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Burrowing as a novel voluntary strength training method for mice: A comparison of various voluntary strength or resistance exercise methods

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HIGHLIGHTS

- Novel and established strength training methods for rodents are compared.
- Burrowing qualifies as a novel voluntary strength training method for mice.
- Resistance running is a multi-modal aerobic-strength exercise method for mice.
- Unloaded tower climbing did not cause functional or muscular improvements.

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ABSTRACT

Background: Voluntary strength training methods for rodents are necessary to investigate the effects of strength training on cognition and the brain. However, few voluntary methods are available.

New method: The current study tested functional and muscular effects of two novel voluntary strength training methods, burrowing (digging a substrate out of a tube) and unloaded tower climbing, in male C57Bl6 mice. To compare these two novel methods with existing exercise methods, resistance running and (non-resistance) running were included. Motor coordination, grip strength and muscle fatigue were measured at baseline, halfway through and near the end of a fourteen week exercise intervention. Endurance was measured by an incremental treadmill test after twelve weeks.

Results: Both burrowing and resistance running improved forelimb grip strength as compared to controls. Running and resistance running increased endurance in the treadmill test and improved motor skills as measured by the balance beam test. Post-mortem tissue analyses revealed that running and resistance running induced *Soleus* muscle hypertrophy and reduced epididymal fat mass. Tower climbing elicited no functional or muscular changes.

Comparison with existing methods: As a voluntary strength exercise method, burrowing avoids the confounding effects of stress and positive reinforcers elicited in forced strength exercise methods. Compared to voluntary resistance running, burrowing likely reduces the contribution of aerobic exercise components.

Conclusions: Burrowing qualifies as a suitable voluntary strength training method in mice. Furthermore, resistance running shares features of strength training and endurance (aerobic) exercise and should be considered a multi-modal aerobic-strength exercise method in mice.

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1. Introduction

1.1. Beneficial effects of aerobic exercise and strength training on brain functioning

Exercise has the potential to increase brain health and brain functioning in mice and men (Hillman et al., 2008; Voss et al., 2013).

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Most studies that investigated the effects of exercise on the brain in animals or humans have focused on the effects of aerobic exercise such as running or walking. This is especially true for rodent studies, in which the use of voluntary running in a running wheel or forced running on a treadmill are standard methods used to investigate the mechanisms underlying the effects of (aerobic) exercise on the brain. And with good reason: a growing body of evidence from human studies shows that aerobic exercise improves cognitive functioning in healthy adults and counters cognitive decline in the context of aging (Hillman et al., 2008; Intlekofer and Cotman, 2013). Moreover, aerobic exercise improved cognition in elderly with Mild-Cognitive Impairment, a proposed intermediate stage of cognitive decline that sits between general age-related decline and dementia-related cognitive decline, as well as in patients suffering from Alzheimer's disease, Parkinson's disease and stroke (Baker et al., 2010; Bossers et al., 2015; Cusso et al., 2016; Intlekofer and Cotman, 2013; Quaney et al., 2009).

However, a recent study performed in dementia patients has shown that a combination of strength training and aerobic exercise is more effective in slowing dementia-related cognitive decline than aerobic exercise alone, underlining the therapeutic potential of strength training (Bossers et al., 2015). A similar effect has been observed in a meta-analysis performed by Colcombe and Kramer which included studies performed on both clinical and non-clinical elderly populations: A combination of strength and aerobic exercise training resulted in higher cognitive improvements than aerobic exercise alone (Colcombe and Kramer, 2003). In other studies, strength training was found to improve cognitive performance in elderly and in older women with Mild-Cognitive Impairment (Cassilhas et al., 2007; Liu-Ambrose et al., 2010; Nagamatsu et al., 2013). Thus, there is recent evidence to show that strength training has beneficial effects on brain functioning both in healthy and clinical populations. However, little is known about the underlying mechanisms involved.

1.2. Lack of animal models of voluntary strength training

In animal experiments, various exercise methods can be applied to investigate potential mechanisms underlying the effects of exercise on the brain. In general, we can differentiate between "forced" and "voluntary" exercise models. Voluntary methods are based on intrinsic motivation of the animal to perform the exercise behavior, such as running in a running wheel, and can be performed in the home-cage of the animal. Forced exercise methods (e.g. treadmill running) rely on negative reinforcers (e.g. shock avoidance) or positive reinforcers (e.g. food reward) to induce exercise behavior and often include extensive handling of the animal. Forced methods can induce stress. However, as touched upon below, the extent to which they do so differs between various forced methods.

Both forced and voluntary exercise methods have benefits and limitations. Forced methods allow researchers to precisely control training duration and intensity of the exercise intervention, this is impossible when using voluntary methods. However, forced methods that employ negative reinforcers such as avoidance of an aversive stimulus can be stressful. Stress is known to be detrimental to the brain, most notably the hippocampus, and can influence learning, memory, and behavior (Kim et al., 2006). The use of positive reinforcers (e.g. food reward) in forced exercise procedures allows researchers to avoid these confounding effects of stress, but can pose other problems. Often, negative reinforcement is needed to enhance the rewarding effects of the positive reinforcer (e.g. food restriction to enable the use of a food reward). Such a negative reinforcer affects the behavior of the animal. For example, food restriction influences physical activity levels in mice, among other behaviors and physiological changes (Overton and Williams, 2004). On the other hand, a more palatable food reward could be

used (e.g. chocolate or sucrose). A downside of such a strategy is that reward-learning could also influence animal behavior in other cognitive tasks. Thus, methods based on positive reinforcement rather than negative reinforcement can affect the physiology and behavior of the animal, albeit not always by inducing high amounts of stress. Voluntary exercise methods avoid the confounding effects of negative and positive reinforcers. In this respect they are often better suited to use in combination with behavioral tests, including cognitive ones.

The use of voluntary running as an aerobic exercise method in rodents has elucidated many of the mechanisms underlying the effects of aerobic exercise on the brain, which include increased hippocampal neurogenesis, enhanced synaptic plasticity, increased angiogenesis, and reduced oxidative stress (Cotman et al., 2007; Mazzola et al., 2015; Olah et al., 2009; Van der Borght et al., 2007; Van Der Borght et al., 2009; Voss et al., 2013). On the other hand, it is largely unknown which underlying mechanisms cause the beneficial effects of strength training on cognition (Strickland and Smith, 2016). This could be studied in rodents too. However, there are few if any voluntary strength training methods available for rodents (Strickland and Smith, 2016).

Strength training, also called resistance exercise, encompasses all training methods which use resistance-induced muscular contraction to increase muscle strength and muscle size (U.S. Department of Health and Human Services, 2008). Strength training relies mainly on anaerobic metabolism and fast twitch (type II) skeletal muscle fibers (Wilson et al., 2012). If performed correctly, strength training leads to muscle hypertrophy (Schoenfeld, 2010). Aerobic exercise, also called endurance exercise, relies mainly on aerobic metabolism and slow twitch (type I) skeletal muscle fibers (Wilson et al., 2012).

One voluntary exercise method for rodents which can partially be considered a form of strength training is resistance wheel running, in which a load is placed on the running wheel to increase the resistance that is needed to overcome the wheel's inertia. Resistance wheel running has differential effects on muscle physiology compared to non-resistance running (Konhilas et al., 2005; Legerlotz et al., 2008; Soffe et al., 2015). These physiological changes resemble some of the effects of strength training (i.e. muscle hypertrophy), but this type of exercise is likely to have a large aerobic component too: Mice still ran three to four hours per day when 5 g of resistance was placed on the running wheel (Konhilas et al., 2005). Exercise over such a prolonged period of time likely relies on aerobic metabolism.

