consists of 7 sequences, lasting 12 min. We used a movement sequence paced at a rate of an average of 0.5 Hz, comparable to a slow walking rate. For additional details see Dotan Ben-Soussan et al. (2013), Venditti et al. (2015).

Walking Training (WT)

The WT group was instructed to make successive steps following the auditory stimulus, keeping the same pace, duration of steps and auditory cue as the QMT, but the movement had to be free in the room space and not within the square. This group was, therefore, told to simply make the first step and then continue in response to the instructions, regardless the number specified by the recording. This reduced the uncertainty regarding the direction of the movement compared to the QMT group. The WT group was not informed about the QMT option relating the numbers to a specific location in the Quadrato space and have thus provided a control performing a task with similar motor demands, but with reduced cognitive ones.

Molecular Examination

Analysis of salivary neurotrophins is a reliable non-invasive procedure (Lee and Wong, 2009; Jang et al., 2011; Jasim et al., 2018). The choice of saliva was taken because several studies reported that neurotrophins have widespread functions in the

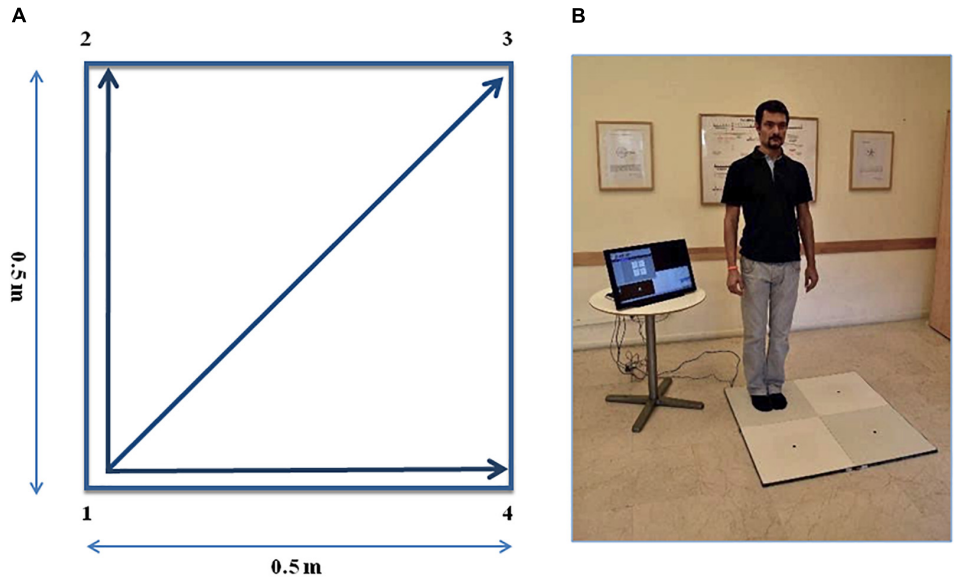

FIGURE 1 | The Quadrato Motor Training (QMT). **(A)** A graphical illustration of the QMT. **(B)** A participant to a QMT session. Written informed consent was obtained for the publication of this image from the person in the picture, who has not taken part in the current research. Adapted from Ben-Soussan et al. (2014).

organism (Rothman et al., 2012; Marosi and Mattson, 2014), that are coordinated by an active communication between brain and periphery. In most instances molecular analysis of brain markers was shown to be conducted from saliva with good reliability (Nohesara et al., 2011; Smith et al., 2015).

Saliva Samples Collection

Salivary proNGF and proBDNF were examined in triplicate to take in consideration the potential variability due to flow rate. Saliva samples were collected at day 1 and after 12 weeks in the morning between 10 and 11 am, and specific instructions were given to the participants including: avoid brushing teeth, using salivary stimulants and consuming a major meal within 1 h prior to collection, avoid consuming acidic or high sugar foods 20 min prior to collection. 10 min before collection the subject was suggested to rinse the mouth with water. Unstimulated whole saliva was collected by passive drool and stored at −80°C. Prior to electrophoresis the samples were subjected to vortex for 30 s and centrifuged at maximum speed for 15 min. Saliva supernatants were transferred to fresh tubes, a protease inhibitor cocktail was added (Roche, 04693116001) and total protein concentration was determined by Bradford assay (BIO-RAD).

Western Blot Analysis

To evaluate the neurotrophin levels in the saliva sample of the participants before and after training, 15 μg of total proteins were subjected to electrophoresis on SDS–PAGE, (4–15% precast gradient gels) under semi-denaturing conditions: samples were loaded on the gels without the previous canonical pre-heating step. Under these conditions, we obtained a better resolution of the neurotrophin protein bands following hybridization with the specific antibody, avoiding interference with the highly concentrated amylase family proteins (**Supplementary Figure S1**). Following electrophoresis, the proteins were transferred onto 0.2 mm PVDF membranes by *Trans*-Blot Turbo Blotting System (BIO-RAD) and hybridized with appropriate amounts of anti-NGF and anti-BDNF antibodies. Antibody anti-NGF: SIGMA-ALDRICH, polyclonal N6655, dilution 1:4000, recognizes both pro and mature NGF (Soligo et al., 2015). Antibody anti-BDNF: Thermo Fisher, oligoclonal 710306, dilution 1:4000, recognizes both pro and mature BDNF. Incubation with both antibodies was followed by anti-rabbit secondary antibody treatment (Jackson ImmunoResearch, 111-035-003, dilution 1:20000).

Quantification and Statistics

The whole family of bands corresponding to proBDNF and proNGF (MW: 50–100 KDa) was quantified relative to the total protein present on the membranes using Image Lab software according to manufacturer instructions. To answer the question regarding the effects of QMT on neurotrophic factors, we ran a Group (QMT, WT) × Training (pre-, post-) analysis of variance (ANOVA) separately for proNGF and proBDNF. We also ran a one-way analysis of covariance (ANCOVA). Then, the Pearson correlation between change in proNGF and proBDNF, was computed. Change in salivary neurotrophins was calculated by dividing their post-value by their pre-value.

RESULTS

proNGF Levels Following 12 Weeks of QMT

In order to evaluate the levels of proNGF in the saliva samples of the participants before and after 12 weeks of training, we

used the western blot technique. The results of this analysis are shown in **Figure 2**. In the panels A and B of **Figure 2** the representative gels for two participants, one control and one QMT, respectively, are illustrated. When looking at these two subjects, it appears that the levels of proNGF increase in the QMT samples after the training (QMT post; **Figure 2**, panel B), while the control samples shows a decrease (control post; **Figure 2**, panel A). As seen in panel A and B, proNGF is not present as a single molecular species, but as a family of bands, ranging from 50 to 100 KDa. This is due to multiple and variegated post-translational modifications, mainly glycosylation, that contribute to the heterogeneity of the molecular weights (Reinshagen et al., 2000). Because they represent different forms of proNGF, all the bands need to be necessarily taken into account when performing the quantification for each single subject. The quantitative analysis of proNGF levels was carried out for all subjects. Given non-normality of the data, these were log-transformed. The histograms in the panel C of **Figure 2** show the post/pre-ratio of the mean values for both groups, indicating a proNGF increase in the QMT group (see also the change in proNGF values for the two separate groups in **Supplementary Figure S2**).

To answer the question regarding the effect of QMT on proNGF, we ran a Group (QMT, WT) × Training (pre, post) analysis of variance (ANOVA). A significant Group × Training interaction was found for proNGF [$F(1,22) = 6.034$, $p = 0.022$]. The effect size using partial η^2 was 0.215.

We then supplemented our analysis with a one-way analysis of covariance (ANCOVA), with pre-QMT proNGF as the covariate, and post-QMT proNGF as the dependent variable. The main effect for Group was significant [$F(1,21) = 6.98$, MSE = 0.021, $p = 0.015$], with a significant proNGF post/pre-ratio increase for QMT compared to the WT group [$t(22) = -2.69$, $p < 0.05$] (see **Figure 2**, panel C).

Taken together, these results indicate that the practice of QMT for 12 weeks significantly increases proNGF, while this is not true for the WT group.

proBDNF Levels Following 12 Weeks of QMT

The same analysis conducted for proNGF by western blot, was also carried out for proBDNF, and the results are shown in **Figure 3**. In the panels A and B of **Figure 3**, two representative gels for proBDNF are displayed, from one control and one QMT subject, respectively. As observed previously for proNGF, the proBDNF profile of the bands is complex, since also this neurotrophin undergoes multiple types of post-translational modifications (Smith et al., 2015). Therefore, even in this case the quantification was done taking into account the whole family of bands. The image shows that the QMT participant presents a higher level of salivary proBDNF following training (**Figure 3**, panel B) compared to the control (**Figure 3**, panel A). The quantitative analysis of proBDNF levels was carried out for all

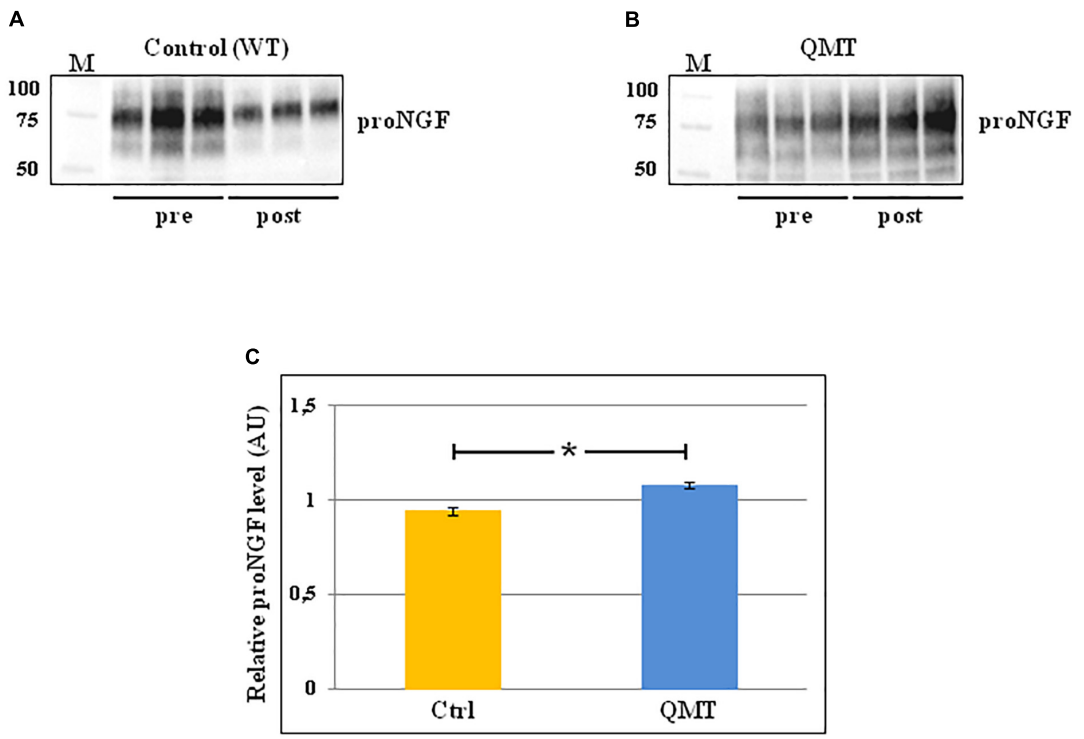

FIGURE 2 | Western blot analysis of changes in proNGF levels for QMT and WT groups. **(A)** Representative gel for one WT participant. **(B)** Representative gel for one QMT participant. **(C)** The histograms show the post/pre-ratio of the proNGF mean log values of all the participants for both groups. Error bars indicate the SEM. *$p < 0.05$. M, Molecular Weight marker.

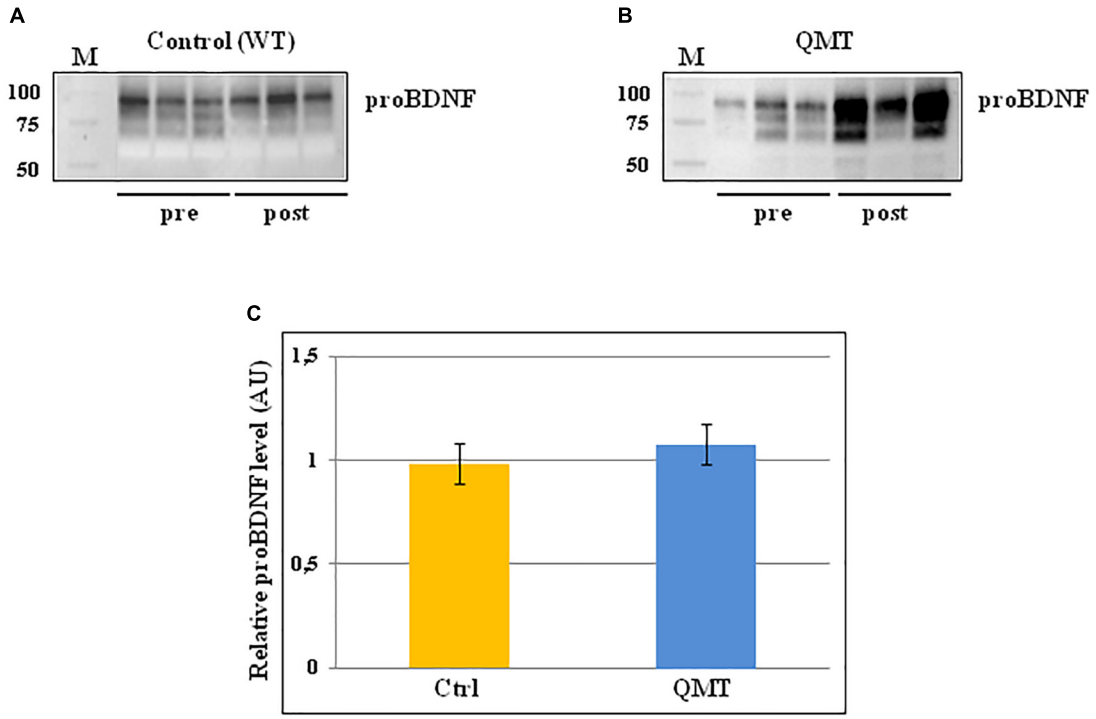

FIGURE 3 | Western blot analysis of changes in proBDNF levels for QMT and WT groups. **(A)** Representative gel for one WT participants. **(B)** Representative gel for one QMT participant. **(C)** The histograms show the post/pre-ratio of the proBDNF mean log values of all the participants for both groups. Error bars indicate the SEM. ns, not significant. M, Molecular Weight marker.

subjects. Given non-normality of the data, these were also log-transformed. The histograms in panel C of **Figure 3** show the post/pre-ratio of the mean values for both groups (see also the change in proBDNF values for the two separate groups in **Supplementary Figure S3**).

When conducting the ANOVA, no significant main effects or interaction were found [$F(1,22) = 0.19, 3.38$ *ns*]. Similarly, the main effect for Group in the ANCOVA was also not significant [$F(1,21) = 2.46$, $MSE = 0.005$, *ns*].

Correlation Between proNGF and proBDNF

We then investigated whether the change in proNGF level was correlated with change in proBDNF. While there was no correlation between proNGF and proBDNF levels prior to training ($r = 0.28$, ns), change in proNGF was significantly and positively correlated with change in proBDNF ($r = 0.49$, $p < 0.05$, $n = 24$, see **Figure 4**).

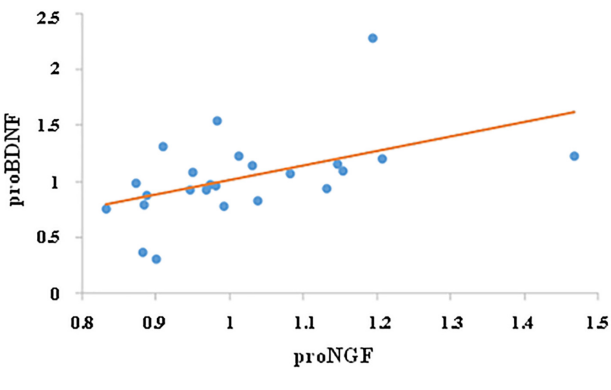

FIGURE 4 | Correlation between proNGF and proBDNF. Change in salivary neurotrophins was calculated by dividing their post-value by their pre-value. Change in proNGF was significantly and positively correlated with change in proBDNF ($r = 0.49$, $p < 0.05$, $n = 24$).

DISCUSSION

Neurotrophins play a key role in the central and peripheral nervous system, and drive neuroplasticity also during adult life. Neuroplasticity is a complex process that can be enhanced by several types of environmental enrichment, including physical and cognitive training, as well as learning-stimulating activities (Ploughman, 2008; Baroncelli et al., 2010; Voss et al., 2013a,b). While a large body of evidences have shown changes of proBDNF levels during training-induced neuroplasticity (Neeper et al., 1995; Rasmussen et al., 2009; Knaepen et al., 2010; Zoladz and Pilc, 2010), information regarding the involvement of proNGF in this process is largely lacking, with only few reports mostly on animal models (Chae et al., 2014; Ando et al., 2016).

In the present study we investigated the modulation of the salivary proNGF and proBDNF levels following 12 weeks comparing two types of training, namely QMT and WT. The main result obtained is increased proNGF following 12 weeks of QMT practice compared to the control group (**Figure 2**). The effect size was 0.215, which is considered a relatively moderate effect. While our previous work reported decreased proNGF after 4 weeks of QMT daily practice (Venditti et al., 2015), the current study suggests that performing the practice for 8 more weeks leads to a different outcome. Our hypothesis is that the proNGF decrease we previously detected after 4 weeks (Venditti et al., 2015), could be due to its fast utilization related to enhanced neuroplasticity, as supported by animal model studies (Bruno and Cuello, 2006). In turn, proNGF consumption stimulates further re-synthesis in the subsequent 8 weeks (**Figure 2**). The combination of the motor and cognitive elements in the QMT requires and reinforces dividing attention, and stimulates neuroplasiticty (Dotan Ben-Soussan et al., 2013; Ben-Soussan et al., 2015a; Lasaponara et al., 2017; Piervincenzi et al., 2017). The present results underline that it is possibly the cognitive component, absent in the WT, that allows QMT to stimulate the increase of proNGF levels. Although a trend to proBDNF enhancement was observed for the QMT group after 12 weeks of training (**Supplementary Figure S3**), it did not reach significance.

Distinct Mechanisms of Change for proNGF and proBDNF

The apparently different behavior of the two molecules could be attributed to the distinct pathways by which they are synthesized and regulated (Mowla et al., 1999). In fact, proNGF is released through both the constitutive and regulatory secretory pathways in cells from peripheral tissues and nerves (Mowla et al., 1999; Costa et al., 2018), while proBDNF follows a regulated pathway that drives its synthesis upon stimulation of neuronal activity (Lu et al., 2005).

We argue that the molecular mechanisms underlying the variation in neurotrophins secretion, induced by QMT practice, are different for the two molecules analyzed. On one hand, proNGF could decrease during the first 4 weeks of training because of enhanced processing of the protein, followed by its transcriptional increase during the subsequent 8 weeks. On the other hand, the proBDNF trend to increase could be due to different regulatory pathways and/or different timing.

Correlation Between Change in proNGF and proBDNF

Most of the evidences on exercise induced modulation of neurotrophins are related to BDNF analysis (for review see Neeper et al., 1995; Rasmussen et al., 2009; Voss et al., 2013b), a few are related to NGF (Chae et al., 2014; Ando et al., 2016), while works describing the relationship between the two are scarce. In fact, several papers have reported concomitant detection of BDNF and NGF levels changes following exercise (Neeper et al., 1996; Hong et al., 2015; Okudan and Belviranli, 2017; Arvidsson et al., 2018), without addressing the correlation between the two. Only one correlation study, outside the exercise field, was reported in rats, regarding the nervous system development, in which BDNF and NGF show reciprocal behavior during development, but parallel increase in the adult brain (Maisonpierre et al., 1990). Consequently, to better understand the possible relationship between these two neurotrophins, we examined the correlation between their changes following training.

While there was no correlation between proNGF and proBDNF before the training, a significant positive correlation was found between change in proNGF and proBDNF. One could hypothesize that the modulation of proNGF induces the variations of proBDNF or, alternatively, that the proBDNF increase stimulates the subsequent resynthesis of proNGF, though this has to be further examined.

Limitations of the Study

There are a few limitations to the study that should be noticed. The first is the small sample size, only 13 and 11 participants for the QMT and control groups, respectively. The second is that the participants performed the training at home, and we checked for the exercise performance only at the end of the experiment, by looking at the daily calendar they had to tick. In the future, a study with a larger sample size should be conducted to extend the present results, using a more efficient method monitoring compliance to the practice (such as video camera recording).

To be able to distinguish between the relative contribution of proNGF and proBDNF to either the cognitive/attentive or the motor components of QMT, it will be important, in addition to the WT group, to introduce a non-practicing control group.

CONCLUSION

The present study increases the current knowledge and understanding related to the involvement of proNGF and proBNDF in the neuroplasticity process induced by training and shows that 12 weeks of daily QMT practice increased proNGF, in contrast to a simple WT. This study highlights the relevance of analyzing molecular parameters, such as neurotrophins, and the dynamic/mutual change in their levels as a result of different motor training paradigms.

AUTHOR CONTRIBUTIONS

The entire project was designed through the concerted contribution of TB-S, LV, MC, and SV. LV, MC, SV, and VV performed the recruiting of the participants and the saliva sample collection. TB-S instructed the participants about the trainings and performed the statistical analysis of data. MC, LV, and VV performed most of the western blot analysis. LV did the quantification of results. SV contributed to the western blot analysis, participated in the interpretation of the results and wrote the manuscript. LV, MC, SV, VV, and TB-S theoretically contributed to the interpretation of the results and critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00058/full#supplementary-material

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Physical Fitness, White Matter Volume and Academic Performance in Children: Findings From the ActiveBrains and FITKids2 Projects

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Objectives: The aims of this study were (i) to examine the association between cardiorespiratory fitness and white matter volume and test whether those associations differ between normal-weight and overweight/obese children (ii) to analyze the association between other physical fitness components (i.e., motor and muscular) and white matter volume, and (iii) to examine whether the fitness-related associations in white matter volume were related to academic performance.

Methods: Data came from two independent projects: ActiveBrains project (n = 100; 10.0 ± 1.1 years; 100% overweight/obese; Spain) and FITKids2 project (n = 242; 8.6 ± 0.5 years; 36% overweight/obese, United States). Cardiorespiratory fitness was assessed in both projects, and motor and muscular fitness were assessed in the ActiveBrains project. T1-weighted images were acquired with a 3.0 T S Magnetom Tim Trio system. Academic performance was assessed by standardized tests.

Results: Cardiorespiratory fitness was associated with greater white matter volume in the ActiveBrain project (P < 0.001, k = 177; inferior fronto-opercular gyrus and inferior temporal gyrus) and in the FITKids project (P < 0.001, k = 117; inferior temporal gyrus, cingulate gyrus, middle occipital gyrus and fusiform gyrus) among overweight/obese children. However, no associations were found among normal-weight children in the FITKids project. In the ActiveBrains project, motor fitness was related to greater white matter volume (P < 0.001, k = 173) in six regions, specifically, insular cortex, caudate, bilateral superior temporal gyrus and bilateral supramarginal gyrus; muscular fitness was associated with greater white matter volumes (P < 0.001, k = 191) in two regions, particularly, the bilateral caudate and bilateral cerebellum IX. The white matter volume

of six of these regions were related to academic performance, but after correcting for multiple comparisons, only the insular cortex remained significantly related to math calculations skills ($\beta = 0.258$; $P < 0.005$). In both projects, no brain regions showed a statistically significant negative association between any physical fitness component and white matter volume.

Conclusion: Cardiorespiratory fitness may positively relate to white matter volume in overweight/obese children, and in turn, academic performance. In addition, motor and muscular fitness may also influence white matter volume coupled with better academic performance. From a public health perspective, implementing exercise interventions that combine aerobic, motor and muscular training to enhance physical fitness may benefit brain development and academic success.

Keywords: aerobic capacity, motor ability, speed-agility, muscular strength, brain structure, academic achievement, obesity, childhood

INTRODUCTION

The brain undergoes significant changes during childhood (Giedd et al., 1999). Further, aspects of cognition, including academic performance, continue to develop throughout the school-aged years. This period of neurodevelopment may be particularly sensitive to health-related factors that influence brain and behavior (Andersen, 2003; Donnelly et al., 2016). In particular, physical fitness is a powerful marker of health that has been associated with brain structure and function, as well as cognition in children (Ortega et al., 2008b; Voss et al., 2011; Esteban-Cornejo et al., 2014; Donnelly et al., 2016). The three main components of physical fitness are cardiorespiratory, motor and muscular fitness, each of them may have different influences on the brain. Cardiorespiratory fitness is the capacity to carry out prolonged strenuous exercise; motor fitness is a combination of speed, agility and coordination, and muscular fitness is the capacity to carry out work against a resistance (Ortega et al., 2008b). Specifically, we have previously shown that cardiorespiratory fitness is associated with greater gray matter volume of the hippocampus and the basal ganglia in both normal–weight and overweight/obese children (Chaddock et al., 2010a,b; Esteban-Cornejo et al., 2017). Additionally, cardiorespiratory fitness and motor fitness, but not muscular fitness, were associated with greater gray matter volume in distinct cortical regions (i.e., frontal, temporal and calcarine cortices) in overweight/obese children (Esteban-Cornejo et al., 2017). In turn, these brain-related associations were coupled with better executive function and academic performance (Chaddock et al., 2010a; Esteban-Cornejo et al., 2017). However, less is known about how the different components of physical fitness (i.e., cardiorespiratory, motor and muscular) may influence white matter tissue, and in turn, academic performance during childhood.

White matter is primarily comprised of glial cells and myelinated neurons. White matter growth is the main source of increased brain volume during child development and continues well into the second decade of life for some brain regions. While cortical gray matter seems to develop in a non-linear trend, with a preadolescent increase followed by a postadolescent decrease, white matter follows a linear trend and continues to mature during childhood and adolescence, increasing its volume and becoming more myelinated (Giedd et al., 1999; Paus et al., 2001). Damage to white matter yields slower processing speed (Kail, 1998), which may impair academic performance (van Eimeren et al., 2008). To date, only two previous studies in youth have examined the association between cardiorespiratory fitness and structure of white matter (Chaddock-Heyman et al., 2014; Herting et al., 2014). Specifically, cardiorespiratory fitness was positively related to the microstructure of white matter fiber tracts (i.e., corpus callosum, corona radiata, and longitudinal fasciculus) in children (Chaddock-Heyman et al., 2014); whereas among adolescents, cardiorespiratory fitness was negatively related to white matter microstructure in the corticospinal tract (Herting et al., 2014). As such, it is difficult to draw a conclusion from those studies due to their contradictory findings. In addition, other dimensions of fitness (i.e., muscular and motor fitness) may have differential effects on white matter, and in turn, academic performance, similar to previous reports in relation to gray matter (Esteban-Cornejo et al., 2017). Lastly, those studies were mainly focused on normal-weight populations; however, obesity has also been associated with alterations in white matter volume and integrity as compared to normal-weight individuals (Kullmann et al., 2015; van Bloemendaal et al., 2016). Indeed, the brain's volumetric structure of individuals with overweight and obesity is 10 years older compared to that of their lean peers, pointing to accelerated aging of white matter structure in overweight/obese (Ronan et al., 2016). For example, obese children have shown white matter reduction in the cerebellar peduncles and lower academic performance than their normal-weight peers (Augustijn et al., 2017; Raine et al., 2018). Therefore, there is a clear need for studies that examine the different components of fitness and their associations with white matter volume in both normal-weight and overweight/obese populations, as well as their coupled influence on academic performance to better determine the relation of health factors on brain structure and cognition during child development.