To our knowledge, apart from resistance running, no other voluntary strength training methods exist. Various forced methods have been applied successfully. Available forced strength training methods for rodents include hanging exercise, loaded ladder climbing, weight lifting in an operant chamber, and weight pulling on a treadmill (Cassilhas et al., 2012; Krüger et al., 2013; Mori et al., 2003; Seo et al., 2014; Strickland and Smith, 2016). In the current study, we proposed and tested the use of two potential (novel) methods of voluntary strength training for rodents: burrowing and tower climbing.

1.3. Burrowing as a potential voluntary strength training method

Burrowing is used as a test, utilizing a natural rodent behavior to detect behavioral abnormalities. (Deacon, 2012, 2009, 2006) As a potential exercise method, burrowing has several interesting properties. Firstly, several lab animal species show spontaneous burrowing behavior when presented with a burrowing tube: i.e. mice, rats, hamsters, and gerbils will readily burrow in food pellets, wood chips or denser materials (Deacon, 2009). Secondly, the amount of substrate removed from the tube, representing the amount of work performed by the animal, is easily and reliably

quantifiable. Thirdly, by using different substrates, intensity can be modified. Finally, our pilot studies revealed that in mice most of the burrowing took place in the first hour after the burrowing tube was presented. During this time, mice spent a total of ~17 min in the burrowing tube, removing ~100 g of pebbles and sand, approximately four times their bodyweight, per minute. (Supplementary Fig. 1) This finding indicates that most burrowing is restricted to a relatively small amount of time. Consequently, burrowing in a dense substrate such as sand and/or pebbles can be viewed as an intensive form of exercise, most likely anaerobic in nature. We therefore hypothesized that burrowing could serve as a voluntary strength training method in mice.

1.4. Tower climbing as a potential strength training method

The successful application of loaded ladder climbing shows that climbing can be used as a strength training method (Duncan et al., 1998; Hornberger and Farrar, 2004; Yarasheski et al., 1990). Unloaded tower climbing has been used as a partially “forced” exercise method which takes place in the home cage, since mice were motivated to climb by placing their water bottle in the top of the tower but otherwise left undisturbed (Mori et al., 2003). Whether the unloaded climbing tower method can be considered strength training is unknown, but tower climbing does increase bone mass (Mori et al., 2003). Mori et al. showed that mice spend approximately 12 min climbing a total of ~60 m per day. In a comparable forced (isometric) strength training method, hanging exercise, as little as 9 min of training per day was sufficient to induce functional and muscular changes (Krüger et al., 2013). This comparison suggests that tower climbing in the home-cage could be performed in sufficient quantity to be a strength training method. Finally, in our pilot study we observed that mice also climbed in climbing towers without being motivated by availability of water in the top. (Data not shown) Therefore, we hypothesized that unloaded tower climbing is a suitable voluntary strength training method.

1.5. Aim and hypotheses

Our aim was to test and characterize burrowing and tower climbing as voluntary strength training methods in mice. Resistance running, as touched upon shortly above, may be used to mimic some aspects of strength training but of aerobic training as well. Functional changes induced by resistance running have scarcely been investigated in mice. Therefore, our second aim was to test and characterize resistance running as a potential voluntary strength training method.

These three different exercise methods will incite the mice to use their muscles in different ways. Burrowing and resistance running are mainly reliant on concentric (shortening) muscle activity. Climbers will rely on concentric as well as isometric (static) and eccentric (lengthening) muscle activity. Therefore, burrowing and resistance running might increase concentric strength whereas climbing may increase isometric strength as well. We also expected resistance running to induce functional changes that overlap with non-resistance running, an established endurance exercise method, such as increased endurance.

Thus, increase in grip strength (concentric strength) was our foremost criterion to define any strength training method. Decrease in muscle fatigue was a second criterion, expected to be elicited by isometric strength training. An increase in endurance capacity was the defining criterion for any aerobic (endurance) training method. Finally, strength training may improve balance in humans (Howe et al., 2007). We expected that any successful strength training method might do the same in our mice.

The current study should be viewed as an important first step in identifying novel voluntary strength exercise methods.

2. Materials and methods

2.1. Experimental groups

Adult male C57Bl6 mice (10 weeks old, 25 ± 1 g, Janvier, $n = 80$) were randomly allocated to seven experimental groups. Three potential voluntary strength exercise groups: Burrowers ($n = 12$), tower Climbers ($n = 12$), and Resistance Runners ($n = 12$). Two sedentary control groups: Home Cage Controls ($n = 10$) and Pseudo-Burrowers ($n = 12$). And an aerobic exercise group, Runners ($n = 12$), and forced isometric strength training group, Hang Exercise mice ($n = 10$). The mice were selected based on age and weight (with minimum variation in both parameters) to ensure variation in body size would not confound results obtained in the grip-strength test or of post-mortem tissue weight. We choose to test in male mice only to reduce variation due to sex differences.

Home Cage Controls served as the sedentary control group for Burrowers, Climbers, Resistance Runners, Runners, and Hang Exercise mice. Since Burrowers were housed in a larger cage to make room for the Burrowing apparatus (see 2.2) and since we wanted to control for all effects of placing the apparatus in the home cage which were not due to burrowing exercise, Pseudo-Burrowers served as a second “sedentary” control group for Burrowers. The only difference between Burrowers and Pseudo-Burrowers was the substrate with which their burrowing tube was filled: Sand/pebbles (~1400 g) for Burrowers and small wood-chips (~140 g) for Pseudo-Burrowers.

Runners were included as an aerobic (endurance) exercise group that could be compared to Resistance Runners. Similarly, Hang Exercise mice were included as an isometric strength training group and served as comparison for Climbers to check for the changes induced by isometric strength training (Krüger et al., 2013).

To balance the groups so that baseline grip strength was equal between them, two mice were allocated to switch groups after the baseline measurements were taken in the first round of behavioral testing. A one-way ANOVA was used to test for differences in grip strength at baseline, after which one out of the three strongest mice of the groups that were significantly stronger was randomly switched with one of the three weakest mice out of one of the other groups.

2.2. Animal housing

Mice were housed individually with *ad libitum* access to food and water. The mice were weighed weekly. Small wood chips were used as bedding material and shredded cardboard was used as nesting material. The mice were housed in a temperature (22°C) and humidity (55%) controlled room. A 12:12 light/dark schedule (lights on at 06:00 GMT + 1 h, 30 lx as measured between cages) was maintained. Cage size differed between experimental groups: Home Cage Controls, Runners and Resistance Runners as well as Climbers and Hang Exercise mice were housed in a type II Makrolon cage ($1 \times w \times h$; $33 \times 15 \times 13$ cm). Burrowers and Pseudo-Burrowers were housed in a type III Makrolon cage ($1 \times w \times h$; $42 \times 26 \times 15$ cm) to make room for a burrowing tube. After arrival, the mice were habituated to handling by the researcher in three 2-min handling sessions and five handling and weighing sessions of 1 min during an 18-day acclimatization period.

This study and all the procedures were approved by the ethical committee for the use of experimental animals of the University of Groningen.

Fig. 1. Burrowing tube and resistance running set-up. The burrowing tube viewed from the side (A) and from the front (B). C The burrowing tube, filled with a mix of sand and pebbles, was placed in the cage of a mouse during the dark phase for five days each week. D–E The resistance running wheel (a) had a pulley (b) connected to the elongated axis. Various weights (c) could be hung underneath the pulley to increase resistance on the wheel. F The wheels were calibrated by hanging a small weight in the wheel at the height of the axis and adjusting the weight underneath the pulley until the wheel moved. The load was increased up to 5 g by the end of the experiment.

2.3. Exercise protocols

All exercise protocols were performed for 14 weeks, including the period of behavioral testing and the week in which the animals were euthanized.

2.3.1. Burrowing and pseudo-burrowing

We based our burrowing protocol on the burrowing method devised by Deacon (Deacon, 2012, 2009, 2006). Burrowers and Pseudo-Burrowers had access to a burrowing tube five nights a week during the dark phase (Fig. 1A–C). The tube was filled with a mix of pebbles and sand (Burrowers, ~1400 g) or small wood chips (Pseudo-Burrowers, ~140 g, bedding material). The amount of substrate removed from the tube was measured 1 h after presentation of the burrowing tube, as well as overnight, on all five days during the first three weeks. During the remaining 10 weeks, the amount burrowed was measured 1 h after presentation of the tube, as well as overnight, on every first day of the week. On the other days, the amount burrowed was measured overnight only. In week 14, the week in which the mice were euthanized, the amount burrowed was measured overnight only.

2.3.2. Resistance and non-resistance running

Non-resistance Runners (Runners) were housed with a running wheel, resistance on this unloaded wheel was <0.5 g. Resistance Runners were housed with the same wheel that was modified so that resistance on the wheel could be increased (Fig. 1D,E). The resistance was increased up to 5 g over the course of the experiment (Fig. 1F). Running was monitored using the Circadian Activity Monitoring System (CAMS by H.M. Cooper, JA Cooper, INSERM U846, department of Chronobiology, Bron, France). Daily external workload (N·m) was calculated as follows: $W = T \times \theta$. Where W is work (N·m), T is torque (N·m) and is θ angular displacement (2 rad per revolution). Torque was calculated as follows: $T = mg \times r$. Where torque is T , m is wheel load (kg), g is Newton's conversion factor (9.81 m/s²) and r is the wheel radius (m). (Konhilas et al., 2005)

2.3.3. Climbing and hanging exercise

Climbers were housed in a cage with a wire-mesh climbing tower (1 m in height, 0.8 mm ϕ wires) based in the lid of the cage. This set-up was based on that of Mori et al., but differed since our mice were not motivated by placing a water bottle in the top. (Mori et al., 2003) Climbers were video monitored during one night (12 h) at the end of every week so there was no disturbance by placement of burrowing tubes or transport of Hang Exercise mice. Videos were analyzed to determine the distance climbed using Ethovision video tracking software (Noldus Information Technology).

Because we expected climbing to contain elements of isometric strength training (partially), we included a forced isometric strength training method for comparison: hanging exercise. A hanging exercise apparatus and protocol similar to that described by Krüger et al. was used. (Krüger et al., 2013) Mice performed three trials of 3 min of hanging exercise with a 1-min break in between during the first hour of the dark phase in a dark room lid by dim red light. The hanging apparatus consisted of a square wooden frame with a 9.5 cm by 9.5 cm square opening in the middle, in which a wire mesh (0.8–1 mm ϕ in diameter) was mounted. Five of these hanging apparatus were mounted parallel to the wall 50 cm above a padded surface so that five mice could be trained at the same time.

2.4. Behavioral tests

Behavioral testing was performed before (Pre), halfway through (Half, week 5–7) and near the end (Post, week 10–12) of the exercise period (Fig. 2). All behavioral tests were conducted in the light phase, between 04:00 and 11:00 Zeitgeber time, in a dimmed light (10 lux) room that was located next to the room where the animals were housed.

2.4.1. Balance beam test

The balance beam test is a sensorimotor integration test, used to test for subtle differences in balance and motor skills after four and nine weeks of exercise. The balance beam apparatus consists of a wooden beam placed 50 cm above a padded surface, with the home cage at the end of the beam and a bright light at the beginning of the beam. This beam could be placed horizontally, or under

Fig. 2. Timeline of the main experiment in weeks. Behavioral tests were conducted before, halfway through, and near the end of a 14-week exercise intervention. The balance beam test (week -3, 5, and 10), grip strength test (week -2, 6, and 11), inverted screen holding test (week -1, 7, and 12) and a treadmill endurance test (week 12) were conducted to determine functional effects of resistance running, climbing, and burrowing. After the mice were euthanized, muscle tissue and fat pads were collected and weighed.

a 30° angle. On the testing day, the animals were habituated to the task using a 14 mm and a 4 mm wide square beam placed horizontally, testing sessions commenced afterwards. Between the 14 mm and 4 mm habituation sessions there was a 90-s break, within both habituation sessions there was a 30-s break between the three trials performed within a session. A square (4 mm width) and round (6 mm ϕ) beam (rod) were used for test sessions. Latency to cross 100 cm was measured during three consecutive sessions on an angled square beam, an angled rod and a horizontally placed square beam, with a minimum 90-s break between sessions. During each session, three trials were performed, with 30-s breaks. If a mouse stopped halfway (e.g. to explore the beam) it was prompted forward by the researcher who moved his hand in the vicinity just behind the mouse and 1 s was deducted from the time needed to cross the beam. Mice that fell were attributed a maximum score of 40 s. We hypothesized that an increase in strength would improve performance on the angled but not the horizontal beams, as moving up at an angle demands more energy.

2.4.2. Grip strength test

The grip strength test measures muscle force in mice *in vivo*. A validated grip strength meter (Columbus instruments) was used to test grip strength before and after five and 10 weeks of exercise. Most tests could be performed routinely and objectively by a non-blinded researcher, but the grip strength test is susceptible for a bias due to subjectivity on the researchers part. Therefore, the researcher performing this test was blinded. Mice were picked up gently by their tails and allowed to grip on to a grid, which was attached to the grip strength meter. Then, the mice were pulled backwards horizontally by their tail until they let go of the grid. Peak force (grams force, gf) was measured. First, forelimb grip strength was measured in four trials separated by 60-s breaks. After a 150-s break, four limb (forelimbs and hindlimbs) grip strength was measured in four trials separated by 60-s breaks. Within each trial, two immediate retries were permitted if a mouse did not hold on to the wired grid. Immediately after the test, the mice were weighed. The maximum peak force measured (highest measurement out of four) was taken as a measure for grip strength.

2.4.3. Inverted screen hanging test

The inverted screen hanging tests was used to assess four limb (forelimbs and hindlimbs) muscle stamina, or muscle fatigue, after six and 11 weeks of exercise. We used a wire grid (wires 2 mm ϕ) surrounded by a small wooden frame. Mice were placed on the grid, and the grid was inverted and placed 50 cm above a padded surface. Five mice were tested at the same time in the same room, paper towels were used to separate them so they could not see each other. The latency until fall was scored using video recordings. Maximum hanging time was set to 20 min. If a mouse fell within 20 min, a second trial was performed after a 5-min break. Immediately after the test, the mice were weighed. Initially, during the first test session (pre-exercise), a 1 mm ϕ wire grids was used, but mice hung on to this grid so easily the test was repeated and consecutively performed with a 2 mm ϕ wire grid at all other time points. The maximum latency to fall and holding impulse were the outcome measures for this test. Holding Impulse was calculated by multiplying body mass (g) by maximum latency to fall (sec).