We have a unique opportunity to test these hypotheses using baseline data from two independently, relatively large, trials conducted on children in Spain and the United States: the ActiveBrains project (Spain) which includes overweight/obese children and the FITKids2 project (United States) which includes both normal-weight and overweight/obese children. As such, our main aim was to examine the association between cardiorespiratory fitness and white matter volume in these two similarly designed, yet independent, studies to better determine the consistency of the relationship between this aspect of fitness and white matter. In addition, we examined whether the abovementioned associations differ between normal-weight and overweight/obese children using data from the FITKids2 project and we analyzed the association between other physical fitness components (i.e., motor and muscular) and white matter volume using data from the ActiveBrains project. To achieve these aims, we performed whole-brain exploratory analyses because, to date, there is no a substantial body of evidence on the associations between physical fitness components and white matter in children. Lastly, we examined whether the fitness-related associations in white matter volume were related to academic performance across these two independent studies.

MATERIALS AND METHODS

Participants

The ActiveBrains and FTIKids2 projects are randomized controlled trials designed to examine the effects of an exercise program on brain, cognition and academic performance in children aged 7–11 years. For the ActiveBrains project[1], a total of 110 overweight/obese children aged 8–11 years were recruited from schools in Granada, Spain (Cadenas-Sanchez et al., 2016). Eligible children were required to: (1) be overweight or obese based on World Obesity Federation cut-off points (2) be 8–11 years-old, (3) not have any physical disabilities or neurological disorder that affects their physical performance, and (4) in the case of girls, not to have started the menstruation at the moment of the assessments. Baseline data were collected from November 2014 to February 2016. Parents or legal guardians were informed of the purpose of the study and written informed parental and child consents were obtained. The ActiveBrains project was approved by the Human Research Ethics Committee of the University of Granada, and was registered in ClinicalTrials.gov (identifier: NCT02295072).

For the FITKids2 project, a total of 252 children aged 7-9 years were recruited from schools in East-Central Illinois, United States. Eligible children were required to (1) report an absence of school related learning disabilities (i.e., individual education plan related learning), adverse health conditions, physical incapacities, or neurological disorders, (2) qualify as prepubescent (Tanner pubertal timing score $\leq$ 2), (3) report no use of medications that influence central nervous system function, and (4) demonstrate right handedness as measured by the Edinburgh Handedness Questionnaire. Data were collected from June 2010 to October 2017. Children signed an informed assent and parents or legal guardians provided written informed consent in accordance with the Institutional Review Board of the University of Illinois at Urbana-Champaign, and was registered in ClinicalTrials.gov (identifier: NCT01619826).

For the present study, we selected children from the ActiveBrains and FITKids2 projects with complete baseline data on physical fitness, academic performance and brain outcomes (i.e., white matter volume). A total of 100 overweight/obese children from the ActiveBrains project (10.0 $\pm$ 1.1 years; 40% girls) met all the criteria. A total of 142 children from the FITKids2 project (8.6 $\pm$ 0.5 years; 54% girls; 36% overweight/obese) met all the criteria; FITKids2 children included in the present study did not differ from those not included across measures of height, weight, peak height velocity (PHV), body mass index (BMI), parental education, cardiorespiratory fitness, total white matter and academic performance data (all $p > 0.05$). The present study includes a total of 242 children.

Physical Fitness

In the ActiveBrains project, physical fitness was assessed following the ALPHA (Assessing Levels of Physical fitness and Health in Adolescents) health-related fitness test battery for youth, a feasible, reliable and valid battery for this age group (Ortega et al., 2008a; Castro-Pinero et al., 2010a; Ruiz et al., 2011). All tests were performed in a single session. The three main physical fitness components were assessed: cardiorespiratory, motor and muscular fitness.

Cardiorespiratory fitness was assessed by the 20-m shuttle-run test. The test was performed once and always at the end of the fitness testing session. Participants were required to run between two lines 20-m apart, while keeping pace with a pre-recorded audio CD. The initial speed was 8.5 km/h, which was increased by 0.5 km/h each minute (1 min = 1 stage). Participants were instructed to run in a straight line, to pivot on completing a shuttle (20-m), and to pace themselves in accordance with the audio signals. The test was finished when the participant failed to reach the end lines concurrently with the audio signals on two consecutive occasions. The last stage completed was recorded and transformed to maximal oxygen consumption (VO_2max, mL/kg/min) using the Léger equation (Leger et al., 1988).

Motor fitness was assessed with the 4 $\times$ 10-m shuttle-run test of speed-of-movement, agility and coordination. The test was performed twice and the fastest time was recorded in seconds (Vicente-Rodriguez et al., 2011). Participants were required to run back and forth twice between two lines 10-m apart. Children were instructed to run as fast as possible and every time they crossed any of the lines, they were instructed to pick up (the first time) or exchange (second and third time) a sponge that had earlier been placed behind the lines. Since a longer time indicates poorer performance (i.e., the person is slower and less agile and coordinated), the variable expressed in seconds was inverted by multiplying by -1, so that a higher score indicates better performance.

Muscular fitness was assessed using maximum handgrip strength and the standing long jump tests (Artero et al., 2012).

[1] http://profith.ugr.es/activebrains

A hand dynamometer with an adjustable grip was used (TKK 5101 Grip D, Takey, Tokyo Japan) for the handgrip strength test. The participant squeezed the dynamometer continuously for at least 2-s, alternatively with right and left hand, with the elbow in full extension (Espana-Romero et al., 2010). The test was performed twice and the maximum score for each hand was recorded in kilograms (kg). The average score of the left and right hands was calculated in kg as an absolute measurement of upper body muscular fitness (Espana-Romero et al., 2008; Espana-Romero et al., 2010). Standing long jump test was performed from a starting position behind a line, standing with feet approximately shoulder width apart (Castro-Pinero et al., 2010b). Children jumped as far forward as possible, landing with feet together. The test was performed three times. The longest distance was recorded in centimeters, and subsequently multiplied by body weight in order to obtain an absolute measurement of lower body muscular fitness. A single muscular fitness score was computed from the two muscular tests. The individual score of each test was standardized as follows: *Z*-standardized value = (value – the sample mean)/SD. The muscular fitness score was calculated as the mean of the two standardized scores.

In the FITKids2 project, only cardiorespiratory fitness was assessed. It was determined by measuring VO_{2max} using a computerized indirect calorimetry system (Parvo Medics True Max 2400, Sandy, UT, United States) during a modified Balke Protocol. Children walked and/or ran on a treadmill at a constant speed with increasing grade increments of 2.5% every 2 min until volitional exhaustion occurred (American College of Sports Medicine, 2014). Average for oxygen consumption and respiratory exchange ratio (RER) assessed every 20 s. A polar heart rate monitor (Polar WearLink+ 31; Polar Electro, Finland) was used to measure heart rate throughout the test, and ratings of perceived exertion (RPE) were assessed every 2 min using the children's OMNI (Utter et al., 2002). VO_{2max} was expressed in mL/kg/min and based upon maximal effort as evidenced by (i) a plateau in oxygen consumption corresponding to an increase of less than 2 mL/kg/min despite an increase in workload; (ii) a peak heart rate $\geq$ 185 beats per minute (American College of Sports Medicine, 2014) and heart rate plateau (Freedson and Goodman, 1993); (iii) RER $\geq$ 1.0 (Bar-Or, 1983); and/or (iv) a score on the children's OMNI ratings of perceived exertion (RPE) scale $\geq$ 8 (Utter et al., 2002).

Magnetic Resonance Imaging (MRI) Procedure

Data Acquisition

Data were collected using a 3.0 Tesla Siemens Magnetom Tim Trio system (Siemens Medical Solutions, Erlangen, Germany) with a 32-channel head coil in the ActiveBrains project and a 3.0 Tesla Siemens Magnetom Tim Trio system (Siemens Medical Solutions, Erlangen, Germany) with a 12-channel head coil in the FITKids2 project. Three-dimensional, high-resolution, T1-weighted images were acquired using a magnetization-prepared rapid gradient-echo (MPRAGE) sequence. In the ActiveBrains project, the parameters were as follows: repetition time (TR) = 2300 ms, echo time (TE) = 3.1 ms, inversion time (TI) = 900 ms, flip angle = 9°, Field of view (FOV) = 256 × 256, acquisition matrix = 320 × 320, 208 slices, resolution = 0.8 × 0.8 × 0.8 mm, and scan duration of 6 min and 34 s. In the FITKids2 project, the parameters were as follows: TR = 1900 ms, TE = 2.32 ms, TI = 900 ms, flip angle = 9°, FOV = 230 × 230 acquisition matrix = 256 × 256, 192 slices, resolution = 0.9 × 0.9 × 0.9 mm, and scan duration of 4 min and 26 s.

Structural Image Processing

Structural imaging data were pre-processed using Statistical Parametric Mapping software (SPM12; Wellcome Department of Cognitive Neurology, London, United Kingdom) implemented in Matlab (The MathWorks, Inc., Natick, MA, United States). Before tissue classification we checked each individual image for acquisition artifacts and alignment along the horizontal anterior commissure and posterior commissure plane.

Detailed information about the pre-processing steps is available elsewhere and is outlined briefly in this section (Esteban-Cornejo et al., 2017). First, T1-weighted structural images of each participant were segmented into gray matter tissue, white matter tissue, and cerebrospinal fluid using the segmentation algorithm implemented in SPM12 (Ashburner and Friston, 2005). Second, we used segmented gray matter/white matter tissues for all participants to create a customized template using Diffeomorphic Anatomical Registration Through Exponentiated Lie algebra (DARTEL) (Ashburner, 2007). DARTEL estimates a best set of smooth deformations from every participant's tissue to their common average and reiterates the process until convergence. The resultant images were spatially normalized to Montreal Neurological Institute (MNI) space with affine transformation to create the DARTEL template. We create a DARTEL template for the ActiveBrains project and a DARTEL template for the FITKids2 project. Subsequently, we normalized each participant's segmented images in each study to each specific DARTEL template via non-linear transformation. To perform a volume change correction, the normalized images were modulated with Jacobian determinants derived from the spatial normalization (Ashburner and Friston, 2000). Finally, the volumetric images were smoothed by convolving them with an isotropic Gaussian kernel of 8 mm full-width at half-maximum (FWHM).

Academic Performance

In the ActiveBrains project, academic performance was assessed via the Spanish version of the Woodcock-Johnson III (i.e., Bateria III Woodcock-Muñoz, pruebas de aprovechamiento). This battery is a well validated measure of academic performance from individuals aged 5–95 years (McGrew and Woodcock, 2001). We applied 12 tests: 11 from the standard battery (i.e., 3 tests of reading, 3 tests of mathematics, 2 tests of oral language and 3 tests of written language) and one from the extended battery (i.e., a test based on science, social science and humanities). All the tests were individually administered by a trained evaluator in one session of 100–120 min. The data collected for each participant was independently checked

by two trained evaluators. In the FITKids2 project, participants completed the Kaufman Test of Educational Abilities (KTEA2) to assess academic performance. Subtests of the KTEA2 were administered to assess their achievements in the content areas of mathematics, reading, and writing. The KTEA2 was individually administrated by a trained evaluator in one session of 60–80 min. The main dependent measures in both projects were the standard scores of 6 academic indicators: mathematics, mathematics calculation skills, reading, writing, written expression and total achievement. Science was also included as an additional academic indicator in the ActiveBrians project.

Covariates

Body Mass Index

Body weight and height were performed with participants having bare feet and wearing underclothes; weight was measured with an electronic scale (ActiveBrains: SECA 861, Hamburg, Germany; FITKids2: Tanita WB-300 Plus digital scale, Tokyo, Japan) and height (cm) with a stadiometer (ActiveBrains: SECA 225, Hamburg, Germany; FITKids2: SECA 240, Hamburg, Germany). Both measurements were performed twice in the ActiveBrains project and three times in the FITIKids2 project in the same session, and averages were used. BMI was expressed as kg/m^2 and children were categorized as normal-weight, overweight and obesity according to Cole and Lobstein (2012).

Biological Maturation

Peak height velocity is a common indicator of maturity in children and adolescents and it used as a maturational landmark due to its relevance in previous studies (Malina et al., 2015). In both projects, PHV was obtained from anthropometric variables (weight, height, and/or seated height) using Moore's equations through validated sex-specific algorithms for children (Moore et al., 2015). Years from PHV were calculated by subtracting the age of PHV from the chronological age. The difference in years was defined as a value of maturity offset.

Parental Education Level

In both projects, socioeconomic status was assessed by the educational level of the mother and father reported as none, elementary school, middle school, high school and university completed. Parent responses were combined as: none of the parents with university studies, one of them had university studies and both had university studies (Huppertz et al., 2016).

Statistical Analysis

All the analyses were performed separately for the ActiveBrains project and the FITKids2 project. In the ActiveBrains project, the analyses were performed for the whole overweight/obese sample together; in the FITKids2 project, the analyses were performed separately for normal-weight and overweight/obese children. Descriptive statistics are presented as means (SD) or percentages using IBM SPSS Statistics (version 18.0 for Windows; P set at < 0.05).

Statistical analyses of imaging data were performed using the GLM approach implemented in SPM12. The individual association between each component of physical fitness (i.e., cardiorespiratory, motor and muscular in the ActiveBrains project and cardiorespiratory in the FITKids2 project) and white matter volume was analyzed using whole-brain voxelwise multiple regression models, adjusted for sex, PHV offset, parent education, and BMI. Additionally, we extracted the eigenvalues from the peak coordinates of each significant cluster. The associations of the extracted mean white matter volumes as predictor variables and academic performance indicators as outcomes, adjusted for sex, PHV offset, parental education and BMI were examined by linear regressions in SPSS. We corrected for assessing multiple white matter-academic performance regressions by defining statistical significance as a Benjamini-Hochberg False Discovery Rate q less than 0.05 (Benjamini and Hochberg, 1995).

The statistical threshold in the imaging analyses was calculated with AlphaSim, as implemented in Resting-State fMRI Data Analysis Toolkit toolbox (RESTplus) (Song et al., 2011). Parameters were defined as follows: cluster connection radius (rmm) = 5 mm and the actual smoothness of the data after model estimation, incorporating a white mask volume of 302567 voxels. The voxel-level alpha significance (threshold, $p < 0.001$ uncorrected) along with the appropriate cluster size for controlling for multiple comparisons in each analysis were indicated in the results. The resulting cluster extents were further adjusted to account for the non-isotropic smoothness of structural images, in accordance with Hayasaka et al. (2004).

RESULTS

Background Characteristics

Table 1 shows the characteristics of the study sample from the ActiveBrains and the FITKids2 projects. The percentage of both parents having completed university studies was 16% in the ActiveBrains Project, 34% in overweight/obese FITKids2 children, and 48% in normal-weight FITKids2 children. In the ActiveBrains project, all participants were overweight/obese (26% overweight children from the total overweight/obese sample). In the FITKids2 project, 64% were normal-weight, 17% were overweight and 19% were obese. BMI was higher in overweigh/obese children from the ActiveBrains project (26.7 $\pm$ 3.7 kg/m^2) relative to their overweight/obese peers from the FITKids2 project (22.5 $\pm$ 3.4 kg/m^2). Cardiorespiratory fitness levels were higher for the normal-weight FITKids2 children (45.4 $\pm$ 6.7 mL/kg/min) than for the overweight/obese FITKids2 children (37.7 $\pm$ 5.6 mL/kg/min) and the ActiveBrains children (40.8 $\pm$ 2.8 mL/kg/min).

White Matter Correlates of Individual Physical Fitness Components

Table 2 and **Figure 1** present the brain regions showing positive associations between each of the components of physical fitness and white matter volume in children, after adjustment for potential confounders.

In the ActiveBrains project, cardiorespiratory fitness (**Figure 1A1**) was associated with greater white matter volume ($P < 0.001$, $k = 177$) in two regions, inferior fronto-opercular

TABLE 1 | Characteristics of samples from the ActiveBrains and FITKids2 projects.

	ActiveBrains (n = 100)	FITKids2 (n = 142)	
	Overweight/ obese	Normal-weight	Overweight/ obese
n	100	91	51
Physical characteristics			
Girls (%)	40	54	55
Age (years)	10.0 ± 1.1	8.6 ± 0.6	8.7 ± 0.5
Peak height velocity offset (years)	−2.3 ± 1.0	−3.3 ± 0.6	−3.0 ± 0.7
Weight (kg)	55.8 ± 11.0	28.7 ± 4.0	43.8 ± 8.8
Height (cm)	143.9 ± 8.3	133.0 ± 5.8	139.0 ± 7.1
Body mass index (kg/m^2)	26.7 ± 3.7	16.2 ± 1.4	22.5 ± 3.4
Overweight (%)	26	–	47
Parental education university level (%)			
Neither parent	66	26	33
One parent	18	26	33
Both parents	16	48	34
Physical fitness components			
Cardiorespiratory fitness (mL/kg/min)*	40.8 ± 2.8	45.4 ± 6.7	37.7 ± 5.6
Motor fitness (s)†	15.1 ± 1.6	–	–
Muscular fitness (*z*-score) ‡	0.0 ± 0.9	–	–
Total brain volume (cm^3)	1200.3 ± 106.7	1199.3 ± 107.4	1217.9 ± 105.5
Academic performance**			
Mathematics	101.7 ± 10.6	109.8 ± 16.5	105.3 ± 13.6
Math calculation skills	103.4 ± 12.0	107.4 ± 16.1	102.6 ± 13.2
Reading	108.2 ± 13.0	112.8 ± 14.6	107.6 ± 15.3
Writing	113.6 ± 12.7	105.6 ± 15.5	105.0 ± 15.6
Written expression	103.4 ± 8.7	102.2 ± 17.6	102.3 ± 17.5
Science	96.6 ± 11.5	–	–
Total achievement	109.1 ± 11.8	110.4 ± 14.7	106.4 ± 14.9

*Values are mean ± SD or percentages. *Measured by the 20-m shuttle run test in the ActiveBrains project and by the treadmill test in the FITKids2 project. †Measured by the 4 × 10-m shuttle run test; values were multiplied by −1 before analyses so that higher values indicate better performance. ‡z-score computed from handgrip strength (kg) and standing long jump (cm*kg) tests. **Assessed by the Spanish version of the Woodcock-Johnson III in the ActiveBrains project and by the Kaufman Test of Educational Abilities in the FITKids2 project.*

gyrus and inferior temporal gyrus; motor fitness (**Figure 1B**) was related to greater white matter volume ($P < 0.001$, $k = 173$) in six regions, specifically, insular cortex, caudate, bilateral superior temporal gyrus and bilateral supramarginal gyrus; muscular fitness (**Figure 1C**) was associated with greater white matter volumes ($P < 0.001$, $k = 191$) in two regions, particularly, the bilateral caudate and bilateral cerebellum IX. In the FITKids2 project, among overweight/obese children, cardiorespiratory fitness (**Figure 1A2**) was associated with greater white matter volumes ($P < 0.001$, $k = 117$) in four regions, namely inferior temporal gyrus, cingulate gyrus, middle occipital gyrus and fusiform gyrus; whereas, no brain regions showed a statistically significant positive association between cardiorespiratory fitness and white matter volume among normal-weight children from the FITKids2 project. In both projects, no brain regions showed a statistically significant negative association between any physical fitness component and white matter volume.

Association Between White Matter and Academic Performance

Table 3 displays the associations between fitness-related associations in white matter volume and academic performance, after controlling for potential confounders.

In the ActiveBrains project, among the brain regions previously associated with cardiorespiratory fitness, both regions were related or demonstrated a trend with an academic indicator; inferior temporal gyrus was related to written expression ($\beta = 0.210$; $P = 0.030$) and inferior fronto-opercular gyrus was marginally related to math calculation skills ($\beta = 0.163$; $P = 0.079$). Regarding the brain regions previously associated with motor fitness, insular cortex was related to mathematics and math calculations skills ($\beta = 0.199$ and $\beta = 0.258$, respectively; both $P < 0.05$); superior temporal gyrus and supramarginal gyrus were related to science ($\beta = 0.194$ and $\beta = 0.208$, respectively; both $P < 0.05$), and supramarginal gyrus was also marginally related to mathematics, math calculation skills, reading and total achievement (β ranging from 0.154 to 0.174; all $P < 0.1$). Regarding the brain regions previously associated with muscular fitness, caudate was related to math calculation skills and science ($\beta = 0.189$ and $\beta = 0.228$, respectively; both $P < 0.05$), and marginally related to writing and total achievement ($\beta = 0.164$ and $\beta = 0.151$, respectively; both $P \leq 0.01$); cerebellum IX was related to science ($\beta = 0.199$; $P = 0.040$). However, after correcting for multiple comparisons, only the insular cortex remained significantly related to math calculations skills ($\beta = 0.258$; $P < 0.005$). In the FITKids2 project, none of the brain regions previously associated with cardiorespiratory fitness were related to academic performance.

DISCUSSION

The main finding of the present study is that cardiorespiratory fitness was positively related to white matter volume in overweight/obese children across two independent studies. In addition, other physical fitness components (i.e., motor and muscular) were also associated with white matter volume. Specifically, cardiorespiratory and motor fitness were related to white matter volume located in association fiber tracts, and muscular fitness was related to white matter regions located in thalamic radiations and projection fiber tracts. Moreover, some of these fitness-related associations in white matter volume were coupled with better academic performance. These results suggest that physical fitness might have the potential to enhance brain development and academic performance during childhood.

There are several possible explanations for the present findings. First, given that physical fitness has been previously

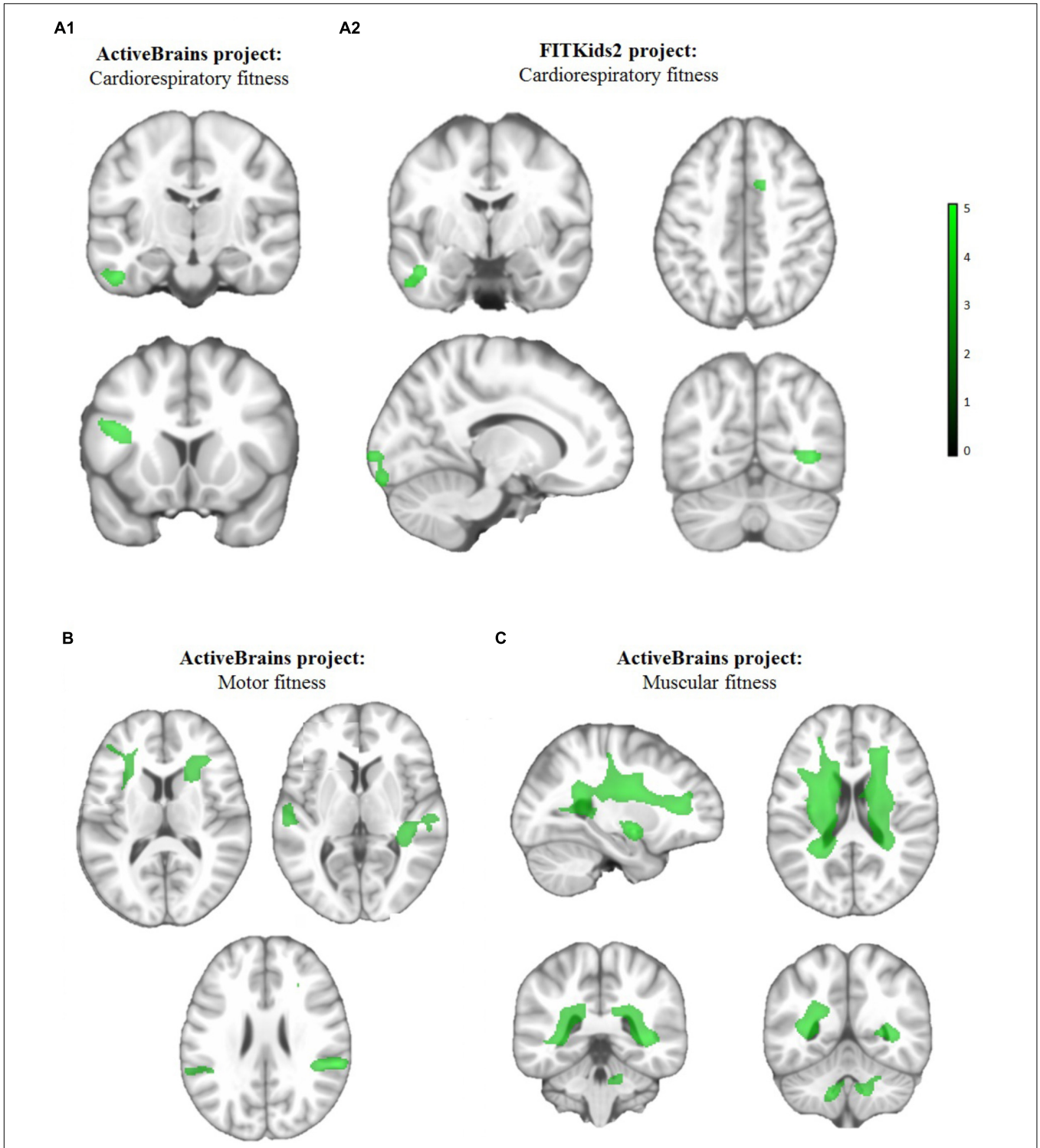

FIGURE 1 | Brain regions showing positive separate associations of **(A1, A2)** cardiorespiratory fitness, **(B)** motor fitness and **(C)** muscular fitness with white matter volume in children from the ActiveBrains **(A1,B,C)** and FITKids2 **(A2)** projects. Analyses were adjusted by sex, peak height velocity offset (years), parent education university level (neither/one/both) and body mass index (kg/m^2). Each physical fitness component was introduced in separate models. Maps were thresholded using AlphaSim at $P < 0.001$ with $k = 177$ voxels for cardiorespiratory fitness in the ActiveBrains project and $k = 117$ voxels in the FITKids2 project, $k = 173$ for motor fitness, and $k = 191$ for muscular fitness, and surpassed Hayasaka correction (see **Table 2**). The color bar represents T-values, with lighter green color indicating higher significant association. Images are displayed in neurological convention, whereby the right hemisphere corresponds to the right side in coronal displays. Sagittal planes show the left hemisphere.

TABLE 2 | Brain regions showing separate positive associations of the components of physical fitness with white matter volume in children from the ActiveBrains and FITKids2 projects.