2.4.4. Treadmill endurance test

Endurance capacity was tested using a progressive running exercise test on a custom-build treadmill after 11 weeks of exercise. Mice were habituated to the treadmill apparatus two days before the treadmill endurance test. During the treadmill endurance test, the mice were placed on the apparatus and started running at 0.15 m/sec; every 3 min speed was increased with 0.05 m/sec (Krüger et al., 2013). Mice ran until exhaustion, which was marked by a period of 20 s during which the mice were unable to stay away from the shock-grid, despite prodding by the researcher. The treadmill test was performed once in week 12 because the stress caused by the shock grid can influence the performance of the mice in the other behavioral tests. Maximum running speed (m/s) and time until exhaustion (sec) were the outcome measures for this test.

2.5. Tissue weight: muscles, fat pads, and heart

In week 14, mice were euthanized via an intraperitoneal injection of pentobarbital, followed by transcardial perfusion with a 0.9% NaCl + heparin solution. Forelimb muscles (*Triceps Brachii*), hindlimb muscles (*M. Soleus*, *Gastrocnemius*, *Tibialis Anterior*, and

Fig. 3. Amount burrowed, running and climbed distances. A) The amount burrowed overnight differed between Burrowers and Pseudo-Burrowers because of the available substrate (~140 g of wood-chips for Pseudo-Burrowers, ~1400 g of pebbles/sand for Burrowers). There was no clear increase or decrease in amount burrowed over the 13-week exercise period. B) Weekly distance run was significantly lower and C) external workload was significantly increased in Resistance Runners as compared to non-resistance Runners. Resistance (g) was increased for Resistance Runners as depicted just above the x-axis and in figure 2F. D) Distance climbed peaked around 200 m per night and declined over time. An "*" indicates a significant difference ($p < 0.05$), an "#" indicates a trend for a difference ($p < 0.10$). Values are mean $\pm$ SEM, $n=12$ mice per group.

Quadriceps Femoris), the epididymal fat pad, and the heart were dissected and weighed.

2.6. Statistical analyses

All statistical analyses were performed using SPSS Statistics 22 (IBM). Distance run and external workload were compared between groups using a mixed repeated measures ANOVA (Within subject factor Time, 13 levels, one for each week) using *a priori* defined contrasts (See supplementary Table 1), differences between groups per time point were tested for using independent sample *t*-tests. Changes in amount burrowed and climbed over time were tested for using a repeated measures ANOVA. Performance on the balance beam, grip strength test, and the inverted screen hanging test were analyzed using a mixed repeated measures ANOVA, comparing the performance over time (within subject factor Time, three levels, one for each time point at which the test was performed) and between exercise groups and relevant controls (between subjects factors) using *a priori* defined contrasts (Supplementary Fig. 2). Greenhouse-Geisser (when $\epsilon \leq 0.75$) or Huyn-Feldt (when $\epsilon > 0.75$) corrections were applied to correct for violations of the assumption of sphericity. Differences between groups per time point were tested for using one-way ANOVA, Kruskal-Wallis one-way analysis of variance was used when normality could not be assumed (Tested by the Shapiro-Wilk test). A one-way ANOVA with *a priori* defined contrasts (Supplementary Fig. 2) was used to detect differences between exercise groups and Home Cage Controls (or Pseudo-Burrowers in the case of Burrowers) in the treadmill test and for tissue weights, Kruskal-Wallis one-way analysis of vari-

ance was used when normality could not be assumed (Tested by the Shapiro-Wilk test). P-values lower than 0.05 were considered to indicate statistical significance, P-values lower than 0.10 were considered to indicate a trend for a difference.

3. Results

3.1. Amount burrowed, running distance, climbed distance, and hanging training adherence

Eight out of 12 Burrowers started burrowing when the burrowing tube was presented for the first night. Within one week all Burrowers burrowed. There was no clear increase or decrease over time in the amount of substrate removed from the tube during the first hour of the dark phase or overnight (Fig. 3A). Burrowers removed an average of 1102 g (± 156 ; SD) of sand and pebbles from the burrowing tube overnight. Pseudo-Burrowers removed an average of 124 g (± 16 ; SD) of wood-chips overnight.

The average distance run per night increased in the first weeks by the Runners and Resistance Runners and peaked around week four and then declined over the remaining weeks. Resistance Runners ran less far than Runners ($p=0.04$), peaking at a distance of 7.4 (± 2.5 ; SD) km/day where Runners peaked at 9.5 (± 2.2 ; SD) km/day (Fig. 3B). During the first week, the distance run was equal between Runners and Resistance Runners. Daily external workload was slightly higher in Runners in week 1 ($p=0.03$). However, external workload was significantly higher in Resistance runners as compared to Runners in week 2–13 ($p < 0.00$). (Fig. 3C)

Table 1
Bodyweight and tissue weights.

Group		Home Cage Controls	Pseudo-Burrowers	Burrowers	Runners	Resistance Runners	Hang Exercise	Climbers
Body weight (g)	Week 0	25.88 ± 1.01	25.49 ± 1.49	25.47 ± 0.72	25.73 ± 1.12	25.91 ± 1.14	25.81 ± 0.99	26.04 ± 0.69
	Week 14	29.20 ± 2.09	28.41 ± 3.28	28.23 ± 0.94	27.65* ± 1.31	28.16* ± 1.32	27.44 ± 1.21	28.85 ± 1.33
Hindlimb muscles (mg)	<i>Tibialis Anterior</i>	49.16 ± 6.74	53.40 ± 7.39	50.28 ± 3.17	50.24 ± 4.41	50.87 ± 5.92	51.91 ± 5.35	52.17 ± 6.61
	<i>Quadriceps Femoris</i>	214.96 ± 26.36	214.93 ± 22.77	215.17 ± 10.02	218.00 ± 18.01	221.21 ± 20.21	211.42 ± 17.43	217.03 ± 14.36
	<i>Gastrocnemius</i>	153.61 ± 8.54	142.61 ± 21.24	149.25 ± 4.28	151.58 ± 13.89	156.41 ± 6.95	147.65 ± 14.42	153.21 ± 5.55
	<i>Muscle Soleus</i>	8.45 ± 0.91	8.51 ± 1.10	8.59 ± 0.61	9.70* ± 1.22	10.56* ± 0.58	8.56 ± 0.68	9.05 ± 0.39
Forelimb muscles (mg)	<i>Triceps Brachii</i>	113.07 ± 13.36	107.58 ± 17.40	108.38 ± 6.72	107.39 ± 7.46	102.87 ± 12.05	109.66 ± 13.07	105.52 ± 9.40
Heart (mg)		165.08 ± 14.26	165.77 ± 16.77	155.33 ± 10.85	164.95 ± 12.71	156.62 ± 8.62	147.97 ± 2.87	154.53 ± 12.50
Epididymal fat pad (mg)		474.06 ± 202.72	469.86 ± 205.00	440.16 ± 99.75	285.54* ± 94.54	313.62* ± 70.43	400.33 ± 110.10	458.90 ± 116.62

The significant difference in bodyweight indicated for the data of week 14 was determined using a mixed repeated measures ANOVA for the 14 weeks during which the animals exercised (the mice were euthanatized in week 14). Values are mean ± SD. An "*" indicates a significant difference ($p < 0.05$) compared to Home Cage Controls, an "#" indicates a trend for a difference ($p < 0.10$) compared to Home Cage Controls, $n = 10$ –12 mice per group for all output parameters but *Tibialis Anterior*, *M. Soleus* and Heart ($n = 9$ –12 mice per group, see Section 3.7).

Climbers were video monitored for one night (12 h) at the end of every week. Due to a technical malfunction video recording failed in week seven and eight. On average, Climbers climbed 131 m per night. The distance climbed decreased over the course of the experiment ($p < 0.01$) as most distance was covered in the first five weeks (a peak of 202 m per night in week three) and declined afterwards (dropping to 64 m in week 12, Fig. 3D).

Hang exercise mice hung for the scheduled amount of time on all days but the first day, during which two mice dropped down from the hanging apparatus.

3.2. Body weight

At the start of the exercise intervention, mice weighed 25.7 g (± 1.0 ; SD) on average. There were no differences between groups. A mixed repeated measure ANOVA revealed that over the course of the 14 weeks of exercise intervention, body weight of all exercise or control groups increased significantly ($p < 0.01$; Table 1). Over the course of the experiment, Runners developed a lower body weight ($p = 0.02$) compared to Home Cage Controls, and weight tended to be lower in Resistance Runners ($p = 0.06$) compared to Home Cage Controls. A One-Way ANOVA showed that the bodyweight of Runners was significantly lower compared to Home Cage Controls from week five onward, with the exception of week nine (data not shown). There was no significant difference in body weight between Burrowers and Pseudo-Burrowers, but there was

a trend for a difference between Burrowers and Home Cage Controls ($p = 0.06$). Finally, there was no significant difference between Climbers or Hang exercise mice and Home Cage Controls.

3.3. Balance beam performance

Balance beam performance of Home Cage Controls and Pseudo-Burrowers did not change over the course of the experiment for any of the tested beams (4 mm square beam or 6 mm $\emptyset$ rod, Table 2). However, Runners and Resistance Runners showed improved performance compared to Home Cage Controls halfway through (week four) and near the end of the exercise intervention (week nine): On the 4 mm wide squared beam both Runners and Resistance Runners were significantly faster to cross the beam than Home Cage Controls ($p < 0.01$; Table 2). When the 4 mm squared beam was placed at an angle, similar but non-significant differences were observed. Neither burrowing, climbing nor hanging exercise improved performance on the 4 mm squared beam placed straight or in an angle (Table 2).

The 6 mm $\emptyset$ rod (which was placed at an angle) makes for a very challenging balance beam test. In fact, only a few mice were able to successfully cross this balance beam when tested before the exercise interventions. However, running and resistance running both significantly improved performance on the 6 mm $\emptyset$ rod trials after four weeks and nine weeks of exercise. The time needed to cross the beam had significantly decreased in both groups compared to

Table 2
Balance beam performance.

Group			Home Cage Controls	Runners	Resistance Runners	Pseudo-Burrowers	Burrowers	Hang Exercise	Climbers
Latency to cross (s)	4 mm habituation (horizontal)	Pre	21.83 ± 4.63	19.79 ± 3.50	23.46 [*] ± 3.13	22.20 ± 3.74	21.36 ± 3.80	22.66 ± 3.92	21.64 ± 3.69
		Half	15.52 ± 2.98	10.94 [*] ± 2.80	11.36 [*] ± 2.67	16.82 ± 4.06	13.24 ^P ± 3.31	12.75 ± 3.09	12.69 ± 2.74
		Post	14.07 ^{P#} ± 2.82	10.20 [*] ± 3.60	10.28 [*] ± 2.33	17.11 ± 3.44	12.33 ^P ± 3.13	12.76 ± 2.94	13.66 ± 2.83
	4 mm (horizontal)	Pre	14.21 ± 3.82	14.39 ± 3.05	15.44 ± 1.92	16.09 ± 3.06	15.24 ± 3.03	16.22 ± 2.89	13.55 ± 2.45
		Half	12.57 ± 2.59	8.04 [*] ± 2.31	8.02 [*] ± 1.64	14.28 ± 4.24	11.43 ^{P#} ± 1.68	12.59 ± 2.70	10.00 ± 1.76
		Post	13.94 ± 2.18	8.58 [*] ± 2.51	8.86 [*] ± 2.46	16.02 ± 4.39	12.53 ^{P#} ± 2.92	12.69 ± 2.64	13.40 ± 2.41
	4 mm (angle)	Pre	16.89 ± 8.34	17.59 ± 5.57	18.64 ± 2.43	17.00 ± 3.61	18.22 ± 3.60	18.49 ± 3.79	18.12 ± 6.82
		Half	16.39 ± 7.97	9.11 ± 2.64	10.30 ± 3.31	15.01 ± 3.26	10.86 ± 2.97	10.98 ± 2.17	11.07 ± 2.94
		Post	14.35 ± 4.36	10.35 ± 4.15	10.48 ± 4.30	16.24 ± 4.62	12.35 ± 3.44	11.62 ± 3.18	10.77 ± 2.35
	6 mm rod (angle)	Pre	38.55 ± 3.05	40	36.73 [*] ± 4.04	39.42 ± 3.87	39.46 ± 1.84	38.97 ± 2.25	40
		Half	35.36 ± 6.77	19.79 [*] ± 8.37	19.75 [*] ± 6.56	34.24 ± 8.74	30.82 ± 9.19	34.32 ± 5.72	33.24 ± 7.75
		Post	38.21 ± 3.75	22.40 [*] ± 11.18	24.32 [#] ± 12.49	36.58 ± 6.53	33.17 ± 8.22	40	37.10 ± 5.89
Distance until fall (cm)	6 mm rod (angle)	Post	36.21 ± 18.62	70.24 [*] ± 30.19	75.45 [*] ± 24.49	43.04 ± 23.08	56.06 ± 26.69	43.77 ± 17.08	48.63 ± 26.64

Values are mean ± SD. An "*" indicates a significant difference ($p < 0.05$) compared to Home Cage Controls, an "#" indicates a trend for a difference ($p < 0.10$) compared to Home Cage Controls. An "p*" indicates a significant difference ($p < 0.05$) compared to Pseudo-Burrowers, an "p#" indicates a trend for a difference ($p < 0.10$) compared to Pseudo-Burrowers, an "r*" indicates significant difference ($p < 0.05$) compared to Runners, $n = 10$ –12 mice per group.

Table 3
Effects of burrowing, resistance running, running and climbing.

Groups	Functional changes				Tissue weights		
	Balance beam performance	Grip strength	Muscle stamina (inverted screen test)	Endurance	Soleus muscle	Other muscles*	Epididymal fat pad
Burrowers	=	↑	=	=	=	=	=
Resistance Runners	↑	↑	=	↑	↑	=	↓
Runners	↑	=	=	↑	↑	=	↓
Climbers	=	=	=	=	=	=	=

Compared to Home Cage Controls and, in the case of Burrowers, to Pseudo-Burrowers as well: "↑" increased, "↓" decreased, "=" unchanged. *Other muscles which were dissected and weighed are the *Tibialis Anterior*, *Quadriceps Femoris*, *Gastrocnemius* and *Triceps Brachii* muscle. No significant differences in muscle mass were found between groups for any of these muscles.