	ActiveBrains (*n* = 100)					FITKids2 (*n* = 142)				
Brain Regions (mm^3)	***X***	***Y***	***Z***	**Peak *t***	**Cluster size**	***X***	***Y***	***Z***	**Peak *t***	**Cluster size**
Normal-weight (*n* = 91)			*n* = 0					*n* = 91		
			No available data					Non-significant regions		
Overweight/obese(*n* = 151)			*n* = 100					*n* = 51		
Cardiorespiratory fitness (mL/kg/min)*										
Inferior temporal gyrus	−53	−12	−33	4.1	312	−53	−6	−32	4.6	357
Inferior fronto-opercular gyrus	−44	17	23	4.2	552	–	–	–	–	–
Cingulate gyrus	–	–	–	–	–	12	9	44	4	138
Middle Occipital Gyrus	–	–	–	–	–	39	−66	2	4.4	342
Fusiform Gyrus	–	–	–	–	–	−26	−92	−9	4.5	951
Motor fitness (s^{-1})†										
Insular cortex	−30.0	32.0	8.0	3.7	1459	No available motor fitness data				
Caudate	33.0	30.0	12.0	3.9	1327					
Superior temporal gyrus	39.0	−32.0	5.0	3.9	1407					
Superior temporal gyrus	−56.0	−21.0	3.0	4.1	510					
Supramarginal gyrus	47.0	−38.0	29.0	5	768					
Supramarginal gyrus	−50.0	−41.0	26.0	3.6	216					
Muscular fitness (z-score)‡										
Caudate	24	26	11	4.4	13261	No available muscular fitness data				
Caudate	−12	11	−15	4.7	17512					
Cerebellum IX	14	−50	−42	3.9	755					
Cerebellum IX	−11	−51	−42	4.2	486					

*Analyses were adjusted by sex, peak height velocity offset (years), parent education university level (neither/one/both) and body mass index (kg/m^2). Each physical fitness component was introduced in separate models. All contrasts were thresholded using AlphaSim at $P < 0.001$with k = 177 voxels for cardiorespiratory fitness in the ActiveBrains project and k = 117 voxels in the FITKids2 project, k = 173 for speed-agility and k = 191 for muscular fitness, and surpassed Hayasaka correction. Anatomical coordinates (X,Y,Z) are given in Montreal Neurological Institute (MNI) Atlas space. *Estimated by the Leger equation in the ActiveBrains project and directly measured by the treadmill test in the FITKids2 study. †The original score of the motor fitness test expressed in seconds was multiplied by −1 to invert the variable, so that a higher score indicate higher fitness performance. ‡z-score computed from handgrip strength and standing long jump tests.*

related to gray matter in cortical and subcortical regions in children (Chaddock et al., 2010a,b; Esteban-Cornejo et al., 2017), it is reasonable that white matter structure connecting gray matter areas might also benefit from physical fitness. Indeed, white matter might be a neural mechanism via which physical fitness enhances integration of regions into networks and facilitates efficient transmission of information to support executive function and academic performance (Mabbott et al., 2006). Second, mouse models show that exercise, a major determinant of physical fitness, increases the number of oligodentrocytes, which are the cells responsible for myelinating axons in white matter tissue (Krityakiarana et al., 2010). Lastly, other biological mechanisms triggered by exercise, such as increased neurotrophic factors and vascularization, have been shown to influence white matter in rodents (Ding et al., 2006). Therefore, based on previous evidence from children and animal models, the positive associations between physical fitness and white matter found in overweight/obese children are neurologically and biologically plausible.

The major finding of the present report suggests that cardiorespiratory fitness may influence white matter volume in overweight/obese children across two independent, relatively large studies conducted in Spain and the United States. To note, although we focused on white matter volume, we superimposed the findings in a white matter tracts atlas for easier comparing and discussing present study with previous studies. Specifically, white matter brain regions that overlap between the ActiveBrains and FITKids project were located in the superior longitudinal fasciculus. Indeed, in the ActiveBrains project, all the white matter brain regions influenced by cardiorespiratory fitness were only located in the superior longitudinal fasciculus, whereas the white matter regions found in the FITKids2 project were located in the superior longitudinal fasciculus and the inferior longitudinal fasciculus. Both superior and inferior longitudinal fasciculi are long association fiber tracts that connect more distant cortical areas within the same cerebral hemisphere, converging in the parietal lobe, and may be involved in common pathways (Schmahmann et al., 2008). The superior longitudinal fasciculus links the frontal and parietal lobes, and may be engaged in attention, inhibition and articulatory aspects of language (Schmahmann et al., 2008; Chaddock-Heyman et al., 2013). The inferior longitudinal fasciculus links the parieto-occipital and temporal lobes, and has been shown to be engaged in object recognition, discrimination and memory (Schmahmann et al., 2008). Therefore, although only one brain region located in the superior longitudinal fasciculus overlapped between the ActiveBrains and FITKids2 projects, possibly due to methodological differences between studies (e.g.,

TABLE 3 | Fitness-related associations in white matter volume and academic performance† in overweight/obese children from the ActiveBrains and FITKids2 projects.

Fitness-related component	Brain Regions (mm^3)	Mathematics		Math calculation skills		Reading		Writing		Written expression		Science		Total achievement	
		b	*P*	b	*P*	b	*P*	b	*P*	b	*P*	b	*P*	b	*P*
ActiveBrains															
Cardiorespiratory fitness (mL/kg/min)	Inferior temporal gyrus	0.034	0.718	−0.006	0.950	−0.095	0.320	0.071	0.465	**0.207**	**0.030**	0.144	0.140	−0.010	0.913
	Inferior fronto-opercular gyrus	0.114	0.217	*0.163*	*0.079*	−0.014	0.886	0.030	0.756	0.036	0.710	0.091	0.350	0.035	0.705
Motor fitness (s^{-1})	Insular cortex	**0.199**	**0.031**	**0.258**	**0.005***	0.063	0.511	0.116	0.233	0.057	0.553	0.150	0.122	0.132	0.155
	Caudate	0.054	0.561	0.093	0.317	0.034	0.720	0.030	0.755	0.100	0.917	0.108	0.264	0.041	0.657
	Superior temporal gyrus	0.132	0.155	0.156	0.093	0.113	0.237	0.080	0.412	0.035	0.719	**0.194**	**0.044**	0.139	0.133
	Supramarginal gyrus	*0.155*	*0.094*	*0.154*	*0.098*	*0.174*	*0.065*	0.083	0.393	0.074	0.443	**0.208**	**0.031**	*0.166*	*0.071*
Muscular fitness (z-score)	Caudate	0.134	0.147	**0.189**	**0.040**	0.107	0.260	*0.164*	*0.087*	0.090	0.346	**0.228**	**0.017**	*0.151*	*0.100*
	Cerebellum IX	0.112	0.230	0.118	0.208	0.142	0.136	0.020	0.838	0.117	0.225	**0.199**	**0.040**	0.115	0.215
FITKids2															
Cardiorespiratory fitness (mL/kg/min)	Inferior temporal gyrus	0.114	0.485	−0.041	0.802	0.092	0.556	−0.043	0.780	−0.016	0.916	–	–	0.117	0.455
	Cingulate gyrus	−0.025	0.889	−0.113	0.521	0.003	0.986	−0.056	0.741	−0.069	0.677	–	–	0.031	0.857
	Middle Occipital Gyrus	0.153	0.365	0.061	0.715	0.070	0.664	0.041	0.799	0.016	0.919	–	–	0.112	0.490
	Fusiform Gyrus	0.192	0.237	0.136	0.398	0.094	0.545	−0.132	0.392	−0.142	0.348	–	–	0.086	0.585

*Values are standardized regression coefficients (β). Analyses were adjusted by sex, peak height velocity offset (years), parent education university level (neither/one/both) and body mass index (kg/m^2). †Academic performance was assessed by the Spanish version of the Woodcock-Johnson III in the ActiveBrains project and by the Kaufman Test of Educational Abilities in the FITKids2 project. Statistically significant values are shown in bold ($P < 0.05$), and borderline significant values are shown in italics ($P < 0.1$); *This was the only association that remained significant when P-values were adjusted for multiple comparisons using the Benjamini and Hochberg method to control for the false discovery rate.*

measurement of cardiorespiratory fitness, differences among the MRI scanners or the MRI sequence acquisition parameters, participant demographics, etc.), the fact that this finding was observed across the two different studies conducted on children strengthens conclusions regarding the specificity of the relations between cardiorespiratory fitness and white matter.

The analysis of the different components of physical fitness, assessed in the ActiveBrains project, allows us to further speculate on the relevance of each fitness component for white matter structure. Our data suggest that not only cardiorespiratory fitness, but also other components, such as motor and muscular fitness, were related to white matter volume among overweight/obese children. This effect in children supports and extends the associations between cardiorespiratory fitness and white matter in the older adults (Sexton et al., 2016). Among youth, there are only two studies examining the association between cardiorespiratory fitness and white matter structure in normal-weight youth and these studies exhibited contradictory findings (Chaddock-Heyman et al., 2014; Herting et al., 2014). Herting et al. (2014) revealed that cardiorespiratory fitness was negatively related to white matter microstructure in the corticospinal tract in male adolescents aged 15–18 years (Herting et al., 2014). In contrast, Chaddock-Heyman et al. (2014) found that cardiorespiratory fitness was positively related to the microstructure of white matter fiber tracts (i.e., body of the corpus callosum, superior corona radiata, and superior longitudinal fasciculus) in 9–10 year old children. The present findings comparing data from the ActiveBrains and FITKids2 project partially concur with the previous report from Chaddock-Heyman et al. (2014) since we found that the white matter brain regions predicted by cardiorespiratory fitness were mainly located in the superior longitudinal fasciculus and the inferior longitudinal fasciculus in overweight/obese children of the same approximate ages. However differences in white matter assessment (i.e., white matter volume vs. white matter microstructure) and in cardiorespiratory fitness levels (i.e., lower fit children vs. higher and lower fit children) among the present and the previous study should be acknowledged. Therefore, our findings shed light on the implications of cardiorespiratory fitness for brain health during childhood.

Specifically, the abovementioned cardiorespiratory fitness-white matter associations were only found in overweight/obese children, but not in their normal-weight peers. The reasons explaining why cardiorespiratory fitness might improve white matter volume only in overweight/obese children cannot be elucidated from the current datasets; yet two mechanisms might be speculated upon. First, moving an excessive amount of body mass is related to various musculoskeletal complaints associated with movement restrictions and motor difficulties during childhood (Paulis et al., 2014), which may have harmful implications for the musculoskeletal system, affecting skeletal neuromuscular function and the brain (Kullmann et al., 2015). For example, obese children have shown lower motor competence and white matter reduction in the cerebellar peduncles than their normal-weight peers (Augustijn et al., 2017). Experimental studies using mice exposed to prolonged movement restrictions has also shown negative effects on neurogenesis and the role of trophic determinants (i.e., nerve growth factor mRNA and brain-derived neurotrophic factor) involved in this phenomenon (Desaphy et al., 2005; Adami et al., 2018). Consequently, overweight/obese children may benefit more from increased cardiorespiratory fitness. Second, in overweight/obese children, not only is there an excess of body mass, but there is also an excess in a particular type of mass (i.e., fat mass vs. lean mass) which may have implications for maturation. That is, overweight/obese children demonstrate higher levels of fat mass than their normal-weight counterparts (Herda et al., 2018), which may confer additional white matter reductions (Kullmann et al., 2015). For example, when comparing correlations between total white matter volume and fat mass among normal-weight and overweight/obese children from the FITKids2 project, we found that fat mass was not associated with white matter in normal-weight children, but it was negatively associated with white matter in overweight/obese children. Therefore, the positive contributions of cardiorespiratory fitness to white matter might be more apparent in individuals with white matter reductions, such as overweight/obese children.

There is no previous evidence linking the other two physical fitness components, motor and muscular fitness, with white matter structure in youth, which hampers comparisons to other studies. The novel findings observed herein indicated that motor fitness was related to greater white matter volume in brain regions located in association fiber tracts (i.e., inferior fronto-occipital fasciculus and superior longitudinal fasciculus), and specifically, with larger white matter regions in the inferior fronto-occipital fasciculus. The inferior fronto-occipital fasciculus is the major white matter tract linking the ventrolateral and medial orbitofrontal cortices to the posterior parietal and occipital cortices. As such, greater white matter volume in brain regions located in this fasciculus could contribute to better prefrontal functioning, and in turn academic performance (Wu et al., 2016). Additionally, we found that muscular fitness was associated with greater white matter volume in brain regions mainly located in thalamic radiations (i.e., anterior thalamic radiation) and projection fiber tracts (i.e., middle cerebellar peduncle) in overweight/obese children. The anterior thalamic radiation is involved in reciprocal communication of limbic regions with prefrontal and anterior cingulate cortex, and the peduncle connects the cerebellum and other parts of the brain (Mori et al., 2005). Whereas these two tracts have been previously shown to be involved in higher-order motor tasks and influence cognitive inhibition (Glickstein and Doron, 2008; Chaddock-Heyman et al., 2013), we provide new support that muscular training aimed at improving upper- and lower-body muscular strength may influence white matter regions located in those motor tracts, and ultimately, academic performance during childhood. However, more research is warranted to understand how different components of physical fitness may effect white matter structures in both normal-weight and overweight/obese children.

Another interesting finding from the present study revealed that the white matter volume of regions related to fitness may influence academic performance. White matter helps

enhance efficiency of neural transmission throughout the brain, and is thought to contribute to enhanced processing speed and executive function resulting in improvements of academic performance (Mabbott et al., 2006). In particular, a previous study in children aged 7–9 years suggested that microstructure in left white matter tracts (i.e., superior corona radiata and inferior longitudinal fasciculus) were related to better mathematical skills (van Eimeren et al., 2008). In addition, Li et al. (2013) showed that the inferior fronto-occipital fasciculus was related to better arithmetic scores. Consonant with those findings, we found that after correcting for multiple comparisons, motor fitness-related changes in white matter volume located in the left inferior fronto-occipital fasciculus was the only region related to better academic performance (i.e., math calculation skills) among overweight/obese children. As such, this finding must be interpreted with caution. Taken together, these findings raise the possibility that a reduction of physical activity opportunities across the school day might confer white matter reduction coupled with academic failure in children.

Limitations and Strengths

Some limitations need to be considered. First, the cross-sectional design does not allow us to draw causal inferences, therefore these findings should be taken with caution; it is also possible that children with higher academic performance, had greater white matter volume and then performed better on physical fitness tests. Moving forward, it is important to replicate these preliminary findings using randomized controlled intervention studies. Second, since we approached the study with voxel-based morphometry, future studies should employ diffusion tensor imaging to examine white matter microstructure using a whole brain approach. Third, while both studies used a 3.0 Tesla Siemens Magnetom Tim Trio system, the head coils differed between studies, with a 32-channel head coil in the ActiveBrains study and a 12-channel head coil in the FITKids2 project. Previous studies have indeed shown signal-to-noise ratio improvements in the cortex for 32-channel head coils compared to 12-channel coils; these differences across coils are reversed for subcortical regions (Kaza et al., 2011). However, the fact that we observed a similar pattern of results across studies with different scan parameters could be seen as a strength as it speaks to the robustness of the observed effects. Because these and other methodological differences between the ActiveBrains and FITKids2 studies exist (e.g., assessment of cardiorespiratory fitness, the MRI sequence acquisition parameters, participant demographics, etc.), data from both projects were analyzed separately instead of pooling the datasets. While this approach limits the power of our findings, it offers replication across and the opportunity to qualitatively compare across studies. Lastly, some confounding variables that may influence the findings (e.g., diet, sleep, or self-discipline) were not available in both projects. Strengths of the present report include the use of data from two independent relatively large studies which speaks to the robustness of the observed findings, the complete and standardized assessment of the three physical fitness components, the whole-brain analysis, and the entire range of the BMI distribution among participants.

CONCLUSION

In conclusion, our findings across two independent studies suggest that cardiorespiratory fitness may positively relate to white matter volume in overweight/obese children, and in turn, academic performance. In addition, other physical fitness components (i.e., motor and muscular) may also influence white matter volume coupled with better academic performance. Specifically, cardiorespiratory and motor fitness were related to white matter volume in brain regions located in association fiber tracts and muscular fitness was related to white matter regions located in thalamic radiations and projection fiber tracts. From a public health perspective, implementing exercise interventions that combine aerobic, motor and muscular training to enhance physical fitness may benefit brain development and academic success.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of 'name of guidelines, name of committee' with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Human Research Ethics Committee of the University of Granada and the Institutional Review Board of the University of Illinois at Urbana-Champaign.

AUTHOR CONTRIBUTIONS

IE-C had full access to all of the data in the studies. CH, FO, AK, KE, and AC conceived and designed the study. IE-C, LC-H, LR, CC-S, JM-G, and MR-A acquired the data. IE-C contributed to statistical analysis. IE-C, CS, MR-A, and JV-R interpreted the data. IE-C drafted the manuscript. All authors critically revised the manuscript for important intellectual content.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Choice of Sports Affects Mental Rotation Performance in Adolescents

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This study investigates mental rotation performance of adolescent female dancers and soccer players in object-based and egocentric mental rotation tasks using human body stimuli. 60 young females, 30 soccer players, and 30 dancers (not twosome), completed a chronometric mental rotation task with object-based and egocentric transformation of male and female figures, which were displayed either in front or back view. During their sport-specific activity soccer-players and dancers very often have to adapt their movements to the movement of a partner or opponent, soccer-players especially in front view positions. While for soccer-players reaction time (RT) often is crucial for sporting success, dancers mainly focus on the accuracy of their movements. Therefore, we expect significantly faster RTs for soccer players for front view stimuli but no differences between soccer players and dancers for back view stimuli. The main result was that soccer-players showed a significantly shorter RT than dancers for stimuli presented in front view in object based and egocentric transformation. There was no such difference, when the stimuli were presented in the back view. Contrary to literature we didn't find significantly higher RTs and error rates for stimuli presented in front view compared to back view in general but only for egocentric transformations. The results of this study show that specific sports affect individual aspects of mental rotation performance.

Keywords: mental rotation, sports, object-based transformations, egocentric transformations, dancing, soccer, visual-spatial abilities

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INTRODUCTION

Physical activity and sports not only benefit bodily constitution, but also cause positive effects on different parts of cognition (Sibley and Etnier, 2003). Enhanced physical activity promotes neuromodulation and the adaption of functional and structural properties of the human brain (Draganski et al., 2004). In sports science meta-analysis data showed a positive relation between motor and cognitive performance for children and adults (Etnier et al., 2006; Raine et al., 2018). Athletes performed better in executive functions (Vestberg et al., 2012), processing speed (Voss et al., 2009) and attention performance (Hüttermann and Memmert, 2014) compared to non-athletes. Especially in the area of visual-spatial capacity, athletes exhibited superior performance in spatial tasks compared to physically inactive people (Pietsch and Jansen, 2012; Voyer and Jansen, 2017). But most studies concerning the relation of spatial and motor performance bother with the influence of general physical activity or sports on visual-spatial abilities as a whole (Tlauka et al., 2008; Steggemann et al., 2011; Voyer and Jansen, 2017), while

the influence of individual sports disciplines on different forms of mental rotation performance requires more precise research. In order to specify prior findings in this study we investigate the specific relationship between different sports and specific kinds of mental rotation tasks with embodied stimuli, namely object-based and egocentric transformations in front and back view in an adolescent sample. These results contribute to specify the relation of motor and visuo-spatial processes in greater detail and give a first hint that mental rotation performance of athletes depends on the sport- specific demands of visual perception.

Mental rotation, which is defined as the mental representation and rotation of objects (Shepard and Metzler, 1971; Linn and Petersen, 1985), is one of the best-investigated spatial abilities. Classic chronometric mental rotation tasks (cMRTs) usually contain two-dimensional (letters, animals, hands) or three-dimensional stimuli (cube figures) rotated in picture-plane and/or picture-depth, which are presented in object-based or egocentric transformation tasks (Zacks et al., 2002; Reed et al., 2006).

In object-based transformations two either identical (that is non-mirror reversed) or different (mirror-reversed) versions of a stimulus are presented side-by-side on a screen. Regarding egocentric transformations, mostly a picture of a single human figure with the left or the right arm raised and rotated in different angular disparities is presented on the middle of the screen. Typically, there exists an increase in response time with ascending angular disparity between the two presented stimuli or the presented stimulus and the stimulus in upright position (Shepard and Metzler, 1971; Wohlschläger and Wohlschläger, 1998; Jola and Mast, 2005). Stimuli presented in front view (facing the participant) resulted in higher reaction times (RTs) and error rates compared to presented in back view, facing away from the participant (Jola and Mast, 2005). This was explained by the additional in-depth rotation.

Independent of the type of stimuli (embodied vs. non-embodied) the left-right-decision participants have to reach in egocentric transformations, induces an internal, embodied experience (Steggemann et al., 2011), while same-different tasks lead to object-based transformations (Voyer et al., 2017).

Object-based and egocentric mental rotation tasks also differ concerning their processing strategies (Jola and Mast, 2005; Keehner et al., 2006). In object-based transformations participants mentally rotate the stimulus like an object with a fixed observer's position. That means that the relationship between the environment and the participant's egocentric frame of reference does not change. In contrast to this, in egocentric transformations participants shift their own perspective to solve the task. To make a left–right decision, participants rotate the representation of the own body (Devlin and Wilson, 2010).

Many studies proved the relation of spatial and motor skills and the trainability of spatial performance through a long term motor activity (see meta-analysis of an experts effect Voyer and Jansen, 2017): quasi-experimental studies showed that physically active people generally seem to have better visual-spatial skills than physically inactive people (Moreau et al., 2012; Pietsch and Jansen, 2012). Experimental studies further verified that there was a positive impact of manual motor training on mental rotation performance that is not bound to a trained object, but improved the process of mental rotation itself (Wiedenbauer and Jansen-Osmann, 2008). Even the practice of specific types of sport like wrestling for 10 month significantly enhanced mental rotation performance of students compared to a control group with running training (Moreau et al., 2012). However, not all types of sport seem to have the same impact on mental rotation performance. Athletes like gymnasts or wrestlers, who have to connect visuospatial and kinesthetic processes during their sporting activity exhibited a better mental rotation performance than athletes with mainly cardiovascular sporting disciplines like running (Moreau et al., 2011; Schmid et al., 2016). In contrast to this, elite team sport athletes didn't show better mental rotation performance of human and abstract figures compared to non-athletes or recreational athletes (Jansen et al., 2012; Heppe et al., 2016).

Research has consistently demonstrated a gender gap in mental rotation performance (Geiser et al., 2008; Hambacha et al., 2014). Men outperform women in typical tasks like the Mental Rotation Test (Peters et al., 1995) which is mostly explained by biological, experiential, and individual factors (Terlecki and Newcombe, 2005; Moé and Pazzaglia, 2006; Yang et al., 2007). To investigate and control a potential influence of gender-stereotype sports on mental rotation performance, in this study we especially compared young female soccer-players and dancers. Soccer mainly sets high visual-spatial challenges on perception and anticipation in terms of the position of teammates, opponents and the ball, especially of that teammates in front view, which are potential passing partners. Further, compared to non-athletes, soccer-players showed faster RTs for mental rotation tasks with embodied stimuli (Jansen et al., 2012). In contrast to that, for dancers movement accuracy of one's own movement compared to that of a teacher, which is positioned in back view to the dancer, is more important for learning new movement sequences, than RT.

The main goal of this study is to investigate differences in mental rotation performance of adolescence female soccer-players and dancers using object-based and egocentric human body stimuli in front and back view to specify the impact of specific forms of physical excellence on certain cognitive skills.

We expect significantly faster RTs for soccer players for front view stimuli but no differences between soccer players and dancers for back view stimuli. Therefore, we don't generally estimate significantly shorter RTs for back view stimuli compared to front view stimuli. While soccer players have to deal with opponents in front view positions during training and competition, dancers, while learning new movement sequences, mainly view their coaches or peer dancers in back view position.

Furthermore, we hypothesize a higher accuracy rate of the dancing-group for object-based transformations tasks with stimuli presented in the back view. One of the most important tasks of adolescent dancers is to adopt new movement sequences precisely by observing and imitating the movements of a teacher who usually is positioned in the same perspective as the learning dancer.

If there is a difference in androgyny assessment between soccer players and dancers, we expect differences in mental rotation performance in favor of the soccer players especially

for male stimuli. Further, we estimate significantly faster RTs for egocentric compared to object-based transformations (Kaltner et al., 2014) and an increasing RT with increasing angular disparity of the stimuli (Wohlschläger and Wohlschläger, 1998; Jola and Mast, 2005).

MATERIALS AND METHODS

Participants

Sixty young females (30 non-twosome dancers and 30 soccer players) aged between 13 and 18 years participated in this study. Participants were recruited from different soccer and dancing sport clubs in Germany. Four participants had to be excluded from the calculation because they did also practice the sports of the other group. The "soccer group" consists of 28 girls (mean age = 15.29, *SD* = 1.35), the "dancing group" comprises 28 girls (mean age = 15.54, *SD* = 1.13). There was no difference in the mean age of both groups, $F(1,54) = 0.558$, n.s. Neither differed the groups in the amount of years, they practice their specific sports [soccer-group: $M = 7.94$, $SD = 3.12$; dancing: $M = 9.00$, $SD = 2.73$, $F(1,54) = 1.800$, n.s.], nor in the sporting hours per week [soccer-group: $M = 4.18$, $SD = 1.47$; dancing: $M = 4.43$, $SD = 2.85$), $F(1,54) = 0.170$, n.s.].

To make sure that dancers and soccer-players do not differ with respect to their cognitive abilities, the *Number Connection test* [Zahlenverbindungstest (ZVT), Oswald and Roth, 1987] was applied to measure cognitive processing speed and executive functions. The test consists of four sheets, on each sheet the numbers 1 to 90 are presented in a matrix in random order. All numbers have to be connected as accurately and fast as possible in ascending order. ZVT-scores are generated by measuring the time for connecting the numbers and are then transferred to corresponding IQ-values. The correlation with other tests of intelligence (e.g., Raven-SPM, CFT-30) moves between $r = 0.60$ to 0.80 (Vernon, 1993). The internal consistency of this test is about 0.90 to 0.95. The results showed no differences in IQ between soccer and dancing group, $F(1,54) = 1.779$, $p = 0.188$, $\eta_p^2 = 0.032$.

To measure differences in gender role identity all participants completed a German version of the Bem Sex-Role-Inventory (BSRI) (Schneider-Düker and Kohler, 1988) and a German version of the Self-Compassion-Scale (SCS) (Hupfeld and Ruffieux, 2011). The BSRI includes questions concerning self-concept and self-attribution of gender-specific features. No significant differences arose between the dancing- and soccer-group, $F(1,54) = 3.630$, $p = 0.062$, $\eta_p^2 = 0.063$. Even the SCS, a measurement tool for self-compassion which has been proven as an effective protective factor, promoting emotional resilience, showed no significant differences between the soccer- and dancing-group, $F(1,54) = 0.921$, $p = 0.342$, $\eta_p^2 = 0.017$.

None of the participants had taken part in a test of mental rotation performance before. All participants gave written informed consent prior to participation. The study was executed in accordance with the declaration of Helsinki for the guidelines of ethical considerations. Ethical approval for this study was not required in accordance with the conditions outlined by the German Research Society (DFG) where research that carries no additional risk beyond daily activities does not require Research Ethics Board Approval. We communicated all considerations necessary to assess the question of ethical legitimacy of the study.

Material

The cMRT was conducted on a laptop with a 17″ monitor, which was located approximately 60 cm in front of the participants. Stimuli were presented by using the software "Presentation" (Neurobehavioral Systems). There was an object-based and an egocentric stimulus condition with male and female stimuli presented in front and back view. In the object-based condition, two same-gender persons with either the left or the right arm raised both in front or back view were shown (see **Figure 1**). In the egocentric condition, one picture of a male or female person in front or back view was displayed, also with the left or the right arm raised (see **Figure 2**). There were two blocks of object-based and egocentric condition, with all blocks containing front and back view stimuli as well as male and female stimuli. The order of the blocks was counterbalanced.

Object-Based vs. Egocentric Transformations

Two male or two female figures of the same person were presented side-by-side in the center of the computer screen in the object-based condition (see **Figure 1**). Requiring a same-different judgment, the comparison figure on the left side was always displayed upright in the normal chirality while the right stimulus was presented in eight different angular disparities (0, 45, 90, 135, 180, 225, 270, 315°). The right figure was rotated in the picture plane in clockwise direction. Half of the trials consisted of identical pairs of objects and in the other half mirror-reversed images were presented. The left and the right stimulus always were displayed both either in front or back view. For the egocentric condition, only one male or female figure was shown in one of the eight orientations mentioned above. For half of the trials the figure raised the left arm while for the other half of the trials the figure was presented with a raised right arm

FIGURE 1 | Example of the stimuli used for the different conditions. Object-based transformation of a female stimulus in front view condition (disparity of 180°). Written informed consent was obtained for the publication of this image (Kaltner and Jansen, 2018).

FIGURE 2 | Example of the stimuli used for the different conditions. Egocentric transformation of a male stimulus in back view condition (0° condition). Written informed consent was obtained for the publication of this image.

(see **Figure 2**). Thus, a left–right decision was required from the participant.