Home Cage Controls ($p < 0.01$; Table 2). Due to high attrition rate during the 6-mm rod balance beam test, we also scored the distance traveled before falling (in cm) the using video recordings for the final balance beam session (week nine). The average distance traveled before falling from the rod had significantly increased for both Runners and Resistance Runners compared to Home Cage Controls. ($p = 0.02$) Neither of the two parameters revealed climbing nor hanging exercise improved performance on the 6 mm $\varnothing$ rod, although there was a trend for improved performance in Burrowers as compared to Pseudo-Burrowers on the horizontal 4-mm beam (Table 2).

Burrowers did significantly improve performance on the balance beam compared to Pseudo-Burrowers during the 4-mm square beam habituation session. Performance on the habituation session improved over the various time-points (Pre, Half, Post) for all groups, whereas performance on non-habituation sessions remains constant over all time-points in control groups (Table 2).

3.4. Grip strength

Overall, forelimb grip strength declined over time. Burrowing significantly improved forelimb grip strength compared to Home Cage Controls ($p = 0.02$) and Pseudo-Burrowers ($p < 0.01$; Fig. 4A). There was no difference between Pseudo-Burrowers and Home Cage Controls. Resistance running significantly improved forelimb grip strength compared to Home Cage Controls ($p < 0.01$; Fig. 4C). Pairwise post-hoc testing (Games-Howell) revealed there was a trend for higher grip strength in Resistance Runners compared to Runners ($p = 0.09$; Fig. 4A). Neither running without resistance, climbing or hanging exercise significantly improved forelimb grip-strength compared to Home Cage Controls, but there was a trend for improved forelimb grip-strength in Runners compared to Home Cage Controls ($p = 0.06$; Fig. 4A,C,D). Four limb grip strength did not decline over time and neither of the exercise methods significantly altered four limb grip strength (Fig. 4B,D,F).

3.5. Inverted screen hanging test

Latency to fall in the inverted screen hanging test declined over time in all groups. Mixed repeated measures ANOVA revealed that although this decline was less pronounced in Burrowers compared to Home Cage Controls ($p = 0.06$) and in week 12 there was a trend for a difference between Burrowers and Pseudo-Burrowers too ($p = 0.06$; Fig. 5A). When Holding Impulse (body mass (g) $\times$ maximum latency to fall (sec)) was compared between groups, a similar pattern was revealed (Fig. 5B). Compared to controls neither running, resistance running, climbing nor hanging exercise altered performance on the inverted screen hanging test (Fig. 5C-F).

One mouse (Home Cage Controls) was excluded from analysis as it let itself drop down within several seconds from the start every time it was tested.

3.6. Endurance treadmill test

Both the time until exhaustion and the maximum running speed were increased in Runners and Resistance Runners compared to Home Cage Controls ($p < 0.01$), suggesting both forms of running exercise increase endurance as measured in this test (Fig. 6C, D). Burrowing, climbing and hanging exercise did not alter performance on the treadmill endurance test (Fig. 6A, B, E, F).

3.7. Muscle tissue, fat pad, and heart weight

Both running and resistance running significantly increased the mass of the *M. Soleus* ($p < 0.01$; Table 1). Running increased the mass of the *Soleus* muscle with 14% whereas resistance running increased *Soleus* muscle mass with 24%. Moreover, both running and resistance running decreased the weight of the epididymal fat pad with 36% and 31%, respectively ($p < 0.01$). This difference was still present when fat pad weight was divided by body-weight ($p < 0.01$; Supplementary Table 2). Running or resistance running did not alter heart weight. Burrowing, climbing or hanging exercise did not alter muscle mass of any of the dissected muscles, fat pad weight or heart weight (Table 1).

One *Tibialis Anterior* weight measurement (Home Cage Control) was excluded from the analysis (outlier, $p < 0.05$) because this sample was frozen before weighing it. One *M. Soleus* weight measurement (Runner) sample was excluded from the analysis because the muscle was damaged during dissection and dissected incompletely (outlier, $p < 0.05$). One Hang exercise mouse heart weight measurement was excluded from the analysis due to a likely weighing error, this sample was an outlier for the entire dataset containing all groups ($p < 0.05$).

4. Discussion

Voluntary strength training methods for rodents are needed to investigate the effects of strength training on the brain and cognition, but few voluntary methods are available. The current study tested functional and muscular effects of two potential (novel) voluntary strength training methods: burrowing and unloaded tower climbing. We also investigated the effects of a more commonly used potential voluntary strength exercise method, resistance running, and compared this to an established endurance (aerobic) exercise method: non-resistance wheel running.

Burrowing and resistance running improved forelimb grip strength compared to sedentary controls, and both qualify as voluntary strength training methods based solely on this output parameter. However, resistance running also induced functional changes similar to those induced by non-resistance running, which is an established endurance (aerobic) exercise method. Climbing did not cause any functional or muscular changes for any of the evaluated parameters. For an overview, see Table 3.

Fig. 4. Grip strength measurements. Grip strength measurements took place in week -2 (Pre), week 6 (Halfway), and week 11 (Post). A) Burrowers had significantly higher forelimb grip strength compared to Pseudo-Burrowers, Pseudo-Burrowers did not differ from Home Cage Controls. B) Resistance Runners had significantly higher forelimb grip strength compared to Home Cage Controls, whereas (non-resistance) Runners did not C) Neither (tower) Climbers nor Hang Exercise mice had higher forelimb grip strength compared to Home Cage Controls. D-F) Grip strength as measured in both forelimbs and hindlimbs was not affected by either of the exercise interventions. An "*" indicates a significant difference ($p < 0.05$), an "#" indicates a trend for a difference ($p < 0.10$). Letters indicate the contrast which was used: i.e. B/HCC * indicates a significant difference between Burrowers (B) and Home Cage Controls (HCC). Other groups are indicated as followed: P=Pseudo-Burrowers, R=Runners, RR=Resistance Runners, C=Climbers and H=Hang Exercise mice. Values are mean $\pm$ SEM, $n=10-12$ mice per group.

4.1. Burrowing and resistance running increase grip strength in the absence of T. Brachii muscle hypertrophy

Although both resistance running and burrowing improved grip strength in the forelimbs as compared to sedentary controls, nei-

ther of the exercise groups showed hypertrophy of the *Triceps Brachii*. The lack of *Triceps Brachii* hypertrophy as a result of resistance running with a 5-g load is in accordance with a previous study performed in adult and aged mice (Soffe et al., 2015). Another study, by Call and co-workers (Call et al., 2010), used a higher resistance

Fig. 5. Muscle fatigue as measured in the inverted screen test. The inverted screen test was performed in week -1 (Pre), week 7 (Halfway), and week 12 (Post). A-C) Neither burrowing (A), nor resistance running or (non-resistance) running (B), nor (tower) climbing or hanging exercise (C) significantly increased the latency to fall (s) compared to the relevant controls. An "*" indicates a significant difference ($p < 0.05$), an "#" indicates a trend for a difference ($p < 0.10$). Letters indicate the contrast which was used: i.e. B/HCC * indicates a significant difference between Burrowers (B) and Home Cage Controls (HCC). Other groups are indicated as followed: P=Pseudo-Burrowers, R=Runners, RR=Resistance Runners, C=Climbers and H=Hang Exercise mice. Values are mean $\pm$ SEM, $n=9-12$ mice per group (see section 3.5).