Procedure and Experimental Design

The individual test session, which lasted about 75 min, took place in a laboratory at University or in a quiet room in the sports club. First of all, the participants filled out a demographic questionnaire, followed by the Number Connection Test (Oswald and Roth, 1987), the FKB, the SCS, the BSRI and the cMRT (see **Figures 1**, **2**) with a standardized task instruction. The experiment contained 12 blocks of 32 experimental trials each, resulting in 384 trials (192 object-based, 192 egocentric) in total. The order of stimulus presentation was randomized.

For the object-based conditions participants had to press the left mouse button (left click) for identical (that is only rotated) stimuli and the right mouse button (right-click) when the two stimuli were "different," that means a mirror version of each other. For the egocentric transformations, participants pressed the left mouse button when the left arm of the figure was raised and respectively the right mouse button when the right arm was raised (see Kaltner et al., 2014).

A fixation cross was displayed at the beginning of each trial. After that, a single figure in the egocentric condition or two human stimuli in the object-based condition appeared and stayed on the screen until participants pressed the mouse button. Within the practice trials for correct responses a "+" appeared in the center of the screen and for incorrect responses a "−" appeared. The next trial began after 1500 ms. During each block, after every 32 trials a pause of 15 s was given.

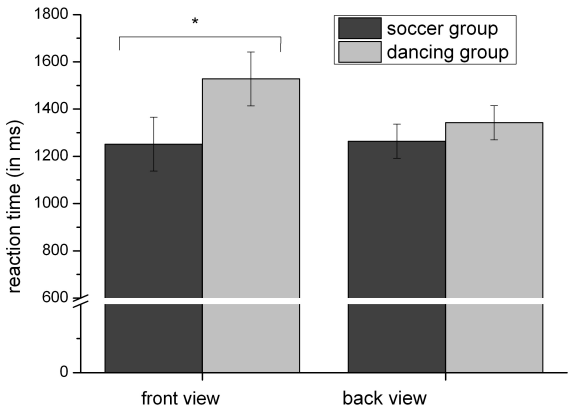

FIGURE 3 | Reaction time (Mean and SE) dependent on group and view: soccer-players show significantly faster reaction times (RTs) for front view stimuli compared to dancers. *Indicates a significant difference between both groups.

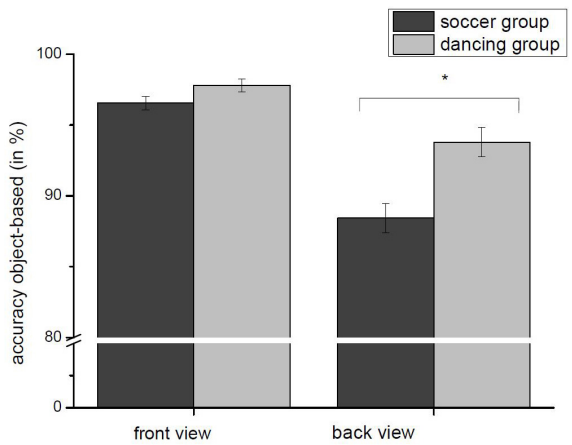

FIGURE 4 | Accuracy (Mean and SE) dependent on group and view for the object-based transformation. Dancers show a significantly higher accuracy rate for back view stimuli compared to soccer-players for object-based transformation. *Indicates a significant difference between both groups.

Statistical Analysis

Reaction time data were trimmed within subjects and means were calculated. Data of no participant had to be excluded because of RTs, which were longer than 2 standard deviations above the mean of the specific stimulus. As mentioned in the participants section, data of two dancers and two football-players were excluded, because in contrast to all other participants, they additionally did other sports similar to dancing (soccer-players) or soccer (dancers) besides their main sports.

Only correct trials were included in the analyses. Due to fact that the dancers and soccer-players showed no significant difference in the Bem Sex-Role-Inventory and the Self-Compassion Scale, the gender of the stimuli was not considered as a factor. Furthermore, we excluded all responses for the mirrored

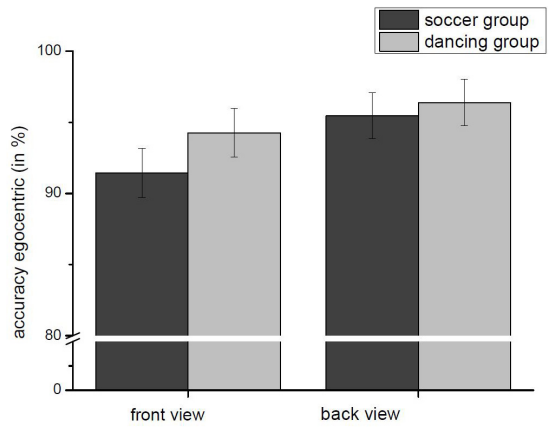

FIGURE 5 | Accuracy (Mean and SE) dependent on group and view for the egocentric transformation. No significant differences between soccer-players and dancers for front and back view stimuli for egocentric transformation.

trials from the analysis, since for mirror-reversed responses angular disparity is not clearly defined (Jolicæur et al., 1985).

Influence of the Factors Angular Disparity, View and Kind of Transformation on Reaction Time and Accuracy

Two repeated measurement ANOVAs with "reaction time" and "accuracy rate" as dependent variables were conducted with the between subject factor "group" and the three within-subject factors "angular disparity" (0, 45, 90, 135, 180, 225, 270, 315°), "view" (front vs. back), and "kind of transformation" (object-based vs. egocentric). The significant interactions were analyzed further with *t*-tests. Due to the multiple-testing, we alpha-corrected in line with Bonferroni, resulting in a corrected significance level of $p < 0.00625$ for the interaction with angular disparity.

RESULTS

Reaction Time

Regarding RT, repeated-measures analysis of variance showed main effects of "kind of transformation," $F(1,54) = 7.080$, $p = 0.01$, $\eta_p^2 = 0.116$, and "angular disparity," $F(7,378) = 9.607$, $p < 0.005$, $\eta_p^2 = 0.151$.

Furthermore, there were two two-way interactions between (a) "view" and "group," $F(1,54) = 4.302$, $p < 0.05$, $\eta_p^2 = 0.074$, and (b) "kind of transformation" and "view," $F(1,54) = 4.306$, $p = 0.05$, $\eta_p^2 = 0.074$.

Concerning our hypotheses, the two-way interaction between "view" and "group" was most relevant. The results revealed that for stimuli presented in the front view, there was a significant difference between dancers and soccer-players, $t(54) = -2.113$, $p < 0.05$. Soccer-players ($M = 1251.49$ ms, $SD = 347.78$) showed a shorter RT than dancers ($M = 1527.99$ ms, $SD = 425.16$). There was no such difference, when the stimuli were presented in the back view, $t(54) = -0.754$, $p = 0.454$, see **Figure 3**.

Accuracy

We found a significant main effect for the factor "angular disparity," $F(7,378) = 2.348$, $p < 0.005$, $\eta_p^2 = 0.127$, and a significant main effect for the factor "view," $F(1,54) = 12.091$, $p = 0.001$, $\eta_p^2 = 0.183$. Further, there were two two-way interactions between (a) "kind of transformation" and "view," $F(1,54) = 71.363$, $p < 0.001$, $\eta_p^2 = 0.569$ and (b) "angular disparity" and "group," $F(7,378) = 2.348$, $p < 0.05$, $\eta_p^2 = 0.42$. Regarding the second interaction, the *t*-tests (Bonferroni corrected) showed, that only the group difference for 225° was significant, $t(54) = -2.889$, $p = 0.006$, all others failed to reach significance (all $p > 0.625$).

Further we found one three-way interaction between "kind of transformation," "view," and "group," $F(1,54) = 7.737$, $p < 0.005$, $\eta_p^2 = 0.125$. Dancers showed a significantly higher accuracy rate for object-based stimuli presented in back view, $t(54) = -3.654$, $p = 0.001$, but not in front view, $t(54) = -1.870$, n.s.) There was no significant difference between both groups for egocentric transformations, see **Figure 4** in front view, $t(54) = -1.165$, n.s., and back view, $t(54) = -0.405$, n.s., see **Figure 5**.

DISCUSSION

In the context of specific types of sports we compared mental rotation performance of female adolescents in front and back view with object-based and egocentric transformations. In contrast to previous studies (Ozel et al., 2004; Heppe et al., 2016) we matched two sports with different challenges in visual cognition concerning the position of group members and/or opponents and one's own body posture. Additionally we tested only female soccer-players and dancers to avoid the factor of gender differences in mental rotation performance.

Regarding RT, we confirmed the frequently verified faster RTs for egocentric compared to object-based transformations and an increase of RT with increasing angular disparity (Jola and Mast, 2005). Contrary to Kaltner et al. (2014) we didn't find a general front-view disadvantage, that means, participants didn't generally show longer RTs for stimuli facing the participant. This holds true for object-based as well as for egocentric conditions. This finding was specified in the main and novel result of our study: soccer-players showed no difference in RT between front and back view stimuli, while dancers revealed slower RTs in front view compared to back view stimuli. Furthermore, soccer-players showed a faster RT than dancers for front view stimuli. This result suggests that the frequency and velocity in which athletes during their sporting activity have to deal with group members and/or opponents in a specific view (front/back) influences the performance in specific mental rotation tasks. While soccer players mainly have to observe facing teammates or opponents and their constantly changing distance (Jendrusch, 2009). Dancers mostly follow the movements of an instructor or group members with the same stance. For object-based as well as for egocentric transformations we found no RT difference between soccer-players and dancers. This may be due to the fact, that athletes generally show shorter RTs concerning signal transmission via visual pathways especially for complex tasks

(Zwierko et al., 2010; Heirani et al., 2012). Additionally during their sporting activity both athletes have to modify their body motions to ensure an upright position during and after fast and difficult movements and have to compare their position with that of partners respectively opponents.

Furthermore, we verified a significantly higher accuracy rate of the dancing-group for object-based transformations tasks with stimuli presented in the back view. Even this result is in accord with the specific requirements of dancing. One of the most important tasks of adolescent dancers is the learning of different basic movement sequences like positions of the feet, arm postures, turns, and jumps (Schack, 2010). To precisely adopt new single and complex movement sequences it is necessary to exactly observe and imitate the movements of the dancer in front of you who usually is positioned in the same perspective as the learning dancer. Thereby the observation accuracy of different body parts is much more important than RT while imitating the new movement. Motor experts are able to adapt their movements even during fast and complex exercises on the basis of incoming perceptual information (Bardy and Laurent, 1998). Especially young dancers often practice and perform in a group and have to adapt their own movement as precisely as possible to the movement of their group members. In contrast to that, for soccer-players it is much more important to observe and react as fast as possible with a freely selectable effective movement (Andok et al., 2001), which could explain the superiority of dancers in accuracy for object-based transformations tasks with stimuli presented in the back view. This result could change for older dancers, especially ballet dancers, which frequently practice and perform long movement sequences all alone and therefore mainly concentrate on their own movements. This could effect a shift of performance in direction of egocentric transformations, which induce an internal, embodied experience (Steggemann et al., 2011). Additionally, frequent exercises in front of a mirror or a teacher instructing face to face in many cases, which normally occurs in advanced dancers, could lead to an accuracy advantage for dancers even in front view object-based transformations.

Those results certainly also would differ if we had tested twosome dancers, because they have to assimilate their dancing steps and body movements to that of their partners in front view position. Additionally types of sport without a partner like swimming or running should show the typical pattern of front-view disadvantage. Even different performances for object-based and egocentric transformation could be found, if athletes of sports without partners or opponents like high diving are compared with team sport athletes.

This is one of the first studies in motor and mental rotation development, which was conducted with adolescents. The high plasticity of the adolescent brain among others facilitates physical exercise to provoke particularly strong effects on cortical development (Konrad et al., 2013). Compared to adult athletes a high temporal amount in training is used for different basic exercises. It is one of the most important targets during the training of adolescent athletes to create a wide and profound sport specific technical and tactical basis and to develop, stabilize and modify basic movement patterns. Concerning to Munzert et al. (2009) real movements and motor mental imagery, that plays an important role for a successful motor result, share the same representations. While dancers learn new movement sequences by adjusting their own movement to that of a person, which is positioned in the same position as the dancers in front of them (back view), soccer training of adolescents contains many different forms of dribbling, passing and kicking tasks. It is one of the main goals to observe and anticipate the movement of teammates and opponents and the distance of the ball as well as to recognize passing structures and keep the orientation on the playing field (Paillard and Noé, 2005). Especially important for a successful passing or shot on goal is the position of team members and opponents, which are facing them (front view). Even during typical training exercises the opposite player mostly is situated in front view position (Brüggemann and Albrecht, 2003). Based on the sport-specific tasks of soccer, adolescent female soccer-players showed an improved mental rotation performance especially for front view tasks compared to adolescent dancers.

To extend the approach that specific types of sport affect different visual-spatial abilities the typology of spatial skills by Uttal et al. (2013) that emphasizes the intrinsic and extrinsic dimension of spatial skills as well as static and dynamic visual-spatial abilities is a valuable basis. For extrinsic visual-spatial abilities the importance of visual perception, like a better exploitation of the visual field in peripheral vision or a high binocular distance eye-sight, which is typical for game sports (Matos and Godinho, 2005; Jendrusch, 2006) is undeniable. In contrast to this, the quality of intrinsic visual-spatial abilities is based on somatosensory information, which is important in sports like artistic gymnastics or wrestling (Perrin et al., 2000). Pietsch (2018) provides a first attempt for the classification of different types of sports in a visual-spatial taxonomy for sports.

The appearance of differences in visual-spatial performance already in adolescents emphasizes actual neuroscientific findings concerning the reorganization of the brain in adolescence, which goes in line with a greater strengthening of structural and functional brain networks and improved cognitive skills (Konrad et al., 2013; Dumontheil, 2016). Our study supports the hypothesis, that specific sports make a different impact on singular visual-spatial abilities.

Limitations

To summarize, our findings indicate, that specific aspects of mental rotation performance can be affected by different forms of physical activity. To range these results in a wider context further types of sports and even male participants have to be tested. Even the results of adult athletes especially of gender-stereotype sports have to be included. Silverman et al. (2007) ascertained that females' spatial abilities are extremely vulnerable to attitudinal and experiential factors. While strenuous and aggressive, competitive team sports like soccer are typically estimated as male sports, dancing, gymnastics or figure skating are classified as typical female sports (Schmalz and Kerstetter, 2006). Because gender-based schematic processing has been shown to affect attitudes and behavior (Chalabaev et al., 2013), the choice of typical male or female sports should be considered as a

factor for mental rotation performance. In our study the results of the Bem Sex-Role Inventory barely didn't reach a significant level, which may be caused by the young age of the participant or the group size. Therefore, a reproduction of this study with female adults and a major sample would be desirable.

CONCLUSION

Our results give a first hint that mental rotation performance of athletes depends on the sport- specific demands of visual perception. The typical RT disadvantage of front view stimuli can be erased by specific forms of motor training. The more precise examination of the impact of specific types of sports on different mental rotation tasks proved to be an interesting research topic that needs further investigation.

AUTHOR CONTRIBUTIONS

SP, PJ, and JL: study concept and design. SP and JL: acquisition of the data. SP and PJ: analysis and interpretation of the data. SP: drafting of the manuscript. PJ and JL: critical revision of the manuscript. PJ and SP: statistical analysis.

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Tyrosine as a Mechanistic-Based Biomarker for Brain Glycogen Decrease and Supercompensation With Endurance Exercise in Rats: A Metabolomics Study of Plasma

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Brain glycogen, localized in astrocytes, produces lactate as an energy source and/or a signal factor to serve neuronal functions involved in memory formation and exercise endurance. In rodents, 4 weeks of chronic moderate exercise-enhancing endurance and cognition increases brain glycogen in the hippocampus and cortex, which is an adaption of brain metabolism achieved through exercise. Although this brain adaptation is likely induced due to the accumulation of acute endurance exercise–induced brain glycogen supercompensation, its molecular mechanisms and biomarkers are unidentified. Since noradrenaline synthesized from blood-borne tyrosine activates not only glycogenolysis but also glycogenesis in astrocytes, we hypothesized that blood tyrosine is a mechanistic-based biomarker of acute exercise–induced brain glycogen supercompensation. To test this hypothesis, we used a rat model of endurance exercise, a microwave irradiation for accurate detection of glycogen in the brain (the cortex, hippocampus, and hypothalamus), and capillary electrophoresis mass spectrometry–based metabolomics to observe the comprehensive metabolic profile of the blood. Endurance exercise induced fatigue factors such as a decrease in blood glucose, an increase in blood lactate, and the depletion of muscle glycogen, but those parameters recovered to basal levels within 6 h after exercise. Brain glycogen decreased during endurance exercise and showed supercompensation within 6 h after exercise. Metabolomics detected 186 metabolites in the plasma, and 110 metabolites changed significantly during and following exhaustive exercise. Brain glycogen levels correlated negatively with plasma glycogenic amino acids (serine, proline, threonine, glutamate, methionine, tyrosine, and tryptophan) ($r < -0.9$). This is the first study to produce a broad picture of plasma metabolite changes due to endurance exercise–induced brain glycogen supercompensation. Our findings suggest that plasma glycogenic amino acids are sensitive indicators of brain glycogen levels in endurance exercise. In particular, plasma tyrosine as a precursor of brain noradrenaline might be a valuable mechanistic-based biomarker to predict brain glycogen dynamics in endurance exercise.

Keywords: endurance exercise, brain glycogen, supercompensation, plasma biomarker, metabolomics

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INTRODUCTION

Brain glycogen localized in astrocytes plays the role of an energy source and/or signaling factor in maintaining neuronal functions, such as memory formation and exercise endurance (Suzuki et al., 2011; Matsui et al., 2017; Magistretti and Allaman, 2018). Chronic exercise enhances memory functions and endurance capacity as well as raises the glycogen levels of the cortex and hippocampus in healthy and type II diabetic rats (Matsui et al., 2012; Shima et al., 2017). This metabolic adaptation of the brain, due to chronic exercise, should be induced by the accumulation of brain glycogen supercompensation after an acute exercise-induced decrease in brain glycogen (Matsui et al., 2011, 2012, 2017). Therefore, brain glycogen dynamics during and following exercise can be a valuable parameter for exercises as training/conditioning for athletes and/or a therapeutic strategy for neurodegenerative diseases.

To date, however, the understanding of human brain glycogen metabolism is still less clear. A previous study using biopsy samples reported that hippocampal glycogen levels were higher compared to other brain regions in patients with epilepsy (Dalsgaard et al., 2007). However, since brain biopsy procedures carry the risk of parenchymal hemorrhage, it is difficult to use in healthy or vulnerable people (Beynon et al., 2018). Although a non-invasive measurement for brain glycogen metabolism has been developed using *in vivo* nuclear magnetic resonance (NMR) in healthy people and type I diabetes patients (Oz et al., 2009, 2017), human brain glycogen metabolism during exercise remains unclear because the head movement can cause noise preventing the accurate measurement of brain glycogen using *in vivo* NMR (Oz et al., 2009). To resolve this issue, non-invasive identification of biomarkers that can predict brain glycogen dynamics with exercise is desirable.

Interestingly, in astrocytes, noradrenaline synthesized from blood-borne tyrosine, activates glycogenolysis through cyclic adenosine monophosphate (cAMP) production in a matter of minutes (Magistretti et al., 1981), but takes hours to induce glycogen synthesis and supercompensation through the expression of protein targeting to glycogen (PTG) (Sorg and Magistretti, 1992; Allaman et al., 2000; Ruchti et al., 2016). We have previously reported that acute endurance exercise decreases brain glycogen levels associated with increased brain tyrosine and noradrenaline levels (Matsui et al., 2011, 2017). We thus hypothesized that blood tyrosine is a mechanistic-based biomarker for the decrease and supercompensation of brain glycogen with acute endurance exercise.

To test this hypothesis, we employed a rat model of acute endurance exercise, high-power microwave irradiation for accurate detection of brain glycogen (10 kW) (Kong et al., 2002; Matsui et al., 2011), and plasma metabolomics using capillary electrophoresis-mass spectrometry (CE-MS). Biomarkers for various disorders, including various types of cancers, have been identified in previous studies using metabolomics, which can analyze comprehensive metabolites (Tomita and Kami, 2012). Its utility has been demonstrated by identifying new biomarkers for prostate cancer (Sreekumar et al., 2009), Parkinson's disease (Bogdanov et al., 2008), type 2 diabetes mellitus (Wang et al., 2005), acute myocardial ischemia (Sabatine et al., 2005), and non-alcoholic fatty liver disease in humans and rodents (Soga et al., 2006, 2011).

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250–300 g) (SLC Inc., Shizuoka, Japan), housed and cared for in an animal facility, were fed a standard pellet diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and given water *ad libitum*. The room temperature was maintained between 22 and 24°C under a 12 h light–12 h dark cycle (lights on: 0700–1900). Fifteen rats were habituated to run on a treadmill (SN-460, Shinano, Tokyo, Japan) for five sessions over 6 days, 30 min/day. The running speed was gradually increased from 5 to 25 m/min (see **Supplementary Table S1**; Matsui et al., 2011, 2012, 2017; Nishijima et al., 2012).

Surgery

Surgery was performed according to methods described by Soya et al. (2007). After habituation to treadmill running, the rats were anesthetized with isoflurane, and a silicone catheter was inserted into the jugular vein and fixed with a silk thread (32 mm). The external distal end of the catheter was fixed at the animal's nape.

Endurance Exercise

Two days after surgery, rats were fasted for 2 h before exercise to obtain stable metabolic conditions. They were exercised to exhaustion on a treadmill at 20 m/min, which is defined as moderate intensity around the lactate threshold in the rat mode of exercise (Ohiwa et al., 2006; Soya et al., 2007; Okamoto et al., 2012; Inoue et al., 2015; Shima et al., 2017). Exhaustion was considered to be achieved when the rat was no longer able to keep pace with the treadmill and when the rat laid flat on the treadmill, and stayed on the grid positioned at the back of the treadmill for a period of 30 s despite being gently pushed with sticks or breathed on (Matsui et al., 2011, 2012, 2017).

Sacrifice of Animals and Tissue Extraction

We collected tissue samples according to a previous study confirming a decrease and supercompensation of brain glycogen with exercise (Matsui et al., 2012). At the pre-exercise, immediately after exercise (post-0 h), and 6 h after exercise (post-6 h), the rats were anesthetized with isoflurane in a bell jar for less than 1 min and sacrificed using focused microwave irradiation (MI) (10 kW, 1.2 s; NJE-2603, New Japan Radio Co., Ltd., Tokyo, Japan). Previous studies have confirmed that brain glycogen levels are unchanged by this duration of isoflurane challenge (Kong et al., 2002; Matsui et al., 2011). After MI, brain tissues (cortex, hippocampus, and hypothalamus) were collected according to Hirano et al. (2006). Skeletal muscle (plantaris) and blood were also collected. All samples were stored at −80°C until analysis.

Blood Glucose and Lactate Assays

Blood glucose and lactate levels were measured using an automated glucose-lactate analyzer (2300 Stat Plus, Yellow Springs Instruments, United States).

Glycogen Assay

Tissues were homogenized with a bead homogenizer (MS-100R, TOMY, Tokyo, Japan) in ice-cold 6% perchloric acid (PA) containing 1 mM EDTA. The glycogen contained in 100 μl aliquots of homogenate was hydrolyzed to glucose by incubation for 3 h at 37°C with 1 ml 0.2 M sodium acetate, 20 μl 1.0 M $KHCO_3$, and 20 U/ml amyloglucosidase. The addition of 0.5 ml PA stopped the reaction. After centrifugation (14,000 × *g* for 10 min at 4°C), the supernatant was neutralized with a solution consisting of 3 M KOH, 0.3 M imidazole, and 0.4 M KCl. The sample was then centrifuged (16,000 × *g* for 10 min at 4°C) and the supernatant was assayed for glucose content. Non-hydrolyzed samples were used to measure endogenous glucose levels. These samples were homogenized and centrifuged (14,000 × *g* for 10 min at 4°C), and the pH of the supernatants was adjusted to 6–8 using the KOH solution. The neutralized samples were then mixed thoroughly, centrifuged (16,000 × *g* for 10 min at 4°C), and assayed for endogenous glucose levels. The glucose assay was performed in 96-well plates using a coupled enzyme assay method modified from previous studies (Matsui et al., 2011, 2012).

Metabolomics by Capillary Electrophoresis-Time-of-Flight Mass Spectrometry

Capillary electrophoresis-time-of-flight mass spectrometry was conducted by Human Metabolome Technologies Co., Ltd., (Yamagata, Japan) to determine the metabolomics (Sugiura et al., 2011). Each frozen sample was homogenized in methanol (500 mL/100 mg tissue) using a bead homogenizer (Micro Smash MS-100R; TOMY, Tokyo, Japan), followed by the addition of an equal volume of chloroform and 0.4 times the volume of Milli-Q water. After centrifugation (3 cycles at 5,000 × *g* for 60 s), the aqueous phases were ultrafiltered using an ultrafiltration tube (Ultrafree-MC, UFC3 LCC; Millipore, United States) and the filtrates were dried. The dried residues were redissolved in 50 mL Milli-Q water and were used for CE-MS. CE-MS experiments were performed using Agilent CE systems equipped with a time-of-flight mass spectrometer (TOF-MS) and a built-in diode-array detector (Agilent Technologies, Santa Clara, United States). Cationic metabolites were analyzed using a fused-silica capillary (50 mm i.d., 680 cm total length) with cation buffer solution (Human Metabolome Technologies) as the electrolyte. The samples were injected at a pressure of 5.0 kPa for 10 s (approximately 10 nL). The applied voltage was set at 30 kV. Electrospray ionization mass spectrometry (ESI-MS) was conducted in the positive ion mode, and the capillary voltage was set at 4,000 V. The spectrometer was scanned from m/z 50 to 1,000. Other conditions were the same as in the cation analysis (Soga and Heiger, 2000). Anionic metabolites were analyzed using a fused-silica capillary (50 mm i.d., 680 cm total length), with anion buffer solution (Human Metabolome Technologies) as the electrolyte. The samples were injected at a pressure of 5.0 kPa for 25 s (approximately 25 nL). The applied voltage was set at 30 kV. ESIMS was conducted in the negative ion mode, and the capillary voltage was set at 3,500 V. The sample in the spectrometer was scanned from m/z 50 to 1,000. Other conditions were the same as described for the anion analysis. Metabolites in the samples were identified by comparing the migration time and m/z ratio with authentic standards, and differences of 60.5 min and 610 ppm, respectively, were permitted. The identified metabolites were quantified by comparing their peak areas with those of authentic standards using ChemStation software (Agilent Technologies).

The metabolomics data were adopted for principal component analysis (PCA) and hierarchical cluster analysis (HCA) using software by Human Metabolome Technologies. Data were also visualized on a metabolome-wide pathway map for glycolysis and the TCA cycle supported by VANTED software (Junker et al., 2006).

Statistical Analysis

Data are expressed as mean ± standard error and were analyzed using Prism 5 (MDF Co., Ltd., Tokyo, Japan). Group comparisons were performed using a one-way ANOVA with Tukey's *post hoc* tests. Correlations were calculated using Pearson's product-moment correlations. Statistical significance was assumed at P-values <0.05.

RESULTS

Endurance Exercise Induces Decrease and Supercompensation in Brain Glycogen

Rats were exercised on the treadmill until exhaustion (20 m/min, time to exhaustion: 89.5 ± 5.2 min). Blood lactate was significantly increased by endurance exercise ($P < 0.01$) and recovered to basal levels within 6 h after exercise (**Figure 1A**). Blood glucose levels were significantly decreased by endurance exercise ($P < 0.01$) and recovered to basal levels within 6 h after exercise (**Figure 1B**). Muscle glycogen levels were depleted by endurance exercise ($P < 0.01$), and it recovered to basal levels within 6 h after exercise (**Figure 1C**). Brain glycogen levels in the cortex, hippocampus, and hypothalamus were significantly decreased by endurance exercise ($P < 0.01$), but were replenished to higher levels in comparison to the pre-exercise group ($P < 0.05$) (**Figure 1C**).

Plasma Metabolomics in Exercising Rats

Plasma metabolomics measured 186 metabolites and revealed that 110 metabolites changed significantly with endurance exercise (**Supplementary Table S2**). PCA and HCA clearly indicated the difference in metabolic profiles between pre-exercise, post-0 h, and post-6 h (**Figure 2**).

A scatter plot of the fold change of the overlapping metabolites in each condition was generated (**Figure 3**). This figure shows

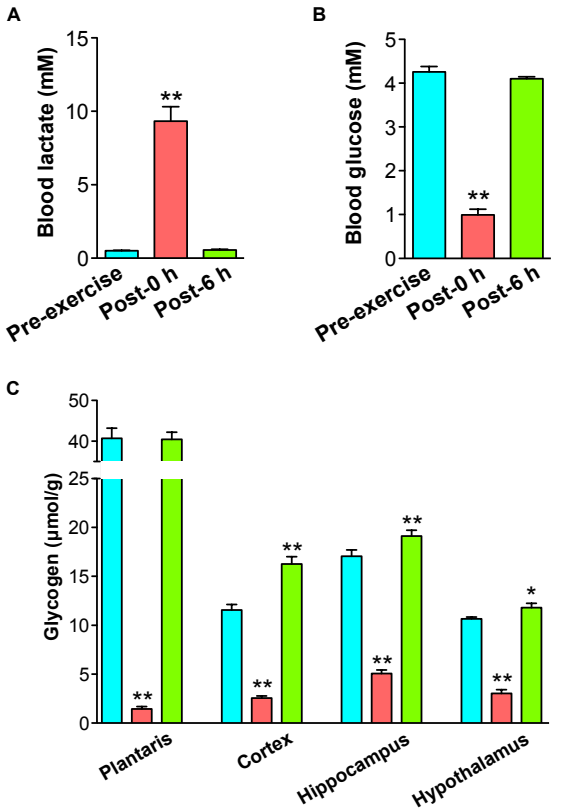

FIGURE 1 | Endurance exercise induces a decrease and supercompensation in brain glycogen. **(A)** Blood lactate levels. **(B)** Blood glucose levels. **(C)** Glycogen levels in the plantaris muscle, cortex, hippocampus, and hypothalamus. Data are expressed as mean ± standard error (n = 5/group). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. pre-exercise group.

that 23 metabolites, including glycogenic amino acids (such as aspartate, tyrosine, and tryptophan), increased immediately after exercise (post-0 h) and were decreased 6 h after exercise (post-6 h). Additionally, a metabolite, acetoacetate, decreased immediately after exercise (post-0 h) and increased 6 h after exercise (post-6 h).

Correlation Between Biomarker Candidates and Brain Glycogen Levels

Correlation analysis between 24 plasma biomarker candidates, including glycogenic amino acids and acetoacetate, and brain glycogen levels in the cortex, hippocampus, and the hypothalamus showed that all candidate metabolites were significantly correlated ($P < 0.05$). Furthermore, glycogenic amino acids (serine, proline, threonine, glutamate, methionine, tyrosine, and tryptophan) showed stronger correlations ($r < -0.9$, $P < 0.05$) (**Figures 4–6**).

DISCUSSION

This study tested the hypothesis that blood tyrosine is a mechanistic-based biomarker that predicts a decrease and supercompensation of brain glycogen with acute endurance exercise. We first reproduced a rat model of endurance exercise to induce a decrease and supercompensation of brain glycogen (**Figure 1**). Our metabolomics of plasma samples from rats, that underwent endurance exercise, showed that plasma glycogenic amino acids, including tyrosine, were increased during exercise and were decreased following exercise associated with brain glycogen dynamics (**Figures 2–6**). These findings support our present hypothesis.

We confirmed hypoglycemia, blood lactate elevation, muscle glycogen depletion, and brain glycogen decrease due to exercise, in the post-0 h group (**Figures 1A–C**). These phenomena are fatig factors that have been reported by previous studies on prolonged exercise in rodents and humans (Gollnick et al., 1974; Nybo and Secher, 2004; Matsui et al., 2011, 2012), indicating the validity of our rat model for acute endurance exercise. During moderate intensity of endurance exercise, glycogen levels in type II fibers or the plantaris muscle, which

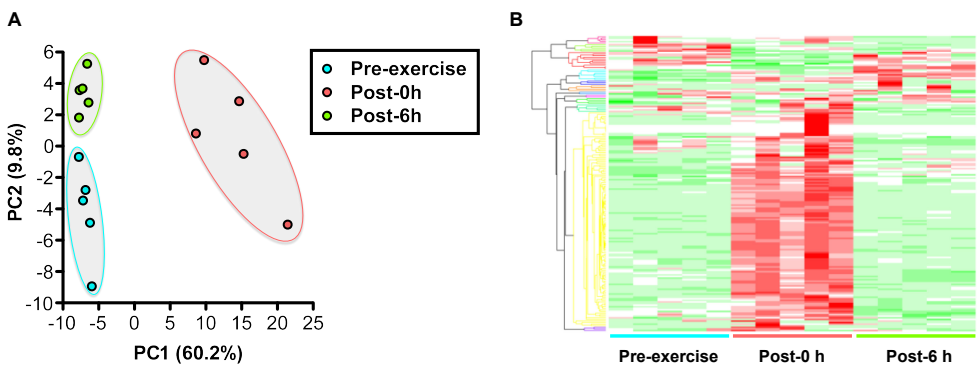

FIGURE 2 | Changes in the plasma metabolic profile with endurance exercise. **(A)** Principal component analysis (PCA) of metabolomics. **(B)** Hierarchical cluster analysis (HCA) of metabolomics. A total of 186 metabolites were measured, revealing that 44 metabolites were changed significantly with endurance exercise. PCA and HCA clearly indicated the difference of metabolic profiles between pre-exercise, post-0 h, and post-6 h.

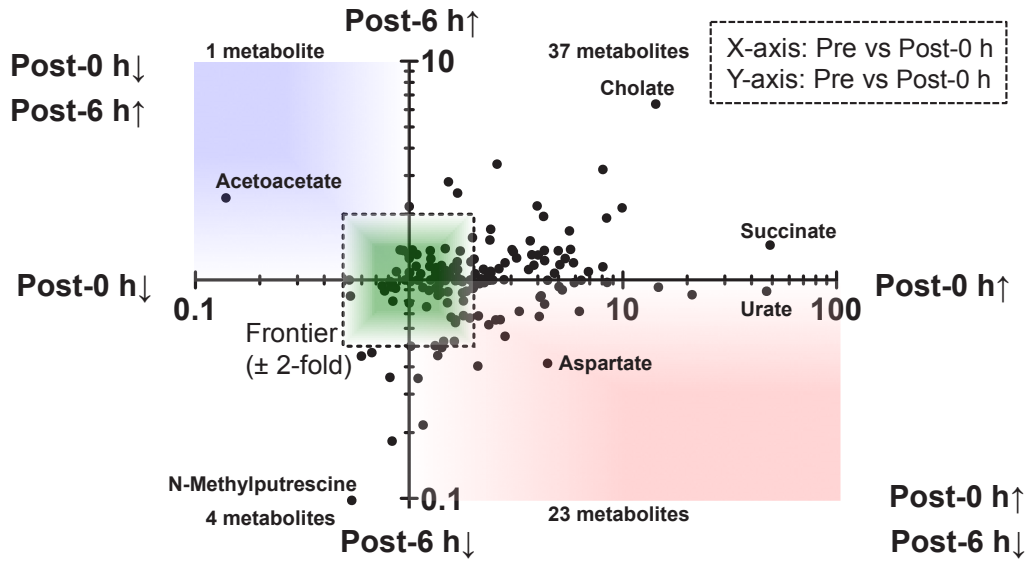

FIGURE 3 | A scatter plot of the fold change of the overlapping metabolites. This figure showed that 23 metabolites, including glycogenic amino acids (such as aspartate, tyrosine, and tryptophan) increased immediately after exercise (post-0 h) and decreased 6 h after exercise (post-6 h) (lower-right quadrant). Also, a metabolite, acetoacetate, decreased immediately after exercise (post-0 h) and increased 6 h after exercise (post-6 h) (upper-left quadrant).

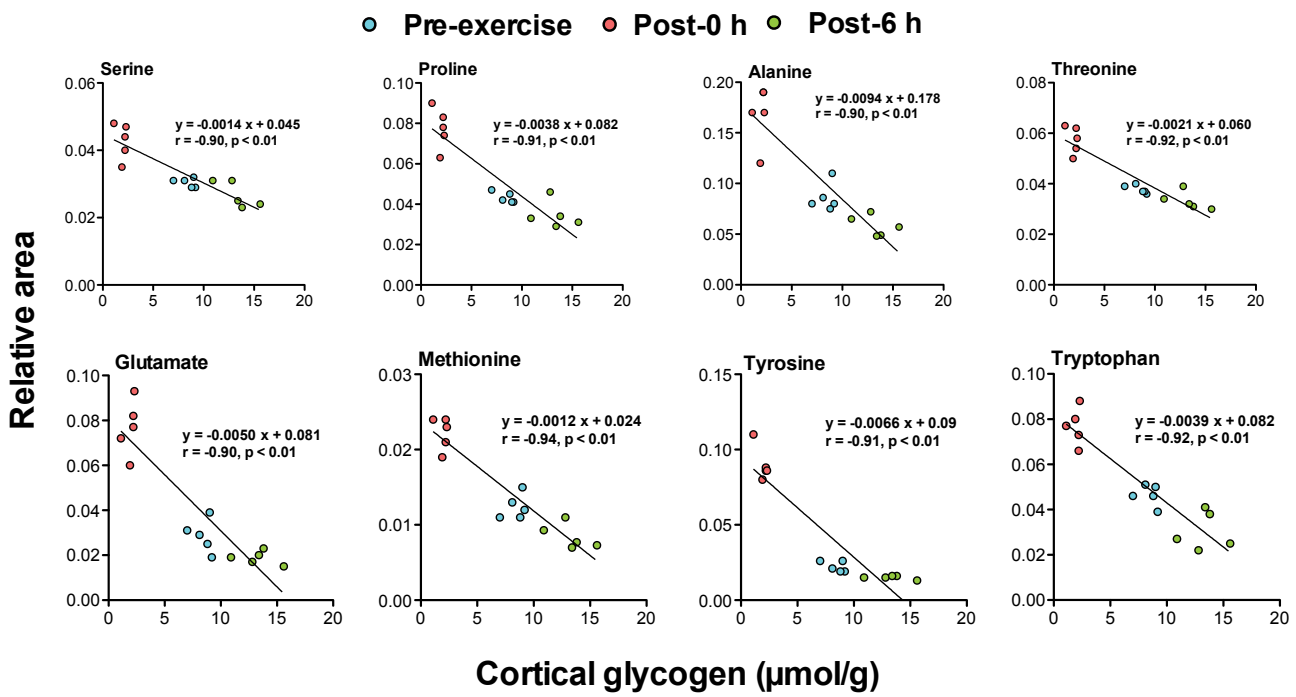

FIGURE 4 | Plasma glycogenic amino acids are associated with cortical glycogen levels during and following endurance exercise. Lines in scatter plots show significant correlation ($P < 0.05$) (Pearson's product-moment correlations test).

consists of over 90% type II fibers, are depleted, similar to that observed in the present study, in rats (Armstrong et al., 1974; Bracken et al., 1988; Matsui et al., 2011, 2012, 2017) and in humans (Gollnick et al., 1974; Vollestad et al., 1984). These previous studies indicate the validity of our glycogen detection. In addition, the decreased brain glycogen due to endurance exercise recovered to higher levels than the basal line within 6 h after exercise, which occurred earlier than muscle glycogen replenishment and supercompensation (**Figure 1C**), reproducing the onset of brain glycogen supercompensation

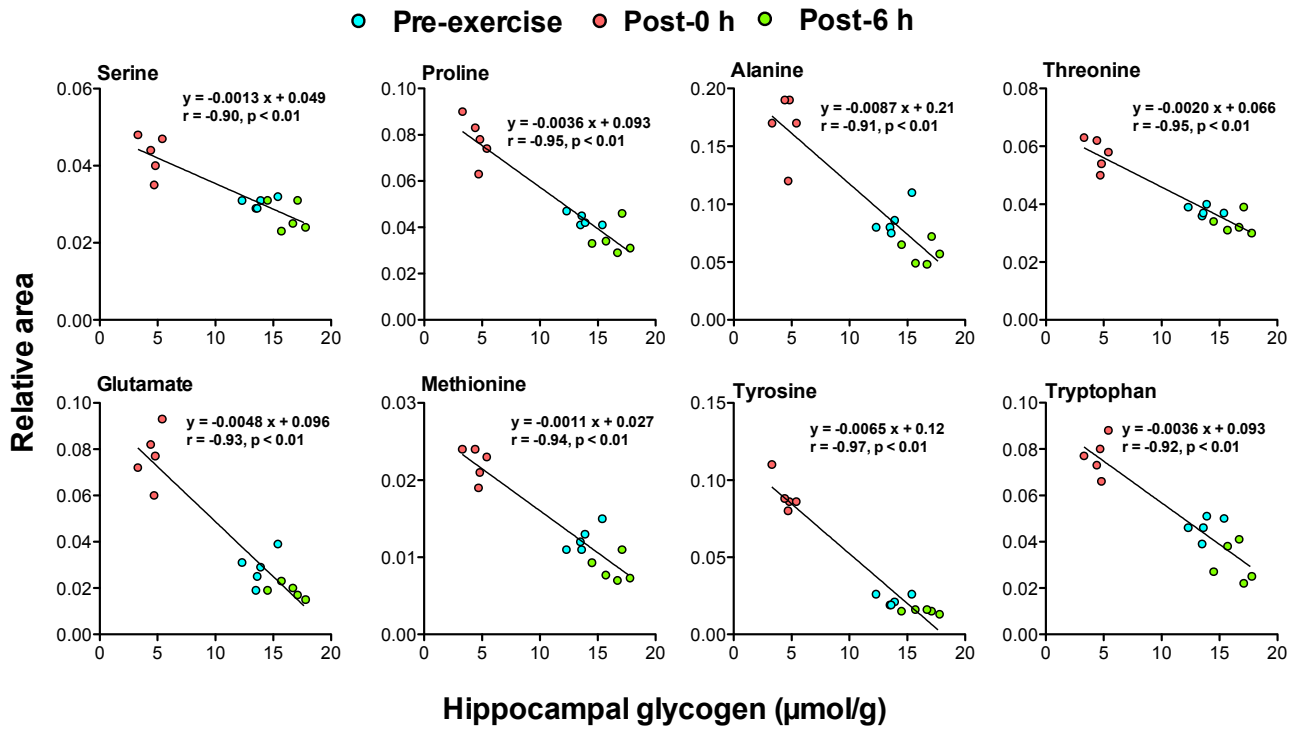

FIGURE 5 | Plasma glycogenic amino acids are associated with hippocampal glycogen levels during and following endurance exercise. Lines in scatter plots show significant correlation ($P < 0.05$) (Pearson's product-moment correlations test).

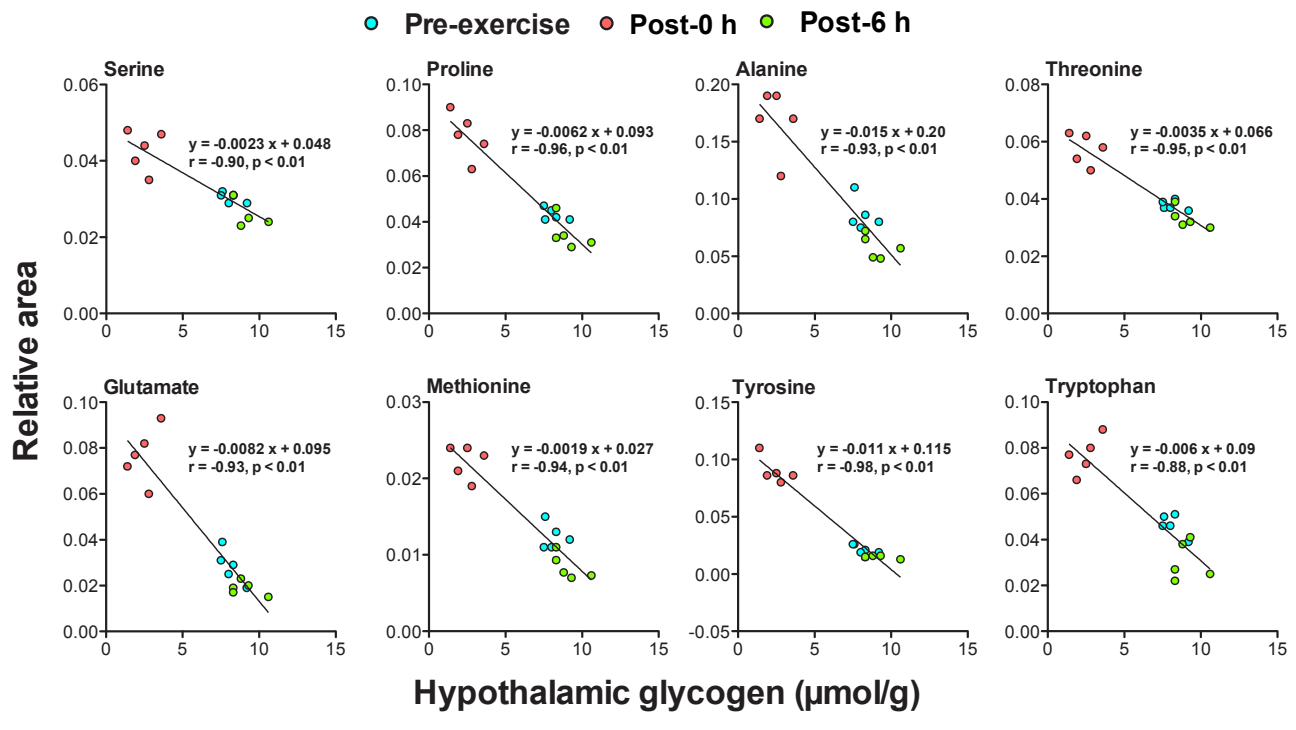

FIGURE 6 | Plasma glycogenic amino acids are associated with hypothalamic glycogen levels during and following endurance exercise. Lines in scatter plots show significant correlation ($P < 0.05$) (Pearson's product-moment correlations test).

after endurance exercise found in our previous study (Matsui et al., 2012).

Plasma metabolomics clearly indicated the difference in metabolic profiles among pre-exercise, post-0 h, and post-6 h (**Figure 2** and **Supplementary Table S2**), and glycogenic amino acids, such as aspartate, alanine, tyrosine, and tryptophan, increased immediately after exercise (post-0 h) and decreased 6 h after exercise (post-6 h), which implies that there is a repulsive interaction between brain glycogen dynamics and endurance exercise (**Figure 3**). Endurance exercise increases blood glycogenic amino acids levels, such as alanine, tyrosine, and phenylalanine derived from protein catabolism in active muscles (Ahlborg et al., 1974), and enhances their splanchnic exchanges to be utilized as hepatic gluconeogenesis sources (Wahren et al., 1971). Furthermore, increased blood glycogenic amino acids recover to basal levels or decrease, compared with pre-exercise levels, to be metabolized by the rest after endurance exercise (Borgenvik et al., 2012; de Godoy et al., 2014). Our present data regarding endurance exercise are supported by these previous findings.

The negative correlations between plasma glycogenic amino acids (serine, proline, threonine, glutamate, methionine, tyrosine, and tryptophan) and brain glycogen levels in the cortex, hippocampus, and hypothalamus were observed ($r < -0.8$, $P < 0.05$) (**Figures 4–6**). These results indicate, for the first time, the possibility that plasma glycogenic amino acids are biomarkers that predict the decrease and supercompensation of brain glycogen with acute endurance exercise.

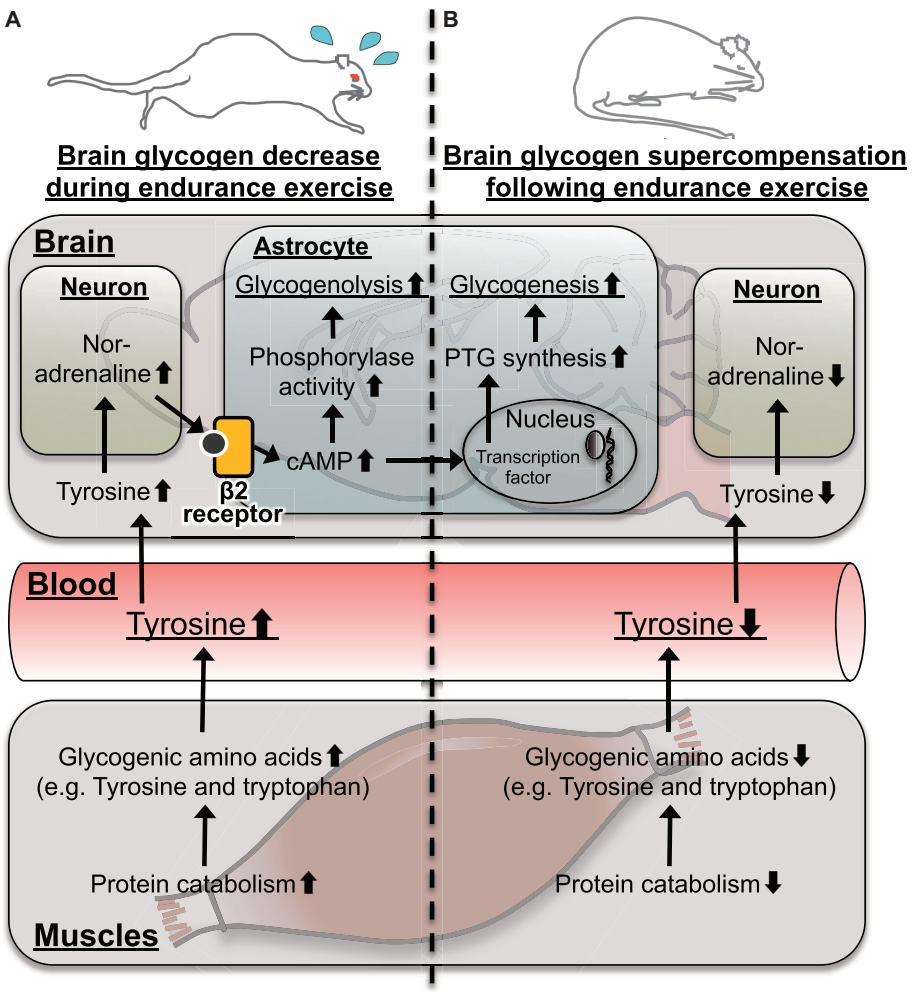

FIGURE 7 | Hypothetical schema for tyrosine as a possible mechanistic-based biomarker predicting brain glycogen dynamics **(A)** during and **(B)** following endurance exercise. In the skeletal muscle, protein catabolism is activated to produce glycogenic amino acids including tyrosine. Tyrosine is released to the blood stream, and its level is increased. Blood tyrosine is taken up by the brain and is converted into noradrenaline in noradrenergic neurons. Noradrenaline activates cyclic adenosine monophosphate (cAMP) production via the β2-adrenaline receptor to activate glycogenolysis in a matter of minutes but takes hours to induce glycogenesis and supercompensation through expression of protein targeting to glycogen (PTG). This late onset of glycogen synthesis likely contributes to supercompensation following endurance exercise.

In particular, tyrosine can be a valuable mechanistic-based biomarker for brain glycogen dynamics with endurance exercise, a concept which is shown in **Figure 7**. In active skeletal muscles during endurance exercise, levels of glycogenic amino acids are increased through protein catabolism (Ahlborg et al., 1974). The increases in levels of blood glycogenic amino acids, including tyrosine, are derived from muscles (Ahlborg et al., 1974). Increased blood tyrosine, a precursor of noradrenaline, is taken up by noradrenergic neurons in the brain (Hufner et al., 2015; Alabsi et al., 2016; Imai et al., 2017). Noradrenergic neurons release noradrenaline into the intracellular fluid, and the noradrenaline activates glycogenolysis through cAMP production mediated by the β2 receptor in the astrocytes (Magistretti et al., 1981; Magistretti, 1988). Actually, endurance exercise decreases glycogen levels associated with activated noradrenergic turnover in the cortex (Matsui et al., 2011). Therefore, tyrosine is a possible biomarker for brain glycogen decrease during endurance exercise (**Figure 7A**).

Furthermore, noradrenaline activates not only glycogenolysis but also glycogenesis and supercompensation through the expression of PTG, mediated by cAMP in astrocytes (Sorg and Magistretti, 1992; Allaman et al., 2000; Ruchti et al., 2016). Following endurance exercise, while glycogen synthesis can be activated through PTG, glycogenolysis is not active, due to decreased brain noradrenaline along with blood tyrosine, and as a result, brain glycogen supercompensation is likely induced. Serotonin, which is synthesized from tryptophan in brains, also activates astrocytic glycogenolysis through a Ca^{2+} influx (Gibbs and Hertz, 2014), but there is no report for PTG induction. Glutamate activates glucose uptake in astrocytes but does not play a direct role in glycogen metabolism (Hamai et al., 1999). Thus, plasma tyrosine, rather than tryptophan and/or glutamate, is a possible biomarker not only of a brain glycogen decrease during endurance exercise but also of brain glycogen supercompensation following endurance exercise (**Figure 7B**).

Furthermore, plasma levels of branched chain amino acids (BCAA) such as leucine, isoleucine, and valine were not significantly changed by endurance exercise (**Supplementary Table S2**). Large-neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tryptophan, and tyrosine share the same transporter, L-type amino acid transporter 1 (LAT1), on the blood-brain barrier (BBB) (Pardridge and Oldendorf, 1977; Boado et al., 1999). Since BCAA levels were unchanged, the LAT1 at the BBB would be ready for use by other neutral amino acids such as tyrosine, which increased in the plasma during exercise.

The use of an acute endurance exercise model in rodents produced new evidence on plasma tyrosine as a mechanistic-based biomarker for brain glycogen dynamics in endurance exercise. In this study, however, glycogen levels after post-6 h was not examined. Therefore, although chronic exercise or brain glycogen loading increases brain glycogen levels in the cortex, hippocampus, and the hypothalamus (Matsui et al., 2012; Soya et al., 2018), it is still unclear whether tyrosine is a useful biomarker not only for acute endurance exercise but also for chronic exercise or glycogen loading. Furthermore, here, we tried to investigate the brain region specificity of biomarker candidates, but it was not revealed because glycogenic amino acids correlated strongly with glycogen levels in all brain loci we detected in the present study (the cortex, hippocampus, and hypothalamus). These important issues should be addressed in future research.

In conclusion, our metabolomics of plasma samples from rats showed quantitative differences in glycogenic amino acids, during and following endurance exercise. In particular, plasma tyrosine, a precursor of noradrenaline, is a possible mechanistic-based biomarker for brain glycogen dynamics during and following endurance exercise. These findings support our present hypothesis that blood tyrosine is a mechanistic-based biomarker that predicts a decrease and supercompensation of brain glycogen with acute endurance exercise. Plasma tyrosine may contribute to the development of a valuable parameter for exercises as a training/conditioning for athletes and/or therapeutic strategy for neurodegenerative diseases.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Tsukuba, and all procedures and methods were performed in accordance with the relevant guidelines laid down by the animal ethics committee (Animal ethical approval number; 15–055). Every effort was made to minimize the number of animals used as well as any pain and discomfort.