load (7 g) and did show that resistance running could induce *Triceps Brachii* hypertrophy, albeit in mdx mice (a mouse model for Duchenne muscular dystrophy). Obviously, *Triceps Brachii* is just one of the many muscles involved in attaining proper grip and power during the grip strength test, and it cannot be excluded that the other muscles that were not dissected show hypertrophy. Moreover, muscle hypertrophy is not the only adaptation that takes place as a consequence of strength or endurance exercise and not

the only means of increasing strength. For example, further analyses of muscle fiber diameter could reveal changes in specific muscle fiber types (fast or slow twitch) that can further characterize the differential effects of burrowing and resistance running. The current study shows that resistance running and burrowing positively affect forelimb strength, but further studies are needed to eluci-

Fig. 6. Endurance as measured on the treadmill test. A) Burrowing did not alter the time until exhaustion on the treadmill test compared to Pseudo-Burrowers. Pseudo-Burrowers did not differ significantly from Home Cage Controls. B) Running and resistance running increase endurance on the treadmill test compared to Home Cage Controls C) Climbing and hanging exercise did not alter the time until exhaustion compared to Home Cage Controls. An “*” indicates a significant difference compared to Home Cage Controls ($p < 0.05$), an “#” indicates a trend for a difference ($p < 0.10$). Values are mean $\pm$ SEM, $n=10$ –12 mice per group.

date the exact muscular responses that underlie this functional improvement.

4.2. Grip strength and muscle stamina declined over time

In all groups, forelimb grip strength and the latency to fall from the inverted screen declined over time. Theoretically, this effect could be due to habituation or the mice learning to deal with the test, or due to an effect of aging. Although protocols similar to our grip strength test and the inverted screen test have been applied

longitudinally, we cannot exclude any habituation effects of our tests with certainty. (Brooks et al., 2012; De Luca et al., 2003) An effect of aging seems unlikely, as previous studies that have applied similar methods have not shown a clear decline in grip strength or an increase in muscle fatigue until mice were over 1.5 years of age. (Brooks et al., 2012; De Luca et al., 2003; Heng et al., 2007; Keeling et al., 2007) Taken together, this indicates that habituation effects may have actually confounded longitudinal measures of forelimb grip strength in the current study to some extent. On the other hand, there was no sign of any habituation effects of the four-limb

grip strength test that was performed in the same test sessions. As far as we know, there are no studies that actually compare mice that were never tested versus mice that were longitudinally tested in either the grip strength test or the inverted screen test at the age of the mice in the current experiment. Although this issue needs to be addressed in future studies, the data from the current study supports the idea that both Burrowing and Resistance Running methods successfully increase or preserve grip strength.

4.3. Resistance running increases endurance and probably has a large aerobic component

Aerobic capacity can be determined using the incremental treadmill running test, often in combination with oxygen uptake measurements. (Krüger et al., 2013) Both running and resistance running increased endurance in the current study, as measured by the maximal speed at which the animal can run (V_{max}) and the time until exhaustion. Although we did not measure oxygen uptake directly, the increase in V_{max} suggests that both Runners and Resistance Runners have increased aerobic capacity compared to controls. In other studies that did measure oxygen consumption, running speed correlated with the oxygen uptake. (Høydal et al., 2007; Krüger et al., 2013). We also found that both running and resistance running decreased the mass of the epididymal fat pad. These results are in line with a recent mouse study that showed that both low and high-resistance running decrease in epididymal fat mass to a similar extend. (Soffe et al., 2015) Loss of fat mass is an effect which is most pronounced in aerobic training. (Willis et al., 2012)

The increased endurance and the decreased epididymal fat mass suggest that resistance running has an important aerobic component, whereas the higher grip strength of Resistance Runners as compared to Home Cage Controls qualifies resistance running as a strength training method. This finding suggests resistance running is a combination of aerobic (endurance) exercise and strength training. Finally, non-resistance Runners tended to have greater forelimb grip strength compared to Home Cage Controls and Resistance Runners tended to have greater strength compared to Runners too. Therefore, a strict dichotomy between running and resistance running is probably inaccurate. One study supporting this point of view showed that both running and resistance running with a 7-g load increases grip strength in mdx mice. (Call et al., 2010) Further investigation of muscular changes could help to characterize these exercise methods (See section 4.7).

Taken together, this means that in order to truly discern the differential effects of the strength training and endurance exercise component on the brain, a resistance running group should always be tested together with a non-resistance running control group. Finally, various resistance running protocols can be used, using different resistance loads at different time-points. Different protocols may be used to induce low-, moderate-, and high-intensity resistance running. Such protocols will likely differ with regard to the balance between endurance and strength exercise components.

4.4. Running and resistance running improve motor coordination on the balance beam

Both running and resistance running improved motor coordination on the balance beam. In contrast, neither burrowing nor climbing improved performance on the balance beam test significantly. The aerobic component is the common denominator between running and resistance running. At first sight it therefore seems, contrary to our expectations, that the aerobic component of (resistance) running is linked to improved motor coordination. In humans, the effect of exercise on balance has been investigated quite extensively in elderly. There is evidence that some exercise

interventions, including strength training, are moderately effective in improving balance in older adults. (Howe et al., 2007) However, there is little evidence to suggest that aerobic endurance increases balance in humans.

Another possibility is that the physiological adaptations that are a consequence of aerobic exercise do not improve balance beam performance in the current study, but the mere act of running does. Since the movement performed in a loaded or unloaded wheel is quite similar, this might explain the similar improvements on the balance beam. Moreover, running wheels allow for the mouse to balance on the axis of the wheel, which could also explain these positive results.

In short, we cannot conclude that there is a causal relation between improved endurance in Runners and Resistance Runners and the observed improvement in the balance beam performance. On the other hand, our balance beam protocol did reveal clear functional differences between both running groups and the Home Cage Controls. This finding warrants further investigation using other aerobic exercise methods, such as running discs, which are less prone to elicit the confounding effects of balancing on the exercise apparatus itself. (Grégoire et al., 2014) Another way to control for some of these non-aerobic effects would be the use of locked running wheels. We can conclude that the increase in strength in Resistance Runners and Burrowers did not alter performance on the balance beam.

Finally, it should be mentioned that Burrowers significantly outperformed Pseudo-Burrowers during the 4-mm habituation session and tended to have a lower latency to cross the 4-mm beam during the test session as well. These results are, however, difficult to interpret. All groups of mice improved performance on the habituation session over the course of the experiment, which indicates that the habituation session probably measures more than motor skills and balance alone: The habituation session includes a habituation or learning component as well. Consequently, it is hard to draw conclusions on the effects of burrowing on motor skills and balance and this matter should be addressed in future studies.

4.5. Resistance running and running induced muscle hypertrophy in absence of cardiac hypertrophy

In our study, both running and resistance running increased muscle mass of the *M. Soleus*, a predominantly type I fiber muscle used while standing and walking. Previous studies have investigated the effects of resistance running on muscle hypertrophy and found similar results.

Konhilas et al. found *M. Soleus* hypertrophy after 12 weeks of running with up to 12 g of resistance in adult mice. (Konhilas et al., 2005) In their study, non-loaded running led to cardiac hypertrophy, whereas low- and high-resistance running did not increase heart weight significantly. In the current study, running and resistance running did not affect heart weight. Soffe et al. studied the effects of resistance running with a 4- and 6-g load in adult and aged mice. (Soffe et al., 2015) Their study showed that while 4 g of resistance induced hypertrophy of the *M. Quadriceps*, *M. Soleus* and *M. Femoris* of adult mice, running with a 6-g load induced hypertrophy of the *M. Soleus* muscle only. These results are consistent with our findings that a 5-g load induces *M. Soleus* hypertrophy.