AUTHOR CONTRIBUTIONS

TM and HS conceived and designed the experiments. TM, Y-FL, MS, and TS collected the data. TM, Y-FL, MS, TS, and HS analyzed and interpreted the data. TM and HS drafted the manuscript and revised it critically for important intellectual content. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00200/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Effects of Different Types of Exercise Training on the Cortisol Awakening Response in Children

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Context: Due to great variability of the hypothalamus-pituitary-adrenal (HPA)-axis, research has to produce better-controlled findings to make a more meaningful statement regarding the effect of exercise training (ET) on the cortisol awakening response (CAR), especially in children.

Objective: The aim of the study was to investigate the effects of different ET interventions on the CAR in children.

Design and setting: We conducted a short-term training study for 10 weeks in primary schools in Westphalia, Germany.

Participants: 71 children (9–10 years old) were randomly assigned to a cardiovascular exercise group ($n = 27$), a motor exercise group ($n = 23$), or a control group ($n = 21$).

Intervention: An experienced instructor trained the children in an after-school setting in 45 min sessions, three times a week over the course of 10 weeks.

Main outcome measure: CAR (0, +30 min) was assessed on 2 schooldays one week apart before and after the 10-week intervention. A Shuttle Run Test was performed to determine the cardiovascular fitness. Motor fitness was assessed using the Heidelberg Gross Motor Test.

Results: Children who enhanced their cardiovascular fitness over the course of the intervention showed an increased CAR after the intervention time ($B = 0.213$), whereas children who underwent a motor exercise intervention and at the same time gained in motor fitness exhibited a decreased CAR after intervention ($B = -0.188$).

Conclusions: It has been speculated that other neurobiological pathways are activated by different exercise interventions. The extent to which these ET effects on CAR can be applied in clinical settings needs further investigation.

Précis: The 10-weeks longitudinal effects of cardiovascular vs. motor exercise interventions (three times a week) on CAR in children show that these interventions exert different effects on hypothalamus-pituitary-adrenal (HPA) axis activity.

Keywords: cortisol, exercise training, physical stress, children, adolescents, motor exercise, cardiovascular exercise

INTRODUCTION

Children and adolescents in industrialized countries today increasingly suffer from inactivity and associated health issues such as obesity and psychological disorders, e.g., as an effect of stress exposure (1). Exercise training (ET), however, is an easy-to-implement intervention, which can be administered in group settings including schools. It has been proven that ET has long-term beneficial effects and is a cost-efficient and sustainable strategy to improve health in various mental and physical disorders (2, 3). ET is defined as a structured exercise program that involves the use of large muscle groups for extended periods of time. ET differs from physical activity (PA) in its planned and structured nature (4). Even though it appears that experts believe prevention of diseases should start in childhood and adolescence, there are rarely studies that focus on the effects of ET on health in children under the age of 12 (5). The present study focuses on an age group that lacks extensive research on stress related hormonal indices (e.g., HPA axis activity).

The HPA axis is a highly stress-responsive system and shows a strong diurnal pattern with the glucocorticoid cortisol as an end product. One suitable marker for determining HPA axis activity is the CAR. The CAR is a reliable measure for the acute responsiveness of the HPA axis and can serve as a useful index of adrenocortical activity (6). Cortisol levels, which are measured during the first 30 min after awakening show an increase of 50–70% in the vast majority of adults but generally seem to be less pronounced in children and adolescence (7). In adults, CAR is generally positively associated with job and general life stress (8). Even though it is still unclear what contributes to a "healthy" CAR, ET has been argued to alter the HPA axis activity in adults depending on the intensity of the intervention (e.g., moderate vs. vigorous) as well as on the intervention type (e.g., aerobic vs. yoga) (6). Thus, exercise intensity is an issue like in other interventions to promote mental health (9, 10).

To our knowledge, there are no randomized and controlled intervention studies focusing on the effects of ET on the CAR in children. Only a few studies investigated the relationship between ET and CAR in children and adolescents in cross-sectional designs presenting insconsistent results. For example, a positive correlation between CAR and vigorous PA was found in a study focusing on 8-year-old girls suffering from metabolic syndrome (11). In contrast, a lower CAR was linked to the duration of acute daytime sport among healthy older adolescents (aged 10–18 years) (12). Finally, a study among healthy 8-year-old children showed no differences in the diurnal salivary cortisol pattern based on the level of the overall daytime PA (13). However, children's general physical fitness level and regular physical activity were not assessed, which could affect cortisol activity. Further, these were all cross-sectional study designs, which limit causal relationships between ET/PA and CAR.

As the exercise intervention type (6) might affect chronic stress levels (e.g., CAR), it could be argued that exercise interventions that focus on improvement of motor abilities might be beneficial for preserving cognitive resources and thus, freeing resources to deal with complex situations in daily life and resulting in a reduced stress response (14). Cardiovascular exercise, by contrast, may lead to stronger neurogenesis, which might result in stronger cortisol responses after a chronic exercise intervention (15). It was previously argued that hippocampal neurogenesis is mainly promoted by cardiovascular exercise interventions (16). Niemann et al. (17) could not reveal changes in hippocampal volume after 6 months of motor demanding training, but found a significant increase in volume after cardiovascular training in elderlies. Thus, specific stressors influence neurogenesis and the HPA axis activity in different ways (18).

Overall, the existing literature does not allow for causal inference because it is unknown if the parameter itself (being more physically fit) caused the changes in HPA axis activity or if other factors might have accounted for these differences. Therefore, controlled intervention studies are required to focus on the causal relationship between exercise and HPA axis activity (e.g., CAR) in a young, pre-adolescent age group. Martikainen et al. (13) stipulated that the exercise intervention type needs to be manipulated. Taken together, the current study aims to fill this gap by investigating the effect of a 10-week ET intervention (cardiovascular vs. motor fitness group) on the HPA axis activity in 8- to 10-year-old children. We hypothesized that cardiovascular exercise leads to an increased CAR response and motor exercise training results in a decreased, or no change in, CAR response among children.

METHODS

Participants

Data[1] of 71 prepubescent primary school children (39 female) between 9 and 10 years (M_{age} = 9.4; SD_{age} = 0.6) with no psychological or physical impairments (e.g., obesity) were randomly assigned to a cardiovascular exercise group (CV, n = 27), a motor exercise group (MO, n = 23), or a control group (CON, n = 21).

Inclusion/Exclusion Criteria

All participants were recruited from local schools and inclusion criteria were 9–10 years of age, right-handedness, corrected-to or normal vision and prepubescent status according to parent and self-report on the Tanner staging system (below a score of 2 on the five-point scale) (20). In case of the presence of mental and physical impairments and/or previous or actual intake of psychoactive substances, participants were deemed ineligible. Before the study commenced, the ethics committee of the German Psychological Society approved the protocol (HB 02201 6_amd_092011). All participants and their legal guardians provided informed written consent after study procedures were explained in detail. The study was conducted following the guidelines set forth in the declaration of Helsinki and registered in the German Clinical Trials Register (DRKS00016590).

[1]Data from this sample (including working memory performance) have been previously reported (19) but neither of the analyses on HPA activity included in the current manuscript.

TABLE 1 | Salivary cortisol raw scores (nmol/l) for each time point.

	Minutes from awakening	
	0	**30**
PRE		
t1	17.99 (7.77)	21.39 (9.68)
t2	17.73 (10.82)	22.24 (8.95)
POST		
t3	17.98 (8.90)	21.34 (12.50)
t4	18.27 (8.30)	23.29 (12.60)

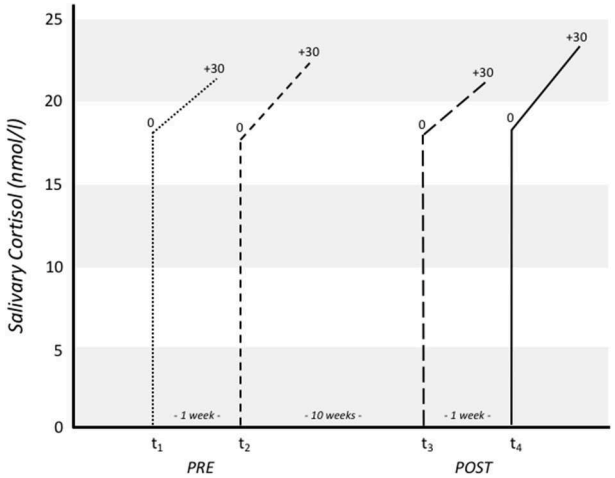

FIGURE 1 | Salivary cortisol raw scores (nmol/l) for each measurement point pre (t_1, t_2) and post (t_3, t_4) intervention.

Measurements

Cortisol Awakening Response (CAR)

The CAR can be defined as the change in cortisol concentration immediately post-awakening: it represents a discrete aspect of the cortisol circadian cycle and has good intra-individual stability across time (21). Morning salivary cortisol levels were assessed at home at two time points. Children obtained saliva samples 0 and 30 min after awakening with the help of their parents, who were instructed personally and with the help of written manuals. Wakening time was 7 o'clock for all children. In order to increase reliability of the measure two cortisol samples (0, +30 min) were taken one week later both on the same day of the week pre- and post-intervention. Children were asked to refrain from physical activity one day before the assessment. Overall salivary cortisol was sampled eight times (see **Figure 1** and **Table 1**). Saliva samples were analyzed from whole saliva collected via the SaliCap® system (IBL, Hamburg, Germany). For each assessment, participants were asked to accumulate saliva in their mouth for 2 min and refrain from swallowing while doing so. The accumulated saliva was then transferred into a pre-labeled vial via a straw. After arriving at school, research staff collected and stored the samples at −20°C until analysis. Cortisol levels (nmol/l) were analyzed using a commercially available enzyme-linked immunoassay (IBL, Hamburg, Germany) at the Biochemical Laboratory of the Department of Clinical Biopsychology, University of Marburg. Intra- and inter-assay coefficients of variation were 6.7 and 7.6%, respectively.

CAR was calculated as the area under the curve (AUCg), in a first step using the mean of both saliva samples (immediately and 30 min after awakening) for pre and post-intervention separately, as it has been shown that awakening cortisol levels might be sensitive to differences in daily activities. In a final step, AUCg was calculated in accordance to Fekedulegn et al. (22). The raw scores for salivary cortisol are illustrated in **Figure 1**.

Cardiovascular Fitness (Card Fit)

Card Fit was tested with the Shuttle Run Test, a standard method for determining cardiorespiratory fitness in school children. Children were asked to run between two lines set 20 m apart. In accordance with the standardization used elsewhere (23), the children ran back and forth continuously with an initial speed of 8.0 km.h-1, increasing the level by 0.5 km h^{-1} each minute. Acoustic signals in a given frequency were used to control the pace. In all stages the students were motivated by cheering and by a pacemaker. We determined the HR_{max} as well as the maximum scores reached, which are the level and number of shuttles reached before fatigue (i.e., unable to maintain pace).

Motor Fitness (Mot Fit)

Mot Fit was assessed using the Heidelberg Gross Motor Test for children. We included the performance of six motor tasks [i.e., balance, rhythm, spatiotemporal orientation, and motor adaptation to moving objects; for further details see Koutsandréou et al. (19)] that were quantitatively measurable and calculated a sum score. For example, in the motor adaptation to moving objects task, points were earned by first throwing a ball backword through straddled legs against a 3 m distant wall and then, catching the rebounding ball (two points) or just touching or dropping it (one point).

Intervention

For 10 weeks, three times a week, for 45 min, an experienced exercise instructor trained the participants after school in groups of 7–14 children. The CV group trained their cardiovascular fitness via running and running-based games, however, varied to avoid boredom. The MO group focused on improving fine and gross motor body coordination through playful coordination exercises with low intensity: for the cardiovascular system. The control group received assisted homework sessions to prevent attention bias and control for retest effects (19). As previously reported (19) the three experimental groups (MO: 125.4 bpm, CV: 138.8 bpm, CON: 79.4 bpm) differed significantly regarding their mean heart rate levels: (recorded by F1 Polar HR monitors; Polar, Kempele, Finland) during exericse with MO and CV scoring significantly higher than CON but also CV scoring slightly higher than MO.

Procedure

For the pre-post-design of this study, cardiovascular and motor fitness were assessed in the week prior to the start

TABLE 2 | Results of the hierarchical regression analysis of post-intervention CAR (cortisol awakening response, log-transformed AUC) with gender, age, pre-intervention CAR, and performance in the shuttle run test (Pre CP) were entered in Model 1.

Variable	Post-CAR											
	Model 1				Model 2				Model 3			
	B	*SE*	*t*	*p*	*B*	*SE*	*t*	*p*	*B*	*SE*	*t*	*p*
Constant	6.716	1.447	4.639	0.000	6.919	1.455	4.754	0.000	7.026	1.416	4.962	0.000
Gender	−0.170	0.160	−1.059	0.293	−0.154	0.164	−0.940	0.351	−0.226	0.162	−1.394	0.169
Pre-CAR	0.284	0.079	3.585	0.001	0.295	0.080	3.666	0.001	0.271	0.077	3.506	0.001
Pre-CP	−0.012	0.045	−0.259	0.796	0.018	0.049	0.371	0.712	0.045	0.047	0.950	0.346
Age	0.094	0.133	0.703	0.485	0.040	0.136	0.292	0.771	0.049	0.132	0.372	0.711
CE					−0.031	0.096	−0.323	0.748	−0.017	0.091	−0.191	0.849
ME					−0.009	0.096	−0.094	0.925	−0.003	0.090	−0.033	0.974
ΔCard Fit					0.167	0.087	1.924	0.059	0.215	0.083	2.572	0.013
ΔMot Fit					0.013	0.082	0.164	0.870	0.032	0.078	0.414	0.680
CE × ΔCard Fit									0.213	0.099	2.157	0.035
ME × ΔCard Fit									0.072	0.100	0.720	0.474
CE × ΔMot Fit									0.018	0.093	0.189	0.851
ME × ΔMot Fit									−0.188	0.093	−2.016	0.049
R^2	0.187				0.236				0.366			
F	3.73				2.35				2.74			
df	(4.65)				(8.61)				(12.57)			
ΔR^2					0.049				0.130			
Δ*F*					0.981				2.914			
df					(4.61)				(4.57)			

PreCP, Pre-cordiocosculor performance. Experimental group assignment (cardiovascular exercise group, CE, and motor exercise group, ME), and increases in cardiovascular fitness (ΔCardFit) and motor fitness (ΔMotFit) were entered in Model 2. Lastly, interaction terms between variables were entered in Model 3.

of the intervention, and 1 week before the last intervention appointment. Following the recommendation by Hellhammer et al. (24), CAR was assessed twice prior and after the intervention: 1 week prior (1st time pre) and on the day of the start of the intervention (2nd time pre), as well as 1 week before the last intervention appointment and at the end of the 10-week intervention.

Statistical Analyses

For statistical analysis cortisol values were log-transformed to achieve normal distribution. A series of hierarchical regression analyses were performed in three blocks to predict post-intervention average CAR value for the two assessments (t_3, t_4), and to control for lower order effects before testing for higher order effects (see **Table 1**). In the first block we controlled for sex, the average value of CAR for the two pre-intervention assessments (t_1, t_2), pre-intervention cardiovascular fitness level, and age. In the second block, the experimental groups (MO vs. CON, CV vs. CON) were included as categorical variables in the model, as well as the change scores for cardiovascular and motor fitness. In the third block, the interaction terms between cardiovascular/motor fitness and intervention condition were added (cardiovascular fitness × CV; cardiovascular fitness × MO; motor fitness × CV; motor fitness × MO). For statistical analysis SPSS 24 software (IBM, Armonk, USA) was used.

RESULTS

In order to test the effects of a 10-week physical exercise intervention on motor vs. cardiovascular fitness on CAR in children, we computed a hierarchical regression analysis using three blocks (see **Table 2**). In the first block, post-intervention CAR was residualized for age, gender, pre-cardiovascular performance, and pre-intervention CAR. Including pre-intervention CAR ($B = 0.284$, $p = 0.001$), age ($B = 0.094$, ns), gender ($B = -0.170$, ns), and pre-cardiovascular performance ($B = -0.012$, ns) rendered the regression analysis on post-intervention CAR significant, $R^2 = 0.187$, $F_{(4, 65)} = 3.730$, $p = 0.009$. In Model 2, we included changes in participants' intervention-induced cardiovascular (ΔCard Fit) and motor fitness (ΔMot Fit), as well as participants' training intervention [cardiovascular exercise training (CV) or motor exercise training group (MO)] compared to the control group in the model. In Model 3, the interaction terms (multiplicative term of z-standardized variables) of training intervention (CV vs. MO) by changes in participants' fitness levels (ΔMot Fit, ΔCard Fit) were added to the regression. Adding the cardiovascular and motor exercise group and the cardiovascular and motor pre-to-post-fitness changes did not result in a significant improvement in Model 2. However, including the interaction terms significantly improved Model 3, $\Delta R^2 = 0.130$, $\Delta F_{(4, 57)} = 2.914$, $p = 0.029$, and rendered the whole model significant, $R^2 = 0.366$, $F_{(12, 57)}$

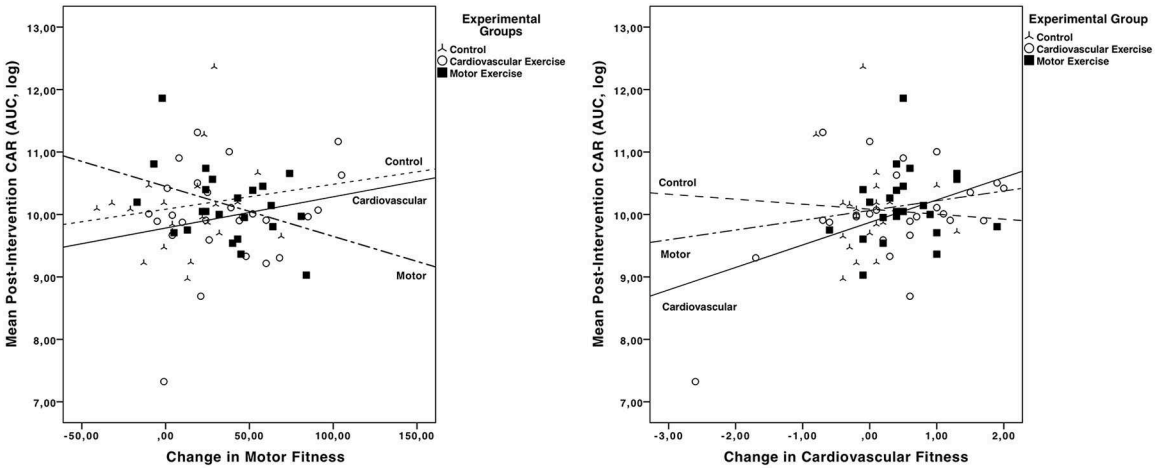

FIGURE 2 | Post-intervention CARs (log-transformed AUC) in the three experimental groups (CV, MO, Control) as function of change in motor fitness (left graph) and change in cardiovascular fitness (right graph).

$= 2.353$, $p = 0.005$ (see **Table 2**). Participants in the CV who enhanced their cardiovascular fitness over the course of the intervention showed an increased CAR after the intervention time ($B = 0.213$), whereas children who underwent a motor exercise intervention and at the same time gained in motor fitness exhibited a decreased CAR after 10 weeks of intervention ($B = -0.188$; see **Figure 2**).

DISCUSSION

The aim of this study was to investigate the effects of cardiovascular vs. motor exercise interventions on CAR in 9 to 10-year-old children. Our results show that cardiovascular and motor exercise exert different effects on HPA axis activity. Thus, specific exercises influence the HPA axis activity in different ways. Whereas, an increase in cardiovascular fitness was accompanied by an increase in HPA axis activity, particularly in children who underwent a cardiovascular exercise program, an increase in motor fitness in children who underwent a motor exercise program was accompanied by a decrease in CAR.

First, it should be kept in mind that it is still challenging to identify whether a large or small CAR is considered "healthy." Second, to our knowledge, there is not enough systematic research on children, who represent an under-studied population. Research in this field is scarce, which makes it challenging to put results into context and highlights the importance of addressing the effects of different exercise interventions on the cortisol activity in children in future research. We base our argumentation on findings in adults showing that a higher increase in CAR is generally positively associated with job and general life stress (8).

Children who did increase their cardiovascular fitness, regardless of their experimental group assignment, showed an increase in cortisol activity. This is in line with previous findings in children indicating vigorous physical activity was positively related with 30 min post-waking cortisol values (11). With respect to children that did increase their cardiovascular fitness and presented an increase in cortisol activity, it could be argued that this is a result of an HPA axis hyper-responsiveness as a biological consequence of the frequent activation of the axis triggered by exercise stress: this may be part of the physiological adaptation of the neuroendocrine system to chronic demands (25).

Utilizing a set of salivary cortisol data in a small sample population of healthy older adults (mean age 65), a robust cortisol awakening response, and increased CAR after exercise training (a 6-month supervised intervention designed to reach 60–70% of their maximum heart rate reserve, 3 days a week, without any mentioned motor demands) were observed (26). However, one has to keep in mind that the cortisol response like many mental health related responses to exercise is depending on age (27).

Children in the motor exercise group that did increase their motor fitness showed a decrease in cortisol activity. This result is in line with research linking lower CAR to the duration of daytime sport (12). In a study with healthy young adults, the impact of long duration and high intensity of exam stress on the CAR supports that the HPA axis is down-regulated by chronic major stress, with this downregulation reflected by a reduction of the CAR (28). Although the mechanisms resulting in hypocortisolism are not yet fully clarified, possible explanations include changes in the biosynthesis of HPA-axis hormones and/or availability and functioning of their receptors at all levels of the HPA-axis [see Heim et al. (29) for discussion].

One could argue that due to improved motor abilities cognitive resources are freed to deal with complex situations in daily life resulting in a reduced chronic stress response (14). It has been further suggested that having control or no control over stress can have opposite effects on neural plasticity (15). Regarding the training of the cognitive system in this group one could speculate that it leads to an improved self-regulation and thus influencing neural input/traffic to the HPA axis.

Results by Blair et al. (30) indicate that moderately higher levels of cortisol are associated with better performance on self-regulation, however it remains to be evaluated if this elevation in cortisol would lead to a change in CAR. One could also argue that the cognitive challenge of this exercise should induce neurogenesis (31). However, these argumentations would impede an explanation of the observed differences. Only the following hypothesis backed up by human data can somehow explain our findings. A recent study about the hippocampal volume in older humans after a 6-month intervention period, indicates that only, cardiovascular but not motor demanding training led to increases in hippocampal volume (17) which is positively related to the magnitude of the CAR (32).

One limitation of the study is that we only used two time points to assess the AUCg for the CAR. However, this procedure has been previously presented by different authors (5). Another limitation is that in the motor exercise group it is not possible to standardize the intensity for the neural nor for the cardiovascular system to compensate for motor demands of the different exercises, which can be challenging for one participant and more difficult for another participant. It cannot be ruled out that training programs matched for cardiovascular load, but with different coordinative demands would have resulted in a different pattern of results. As we previously reported both experimental exercise groups differed in intensity (19, 33).

Also, even though the children in the MO group were provided with playful exercises, and children in the CV group completed a variation of running exercises, it remains unclear how they were perceived by the children themselves. Finally, we did not control for subjectively perceived (chronic) stress prior or post-intervention. Future research, especially when focusing on children, should do so in order to control for potential confounding variables, as general life stress in adults has been associated with differences in CAR (8).

Another limitation of our study is that we did not assess abdominal fat in these children. It is known that abdominal fat may affect neuroendocrine responses mainly to psychological stress (34). However, significant changes in abdominal fat usually take place during puberty and children in our study were on average 9.4 years old which might diminish the effect of abdominal fat on HPA axis reactivity. Also, there is not enough systematic research on this specific understudied population and research in this field has not been well-discussed in the literature yet. This should be addressed in future studies and encourage researchers to add abdominal fat as a covariate.

One advantage of the present study though is that the AUCg used presents a more stable measure of the CAR because we used two AUCg measurements 1 week apart as previously recommended in the CAR guidelines by Stalder et al. (35). However, as training intensity and volume play a crucial role for the effects on CAR (6), future research should implement follow-up measurement points.

Overall, our results show that cardiovascular and motor exercise training in school exerts different effects on HPA axis activity. Whereas, an increase in cardiovascular fitness was accompanied by an increase in HPA axis activity, an increase in motor fitness in children was lead to a decrease in CAR.

Research has yet to produce more detailed and consistent findings to make a more meaningful statement regarding ET and its role on CAR, especially in children. The current study raises questions that future research needs to address in order to increase prevention of potential pathological diseases in childhood and adolescence.

Unfortunately, we simply do not know under what circumstances, for whom, and at what developmental periods under- vs. over-activation of the HPA-axis are most likely and how this is expressed by changes in CAR in this age group. While we suspect that under-activation of the HPA-axis may in fact be a reflection of more severe stress exposure and have more serious consequences than hyperactivation (36), however it needs to be established what the consequences are and under which cortisol concentration they occur.

ETHICS STATEMENT

Before the study commenced, the ethics committee of the German Psychological Society approved the protocol (HB 02201 6_amd_092011). All participants and their legal guardians provided informed written consent after study procedures were explained in detail. The study was conducted following the guidelines set forth in the declaration of Helsinki and registered in the German Clinical Trials Register (DRKS00016590).

AUTHOR CONTRIBUTIONS

MW, HB, and FK contributed conception and design of the study. FK organized the database. FK and MW performed the statistical analysis. FK, MW, HB, and FL wrote the first draft of the manuscript. MW, HB, FK, FL, and AM-A wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Acute Sprint Interval Exercise Increases Both Cognitive Functions and Peripheral Neurotrophic Factors in Humans: The Possible Involvement of Lactate

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There is increasing attention to sprint interval exercise (SIE) training as a time-efficient exercise regime. Recent studies, including our own (Kujach et al., 2018), have shown that acute high-intensity intermittent exercise can improve cognitive function; however, the neurobiological mechanisms underlying the effect still remain unknown. We thus examined the effects of acute SIE on cognitive function by monitoring the peripheral levels of growth and neurotrophic factors as well as blood lactate (LA) as potential mechanisms. Thirty-six young males participated in the current study and were divided into two groups: SIE (n = 20; mean age: 21.0 ± 0.9 years) and resting control (CTR) (n = 16; mean age: 21.7 ± 1.3 years). The SIE session consisted of 5 min of warm-up exercise and six sets of 30 s of all-out cycling exercise followed by 4.5 min of rest on a cycling-ergometer. Blood samples to evaluate the changes of serum concentrations of brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and blood LA were obtained at three time points: before, immediately after, and 60 min after each session. A Stroop task (ST) and trail making test (TMT) parts A and B were used to assess cognitive functions. Acute SIE shortened response times for both the ST and TMT A and B. Meanwhile, the peripheral levels of BDNF, IGF-1, and VEGF were significantly increased after an acute bout of SIE compared to those in CTR. In response to acute SIE, blood LA levels significantly increased and correlated with increased levels of BDNF, IGF-1, and VEGF. Furthermore, cognitive function and BDNF are found to be correlated. The current results suggest that SIE could have beneficial effects on cognitive functions with increased neuroprotective factors along with peripheral LA concentration in humans.

Keywords: sprint interval exercise, BDNF, IGF-1, VEGF, blood lactate, cognitive function

INTRODUCTION

There is increasing attention to the beneficial effects of exercise on human cognition. Among various types of exercise, many studies have pointed out that moderate-intensity exercise has beneficial effects on cognitive abilities such as information processing or control of inhibition (Knaepen et al., 2010; Alves et al., 2012). However, these recommended aerobic exercise regimens require people to make a considerable time commitment, which is known as a limiting factor of physical activity in the modern era (Gibala and Little, 2010). Recently, high-intensity interval training (HIT) has become very popular among people as a time-efficient exercise regime (Gibala and Little, 2010; Astorino and Sheard, 2019). In addition, recent evidence suggests that HIT is more enjoyable than moderate-intensity endurance exercise (Bartlett et al., 2011; Little et al., 2011a). Since some reported that HIT could also be uncomfortable, causing effects such as active displeasure (i.e., distressed, upset) and lower enjoyment assessed with the Physical Activity Enjoyment Scale, especially for the sedentary population (Decker and Ekkekakis, 2017; Farias-Junior et al., 2019), various transferable forms of high-intensity intermittent exercise have been developed and it was revealed that these exercise regimes have beneficial effects not only on cardiovascular and metabolic adaptation but also on cognitive function (Little et al., 2010; Kujach et al., 2018). Sprint interval exercise (SIE) as a Wingate test-based modality consists of a number of supramaximal "all out" exercise bouts interspersed with recovery periods, achieving ∼20 min of activity in a single session (Burgomaster et al., 2005; Gibala and Little, 2010). This training protocol has been widely used and demonstrated to positively influence cardio-metabolic health parameters (Sloth et al., 2013). However, there is less evidence for how acute SIE improves cognitive functions and underlying neurobiological mechanisms.

Numerous animal and human studies have revealed that exercise enhances human cognition via exercise-enhanced neurotrophins and catecholamine production, which is known to mediate neural plasticity and energy metabolism in the brain (Kohut et al., 2006; Gomez-Pinilla and Hillman, 2013). Several neurochemicals, including brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF), are currently considered key proteins that mediate downstream effects of exercise on the brain and cognition (Cotman et al., 2007). As a single bout of physical exercise may lead to an increase in BDNF level (Ferris et al., 2007; Griffin et al., 2011), these exercise-increased neurotrophins may contribute to a reduction in mood disorders and to the protection and regeneration of various tissues resulting in increased cognitive performance in humans (Hansen et al., 2001; Rojas Vega et al., 2008; Zoladz and Pilc, 2010).

Interestingly, previous research has indicated that SIE facilitates muscle remodeling, which in turn may lead to the production of BDNF (Little et al., 2011b; Wrann et al., 2013; Phillips et al., 2014). In fact, high-intensity exercises and strength training both stimulate the secretion of IGF-1 (Bermon et al., 1999; Cassilhas et al., 2007), which is needed to transform pro-BDNF into BDNF in the central nervous system and can easily cross the blood–brain-barrier affecting neurogenesis and synaptic plasticity (Ding et al., 2006; Nishijima et al., 2010). In addition, recent animal studies have revealed that IGF-1 mediates exercise-induced angiogenesis, increased central BDNF, and VEGF production (Lopez-Lopez et al., 2004; Ding et al., 2006). Similar to IGF-1, peripheral VEGF also increases during exercise, in part mediating exercise-induced angiogenesis and neurogenesis (Lopez-Lopez et al., 2004). Circulating VEGF may promote neurogenesis and synaptic plasticity by stimulating neural stem cell proliferation and differentiation (Zacchigna et al., 2008; Ruiz de Almodovar et al., 2009) and also increases central endothelial cell and astrocytic productions of VEGF, BDNF, and IGF-1 (Zacchigna et al., 2008; Ruiz de Almodovar et al., 2009).

Moreover, SIE is related to the increase in glucose metabolism and lactate (LA) production, where glucose as well as LA are important energy sources for the human brain (van Hall et al., 2009; Tsukamoto et al., 2016; Hashimoto et al., 2018). Further, among various HIT protocols, SIE is well recognized as inducing important elevation in blood LA concentration (Wood et al., 2016; Olney et al., 2018). Glenn et al. (2015) found that peripherally produced LA is available as a cerebral energy supply after traumatic brain injury. Although the brain metabolism relies mainly on glucose while at rest, the cerebral consumption of glucose decreases during high intensity exercise, along with an increase in blood LA of consequence for the cerebral uptake (Kemppainen et al., 2005). Furthermore, improvement in executive functions may also be related with neuronal activation induced by high intensity intermittent exercise (Kujach et al., 2018). Neuronal activation is associated with an increase in energy requirement (Dalsgaard et al., 2002). Elevated peripheral LA concentration in response to intense exercise promotes the supply of LA as an energy substrate to meet acute neuronal energy requirements (Barros, 2013; Dienel, 2017). Thus, SIE modulating blood LA concentration and neuronal activation could affect cognitive function.

Although SIE may lead to progressive fatigue, causing excessive activation of the central nervous system and subsequent cognitive impairments (Chmura et al., 1994; Tomporowski, 2003), it might also induce the production of neurotrophins such as BDNF or IGF-1, positively affecting neurogenesis and synaptic plasticity in the brain (Lista and Sorrentino, 2010).

Given this evidence, we hypothesized that BDNF, IGF-1, and VEGF would increase in response to SIE and that this may be related to acute-exercise-induced cognitive benefits. Here, we have investigated whether acute SIE affects circulating neuroprotein concentrations, which may play a critical role in the enhancement of cognitive abilities.

MATERIALS AND METHODS

Subjects

Thirty-six healthy, right-handed Polish-speaking male subjects participated in the study. All volunteers had normal vision (including color vision). No subject had a history of neurological, major medical, or psychiatric disorders, and none were taking medication at the time of measurement. Additionally, they were

required to refrain from consumption of caffeine for 12 h prior to the testing session. All the subjects provided written informed consent prior to the study procedures. The participants were assigned to either the SIE group (SIE; n = 20, 21.0 ± 0.9 years) or the control group (CTR; n = 16, 21.7 ± 1.3 years) based on their age, weight, and physical fitness level ($\dot{V}O_{2max}$). All the procedures were approved by the Bioethical Committee of the Regional Medical Society in Gdańsk.

Study Design

Participants visited the study site three times. One week before starting the experiment the subjects were asked to come to the laboratory for a familiarization session to learn about the testing procedures. Next, participants completed the same set of body composition, aerobic, and cognitive assessments before the main testing session. An overview of the experimental protocol is presented in **Figure 1**.

Body Composition Measurements

Body mass (BM) and body composition were estimated using a multi-frequency impedance plethysmograph body composition analyzer (InBody 720, Biospace, South Korea). This analyzer accurately measures body water and body composition, including fat mass, free fat mass, skeletal muscle mass, and soft lean mass (Ziemann et al., 2013).

Maximal Oxygen Uptake ($\dot{V}O_{2max}$) Test

To determine $\dot{V}O_{2max}$, participants performed a graded cycle ergometry test on a mechanically braked cycle ergometer (884E Sprint Bike, Monark, Sweden). Subjects were allowed a 5-min warm-up period at an intensity of 1.5 W × kg^{-1} with a pedaling cadence of 60 rpm. Immediately after the warm-up the participants began $\dot{V}O_{2max}$ testing by cycling at increasingly difficult workloads in which resistance was increased by 25 W × min^{-1} until the participant reached the point of volitional exhaustion. $\dot{V}O_{2max}$ was determined when at least two of the following criteria were satisfied: (1) the respiratory exchange ratio (RER) exceeded 1.05, (2) achievement of 90% of age-predicted peak HR (220–age), and (3) an ratings of perceived exertion (RPE) of 19 or 20 (Suwabe et al., 2017). Breath-by-breath pulmonary gas exchange was measured (MetaMax 3B, Cortex, Germany) throughout the $\dot{V}O_{2max}$ test; the O_2 and CO_2 analyzers were calibrated before each test using standard gases of known concentrations in accordance with manufacturer guidelines.

SIE Sessions

Sprint interval exercise is a subcategory of interval exercise, involving "all out" supramaximal intensity (>100% $\dot{V}O_{2max}$) (Weston et al., 2014). The SIE sessions were performed on a mechanically braked cycle ergometer (884E Sprint Bike, Monark, Sweden). Exercise protocol started with a standard 5-min warm up at 1.5 Watts × kg^{-1} of BM. After the warmup, subjects performed the interval exercise, which included six sets of 30 s of "all out" sprint cycling exercise. Flywheel resistance equaled 0.075 kG × kg^{-1} of BM (i.e., Wingate test based) which corresponded to 7.5% of each individual's BM and was applied on the onset of the SIE (Burgomaster et al., 2008). The interval rest periods between the 30-s bouts were 4 min and 30 s. The participants were instructed to accelerate until they reached their maximal pedaling rate and were verbally encouraged to maintain this pedaling cadence as long as possible throughout the SIE compilation. Only during the first few initial movements of each bout of the test was each participant allowed to pedal in a standing position; this was to help overcome the resistance and to quickly achieve the maximal pedaling rate. During the testing session, oxygen uptake was also monitored. For this purpose, a breath-by-breath pulmonary gas exchange method was used, where MetaMax 3B (Cortex, Germany) was applied. The O_2 and CO_2 analyzers were calibrated before each test using standard gases of known concentrations in accordance with manufacturer guidelines. Using MetaMax 3B, lung minute ventilation (VE L × min^{-1}) and relative maximal oxygen uptake ($\dot{V}O_{2max}$ mL × min^{-1} × kg^{-1}) were obtained. Heartrate (HR b × min^{-1}) was monitored continuously via telemetry (S-625, Polar Electro-Oy, Finland). All training sessions were performed at similar times in the morning at least 2 h after breakfast.

Cognitive Assessments

The assessment of cognitive functions was made using selected neuropsychological timed tasks. TMT A and B were used to measure working memory and the ST was adopted to assess executive functions. Since in meta-analyses, Lambourne and Tomporowski (2010) and Chang et al. (2012) suggest that more pronounced cognitive function facilitation could be observed 11–20 min following acute high-intensity exercise, each task was assessed before and 20 min after the SIE session for the SIE group, and before and 20 min after the resting session for the control group.

Stroop Test

The Polish paper version of the ST was used in this study. The ST consisted of 71 words written in colored ink. The participants' task was to name the color of the font regardless of the word written. At the beginning of the test the meaning of each word was consistent with the color of the font in which it appeared (e.g., the word "blue" written in blue or "red" written in red; congruent) in order to activate the automatism of reading, and as the test progressed words written in incongruent fonts (e.g., the word "blue" written in green or "red" written in blue; incongruent) were randomly mixed in with the congruent words, requiring cognitive control in order to respond accurately. The numbers of "congruent" and "incongruent" stimuli were the same. The need to inhibit the automatic reading response, rather than to only name the color in which the word was printed, elicits a significant slowing in reaction time called the Stroop interference effect; thus, cognitive control is reflected in the time taken to execute the test and the number of errors. Consequently, the ST is a sensitive measure of cognitive inhibition and information processing speed (Stroop, 1935; Strauss et al., 2006).

Trail Making Test

The trail making test (TMT) is one of the most commonly used tests assessing working memory. This test includes

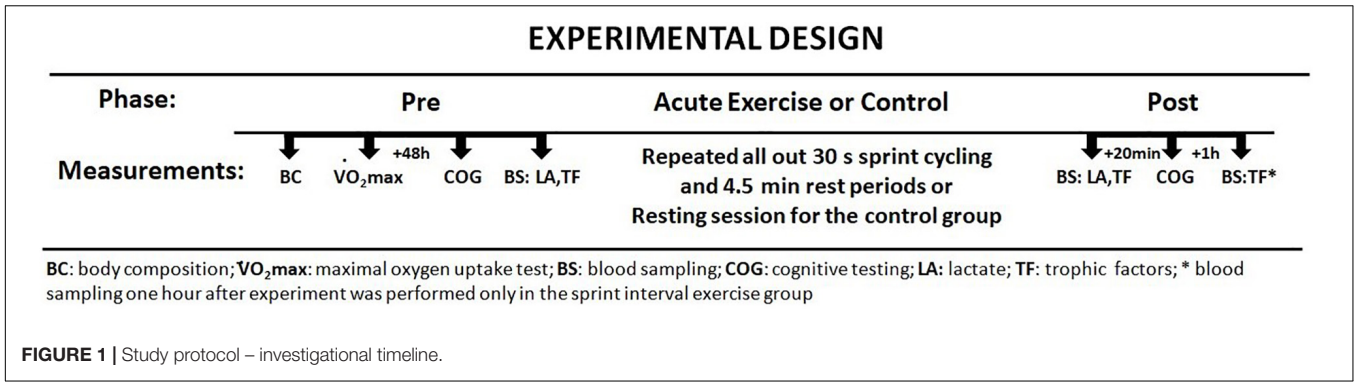

FIGURE 1 | Study protocol – investigational timeline.

two conditions (i.e., "A" and "B"), where the "A" condition reflects psychomotor speed and the "B" condition requires additional executive control needed to switch between number and letter sequences. TMT-A consists of drawing lines as quickly as possible to link consecutively numbered circles. In contrast, in TMT-B participants must connect circles while alternating between numbers and letters. Performance in both conditions is assessed based on the time (seconds) taken to complete each task (Chang and Etnier, 2009; Alves et al., 2012). The difference in time needed to complete part B and part A reflects the efficiency of the working memory, as both parts of the test are comparable in terms of visual search and motor requirements but do differ in cognitive complexity.

Blood Sampling and Analysis

Samples were collected from the antecubital vein (*v. mediana cubiti*) between 8:00 and 10:00 a.m. to establish a baseline. Follow-up samples were taken before the warm-up, directly after the last (sixth) 30-s bout, and 1 h after the completion of all SIE bouts in order to evaluate serum concentrations of BDNF, IGF-1, VEGF, and cortisol. Samples for the CTR group were taken following the same timeline as those for the SIE group, except 1 h after the experiment.

The samples were centrifuged at 2000 × *g* for 10 min at 4°C. The separated serum samples were frozen and kept at −80 °C until later analysis. Serum BDNF, IGF-1, and VEGF were determined by enzyme immunoassay methods using commercial kits (R&D Systems, United States, catalog no. DBD00, DG100, DVE00). The average intra-assay CV was 8.0% for all proteins. Serum cortisol concentration was evaluated using a Demeditec (Germany) ELISA kit. Detection limits were 2.5 ng mL^{-1}, and the intra-assay coefficient of variation for the kits was <7%. For blood LA analysis samples were collected from capillary blood taken from the finger as part of the baseline and after completion of last SIE bout. Immediately after collection, the blood was deproteinized by the addition of ice-cold 0.4 M perchloric acid. After being thoroughly mixed, the samples were centrifuged at 12,000 × *g* for 10 min. Blood LA was determined using a standard Randox (Crumlin, United Kingdom) kit based on the LA oxidase method (LC2389); assays were performed on a Cecil CE9200 spectrophotometer (Cambridge, United Kingdom).

TABLE 1 | Demographic and clinical characteristics.

Variable	SIE (*n* = 20)	CTR (*n* = 16)
Age (years)	21.0 ± 0.9	21.7 ± 1.3
Height (cm)	181.4 ± 6.4	183.3 ± 5.6
Weight (kg)	79.9 ± 7.9	83.1 ± 11.2
Fat (%)	13.0 ± 4.2	14.3 ± 6.4
Fat (kg)	10.5 ± 4.3	12.4 ± 6.5
SMM (kg)	39.9 ± 3.5	40.5 ± 4.1
BMI (kg × m^{-2})	24.2 ± 2.0	24.6 ± 2.5
TBW (kg)	50.8 ± 4.2	51.7 ± 5.0
$\dot{V}O_{2max}$ (mL × min^{-1} × kg^{-1})	48.6 ± 5.1	49.4 ± 6.2
Aerobic power (W)	285.7 ± 31.4	294.0 ± 35.6

SIE, sprint interval exercise group; CTR, control group; values are mean ± SD expressed in absolute or relative values; Fat (%), fat percentage; Fat (kg), fat mass; SMM, skeletal muscle mass; BMI, body mass index; TBW, total body water; $\dot{V}O_{2max}$, maximal oxygen uptake; aerobic power (W), maximal aerobic power achieved during $\dot{V}O_{2max}$ test.

Statistical Analysis

The statistical calculations were performed using STATISTICA 13. The results are expressed as mean and standard deviation (SD), or standard error of mean (SEM). The normality of data distribution was established using the Shapiro–Wilk *W*-test. The level of significance was set as $p = 0.05$ for all of the analyses. Additionally, two-way analysis of variance (ANOVA) with repeated measures was used to investigate the significance of differences between groups and time. For peripheral neurotrophic factors concentration response to acute SIE a one-way repeated measures ANOVA was applied. Significant main effects were further analyzed with the Bonferroni or Tukey's *post hoc* test. Changes (delta) in both groups were compared using an independent samples *t*-test or a Mann–Whitney *U* test, according to the data distribution. Correlations between variables were evaluated using the Spearman correlation coefficient for non-normally distributed data.

RESULTS

The study participants were physical education students who experience various forms of physical activity during the course of their studies (swimming, gymnastics, athletics,

football, or basketball). Moreover, the results of body fat and $\dot{V}O_{2max}$ values demonstrate that the participants were physically active. All participants completed the study. The anthropometric and physical activity parameters are presented in **Table 1**. At baseline, there were no significant differences in basic anthropometric characteristics or in aerobic performance between groups (**Table 1**).

Physiological Response to SIE

The evolution of VO_2, VE, and HR during the six bouts of SIE are displayed in **Figure 2**. The obtained data indicate a significant increase in cardio-respiratory parameters compared to the baseline in the SIE group (**Figure 2**). The average $\dot{V}O_2$, VE, and HR responses to SIE correspond to ∼85, 90, and 90%, respectively, compared to the values reached in the maximal graded exercise test ($\dot{V}O_{2max}$ test). Moreover, the SIE group had RPE after acute SIE that averaged between 17–18 ± 2 (mean ± SD).

Cognitive Performance in Stroop Test and Trail Making Test

The analysis revealed no statistical differences between the SIE and CTR groups in ST performance ($p = 0.85$), TMT-A ($p = 0.24$), and TMT-B ($p = 0.39$) for the pre-sessions (**Figure 3**). There was significant interaction between group (SIE/CTR) and time (PRE/POST) factors when we performed a two-way ANOVA with repeated measures for ST execution time ($p < 0.05$; $F_{(1,34)} = 9.45$). Next, to examine the interaction, we calculated the difference of the degree of ST performance between post- and pre-sessions (delta), contrasted for both the SIE and CTR groups separately, and compared the difference between them. The delta ST performance difference was significantly more negative in the SIE than in the CTR group ($z = 62.0$; $p < 0.001$, Mann–Whitney U test) (**Figure 3B**).

Moreover there was a significant main effect of time in the TMT parts A and B ($p < 0.05$; $F_{(1,68)} = 21.23$ and $p < 0.01$; $F_{(1,68)} = 6.42$, respectively; two-way ANOVA), whereas neither main effect of group nor interaction of the factors was significant. *Post hoc* Tukey's test analysis revealed a significant decrease in the both test version A ($p < 0.01$) and B ($p < 0.05$) execution time in the SIE group 20 min post-exercise (**Figures 3C,D**).

The Response of Blood Lactate and Cortisol Levels Following Acute SIE

Blood LA concentration and serum cortisol concentration were significantly affected by SIE (**Figure 4**). The SIE resulted in a rapid increase in blood LA concentration ($t = 35.5$; $p < 0.01$). Acute SIE induced an almost twofold cortisol level increase ($p < 0.01$) sustained to 1 h post exercise ($p < 0.05$) relative to baseline: one-way repeated measures ANOVA with *post hoc* Bonferroni test results were $p < 0.01$; $F_{(2,38)} = 18.51$.

Effect of Acute SIE on Peripheral Levels of BDNF, IGF-1, and VEGF

Analyzing the effect of SIE on peripheral trophic factor concentration, a two-way ANOVA for repeated measures with *post hoc* Bonferroni test revealed a significant interaction (Group x Time) ($p < 0.01$; $F_{(1,34)} = 10.18$) in BDNF and VEGF ($p < 0.01$; $F_{(1,34)} = 7.51$) peripheral concentration (**Figures 5A,C**). Further, there was a significant main effect of time ($p < 0.05$; $F_{(1,34)} = 3.87$), whereas neither the main effect of group nor interaction of the factors was significant in peripheral IGF-1 concentration (**Figure 5B**). Moreover, statistically significant differences between the SIE and CTR groups in delta BDNF ($t = 3.19$; $p < 0.01$) and VEGF ($t = 2.60$; $p < 0.01$) concentrations were found (**Figures 5D,F**). Despite the increase in IGF-1 following SIE, statistical analysis did not show any significance effect versus the CTR group ($t = 1.77$; $p = 0.08$) (**Figure 5E**).

The Response of Peripheral BDNF, IGF-1, and VEGF Following Acute SIE

To verify the peripheral BDNF, IGF-1, and VEGF concentration response to acute SIE a one-way repeated measures ANOVA and *post hoc* Bonferroni was applied. Acute SIE induced an increase in serum BDNF, IGF-1, and, VEGF concentration (**Figure 6**). There was a significant difference between time points in BDNF, IGF-1, and VEGF concentration ($p < 0.01$; $F_{(2,38)} = 9.97$, $p < 0.01$; $F_{(2,38)} = 5.54$, and $p < 0.01$; $F_{(2,38)} = 5.23$, respectively; one-way repeated measures ANOVA and *post hoc* Bonferroni test). *Post hoc* analysis revealed a significant increase in serum BDNF concentration immediately post-SIE ($p < 0.01$), sustained to 1 h post-SIE ($p < 0.01$), relative to baseline, as well as for VEGF concentration immediately post-SIE ($p < 0.05$), sustained to 1 h post-SIE ($p < 0.05$), relative to baseline. Acute SIE also significantly altered serum IGF-1 concentration immediately post exercise ($p < 0.01$) whereas at 1-h post-SIE serum IGF-1 concentration decreased and was not significantly different from the baseline. Changes in serum BDNF, IGF-1, and VEGF concentration after SIE are displayed in **Figure 6**.

Correlation Analyses

Positive correlations were identified between LA and BDNF concentration ($r = 0.35$, $p < 0.05$), between LA and IGF-1 concentration ($r = 0.31$, $p < 0.05$), and between LA and VEGF concentration ($r = 0.33$, $p < 0.05$) in the pre- and post-session in the SIE group.

In addition, we performed Spearman correlation analysis to examine the association between Stroop execution time and BDNF concentration ($r = -0.26$, $p < 0.05$), and a blood LA concentration ($r = -0.40$, $p < 0.01$) (**Figures 8A,B**).

DISCUSSION

In the present study, acute SIE led to an improvement in prefrontal-dependent cognitive performance. Also, SIE resulted in significant elevations of serum BDNF, IGF-1, and VEGF levels and these changes are accompanied by an increased peripheral LA concentration. These findings suggest that acute bouts of SIE are beneficial to improve cognitive performance 20 min following exercise in young adults.

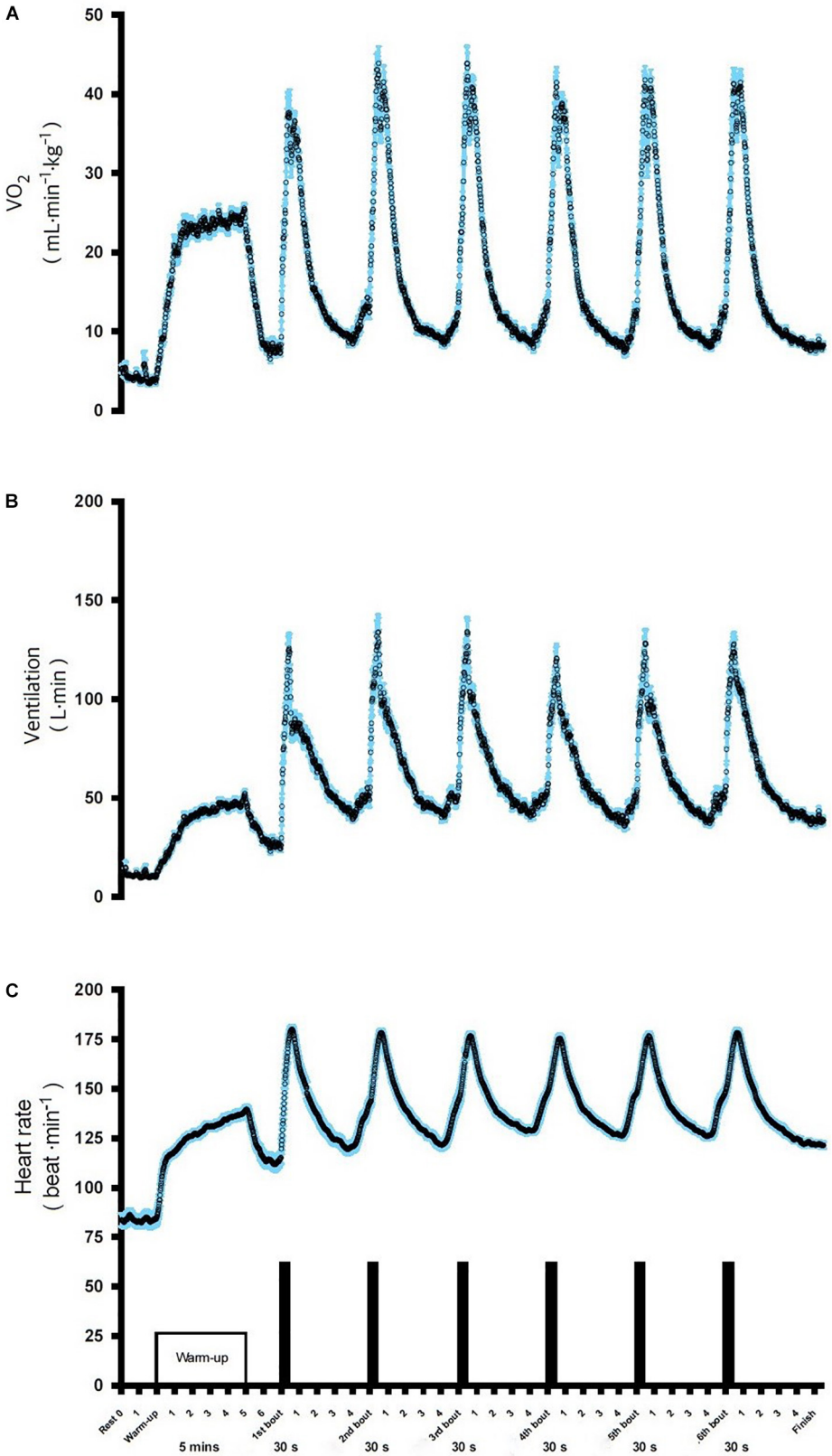

FIGURE 2 | Oxygen consumption VO_2 **(A)**, ventilation VE **(B)**, and heart rate HR **(C)**, during SIE. Data shown are means.