In our study, we also found *M. Soleus* muscle hypertrophy induced by non-resistance running. Other studies investigated the effects of running without additional resistance on muscle hypertrophy in mice (Landisch et al., 2008; Pellegrino et al., 2005). Pellegrino et al. found no effect of non-resistance running on *M. Soleus* muscle mass. Landisch et al. showed that non-resistance running increases *Soleus* muscle mass by 28% in adolescent C57Bl10 mice, but it should be noted the effect of running on *Soleus* muscle mass was found to be significant in an analysis in which wild-type

mice and mdx mice were pooled together. Finally, a study by Call et al. (2010) that was conducted in male mdx mice (no wild-type controls were used), showed that a 12-week running program with up to 7 g of resistance induced *Triceps Brachii* hypertrophy, whereas non-resistance running induced hypertrophy of the *M. Soleus* and *T. Anterior* (Call et al., 2010).

In summary, although *M. Soleus* hypertrophy is more pronounced in resistance running, it has been observed as an effect of non-resistance running as well. In addition, various muscles were affected differently between different studies (for an overview, see Soffe et al. (2015)). The use of mice of different ages and of different strains, as well as the use of different running wheels and resistance running protocols may partially explain these variable outcomes between studies. The lack of consistent findings prohibits us to make any clear-cut conclusion about how different (resistance) running exercise methods affect muscle adaptation in the hindlimbs. Future studies testing resistance running exercise protocols with different resistance loads could provide a clearer view on this matter.

4.6. Climbing without additional load does not qualify as voluntary strength training

Climbing did not lead to functional changes and therefore seems unsuitable as a strength training model in the current form. Mice climbed up to 202 m per night on average in week three, after which climbing distance steadily declined over the following weeks. A similar pattern was seen in running mice and has been observed in other studies as well (Call et al., 2010; Soffe et al., 2015). These findings suggest that intrinsic motivation to climb or run declines over time. An alternative explanation is that the decline in grip strength that was observed in the current study caused a decline in distance climbed or run. However, it is difficult to interpret the meaning of the observed decline in grip strength (see Section 4.2). Contrary to our hypothesis, neither climbing nor hanging exercise caused an improvement in muscle stamina in the inverted screen hanging test.

Previously, Krüger et al. have shown that hanging exercise improved holding time in a maximum holding test, comparable to our inverted screen test. (Krüger et al., 2013) This functional adaptation was accompanied by increased muscle fiber type II diameter, and with improved glucose metabolism in an additional group of mice that were put on a high-fat diet. In the current study, we were not able to detect any functional improvements after (isometric) hanging exercise. This difference could be due to the use of slightly different behavioral tests used in the current study and the study of Krüger et al. In their original study, the maximum holding test was performed on the exact same set-up that was used to perform the training procedure (hanging exercise). In the current study, the mice were not trained and tested on the same apparatus in order to prevent any effects of habituation or adapted behavior from confounding the muscle fatigue test. It is hard to tell apart increased skill from increased stamina when one group of mice is trained on the apparatus five days a week, while the controls have not or hardly encountered the apparatus before the actual test. For this reason, we used a wire mesh of a different diameter in the inverted screen test (2 mm) compared to the wire diameter used on the apparatus to train the mice (~1 mm). The thicker wire diameter increases the difficulty of the inverted screen test. Moreover, our inverted screen was oriented horizontally (upside-down) while the hanging exercise apparatus was oriented vertically. In accordance with the original publication, we did not detect hypertrophy in any of the investigated muscles as a result of isometric hang exercise training.

In summary, in the current study neither climbing nor hanging exercise led to functional improvements that suggest either one

is suitable as a strength training method. The lack of functional changes induced by hanging exercise is a limitation to the current study as it disqualifies the use of Hang Exercise mice as a positive control group in the current set-up. All mice did, however, voluntarily climb in the towers throughout the experiment. This shows that climbing behavior could potentially be utilized as a voluntary exercise method. Modifications to the protocol, such as having mice climb at an overhanging angle, with extra weight load or in towers made from thicker wires, could combine the voluntary aspect of the climbing towers with the strength training component that is already present in ladder climbing methods. (Duncan et al., 1998; Hornberger and Farrar, 2004; Yarasheski et al., 1990)

4.7. Limitations and recommendations

Although the current study successfully characterizes functional and structural effects of various resistance exercise methods, it has three limitations. First, although gross muscular adaptations were investigated (weight differences), we did not perform a detailed analysis of muscle fiber adaptation or of possible metabolic effects or adaptations (considered beyond the scope of the current study). Secondly, the current study investigated the effects of burrowing and other exercise methods in male mice. Previous studies have shown that voluntary exercise behavior and the effects of exercise can differ between sexes in both mice and rats. (Eikelboom and Mills, 1988; Novak et al., 2012) Although we have observed that female mice will readily burrow when presented with a burrowing tube (pilot study), further characterization of burrowing and resistance running in female mice is necessary in order to facilitate studies that (also) use female mice. Furthermore, the usefulness of burrowing as a strength exercise method in rats or other species has not been investigated. Thirdly, the current study made use of a limited number of mice per group ($n = 10$ – 12). The use of *a priori* contrasts enhances statistical power, but this study was set-up to detect differences of a large and not medium or small effect size. For example, the trend for a difference in grip strength between Resistance Runners and Runners or Runners and Home Cage Controls could prove significant when investigated in more detail using a larger number of animals.

5. Conclusions

The current study should be viewed as an important first step in identifying novel voluntary strength exercise methods.

Natural burrowing behavior of rodents can be utilized as a voluntary strength training method. This novel strength training method increased forelimb strength and showed potential to improve muscle stamina. Resistance running is also suitable as a strength training method, but its use warrants caution as it shares many features with non-resistance wheel running and likely has a substantial aerobic component too. When using a 5-g load, resistance running is a combination of strength training and aerobic exercise as it increases strength but also endurance. Finally, voluntary climbing did not cause any functional or muscular changes and does not seem a suitable strength training method in the current form, but the method could be modified since climbing behavior was ubiquitously shown by all our mice.

5.1. Perspectives

The use of voluntary running as an endurance exercise method has vastly improved our knowledge on the effects of endurance exercise on the brain and on cognition. Far less is known about the effects of strength training on the brain. In a recent review, Strickland and Smith (2016) summarized the available strength

training methods that are currently being used to study the neurobiological mechanisms underlying the effects of strength training on the brain in rodents. They conclude that the use of animal strength training methods that limit the use of stressful stimuli and that minimize the contribution of aerobic factors will be of pivotal importance to examine the effects of strength training on central nervous system functioning. We show that burrowing can be used as a strength exercise method that fits these criteria. This method can be used, solely or combined with cognitive tests, to investigate the effects of strength training on brain functioning without the confounding factors of stress or handling.

Such studies are of significant importance in the fields of healthy (brain) aging and battling or slowing down the progression of neurodegenerative diseases by way of optimal exercise interventions.

Conflict of interest

None of the authors declare any actual or potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jneumeth.2017.05.027>.

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