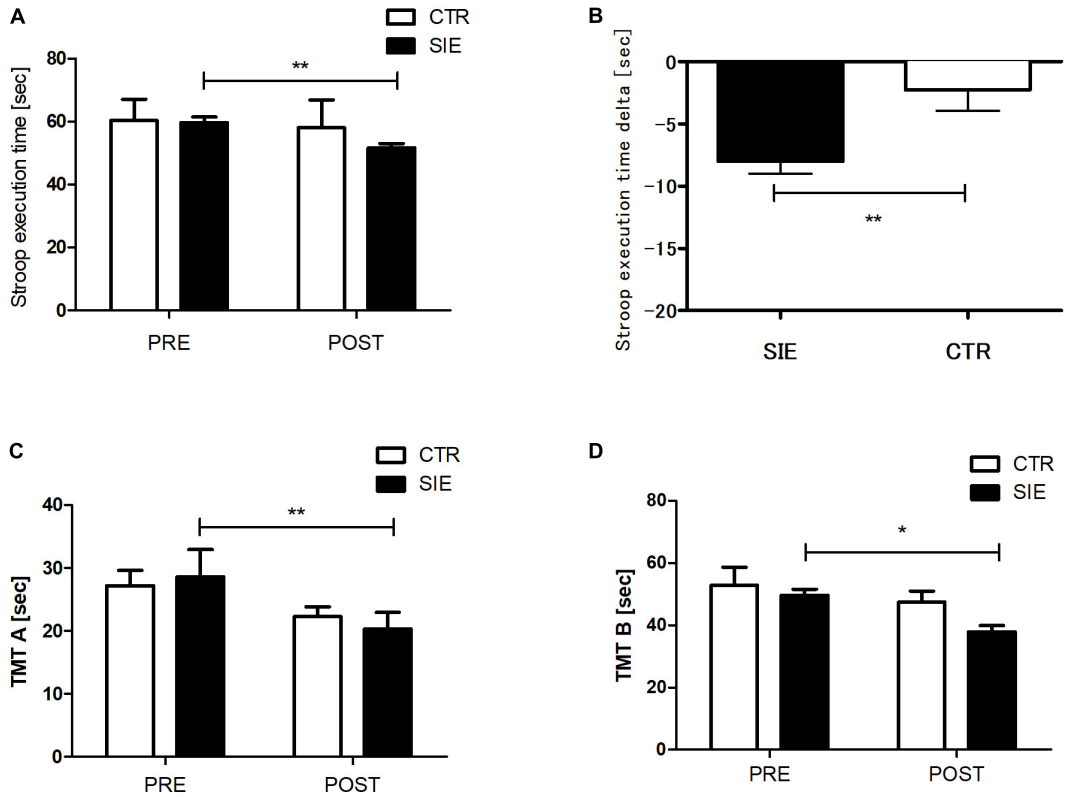

FIGURE 3 | Stroop task **(A)** and trial making test, parts A and B **(C,D)** execution times. Contrast between SIE delta (post–pre) and CTR delta (post–pre) for Stroop task performance **(B)**. Values are means. Error bars indicate ± SEM (standard error of mean). $^{*}p < 0.05$, $^{**}p < 0.001$.

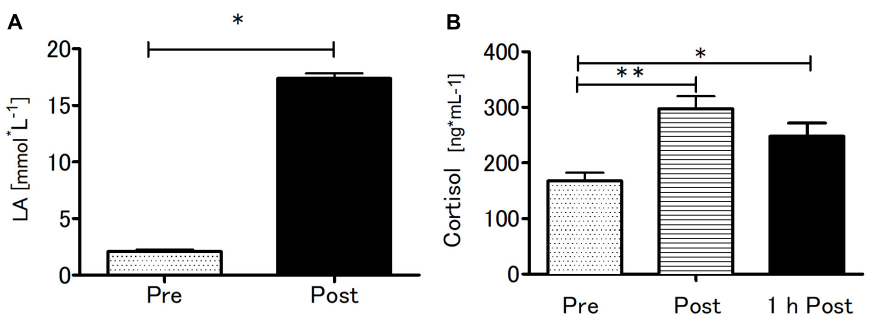

FIGURE 4 | Blood lactate at baseline and after SIE **(A)** and serum cortisol concentrations pre, immediately post, and 1-h post-SIE **(B)** for the exercise group. Values are means. Error bars indicate ± SEM. $^{*}p < 0.05$, $^{**}p < 0.001$.

We illustrated that acute SIE improved cognitive performance as indicated by shorter response times for the ST and faster completion time of the TMT parts A and B. These results are consistent with the findings of recent neuroimaging studies revealing improved cognitive performance on the ST and TMT A and B after acute aerobic exercise as well as with SIE, both of which are associated with changes of neural activation in the prefrontal cortex (Yanagisawa et al., 2010; Lee et al., 2014; Kujach et al., 2018). Therefore, we can postulate that the current SIE model is beneficial to the prefrontal-dependent cognitive functions in young, healthy adults.

Blood LA elevated through SIE is, potentially, a factor that leads to improved cognitive function together with SIE-induced neuroprotective protein levels. It has been demonstrated that glucose and LA are important energy sources not only in muscle, but also in the human brain (van Hall et al., 2009). At rest, the brain mainly relies on glucose, whereas during high-intensity exercise, glucose uptake significantly decreases with increased blood LA concentration (Kemppainen et al., 2005). In contrast, LA is used by the brain in order to compensate for the increased energy required to maintain neuronal activity during high-intensity exercise (Kemppainen et al., 2005; Weston et al., 2014).

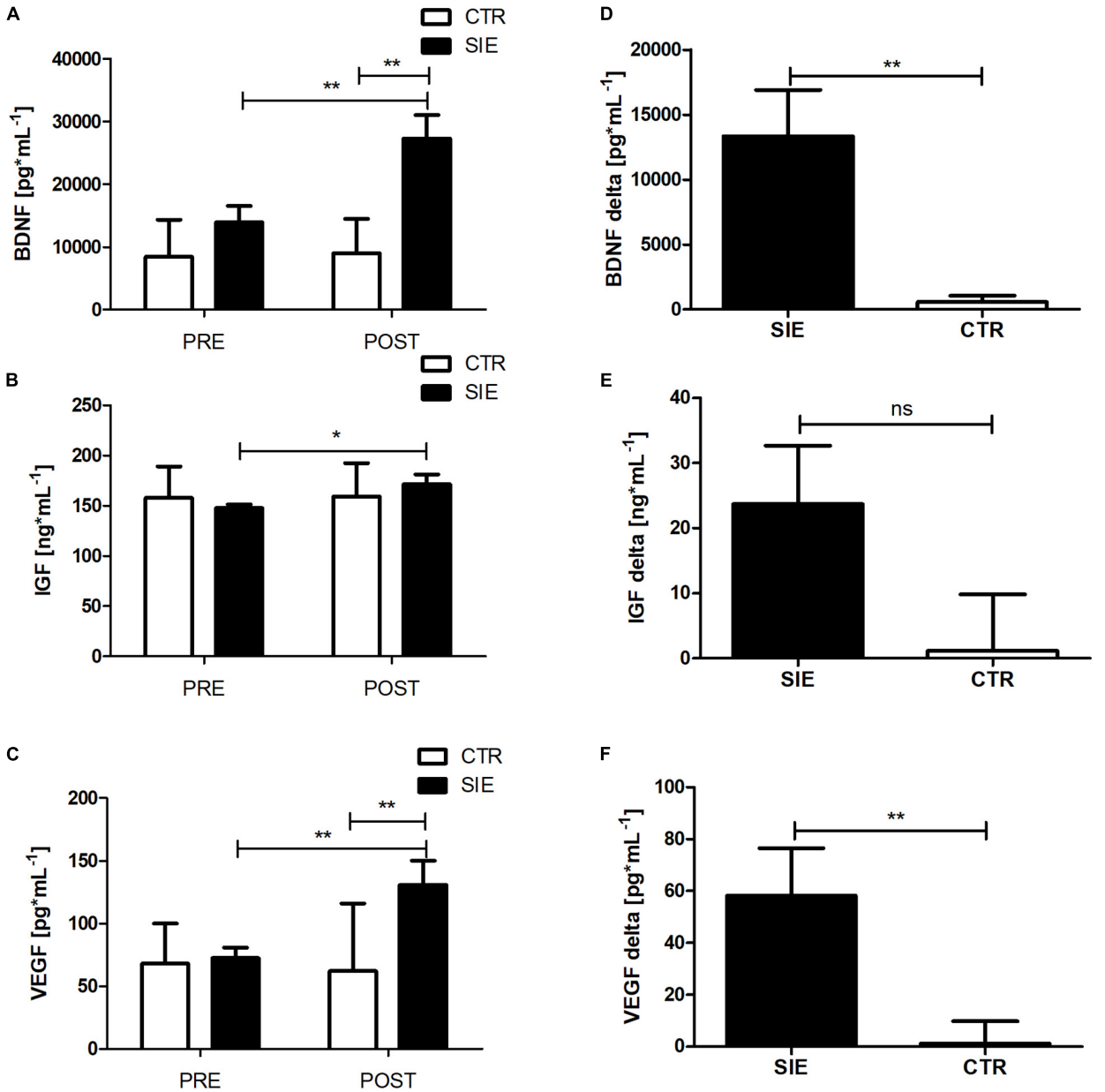

FIGURE 5 | Effect of acute SIE on peripheral BDNF **(A)**, IGF-1 **(B)**, and VEGF **(C)** concentrations and contrast between SIE versus CTR deltas (post–pre) **(D–F)**. Values are means. Error bars indicate ± SEM. *$p < 0.05$; **$p < 0.001$; ns, non-significant.

Recently, Hashimoto et al. (2018) showed that arterial LA and brain LA uptake (arterial–venous differences across the brain) increases after SIE, suggesting that systemic LA affects brain LA uptake and influences executive function after exercise (Weston et al., 2014). Moreover, we found a positive correlation between blood LA and neuro-supporting protein concentrations. Similarly, Ferris et al. (2007) found that the increased blood LA level induced by acute exercise correlated with blood BDNF level. Furthermore, it has been pointed out, that LA is the "missing exercise factor" inducing BDNF synthesis (El Hayek et al., 2019). Very recently, El Hayek et al. (2019) observed that LA modulates the redox status of neurons by altering the NAD^+/NADH ratio which leads to SIRT1 activation and in turn engages the hippocampal PGC1-α/FNDC5 pathway to induce BDNF expression (Koltai et al., 2010; Wrann et al., 2013). Moreover LA released from exercising muscles mediates cerebral angiogenesis through the activation of the LA receptor HCAR1, a key regulator of VEGF (Morland et al., 2017). Additionally, LA induces IGF-1 mRNA expression via the somatotropic axis stimulation (Salgueiro et al., 2014). Since blood LA following SIE greatly increased in the present study, we cannot rule out that the improvement in cognitive functions

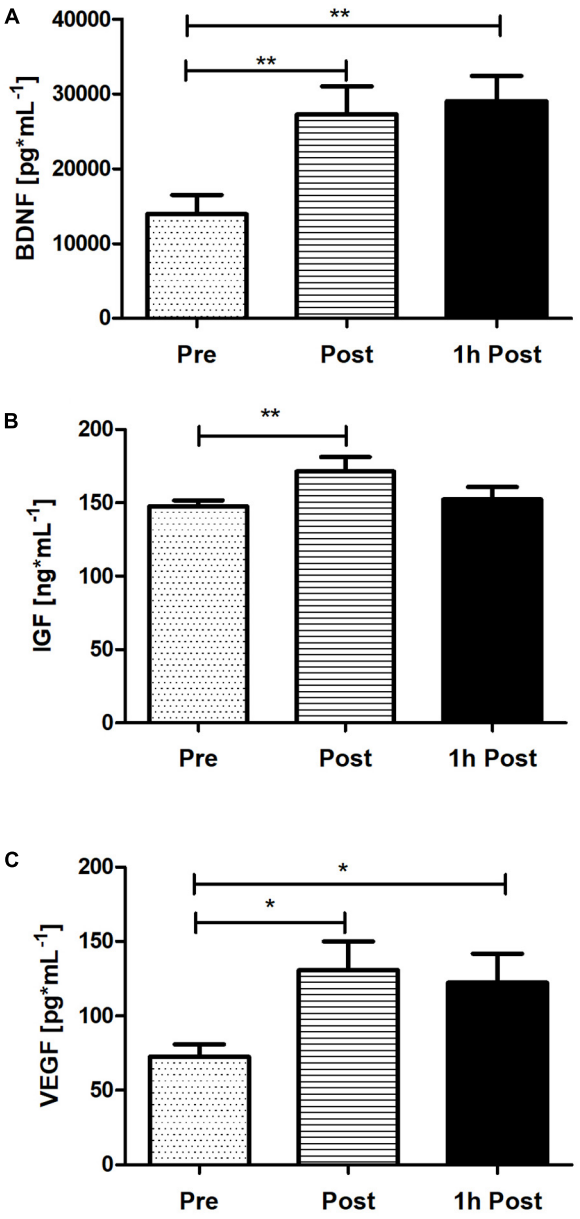

FIGURE 6 | The response of serum BDNF **(A)**, IGF-1 **(B)**, and VEGF **(C)** concentration immediately post and 1 h following acute SIE. Values are means. Error bars indicate ± SEM. $^{*}p < 0.05$, $^{**}p < 0.001$.

resulted from the acceleration of cerebral LA metabolism along with neuroprotective proteins induction. However, various high-intensity interval training protocols could differently affect blood LA synthesis (Wood et al., 2016; Warr-di Piero et al., 2018). SIE is characterized by high LA production whereas longer protocols could rely more on aerobic metabolism, with lower LA synthesis (Astorino and Sheard, 2019). Therefore, interval training protocols could differently modulate the central nervous system activation and cognitive performance.

The present data also revealed that peripheral BDNF, IGF-1, and VEGF secretion are modulated by acute SIE. Recent studies proposed a hypothesis of the role of exercise-induced BDNF synthesis (Kemppainen et al., 2005; Wrann et al., 2013; Saucedo Marquez et al., 2015). Wrann et al. (2013) suggested that the activation of peroxisome proliferator-activated receptor-alfa coactivator (PGC)-1α is induced by exercise in skeletal muscle cells, and that it could activate FNDC5 gene expression, which is a positive regulator of BDNF levels in the brain, mainly in the hippocampus. Interestingly, an increase in PGC1-α has also been observed after exercise adopted in an interval exercise protocol (Little et al., 2011b). Moreover, Ferris et al. (2007) indicated that intensive exercise leads to greater increases in peripheral BDNF concentration than does low-intensity, continuous exercise, suggesting that exercise intensity could be a key factor. Saucedo Marquez et al. (2015) have speculated that skeletal muscle contractions during high-intensity exercise may trigger this biochemical pathway, inducing elevated BDNF levels in the brain. They revealed that the SIE protocol (10 × 1-min bouts at 90% of maximal work load, alternating with 1-min rest at 60 W for a total duration of 20 min) is a more effective and preferred intervention for elevating BDNF levels than traditional, continuous, moderate-intensity exercise (Saucedo Marquez et al., 2015). Furthermore, the high-intensity interval exercise protocol could also have induced optimal short bursts of oxidative stress and inflammation, leading to the activation of the prefrontal cortex a brain region involved in cognitive processing, including executive function (Gomez-Pinilla and Hillman, 2013). Our protocol was "all out" and characterized by a very high intensity which on the one hand stimulates dramatic BDNF increase and on the other hand could increase oxidative stress and inflammation to a greater extent. Thus, high-intensity exercises such as SIE, which is associated with increased inflammation and oxidative stress, will certainly be better tolerated by young active people in comparison to inactive persons or the elderly.

Moreover, it has been revealed that BDNF could modulate presynaptic neurotransmitter release and evoke excitatory postsynaptic currents via TrkB receptors, directly inducing neuronal depolarization (Kafitz et al., 1999; Jovanovic et al., 2000). Furthermore, we found a positive association between improved ST performance and BDNF concentration. Accordingly, other animal and human studies have shown significant, positive associations between serum BDNF and cortex BDNF ($r = 0.81$) at rest as well as elevated BDNF and improved cognitive performance in response to acute exercise (Karege et al., 2002; Kraus et al., 2004; Winter et al., 2007; Griffin et al., 2011). These findings suggest that acute SIE stimulates BDNF and may induce cognitive enhancement related to the above-mentioned changes in neural activation in the prefrontal cortex (Yanagisawa et al., 2010; Lee et al., 2014).

According to our initial hypothesis, acute SIE significantly increased peripheral IGF-1 concentration. Although IGF-1 concentration in the serum has been reported to increase following high-intensity exercise (Kraemer et al., 2004) other studies have reported a lack of post-exercise increase in peripheral IGF-1 concentration (Jahreis et al., 1989; Schiffer et al., 2009). Circulating IGF-1 is mainly derived from the liver, but its secretion is also found in the brain, skeletal muscle, or bones (Yakar et al., 1999; Dall et al., 2001). Therefore, it is assumed that

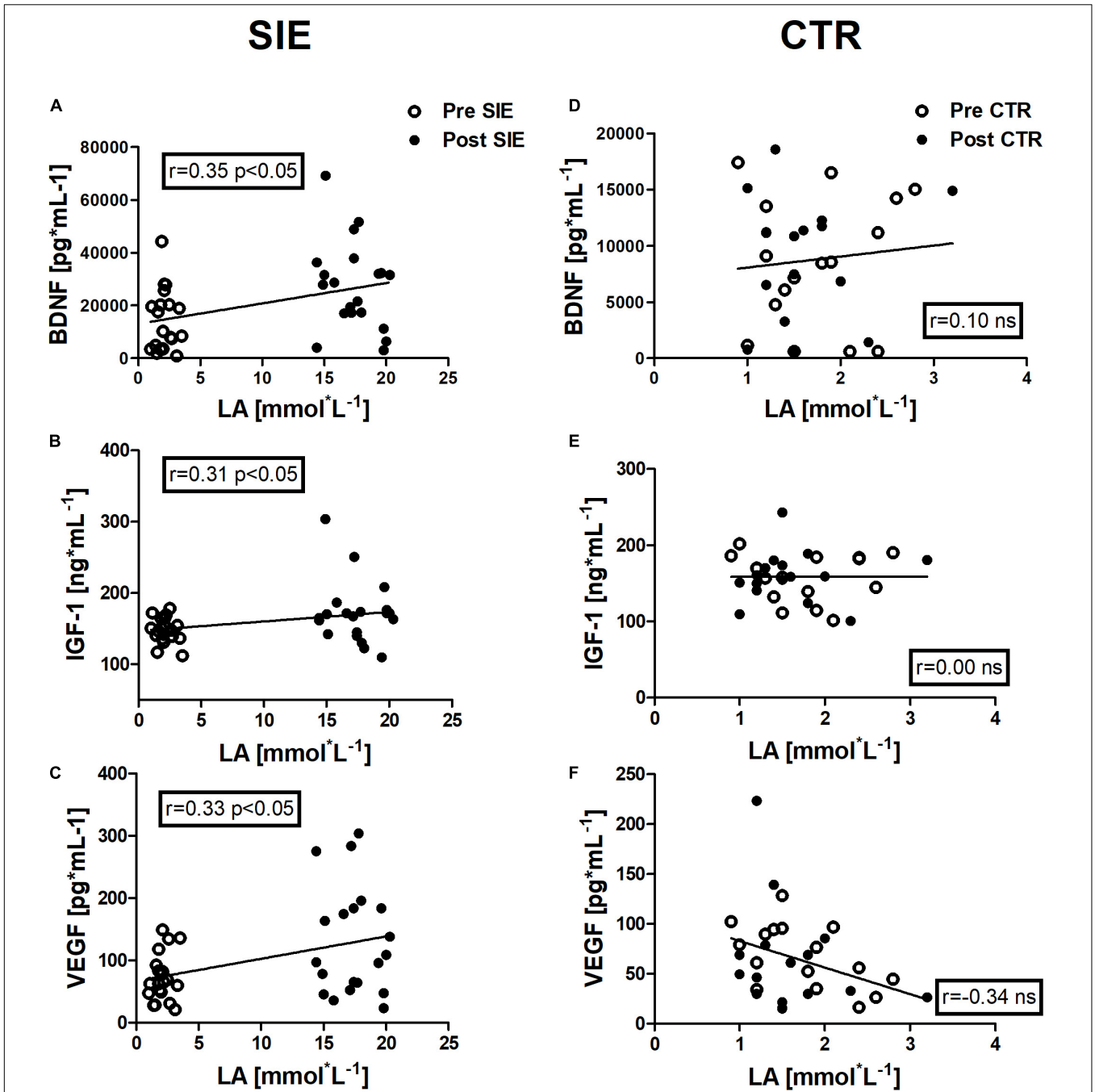

FIGURE 7 | Association between LA and BDNF, IGF-1, and VEGF concentrations, of pre- and post-sessions in the SIE and CTR groups. Significant positive correlations between LA and BDNF concentration **(A)**, LA and IGF-1 concentration **(B)**, and LA and VEGF concentration **(C)** were found. No significant associations were observed in the concentration of neurotrophic factors and LA in the control group **(D–F)**. Data are presented as open circles: pre, and closed circles: post-SIE or CTR. ns, non-significant.

for post-exercise IGF-1 concentration, peripheral and cerebral sources are partly responsible (Dall et al., 2001). Additionally, acute exercise stimulates the expression and release of liver IGF-1 and results in elevated brain uptake of IGF-1 (Carro et al., 2000). Also, peripheral IGF is necessary for exercise-induced hippocampal neurogenesis and for functional recovery after brain injury in rodents (Trejo et al., 2001; Duman et al., 2009). Similarly, to IGF-1 we found significant increase in VEGF concentrations. The effects of acute exercise on peripheral VEGF have produced conflicting results with some investigators finding an increase after acute exercise (Kraus et al., 2004; Wahl et al., 2011) and others finding no differences in VEGF

concentration in healthy subjects after physical exercise (Landers-Ramos et al., 2014). Interestingly the level of VEGF in the hippocampus decreases with age, while post-exercise VEGF elevation could play an important therapeutic role increasing brain functions (Shetty et al., 2005). Peripherally circulating VEGF can affect neurogenesis and synaptic plasticity by inducing proliferation and differentiation of neural stem cells (Zacchigna et al., 2008; Ruiz de Almodovar et al., 2009). It appears that the IGF-1 as well as VEGF response are largely dependent on exercise intensity and a lack of consistency in study designs as far as exercise type, intensity, duration, and timing of blood sampling after exercise may explain some of these discordant findings (Griffin et al., 2011; Wahl et al., 2011; Skriver et al., 2014).

There is strong evidence from studies of both human and animal subjects that BDNF, IGF-1, and VEGF are important pathways by which chronic exercise modulates brain function (Cotman et al., 2007; Voss et al., 2013). Recent studies suggest that the three growth factors have an impact on functional brain connectivity in the medial and lateral temporal cortices (Voss et al., 2013). Moreover, BDNF may also influence functional connectivity by increasing synaptogenesis and dendritic spine density, therefore improving long-term potentiation (LTP) via increased synaptic plasticity (Schinder and Poo, 2000; Rex et al., 2006; Vaynman et al., 2006; Stranahan et al., 2007). In addition, periphery-produced IGF-1 and VEGF support exercise-induced neurogenesis and angiogenesis (Ding et al., 2006). Further, exercise-induced neurovascular adaptations in the hippocampus have been associated with cognitive function (Clark et al., 2009). Taken together, a growing number of evidence supports the importance of neurotrophic factors for synaptic plasticity and structural brain changes. However, these findings are mostly observed in chronic exercise intervention and were not replicated in the current study.

It has been revealed that exercise intensity may differently affect cognitive performance (Tomporowski, 2003; Kohut et al., 2006). Low- and moderate-intensity exercise improves cognition, whereas high-intensity exercise may lead to increased arousal resulting in impaired cognitive performance. For example, Chmura et al. (1994) observed gradual shortening in reaction time during a multiple-choice reaction task with an exercise intensity of up to ∼75% $\dot{V}O_{2peak}$, beyond which reaction time was rapidly impaired, suggesting that exercise intensity plays a role in the effects of acute exercise on some aspects of cognitive performance (Chmura et al., 1994). Moreover, prolonged high-intensity exercise exposure causes impairments in cognitive control and has neurotoxic effects in the human brain (Farias-Junior et al., 2019). In contrast, not only did our study not reveal any decrements in cognitive performance after an SIE session, but it also identified improvements in cognitive control and working memory. This is consistent with previous research adopting higher-intensity intermittent exercise, in which cognition parameters remained unaffected or even improved (Winter et al., 2007; Salgueiro et al., 2014). In addition, these findings are generally in agreement with other studies revealing that a single bout of HIT could improve executive performance and that this facilitation is sustained even for as long as 30 min (Yakar et al., 1999; Duman et al., 2009; Alves et al., 2014).

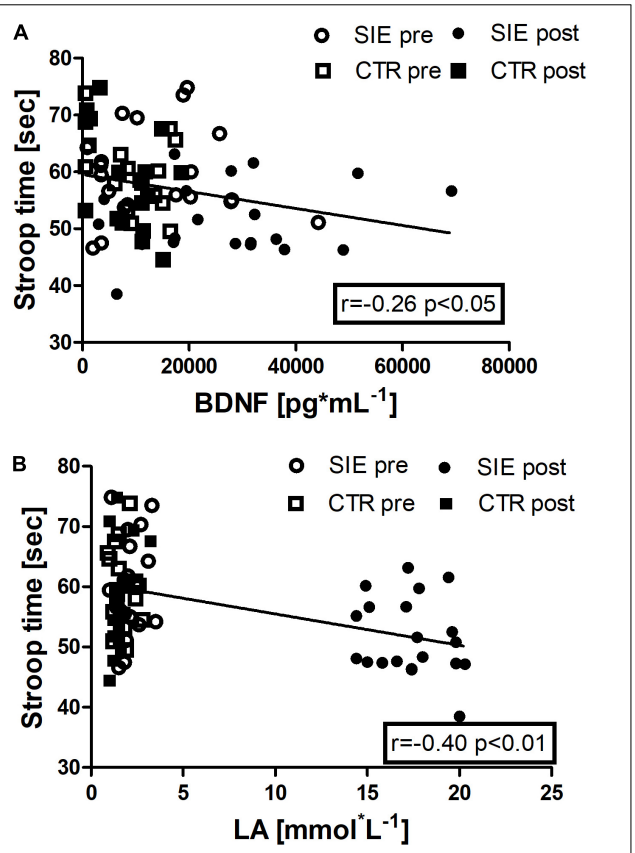

FIGURE 8 | Association between Stroop execution time and BDNF concentration ($r = -0.26$, $p < 0.05$) **(A)**, and Stroop execution time and blood LA concentration ($r = -0.40$, $p < 0.01$) **(B)**. Data are presented as open circles: pre, and closed circles: post-SIE and open squares: pre, and closed squares: post-CTR.

The heterogeneity in the pattern of results of previous studies maybe due to differences in methodologies involving exercise mode and protocol, the participants' fitness levels, cognitive task type, post-cognitive test timing, and other confounding factors (Chang et al., 2012).

It should also be noted that the high intensity of SIE could produce negative affective responses, thus making people unlikely to habitually perform the exercise (Hardcastle et al., 2014; Biddle and Batterham, 2015). We did not determine the mood state (enjoyment, pleasure, or affect) of our participants, so it is difficult to conclude whether they would like to repeat this kind of exercise in the future. However, Olney et al. (2018) demonstrated that healthy adults unaccustomed to interval training perceived high-intensity and SIE to be as enjoyable as time-consuming moderate-intensity continuous exercise. It is worth mentioning that the structure of interval training can be easily modified, allowing its application in people with diverse physical fitness levels. Therefore, there is a need for further research verifying the impact of various SIE protocols, especially since Townsend et al. (2017) showed that shorter sprint bouts (<30 s) in SIE produce greater pleasure among subjects. The proposed SIE can be successfully

and safety applied by subjects with physical activity experience, such as physical education students or athletes. Collectively, we propose that even high intensity exercise, such as SIE, can be a fruitful and time-efficient intervention providing cognitive benefits to young, physically active people.

LIMITATIONS

In this study we tested young, active, healthy men, making it difficult to generalize our results to the general population. Moreover, we did not control for BDNF polymorphism, which could also influence BDNF secretion in response to exercise.

CONCLUSION

In summary, the current findings indicate that acute SIE enhances human cognitive function. The improvement in cognitive performance may result from the synthesis nor release of neuroprotective proteins modulated by high post-exercise blood LA concentration.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bioethical Committee of the Regional Medical Society in Gdańsk. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SK, RO, and RL conceived of and designed the experiment. SK, RO, EZ, and RL collected the data. SK, KB, and ES performed the statistical analyses and interpreted the data. SK, RO, KB, ES, EZ, RL, and HS participated in drafting the article or revising it critically for important intellectual content. SK, RO, KB, KS, EZ, ES, RL, and HS approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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